Gene Ther Mol Biol Vol 1, 1-172. March, 1998.

Status of gene therapy in 1997: molecular mechanisms, disease targets, and clinical applications

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Key words: gene therapy, gene transfer, clinical trials, cancer, immunotherapy, p53, adenovirus, retrovirus, adeno-associated virus, HIV-1, HSV-1, EBV, AIDS, tumor vaccines, IFN-γ, TNF-α, VEGF, retinoblastoma, purine nucleoside phosphorylase, HSV-tk, E1A, E1B, Cre, LoxP, recombination, HIV vectors, liposomes, fusogenic peptides, plasmovirus, transcription factor, TIL, IL-2, IL-3, IL-7, IL-12, GM-CSF, prostate cancer, p21, p16, apoptosis, Bcl-2, Bax, Bcl-xs, E2F, bystander effect, MDR1, IGF-I, antisense, triplex DNA, Parkinson's disease, lysosomal storage disease, hemophilia, cystic fibrosis, CFTR, rheumatoid arthritis, hypertension, familial hypercholesterolemia, LDL, angiopoietin, restenosis, angiogenesis, TGF-β, arterial injury, atherosclerosis, ADA deficiency, obesity, leptin.

Summary

Gene therapy is a newly emerging field of biomedical research aimed at introducing therapeutically important genes into somatic cells of patients; a new and revolutionary era in molecular medicine has begun. Diseases already shown to be amenable to therapy with gene transfer in clinical trials include cancer (melanoma, breast, lymphoma, head & neck, ovarian, colon, prostate, brain, chronic myelogenous leukemia, non-small cell lung, lung adenocarcinoma, colorectal, neuroblastoma, glioma, glioblastoma, astrocytoma, and others), AIDS, cystic fibrosis, adenosine deaminase deficiency, cardiovascular diseases (restenosis, familial hypercholesterolemia, peripheral artery disease), Gaucher disease, α1-antitrypsin deficiency, rheumatoid arthritis and a few others. Human diseases expected to be the object of clinical trials include hemophilia A and B, Parkinson's disease, ocular diseases, xeroderma pigmentosum, high blood pressure, obesity and many others. The establishment of novel animal models for human disease, the discovery of new genes, and improvements in successful gene delivery open bright new prospects for molecular medicine. A wide variety of delivery vehicles for genes have been tested including murine retroviruses, recombinant adenoviral vectors, adeno-associated virus, HSV, EBV, HIV vectors, and baculovirus. Nonviral gene delivery methods use cationic or neutral liposomes, direct injection of plasmid DNA, and polymers. Various strategies to enhance efficiency of gene transfer have been tested such as fusogenic peptides in combination with liposomes, or polymers, to enhance the release of plasmid DNA from endosomes. Recombinant retroviruses stably integrate into the DNA and require host DNA synthesis; adenoviruses can infect nondividing cells but cause immune reactions leading to the elimination of therapeutically transduced cells. Adeno-associated virus (AAV) is not pathogenic, does not elicit immune responses but new strategies are required to obtain high AAV titers for preclinical and clinical studies. Wild-type AAVs integrate into chromosome 19 whereas recombinant AAVs are deprived of site-specific integration and may also persist episomally; HSV vectors can infect nonreplicating cells such as neuron cells, have a high payload capacity for foreign DNA but inflict cytotoxic effects. It seems that each delivery system will be developed independently of the others and that each will prove its strengths for specific applications. At present, retroviruses are most commonly used in human clinical trials followed by adenoviruses, cationic liposomes and AAV. Polymer-encapsulated syngeneic or allogeneic cells implanted into a tissue of a patient can be used to secrete therapeutic proteins; the method is in trials for

amyotrophic lateral sclerosis using the ciliary neurotrophic factor gene, and can be extended to Factor VIII and IX for hemophilia, interleukin genes, dopamine-secreting cells to treat Parkinson's disease, nerve growth factor for Alzheimer's disease and other diseases. Ingenious techniques under development with great future prospects for human gene therapy, include the Cre-LoxP recombinase system to rid of undesirable viral DNA sequences used for gene transfer, use of tissue-specific promoters to express a gene in a particular cell type or use of ligands, such as peptides selected from random peptide libraries, recognizing surface molecules to direct the gene vehicle to a particular cell type, designing p53 "gene bombs" that explode into tumor cells, exploit the HIV-1 virus to engineer vectors for gene transfer, the combining of viruses with polymers or cationic lipids to improve gene transfer, the attachment of nuclear localization signal peptides to oligonucleotides to direct them to nuclei, and the invention of molecular switch systems allowing genes to be turned on or off at will.

Although many human tumors are non- or weakly immunogenic, the immune system can be reinforced and instructed to eliminate cancer cells after transduction of patient's cells ex vivo with the cytokine genes GM-CSF, IL-12, IL-2, IL-4, IL-7, IFN-γ, and TNF-α, followed by cell vaccination of the patient (e.g. intradermally) to potentiate T-lymphocyte-mediated antitumor effects (cancer immunotherapy). DNA vaccination with genes encoding tumor antigens and immunotherapy with synthetic tumor peptide vaccines are further developments in this exciting field. The genes used for cancer gene therapy in human clinical trials include a number of tumor suppressor genes (p53, RB, BRCA1, E1A), antisense oncogenes (antisense c-*fos*, c-*myc*, K-*ras*), and suicide genes (HSV-tk, in combination with ganciclovir, cytosine deaminase in combination with 5-fluorocytosine). Important in gene therapy are also the genes of bcl-2, MDR-1, p21, p16, bax, bcl-xs, E2F, IGF-I VEGF, angiostatin, CFTR, LDL-R, TGF-β, and leptin. Reports on human clinical trials using adenoviral and retroviral injections of the p53 gene have been very encouraging; future directions might go toward the use of genes involved in the control of tumor progression and metastasis. The molecular mechanisms of carcinogenesis have been largely elucidated and improvements in gene delivery methods are likely to lead to the final victory of the human race in the fight against cancer and other deadly diseases.

Abbreviations:

α1-AT, α1-antitrypsin
5FC, 5-fluorocytosine
5FU, 5-fluorouracil
aa, amino acid
AAV, adeno-associated virus
Ad, adenovirus
ADA, adenosine deaminase
aFGF, acidic fibroblast growth factor
AIDS, acquired immunodeficiency syndrome
APCs, antigen-presenting cells
bFGF, basic fibroblast growth factor
bp, base pairs
CAT, chloramphenicol acetyltransferase
CD, cytosine deaminase
CDKs, cyclin-dependent kinases
CEA, carcinoembryonic antigen
CF, cystic fibrosis
CFTR, cystic fibrosis transmembrane regulator
cfu, colony forming units
CMV IE, cytomegalovirus immediate-early
CMV, cytomegalovirus
CNS, central nervous system
CNTF, ciliary neurotrophic factor
CTLs, cytotoxic T lymphocytes
DBD, DNA-binding domain
DSBs, double-strand DNA breaks
EBV, Epstein-Barr virus
EGF, epidermal growth factor
EGFR, epidermal growth factor receptor
FH, familial hypercholesterolemia
GCV, ganciclovir
GFP, green fluorescent protein
GM-CSF, granulocyte-macrophage colony stimulating factor
HIV-1, human immunodeficiency virus type 1
HPV, human papillomavirus
HSC, hematopoietic stem cells
HSV, herpes simplex virus
i.m., intramuscular
i.p., intraperitoneal
i.v., intravenous
ICE, interleukin-1β converting enzyme
IFN-γ, interferon-γ
IGF-I, insulin-like growth factor I
IGF-IR, insulin-like growth factor I receptor
IL, interleukin
IL-1β, interleukin-1β
ITR, inverted terminal repeat
LAK, lymphokine-activated killer cells
LDL-R, low density lipoprotein receptor
LTR, long terminal repeat
mAb, monoclonal antibody
MAR, matrix-attached region
MeP-dR, 6-methylpurine-2'-deoxyriboside
MHC, major histocompatibility complex
MLV, murine leukemia virus
MMTV, mouse mammary tumor virus
Mo-MLV, Moloney murine leukemia virus
MOI, multiplicity of infection
MT, metallothionein
Neo^R, neomycin phosphotransferase
NK, natural killer cells
nt, nucleotides
ODNs, oligodeoxynucleotides
ORFs, open reading frames
ORIs, origins of replication
PAI-1, plasminogen activator inhibitor-1
PARP, poly(ADP-ribose) polymerase
PBL, peripheral blood lymphocytes
PCNA, proliferating cell nuclear antigen
PDGF, platelet-derived growth factor
PGF, placenta growth factor
PKC, protein kinase C
PMGT, particle-mediated gene transfer
PNP, purine nucleoside phosphorylase
PSA, prostate specific antigen
PVR, proliferative vitreoretinopathy
RA, rheumatoid arthritis
RA-SF, rheumatoid arthritis synovial fibroblasts
rAAV, recombinant adeno-associated virus
RAC, recombinant advisory committee
RB, retinoblastoma
RLU, relative luciferase units
RSV, Rous sarcoma virus
s.c., subcutaneous
SCID, severe combined immunodeficient
SMC, smooth muscle cell
TAD, transactivation domain
TGF-β, transforming growth factor-β
TIL, tumor-infiltrating lymphocyte
TK, thymidine kinase
TNF-α, tumor necrosis factor α
tPA, tissue plasminogen activator
uPA, urokinase plasminogen activator
VEGF, vascular endothelial growth factor
VSMC, vascular smooth muscle cell
VSMC, vascular smooth muscle cells
VSV, vesicular stomatitis virus
wt, wild-type

I. Introduction

Monumental progress in several fields including DNA replication, transcription factors and gene expression, repair, recombination, signal transduction, oncogenes and tumor suppressor genes, genome mapping and sequencing, and on the molecular basis of human disease are providing the foundation of a new era of biomedical research aimed at introducing therapeutically important genes into somatic cells of patients. The main targets of gene therapy are to repair or replace mutated genes, regulate gene expression and signal transduction, manipulate the immune system, or target malignant and other cells for destruction (reviewed by Anderson, 1992; Nowak, 1995; Boulikas, 1996a,b; Culver, 1996; Ross et al, 1996).

Two main approaches have been pursued for gene transfer to somatic cells **(i)** direct gene delivery using murine retroviruses, adenoviruses, adeno-associated virus, HSV, EBV, liposomes, polymers, or direct plasmid injection (gene therapy *in vivo*); and **(ii)** ex vivo gene therapy involving removal of syngeneic cells from a specific organ or tumor of an individual, genetic correction of the defect in cell culture (ADA deficiency, LDL-R for FH) or transfer of a different gene (IL-2 to tumor infiltrating lymphocytes to potentiate the cytotoxicity to tumors, cytokine genes to tumor cells from a patient for cancer immunotherapy, multidrug resistance gene transfer to render bone marrow cells resistant to certain antineoplastic drugs), followed by reimplantation of the cells. The reimplanted cells produce the therapeutic protein.

Several key factors or steps appear to be involved for the effective gene transfer to somatic cells in a patient or animal model: **(i)** the type of vehicle used for gene delivery (liposomes, adenoviruses, retroviruses, AAV, HSV, EBV, polymer, naked plasmid) which will determine not only the half-life in circulation, the biodistribution in tissues, and efficacy of delivery but also the route through the cell membrane and fate of the transgene in the nucleus; **(ii)** interaction of the gene-vehicle system with components in the serum or body fluids (plasma proteins, macrophages, immune response cells); **(iii)** targeting to the cell type, organ, or tumor, and binding to the cell surface; **(iv)** port and mode of entrance to the cell (poration through the cell membrane, receptor-mediated endocytosis), **(v)** release from cytoplasmic compartments (endosomes, lysosomes), **(vi)** transport across the nuclear envelope (nuclear import); **(vii)** type and potency of regulatory elements for driving the expression of the transferred gene in a particular cell type including DNA sequences that determine integration versus maintenance of a plasmid or recombinant virus/retrovirus as an extrachromosomal element; **(viii)** expression (transcription) of the transgene producing heterogeneous nuclear RNA (HnRNA) which is then **(ix)** spliced and processed in the nucleus to mature mRNA and is **(x)** exported to the cytoplasm to be **(xi)** translated into protein. Additional steps may include posttranslational modification of the protein and addition of a signal peptide (at the gene level) for secretion.

All steps can be experimentally manipulated and improvements in each one can enormously enhance the level of expression and therapeutic index of a gene therapy approach. It has been proposed that the plasmid vector is unable to translocate to the nucleus unless complexed in the cytoplasm with nuclear proteins possessing nuclear localization signals (NLSs). NLSs are short karyophilic peptides on proteins destined to function in the nucleus used for binding to specific transporter molecules in the cytoplasm, mediating their passage through the pore complexes to the nucleus (see Boulikas, 1998, this volume). NLS are present on histones, transcription factors, nuclear enzymes, and a number of other nuclear proteins; nascent chains of DNA-binding polypeptides could bind to the supercoiled plasmid in the cytoplasm mediating its translocation to the nucleus.

During delivery of foreign DNA *in vivo* vehicles may be attacked by macrophages, lymphocytes, or other components of the immune system and the vast majority will be cleared from blood, intracellular, or other body fluids before it is given the chance to reach the membrane of the cell target; the half-life of naked plasmids injected intravenously into animals is about 5 min (Lew et al, 1995). Cationic lipids, other than being very toxic, mediate efficient gene delivery passing through biological membranes; those lipid-DNA complexes surviving the immediate neutralization by serum proteins in the blood can reach the lung, heart and other tissues after vein or artery injection with one heart beat and transform endothelial vascular cells (reviewed by Boulikas, 1996d).

A variety of viral vectors have been developed to exploit the characteristic properties of each group to maintain persistence and viral gene expression in infected cells. Retroviral vectors and AAV integrate into target chromosomes and the transgene they carry can be inactivated from position effects from chromatin surroundings. Vectors with persistence/integration functions may not result in high levels of gene delivery in vivo.

Adenoviruses and retroviruses which are of the most frequently used vehicles for gene transfer can accommodate up to 7kb of total foreign DNA into their genome because of packaging limitations. This precludes their use for the transfer of large genomic regions. Transfer of intact yeast artificial chromosome (YAC) into transgenic mice will enable the analysis of large genes or multigenic loci such as human β-globin locus (reviewed by Peterson et al, 1997).

A small portion of plasmid molecules crossing the cell membrane will escape degradation from nucleases in the lysosomes and become released to the cytoplasm; even a smaller portion of these molecules will enter nuclei; finally, after successfully reaching the nucleus, plasmids with therapeutic genes are usually degraded by nuclear enzymes and transgene expression is permanently lost after about 2-7 days from animal tissues following successful

gene delivery. During the peak of transgene expression (usually 7-48 h from injection) the transgene transcript can follow the normal fate of other nuclear transcripts when proper polyadenylation signals are provided; its processed mRNA will be exported to the cytoplasm and translated into the therapeutic protein.

The choice of the appropriate delivery system for successful somatic gene transfer demands understanding of the drawbacks and advantages of each delivery system, such as limitations in the total length of the DNA that can be introduced, including the cDNA of the therapeutically important gene and control elements. Understanding the pathophysiology of the disease and the cell targets can give clues on the way of introducing the gene (i.v., i.p., intratumoral, s.c. injection) or direct the gene therapist to designing methods to target and secrete a therapeutic protein from a tissue which is not the normal site of production of a therapeutic protein. The type of control elements required for the anticipated tissue-specific expression of the construct, the presence of viral or other origins of replication as well as of the cDNA encoding the viral replication initiator protein for an episomal replication of the transgene, sequences that prompt integration and others that insulate the gene from the chromatin surroundings at the integration site, are also important for successful gene transfer.

Cancer gene therapy and immunotherapy has been the first priority of human gene therapy protocols. New gene targets are being defined and new clinical protocols are being proposed and approved. Effective eradication of a great variety of tumors with drugs which inhibit angiogenesis has been extraordinarily successful on animal models and the method moves fast to clinical trials; transfer of anti-angiogenesis genes will be the next step. A number of anticancer genes are being tested in preclinical or clinical cancer trials including p53, RB, BRCA1, E1A, bcl-2, MDR-1, HER2, p21, p16, bax, bcl-xs, E2F, antisense IGF-I, antisense c-fos, antisense c-myc, antisense K-ras and the cytokine genes GM-CSF, IL-12, IL-2, IL-4, IL-7, IFN-γ, and TNF-α. A promising approach is transfer of the herpes simplex virus thymidine kinase (HSV-*tk*) gene (suicide gene) and systemic treatment with the prodrug ganciclovir which is converted by HSV-tk into a toxic drug killing dividing cells. Theoretically, expression of therapeutic genes preferentially in cancer cells could be achieved by regulatory elements from tumor-specific genes such as carcinoembryonic antigen.

The first gene therapy products are expected to receive FDA approval by the year 2000; the market for gene therapy products is expected to exceed $45 billion by 2010.

This article reviews the molecular mechanisms and recent developments for the gene therapy of cancer, HIV, ADA deficiency, Parkinson's disease, lysosomal storage disease, hemophilia A and B, α1-antitrypsin deficiency, cystic fibrosis, rheumatoid arthritis, hypertension, familial hypercholesterolemia, atherosclerosis/restenosis, wound healing, and obesity including the treatment of cancer and heart diseases with angiogenesis inhibitors and gene transfer to the arterial wall. It is my intention to give a general overview rather to exhaust the field.

DIVISION ONE: GENE DELIVERY SYSTEMS AND GENE EXPRESSION

II. Gene delivery using retroviruses

A. Recombinant murine retroviruses

The recombinant Moloney murine leukemia virus (Mo-MLV or MLV) has been extensively used for gene transfer. Retroviral vectors derived from Mo-MLV promote the efficient transfer of genes into a variety of cell types from many animal species; up to 8 kb of foreign DNA can be packaged in a retroviral vector. Recombinant retroviruses have been the most frequently used and promising vehicles for the delivery of therapeutic genes in human gene therapy protocols (**Appendix 1**). Retroviral vectors cause no detectable harm as they enter their target cells; the retroviral nucleic acid becomes integrated into chromosomal DNA, ensuring its long-term persistence and stable transmission to all future progeny of the transduced cell.

The life cycle of the retrovirus is well understood and can be effectively manipulated to generate vectors that can be efficiently and safely packaged. An important contribution to their utility has been the development of retrovirus packaging cells, which allow the production of retroviral vectors in the absence of replication-competent virus.

Recombinant retroviruses stably integrate into the DNA of actively dividing cells, requiring host DNA synthesis for this process (Miller et al, 1990). Although this is a disadvantage for targeting cells at G_0, such as the totipotent bone marrow stem cells, it is a great advantage for targeting tumor cells in an organ without affecting the normal cells in the surroundings. This approach has been used to kill gliomas in rat brain tumors by injection of murine fibroblasts stably transduced with a retroviral vector expressing the HSV-tk gene (Culver et al, 1992; see below).

B. Retrovirus packaging cell lines

The use of retroviral vectors in human gene therapy requires a packaging cell line which is incapable of producing replication-competent virus and which produces high titers of replication-deficient vector virus. The packaging cell lines have been stably transduced with viral genes and produce constantly viral proteins needed by viruses to package their genome. Wild-type virus can be produced through recombinational events between the helper virus and a retroviral vector. Methods are also available for generating cell lines which secrete a broad host range retrovirus vectors in the absence of helper virus.

Retrovirus packaging cell lines containing the gag-pol genes from spleen necrosis virus and the env gene from

spleen necrosis virus or from amphotropic murine leukemia virus on a separate vector have been used; retrovirus vectors were produced from these helper cell lines without any genetic interactions between the vectors and sequences in the helper cells (Dougherty et al, 1989). An ecotropic packaging cell line and an amphotropic packaging cell line, in which the viral gag and pol genes were on one plasmid and the viral env gene were on another plasmid have been constructed; both plasmids contained deletions of the packaging sequence and the 3' LTR; when the fragmented helper virus genomes were introduced into 3T3 cells they produced titers of retrovirus which were comparable to the titers produced from packaging cells containing the helper virus genome on a single plasmid (Markowitz et al, 1990).

The pBabe retroviral vector constructs which transmit inserted genes at high titers and express them from the Mo-MLV LTR have been designed with one of four different dominantly acting selectable markers, allowing the growth of infected mammalian cells in the presence of G418, hygromycin B, bleomycin/phleomycin or puromycin, respectively. The packaging cell line, omega E, generated with separate gag/pol and ecotropic env expression constructs, was designed in conjunction with the pBabe vectors to reduce the risk of generation of wild type Mo-MLV via homologous recombination events (Morgenstern and Land, 1990).

C. Pseudotyped retroviral vectors

The traditional retroviral vector enters the target cell by binding of a viral envelope glycoprotein to a cell membrane viral receptor. Coinfection of cells with a retrovirus and VSV (vesicular stomatitis virus) produces progeny virions containing the genome of one virus encapsidated by the envelope protein of the other (pseudotypes of viruses); this led to the development of pseudotyped retroviral vectors where the Moloney murine leukemia *env* gene product is replaced by the VSV-G protein able to interact with other membrane-bound receptors as well as with some components of the lipid bilayer (phosphatidylserine); because of the ubiquitous distribution of these membrane components pseudotyped particles display a very broad host range (Friedmann and Yee, 1995). Use of pseudotyped vectors has been a significant advancement for retroviral gene transfer.

Pseudotypes of VSV and Mo-MLV, are released preferentially at early times after infection of MuLV-producing cells with VSV; at later times, after synthesis of M-MLV proteins has been inhibited by the VSV infection, neither Mo-MLV virions nor the VSV (Mo-MLV) pseudotypes are made. There appears to be a stringent requirement for recognition of the viral core by homologous envelope components for the production of VSV (M-MLV) pseudotypes (Witte and Baltimore, 1977).

The finding that the G protein of vesicular stomatitis virus (VSV) can serve as the exclusive envelope protein component for one specific retroviral vector that expresses VSV G protein was extended to a general transient transfection scheme for producing very high-titer VSV G-enveloped pseudotypes from any Moloney murine leukemia-based retroviral vector (Yee et al, 1994). Pseudotyping of MuLV particles with VSV-G expressed transiently in cells producing MLV Gag and Pol proteins, has yielded vector preparations with a broader host range that could be concentrated by ultracentrifugation. For example, this technology allowed for efficient concentration of vector by ultracentrifugation to titers > 10^9 colony-forming units/ml and offers hope for potential use for gene transfer in vivo. Furthermore, these vectors could infect cells, such as hamster and fish cell lines, that are ordinarily resistant to infection with vectors containing the retroviral envelope protein (Burns et al, 1993).

A human 293-derived retroviral packaging cell line was generated by Ory et al (1996) capable of producing high titers of recombinant Mo-MLV particles that have incorporated the VSV-G protein. This new packaging cell line may be used for direct in vivo gene transfer using retroviral vectors because the retroviral/VSV-G pseudotypes generated with these cells were significantly more resistant to human complement than commonly used amphotropic vectors.

A human immunodeficiency virus type 1 (HIV-1)-based retroviral vector containing the firefly luciferase reporter gene could be pseudotyped with a broad-host-range VSV envelope glycoprotein G; higher-efficiency gene transfer into $CD34^+$ cells was achieved with a VSV-G-pseudotyped HIV-1 vector than with a vector packaged in an amphotropic envelope (Akkina et al, 1996).

Because the VSV-G protein is toxic to cells when constitutively expressed, Yang et al (1995) have used steroid-inducible and tetracycline-modulated promoter systems to derive stable producer cell lines capable of substantial production of VSV-G pseudotyped MLV particles. Similarly, the toxic G protein of VSV could be induced in a cell line by the removal of tetracycline and the addition of estrogen; this cell line was transduced with a modified tTA transactivator gene engineered with the ligand-binding domain of the estrogen receptor to the carboxy terminus of the tTA transactivator; a single retroviral vector could transduce both the transactivator gene and the VSV-G protein gene controlled by the tTA-inducible promoter into mammalian cells (Iida et al, 1996). The tetracycline-inducible system was modified by fusing the ligand binding domain of the estrogen receptor to the carboxy terminus of a tetracycline-regulated transactivator to regulate VSV-G expression in a tetracycline-dependent manner that could be modulated by β-estradiol in stable packaging cell lines (Chen et al, 1996).

D. Limitations and advancements using retroviral vectors

Before the in vivo gene therapy with retroviruses becomes a successful reality a number of problems must

be overcome. Despite the extensive use of retroviral vectors in gene therapy, there are still problems to be solved and there is an ultimate need for the development of new, improved retroviral vectors and packaging systems to fuel further advances in the field of human gene therapy. The principle limitation of retroviruses has been poor gene expression in vivo which has been overcome through the use of tissue-specific promoters. Use of internal ribosome entry sites from picornaviruses in retroviral vectors has provided stable expression of multiple gene enhancers (reviewed by Naviaux and Verma, 1992; Boris-Lawrie and Temin, 1993).

Little is known about the factors that influence the efficiency of retroviral infection in vivo. Many commonly used experimental animal strains, such as mice, harbor endogenous C-type proviruses, some of which are expressed and have circulating antibodies against the viral envelope glycoproteins that cross-react with the Mo-MLV; the efficiency of retrovirus-mediated transfection in vivo using a variety of mouse strains was affected by humoral immune competence and interference between endogenous MLVs and exogenous recombinant Mo-MLV (Fassati et al, 1995).

One of the drawbacks of retroviruses for their exploitation in gene therapy has been the low **viral titers** obtained, too low to achieve therapeutic levels of gene expression; methods for the efficient concentration from large volumes of supernatant and purification of amphotropic retrovirus particles have been developed in several laboratories. For example, Bowles et al (1996) have used concentration and further purification of virus particles by sucrose banding ultracentrifugation; animal studies have shown that viral transduction increased proportionally with titer of the retrovirus.

Transduced cells producing retrovirus are tissue-incompatible and are, therefore, expected to be attacked by the immune system; this will lead to the elimination of therapeutic cells from the body, a phenomenon markedly associated also with adenoviral gene transfer. A privileged exception are brain tumor cells expressing recombinant retrovirus which persist without immunologic rejection (Culver et al, 1992).

Sodium butyrate treatment of murine retrovirus packaging cells producing a CFTR vector increased the production of the retrovirus vector between 40- and 1,000-fold (Olsen and Sechelski, 1995).

The Cre/LoxP recombinase strategy (see below) has been used to generate retroviral vectors that have the ability to excise themselves after inserting a gene into the genome, thereby avoiding problems encountered with conventional retrovirus vectors, such as recombination with helper viruses or transcriptional repression of transduced genes (Russ et al, 1996). Retroviral vectors with the Cre/LoxP technology have also been used to deliver the GM-CSF gene to K562 cell culture (Fernex et al, 1997), for the development of retroviral suicide vectors for gene therapy using the HSV-tk gene (Bergemann et al, 1995), and for the production of a high-titer producer cell line containing a single LoxP site flanked by the viral LTRs (Vanin et al, 1997).

Because retrovirus vectors are integrated into the genome, transcriptional repression of transduced genes will often take place from position effects exerted from neighboring chromatin domains; two matrix-attached regions (MARs), one at either flank of the transgene, are proposed here to insulating the gene in the retrovirus vector from chromatin effects at the integration site by creating an independent realm of chromatin structure harboring the transgene. **MAR insulators** have been used and can enhance up to 2,000-fold the expression of genes in transgenic animals and plants (McKnight et al, 1992; Breyne et al, 1992; Allen et al, 1993; Brooks et al, 1994; Thompson et al, 1994; Forrester et al, 1994).

E. Targeting of retrovirus to specific cell types

A number of approaches have been directed to develop retroviral vectors that are able to target particular cell types; also efforts focus toward retroviral vectors that incorporate nonretroviral features and are tailored to desired needs for specific uses (reviewed by Vile and Russell, 1995; Gunzburg and Salmons, 1996).

Ideally, therapeutic genes should be delivered only to the relevant cell type and/or expressed in this cell type. Viral and nonviral vectors can be targeted through ligand-receptor interactions. Retroviral targeting through protease-substrate interactions has also been described; epidermal growth factor (EGF) was fused to a retroviral envelope glycoprotein via a cleavable linker comprising a factor Xa protease recognition signal. Vector particles displaying the cleavable EGF domain could bind to EGF receptors on human cells but did not transfer their genes until they were cleaved by factor Xa protease (Nilson et al, 1996).

A retroviral vector that infects human cells specifically through recognition of the low density lipoprotein receptor has been described by adding onto the ecotropic envelope protein of M-MLV a single-chain variable fragment derived from a monoclonal antibody recognizing the human LDL-R; the chimeric envelope protein was used to construct a packaging cell line producing a retroviral vector capable of transfer of the lacZ gene to human cells expressing LDL-R (Somia et al, 1995).

F. Other retroviruses

Viruses that contain RNA as their genetic material may be either negative- or positive-strand RNA viruses. The very large group of negative-strand RNA viruses includes some of the most serious and notorious pathogens subdivided into those with segmented RNA (influenza viruses, comprising eight separate segments of RNA and bunyaviruses containing three segments of single-stranded RNA, the large, L, the medium, M, and the small, S) and those with nonsegmented RNA (VSV, rabies, measles,

Sendai, respiratory syncytial virus, Ebola viruses). Positive-strand RNA viruses include poliovirus.

Cloned positive-strand poliovirus cDNA is infectious but neither isolated genome nor antigenome RNA of negative-strand viruses is infectious; this is because the negative-strand viral RNA is assembled with viral nucleoprotein into an RNP complex that becomes the template for the viral RNA-dependent RNA polymerase. Helper influenza virus-dependent procedures have been developed in which an influenza virus-like RNA molecule, containing a reporter gene, was mixed with disrupted virion core proteins to reconstitute RNP complexes in vitro which were then transfected into influenza virus-transfected cells. Recombinant nucleocapsid and polymerase proteins for the unsegmented RNA viruses have also been used to produce infectious virus without help from an homologous virus using full-length cDNA clones of intracellularly transcribed antigenomes (rabies, VSV, measles, Sendai) (see Bridgen and Elliott, 1996 and the references cited therein).

Plasmids containing full-length cDNA copies of the three RNA genome molecules of Bunyamwera bunyavirus and a negative-sense copy of the GFP gene, flanked by T7 promoter and hepatitis delta virus ribozyme sequences, were used to produce infectious virus particles without helper virus; these plasmids were used to transfect HeLa cells which expressed T7 RNA polymerase and recombinant Bunyamwera bunyavirus proteins by previous transfection with the appropriate plasmids; 24 h after infection about 1 in 1,000 HeLa cells displayed fluorescence indicative of transcription and replication of the reporter RNA (Bridgen and Elliott, 1996).

III. Adenoviral gene delivery

A. Adenovirus replication, transcription, and attachment to the nuclear matrix

Before understanding the principle of adenoviral gene transfer, it is essential to comprehend the molecular events which are involved in the life cycle of the adenovirus. Adenoviruses posses a well-defined origin of replication which is stimulated by transcription factors NFI and NFIII (Hay, 1985; Pruijn et al, 1986). The transcription factor NF-I (also called CTF, CCAAT box-binding protein, or C/EBP) stimulates replication of adenovirus DNA in vitro (Pruijn et al, 1986; Jones et al, 1987; Santoro et al, 1988; Coenjaerts et al, 1991) by establishing cooperative interactions with **Ad-DBP** (Adenovirus DNA-binding protein) (Cleat and Hay, 1989). The transcription factor NFIII (also called Oct-1 or OTF-1), involved in the regulation of the histone H2B and immunoglobulin genes, can stimulate initiation of adenovirus DNA replication *in vitro* (O'Neil et al., 1988; Mul et al, 1990; Verrijzer et al, 1990; Coenjaerts et al, 1991).

The adenovirus 5 protein Ad-DBP is a single-stranded DNA binding protein product of the viral E2A absolutely required for chain elongation during Ad5 DNA replication; other than facilitating unwinding of the DNA, Ad-DBP might also protect single-stranded DNA at the replication fork from nuclease attack, increase the rate of processivity of the viral DNA polymerase, and increase binding of NFI of the core origin of Ad5 (Cleat and Hay 1989). This protein has a size of 529 amino acids, is phosphorylated and apart from its role in DNA replication is also involved in transcription, recombination, transformation, and virus assembly (see Tucker et al 1994). Crystal structure at 2.6 A resolution of Ad-DBP shows that a 17 aa C-terminal domain hooks onto a second Ad-DBP molecule thus promoting its cooperativity during DNA binding; Ad DBP was proposed to act by forming a core around which single-stranded DNA winds (Tucker et al, 1994).

Adenoviruses replicate episomally; they need to attach to the nuclear matrix of the host cell for their replication. Two adenoviral proteins have been found attached to the nuclear matrix and presumably mediating the anchorage of the adenovirus: **(i)** the E1a protein (11 kDa), a transcription and replication factor sufficient to immortalize primary rodent cells, which was crosslinked to matrix proteins with oxidation with o-phenanthroline/Cu^{2+} (Chatterjee and Flint, 1986) and **(ii)** the adenovirus terminal protein (55 kDa) which is covalently attached to the 5' end of Ad DNA and initiates DNA replication; the adenovirus terminal protein mediated adenovirus anchorage to nuclear matrix was resistant to 1M guanidine extraction (Bodnar et al, 1989; Schaack et al, 1990; Fredman and Engler, 1993).

Three types of internal matrix structures were recognized in HeLa cells infected with adenovirus 2; an amorphously dense region; granular regions representing virus capsid assembly structures; and filaments connecting these regions to one another and to the peripheral lamina (Zhonghe et al, 1987); the perinuclear matrix was also rearranged after adenovirus infection.

Electron micrographs of thin sections through nuclei of adenovirus-infected HeLa cells showed that the ^{3}H-deoxyuridine grains were located at the periphery as well as in the interior of nuclei. Simultaneous visualization of adenovirus transcription and replication showed that the two processes occurred in adjacent, yet distinct, foci throughout the interior and periphery of nuclei presumably in association with the nuclear matrix; DNA molecules were found to be displaced from the replication foci and to become spread in the surrounding nucleoplasm serving as templates for transcription (Pombo et al, 1994).

Adenovirus infection provokes dramatic rearrangements to the nuclear matrix. A reorganization in both internal and peripheral NM was also observed in HeLa cells after infection with adenovirus 2 giving structures able to support the increased replication demands and capsid assembly of the virus (Zhonghe et al, 1987). Splicing of adenoviral HnRNA takes place on the nuclear matrix. All adenovirus 2 polyadenylated RNAs could be UV crosslinked to two host HnRNP proteins that are involved in the association of HnRNA to the matrix (Mariman et al, 1982).

Adenovirus establishes foci called replication centers within the nucleus, where adenoviral replication and transcription occur; although the rAAV genome was faintly detectable in a perinuclear distribution after successfully entering the cell, AAV was mobilized to the adenovirus replication centers when the cell was infected with adenovirus; thus AAV colocalizes with the adenovirus replication centers (Weitzman et al, 1996).

B. Adenovirus E1A and E1B proteins in apoptosis and control of the host cell cycle

Viruses have developed strategies to shut down protein synthesis in the host and subdue its protein synthesizing machinery to produce progeny virus when infecting cells. In response, many cell types commit suicide after viral infection to protect the organism from further infection. Striking back, viruses have evolved mechanisms to prevent infected cells from perishing using mechanisms that inhibit apoptosis of the host cell; adenoviruses synthesize the 19 kDa E1B protein which has a domain similar to that of the cellular protein Bcl-2, the apoptosis inhibitor (Sarnow et al, 1982; van den Heuvel et al., 1990). p53 can be complexed with adenovirus E1B (Sarnow et al, 1982; van den Heuvel et al., 1990).

Expression of the adenovirus E1A protein stimulates host DNA synthesis and induces apoptosis; on the contrary E1B 19 kDa associates with Bax protein and inhibits apoptosis (**Figure 1**). The E1A oncogene of adenovirus exerts its effect via p53 protein (Debbas and White, 1993; White, 1993). Indeed, expression of E1A increases the half-life of p53 resulting in accumulation of p53 molecules in adenovirus-infected cells leading to apoptosis. Although induction of host DNA synthesis by E1A provides a suitable environment for virus replication, the induction of apoptosis by the same protein impairs virus production since virus-infected cells are eliminated (see Han et al, 1996 for references). p53-deficient cells are transformed by E1A because of absence of the pathway for induction of apoptosis by p53 (Lowe et al, 1994).

E1A represses HER-2/neu transcription and functions as a tumor suppressor gene in HER-2/neu-overexpressing cancer cells. Transfer the E1A gene into cancer cells that overexpress HER-2/neu is an interesting aspect of gene therapy (see E1A in gene therapy; Yu et al, 1995; Chang et al, 1996; Ueno NT et al, 1997; Rodriguez et al, 1997; Xing et al, 1997).

The E1B oncogene products inhibit apoptosis induced by E1A expression thus preventing premature death of host cells during adenovirus infection. This gives an advantage to virus for its proliferation and E1B proteins (19 kDa and 55 kDa) are necessary for transformation of primary rodent cells by E1A. E1A alone is unable to transform primary rodent cells (White, 1993).

The E1B 19K protein of adenovirus is the putative viral homolog of the cellular Bcl-2 protein; using the yeast two-hybrid system for the identification of proteins interacting with E1B, Han and coworkers (1996) have identified Bax as one of the seven 19k-interacting clones. The 50-78 amino acid domain of Bax contains a conserved region homologous to Bcl-2 which is able to interact specifically with either Bcl-2 or E1B. In p53 mutant cells expression of Bax induced apoptosis; inhibition of apoptosis by Bcl-2 may proceed via its ability to bind the death-promoting Bax protein (Han et al, 1996). The *bax* gene is upregulated by p53.

Expression of p53 and of adenovirus E1A induce apoptosis (Debbas and White, 1993; Lowe and Rudley, 1993). A number of proteins when expressed at sufficient amounts block apoptosis; these include Bcl-2 and E1B 19 kDa protein of adenovirus (Debbas and White, 1993; Chiou et al, 1994). All four protein molecules act upstream of Bax which is a potent inducer of apoptosis: both the cellular Bcl-2 and the 19 kDa protein E1B of adenovirus are able to interact with Bax inhibiting its involvement in induction of apoptosis (Han et al, 1996; **Figure 1**). E1A acts upstream of p53 by increasing the half-life of p53 resulting in an accumulation of p53 molecules in the nucleus (Lowe and Ruley, 1993); increased levels of p53 are then believed to upregulate the *bax* gene.

The transcription factor E2F was originally identified as an activator of the adenovirus E2 gene and is implicated in the regulation of DNA replication (Shirodkar et al., 1992). Following infection of cells with adenovirus, the DNA binding activity of E2F increases and as a consequence transcription of the E2 gene of adenovirus increases (Kovesdi et al., 1987). These changes in E2F are mediated by E1A protein of adenovirus. RB forms specific complexes with E2F keeping E2F in a form unable to upregulate its target regulatory sequences. E2F can form specific complexes also with cyclin A during S-phase in NIH 3T3 cells (Mudryj et al., 1991). Both types of complexes, E2F-RB and E2F-cyclin A, can be dissociated by the adenovirus E1A protein (Chellappan et al., 1991; Bagchi et al., 1990; reviewed by White, 1998 this volume) but also by phosphorylation of RB at G1/S causing release of E2F and stimulation in transcription of genes required for DNA replication (*myc*, *DHFR*). These events contribute to the uncontrolled proliferation of adenovirus-transformed cells (Mudryj et al., 1990, 1991). Release of E2F from RB induced by E1A is critical for transformation of cells by E1A (for references see Hiebert et al, 1995).

C. Strategies of adenoviruses to enter the cell

In order to enter the host cell the adenovirus first attaches with a high affinity to a cell surface receptor, whose nature still remains elusive, using the head domains of the protruding viral fibers; the fibronectin-binding integrin on the cell surface then associates with the penton base protein on the adenovirus triggering endocytosis of the virus particle via coated pits and coated vesicles (Svensson and Persson, 1984; Greber et al, 1996). The third step in adenovirus entry into the host cell includes

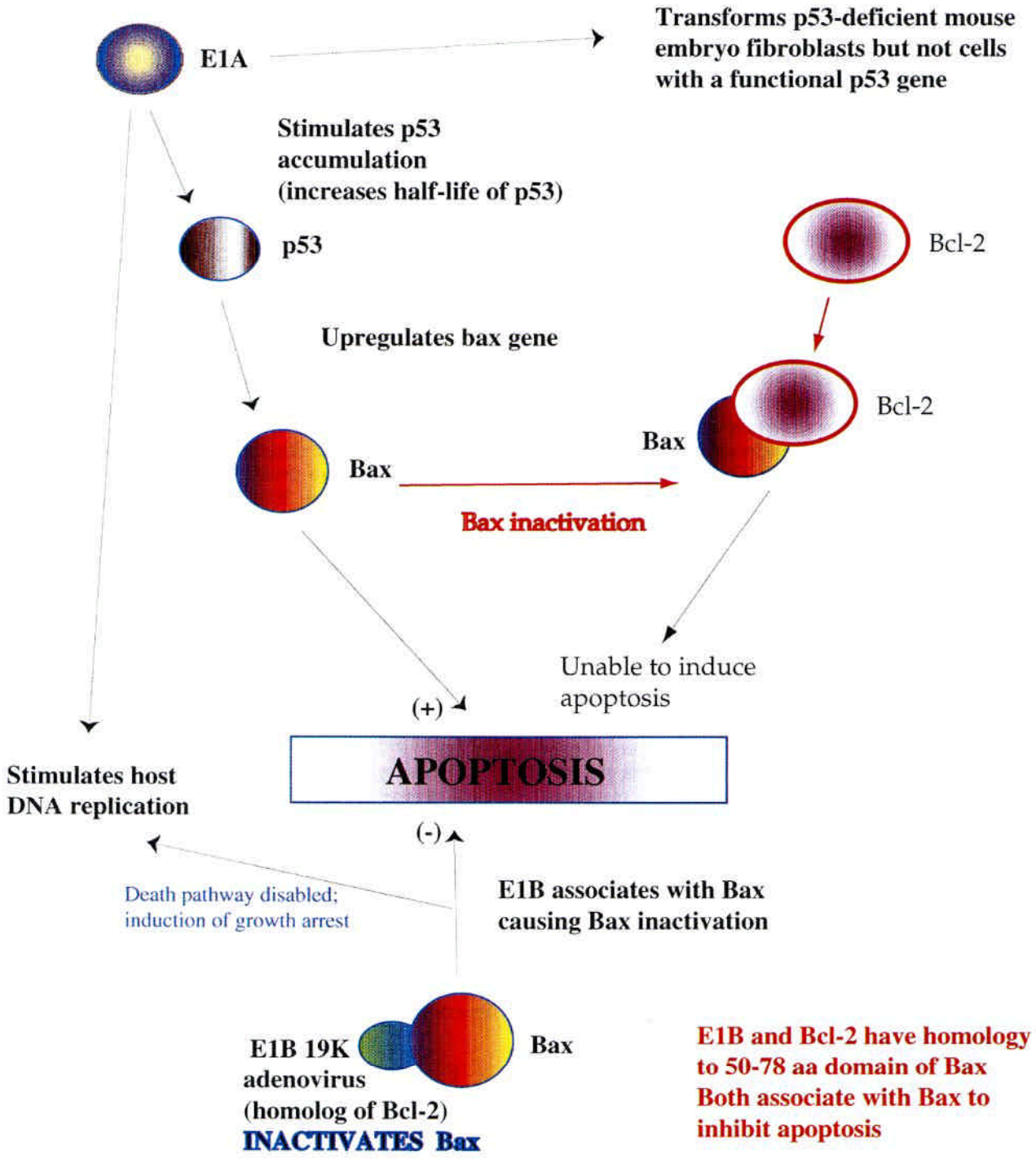


Figure 1. Role of E1A and E1B19-kDa proteins of adenovirus in apoptosis.

penetration of the adenoviral particles by acid-catalyzed rupture of the endosomal membrane involving the penton protein and the integrins and allowing escape to the cytoplasmic compartment; a decrease in endosome pH during internalization expose hydrophobic domains of these adenoviral capsid proteins which permits these proteins to insert into the vesicle membrane in a fashion that ultimately disrupts its integrity (Seth et al, 1984). At the final step the adenoviral particle is attached to the cytoplasmic side of pore complexes and the DNA is released to the interior of pore annuli entering the nucleoplasm.

These highly ordered processes are accompanied by losses or protease degradation of specific proteins on the viral particles; the fibers and some of the penton base complexes on the adenovirus surface are already lost during the process of endocytosis; a viral protease, L3/p23, located inside the capsid at 10 copies per virion, plays a key role in the stepwise dismantling and in the weakening of the capsid structure culminating with the release of the adenovirus DNA by degrading of the viral capsid protein VI (Greber et al, 1996). The mechanism of disruption of endosomes by the adenoviral particles has been exploited to augment efficiency of transfection with transferrin-polylysine-DNA complexes (see fusogenic peptides and Curiel et al, 1991; Cotten et al, 1992; Wagner et al, 1992b; Cristiano et al, 1993; Morishita et al, 1993; Harries et al, 1993; Curiel, 1994).

To overcome one of the major limitations to the clinical utility of adenoviruses which is the low efficiency of gene transfer achieved in vivo, Arcasoy et al (1997) found that the presence of the polycations polybrene, protamine, DEAE-dextran, and poly-L-lysine significantly increased the transfection efficiency in cell culture using the lacZ gene; because the polyanion heparin did not significantly alter gene transfer efficiency, but completely abrogated the effects of polycations it supports the idea that the negative charges presented by membrane glycoproteins reduce the efficiency of adenovirus-mediated gene transfer, an obstacle that can be overcome by polycations.

D. Advantages and drawbacks of adenoviral vectors in gene delivery

Adenoviruses possess a linear double-stranded genome which can be manipulated to accommodate up to 7.5 kb of DNA. Adenoviruses have the advantage of being able to infect nondividing cells. Other advantages are the rarity of recombination events between adenoviral vectors and the host chromosomes, the absence of induction of human malignancies by adenoviruses, and the relative safety of their use as vaccines (e.g. Ballay et al, 1985; Haj-Ahmad and Graham, 1986). For safety, replication-deficient, infectious adenoviruses are being used in somatic gene transfer; for example deletion in a portion of the E3 region of the virus permits encapsidation whereas deletion of a portion of the *E1A* coding sequence impairs viral replication (Gilardi et al, 1990; Rosenfeld et al, 1991).

E. Deletion of adenoviral DNA sequences for gene delivery

First generation recombinant adenoviruses were rendered defective by deletion of sequences spanning the E1A and E1B genes; these adenoviruses expressed low levels of early and late viral genes responsible for activating destructive cellular immune responses. Further deletion of other essential genes and growth in new packaging cell lines or incorporation of temperature sensitive mutations which allow propagation of the virus in available packaging cell lines at permissive temperatures are strategies for improving the therapeutic efficacy of recombinant adenoviruses and for minimizing the immune response elicited to the host (Fisher et al, 1996).

E1-defective, recombinant adenoviruses can be replication-enabled by the codelivery of a plasmid encoding the deleted E1 functions, a strategy now designated as “conditional replication-enablement system for adenovirus” (CRESA); when the original replication-enabling plasmid was replaced by two separate plasmids that encoded the necessary E1A and E1B functions the E1-defective adenovirus could become conditionally replication-enabled by an RNA transcript encoding the required E1 functions. The RNA transcript of E1A enhanced the therapeutic efficacy of the E1-defective adenovirus: subcutaneous human tumor nodules containing a fraction of cells cotransduced with the replication-enabling RNA + DNA and an HSV-tk adenovirus were reduced to a greater extent than control nodules generated from the same fraction of cells cotransduced with the HSV-tk adenovirus and an irrelevant plasmid (Dion et al, 1996).

A new type of recombinant adenovirus, (called delta-rAd), deprived of all viral open reading frames and retaining only the essential cis elements (i.e., ITRs and contiguous packaging sequence), was propagated in 293 cells in the presence of E1-deleted helper virus (Fisher et al, 1996). This adenovirus was packaged as concatamers into virions and was used to deliver successfully the CFTR gene to human airway epithelial cells in culture derived from a cystic fibrosis patient. The new delivery system needs improvements in its production and purification to allow its evaluation and use in vivo.

F. Immune response to adenoviruses eliminate therapeutic cells

Adenoviruses can achieve high levels of gene transfer (Haffe et al, 1992; Morsy et al, 1993; Herz and Gerard; Wilson, 1995; Kozarsky et al, 1996). However, the duration of transgene expression is limited **(i)** by clearance of the infected cells because of the cellular and humoral immune response (including those mediated by cytotoxic T lymphocytes) to adenoviral antigens (Yang Y

et al, 1994, 1995) and **(ii)** by loss of adenoviral episomes in progeny cells (Feng et al, 1997). To circumvent this problem adenoviral/retroviral chimeric vectors were constructed where the nonintegrative adenoviral vector was able to induce target cells to function as transient retrovirus producer cells and the retroviral particles were able to transduce neighboring cells; thus the recombinant adenovirus became integrative via the intermediate generation of a retroviral producing cell (Feng et al, 1997). First generation adenovirus-mediated gene transfer of CFTR to the mouse lung resulted in the expression of viral proteins leading to the elimination of the therapeutic cells expressing CFTR by cellular immune responses and repopulation of the lung with nontransgene containing cells; second generation E1-deleted viruses, also crippled by a temperature sensitive mutation in the E2A gene, displayed substantially longer recombinant gene expression and induced a lower inflammatory response (Yang et al, 1994).

In order to circumvent the elimination of adenovirus-transduced cells by immune responses and for achieving persistence of transgene expression strategies to reduce the potential for viral gene expression have been developed; for example, an E4 modified adenovirus which was replication defective in cotton rats and displayed a reduced potential for viral gene expression in vivo was engineered (Armentano et al, 1997). Vectors containing a wild-type E4 region, E4 open reading frame 6, or a complete E4 deletion were compared in the lungs of BALB/c mice for persistence of CFTR or lacZ expression; expression was transient from the E1a promoter with all vectors but persisted from the CMV promoter only with a vector containing a wild-type E4 region; thus, transient expression from adenoviral vectors may result from the down-regulation of a promoter and not necessarily from immune response-related factors (Armentano et al, 1997).

The elimination of therapeutically important cells from the body after recombinant adenovirus-mediated delivery seems to be a great limiting factor for the use of adenoviruses in long-term gene therapy (Dai et al, 1995). This problem can be partially circumvented by daily administration of the immunosupressant cyclosporin A prohibiting the elimination of virally-transduced cell by activated T lymphocytes (Fang et al, 1995). A different approach to suppress elimination of therapeutically-transduced cells after intra-articular delivery of genes to treat RA is by pretreatment of the joints with the anti-T cell receptor monoclonal antibody H57, a treatment which resulted in a significant reduction in lymphocytic infiltration and a persistence of transgene expression (Sawchuk et al, 1996).

The prokaryotic Cre-LoxP recombination system was adapted to generate recombinant adenoviruses with extended deletions in the viral genome in order to minimize expression of immunogenic and/or cytotoxic viral proteins. An adenovirus was produced with a 25-kb deletion that lacked E1, E2, E3, and late gene expression; this vector exhibited viral titers similar to those achieved with first-generation (E1a-deleted) vectors which was efficient for gene transfer to cell culture but gene expression declined to undetectable levels much more rapidly than that sustained from first-generation vectors. Vectors deleted only at E1a were sustaining a better reporter gene expression because of their ability to replicate (Lieber et al, 1996).

A clinical protocol proposed recently for the therapy of amyotrophic lateral sclerosis uses a semipermeable membrane to enclose the *ex vivo* modified xenogenic BKH cells which is implanted intrathecally to provide human ciliary neurotrophic factor; the membrane prevents immunologic rejection of the cells interposing a virus impermeable barrier between the transduced cells and the host (Deglon et al, 1996; Pochon et al, 1996); the method has been applied before for cross-species transplantation of a polymer-encapsulated dopamine-secreting cell line to treat Parkinson's disease and for the delivery of nerve growth factor in rat and primate models of the Alzheimer's disease (Kordower et al, 1994; see Pochon et al, 1996 for more references). Evidently, similar approaches could be used to protect adenovirus- and retrovirus-transduced syngeneic cells from immunologic rejection provided that the therapeutic protein is secreted.

A new area of investigation is directed toward surface modification of recombinant adenoviruses to render them safer and to minimize the strong immune responses against the virus and virus-infected cells; to this end Fender et al (1997) proposed a dodecahedron made of adenovirus pentons or penton bases and having only one or two adenovirus proteins instead of the 11 contained in an adenovirus virion; the penton is a complex of two oligomeric proteins, a penton base and fiber, involved in the cell attachment, internalization, and liberation of virus from endosomes.

It is certain that great improvements in adenoviral gene delivery will solve many of the current problems and permit a higher therapeutic efficacy in the near future.

G. Examples of adenoviral gene transfer

Recombinant adenovirus vectors have been used: for the transfer of factor IX gene in hemophilia B dogs via vein injection (Kay et al, 1994) and in mice (Smith et al, 1993); for the transfer of genes into neurons and glia in the brain (le Gal la Salle, 1993); for the transfer of the gene of ornithine transcarmylase in deficient mouse and human hepatocytes (Morsy et al, 1993); for the transfer of the VLDL receptor gene for treatment of familial hypercholesterolaemia in the mouse model (Kozarsky et al, 1996); for the transfer of low density lipoprotein receptor gene in normal mice (Herz and Gerard, 1993); and for the ex vivo transduction of T cells from ADA-deficient patients (Blaese et al, 1995; Bordignon et al, 1995). The adenovirus major late promoter was linked to a human α1-antitrypsin gene for its transfer to lung epithelia of cotton rat respiratory pathway as a model for the treatment of α1-antitrypsin deficiency; both in vitro and in vivo infections

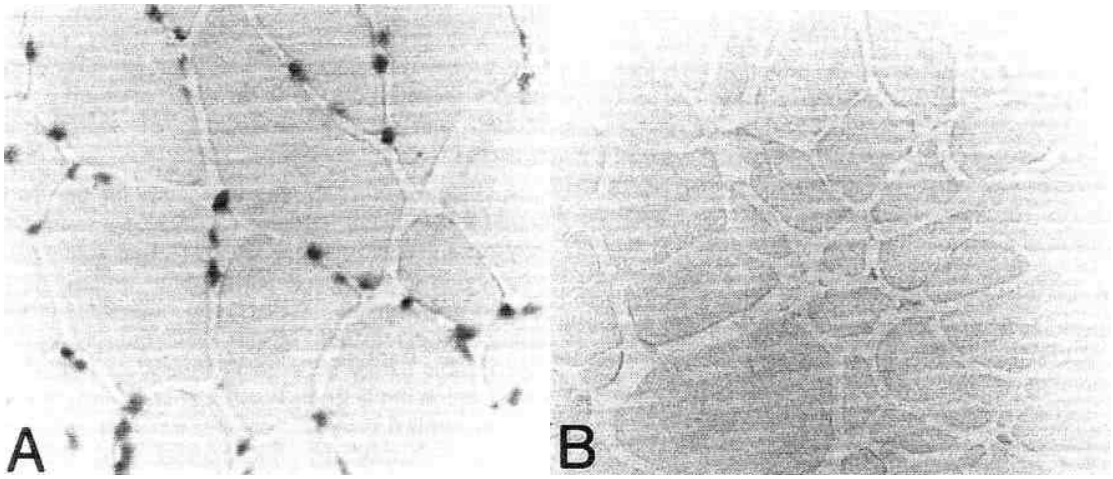
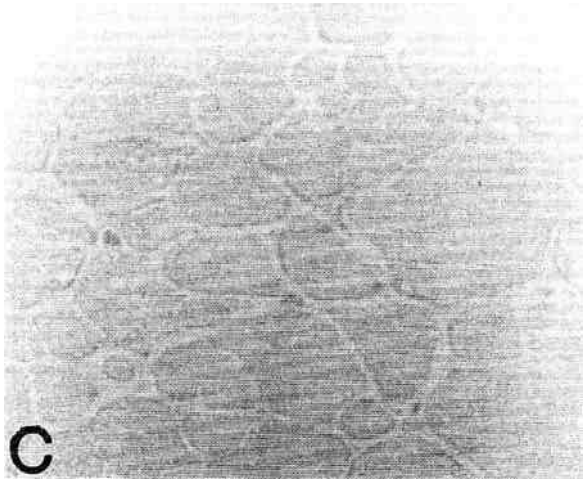


Figure 2. Localization of a recombinant adenoviral vector carrying 6.3 kb of dystrophin cDNA by in situ PCR following intramuscular injection to immunosuppressed mdx mice. Shown are transverse cryostat sections of mdx tibialis anterior muscle. Panel **A** shows a strong in situ hybridization signal (an E4 adenoviral sequence was amplified and an E4 probe was used) in myonuclei of an immunosuppressed animal injected with E1, E3-deleted adenovirus at 30 days postinjection (magnification 650x). Panel **B** was produced without Taq polymerase during PCR as a negative control. Panel **C** shows an uninjected muscle processed as described in panel A showing no hybridization signal. From Zhao JE, Lochumuller H, Nalbantoglu J, Allen C, Prescott S, Massie B, Karpati G (**1997**) Study of adenovirus-mediated dystrophin minigene transfer to skeletal muscle by combined microscopic display of adenoviral DNA and dystrophin. **Hum Gene Ther** 8, 1565-1573. With kind permission of the authors (George Karpati, Montreal Neurological Institute, Canada) and Mary Ann Liebert, Inc.



have shown production and secretion of α1-antitrypsin by the lung cells (Rosenfeld et al, 1991).

A transductional preference of adenovirus-polylysine-DNA complexes and E1A/B-deleted replication-deficient adenoviruses was demonstrated for the prostate carcinoma cell lines DU145, LNCaP, and PC-3 over primary human bone marrow cells and the leukemia cell line KG-1; this finding led to a strategy to purge bone marrow of a specific subset of prostate carcinoma cells (Kim et al, 1997).

Figure 2 shows the localization of a recombinant adenoviral vector carrying 6.3 kb of dystrophin cDNA, driven by the CMV promoter, by in situ PCR following intramuscular injection to immunosuppressed mdx mice. **Figure 3**. shows a comparison of the persistence of dystrophin expression and adenoviral genomes in immunosuppressed versus immunocompetent mdx mice. The maximum number of fibers containing recombinant adenovirus was maintained until 60 days in immunosuppressed mice but for only 10 days in immunocompetent animals. Thus, optimization of immunosuppression could assure successful long term dystrophin gene transfer for gene therapy of Duchenne muscular dystrophy (Zhao et al, 1997).

A number of RAC-approved protocols for gene transfer to humans use recombinant adenoviruses (**Appendix 1,** protocols 118-157). Genes transferred to patients with recombinant adenoviruses include p53 (#130, 131, 147, 148, 152-156), RB (#140), CFTR (#118-123, 125, 128, 129), HSV-tk (126, 127, 132, 136, 139, 141, 143, 145, 146), cytosine deaminase (#134, 151), VEGF (#157), IL-2 (#135), GM-CSF (#149, 150), anti-erbB-2 single chain antibody (#133), ornithine transcarbamylase (#137), and GP100 melanoma antigen (#142).

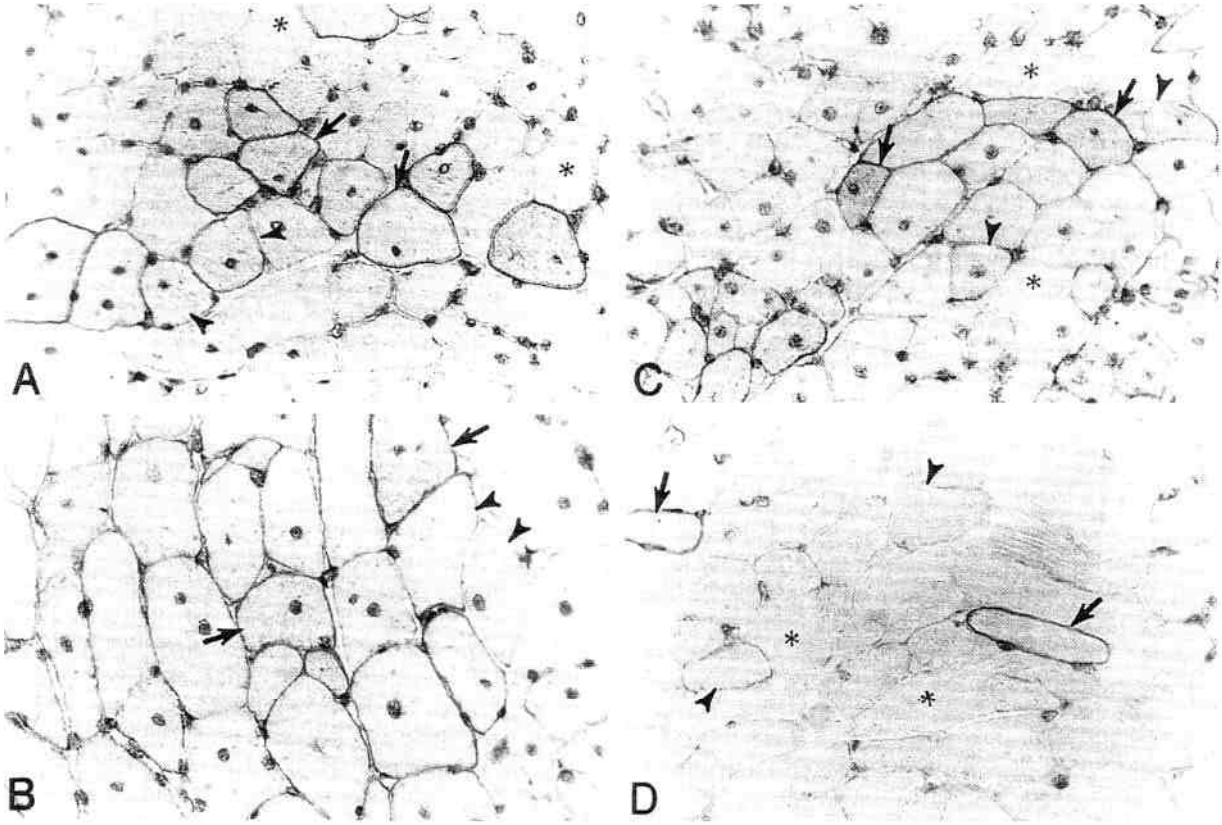


Figure 3. Comparison of the persistence of dystrophin expression and adenoviral genomes in immunosuppressed versus immunocompetent mdx mice. Shown are combined dystrophin immunostaining and in situ PCR in tibialis anterior muscles of mdx mice at 10 days (**A** and **C**) and 60 days (**B** and **D**) postinjection. In A and B, FK506 was used as an immunosuppressant, whereas in C and D no immunosuppression was employed. At 10 days there was no significant difference in adenovirus positive nuclei (arrows) fibers between the immunosuppressed and the immunocompetent groups. At 60 days, however, there was a dramatic decline in the number of positive nuclei in the immunocompetent muscle. Magnification 650X. From Zhao JE, Lochumuller H, Nalbantoglu J, Allen C, Prescott S, Massie B, Karpati G (**1997**) Study of adenovirus-mediated dystrophin minigene transfer to skeletal muscle by combined microscopic display of adenoviral DNA and dystrophin. **Hum Gene Ther** 8, 1565-1573. With kind permission of the authors (George Karpati, Montreal Neurological Institute, Canada) and Mary Ann Liebert, Inc.

IV. Gene delivery with Adeno-Associated Virus (AAV)

A. Replication of AAV and rAAV: the role of the inverted terminal repeats

AAVs are replication-defective parvoviruses, not associated with any human disease (nonpathogenic), requiring cotransfection with a helper virus to produce infectious virus particles; they can replicate in cell culture only in the presence of coinfection with adenovirus or herpes virus. Five serotypes of distinct AAV isolates have been recovered from human and other primates. AAV infections in humans are asymptomatic acquired with other viral infections such as adenovirus or HSV infections; 80-90% of adults are seropositive for antibodies against AAV (for references see Clark et al, 1995; Berns and Linden, 1995).

The replication of the AAV is dependent on two copies of a 145-bp inverted terminal repeat (ITR) sequence that flanks the AAV genome which is the primary cis-acting element required for productive infection and the generation of recombinant AAV (rAAV) vectors.

In the absence of helper virus, the AAV particle can penetrate cells and find its way to the cell nucleus where the linear genome is uncoated and becomes integrated at a specific site on chromosome 19q13.3; several copies of AAV may integrate in tandem arrays. Thus, the AAV establishes a latent infection; the integrated viral genome can be activated and rescued by superinfection with helper virus (either adenovirus or any type of herpes virus). Inverted repeats at the ends of the viral DNA serve for the integration appearing near the junctions with cellular DNA sequences (Bohenzky et al, 1988).

Adenovirus establishes foci called replication centers within the nucleus, where adenoviral replication and transcription occur; AAV was colocalized with the adenovirus replication centers using in situ hybridization and immunocytochemistry; AAV may, thus, utilize adenovirus and cellular proteins for its own replication; the rAAV genome was faintly detectable in a perinuclear distribution after successfully entering the cell; however, rAAV was mobilized to replication centers when the cell was subsequently infected with adenovirus (Weitzman et al, 1996).

Xiao et al (1997) have engineered the pDD-2 plasmid containing two copies of the D element, a unique sequence adjacent to the AAV nicking site, flanking a single ITR (a total of only 165 bp of AAV sequence); this modified hairpin was sufficient to sustain replication of the plasmid vector when Rep and adenovirus helper functions were supplied in trans. This plasmid has a significant prospect in gene transfer because is replicated more efficiently than infectious AAV clones; as a prelude to its replication the input circular plasmid was converted into a linear substrate by resolution of the AAV terminal repeat through a Holliday-like structure, a process most likely mediated by host factors. Linear monomer, dimer, and other higher-molecular-weight replicative intermediates were generated during the replication of pDD-2, a feature characteristic of AAV replication. The replicative intermediates of this plasmid substrate were competent for AAV DNA replication, encapsidation, infection, integration, and subsequent rescue from the chromosome when superinfected with Ad and wild-type AAV (Xiao et al, 1997). The elucidation of the important role of this 165-bp ITR sequence for AAV replication and the entire life cycle invigorates the important role of inverted repeats at the origin of replication not only of viruses but also of cellular origins of replication (Boulikas, 1996e).

B. Packaging capabilities of AAVs

AAVs posses a 4.7 kb single-stranded DNA genome. Hermonat et al (1997) have examined the maximum amount of DNA which can be inserted into the wild-type AAV genome without compromising packaging into an infectious virus particle; the maximum effective packaging capacity of AAV, examined as increments of 100 bp ligated at map unit 96 of AAV, is approximately 900 bp larger than wild type. Thus, wtAAV therapy vectors can be generated carrying a foreign gene of 900 bp or less with the advantages of wtAAV such as the ease in which high titers of infectious virus can be generated and the ability to specifically integrate in chromosome 19.

On the contrary, the payload capacity of recombinant AAV, which has been deprived of its viral genes and bears only the ITRs is in the order of 4.5-4.7 kb; this means that a cDNA up to this size can be inserted into a rAAV; for example the size of the CFTR cDNA is 4.5 kb and thus, the combined length of the promoter that drives CFTR expression and ITRs needs to be kept under 500 bp (Dong et al, 1996).

Similar results were reported by Dong et al (1996) who have estimated that the optimal size of AAV vector is between 4.1 and 4.9 kb; the packaging efficiencies were sharply reduced above 5.2 kb and below 4.1 kb; two copies of the vector were packaged into each virion when vectors of 2.2-2.5 kb were provided.

C. Integration of wtAAV but not of rAAV is site-specific

Wild-type AAV is able to undergo targeted integration on chromosome 19 after infection in 15 out of 22 clones examined (Kotin et al, 1990, 1992). Of 51 integrations examined by fluorescence in situ hybridization (FISH) 48 (94%) were to chromosome 19 after infection of IB3-1 bronchial epithelial cells with wild-type AAV (Kearns et al, 1996). Site-specific integration has been reported for other viruses including avian leukosis virus (ALV) integrating adjacent to cellular oncogenes in tumors; however, the mechanism of ALV integration involves a process of selection of cells able to form tumors by overexpression of the oncogene due to virus integration rather than exclusive integration of the ALV at unique sites of the genome (Hayward et al, 1981). RSV also appears to be integrated at a limited number of sites (Shih et al, 1988). Adenovirus integration, a more rare event compared to the majority of episomal molecules, may also occur at a number of preferred sites (Jessberger et al, 1989). A larger number of recombinase molecules than those known today may be present in mammalian cell nuclei and promote site-specific integration and recombination events.

Although the human wild-type AAV (**wtAAV**) is unique in its ability to target viral integration to a specific site on chromosome 19, the recombinant AAV (**rAAV**) vectors have lost the site-specific integration and targeting ability; furthermore, rAAVs have incapacitated ability to integrate, and can be found as episomes. When wtAAV-2 was used to infect IB3-1 bronchial epithelial cells all metaphase spreads examined by fluorescence in situ hybridization (FISH) had integrated copies and 94% of the integrations were to chromosome 19; furthermore, 36 of 56 metaphase spreads had a single copy of wtAAV integrated and 20 of 56 showed two sites within chromosome 19 (Kearns et al, 1996). On the contrary, when a recombinant AAV containing the CFTR cDNA was used to infect the same cells, examination of 67 metaphase chromosome spreads identified four integrations (only 6% of total) to different chromosomes. No integration was to chromosome 19. When these studies were repeated on the A35 epithelial cell line selected for stable CFTR expression, the episomal AAV-CFTR sequences were abundant in the low molecular weight DNA fraction (Kearns et al, 1996).

Yang et al (1997) have cloned over 40 AAV and rAAV integration junctions to determine the terminal-repeat

sequences that mediate integration. These studies have shown that in both immortalized and normal diploid human cells, wt AAV targeted integration to chromosome 19 in head-to-tail tandem arrays; the majority of the junction sequences were involving incomplete copies of the AAV inverted terminal repeats (ITRs); inversions of genomic and/or viral DNA sequences at the wt integration site took place. The viral integration event was found to be mediated by terminal repeat hairpin structures and cellular recombination pathways. In contrast, rAAV provirus integrated on chromosome 2 and at the same locus in two independent cell lines, in both the flip and flop orientations; genomic rearrangements took place at the integration site of rAAV, mainly involving deletions and/or rearrangement-translocations.

Similar data were reported by Rutledge and Russell (1997): recombinant AAV vectors were found to be integrated by nonhomologous recombination as single-copy proviruses in HeLa cells and at random chromosomal locations; the recombination junctions were scattered throughout the vector terminal repeats with no apparent site specificity; the flanking HeLa DNA at integration sites was not homologous to AAV or to the site-specific integration locus of wild-type AAV. Furthermore, vector proviruses with nearly intact terminal repeats were excised from the genomic HeLa DNA and were amplified after infection of cells with wild-type AAV and adenovirus.

The integration patterns of four recombinant AAV-2 genomes in individual clonal isolates of the human nasopharyngeal carcinoma cell line (KB) were different; the difference between the recombinant AAV-2 genomes were in the combinations of the genes for resistance to tetracycline, to neomycin, to ampicillin, with the genes for AAV replication, and the AAV capsid genes. None of the KB cell clones examined had the proviral genome covalently linked to the specific-site of integration of the wt AAV on chromosome 19 (Ponnazhagan et al, 1997a,b).

D. Drawbacks of AAV in gene therapy and their remedy

Gene transfer with AAV vectors has typically been low. Difficulties in generating recombinant virions on a large scale sufficient for preclinical and clinical trials and in obtaining high-titer virus stocks after the initial transfection into producer cells is a limiting factor for the widespread usage of AAV vectors; this obstacle is expected to be overcome in the near future. The high viral titers required for preclinical and clinical studies have been achieved by a new strategy developed by Tamayose et al (1996); AAV vector particles in cell lysates could be concentrated by sulfonated cellulose column chromatography to a titer higher than 10^8 cfu/ml or 5 x 10^{10} particles/ml. A method for transfecting cells at extremely high efficiency with a rAAV vector and complementation plasmid while simultaneously infecting those cells with replication competent adenovirus using adenovirus-polylysine-DNA complexes has been developed by Mamounas et al (1995).

The difficulties in developing packaging cell lines for AAV relate to low levels of rep gene expression from the AAV-p5 promoter and to the propensity of Rep proteins to suppress continued growth of immortalized cell lines; expression of AAV rep under control of the LTR of the human HIV together with the development of cell populations containing rescuable AAV recombinant genomes increased 50-fold the packaging efficiency of AAV vectors (Flotte et al, 1995).

After infection of cell cultures with recombinant AAV there is a decline in the percentage of cells expressing the transferred gene with time in culture. This decline was associated with ongoing losses of vector genomes (Malik et al, 1997). For example, transfer to cultures of K562 human erythroleukemia cells of a truncated rat nerve growth factor receptor (tNGFR) cDNA as a cell surface reporter under control of the LTR of the Moloney murine leukemia virus showed that about 30% of cells expressed tNGFR on the surface early after transduction at a multiplicity of infection (MOI) of 13 infectious units (IU), which declined to 3% over 1 month of culture. At an MOI of 130 IU, nearly all cells expressed tNGFR immediately and the proportion of cells expressing tNGFR declined to 62% over 2 months of culture (Malik et al, 1997).

Another obstacle of rAAV vectors is the low rate of integration of rAAV into the host genome (which can be improved at high MOI). The efficiency of integration was about 2% at low MOI (1.3 IU) and increased to at about 49% at an MOI of 130 (Malik et al, 1997).

E. Advantages using AAV and improvements in AAV gene delivery

AAV does not elicit an immune reaction and is a nonpathogenic virus to humans. AAV contains normally a single-stranded copy of its genome. Transduction with AAV can be enhanced in the presence of adenovirus gene products through the formation of double stranded, non-integrated AAV genomes.

AAV has been reported to have advantages over other viruses for gene transfer to hematopoietic stem cells due to their high titers and relative lack of dependence on cell cycle for target cell integration. A robust CMV/LacZ reporter gene expression in primary human $CD34^+CD2^-$ progenitor cells induced to undergo T-cell differentiation was obtained without toxicity or alteration in the pattern of T-cell differentiation. 70% to 80% of the cells isolated from either adult bone marrow or umbilical cord blood were efficiently transduced with AAV; however, the expression was transient without integration; this limits the potential use of AAV in gene therapy strategies for diseases such as AIDS (Gardner et al, 1997).

Gene transduction by AAV vectors in cell culture can be stimulated over 100-fold by treatment of the target cells with agents that affect DNA metabolism, such as irradiation or topoisomerase inhibitors (Russell et al, 1995); great improvements in transduction efficiency can also be achieved in vivo: previous γ-irradiation increased

the transduction rate in mouse liver by up to 900-fold, and the topoisomerase inhibitor etoposide increased transduction by about 20-fold after direct liver injection or after systemic delivery via tail vein injection; up to 3% of hepatocytes could be transduced after a single systemic vector injection (Koeberl et al, 1997). This is a significant advantage compared to stealth liposomes which , although concentrating in the liver, spleen and tumors can transduce Kupffer cells but not hepatocytes after systemic delivery (Martin and Boulikas, 1998, following article).

A combination of the adenovirus-5 capsid protein or the Fiber protein of adenovirus with liposomes, termed adenosomes (adenovirus protein-cationic liposome complexes) improved the efficiency of gene transfer. This complex was able to mediate efficient transfer of a AAV/CMV-LacZ construct to endothelial cells (Zhou et al, 1995).

Clark et al (1996) have developed a sensitive assay system to determine infectivity of AAV vectors based on the replication of input rAAV genomes rather than transgene expression which depends on the type of promoter which drives the foreign gene; this system uses a cell line that expresses AAV helper functions (rep and cap) upon induction by adenovirus infection.

F. Examples using AAV for gene transfer

AAV will infect a broad number of mammalian cell lines and has been used as a cloning vector to transduce the *Neo*^{R} gene into mammalian tissue culture cells (Hermonat and Muzyczka 1984). Antisense AAV vectors have been used to inhibit HIV replication (Chatterjee et al, 1992), and to correct Fanconi's anemia in human hematopoietic cells (Walsh et al, 1994). AAVs transduce preferentially cells in S phase; topoisomerase inhibitors increase transduction efficiency (Russell et al, 1995).

Using AAV, the genomic copy of a normal human β-globin gene under control of the DNase l-hypersensitive site 2 (HS-2) from the locus control region was expressed in K562 human erythroleukemia cells, which normally lack the β-globin gene; following selection with G418 by virtue of the neo-resistance function which was provided in the rAAV vector, stable integration of the exogenous β-globin allele was determined (Zhou et al, 1996). Similar data were reported by Einerhand et al (1995) transferring a recombinant AAV-vector containing a human β-globin gene together with the DNase1 hypersensitive sites 4, 3 and 2 of the human β-globin locus control region as an approach for the gene therapy of β-thalassemia and sickle cell anemia. The vector replicated to high titers and could efficiently transduce hematopoietic stem cells isolated from patients. In order to treat sickle cell anemia Lubovy et al (1996) have transferred lacZ with a recombinant AAV vector and stably transduced hematopoietic stem cells purified from normal and homozygous sickle cell anemia patients.

AAV was able to promote delivery of functional levels of glial cell line-derived neurotrophic factor (GDNF), in a degenerative model of Parkinson's disease (Mandel et al, 1997). AAV has also been used for the transduction of the mouse liver in vivo with Factor IX cDNA as a prelude to treatment of hemophiliacs (Snyder et al, 1997; Herzog et al, 1997) and for the human intratracheal instillation of CFTR cDNA into neonatal New Zealand white rabbits (Rubenstein et al, 1997), and to the lungs of rhesus macaques without eliciting inflammation (Conrad et al, 1996).

AAV has also been used for the transfer of the human multidrug resistance gene (hMDR1) cDNA to NIH-3T3 cells followed by selection of successfully transfected cells based on the drug-resistant phenotype conferred by the P-glycoprotein efflux pump (see below and Lee et al, 1997, this volume); integration of MDR1 sequences into the host cell genome was demonstrated by fluorescent in situ hybridization (FISH) but also the persistence of nonintegrated AAV-MDR1 episomal plasmids (Baudard et al, 1996).

Introduction of a human globin gene into murine hematopoietic bone marrow cells ex vivo with a recombinant AAV vector followed by transplantation of these cells into lethally irradiated congenic mice sustained a long-term repopulating ability: human globin gene sequences were detected in the bone marrow and spleen in primary recipient mice for at least 6 months.

Kessler et al (1996) have shown that following a single intramuscular administration of a recombinant adeno-associated virus (rAAV) vector, carrying either the lacZ or the human erythropoietin gene into adult BALB/c mice leads to the local production of the foreign protein in the muscle for at least 32 weeks; furthermore, human erythropoietin was secreted and stimulated red blood cell production in the mouse for up to 40 weeks. This finding was extended by Fisher et al (1997) who arrived to the unexpected finding that intramuscular injection of highly purified recombinant AAV can sustain a high level of transgene expression in the absence of adenovirus after direct injection to the muscle in mice (**Figure 4**); this expands the potential of AAV for the treatment of inherited and acquired diseases. Using this approach no humoral or cellular immune responses were elicited after transfer of lacZ against the neoantigenic *E. coli* β-galactosidase. The rAAV genome was integrated at single sites as head-to-tail concatamers into nuclei of differentiated muscle fibers. Transfer of the lacZ gene using a highly purified preparation of AAV which was injected into the skeletal muscle of adult mice in the presence of E2a-deleted adenovirus to enhance transduction followed by direct visualization of the β-galactosidase by X-gal histochemistry revealed high transduction of muscle fibers by day 17 associated with inflammation (**Fig 4a and b**). Animals that received the same AAVlacZ in the absence of adenovirus demonstrated higher levels of transduction that persisted for 240 days (**Fig 4c-h**).

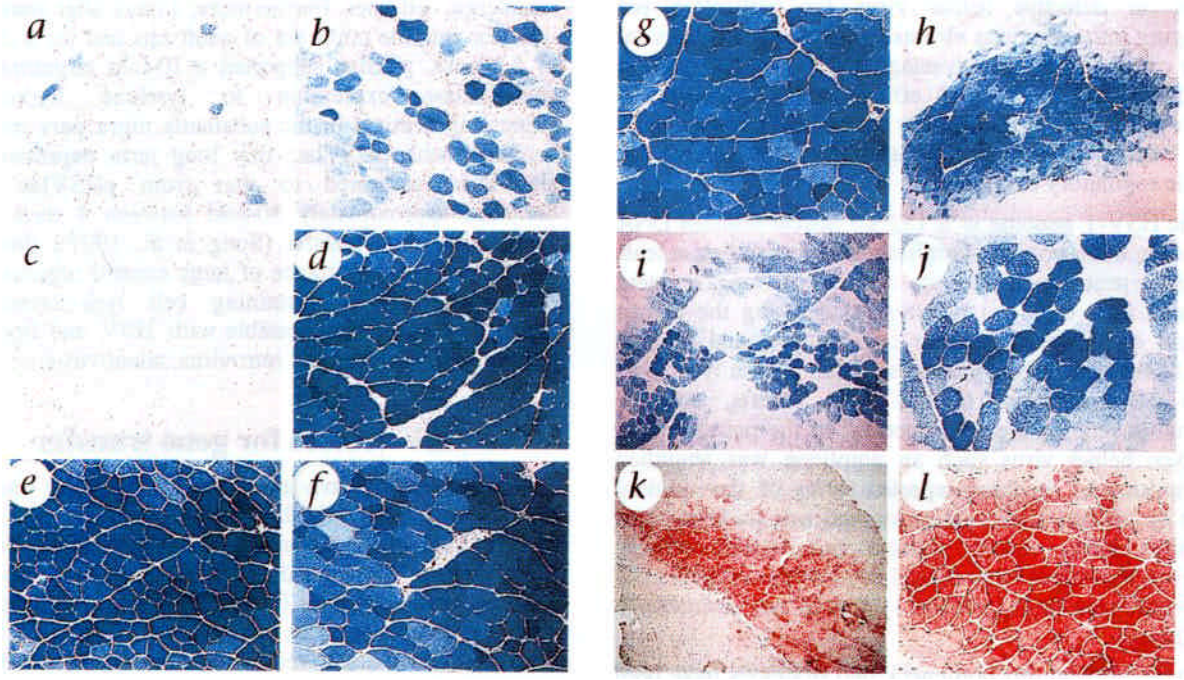


Figure 4. Purified recombinant AAV-mediated lacZ gene transfer to the muscle in adult mice sustains a high level of expression and is inflammation-free. Purified AAVlacZ ($1x10^9$ genomes in 25 µl) was injected into the tibialis anterior of 5-week-old C57BL/6 mice and tissue was harvested at days 3 (**c**), 17 (**d**), 30 (**e**), 64 (**f**), and 180 (**g, h**) post-injection and analyzed by X-gal histochemistry. Samples of AAVlacZ ($1x10^9$ genomes in 25 µl) were also supplemented with an E2a mutant adenovirus dl802 ($5x10^{10}$ A_{260} particles) just prior to injection and tissue was harvested at days 3 (**a**) and 17 (**b**) post-injection. Magnification: a-g, X10; h, X5.

Purified AAVlacZ (175 µl , $1x10^{12}$ genomes/ml) was injected into the tibialis anterior of a male rhesus monkey. Biopsies were taken 14 days post-injection and frozen sections were cut and stained for β-galactosidase activity (**i** and **j**); magnification: I, X5; j, X10.

k and **l**: rAAV vector expressing human β-glucoronidase was injected into the tibialis anterior of 5-week-old C57BL/6 mice ($1x10^9$ genomes in 25 µl). 30 days post-injection the muscle was harvested and frozen sections were cut and stained for β-glucoronidase. From Fisher KJ, Jooss K, Alston J, Yang Y, Haecker SE, High K, Pathak R, Raper SE, Wilson JM (**1997**) Recombinant adeno-associated virus for muscle directed gene therapy. **Nat Med** 3, 306-312. Reproduced with the kind permission of the authors and Nature America, Inc.

AAV-mediated delivery of the lacZ gene by direct injection to brain tumors which were induced from human glioma cells in nude mice showed that 30-40% of the cells along the needle track expressed β-galactosidase; subsequent delivery of the HSV-tk/IL-2 genes to these tumors with AAV and administration of GCV to the animals for 6 days resulted in a 35-fold reduction in the mean volume of tumors compared with controls by a significant contribution from the bystander effect (Okada et al, 1996).

A phase I clinical trial for CF is being conducted at Johns Hopkins Hospital using AAV (see Kearns et al, 1996, and protocols #165, 166 in **Appendix 1**).

V. Herpes Simplex Virus-1 (HSV-1) and miniviral vectors

HSV-1 has a capacity of inserting up to 30 kb of exogenous DNA which is a clear advantage over the adenovirus (up to 7.5 kb of exogenous DNA). High titer viral stocks can be prepared from HSV-1. HSV-1 also displays a wide range of host cells and can infect nonreplicating cells such as neuron cells in which the vectors can be maintained indefinitely in a latent state. However, infection with HSV-1 is cytotoxic to cells because of residual viral proteins produced by the virus. Strategies to circumvent this drawback led to the development of viral vectors with a very large capacity for insertion (almost as large as the size of the virus) which depend on defective helper virus for replication and

packaging into infectious virions (see below). A miniviral vector can combine the advantage of cloning the gene in bacterial plasmids, the high efficiency of virus-mediated gene transfer, and the possibility to transfer large genomic DNA fragments including far upstream, downstream and intronic regulatory elements.

The HSV-1 genome is a 152 kb double-stranded DNA containing three origins of replication and encoding at least 72 unique proteins; it consists of a unique long segment replicated from oriL and two repeats flanking the unique segment each replicated from oriS. Spaete and Frenkel (1982) have constructed plasmids containing the lytic viral origin of replication, foreign DNA inserts, and the terminal packaging signal sequences; in the presence of a wild-type helper virus such an amplicon was amplified into multimeric tandemly-repeated forms of the original vector by rolling-circle replication and was packaged into infectious HSV virions (Spaete and Frenkel, 1982). However, the helper virus caused death of the infected cells due to lytic replication and this system is not amenable to gene therapy.

To circumvent this bottleneck two strategies have been developed leading to replication-defective helper HSV: **(i)** a temperature-sensitive system permitted production of virion stocks at 31^{o} C whereas infection of cells at 37^{o} C caused inactivation of the helper virus which was incapable of entering the lytic cycle and allowed delivery of the miniviral vector to the target cell without causing its death. **(ii)** In a different system, the immediately early gene *IE3* was deleted from the helper virus; *IE3* encodes for a protein (ICP4) essential for early and late viral gene expression and replication; the helper cell line used for packaging had a genomic insertion of the *IE3* gene of HSV which was functionally expressed allowing for complementation and for lytic infection using the *IE3*-defective HSV virus (DeLuca and Schaffer, 1987; Geller and Freese, 1990).

Two types of viral vectors have been used for gene transfer to cancer cells: replication-incompetent vectors expressing a gene product that leads to the destruction of the tumor or replication-competent vectors that are inherently cytotoxic to the tumor cells. In order to combine the two modes of action Miyatake et al (1997) used a defective HSV vector that consisted of a defective particle, containing tandem repeats of the HSV-tk gene, and a replication-competent, non-neurovirulent HSV mutant as a helper virus. When glioma GL261 cells were infected with the tk-defective vector/helper virus the HSV-TK activity was significantly higher than that in helper virus-infected cells which contained a single copy of HSV-tk; subcutaneous injection of these cells to C57BL/6 mice inducing gliomas led to a significant decrease in tumor size after GCV treatment.

An HSV-1 vector containing a 6.8-kb fragment of the rat tyrosine hydroxylase promoter (pTHlac) supported a seven- to 20-fold increase in reporter gene expression in catecholaminergic cell lines compared to noncatecholaminergic cell lines. Furthermore, 4 days after stereotactic injection into the midbrain of adult rats and for a duration of 6 weeks, pTHlac supported a 10-fold targeting of β-galactosidase expression to tyrosine hydroxylase-expressing neurons in the substantia nigra pars compacta compared with pHSVlac; this long term expression was significant compared to that from pHSVlac which decreased approximately 30-fold between 4 days and 6 weeks after gene transfer (Song et al, 1997); this study also shows the importance of large control regions in the order of 7 kb in sustaining cell type-correct gene expression, something feasible with HSV and liposomes but nor with recombinant retrovirus, adenovirus, or AAV.

VI. HIV vectors for gene transfer

Recent studies have succeeded in exploiting the deadly HIV-1 virus, after crippling some functions, as a gene delivery vehicle. An advantage of HIV vectors has been the broad range of tissues and cell types they can transduce, a property granted because lentiviral vectors are pseudotyped with vesicular stomatitis virus G glycoprotein. Human lentiviral (HIV)-based vectors can transduce non-dividing cells in vitro and deliver genes in vivo; expression of transgenes in the brain has been detected for more than six months. HIV vectors have been also used to introduce genes directly into liver and muscle; 3-4% of the total liver tissue was transduced by a single injection of 1-3 x 10^{7} infectious units (I.U.) of recombinant HIV with no inflammation or recruitment of lymphocytes at the site of injection. Whereas expression of green fluorescent protein (GFP), used as a surrogate for therapeutic protein, was observed for more than 22 weeks in the liver and for over 8 weeks in the muscle using lentiviral vectors, little or no GFP could be detected in liver or muscle transduced with the Moloney murine leukemia virus (Mo-MLV), a prototypic retroviral vector (Kafri et al, 1997).

The development of a stable noninfectious HIV-1 packaging cell line capable of generating high-titer HIV-1 vectors is another important step towards use of HIV vectors in gene therapy (Corbeau et al, 1996). A hybrid murine leukemia virus-based vector containing U3 and R sequences from HIV-1 in place of the MLV U3 and R regions gave single transcriptional unit retroviral vectors under the control of Tat; this vector has advantages for anti-HIV gene therapy (Cannon et al, 1996).

Although replication-incompetent HIV vectors displayed a strict $CD4^{+}$ T cell tropism for gene transfer, a feature important for AIDS therapy, it was thought to preclude HIV-based vectors for other gene transfer applications; a two-step gene transfer system, however, was developed to expand the host range of the HIV vector: in the first step, the CD4 gene was introduced into target cells using a replication-defective adenoviral vector; in the second step the CD4-transfected cells were incubated with HIV vectors which resulted in stable integration and HIV-mediated gene transfer (Miyake et al, 1996).

An HIV multiply attenuated vector in which the virulence genes env, vif, vpr, vpu, and nef were deleted

was able to deliver genes in vivo into adult neurons (Zufferey et al, 1997).

HIV-mediated gene transfer was used to transfer the GFP gene under control of CMV to retinal cells by injection into the subretinal space of eyes in rats; the GFP gene was efficiently expressed in both photoreceptor cells and retinal pigment epithelium; predominant expression in photoreceptor cells was achieved using the rhodopsin promoter. The transduction efficiency was high and photoreceptor cells in >80% of the area of whole retina were expressing GFP (Miyoshi et al, 1997).

VII. Epstein-Barr virus (EBV) and baculovirus vectors

EBV is an episomaly-replicating virus in synchrony with the cell cycle. EBV infects human cells causing mononucleosis; the presence of the unique latent origin of replication (oriP) in EBV allows for episomal replication of the virus in human cells without entering the lytic cycle. The presence of oriP and of the replication initiator protein EBNA1 cDNA on a vector allows episomal replication in human cells; in addition, plasmids containing only oriP can replicate episomally into cell lines expressing EBNA-1 (Sun et al, 1994; Banerjee et al, 1995).

A hybrid HSV-1/EBV vector has been developed by Wang and Vos (1996), which combines **(i)** the HSV-1 lytic oriS; **(ii)** an HSV-1 packaging sequence which allows replication and packaging in the presence of defective helper virus carrying a deletion in the *IE3* gene in the E5 cell line expressing the *IE3* gene; **(iii)** the latent oriP of EBV and **(iv)** the *EBNA-1* cDNA allow episomal replication of the infectious vector in the E5 cell line so that viral stocks of high titer can be made. Infection of tumor-derived fibroblast and epithelial cell lines in culture and local injection of human liver tumors in nude mice was used to demonstrate 95-99% efficiency of infection and transfer of the reporter β-galactosidase gene.

Genetically modified baculoviruses (Autographa californica nuclear polyhedrosis virus) were used to efficiently deliver genes into cultured hepatocytes of different origin; delivery into human hepatocytes with baculovirus vectors approached 100% efficiency in cell culture and expression levels were high when mammalian promoters were chosen. A number of drawbacks preclude their direct application in vivo; nevertheless gene transfer was feasible in ex vivo perfused human liver tissue (Sandig et al, 1996; Hofmann et al, 1998 this volume).

VIII. Liposomal gene delivery

Abbreviations:

DC-CHOL: 3β [N-(N',N'-dimethylaminoethane)carbamoyl]cholesterol

DDAB: dimethyldioctadecyl ammonium bromide

DMRIE: N-[1-(2,3-dimyristyloxy)propyl]-N,N-dimethyl-N-(2-hydroxyethyl) ammonium bromide

DMTAP: 1,2-dimyristoyl-3-trimethylammonium propane

DOGS: Dioctadecylamidoglycylspermine (Transfectam, Promega)

DOPE: dioleyl phosphatidylethanolamine

DOSPA: 2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl -1-propanaminium trifluoroacetate

DOTAP: N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride

DOTMA: N-[1-(2,3-dioleyloxy) propyl]-n,n,n-trimethylammonium chloride

DPTAP: 1,2- dipalmitoyl-3-trimethylammonium propane

DSTAP: 1,2-disteroyl-3-trimethylammonium propane

Lipofectin: DOTMA:DOPE 1:1 (GIBCO BRL)

A. Immune responses and toxicity of cationic lipid-DNA complexes

Cationic lipids have been widely used for gene transfer; a number of clinical trials (34 out of 220 total RAC-approved protocols as of December 1997) use cationic lipids (see **Table 4** in Martin and Boulikas, 1998, this volume, pages 203-206). Although many cell culture studies have been documented, systemic delivery of genes with cationic lipids in vivo has been very limited. All clinical protocols use subcutaneous, intradermal, intratumoral, and intracranial injection as well as intranasal, intrapleural, or aerosol administration but not i.v. delivery because of the toxicity of the cationic lipids and DOPE (see **Table 4** in Martin and Boulikas, 1998, this volume, pages 203-206).

Liposomes formulated from DOPE and cationic lipids based on diacyltrimethylammonium propane (dioleoyl-, dimyristoyl-, dipalmitoyl-, disteroyl-trimethylammonium propane or DOTAP, DMTAP, DPTAP, DSTAP, respectively) or DDAB were highly toxic when incubated in vitro with phagocytic cells (macrophages and U937 cells), but not towards non-phagocytic T lymphocytes; the rank order of toxicity was DOPE/DDAB > DOPE/DOTAP > DOPE/DMTAP > DOPE/DPTAP > DOPE/DSTAP; the toxicity was determined from the effect of the cationic liposomes on the synthesis of nitric oxide (NO) and TNF-α produced by activated macrophages (Filion and Phillips, 1997).

Another factor to be considered before i.v. injections are undertaken is that negatively charged serum proteins can interact and cause inactivation of cationic liposomes (Yang and Huang, 1997). Condensing agents used for plasmid delivery including polylysine, transferrin-polylysine, a fifth-generation poly(amidoamine) (PAMAM) dendrimer, poly(ethyleneimine), and several cationic lipids (DOTAP, DC-Chol/DOPE, DOGS/DOPE, and DOTMA/DOPE) were found to activate the complement system to varying extents. Strong complement activation was seen with long-chain polylysines, the dendrimer, poly(ethyleneimine), and DOGS; complement activation was considerably reduced by modifying the surface of preformed DNA complexes with polyethyleneglycol (Plank et al, 1996).

B. Mechanism of liposome entry to cells

Cationic lipids increase the transfection efficiency by destabilizing the biological membranes including plasma, endosomal, and lysosomal membranes; indeed, incubation of isolated lysosomes with low concentrations of DOTAP caused a striking increase in free activity of β-galactosidase, and even a release of the enzyme into the medium demonstrating that lysosomal membrane is deeply destabilized by the lipid; the mechanism of destabilization was thought to involve an interaction between cationic liposomes and anionic lipids of the lysosomal membrane, allowing a fusion between the lipid bilayers; the process was less pronounced at pH 5 than at pH 7.4 and anionic amphipathic lipids were able to prevent partially this membrane destabilization (Wattiaux et al, 1997).

In contrast to DOTAP and DMRIE which were 100% charged at pH 7.4, DC-CHOL was only about 50% charged as monitored by a pH-sensitive fluorophore; this difference decreases the charge on the external surfaces of the liposomes and was proposed to promote an easier dissociation of bilayers containing DC-CHOL from the plasmid DNA and an increase in release of the DNA-lipid complex into the cytosol from the endosomes (Zuidam and Barenholz, 1997).

C. Tissue targets using cationic liposomes in vivo

Although cationic lipids have been used widely for the delivery of genes very few studies have used systemic i.v. injection of cationic liposome-plasmid complexes because of the toxicity of the lipid component and certainly in animal models, not humans. Administration by i.v. injection of two types of cationic lipids of similar structure, DOTMA and DOTAP, has shown that the transfection efficiency was determined mainly by the structure of the cationic lipid and the ratio of cationic lipid to DNA; the luciferase and GFP gene expression in different organs was transient, with a peak level between 4 and 24 hr, dropping to less than 1% of the peak level by day 4 (Song et al, 1997).

Figure 5 shows the effect of cationic lipid:DNA ratio on transfection efficiency after i.v. tail injection. Luciferase activity was detected in all organs examined with the highest level in lung. In the absence of neutral lipid both DOTMA and DOTAP promoted a linear increase in luciferase activity in the lung with increasing lipid:DNA from 12:1 to 36:1 nmol lipid: μg of DNA. DOTMA was 10 times more efficient than DOTAP (10^6 versus 10^7 relative luciferase units (RLU) per mg protein. Cholesterol (Chol) mixed with DOTMA (1:1 molar ratio) decreased the level of gene expression in the lung whereas cholesterol did not affect the transfection efficiency of DOTAP liposomes. Inclusion of DOPE into either DOTAP or DOTMA liposomes significantly decreased the transfection efficiency by 100-fold in the lung.

When a group of four cationic lipids with identical head group but of different fatty acyl chains were tested for their transfection efficiencies (**Figure 6**); these included DOTAP, DMTAP, DPTAP, and DSTAP. The C_{14} acyl chain-lipid DMTAP had a similar transfection efficiency as DOTAP which has 18 carbon atoms in the acyl chain and one double bond ($C_{18}\Delta_9$); on the contrary, the transfection efficiencies of DPTAP (C_{16}) was 10-100 fold lower and that of DSTAP (C_{18}) was 100 to 1000 fold lower.

Confocal microscopy of lung tissue after injection of 25 μg pCMV-GFP plasmid DNA complexed with DOTMA liposomes to mice (**Figure 7**) has shown that the type of cells that express the transgene are the endothelial cells that have typical characteristics of neighboring multiple air-sac structures (**Figure 7D**).

A number of different organs in vivo can be targeted after liposomal delivery of genes or oligonucleotides. Intravenous injection of cationic liposome-plasmid complexes by tail vein in mice targeted mainly the lung and to a smaller extend the liver, spleen, heart, kidney and other organs (Zhu et al, 1993). Intraperitoneal injection of a plasmid-liposome complex expressing antisense K-ras RNA in nude mice inoculated i.p. with AsPC-1 pancreatic cancer cells harboring K-ras point mutations and PCR analysis indicated that the injected DNA was delivered to various organs except brain (Aoki et al, 1995).

A number of factors for DOTAP:cholesterol/DNA complex preparation including the DNA:liposome ratio, mild sonication, heating, and extrusion were found to be crucial for improved systemic delivery; maximal gene expression was obtained when a homogeneous population of DNA:liposome complexes between 200 to 450 nm in size were used. Cryo-electron microscopy showed that the DNA was condensed on the interior of invaginated liposomes between two lipid bilayers in these formulations, a factor that was thought to be responsible for the high transfection efficiency in vivo and for the broad tissue distribution (Templeton et al, 1997).

Steps to improve for successful liposome-mediated gene delivery to somatic cells include persistence of the plasmid in blood circulation, port of entry and transport across the cell membrane, release from endosomal compartments into the cytoplasm, nuclear import by docking through the pore complexes of the nuclear envelope, expression driven by the appropriate promoter/enhancer control elements, and persistence of the plasmid in the nucleus for long periods. A number of strategies for liposomal delivery and for enhancing the efficiency of uptake by the cells and release from endosomal compartments of plasmid or oligonucleotide DNA are reviewed in the following article (Martin and Boulikas, 1998).

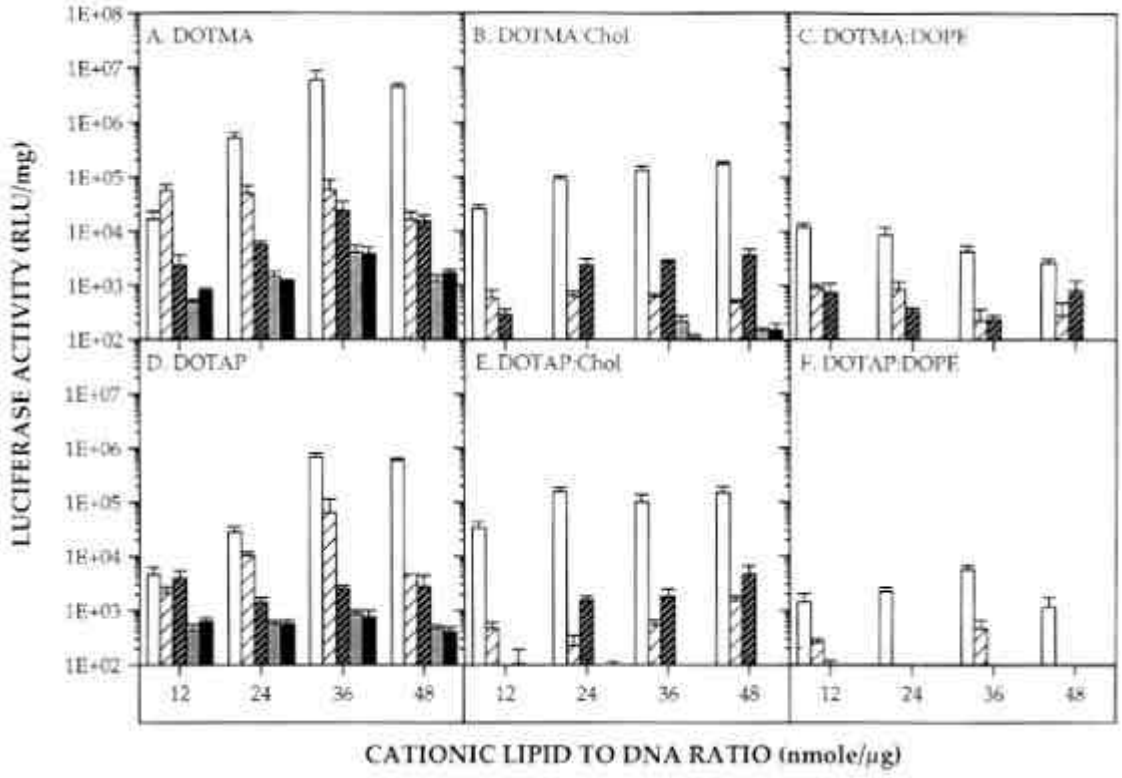
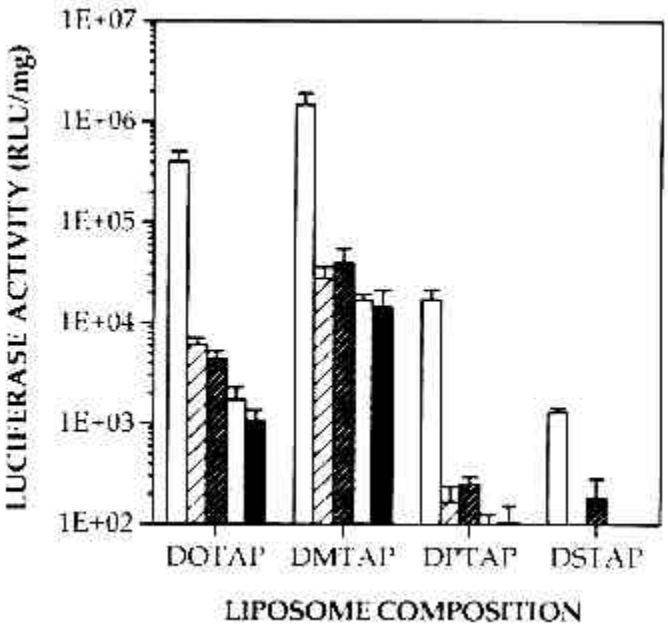


Figure 5. Effect of cationic lipid:DNA ratio on transfection efficiency after i.v. tail injection. Each mouse received 25 μg of pCMV-Luciferase plasmid DNA complexed with various amounts of liposomes indicated on the charts (at 1:1 ratio when two lipids were used). Luciferase activity was assayed 20 h after i.v. injection in up to 5 different tissues represented with different bar forms: the empty bar is lung, the large stripe bar is spleen, the small stripe is heart, gray bar is liver, and black bar is kidney. Four time points (12h, 24h, 36h, and 48h from i.v. injection) of luciferase activity are shown. Numbers +02 to +08 to the left of the figure indicates 10^2 to 10^8 relative luciferase units (RLU) per mg protein in the tissue. From Song YK, Liu F, Chu S, Liu D (**1997**) Characterization of cationic liposome-mediated gene transfer in vivo by intravenous administration. **Hum Gene Ther** 8, 1585-1594 with the kind permission of the authors (Dexi Liu, University of Pittsburgh) and Mary Ann Liebert, Inc.

Figure 6. Effect of fatty acyl chain composition on transfection efficiency. Luciferase activity was assayed 20 h post-injection in the lung, spleen, heart, liver, and kidney (in the order shown, see legend to previous figure for bar symbols). From Song YK, Liu F, Chu S, Liu D (**1997**) Characterization of cationic liposome-mediated gene transfer in vivo by intravenous administration. **Hum Gene Ther** 8, 1585-1594 with the kind permission of the authors (Dexi Liu, University of Pittsburgh) and Mary Ann Liebert, Inc.



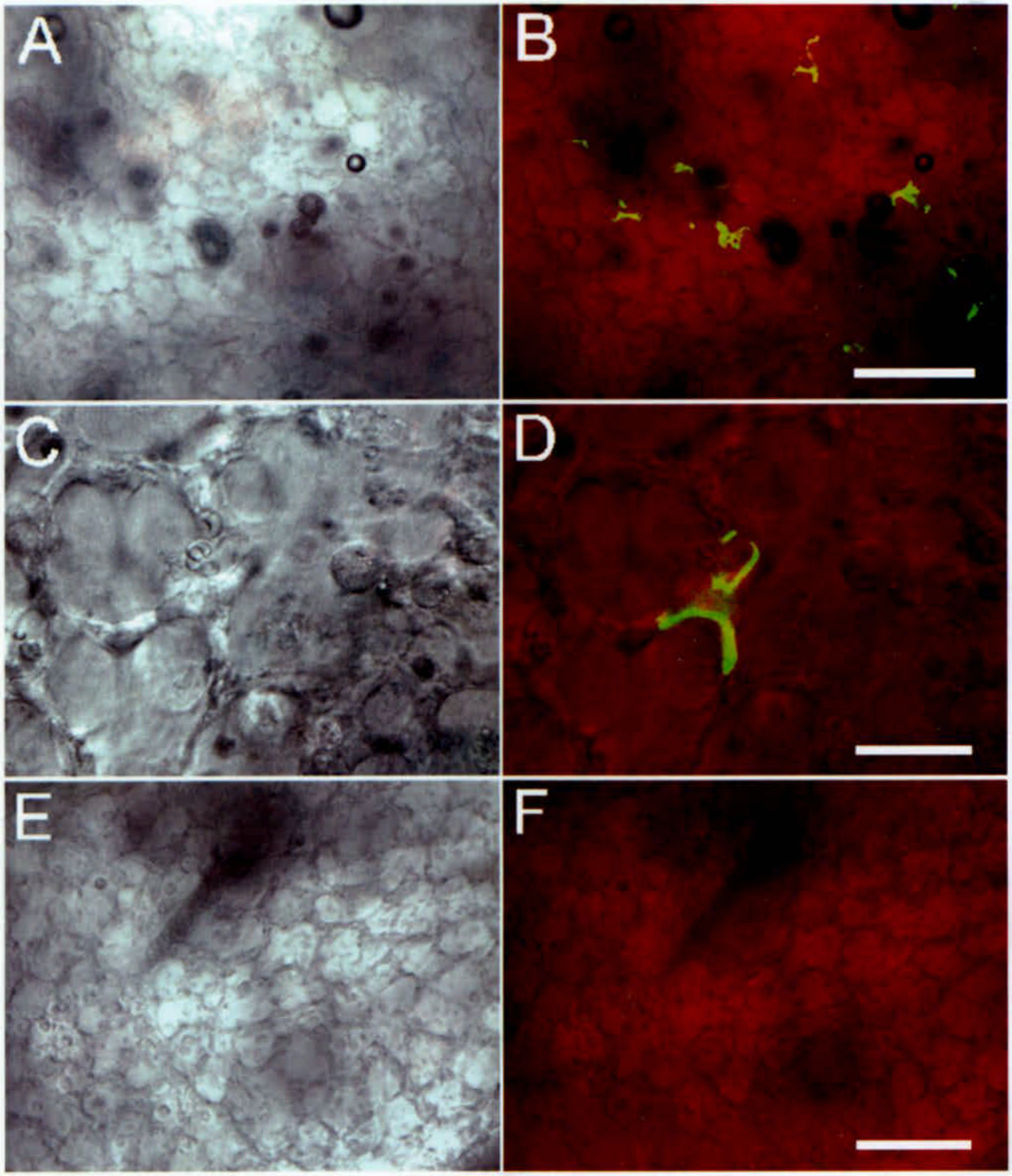


Figure 7. Analysis of green fluorescence protein (GFP) expression in the lung using confocal microscopy. 25 μg pCMV-GFP plasmid DNA complexed with DOTMA liposomes were injected to mice and GFP expression in the lung was examined 14 h post-injection . (**A**): transmitted light image and (**B**): fluorescence image (green) were observed at low magnification (Bar 100 μm). **C** and **D** are the images obtained at higher magnification showing the localization of GFP in endothelial cells (Bar 25 μm). **E** and **F** are the images from control animals injected with pCMV-Luc plasmid rather than GFP plasmid. From Song YK, Liu F, Chu S, Liu D (**1997**) Characterization of cationic liposome-mediated gene transfer in vivo by intravenous administration. **Hum Gene Ther** 8, 1585-1594 with the kind permission of the authors (Dexi Liu, University of Pittsburgh) and Mary Ann Liebert, Inc.

D. Cationic lipids in oligonucleotide transfer

Encapsulation of oligonucleotides into liposomes increased their therapeutic index, prevented degradation in cultured cells and in human serum and reduced toxicity to cells (Thierry and Dritschilo, 1992; Capaccioli et al, 1993; Morishita et al, 1993; Williams et al, 1996; Lewis et al, 1996); conjugation to a fusogenic peptide enhanced the biological activity of antisense oligonucleotides (Bongartz et al, 1994). However, most studies have been performed in cell culture, and very few in animals in vivo; there is still an important number of improvements needed before these approaches can move to the clinic.

Zelphati and Szoka (1997) have found that complexes of fluorescently labeled oligonucleotides with DOTAP liposomes entered the cell using an endocytic pathway mainly involving uncoated vesicles; oligonucleotides redistributed from punctate cytoplasmic regions into the nucleus; this process was independent of acidification of the endosomal vesicles. The nuclear uptake of oligonucleotides depended on several factors such as charge of the particle where positively charged complexes were required for enhanced nuclear uptake; DOTAP increased over 100 fold the antisense activity of a specific anti-luciferase oligonucleotide. Physicochemical studies of oligonucleotide-liposome complexes of different cationic lipid compositions indicated that either phosphatidylethanolamine or negative charges on other lipids in the cell membrane are required for efficient fusion with cationic liposome-oligonucleotide complexes to promote entry to the cell (Jaaskelainen et al, 1994).

Similar results were reported by Lappalainen et al (1997); digoxigenin-labeled oligodeoxynucleotides (ODNs) complexed with the polycationic DOSPA and the monocationic DDAB (with DOPE as a helper lipid) were uptaken by CaSki cells in culture by endocytosis. The nuclear membrane was found to pose a barrier against nuclear import of ODNs which accumulated in the perinuclear area. Although DOSPA/DOPE liposomes could deliver ODNs into the cytosol, they were unable to mediate nuclear import of ODNs; on the contrary oligonucleotide-DDAB/DOPE complexes with a net positive charge were released from vesicles into the cytoplasm; it was determined that DDAB/DOPE mediated nuclear import of the oligonucleotides.

DOPE-heme (ferric protoporphyrin IX) conjugates, inserted in cationic lipid particles with DOTAP, protected oligoribonucleotides from degradation in human serum and increased oligoribonucleotide uptake into 2.2.15 human hepatoma cells; the enhancing effect of heme was evident only at a net negative charge in the particles (Takle et al, 1997). Uptake of liposomes labeled with ^{111}In and composed of DC-Chol and DOPE was primarily by liver, with some accumulation in spleen and skin and very little in the lung after i.v. tail injection; preincubation of cationic liposomes with phosphorothioate oligonucleotide induced a dramatic, yet transient, accumulation of the lipid in lung which gradually redistributed to liver. The mechanism of lung uptake involved entrapment of large aggregates of oligonucleotides within pulmonary capillaries at 15 min post-injection via embolism; labeled oligonucleotide was localized primarily to phagocytic vacuoles of Kupffer cells at 24 h post-injection; nuclear uptake of oligonucleotide in vivo was not observed (Litzinger et al, 1996).

Phosphorothioate oligonucleotides were found in most tissues 48 h after i.p. administration with highest concentrations in kidney and liver; complexation of the oligonucleotide with DOTMA did not affect neither the oligonucleotide uptake nor its tissue distribution in normal mice but increased the oligonucleotide cellular uptake (4-10 times) in LOX ascites tumors (Saijo et al, 1994).

Triplex-forming ODNs were delivered to cells in culture using adenovirus-polylysine-ODN complexes designed to take advantage of the receptor mediated endocytosis of adenoviruses to transfer the ODNs to the cell nucleus; nuclear uptake peaked at 4 h and intact ODN persisted in the nucleus with a half-life of 12 h (Ebbinghaus et al, 1996).

E. Fusogenic peptides enhance gene transfer efficiency

Enveloped viruses have evolved efficient mechanisms to release their genomes from the endosomes into the cytoplasm of the host cells; specific envelope proteins of the nucleocapsid are capable of destabilizing the endosomal membrane. Therefore, inactivated viruses have been used to enhance the transfer of plasmids. Addition of adenoviral particles capable of inducing endosome lysis (Blumenthal et al, 1986), mediated by a conformational change in the adenovirus penton protein induced at the lower pH of endosomes (Seth, 1994) can increase transfection efficiency 100-1000 fold using 10^9 adenoviral particles/ml and the transferrin receptor (Curiel et al, 1991; Cotten et al, 1992; Wagner et al, 1992b; Cristiano et al, 1993; Morishita et al, 1993; Harries et al, 1993; Curiel, 1994; reviewed by Ledley, 1995).

Use of fusogenic peptides from influenza virus hemagglutinin HA-2 enhanced greatly the efficiency of transferrin-polylysine-DNA complex uptake by cells; in this case the peptide was linked to polylysine and the complex was delivered by the transferrin receptor-mediated endocytosis (Wagner et al, 1992a; Plank et al, 1994). This peptide had the sequence: GLFEAIAGFIENGWEGMID GGGYC and was able to induce the release of the fluorescent dye calcein from liposomes prepared with egg yolk phosphatidylcholine which was higher at acidic pH; this peptide was also able to increase up to 10-fold the anti-HIV potency of antisense oligonucleotides, at a concentration of 0.1-1 mM, using CEM-SS lymphocytes in culture (Bongartz et al, 1994). This peptide changes conformation at the slightly more acidic environment of the endosome destabilizing and breaking the endosomal membrane (Murata et al, 1992; Bullough et al, 1994). Fusogenic peptides have been used by other investigators

(Midoux et al, 1993; Kamata et al, 1994). It is thought that several fusogenic peptides self-assemble following their conformational change forming a transmembrane channel (Bongartz et al, 1994).

Sendai virosomes were effective for delivering AAV neuropeptide Y (NPY) cDNA constructs in vivo. Injections into brain neocortex of Sendai-virosome encapsulated rAAV construct expressing NPY increased NPY-like immunoreactivity in neurons but not glia; injections into the rat hypothalamic para-ventricular nucleus increased body weight and food intake for 21 days (Wu et al, 1996). Tomita et al (1996) have found that newborn mice can sustain expression of the insulin gene delivered by Sendai virus-liposome complexes for at least 8 weeks as assayed by reverse transcriptase PCR and radioimmunoassay, compared to 2 weeks in adult animals.

A 27 residue peptide vector, containing the fusion sequence of HIV gp41 and the nuclear localization sequence of SV40 T antigen was used to deliver oligonucleotides to cell nuclei very rapidly in cell culture (1h). The complexes formed strongly increased the stability of the oligonucleotide to nucleases, enhanced passage through the plasma membrane, and led to endosomal internalization (Morris et at, 1997).

Certain cationic lipids are endowed with a better ability to disrupt the endosomal membrane and promote release of the plasmid to the cytoplasm, a prelude for its nuclear import. Presentation of plasmid DNA to COS cell cultures using three different lipid formulations: **(i)** vectamidine (3-tetradecylamino-N-tert-butyl-N'-tetradecylpropionamidine), **(ii)** DOTMA:DOPE (Lipofectin), and **(iii)** DMRIE-Chol (1:1) resulted in complex entry via endocytosis for all three cationic lipids as revealed using transmission electron microscopy. However, the endosomal membrane in contact with complexes containing vectamidine or DMRIE-Chol, but not Lipofectin, often exhibited a disrupted morphology (El Ouahabi et al, 1997).

F. Plasmid condensation with spermine, polylysine, protamine, histones enhances the transfection efficiency

DNA can be presented to cells in culture as a complex with polycations such as polylysine, or basic proteins such as protamine, total histones or specific histone fractions (Fritz et al, 1996), cationized albumin, and others (Smull and Ludwig, 1962). These molecules increase the transfection efficiency. In addition to HMG1, also histone H1 and HMG17 were identified as transfection-enhancing proteins in cell culture (Zaitsev et al, 1997). Histone H2A significantly enhanced in vitro DNA transfection whereas other histones and anionic liposomes did not (Balicki and Beutler, 1997). Gene transfer through the asialoglycoprotein receptor-mediated endocytosis pathway was enhanced with the histones H1, H2a, H2b, H3, and H4 which were galactosylated with the crosslinker agent, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, conjugated to DNA and then used to transfect HepG2 cells, which display the asialoglycoprotein receptor (Chen et al, 1994). Plasmid DNA and HMG1 were efficiently co-encapsulated in liposomes by agitation and sonication, and were co-introduced into cells by hemagglutinating virus of Japan (HVJ)-mediated membrane fusion; the presence of HMG1 enhanced 3-fold the transfection efficiency (Kato et al, 1991).

The interaction of plasmid DNA with protamine sulfate followed by the addition of DOTAP cationic liposomes offered a better protection of plasmid DNA against enzymatic digestion and gave consistently higher gene expression in mice via tail vein injection compared with DOTAP/DNA complexes; 50 μg of luciferase-plasmid per mouse gave 20 ng luciferase protein per mg extracted tissue protein in the lung which was detected as early as 1 h after injection, peaked at 6 h and declined thereafter. Intraportal injection of protamine/DOTAP/DNA led to about a 100-fold decrease in gene expression in the lung as compared with i.v. injection; endothelial cells were the primary locus of lacZ transgene expression (Li and Huang, 1997). Protamine sulfate enhanced plasmid delivery into several different types of cells in vitro using the monovalent cationic liposomal formulations (DC-Chol and lipofectin); this effect was less pronounced with the multivalent cationic liposome formulation, lipofectamine (Sorgi et al, 1997).

Spermine has been found to enhance the transfection efficiency of DNA-cationic liposome complexes in cell culture and in animal studies; this biogenic polyamine at high concentrations caused liposome fusion most likely promoted by the simultaneous interaction of one molecule of spermine (four positively charged amino groups) with the polar head groups of two or more molecules of lipids. At low concentrations (0.03-0.1 mM) it promoted anchorage of the liposome-DNA complex to the surface of cells and enhanced significantly transfection efficiency (Boulikas et al, in preparation).

Because the receptor for ecotropic viruses is a transporter for basic amino acids, use of a histone as a facilitator increased the efficiency of retroviral infection (Singh and Rigby, 1996). Polybrene is the usual agent employed during retroviral infection. For supernate infections, concentrations of 5-10 μg/ml of protamine provided essentially the same infection efficiency as polybrene; protamine displayed low toxicity on a range of cell types and increased 7-fold the efficiency of retroviral infection (Cornetta and Anderson, 1989).

The polycations polybrene, protamine, DEAE-dextran, and poly-L-lysine significantly increased the efficiency of adenovirus-mediated gene transfer in cell culture; this was thought to act by neutralizing the negative charges presented by membrane glycoproteins which reduce the efficiency of adenovirus-mediated gene transfer (Arcasoy et al, 1997).

G. Targeted gene delivery

Targeting a specific cell type or animal tissue is an important goal of gene therapy. Many different approaches have been undertaken to achieve targeting. A recombinant adenovirus encoding an anti-erbB-2 intracellular single-chain antibody (sFv) displayed a genetic selectivity for the erbB-2-positive prostate carcinoma cell lines DU145 and LNCaP; delivery of this recombinant adenovirus resulted in cytotoxicity to the DU145 and LNCaP, but not PC-3, cell lines and reduced the clonogenic capacity of DU145 cells cultured alone or mixed with various ratios of irradiated human bone marrow. This finding led to a strategy for effectively reducing DU145 and erbB-2-positive primary prostate tumor contamination in bone marrow cultures (Kim et al, 1997). Delivery of an anti-erbB-2 single chain (sFv) antibody gene for previously treated ovarian and extraovarian cancer patients is in clinical trials using adenoviral gene delivery (protocol #133).

A luciferase expression vector (pRSVLuc) non-covalently linked to a humanized HER2 antibody (rhuMAbHER2) covalently modified with poly-L-lysine bridges was able to direct gene transfer to HER2 expressing cells in vitro (Foster and Kern, 1997).

A targeting gene therapy approach for hematopoietic stem/progenitor cells has been directed to cell lines expressing the c-kit receptor; plasmid DNA containing a luciferase reporter gene was condensed with polylysine covalently linked to streptavidin (which binds biotinylated ligand) and with polylysine covalently linked to adenovirus (to achieve endosomal lysis) with the final addition of biotinylated steel factor; omission of the adenovirus endosomalytic agent from the vector resulted in the loss of gene expression (Schwarzenberger et al, 1996).

Systemic administration of a c-fos antisense, regulated by mouse mammary tumor virus (MMTV) control elements in a retroviral vector, showed expression only in breast epithelium although the vector could be detected in several tissues thus supporting targeting to MMTV-regulated tissues (Arteaga and Holt, 1996).

Liposomes coated with polyethyleneglycol (PEG) can be efficiently targeted to tumor cells that express folate receptors (KB cells) via conjugation of folate to a PEG spacer of 25 nm in length; shorter PEG spacers were not efficient in mediating binding of the liposomes to KB cells (Lee and Low, 1995).

Neri and coworkers (1997) were able to target an angiogenesis-associated oncofetal fibronectin (B-FN) isoform by affinity-matured recombinant antibody fragments. B-FN is present in vessels of neoplastic tissues during angiogenesis but is absent from mature vessels and could provide a target for diagnostic imaging and therapy of cancer. Phage display libraries were screened to isolate human antibody fragments able to recognize this isoform across species; imaging of F9 murine teratocarcinomas grafted in nude mice is shown on **Figures 8 and 9.**

H. Targeted gene delivery with peptide-displaying phages

Development of methods to display and select collections of peptides specific for binding a target provide valuable tools to identification of peptide drugs; peptides could be selected for binding biological targets including cell surface receptor molecules, DNA, antibodies, or whole cells. The technique of peptide-displaying phages has been developed for targeted gene delivery. Selection of cell surface-binding peptides, ideally specific for each type of cell in the human body, will be used for incorporation into gene delivery vehicles to achieve the long-searched tissue specificity of the vector (reviewed by Russell, 1996).

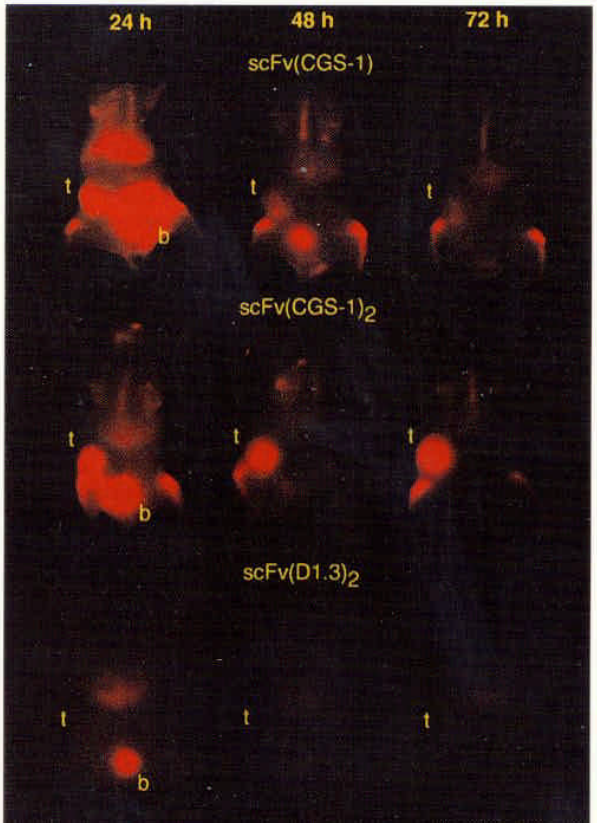
Development of the random peptide library as a source of specific protein binding molecules (Devlin et al, 1990) and exposure of random peptides on the surface of phages (Cwirla et al, 1990) has been the catalyst for progress in this promising field. Libraries of random 8 to 12 amino acid peptides expressed on the N-terminus of the pIII protein of the fd phage or on the N-terminus of the pVIII major coat protein of the same phage have been selected that bind the extracellular domain of human IL-1 receptor; screening was against immobilized IL-1 receptor extracellular domain. Two families of peptides could act as antagonists blocking triggering of the IL-1 signaling pathway; because IL-1 levels become elevated in autoimmune and inflammatory disorders, these peptide antagonists of IL-1 receptor could provide novel drugs for these diseases (Yanofsky et al, 1996).

Phages displaying known integrin-binding peptides have been shown to bind and enter mammalian cells (Hart et al, 1994). A peptide antagonist to thrombin receptor has been identified using phage display (Doorbar and Winter, 1994). Production of cell-targeting ligands has been achieved by cell-binding peptides specific for different cell types in culture; these peptides are selected through six rounds of binding (and amplification of phage clones) to a particular cell type from random peptide-presenting phage libraries; the selected peptides are apparently recognizing specific surface receptor molecules. For example, the 20mer peptide KTLTLEAALRNAWLREVGLK has been selected for its high affinity for PEA10 mouse fibroblast cells binding 1000 more efficiently to the cells than random peptides (Barry et al, 1996).

IX. Gene delivery with polymers, peptides and other means

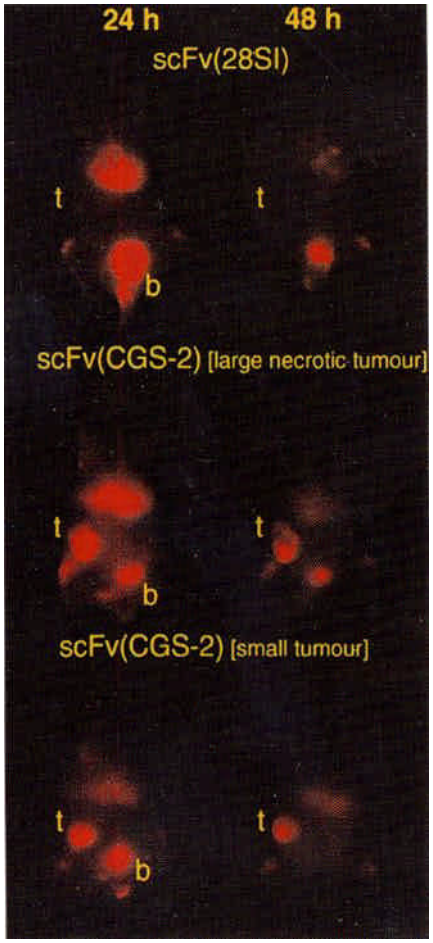
A. Delivery of transferrin-polylysine-DNA complexes

A number of polymers have been tested and shown to enhance significantly the transfection efficiency of plasmids but also of viruses; the enhancement in transfection results from a facilitation in the interaction of plasmids with the cell surface, transport to endosomes, release to the cytoplasm, and in some cases nuclear import (reviewed by Behr, 1994).



← **Figure 9**. Role of antibody affinity. Targeting of fluorescently-labeled antibody fragments to the F9 murine teratocarcinoma grafted in nude mice using the affinity-matured scFv(CGS-2) and the lower affinity scFv(28SI) directed to the same epitope of oncofetal fibronectin; Tte dimeric scFv(D1.3)$_2$ with a binding specificity to lysozyme was used as a negative control. t: tumor; b: bladder. From Neri D, Carnemolla B, Nissim A, Leprini A, Querze G, Balza E, Pini A, Tarli L, Halin C, Neri P, Zardi L, Winter G (**1997**) Targeting by affinity-matured recombinant antibody fragments of an angiogenesis associated fibronectin isoform. **Nat Biotechnol** 15, 1271-1275. Reproduced with the kind permission of the authors (Dario Neri, Inst for Mol Biology and Biophysics, Zürich) and Nature America, Inc.

Figure 8. Role of antibody valence. → Targeting of fluorescently-labeled antibody fragments to the F9 murine teratocarcinoma grafted in nude mice using the monomeric scFv(CGS-1) and dimeric scFv(CGS-1)$_2$ directed to oncofetal fibronectin; the dimeric scFv(D1.3)$_2$ with a binding specificity to lysozyme was used as a negative control. t: tumor; b: bladder. From Neri D, Carnemolla B, Nissim A, Leprini A, Querze G, Balza E, Pini A, Tarli L, Halin C, Neri P, Zardi L, Winter G (**1997**) Targeting by affinity-matured recombinant antibody fragments of an angiogenesis associated fibronectin isoform. **Nat Biotechnol** 15, 1271-1275. Reproduced with the kind permission of the authors (Dario Neri, Inst for Mol Biology and Biophysics, Zürich) and Nature America, Inc.



Curriel and coworkers (1991) have used the transferrin receptor on the surface of mammalian cells to deliver plasmid-polylysine-transferrin complexes to cells. These complexes are taken up by endosomes following receptor binding, a method which suffers from that the endocytosed DNA is trapped in the intracellular vesicle and is later largely destroyed by lysosomes; use of the capacity of the adenoviruses to disrupt endosomes as part of their entry mechanism to the cells have augmented over 1000-fold the efficiency of gene transfer. This method has been further developed in collaboration with Max Birnstiel; true chemical coupling rather than simple addition of replication-defective adenovirus particles has shown a further increase in transfection efficiency (Cotten et al, 1992; Wagner et al, 1992a,b).

A monoclonal antibody against the CE7 antigen (chCE7) covalently linked to polylysine in the presence of chloroquine was able to transfect NB cells as efficiently as DOTAP, transfectam, TF-X50, or lipofectamine; furthermore, transfection was not observed in cell lines negative for the CE7 antigen (Coll et al, 1997).

B. Polyethylenimine (PEI, ExGen500)

Polyethylenimine, H_2N-(CH_2-CH_2-$NH)_n$-H, is an organic polymer with a potential for high cationic charge. PEI enhanced transfection efficiency in cell culture (Boussif et al, 1996). ExGen500 is a linear 22 kDa form of PEI, which was found to be more efficient than lipofectin, DOTAP and DOGS in delivering the luciferase reporter gene in both newborn and adult rabbit lungs (Ferrari et al, 1997). The PEI 800 kDa and PEI 25 kDa branched polymers have also been used to transfer marker genes to the newborn and adult mouse brain (Boussif et al, 1995; Abdallah et al, 1996). Another advantage of PEI is that it yields high transfection efficiencies with a charge ratio of DNA:PEI close to neutral; this is an advantage as particles with a net positive charge (cationic lipid-DNA complexes) interact with circulating serum proteins or anionic components of the extracellular matrix in the various tissues hindering their bio-availability (Schwartz et al, 1995).

The high transfection efficiency of ExGen 500 was suggested to arise from the “proton sponge” effect which leads to osmotic swelling of endosomes which have uptaken the DNA complexes (Ferrari et al, 1997).

Different cationic compositions may result in different targeting and transfection abilities to specific organs; the branched, 25-kD polyethylenimine polymer (PEI 25k) was superior over DOTAP and DOGS (Transfectam) in the efficiency of transfection of the kidney when complexes of these cations with luciferase plasmid were injected into the left renal artery of rats; luciferase activity peaked at 2 days, was still significantly higher than controls at 7 days, but was undetectable at 14 days post-injection (Boletta et al, 1997).

Scanning force microscopy allowed plasmid DNA strands to be visualized without drying in incomplete condensates prepared with varying stoichiometries of lipospermine or polyethylenimine in physiological solution; discrete nucleation centers of condensation were observed often surrounded by folded loops of DNA using either condensing agent; increasing the amount of lipospermine or polyethylenimine led to further aggregation through folding rather than winding of the DNA (Dunlap et al, 1997).

C. APL PolyCat57 and other polymers

APL PolyCat57 is a synthetic polyamino derivative (nonpeptide, nonlipid polymer) with a glucose backbone which was used by Goldman and coworkers (1997) for gene transfer in vivo and in vitro. A variety of human carcinoma cell lines were transfected with an efficiency superior to that of Lipofectamine. The polymer-plasmid complex was resistant to inhibition by serum allowing for efficient gene transfer in vivo. The level of the luciferase and β-galactosidase reporter gene expression after intrathecal injection, evaluated in animal models bearing stereotactically implanted D54-MG human glioma cell xenografts, was comparable to that obtained with an adenoviral vector.

Liposomes composed of the cationic peptide amphiphile N,N-dihexadecyl-N α-[6-(trimethyl ammonio)-hexanoyl]-L-alaninamide bromide comprising an L-alanine residue interposed between a charged head group and a double-chain segment were more effective and less toxic than lipofectin, and DOTAP for the transfection of COS-7 cells (Kato et al, 1996).

D. Adenovirus-polymer complexes

An adenovirus/DNA complex was constructed by chemically linking poly-L-lysine to the capsid of the replication-defective adenovirus dl312; this complex was then coupled with plasmid DNA via ionic interaction. This system was used to deliver the tumor suppressor protein p53 to the $p53^-$ human lung cancer cell line H1299, both in vitro and in vivo, leading to induction of apoptosis; injection of the complex carrying the p53 gene to subcutaneous tumor sites 5 days after tumor cell implantation resulted in a significant inhibition of tumorigenicity as measured by the number and size of tumors that developed 21 days after treatment (Nguyen et al, 1997a,b).

Complexes of cationic polymers and cationic lipids with adenovirus increased adenovirus uptake and transgene expression in cells that were inefficiently infected by adenovirus alone; infection by both complexes was independent of adenovirus fiber and its receptor, occurred via a different cellular pathway than adenovirus alone, and enhanced gene transfer to the nasal epithelium of cystic fibrosis mice in vivo (Fasbender et al, 1997).

E. Peptides in transfer of oligonucleotides

Peptide/oligonucleotide complexes containing a peptide vector and single or double stranded oligonucleotides were delivered into cultured mammalian cells in less than 1 h with relatively high efficiency (90%) at a peptide/oligonucleotide ratio of 20/1. The peptide vector, termed MPG (27 residues), contained a hydrophobic domain derived from the fusion peptide of HIV gp41 and a hydrophilic domain derived from the nuclear localization sequence of SV40 T-antigen. The complexes involved electrostatic interactions between basic peptide residues and phosphate groups from the oligos, as well as additional peptide-peptide interactions yielding oligonucleotides most likely coated with several molecules of MPG; these complexes, which strongly increased the stability of the oligonucleotide to nucleases, enhanced passage through the plasma membrane, and did lead to endosomal internalization; such complexes are promising delivery systems for oligos (Morris et at, 1997).

The cationic amphipathic peptide WEAKLAKALA KALAKHLAKALAKALKACEA was synthesized by Wyman et al (1997) to display hydrophobic leucine residues on one side and hydrophilic lysine residues on the other after coiling to an amphipathic α-helix at pH 7.5; this peptide was suited for oligonucleotide nuclear delivery when complexes were prepared at a 10/1 (+/-) charge ratio and was endowed with the additional property of destabilizing membranes in cell culture.

F. Plasmoviruses

Plasmoviruses are plasmids capable of expressing all the viral genes required for generating infectious particles and packaging a defective genome; transfected as plasmids, plasmoviruses transform the transduced cells into packaging cells; the cells then release infectious replication-defective retrovirus particles of typical type C as revealed by electron microscopy, with the gag proteins correctly processed in the released particles and containing the transgene to be transferred. Released particles are capable of infecting nearby cells and to propagate the transgene in the culture, resulting in stable integration of plasmovirus proviral DNA into the host genome of infected cells. Nonintegrated plasmovirus DNA was not toxic for the cells. Plasmoviruses have been used for the propagation of the HSV-tk gene in cell culture resulting in a major improvement in therapeutic efficacy after ganciclovir treatment, when compared to that of plasmovirus constructs that cannot propagate (Morozov et al, 1997).

G. Particle-mediated gene transfer (PMGT) or gene gun

The particle-mediated gene transfer (PMGT) technique, unlike retroviral transfection, does not require tumor cell proliferation in vitro for gene transfer; instead, tumor tissue can be dissociated into small tissue clumps or cell aggregates and then immediately transfected using the gene gun; plasmid-coated gold particles are delivered to tumor cells using helium pressure with a hand-held gene delivery device overcoming the cumbersome exposure of the patient to viral antigens. PMGT with gold particles coated with human GM-CSF plasmid DNA is being used to transfect melanoma or renal carcinoma tissue from patients; tumor cells are then lethally-irradiated and patients are intradermally vaccinated to elicit anti-tumor immune responses (Mahvi et al, 1997).

Gene gun-mediated DNA delivery into the epidermis overlying an established intradermal murine tumor was used to compare the antitumor effect of several transgene expression plasmids encoding the cytokines IL-2, IL-4, IL-6, IL-12, IFN-γ, TNF-α, and GM-CSF; IL-12 was superior (see IL-12) (Rakhmilevich et al, 1997).

X. Direct injection of naked plasmid DNA

Naked plasmid has been injected to various tissues and has shown transfection efficiency. Muscle has been the classical tissue in a number of studies. Intramuscular (i.m.) administration of expression plasmids may directly deliver the plasmid to the cytoplasm by damaging the myofibril along the injected area. Direct i.m. injection of naked VEGF plasmid DNA was used in rabbits to optimize treatment of acute limb ischemia; after ligation of distal external iliac artery in New Zealand White rabbits, direct injection of 500 μg of a $VEGF_{165}$ expression vector into the ischemic thigh muscles resulted in more angiographically recognizable collateral vessels at 30 days posttransfection (Tsurumi et al, 1996, 1997). Injection, guided by intense illumination along the longitudinal axis of the mouse quadriceps muscle and parallel to the myofibers, yielded 200-fold higher levels of luciferase expression than perpendicular injection (Levy et al, 1996).

Other tissues including skin, liver, brain and the gastric submucosa have been successfully transduced with reporter gene cDNA using naked plasmid delivery. Skin from transglutaminase 1 (TGase1)-deficient patients suffering with lamellar ichthyosis was regenerated on nude mice; repeated in vivo direct injections of naked DNA using a TGase1 expression plasmid showed restoration of TGase1 expression in the correct tissue location (Choate and Khavari, 1997).

A naked luciferase expression vector injected intracerebrally in mice provided expression of the luciferase transgene, in both neurons and glia cells (Schwartz et al, 1996). Naked plasmid DNA in hypertonic solutions, injected intraportally in mice whose hepatic veins were transiently occluded, resulted in high levels of luciferase and β-galactosidase expression in 1% of the hepatocytes throughout the entire liver using 100 μg DNA (Budker et al, 1996).

A single injection through the tail vein of a naked endothelium-derived nitric oxide cDNA plasmid caused a significant reduction of systemic blood pressure for 5 to 6

weeks in spontaneously hypertensive rats (Lin et al, 1997). In vivo delivery of a luciferase gene under control of the human cytomegalovirus immediate early gene promoter after intravenous injection (50 μg DNA) via the tail vein into ICR mice has shown that the DNA was degraded with a half-life of less than 5 min from the blood; plasmid DNA was differentially retained in the lung, spleen, liver, heart, kidney, bone morrow, and muscle up to 24 h postinjection; femtogram levels of plasmid were detected only in muscle at 6 months post infection (Lew et al, 1995). pCAT was rapidly degraded after incubation with mouse whole blood in vitro with a half-life of approximately 10 min and much faster after intravenous injection; i.v. injection of radioactively-labeled pCAT showed rapid elimination from the plasma due to extensive uptake by non-parenchymal cells in the liver, a process thought to be mediated via scavenger receptors on these cells (Kawabata et al, 1995).

Direct injection of plasmids carrying reporter genes into the gastric submucosa of adult rats resulted in transient expression (1-3 days and in exceptional cases for up to 21 days) in smooth muscle cells of the muscularis mucosae and the muscular layer and mesenchymal cells in the lamina propria. These studies indicate that the gastrointestinal nonepithelial tissue, a useful target for in vivo gene transfer, can be transfected with naked DNA (Takehara et al, 1996).

Clinical protocols #158-161 use naked plasmid DNA. Protocol #158 proposes transferring the carcinoembryonic antigen to autologous tumor cells in patients with metastatic colorectal cancer for cancer immunotherapy (**Appendix 1**, page 170). Protocols 159 and 160 use an intraarterial angioplasty catheter to deliver VEGF cDNA plasmid to patients with peripheral artery disease or restenosis. Plasmid DNA coding for tumor idiotype is being used for intramuscular injection for immumotherapy of non-Hodgkin's B-cell lymphoma (protocol #161).

Table 1 summarizes the advantages and disadvantages of the principal gene delivery methods.

Table 1. Advantages and drawbacks of delivery systems

Gene deliv. system	Advantages	Drawbacks
Murine retroviral vectors	Very safe; may achieve high efficiency of transduction; infects only dividing cells; integrates into host DNA.	Loss in expression soon after infection; low efficiency in vivo; up to 8 kb of DNA; high titers required for in vivo gene delivery; immunogenicity.
Recombinant adenoviruses	Infect nondividing cells; rarity of recombination events between adenoviral vectors and the host chromosomes; high efficiency of transduction; adenovirus vectors efficiently escape from the endosome and enter the nucleus; episomaly-replicating virus.	Induction of immune responses that eliminates therapeutic cells; may induce unwanted infections to humans; only up to 7.5 kb of exogenous DNA can be inserted; loss of adenoviral episomes in progeny cells.
AAV	Does not stimulate inflammation or immune reaction; enters nondividing cells and does not replicate; nonpathogenic virus.	Low efficiency of gene transfer; only up to 4.1 and 4.9 kb can be incorporated; wt AAV integrates on chromosome 19 but recombinant AAV integrates at different sites (e.g. chromosome 2); integration may cause inactivation of the transgene by chromatin effects.
HSV-1	Can take up to 30 kb of exogenous DNA; high titer viral stocks; wide range; can infect nonreplicating cells	Infection with HSV-1 is cytotoxic.
Baculovirus	Specificity for hepatocytes; high efficiency of infection	Not applicable in vivo at present.
EBV	Episomaly-replicating virus	wt EBV infects human cells causing mononucleosis.
HIV-1	Transduces non-dividing cells; broad range of tissues and cell types; no inflammation; sustains expression of GFP for 8-22 weeks in muscle and liver after injection to animals	Start up technology, not broadly tested.
Hybrid HSV/EBV	High efficiency of infection (95-99% after intratumoral liver injection)	Not broadly tested.
Cationic lipids	High efficiency of transfection via membrane destabilization (cell membrane and endosomal); destabilize lysosomal membranes and promote release of plasmid in the cytoplasm.	Toxic, not suited for i.v. injection; can interact with negatively charged serum proteins in vivo causing transgene inactivation; gene expression is transient; i.v. injection targets mainly the lung
Stealth liposomes	Non toxic, escape immune surveillance and concentrate into solid tumors by extravasation.	Not taken up by tumor cells but remain in the extracellular space.
Naked plasmid DNA	Suited for intramuscular injection and DNA vaccination; easy to use; no viral antigens.	Low transfection; not widely applicable method; naked plasmid is cleared from blood rapidly.
Gene gun	Easy to use (plasmid-coated gold particles are delivered to tumor cells using helium pressure); rapid, suited for gene transfer to tumor specimens from patients for	Not broadly tested.

immunotherapy.

XI. Promoters and enhancers for transgene expression

A. Viral promoters

After escaping serum components and immune cells, crossing the cell membrane, released from endosomes to the cytoplasm and transported through the nuclear pores to the nucleus the transgene has to accomplish two additional tasks: (i) to be efficiently transcribed and (ii) its expression to last for long periods. These two very important factors depend on the DNA regulatory elements that drive the expression of the therapeutic gene. The use of mammalian gene expression vectors has revolutionized the field of direct gene delivery. The proper choice of promoter and enhancer elements linked to the gene of interest is decisive for the successful expression of the gene in the desired tissue or cell type in gene therapy.

The majority of mammalian expression vectors make use of promoter/enhancer elements from pathogenic viruses including the immediately early promoter of the human cytomegalovirus (CMV), the Rous sarcoma virus (RSV) promoter, the enhancer/origin of replication of SV40, the adenovirus type 2 major late promoter (Ad-MLP), as well as promoters from the mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV), herpes simplex virus (HSV), Epstein-Barr virus (EBV), and others.

Many studies have compared the strength of different promoters in driving a therapeutic gene both in cell culture and in vivo. I will mention a few sample studies here. Recombinant adenoviruses carrying the HSV-tk gene under control of the human cytomegalovirus (CMV) immediate early gene promoter or the adenovirus type 2 major late promoter (Ad-MLP) were compared for their killing efficiency in combination with GCV treatment; the rat 9L model for brain tumor and leptomeningeal metastases was used; the adenovirus containing the CMV promoter showed greater cell killing efficiency compared to the Ad-MLP promoter; animals with brain tumors showed significantly longer survival time and animals with leptomeningeal metastases had symptom-free periods (Vincent et al, 1997).

Doll et al (1996) have compared the efficiency of expression of the β-galactosidase gene flanked by the AAV ITRs in brain tumors and primary brain cell cultures driven by four different promoters. The human CMV immediate-early enhancer/promoter was always the strongest, generally by at least one order of magnitude, compared with the SV40 early enhancer/promoter, the JC polymovirus promoter, and the chicken β-actin promoter coupled to the CMV enhancer. High level of expression was usually seen within 24 h of transgene delivery by either transfection or infection, but dropped dramatically within days; all four promoters showed the same decline in sustaining gene expression of β-galactosidase with time (Doll et al, 1996).

The type of regulatory elements on plasmid vectors, including promoter, enhancer, intron, and polyadenylation signals, were systematically evaluated by Yew et al (1997) by constructing a series of plasmids. **Figure 10** shows the effect of different introns (panel A) and different poly(A) signals (panel B) on CAT expression. A hybrid intron (HI) appeared to be the most effective. There was a 4-fold increase in CAT expression from the bovine growth hormone (BGH) poly(A) signal vector compared to the SV40 poly(A) signal vector.

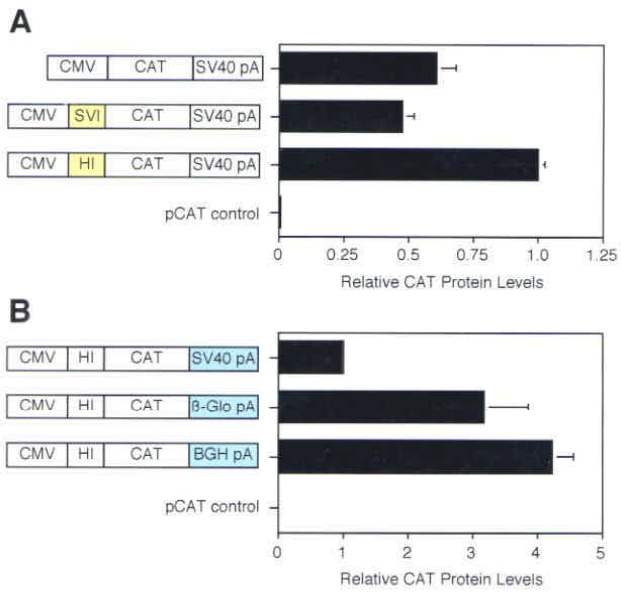


Figure 10. Effect of different introns (**A**) and polyadenylation signals (**B**) on CAT expression. ELM cells were co-transfected with equimolar amounts of each plasmid using DMRIE:DOPE and CAT protein levels in cell lysates were assayed 48 h after transfection; pCMVβ was used as an internal control. SVI is the SV40 19S/16S intron; HI, hybrid intron, SV40 pA, SV40 late polyadenylation signal; BGH, bovine growth hormone polyadenylation signal; β-Glo, rabbit β-globin polyadenylation signal. The data are expressed as mean ±SD (n=3). From Yew NS, Wysokenski DM, Wang KX, Ziegler RJ, Marshall J, McNeilly D, Cherry M, Osburn W, Cheng SH (**1997**) Optimization of plasmid vectors for high-level expression in lung epithelial cells. **Hum Gene Ther** 8, 575-584. Reproduced with kind permission of the authors (Nelson Yew, Genzyme Corp., Framingham, MA) and Mary Ann Liebert, Inc.

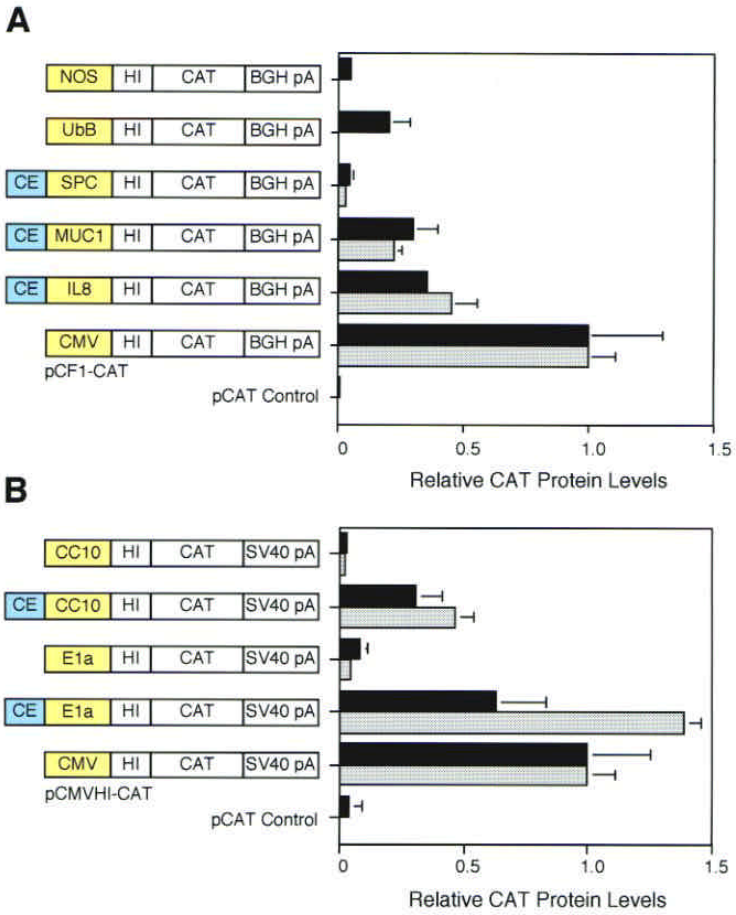


Figure 11. Comparison of CAT expression from different promoters in vitro. ELM cells (solid bars) or CFT1 cells (stippled bars), a human airway epithelial cell line derived from a CF patient, were transfected as described in Figure 10. CAT ELISA assays were carried out 48 h after transfection (an average of 6 assays). CAT protein levels were normalized to pCF1-CAT (in A) or pCMVHICAT (in B). **A**. Expression from plasmids containing the BGH poly(A) signal. SPC, Surfactant protein C promoter; NOS, nitric oxide synthase promoter; UbB, ubiquitin B promoter; MUC1, mucin 1 promoter; IL8, interleukin 1 promoter; CE, CMV enhancer; pCAT control is a promoterless CAT plasmid. **B**. Expression from plasmids containing the SV40 poly(A) signal. CC10, Clara cell 10 kDa protein promoter; E1a, adenovirus E1a promoter. The data are expressed as mean ±SD (n=3-12). From Yew NS, Wysokenski DM, Wang KX, Ziegler RJ, Marshall J, McNeilly D, Cherry M, Osburn W, Cheng SH (**1997**) Optimization of plasmid vectors for high-level expression in lung epithelial cells. **Hum Gene Ther** 8, 575-584. Reproduced with kind permission of the authors (Nelson Yew, Genzyme Corp., Framingham, MA) and Mary Ann Liebert, Inc.

Figure 11 compares the strength of different promoters from CAT constructs containing the bovine growth hormone (BGH) poly(A) signal (panel A) or the SV40 poly(A) signal (panel B) and the hybrid intron. The promoters were chosen for lung targeting. CMV yielded the highest expression in vitro. To determine whether or not incorporating two CMV enhancers could produce higher levels of CAT expression than one, a second CMV enhancer (from -118 to -522 relative to the transcription start site) was inserted 186 bp upstream of the CMV promoter and its associated enhancer; in the context of the SV40 poly(A) signal the second CMV enhancer (CE in Figure 11) increased expression 3-fold; however, when the BGH poly(A) signal was present, the second copy of CMV did not increase CAT expression (**Figure 12**).

Because of its wide use and the more potent effect, the CMV IE enhancer/promoter deserves some special attention. In order to understand the potent effect of the CMV promoter in the expression of foreign genes we need to understand the transcription factors (TFs) that activate this regulatory region; TFs in the transfected cell will be responsible for binding to the CMV promoter leading to the activation of the transgene. At present not all TF regulatory circuits leading to activation of CMV have been deciphered. **Figure 13** shows two CMV promoters retrieved from Genbank which are being used in expression vectors.

The CMV IE promoter includes the 10-bp palindromic sequence ***CCATATATGG*** (**Figure 13**) which resembles the core motif of serum response elements and proved to bind specifically to the cellular nuclear protein serum response factor (SRF). Reporter gene constructs containing four tandem copies of these elements displayed up to 13-fold increased basal enhancer activity and 18-fold tetradecanoyl phorbol acetate responsiveness in U937 cells (Chang et al, 1993).

B. Transcription factor binding sites within the CMV promoter

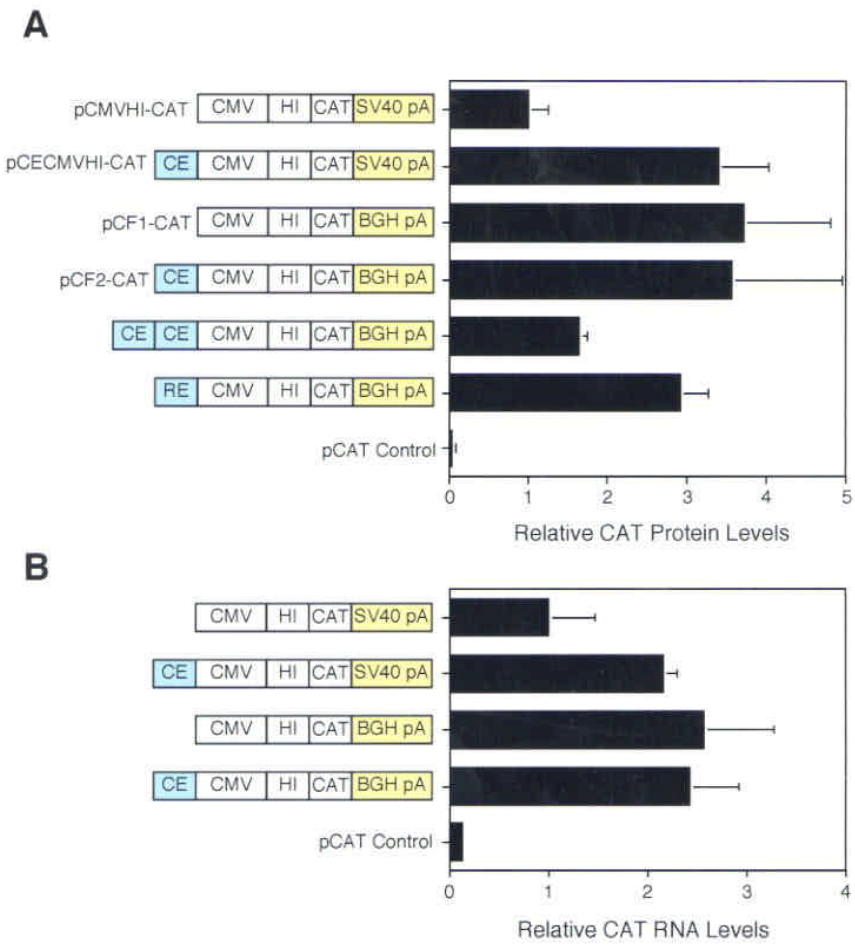


Figure 12. Effect of a second CMV enhancer region on CAT expression from the CMV promoter. Plasmids were transfected into ELM cells and the cells were harvested 48 h after transfection. Expression was normalized to pCMVHICAT (in B). **A**. CAT protein levels in cell lysates. RE, RSV LTR enhancer. **B**. Levels of CAT RNA. Total RNA was isolated from the transfected cells and a quantitative RNA protection assay was performed. The data are expressed as mean ±SD (n=3-9). From Yew NS, Wysokenski DM, Wang KX, Ziegler RJ, Marshall J, McNeilly D, Cherry M, Osburn W, Cheng SH (**1997**) Optimization of plasmid vectors for high-level expression in lung epithelial cells. **Hum Gene Ther** 8, 575-584. Reproduced with kind permission of the authors (Nelson Yew, Genzyme Corp., Framingham, MA) and Mary Ann Liebert, Inc.

Two multicopy basal enhancer motifs within the simian CMV IE enhancer, namely, 11 copies of the 16-bp cyclic AMP response element (CRE) and 3 copies of novel 17-bp serum response factor (SRF) binding sites referred to as the **SNE** (SRF/NF-κB-like element), as well as four classical NF-κB sites within the human CMV promoter, contributed to TPA responsiveness; the SNE sites of the simian CMV promoter contain potential overlapping core recognition binding motifs for SRF, Rel/ NF-κB, ETS, and YY1 class transcription factors but fail to respond to either serum or tumor necrosis factor α; the TPA responsiveness of both human and simian CMV elements proved to involve synergistic interactions between the core SRF binding site (***CCATATATGG***) and the adjacent inverted ETS binding motifs (TTCC), which correlated directly with formation of a bound tripartite complex containing both the cellular SRF and ELK-1 proteins. This protein complex was more abundant in U-937, K-562, and HeLa cell extracts than in Raji, HF, BALB/c 3T3, or HL-60 cells. A 40-fold stimulation of chloramphenicol acetyltransferase activity mediated by four tandem repeats of the SNE could be induced within 2 h (and up to 250-fold within 6 h) after addition of TPA in DNA-transfected U-937 cells, indicating that the stimulation appeared likely to be a true protein kinase C-mediated signal transduction event rather than a differentiation response (Chan et al, 1996). These studies demonstrate that different cell types are expected to sustain different levels of expression from CMV and that, for cell culture transfections, PKC transduction pathways are likely to stimulate transgene expression from CMV promoters. These findings have important implications for promoter choice in gene therapy.

7TCAATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATCAATATTGGCTAT
TGGCCATTGCATACGTTGTATCTATATCATAATATGTACATTTATATTGGCTCATGTCCAATA
TGACCGCCATGTTGGCATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTA
GTTCATAGC***CCATATATGG***AGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCT
GACCGCCCAACGACCCCCGCC**CATTGACG**TCAATAATGACGTATGTTCCCATAGTAACGCC
AATAGGGACTTTC**CATTGACG**TCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCA
GTACATCAAGTGTATCATATGCCAAGTCCGCCCCCTA**ttgacgtcaa**TGACGGTAAATGGCCCGC
CTGGCATTATGCCCAGTACATGACCTTAC**GGGACTTTCC**TACTTGGCAGTACATCTACGTA
TTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACACCAATGGGCGTGGATAGC
GGTTTGACTCACGGGGATTTCCAAGTCTCCACCC**CATTGACG**TCAATGGGAGTTTGTTTTG
GCACCAAAATCAAC**GGGACTTTCC**AAAATGTCGTAATAACCCCGCCCCGTTGACGCAAAT
GGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCA
GATCACTAGAAGCTTTATTGCGGTAGTTTATCACAGTTAAATTGCTAACGCAGTCAGTG803

Figure 13A. The CMV promoter sequence from plasmid pRL-CMV, 4079 bp (nucleotides 7-803, Promega) (LOCUS AF025843)

37TCTGCTTATATAGACCTCCCACCGTACACGCCTAC**CGCCCATT**TGCGTCAATGGGGC
GGAGTTGTTACGACATTTTGGAAAGTCCCGTTGATTTTGGTGCCAAAACAAACTCC**CATTG**
ACGTCAATGGGGTGGAGACTTGGAAATCCCCGTGAGTCAAACCGCTATCCA**CGCCCATT**G
ATGTACTGCCAAAACCGCATCACCATGGTAATAGCGATGACTAATACGTAGATGTACTGCCA
AGTAGGAAAGTCCCATAAGGTCATGTACTGGGCATAATGCCAGGCGGGCCATTTACCGT**CA**
TTGACGTCAATAGGGGGCGTACTTGGCATATGATACACTTGATGTACTGCCAAGTGGGCA
GTTTACCGTAAATACTCCACC**CATTGACG**TCAATGGAAAGTCCCTATTGGCGTTACTATGG
GAACATACGTCATTATTGACGTCAATGGGCGGGGGTCGTTGGGCGGTCAGCCAGGCGGGC
CATTTACCGTAAGTTATGTAACGCGGAACT***CCATATATGG***GCTATGAACTAATGACCCCGTA
ATTGATTACTATTAATAACTAGTCAATAATCAATGTCAACATGGCGGTAATGTTGGACATGA
GCCAATATAAATGTACATATTATGATATGGATACAACGTATGCAATGGGCCAA695

Figure 13B. The CMV IE promoter (nucleotides 37-695, 658 bp) from the expression vector pCMVtkLUC$^+$ (ACCESSION AF027129). **ttgacgtcaa** is the binding site of HB16; **GGGACTTTCC** is the binding site for HIVEN 86A (two sites); ***CCATATATGG*** is the SRF binding site; TTCC is the ETS core motif; three CATTGACG motifs in each sequence are in bold-face (see Boulikas, 1994 for more references).

A closely related family of ubiquitous DNA binding proteins, called MDBP, binds with high affinity to two 14 base pair (bp) sites within the human cytomegalovirus immediate early gene 1 (CMV IE1) enhancer; these MDBP sites did not require cytosine methylation for optimal binding; mutation of one of the enhancer MDBP sites to prevent MDBP recognition modestly increased the function of a neighboring CREB binding site in a transient transfection assay (Zhang et al, 1995). Furthermore, the CMV promoter competed with the Egr1 promoter for transcription factors or co-factors which might be required for activation by WT1; WT1 was converted from an activator to a repressor by co-transfection of an excess of the parental CMV-based vector (Reddy et al, 1995).

C. Tissue-specific promoters in gene therapy

A number of studies have used tissue-specific promoters and enhancers from mammalian genes in order to attain a cell type-specific expression of the transgene. The discovery of genes which are expressed at high levels in specific tumor cell types has prompted the idea of the use of their promoter or enhancer DNA sequences to express in this particular cancer cell type therapeutically important genes (Venkatesh et al, 1990; Brady et al, 1994; Dimaio et al, 1994; Osaki et al, 1994; Pang et al, 1995).

Examples include the expression of the suicidal *CD* gene under control of the regulatory regions of the tumor marker gene carcinoembryonic antigen (Richards et al, 1995), the expression of HSV-*tk* gene, under control of α-fetoprotein enhancer and albumin promoter, into adult liver cells in transgenic animals (Su et al, 1996), the expression of β-galactosidase in tyrosine hydroxylase-expressing neurons in the substantia nigra midbrain of adult rats using the tyrosine hydroxylase promoter (Song et al, 1997), and

the expression of the lacZ marker gene under control of the murine pancreatic amylase promoter in the pancreas in neonatal and adult mice (Dematteo et al, 1997). Transduction of the human LDL-R cDNA under the transcriptional control of the liver-type pyruvate kinase promoter allowed high and tissue specific expression of the gene in primary hepatocytes (Pages et al, 1996b).

Fibroblasts, infected with recombinant retroviruses and selected with G418 for the expression of the vector carrying the therapeutic gene, have been used for the ex vivo treatment in animal models; when the therapeutic gene was either under control of the viral LTR or an heterologous internal promoter, expression of the transgene from the integrated retrovirus was shut off (Scharfmann et al, 1991). The use of the dihydrofolate reductase housekeeping gene promoter which is expressed in all cell types, led to sustained expression, albeit at very low levels (Scharfmann et al, 1991); it appears that the combination of a suitable enhancer and promoter for a particular cell type and the method of introduction of the transgene is crucial for sustained expression.

Combination of the mouse muscle creatine kinase enhancer with the human cytomegalovirus promoter to drive the expression of the canine *factor IX* gene in *ex vivo* infected mouse primary myoblasts led to the expression of factor IX and its secretion in the blood of mice transplanted with these myoblasts for over 6 months; however, the levels of factor IX protein secreted into the plasma (10 ng/ml for 10^7 injected cells) were not sufficient to be of therapeutic value (Dai et al, 1992).

Joki and coworkers (1995) have used the promoter of the early growth response gene 1 (EGR-1, also known as Zif/268, TIS-8, NFGI-A, or Krox-24) to confer selective expression of the luciferase gene in glioma cell lines exposed to ionizing radiation; a 10-fold higher activity in luciferase activity was found after irradiation of the cells which was detectable at 1-3 h after stimulation with 20 Gy (stereotactic radiosurgery during treatment of isolated brain metastases, arteriovenous malformations, meningiomas, craniopharyngiomas, and glioblastomas uses a single dose of 20-30 Gy). Transfection of the HSV-*tk* gene under control of the *EGF-1* promoter rendered irradiated, but not nonirradiated, cells sensitive to GCV. Irradiation induces DNA repair, cell cycle arrest, and reinitiation of DNA synthesis in surviving cells; γ-radiation also induces higher levels of a number of proteins including p53, AP-1, NF-κB, TNF, IL-1, and EGF-1. Therefore, use of the *EGF-1* promoter can activate gene expression selectively in radiation fields and could be used to drive the expression of cytotoxic genes (HSV-tk) for the killing of cancer cells.

Peptides containing the three zinc fingers of Zif268 could efficiently repress activated transcription from promoter constructs prepared with Zif268 binding sites inserted at various positions with respect to the TATA box (Kim and Pabo, 1997); such strategies could find important applications in gene therapy leading to construction of artificial promoters able to activate or repress transcription of transferred genes. A potent hybrid CAG promoter was used to drive the HSV-tk gene and showed effective eradication of pancreatic tumors in animal xenografts (Aoki et al, 1997).

D. Molecular switch systems

The ability to regulate gene expression via exogenous stimuli will facilitate the study of gene functions in mammalian cells. Molecular switch systems have been devised (Wang et al, 1994) allowing the researcher to turn on or off individual genes; the switch used by Delort and Capecchi (1996) is composed of three elements: **(i)** the inducible UAS promoter, a synthetic promoter containing five GAL4 response elements, normally absent from mammalian genomes; **(ii)** the synthetic hybrid steroid receptor (TAXI), composed of the GAL4 DNA -binding domain, a truncated human progesterone receptor, and the acidic region from VP16 protein of HSV; the hybrid molecule activates transcription from the UAS promoter when bound to an inducer drug, and **(iii)** the synthetic nontoxic drug inducer RU486 which is permeable to blood-brain and placental barriers; this model allows up to 100-fold induction of a gene linked to this system and can be finely tuned to lower levels of induction (Delort and Capecchi, 1996).

Transient cotransfection of HeLa cells with the UAS-CAT and the hybrid receptor expression vector showed that the hybrid TAXI protein bound to the UAS promoter only after treatment with RU486 but not progesterone; the TAXI/UAS system was successfully used in transgenic mice to regulate the expression of a human growth hormone gene; the ex vivo approach, however, did not sustain long-term expression of the transgene. This system might allow physicians to alter the level of expression of foreign genes during somatic cell transfer in response to the clinical state of the patient (Delort and Capecchi, 1996).

Iida et al (1996) have modified the tetracycline-controlled inducible system by the addition of the ligand-binding domain of the estrogen receptor to the carboxy terminus of the tTA transactivator; a single retroviral vector could transduce both the transactivator gene and the gene of interest controlled by the tTA-inducible promoter into mammalian cells; cell lines expressing the transactivator were established where the expression of a gene (the toxic G protein of vesicular stomatitis virus) depended on the removal of tetracycline and the addition of estrogen.

A different genetic switch used consisted of the cytochrome P450 1A1 promoter driving the expression of the human apolipoprotein E (apoE) gene in transgenic mice; this switch system was induced by β-naphthoflavone; the inducer could pass transplacentally and via breast milk from an injected mother to her suckling neonatal pups, giving rise to the induction of human apoE in neonate plasma and lowering the cholesterol levels in hypercholesterolemic pups (Smith et al, 1995).

XII. DNA recombination in gene therapy

A. Mechanisms of DNA recombination

Genetic recombination, i.e., exchange of segments of DNA between two molecules of DNA, is a very frequent event. It often occurs during meiosis and also between homologous chromosomes in mitosis. Homologous recombination involving double-strand DNA breaks (DSBs), has similarities to mechanisms of repair of DSB lesions by cells. Specific recombinases have played and continue to play an important role in molecular evolution and genome shuffling; deregulation in recombination procecess is connected to chromosomal aberrations (inversions, translocations) in cancer. The double-strand-break repair model was put forward by Szostak and collaborators (1983) to explain genetic recombination in yeast. Recent studies (reviewed by Stahl, 1996) have isolated the recombination intermediate molecules predicted by the DSB repair model; in this model, a 5'-3' exonuclease is responsible for the removal of segments of single strands starting bidirectionally from the DSB followed by invasion, repair synthesis and ligation to give the joint molecule which is then reduced to a pair of duplexes by a Holliday junction resolvase.

The development of mature lymphocytes in mammals results from a complex combination of genetically preprogrammed events and interactions with antigens. Shared in its general mechanisms by both B (bone marrow) and T (thymus) lymphocytes this developmental program involves a series of cell migration gene rearrangements, cell-to-cell contacts, as well as positive and negative selection processes; recombination mechanisms take place at the immunoglobulin and the T cell receptor genes to generate a large number of immunoglobulin genes in different lymphocyte clones. One site-specific recombination event brings together the V and the J segments of the light chain immunoglobulin genes. In the case of the heavy chain genes, one recombination event joins a V to a D segment, sequentially followed in a time frame by the joining of the recombined V-D segment to a J segment. Recent studies have shown that the mechanism of V(D)J recombination is a two-step process involving: **(i)** site-specific DNA cleavage at the 7mer sequence and at the first nucleotide of the coding sequence, implicating the RAG-1 and RAG-2 proteins which are necessary and sufficient for this step (van Gent et al, 1996); **(ii)** joining of broken ends in a mechanism similar to the repair of double strand breaks. The murine SCID locus has provided crucial information in the elucidation of the second step in V(D)J recombination: thymocytes in SCID mice are able to catalyze joining of signal ends but display an accumulation of hairpin coding ends (Zhu et al, 1996). The murine SCID locus has been mapped to the gene encoding the catalytic subunit of DNA-dependent protein kinase (DNA-PK) (Kirchgessner et al, 1995).

Group I introns from a variety of organisms contain long open reading frames (ORFs) that encode site-specific DNA endonucleases which promote integration of their DNA into cognate sites via homologous recombination. These endonucleases typically cleave intron-lacking DNA near the site of intron insertion (exon-exon junction) creating a staggered DSB which facilitates intron invasion (intron homing). This mechanism has been demonstrated in mitochondria, chloroplasts and nuclei of eukaryotic cells. I-CreI is a member of this class of molecules that promotes homing of the chloroplast 23S rRNA intron in *Chlamydomonas reinhardtii* ; I-CreI contains once the LAGLI-DADG motif (whereas other members of the family contain two copies of this motif separated by 90-120 amino acids); this motif is important for the endonuclease activity of the molecule. DNA cleavage by I-CreI requires Mg^{2+} or Mn^{2+} and is inhibited by monovalent cations, has an optimum for catalytic activity of 50-55ºC, is stabilized by DNA and binds to 12 nt on each target strand (Wang et al, 1997).

B. Aberrant recombinations can result in human disease

Mammals carry about 1,000,000 copies of Alu sequences and 10,000 to 100,000 copies of complete and truncated versions of the L1 class of LINEs. Such sequences promote homologous recombination causing translocations of genes and have been hold responsible for a number of human disorders. Alu sequences are found in introns. One type of mutation in the LDL receptor gene responsible for familial hypercholesterolemia has incurred by Alu-Alu recombination deleting several exons and thus producing a truncated receptor molecule with loss of function (Lehrman et al, 1987). *De novo* insertions of an L1 element into the factor VIII gene can cause hemophilia A in humans (Kazazian et al, 1988).

Foreign DNA transferred to host cells may be rejected (degraded), integrated at random sites by illegitimate recombination, integrated at homologous sites by legitimate recombination, or remain extrachromosomal and replicate autonomously. The homologous recombination between chromosomal DNA and transfected DNA sequences, an event termed "gene targeting," can be used to correct mutated genes in cultured cells.

It has been known for a long time that the translocation of an active gene to the neighborhood of heterochromatin (transcriptionally inert part of the genome) results in silencing of the translocated gene, a process known as "position effect variegation", first described in *Drosophila* (Lewis, 1950; Wilson et al, 1990).

Chromosomal translocations seem to contribute to tumorigenesis either by activating proto-oncogenes to oncogenes or by inactivating tumor suppressor genes. Mammalian chromosomes contain a number of break-susceptible or fragile sites where breakage can be induced reproducibly by experimental manipulations. Such fragile

sites might lie in the neighborhood of transposable elements, hypervariable minisatellites or other DNA structural peculiarities such as Z-DNA, and other hotspots of recombination (reviewed by Haluska et al., 1987).

Nonrandom chromosome rearrangements, observed in a variety of human and animal tumors are associated with the enhanced expression or deregulation of cellular oncogenes. For example, the human c-*myc* oncogene becomes active following its translocation close to the enhancer sequence within the immunoglobulin heavy chain gene locus (Hayday et al., 1984). The chromosomal translocation (17;19) in acute lymphoblastic leukemia produces a chimeric transcription factor consisting of the amino-terminal portion of the helix-loop-helix proteins E12/E47 fused to the DNA binding and leucine zipper dimerization motifs of the liver-specific protein factor Hlf (Hepatic leukemia factor), normally not expressed in lymphoid cells (Hunger et al., 1992). In pre-B cell acute lymphoblastic leukemia (ALL) the t(1;19) translocation brings together two gene fragments encoding for transcription factors and results in the synthesis of a chimeric transcription factor composed of truncated E2A and Pbx1 (Kamps et al., 1991).

C. Exploitation of recombinases in gene therapy: the Cre/LoxP system

A strategy has recently been developed which facilitates culturing of human cells derived from primary tumors. This method is based on the transient expression of T antigen of SV40 which has been shown to immortalize primary cells of human and murine origin and on the use of bacteriophage recombinase Cre which catalyzes sequence-specific recombination at the LoxP sequence inducing permanent deletion of T antigen cDNA; indeed, if two LoxP sequences were provided as direct repeats the intervening sequence could be deleted during Cre recombination and lost from cells. This is an advantage for primary cell cultures because the continuous expression of SV40 large T antigen may alter the antigenicity of the cells and induce other type of mutations not associated with the original tumor; according to this strategy large T antigen can be expressed in a time-dependent way (Li et al, 1997).

Figure 14 shows the structure of the two retroviral vectors used by Li et al (1997) to facilitate culturing of primary tumor cells and **Figure 15** the change in morphology as a result of Cre-Puro retrovirus-induced loss of expression of T antigen in T antigen-immortalized primary cell cultures of mouse breast cancer cells.

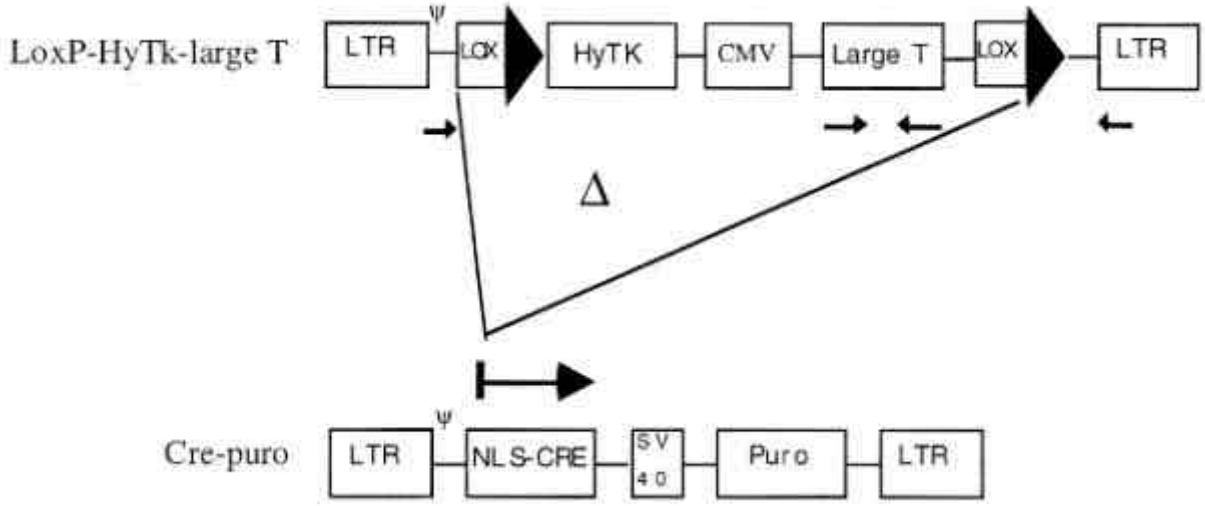


Figure 14. Structure of retroviral vectors LoxP-HyTK-large T and Cre-puro allowing expression of T antigen and Cre/LoxP-catalyzed deletion of T antigen cDNA. The 4 small arrows indicate the primers used for PCR analysis; the large arrow above the NLS-CRE box indicates the Cre recombinase mediating LoxP specific deletion. LTR are the long terminal repeats of Moloney Murine Leukemia virus; LOX is the 34 bp sequence identical to the recognition site of Cre recombinase; HyTK is the hygromycin/thymidine kinase fusion gene; NLS-CRE is the Cre recombinase gene targeted to the nucleus by a nuclear localization signal (NLS); SV40 is the SV40 promoter; Puro is a puromycin selection marker. From Li LP, Schlag PM, Blankenstein T (**1997**) Transient expression of SV 40 large T antigen by Cre/LoxP-mediated site-specific deletion in primary human tumor cells. **Hum Gene Ther** 8, 1695-1700. Reproduced with the kind permission of the authors and Mary Ann Liebert, Inc.

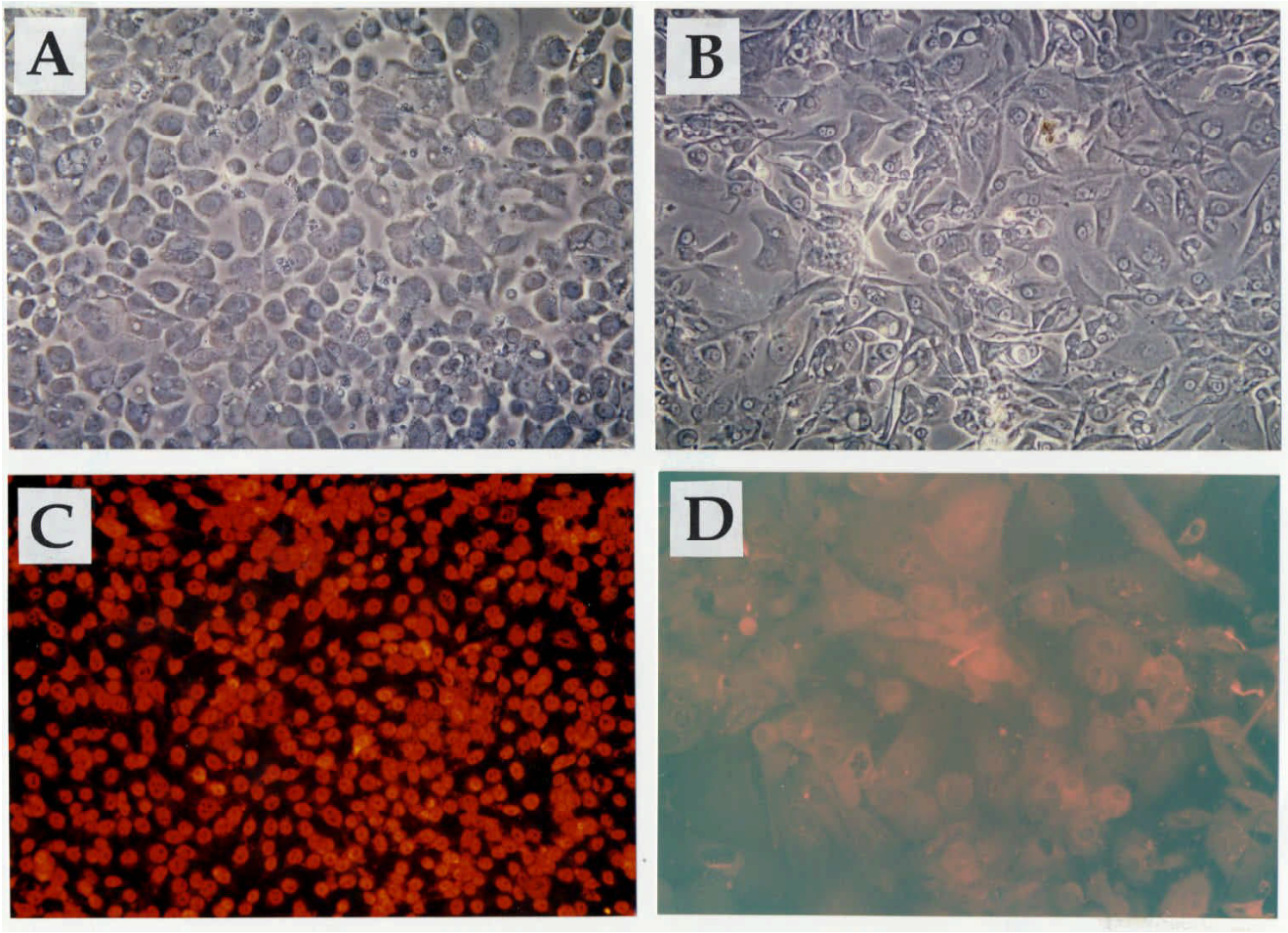


Figure 15. Large T antigen-immortalized breast cancer cells change morphology and lose expression of T antigen after infection with Cre-Puro retrovirus: after prolonged culture the cells were infected with Cre-Puro retrovirus (**B, D**) or mock-infected (**A, C**), selected for Puromycin resistance (A, B, C, D) and resistance to ganciclovir (B, D) and analyzed by light microscopy (A, B) and staining with a large T-antibody (C, D). From Li LP, Schlag PM, Blankenstein T (**1997**) Transient expression of SV 40 large T antigen by Cre/LoxP-mediated site-specific deletion in primary human tumor cells. **Hum Gene Ther** 8, 1695-1700. Reproduced with the kind permission of the authors and Mary Ann Liebert, Inc.

Reversible immortalization of primary cells was achieved by Westerman and Leboulch (1996) using retrovirus-mediated transfer of an oncogene that could be subsequently excised by site-specific Cre/LoxP recombination; the FLP/FRT recombination was not efficient in primary cells. Pure populations of cells in which the oncogene was permanently excised were obtained which reverted to their preimmortalized state. Using the Cre/LoxP recombination strategy primary cells could be cultured and expanded; the method was proposed to be applicable for facilitating gene transfer to cells unresponsive to exogenous growth factors.

A retroviral vector, containing both a neomycin resistance expression unit flanked by loxP sites and GM-CSF cDNA, was used to transduce the human hematopoietic K-562 cell line. Superinfection of K562 cell clones with a retrovirus containing a Cre recombinase expression unit and molecular analyses of 30 doubly transduced subclones showed a strict correlation between Cre expression and LoxP-flanked selectable cassette excision; excision of the selectable cassette resulted in a significant increase of GM-CSF transcription driven by the retroviral promoter (Fernex et al, 1997).

Novel retroviral vectors for gene transfer were developed by Bergemann et al (1995) by inserting two LoxP sites into a retroviral vector also containing the HSV-tk gene; Cre expression in cells infected with this vector was followed by BrdU selection for cells in which site-specific recombination took place. Furthermore, replacement of the enhancer/promoter elements in both LTRs by Lox sequences led to the development of retroviral suicide vectors for gene therapy. Vanin et al (1997) have used the Cre/LoxP recombinase system to generate high-titer retroviral producer cell lines;

incorporation of LoxP sites at the flanks of a Neo^R-HSV-tk cassette in the proviral DNA allowed excision of these selectable markers through expression of Cre recombinase and the production of a high-titer producer cell line containing a single LoxP site flanked by the viral LTRs. Retransfection of this cell line with a plasmid containing a gene of interest flanked by LoxP sites and the Cre expression vector allowed insertional LoxP/LoxP recombination of the gene into the favorable preexisting site in the genome and the generation of a new line with a titer equivalent to that of the parental producer cell line. The Cre/LoxP recombinase strategy has been used to generate retroviral vectors with the ability to excise themselves after inserting a gene into the genome (Russ et al, 1996).

Bushman and Miller (1997) fused retroviral integrase enzymes to sequence-specific DNA-binding domains and investigated target site selection by the resulting proteins. A fusion protein composed of HIV integrase linked to the DNA-binding domain of λ repressor was able to direct selective integration of retroviral cDNA in vitro into target DNA containing λ repressor binding sites. A fusion of HIV integrase to the DNA binding domain of the zinc finger protein Zif268 also directed increased integration near Zif268 recognition sites.

Introduction of foreign DNA into cell nuclei with recombinase cDNA and appropriate sequences to promote recombination may promote nor only insertion of a therapeutic gene into a specific chromosomal site but also chromosomal rearrangements that could convert therapeutically transduced cells into malignant. There is a great deal of knowledge to be derived from these very promising strategies of gene therapy before they can be successfully applied to humans.

XIII. Fate of the transgene in the nucleus

A. How to sustain transgene expression?

A major drawback in gene therapy applications is loss in gene activity within a few days from gene transfer although all previous steps were successful. In other words, the transferred gene is transiently expressed for 1-4 days and its expression thereafter declines dramatically. This is due **(i)** to the degradation of the gene in the nucleus; **(ii)** the dilution of the plasmid during replication of the cells from its inability to replicate; **(iii)** its inactivation by position effects from chromatin surroundings after its integration into the chromosomal DNA; **(iv)** the elimination of the therapeutic cells expressing the transgene by the immune system of the organism either because of the antigenicity of the expressed protein or because of the antigenicity of viral proteins, an effect often associated with adenoviral and retroviral gene delivery.

A number of strategies are being pursued to solve these problems. Sustaining the expression of a transgene into somatic cells for, lets say, 6 months would mean than a gene therapy treatment would need to be repeated twice a year, for example to a hemophilia patient or to a patient who has undergone balloon treatment after coronary heart disease and is being treated via arterial gene transfer.

An approach to sustain expression of the transgene is via episomal replication of the plasmid carrying the transgene for long periods of time, maintaining the plasmid in high copy numbers, and in a form replicating in synchrony with the cell cycle; even better a plasmid can be replicated continuously independently of the cell cycle, an approach to find application in the transfection of nondividing cells by plasmids (which to date is a virtue of adenoviruses, AAV, and HIV-1 vectors; see **Table 1**).

A way to sustain expression of the transgene could be achieved via targeted integration into one or several different chromosomal locations and the insulation of the transgene from neighboring chromatin domains using special classes of DNA sequences able to act as insulators and maintain independent realms of gene activity (such as matrix-attached regions, MARs). In this case flanking of the foreign gene by two MAR sequences is expected to insulate it against position effect variegation and prevent inactivation of the gene at the chromatin level by chromatin condensation or other mechanisms propagated from the neighboring domains at the integration site (Boulikas, 1995b).

Several studies have shown that linearization of plasmids with restriction enzymes favor highly their integration into the host's genome compared with supercoiled, covalently-closed plasmid DNA. Free ends of DNA are known to promote recombination and a number of nuclear proteins including p53, poly(ADP-ribose) polymerase, ligases I and II, Ku antigen, DNA-dependent protein kinase are known to bind to free ends of DNA, whereas other molecules such as helicases and endonucleases are known to function during repair of lesions in DNA inducing the appearance of strand breaks; especially important in this aspect are members of the RAD50-57 family of proteins involved in recombination and in repair of double-strand breaks.

B. Episomal plasmids for gene transfer

Integration or replication of a foreign gene introduced as a plasmid into mammalian cells is a very rare event; plasmid DNA resides transiently in the nucleus as an episomal, extrachromosomal element for short periods of time after transfection of cells in culture (usually up to one or very few days) during which transcription can take place; after that the episomal DNA is degraded and lost permanently from the cells.

Viral origins of replication have been introduced into the same plasmid as the reporter gene and found to increase the persistence of expression. A polyoma virus-based plasmid containing the polyoma virus origin of replication and the T antigen gene, as well as the neo^R gene was maintained extrachromosomally in mouse embryonic stem (ES) cells at 10-30 copies per cell for at least 74 cell

generations in the presence of G418 (Gassmann et al, 1995).

Prolonged episomal persistence may be an advantage for gene therapy of nondividing cells. A limited number of studies in gene transfer have used plasmids able to replicate episomally. Most of the plasmids used contain viral origins of replication but also the gene of the replication initiator protein that after its expression in the host will interact with the origin of the plasmid to maintain a relatively high copy number of plasmids which will persist for some time. The advantage using episomal replication of plasmids is enormous in somatic human gene therapy as it can sustain expression of a transgene for a few months after a single injection of the plasmid as compared to the loss of expression after about 1-10 days (maximum at day 2) following injection of nonepisomal plasmids (Zhu et al, 1993). Thierry and coworkers (1995) have succeeded in sustaining the expression of the luciferase reporter gene in mice for up to 3 months after a single intravenous injection of a plasmid including the human papovavirus BKV early region and origin of replication, the large tumor antigen (T antigen) as the replication initiator protein, and the late viral capsid proteins in the same construct harboring the luciferase gene; this plasmid was shown to be replicated extrachromosomally for 2 weeks in the lung.

Episomal replication of a hybrid HSV-1/EBV vector was achieved when the latent oriP of EBV and the *EBNA-1* cDNA, which encodes for the replication initiator protein of EBV, were included in the vector (Wang and Vos, 1996).

Expression of viral replication initiator proteins (e.g. T antigen) is oncogenic. Of special interest in human gene therapy is to determine human DNA sequences able to sustain the extrachromosomal replication of plasmids into permissive human cells for longer periods. Such DNA sequences known to act as origins of replication, although poorly understood, have been found in human, monkey, and other mammalian genomes and could be used to sustain the replication of the plasmid thus increasing its copy number in the cell and the time of its persistence (see page 122-123).

To this end, a technology has been developed in our laboratory that permits us to isolate human origins of replication (ORIs) and to include selected ORIs together with the cDNA of the replication initiator protein responsible for activating this particular ORI, in plasmids with therapeutic genes (Boulikas et al, in preparation).

C. Considerations of chromatin structure of plasmids during gene delivery

Almost all supercoiled plasmids used in gene transfer, as produced in bacteria, are under negative supercoiling. Immediately after their import into nuclei plasmids are packaged into nucleosomes that absorb and constrain part or most likely all of the negative supercoils. This is true assuming that no cuts on the DNA are introduced during its passage through the cell membrane barrier to cytoplasmic lysosomes before entering nuclei; if DNA is cut the supercoils on the plasmid will be relaxed. Nicked DNA might be repaired and ligated in nuclei by DNA ligases and be subject to the same constrains as chromosomal DNA. Use of linear plasmids is expected to stimulate recombination during repair of double strand breaks (also would increase degradation of the plasmid in the nucleus and loss of the transgene) ultimately resulting in plasmid integration at variable chromosomal loci, determined to some extend by the nature of the free ends of DNA and the short terminal sequence of the DNA at the ends as well as the type of recombinase molecules in the cell type used.

Treatment of cell cultures with sodium butyrate inducing hyperacetylation of core histones would reverse in part the relieving of the negative torsional strain by the wrapping of the plasmid around histone octamers and will provide DNA in a negatively superhelical of underwound form able to sustain transcription of the template (Schlake et al, 1994).

D. Overcoming the influences of chromosomal surroundings at plasmid integration sites

Use of two MARs each flanking the reporter gene on either side is expected to form a minidomain after integration of the foreign gene into a chromosomal site. MARs potentiate the effect of promoters and enhancers when two MAR elements are placed one upstream and the other downstream from control elements but not between them. MARs will **(i)** shield reporter genes from the influences of chromosomal surroundings that most often cause inactivation of foreign genes. This effect of chromatin structure on neighboring sequences is known as position effect variegation. Indeed about 85% of the chromosomal sites are transcriptionally inactive assuming that 15% of the genomic DNA is transcribed; however, even integration of a foreign gene into an active chromatin locus may not warrantee its transcriptional activation as other parameters, such as proximity of the integration site to the natural promoter and enhancer elements of the active chromatin domain, or orientation of the integrated gene with respect to the active gene in the chromosomal DNA may determine its level of expression. **(ii)** MARs will maintain a supercoiled DNA topology within the domain thus increasing the negative supercoiling at local promoter and enhancer sites, a prerequisite for efficient transcription (see Boulikas, 1995b).

XIV. Transfer of reporter genes

A. Transfer of the β-galactosidase (lacZ) reporter gene

Before a gene therapy preclinical study or even gene transfer to cells in culture begins it is essential to test the variables and pinpoint the conditions leading to the success of the operation using reporter gene transfer. *LacZ*,

encoding the β-galactosidase (β-Gal) from *E. coli* is one of the most commonly used reporter genes. A staining procedure for this enzymatic activity can result in the generation of blue color using X-Gal as a substrate leading to the direct visualization of its activity, for example, in thin sections through animal tissues.

Transfer of the reporter β-galactosidase gene to human liver tumors in nude mice was performed by Wang and Vos (1996) using a hybrid HSV-1/EBV vector which replicates episomally when the latent oriP of EBV and the *EBNA-1* cDNA were included.

Many mammalian tissues, especially intestine, kidney, epididymis, and lung contain endogenous β-Gal, a lysosomal enzyme participating biochemically in glycolipid digestion. Weiss et al (1997) were able to detect mammalian β-Gal activity on histochemical preparations of mouse, rat and baboon lung tissue (**Figure 16**) and also to distinguish between the endogenous and bacterial β-Gal activity in airway epithelial cells in the transgenic Rosa-26 mouse in based upon the differences in pH optima between the mammalian and bacterial enzymes (**Figure 17**). Time and temperature of exposure to X-Gal could not be used to distinguish between endogenous and exogenous β-Gal activity; thus, exposure of tissue preparation to pH 8.0-8.5, which minimized detection of the endogenous activity allowed unambiguous discrimination and was the method of choice to detect reporter β-Gal activity.

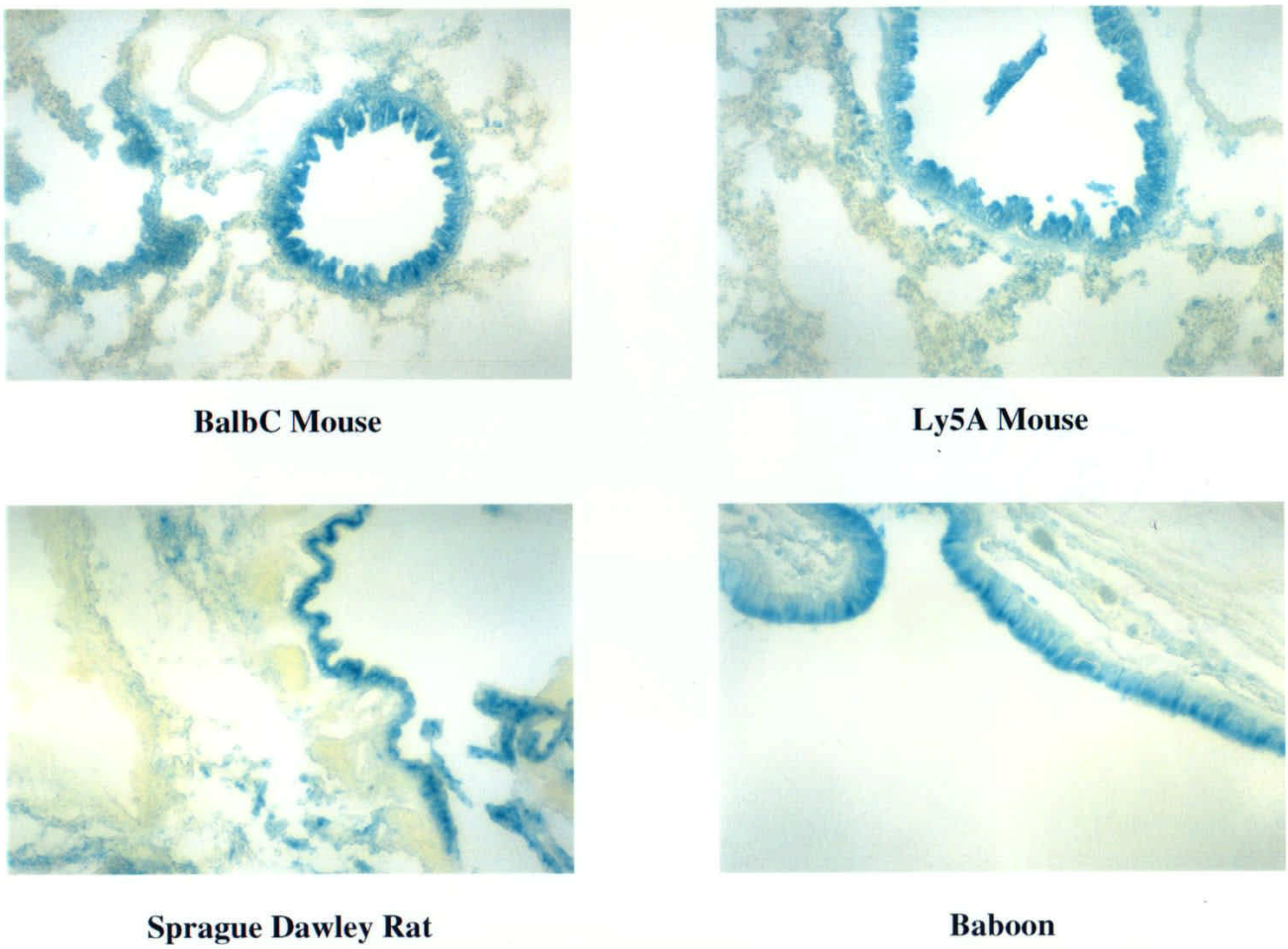


Figure 16. Endogenous mammalian β-Gal activity was detected in minced lung preparations from a variety of species following incubation with X-Gal in PBS. Representative fields from paraffin-embedded sections are shown. Original magnification 400X. Airway epithelium, alveolar macrophages and several unidentified cell types are stained blue and are, therefore, positive for β-Gal activity. From Weiss DJ, Liggitt D, Clark JG (**1997**) In situ histochemical detection of β-galactosidase activity in lung: assessment of X-Gal reagent in distinguishing lacZ gene expression and endogenous β-galactosidase activity. **Hum Gene Ther** 8, 1545-1554. With the kind permission of the authors (Daniel Weiss, Fred Hutchinson Cancer Research Center) and Mary Ann Liebert, Inc.

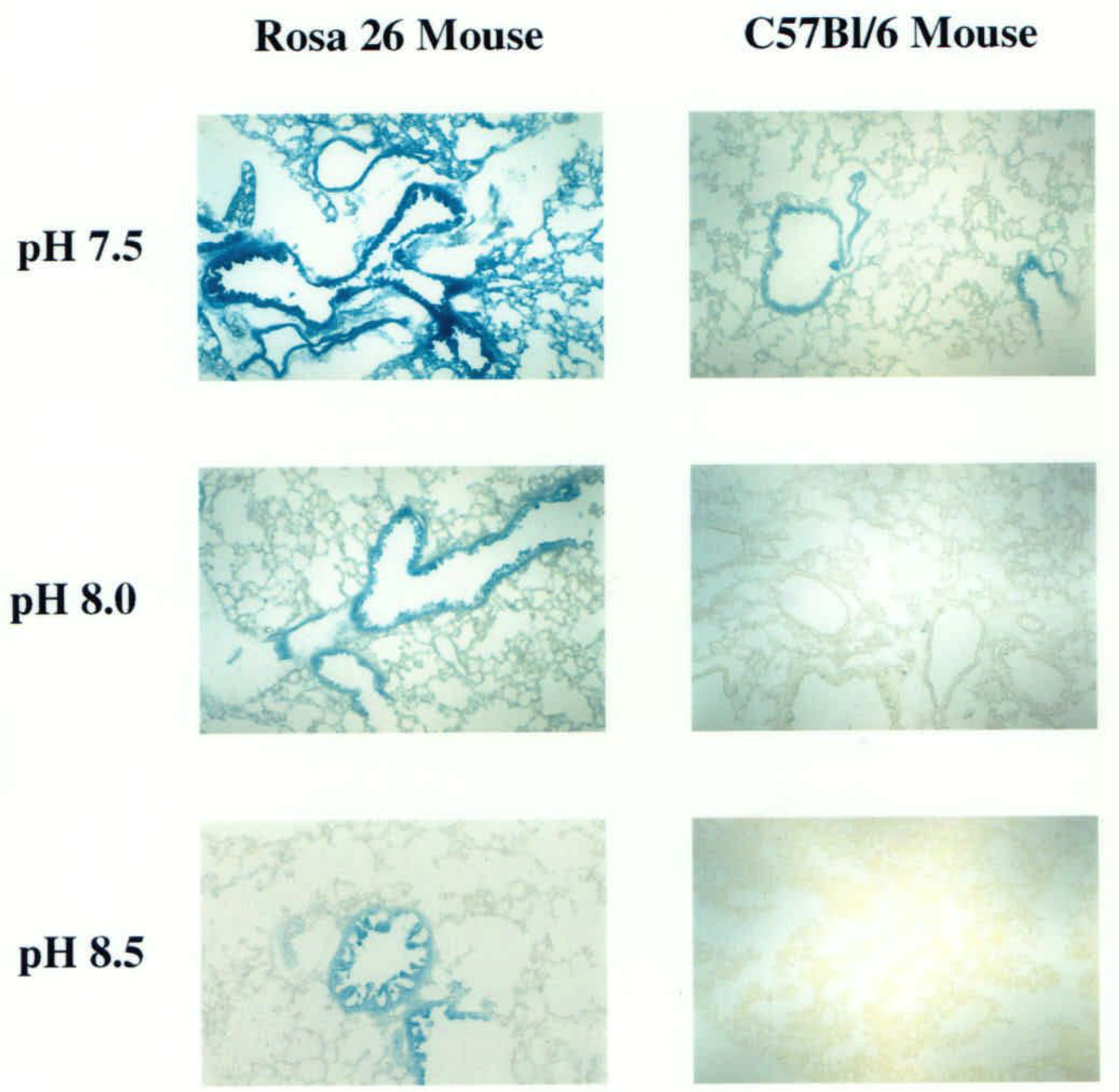


Figure 17. Endogenous mammalian β-Gal activity was not detected following incubation with X-Gal at pH>7.5 whereas bacterial β-Gal activity was detected at pH 8.0-8.5 in airway epithelial cells in the transgenic Rosa-26 mouse. Original magnification 250X. From Weiss DJ, Liggitt D, Clark JG (**1997**) In situ histochemical detection of β-galactosidase activity in lung: assessment of X-Gal reagent in distinguishing lacZ gene expression and endogenous β-galactosidase activity. **Hum Gene Ther** 8, 1545-1554. With the kind permission of the authors (Daniel Weiss, Fred Hutchinson Cancer Research Center) and Mary Ann Liebert, Inc.

Table 2. Reporter gene transfer in vivo

Gene delivered	Human disease	Vector	Method/Goal	Results	Reference
CAT and luciferase	none	DOTMA: DOPE	Expression of reporter genes in mouse tissues after a single intravenous injection.	CMV promoter was most effective; expression in vascular endothelia, extravascular and parenchymal cells present in lung, spleen, and heart; lower expression in all other tissues; persisted for 9 weeks.	Zhu et al, 1993
Luciferase/ CMV construct and lacZ	none	Transfectam (DOGS :DOPE)	To show effectiveness in gene transfer after intracranial injection of liposome-plasmid complexes into the newborn mouse.	Transient expression of luciferase in striatal parenchymal cells; lipospermine:DNA charge ratio of 2 or smaller was most effective in vivo.	Schwartz et al, 1995
Luciferase/ CMV	none	Cationic liposomes	Distribution of luciferase gene expression among tissues and persistence of expression after systemic injection.	Use of viral origins of replication and T antigen cDNA in vector sustained expression up to 3 months primarily in the lung in mice.	Thierry et al, 1995
lacZ and HSV-tk/IL-2	glioma	AAV	Direct injection of tumors induced from human glioma cells into the brains of nude mice.	30-40% of the cells along the needle track expressed β-galactosidase; administration of GCV to the HSV-tk/IL-2 treated animals for 6 days, resulted in a 35-fold reduction in the mean volume of tumors compared with controls.	Okada et al,. 1996
lacZ and human erythropoietin	none	AAV	Single intramuscular injection into adult BALB/c mice.	Protein expression was detected in myofibers for at least 32 weeks; dose-dependent secretion of erythropoietin and corresponding increases in red blood cell production in mice persisted for up to 40 weeks.	Kessler et al, 1996
lacZ	Retinal degeneration ; retinitis pigmentosa	AAV	Subretinal injection of recombinant AAV particles encoding lacZ.	Successful transduction of all layers of the neuroretina as well as the retinal pigment epithelium; the efficiency of transduction of photoreceptors was significantly higher than that achieved with an equivalent adenoviral vector.	Ali et al, 1996
lacZ	Inherited hearing disorders	AAV	To assess the feasibility of introducing genetic material directly into the peripheral auditory system; infusion into the cochlea of guinea pigs.	Thin sections of cochleae showed intense immunohistochemical reactivity in the spiral limbus, spiral ligament, spiral ganglion cells and the organ of Corti but much weaker staining in the contralateral ear.	Lalwani et al, 1996
lacZ	Parkinson's disease	HSV-1	Stereotactic injection into the midbrain of adult rats.	A 6.8-kb fragment of the rat tyrosine hydroxylase promoter supported a 7- to 20-fold increase in reporter gene expression in catecholaminergic tyrosine hydroxylase-expressing neurons in the substantia nigra.	Song et al, 1997
lacZ	Leptomeningeal cancer	AAV	To test the feasibility of AAV-mediated gene therapy.	Successful transduction of medulloblastoma (DAOY) cells in a nude rat model of leptomeningeal disease.	Rosenfeld et al, 1997
lacZ	vascular disorders	AAV	To develop gene therapies for vascular disorders by gene transfer into isolated segments of normal and balloon-injured rat carotid arteries.	Comparable gene transfer into medial and adventitial cells, with significantly higher efficiency of transduction in injured compared with normal vessels.	Rolling et al, 1997
human placental alkaline phosphatase (AP)	lung disease	AAV	To assess the ability of AAV vectors to transduce airway cells; AP gene was delivered to one lobe of the rabbit lung using a balloon catheter.	AP staining was almost exclusively in the epithelial and smooth muscle cells in the bronchus at the region of balloon placement; staining was in ciliated cells but was also in basal cells and airway smooth muscle cells.	Halbert et al, 1997

A phase I study involving six patients with inoperable lung cancer and an endobronchial lesion accessible by bronchoscopy was initiated to evaluate the feasibility, tolerance, and clinical effects using adenoviral delivery of the *Escherichia coli* lacZ marker gene driven by the RSV promoter; biopsy specimens of the tumor and surrounding mucosa in 5 patients were tested positive for β-galactosidase expression (Tursz et al, 1996).

B. Transfer of the luciferase and green fluorescent protein (GFP) reporter genes

A synthetic polyamino derivative was used by Goldman and coworkers (1997) to transfer the luciferase and β-galactosidase reporter genes in animal models bearing stereotactically implanted human glioma cell xenografts. The luciferase reporter gene was transferred in both newborn and adult rabbit lungs using polyethylenimine (Ferrari et al, 1997).

Thierry and coworkers (1995) have succeeded in sustaining the expression of the luciferase reporter gene in mice for up to three months using episomal vectors (**Table 2**).

GFP has been used as a reporter molecule for gene expression because it fluoresces green after blue-light excitation. However, many attempts by Hanazono et al (1997) to isolate stable retroviral producer cell clones secreting vectors containing GFP (after transfer of the neoR gene and selection in G418) have failed because stable GFP-clones were undergoing major rearrangements or other mutations which abrogated GFP expression and prevented vector production.

Additional studies using reporter gene transfer are summarized on **Table 2**.

DIVISION TWO: GENE THERAPY OF CANCER

XV. Cancer immunotherapy and tumor vaccines

A. The molecular basis of cancer immunotherapy

Many human tumors are nonimmunogenic or weakly immunogenic. The immune system, evolved to rid the body of unwanted intruders, could be instructed and reinforced to eliminate cancer cells. Increasing the immunogenicity of tumors by causing local cytokine production or by increase in the expression in MHC antigen can lead to local antitumor effect (Tepper et al, 1989; Fearon et al, 1990). Indeed, immune surveillance is of the major defense mechanisms against cancer; immunosuppressed individuals are more prone to cancer and nude mice, lacking immune response, are exploited in the lab to elicit tumors after injection of tumorigenic cells, a response that, in many cases, cannot be elicited in normal mice.

Cells undergoing malignant transformation are believed to be eliminated from the body by white blood cells including natural killer cells (NK), lymphokine-activated killer cells (LAK), cytotoxic T lymphocytes (CTL), tumor-infiltrating lymphocytes (TIL), and activated macrophages; since established cancers in the human body may escape this potential defense mechanism of immunologic surveillance, cancer patients have been treated with IL-2 to stimulate their cellular immune mechanisms to kill cancer cells; lengthy and complete remissions, however, were at a low rate and complications were encountered by the toxicity caused by the systemic administration of IL-2 (Rosenberg, 1992).

Transfection of the *IL-2* gene into human melanoma cells increased cellular immune response (Uchiyama et al, 1993). This and similar approaches have established the foundation of the ex vivo cancer immunotherapy by transfer of autologous (cancer patient's) cells after transduction in vitro with cytokine genes (see below). The ultimate goal is the activation of tumour-specific T lymphocytes capable of rejecting tumour cells from patients.

B. Cancer immunotherapy with tumor infiltrating lymphocytes (TILs)

Ex vivo approaches in immunotherapy have been aimed at isolating T cells directly from tumors (known as tumor infiltrating lymphocytes or TILs), stimulate TILs to proliferate in cell culture with IL-2 followed by their reintroduction into the blood stream of advanced cancer patients (Rosenberg et al, 1988). The adoptive transfer of TILs was 50-100 times more potent than that of lymphokine-activated killer (LAK) cells isolated from the patient's tumors. Treatment of 20 patients with TILs after their expansion in vitro, plus IL-2, resulted in objective regression of metastatic melanomas in lungs, liver, bone, skin, and subcutaneous sites which lasted for several months (Rosenberg et al, 1988).

TILs were also transfected in vitro with the bacterial neomycin-resistance gene and were reintroduced into patients with advanced cancer in order to follow their persistence in blood circulation with PCR methods (Aebersold et al, 1990). Such "gene marking" clinical protocols using TILs are numbers 1, 3, 9. 13, 57, and 169 in **Appendix 1**, pages 159-172**.** Having shown safety in the NeoR-modified TIL protocol, the gene for tumor necrosis factor (*TNF*) was added to the vector for therapy of malignant melanoma in advanced cancer patients; the first patient began treatment in January 1991. TNF, however, is effective as an anticancer agent in mice at 400 mg/kg body weight, but in humans, TNF is toxic at 8 mg/Kg and so far of no proven therapeutic value at this low concentration (reviewed by Anderson, 1992). In a similar approach, the *TNF* gene was replaced by the gene of

interleukin-2 (*IL-2*) in order to develop locally high doses of IL-2 at the tumor site by immunization with TIL cells from the patient producing systemic antitumor immunity (Rosenberg et al, 1992).

TNF-α, (also IL-1β, IFN-γ, and vitamin D3) after binding to their transmembrane receptors stimulate the production of the second messager ceramide from sphingomyelin in the plasma membrane by activating sphingomyelinase; this results in a cascade of signal transduction events that result in down regulation of c-myc and induction of apoptosis, to terminal differentiation, or to RB-mediated cell cycle arrest (see apoptosis further below).

IL-2 stimulates the differentiation of precursor lymphocytes into LAK cells and further stimulates LAK cell proliferation; LAK cells are probably produced by activation of NK cells or from activated T cells by IL-2. Administration of IL-2 plus amplified LAK cells into mice models led to marked regression of disseminated cancers and leukemia. LAK cells are able to destroy tumor cells that express only weakly histocompatibility antigens. IL-2, however, has several pleiotropic effects: stimulation of B cell proliferation; activation of HLA class II antigen expression on endothelial cells, TILs, and melanoma cells; and enhanced production and release of TNF-α, and IFN-γ (see Cassileth et al, 1995 and the references cited therein).

However, the use of large numbers of adoptively transferred, broadly cytotoxic LAK cells in combination with IL-2 has been effective for only small subsets of cancer patients (reviewed by Wiltrout et al, 1995).

TILs, which could potentially kill tumor cells, are found in many tumors but remain suppressed or anergic; this anergy may arise from the absence of lymphokines which provide signals for TIL cell activation and stimulation to proliferation although ligands may be bound to the variable region of the T cell receptor; indeed, nonimmunogenic tumors are rejected by syngeneic mice upon transfection by *IL-2* or *IL-4* genes; IL-2 lymphokine production by the tumor cells bypasses T helper function in the generation of an antitumor response rendering the tumor cells immunogenic; nontransfected tumors are not rejected by the animal and grow causing its death (Tepper et al, 1989; Fearon et al, 1990).

Ex vivo gene therapy trials using cytokine gene transfer (see below) circumvent the problem of toxicity of IL-2 administration; for non-gene transfer therapies, white blood cells drawn from patients are fractionated, cultured, stimulated with IL-2 or other cytokines, and reintroduced in much higher numbers into the blood of the patient.

C. Cancer immunotherapy with cytokine genes

The combination of immunotherapy with conventional treatments such as radio- and chemotherapy may be necessary to eradicate minimal residual disease. Advanced therapies involve the transfection of lymphocytes in culture with cytokine genes followed by selection of the successfully transfected cells with a selectable marker such as the bacterial neomycin-resistance gene (Cassileth et al, 1995). Numerous phase I clinical trials employing either syngeneic genetically modified or allogenic tumor vaccines are in progress (see immunotherapy in **Appendix 1**, page 159-172). The development of tumor cells transduced with cytokine genes and their exploitation as tumor vaccines in patients with cancer is a very promising field (reviewed by Jaffee and Pardoll, 1997).

Cytokine genes used for cancer immunotherapy include those of IL-2, IL-4, IL-7, IL-12, IFNs, GM-CSF, TNF-α in combination with genes encoding co-stimulatory molecules, such as B7-I. The major goal of the use of immunostimulatory cytokines is the activation of tumour-specific T lymphocytes capable of rejecting tumour cells from patients with low tumour burden or to protect patients from a recurrence of the disease. As distant metastasis is the major cause for therapeutic failures in clinical oncology, treatment of patients having a low tumor volume with immunotherapy could protect the patient from recurrence of disease. Treatment of rodent tumor models with little or no intrinsic immunogenicity with this approach resulted in regression of preexisting tumors and cure of the animals from their disease; furthermore, in some instances cured animals had retained immunological memory and resisted a second challenge with the parental tumor cells (reviewed by Gilboa, 1996; Mackensen et al, 1997).

The transduction of the tumor cells of the patient with cytokine genes ex vivo and the development of tumor vaccines depends on the establishment of primary cell culture from the solid tumor. Although malignant melanomas are easy to culture, it is difficult to establish cell lines from most other primary human tumors using convenient methods; primary tumor cultures are being used **(i)** for the transduction of autologous cells from the cancer patient with cytokine genes to develop cancer vaccines after intradermal implantation to the patient; **(ii)** for characterization of tumor-specific cytotoxic T lymphocytes in order to identify specific antigens on the human primary culture; **(iii)** for extensive phenotypic characterization of the tumor in cell culture. The Cre/LoxP system (see recombinases in gene therapy) has been used to facilitate the establishment of primary cell lines from human tumors (Li et al, 1997).

Human gene therapy protocols 3 and 10 (**Appendix 1**) use immunization of cancer patients with autologous cancer cells transduced with the gene for **tumor necrosis factor** (TNF).

D. Cancer immunotherapy with the IL-2 gene

Active immunization with pancreatic tumor cells genetically engineered to secrete IL-2 were shown to inhibit pancreatic tumor growth in vivo; this was shown using a poorly immunogenic subcutaneous model of murine ductal pancreatic cancer by implanting tumor

Panc02 cells in C57BL/6 mice; whereas 90% of animals vaccinated with irradiated parental Panc02 and subsequently challenged with parental Panc02 cells developed tumors by 48 days only 40% of animals vaccinated with irradiated Panc02 cells engineered to secrete IL-2 and challenged with parental Panc02 cells developed tumors by 48 days (Clary et al, 1997).

According to a RAC-approved clinical protocol the gene for human interleukin-2 (IL-2) was transduced into a cell line established from the neoplastic cells of a patient with malignant melanoma; this procedure established an IL-2-secreting cell line with integration of the IL-2 gene into genomic DNA. The IL-2-secreting cells were irradiated, in a manner sufficient to inactivate 100% of the cells but insufficient to completely inhibit IL-2 synthesis, and administered to 12 patients with metastatic malignant melanoma in a Phase I toxicity study. These cells have the capacity to induce an antimelanoma response as shown in animal studies (Das Gupta et al, 1997).

A significant number of RAC-approved clinical protocols use IL-2 cDNA transfer. These include protocols 11, 16, 19, 20, 46, 48, 50, 61, 71, 102, and 135, in **Appendix 1** and protocols 190, 197, 198, 200, 204, 211, 213, 215, and 219 using cationic lipids for gene transfer (**Table 4** in Martin and Boulikas, 1998, this volume, pages 203-206).

E. Cancer immunotherapy with the IL-3 gene

IL-3 was found to enhance the development of cytotoxic T lymphocytes; during antitumor response, macrophages could ingest whole tumor cells, cell fragments, or heat shock proteins complexed to antigenic peptides and then process the tumor antigens for presentation; IL-3 stimulated antigen-presenting cells (APCs), which are macrophage-like, within the tumor leading to generation of cytotoxic T lymphocytes (CTLs). This constitutes a plausible pathway for enhancement in tumor rejection by IL-3 stimulation (Pulaski et al, 1996).

IL-3 signaling proceeding either via the JAK-STAT or the Ras-Raf pathways, stimulates a number of genes such as the *DUB-1* encoding a deubiquitinating enzyme the overexpression of which leads to G1 arrest (Zhu et al, 1996); deubiquitination might be an additional mechanism to couple extracellular signaling to cell growth. IL-3 signaling leads to stimulation in myeloid cell proliferation.

F. Cancer immunotherapy with the IL-7 gene

Primary cell cultures from 45 patients with malignant melanoma were transfected via electroporation with the gene encoding for human interleukin-7 (IL-7) resulting in the production of biologically active IL-7 without altering the expression of HLA class I and II, ICAM-1, and of a melanoma-associated antigen. Irradiation of the transfected cells with 10,000 cGy, which arrested tumor cell growth in vitro, did not affect the ability of the cells to secrete IL-7 in the culture medium; this approach, which does not use retroviruses, could be applicable in vaccination protocols for melanoma patients (Finke et al, 1997).

Transfer of the IL-7 cDNA for cancer immunotherapy is being used in a human clinical trial (protocol 70, Appendix 1).

G. Cancer immunotherapy with the IL-12 gene

IL-12 gene therapy is one of the more novel and promising approaches in cancer therapy. IL-12 is a heterodimeric cytokine composed of two subunits, p40 and p35, that requires the simultaneous expression of both the p35 and p40 chain genes from the same cell for production of biologically active IL-12. Coordinate expression of the IL-12 p40 and p35 genes in several solid tumor models has been found to induce strong and specific antitumor immune responses. A variety of biological functions have been attributed to IL-12 including the induction of IFN-γ and the promotion of predominantly Th1-type immune responses to antigens (Tahara et al, 1996).

The local secretion of IL-12 achieved by gene transduction suppressed tumor growth and promoted the acquisition of specific antitumor immunity in mice. This was shown by intradermal inoculation of mice with NIH3T3 cells transduced with expression plasmids or a retroviral vector expressing the murine IL-12 gene admixed with murine melanoma BL-6 cells; $CD4^+$ and $CD8^+$ T cells, as well as NK cells, were responsible for the observed antitumor effects resulting from IL-12 paracrine secretion. Transduction of tumor cells with B7.1 gene enhanced the antitumor immune response (Tahara et al, 1996).

The antitumor effect of several transgene expression plasmids encoding the cytokines IL-2, IL-4, IL-6, IL-12, IFN-γ, TNF-α, and GM-CSF was tested using the gene gun-mediated DNA delivery into the epidermis overlying an established intradermal murine tumor; this study showed that IL-12 gene therapy was much more effective than treatment with any other tested cytokine gene for induction of tumor regression as determined from the increased $CD8^+$ T cell-mediated cytolytic activity in the draining lymph nodes of tumor-bearing mice; treated animals were able to eradicate not only the treated but also the untreated solid tumors at distant sites; elevated systemic levels of IFN-γ, were found after IL-12 gene therapy. This approach is providing a safer alternative to IL-12 protein therapy for clinical treatment of cancers (Rakhmilevich et al, 1997).

Lieu et al (1997) have evaluated three IL-12 retroviral vector designs for their level of IL-12 expression in leukemia/lymphoma cells; these retroviral vectors were based on the murine stem cell virus (MSCV) which efficiently transduces functional genes into normal hematopoietic cells. MSCVpac-mIL-12 and MIPV-mIL-12

contained an encephalomyocarditis virus internal ribosome entry site for internal translation of bicistronic mRNA transcripts, while MDCVpac-mIL-12 carried an expression cassette in the U3 region of the 3' LTR. The MSCVpac-mIL-12 vector was more efficient and directed robust expression of both p40 and p35 IL-12 genes in several murine tumor cell lines of hematopoietic origin, including a T-cell lymphoma, a B-cell lymphoma, and a plasmacytoma/myeloma.

Adenoviral delivery of the IL-12 gene was effective against breast tumors (Bramson et al, 1996) or metastatic colon carcinoma (Caruso et al, 1996) in animal models: mice bearing breast tumors, injected intratumorally with a single dose of an adenovirus expressing IL-12 showed regressions in greater than 75% of the treated tumors; this effect was accompanied with a maximum expression of IL-12 within the tumor between 24 and 72 hr post-injection which lasted for 9 days and an elevation in IFN-γ within the tumor; local production of IL-12 also stimulated IFN-γ production in tumor-draining lymph node cells (Bramson et al, 1996). Whereas intratumoral adenoviral transfer of the HSV-tk and the murine IL-2 genes resulted in substantial hepatic tumor regression, induced an effective systemic antitumoral immunity in the host and prolonged the median survival time of the treated animals from 22 to 35 days a recombinant adenovirus expressing the murine IL-12 gene was much more effective: intratumoral administration of the IL-12 vector alone increased significantly survival time of the animals; 25% of the treated animals lived over 70 days (Caruso et al, 1996).

The immunological host response to syngeneic murine mammary carcinoma cell line variants, genetically modified to express B7-1 or secrete GM-CSF and IL-12, was examined by Aruga et al (1997). The mammary adenocarcinoma MT-901 subline was weakly immunogenic by immunization/challenge experiments and induced tumor-specific T-cell responses in lymph nodes draining progressive subcutaneous tumors; however, tumor clones from this cell line expressing B7-1 or secreting GM-CSF exhibited reduced tumorigenicity and resulted in significantly enhanced T-cell reactivity to tumor-draining lymph node (TDLN) cells as compared to wild-type TDLN cells. In contrast, transduction with the IL-12 gene led to complete tumor growth inhibition.

An adenovirus vector, AdIL12-B7-1, encoding the two IL-12 subunits in early region 1 (E1) and the B7-1 gene in E3 of adenovirus under control of the murine CMV promoter was used to treat mice tumors derived from a transgenic mouse mammary adenocarcinoma. A single intratumoral injection with a low dose (2.5×10^7 pfu/mouse) mediated complete regression in 70% of treated animals whereas a similar dose of recombinant virus encoding IL-12 or B7-1 alone resulted in only a delay in tumor growth. Coinjection of two different viruses expressing either IL-12 or B7-1 induced complete tumor regression in only 30% of animals treated (Putzer et al, 1997).

Human peripheral blood lymphocytes (HuPBLs), injected s.c. in mixture with human lung tumor cells into severe combined immunodeficient (SCID) mice, engrafted and displayed antitumor cytotoxic activity; this antitumor activity was dependent upon both $CD8^+$ T cells and $CD56^+$ natural killer cells in the donor HuPBLs. IL-12 enhanced the human peripheral blood lymphocyte-mediated tumor suppression; this implies that transfer of the IL-12 gene has a prospect in this type of immunotherapy. This could be significant under the light of studies showing that PBLs isolated from a lung cancer patient also suppressed the growth of the patient's (autologous) tumor when coinjected s.c. with the tumor cells into SCID mice (Iwanuma et al, 1997).

Tumor cell vaccines were transduced with IL-12 or IL-2 genes and the antitumor response induced in mice bearing lung metastases of the BALB/c colon carcinoma C51 were compared by Rodolfo et al (1996). The cells used for transduction with the IL-12 or IL-2 genes were the histologically related, and antigenically cross-reacting C26 tumor cells which were irradiated and injected s.c. Vaccination with C26/IL12 cells cured 40% of mice, while vaccination with C26/IL2 cells reduced the number of metastatic nodules without affecting survival; both cell vaccination regimens showed similar antitumor CTL activation in mice. Both treatments induced antibodies directed against tumor-associated antigens, but only sera from mice treated with C26/IL12 contained antibodies that lysed tumor cells. The better therapeutic efficacy of vaccination with C26/IL12 was found to be associated, among other factors, with an early infiltration of the metastatic lungs by activated T lymphocytes (Rodolfo et al, 1996).

Transfer of the IL-12 cDNA for cancer immunotherapy is being used in human clinical trials (protocols 62, 111, 180, and 183, Appendix 1).

H. Adoptive immunotherapy with GM-CSF

1. Cell culture experiments

The human hematopoietic growth factor, granulocyte-macrophage colony-stimulating factor (GM-CSF), is important in the management and gene therapy of a variety of malignant disorders of the human hematopoietic system. Infection of COS-1 monkey kidney cells with a recombinant AAV vector containing the GM-CSF gene resulted in the release of recombinant GM-CSF protein into the supernatant; the released GM-CSF was able to sustain the active proliferation of the GM-CSF-dependent human megakaryocytic leukemia cell line, M07e, (Luo et al, 1995).

2. Animal studies

The Dunning rat R3327-MatLyLu prostate tumor model (an anaplastic androgen-dependent, nonimmunogenic tumor that metastasizes to the lymph nodes and the lung)

has been used for GM-CSF therapy; IL-2- or GM-CSF-secreting human tumor cell preparations (tumor vaccines) were used for the treatment of advanced human prostate cancer in rats. All animals with subcutaneously established tumors were cured; the cancer vaccine induced immunological memory that protected the animals from subsequent tumor challenge; GM-CSF was less effective than IL-2 (Vieweg et al, 1994). Using the Dunning rat prostate carcinoma model, animals with hormone refractory prostate cancer treated with irradiated prostate cancer cells genetically engineered to secrete human GM-CSF showed longer disease-free survival compared to untreated control rats.

To further test the clinical feasibility of the prostate cancer cell vaccine, cancer cells from patients with stage T2 prostate cancer undergoing radical prostatectomy were successfully transduced with MFG-GM-CSF, achieving a significant human GM-CSF secretion in each of 10 consecutive cases (Sanda et al, 1994).

Continuous secretion of GM-CSF and activation of macrophages may contribute to the antitumor effects of a recombinant vaccinia virus expressing the gene for murine GM-CSF injected to solid melanoma tumors twice weekly for 3 weeks; this injection regimen resulted in growth inhibition of the subcutaneous tumor and enhanced the survival of the animals (Ju et al, 1997).

A recent effort has been toward potentiation of T-lymphocyte-mediated antitumor effects. T-lymphocyte response incapacitation in the murine renal cancer model could arise from an impairment of critical nuclear transcription factors. A vaccine-oriented gene therapy approach used T cells and antigen-presenting dendritic cells which were recruited through the use of antigen, chemokines and GM-CSF and further potentiated by fibroblasts expressing IL-2, IL-4, IL-7, or IL-12; the goal of this approach was to optimize MHC class I- and class II-dependent pathways for induction of T-lymphocyte-mediated responses to cancer in animal models (Wiltrout et al, 1995).

Chen et al (1996) found that adenoviral delivery of a combination of HSV-tk, mouse IL-2, and mouse GM-CSF is much more effective for the treatment of metastatic colon carcinoma in the mouse liver than HSV-tk alone or HSV-tk combined only with IL-2; a fraction of the animals developed long-term antitumor immunity and survived for more than 4 months without tumor recurrence in the three gene combination regimen; thus, local expression of GM-CSF in the hepatic tumors and prolonged IL-2 expression were necessary to generate persistent antitumor immunity.

A gene gun device was used to accelerate and introduce gold particles coated with GM-CSF cDNA plasmids into mouse and human tumor cells. Transfected and irradiated murine B16 melanoma cells produced about 100 ng/ml murine GM-CSF/million cells per 24 hr in vitro for at least 10 days. Toward development of a tumor vaccine, irradiated B16 tumor cells expressing murine GM-CSF cDNA were then injected into mice. Subsequent challenge of these mice with nonirradiated, nontransfected B16 tumor cells showed that 58% of the animals were protected from the tumor by the prior vaccine treatment compared to only 2% of control animals inoculated with irradiated B16 cells transfected with the luciferase gene (Mahvi et al, 1996).

Human tumor tissue transfected within 4 hr of surgery produced significant levels of transgenic human GM-CSF protein in vitro. Human GM-CSF was readily detectable in serum and at the injection site following subcutaneous implantation of these transfected tumor cells into nude mice (Mahvi et al, 1996).

The autocrine secretion of GM-CSF by transduced tumor cells was found to serve as an effective immune adjuvant in the host response to a weakly immunogenic murine mammary carcinoma tumor: transfer of activated lymph node cells derived from mice inoculated with GM-CSF-secreting (240 ng/million cells/24 hours) murine mammary carcinoma cells resulted in the prolonged survival of animals with macroscopic metastatic disease; this was not evident utilizing lymph node cells from mice inoculated with wild-type tumor (Aruga et al, 1997).

3. Clinical trials

Autologous cells (sensitized T cells) genetically-modified to secrete GM-CSF have been used for adoptive immunotherapy on humans. GM-CSF has been used for the treatment of advanced melanoma or renal cell cancers (Chang et al, 1996). The steps included retrieval of tumor from the patient for use as a vaccine; the tumor cell line was transduced with a retroviral/GM-CSF vector; cells were reintroduced into the patient (tumor vaccination). Removal of draining lymph nodes after 7-10 days and activation of lymph node cells with a monoclonal antibody directed against CD3 and expansion of the cell population with IL-2 gave anti-$CD3^+$/IL2-activated cells which were exquisitely tumor-specific and mediated the regression of established tumors in animal models (**Figure 18**).

According to a phase I clinical trial cancer patients are intradermally vaccinated with lethally-irradiated tumor cells that have been transfected by particle-mediated gene transfer with gold particles coated with human GM-CSF plasmid DNA; this is based on preclinical studies showing that vaccination of mice with irradiated, GM-CSF-transfected melanoma cells provided protection from subsequent challenges with non-irradiated, non-transfected tumor cells. Human tumor immunotherapy studies in course use patients' fresh specimens of melanoma or renal carcinoma; cells are dissociated, lethally-irradiated and transfected with GM-CSF plasmid DNA-coated gold particles resulting in the subsequent production of biologically active GM-CSF protein by the patient’s cells. Patient’s cells are used intradermally as a vaccine to elicit anti-tumor immune responses. Surgical excision of the vaccination sites will assess GM-CSF production and infiltration of immune effector cells; patients are being subjected to an intradermal injection in their opposite extremity of 5 million irradiated cryopreserved tumor cells taken from the patient at the time of vaccine preparation to asses immune reactions

(DTH testing); if a positive reaction is noted on day 28 the DTH site will be surgically removed (Mahvi et al, 1997).

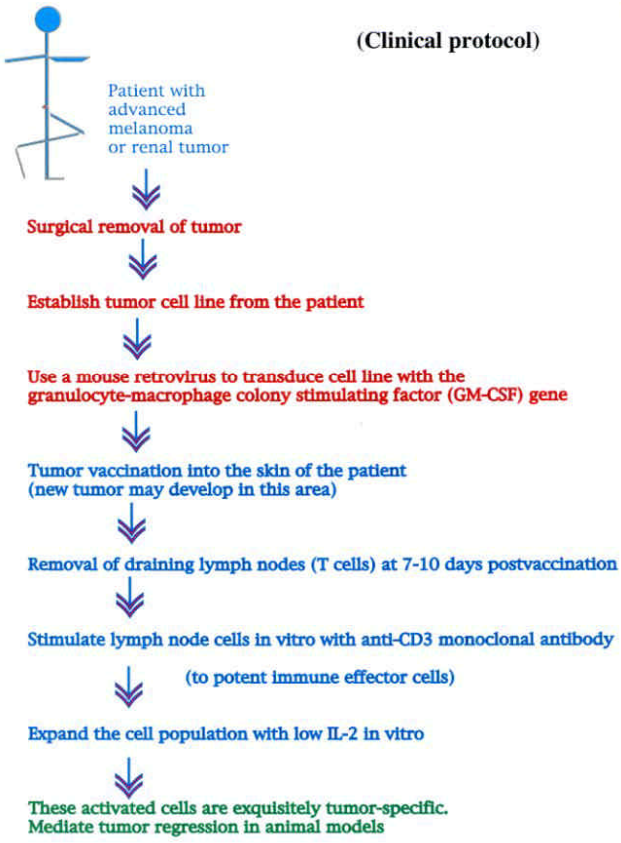


Figure 18. A clinical protocol for adoptive immunotherapy of advanced melanoma patients. Adapted from Chang et al (1996).

A number of RAC-approved human gene therapy protocols use GM-CSF cDNA transfer. These are protocols 35, 53, 63, 113, 149, 150, 162, and 181 in **Appendix 1**.

I. Cancer immunotherapy with the IFN-γ gene

Solid tumors in nude mice have been successfully eradicated with treatment with tumor cell lines stably transfected with an IFN gene. A number of human tumor cell lines including 293, HeLa, K562, and Eskol (a malignant immunoblastic lymphoma) were infected with a rAAV carrying a synthetic type I interferon gene and the bacterial neomycin-resistant gene and geneticin-resistant cells were selected; when injected into nude mice, 293, K562, and Eskol cells failed to form tumors for a duration of up to 3 months; on the contrary, mice receiving nontransduced cells developed tumors within 7 to 10 days; in addition, treatment of an established Eskol tumor with transduced 293 cells resulted in tumor regression (Zhang et al, 1996).

Three RAC-approved human gene therapy protocols use IFN-γ cDNA transfer. These are protocols 36, 54, and 71 in **Appendix 1**.

J. Immunotherapy with synthetic tumor peptide vaccines

Progress in the identification of tumor-specific antigens, that is proteins expressed at high levels by a specific tumor cell type such as prostate or breast cancer, most of which are surface glycoproteins easily recognizable by the immune system, as well as the deciphering of the mechanisms for enhancing the response of cytotoxic T cell lymphocytes have advanced the potential for developing cancer vaccines.

Cancer immunotherapies based on synthetic tumor peptide vaccines have been developed. Tumor-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) recognize short peptide epitopes presented by MHC class I molecules that are expressed on the surface of cancer cells. Bone marrow-derived dendritic cells, grown in vitro in media containing combinations of GM-CSF + IL-4, when pulsed with synthetic tumor peptides (which are loaded on the surface of the dendritic cells) became potent antigen-presenting cells (APCs) capable of generating a protective antitumor immune response. Injection of these cells into naive mice protected the mice against a subsequent lethal tumor challenge; in addition, treatment of mice bearing C3 sarcoma or 3LL lung carcinoma tumors with the same type of cells resulted in sustained tumor regression in over 80% of the animals (Mayordomo et al, 1995).

One of the obstacles of this method has been the difficulty in obtaining sufficient numbers of APCs; dendritic APCs have been isolated from $CD34^+$ hematopoietic progenitor cells drawn from cord blood and expanded in cell culture in the presence of GM-CSF and TNF-α; TNF-α inhibits the differentiation of dendritic cells into granulocytes. Human peripheral blood mononuclear cells or mouse bone marrow cells depleted of lymphocytes could also yield dendritic cells when cultured in the presence of GM-CSF + IL-4 (Mayordomo et al, 1995).

K. DNA vaccines

Vaccines may be one of the first successful applications of foreign genes into mammalian cells under control of heterologous promoters and enhancers (Felgner and Rhodes, 1991; Thompson, 1992; Gilboa and Smith, 1994). Vaccination with DNA has been shown to be a promising approach for immunization against a variety of infectious diseases (Wang et al, 1993; Michel et al, 1995; Huygen et al, 1996; Kuhober et al, 1996). The method consists in introducing the gene of a viral or bacterial antigen which is uptaken and expressed by the host's cells to elicit an antigen-specific immune response. DNA coding for an antigen can be directly injected into muscle or skin and stimulate an immune response against the expressed antigen; the gene can either code for surface

molecules, which are often used for conventional peptide vaccines, or from internal microbial proteins.

During this approach the antigens are produced intracellularly where they are correctly folded and can be presented to the immune system to stimulate cytotoxic T cells; the method is safe and simple and has shown promising results on animals (reviewed by Moelling, 1997). For example, mice injected intramuscularly with an HIV-1 envelope DNA construct developed anti-HIV envelope immune responses (Wang et al, 1993); intramuscular injection of plasmid DNA expression vectors encoding the three envelope proteins of the hepatitis B virus (HBV) induced humoral responses in C57BL/6 mice specific to several antigenic determinants of the viral envelope (Michel et al, 1995). Immunization of mice with plasmid DNA constructs encoding one of the secreted components of *Mycobacterium tuberculosis*, antigen 85 gene induced substantial humoral and cell-mediated immune responses (Huygen et al, 1996).

Because immunization of cancer patients with tumor antigen proteins is a very promising approach used extensively in cancer therapy (e.g. Karanikas et al, 1997) many of these approaches could be transferred to the DNA level using the gene encoding the tumor antigen.

As an extension, this method could find application using human tumor antigen genes rather than bacterial/viral antigen genes, that is genes encoding for proteins expressed in tumor but not in normal cells leading to development of tumor vaccines (Graham et al, 1996; Okamoto et al, 1997); this method mimics the infection of the cell in the host by a pathogenic virus resulting in the intracellular processing of the viral proteins and their presentation on the cell surface. Human tumor antigens are, however, weakly immunogenic compared to microbial antigens a problem connected with polymorphism in the major histocompatibility complex proteins of the host and in antigen presentation.

Development of a fusigenic viral liposome vector was made possible using the HVJ (hemagglutinating virus of Japan, a Sendai virus) renowned for its cell fusion ability; plasmid DNA containing the human tumor antigen genes MAGE-1 and MAGE-3 was mixed with HMG-1 nonhistone protein (to increase nuclear import and expression of the plasmid after transfection) and was encapsulated into anionic liposomes (phosphatidylserine, phosphatidylcholine, cholesterol) followed by the addition of inactivated HVJ; intramuscular injection into mice resulted in production of MAGE-1 and -3 IgG antibodies (Okamoto et al, 1997).

XVI. Gene therapy of cancer and candidate genes

A. Mechanisms of carcinogenesis

Whereas for inborn errors of metabolism transfer of a single gene can correct the disorder, cancer is a complex disease involving mutations in a number of proto-oncogenes and tumor suppressor genes as well as an imbalance and disarray in phosphorylation events and regulatory circuits of the cell cycle. As a result of transformation, tumor cells acquire a proliferation advantage compared with normal cells, most of which are quiescent in the adult organism; cancer cells acquire partial independence from regulatory signals from neighboring cells for restricted cell growth. A crucial step in cancer development is the nonelimination of pre-cancer cells by apoptosis (usually a subsequence of a mutation in the p53 gene); such cells acquire a number of unrepaired damage in their DNA, such as strand breaks, which induce chromosomal translocations and result in clonal expansion of this cell population.

Tumor cells are able to survive after DNA damage, and display an increase in mutation rate; cancer cell populations are heterogenous with respect to translocations, loss of heterozygosity, point mutations and transpositions in various genes. Whenever the mutated cell acquires an advantage for rapid growth over other cells in the tumor mass, escaping cell cycle checkpoints, it may replace the original population, a phenomenon known as tumor progression; this may lead to appearance of a more malignant phenotype. As a result, tumor cells are of different genotypes and clones obtained from the same solid tumor may differ in the level of malignancy.

A number of candidate genes, when become mutated or overexpressed, may lead to tumor phenotype: p53, RB, and p21 appear to be the most important. The deregulation of other genes is connected to tumor progression whereas different groups of genes are associated with tumor cell metastasis. These facts make a single gene transfer approach to tumor cell mass to inhibit its growth or change its phenotype from malignant to normal very challenging.

B. Human clinical trials

The genes used for cancer gene therapy in human clinical trials include a number of tumor suppressor genes (p53, RB, BRCA1, E1A), antisense oncogenes (antisense c-*fos*, c-*myc*, K-*ras*), suicide genes (HSV-tk, in combination with ganciclovir, cytosine deaminase in combination with 5-fluorocytosine) which have been very effective in eradicating solid tumors in animals. Also the cytokine genes (IL-2, IL-7, IFN-γ, GM-CSF) are being used for the ex vivo treatment of cancer cells isolated from human patients and are able to elicit an immunologic regression especially on immunoresponsive malignancies (melanomas, colorectal carcinomas, renal cell carcinomas) (Culver, 1996). Future directions might be toward use of genes involved in the control of tumor progression and metastasis. Discovery of new genes which are over- or under-expressed during transformation and metastasis is a promising approach for the identification of novel gene targets in cancer gene therapy (Georgiev et al, 1998, this volume).

Diseases amenable to therapy with gene transfer in clinical trials (**Appendix 1** and **Table 4** in Martin and Boulikas, this volume) include cancer (melanoma, breast,

lymphoma, head and neck, ovarian, colon, prostate, brain, chronic myelogenous leukemia, non-small cell lung, lung adenocarcinoma, colorectal, neuroblastoma, glioma, glioblastoma, astrocytoma, and others), AIDS, cystic fibrosis, adenosine deaminase deficiency, cardiovascular diseases (restenosis, familial hypercholesterolemia, peripheral artery disease), Gaucher disease, Hunter syndrome, chronic granulomatous disease, PNP deficiency, α1-antitrypsin deficiency, leukocyte adherence deficiency, partial ornithine transcarbamylase deficiency, Cubital Tunnel syndrome, Canavan disease and rheumatoid arthritis. Several RAC-approved protocols use gene marking rather than gene therapy . An important number of protocols in cancer use ex vivo immunotherapy (**Appendix 1**, pages 159-172 & 203-206).

XVII. Gene therapy strategies based on p53

A. p53 as a tumor suppressor protein

The p53 has been a fascinating subject in cancer biology since its discovery (Lane and Crawford, 1979; Linzer and Levine, 1979). Originally assigned in the constellation of oncogenes was later shown to exert suppressive effects on cell growth (Finlay et al, 1989); indeed, the mutated p53 has many characteristics of an oncogene (Will and Deppert, 1998, this volume). Mutations in the p53 gene contribute to the emergence of the malignant phenotype (Diller et al., 1990; Baker et al., 1990). Alterations in the p53 tumor suppressor gene appear to be involved, directly or indirectly, in the majority of human malignancies (Vogelstein, 1990). For example, human lung cancer cell lines and specimens showed allelic loss for chromosome regions 3p and 17p (p53 is assigned to 17p13); these specimens displayed homozygous deletions of *p53*, DNA rearrangements involving the *p53* gene, or expression of truncated p53 transcripts suggesting abnormal splicing, initiation, and termination arising from point or other mutations (Takahashi et al, 1989; Nigro et al, 1989).

An interesting approach to unravel the molecular mechanism of action of p53 in restricting cell growth and in inducing apoptosis was the cloning of genes induced by p53 before the onset of apoptosis; this led to the identification of a group of 14 genes (out of 7,202 transcripts examined) which were markedly increased in p53-expressing cells compared with control cells many of which were predicted to encode proteins that could generate oxidative stress or respond to oxidative stress (Polyak et al, 1997). Additional studies in this line have suggested that the induction of the apoptotic pathway by p53 involves **(i)** transcriptional induction of redox-related genes; **(ii)** formation of reactive oxygen species; and **(iii)** the oxidative degradation of mitochondrial components (Polyak et al, 1997).

p53 can inhibit transformation of rat embryo fibroblasts mediated by adenovirus *E1A* plus activated *ras* and can also suppress focus formation mediated by *myc* plus activated *ras* (Finlay et al, 1989; Eliyahu et al, 1989). Both alleles of p53 need to be mutated or altered for transformation. Introduction of a null mutation by homologous recombination in murine embryonic stem cells gave mice which appeared normal but were susceptible to a variety of neoplasms by 6 months of age (Donehower et al, 1992).

The tumor suppressive activity of p53 seems to involve at least six independent pathways: **(i)** induction by p53 of the *p21/Waf-1/Cip-1* gene which causes growth arrest both via inhibition of cyclin-dependent kinases and via inactivation of PCNA; PCNA is the accessory molecule to DNA polymerases α and δ and its absence causes arrest of DNA synthesis at the replication fork; **(ii)** induction of the death-promoting *bax* gene by p53 as a mechanism which eliminates oncogenic virus-infected and transformed cells; **(iii)** by a direct interaction of p53 with origins or replication preventing firing and initiation of DNA replication; **(iv)** via binding of p53 to a number of important molecules involved in transcription (TATA box-binding protein or TBP, TFIIH); **(v)** by the role of p53 in DNA repair via its patrolling the genome for small insertion deletion mismatches or free ends of DNA; **(vi)** p53 is able to attract RPA, an accessory to DNA polymerases α and δ as well as TFIIH and RAD51 at the damaged DNA sites; TFIIH, RAD51, and RPA have a demonstrated role in DNA repair (**Figure 19**). Additional properties of p53 include the induction of Gadd45 involved in the arrest of the cell cycle and induction of Mdm2 which, after exceeding a threshold value in the cell associates with p53 to restrict its regulatory functions; thus, Mdm2 acts as a feedback loop for p53 to moderate its apoptotic and cell cycle restrictive functions (**Figure 20**).

B. Genes regulated by wild-type p53

Protein p53 appears to be a transcription factor able to recognize specific regulatory regions in a number of genes via its central DNA-binding domain; the DNA sequence-specific binding of wt p53 is regulated by the C-terminal domain of p53 and is activated by a variety of posttranslational modifications (Hupp et al, 1992). p53 is phosphorylated and is constitutively expressed at low levels in most normal tissues (Lane and Crawford, 1979; Linzer and Levine, 1979).

The sequence specificity of p53 has been determined using random synthetic oligonucleotides followed by selection by wtp53 and cloning; these studies revealed the 10 bp motif $RRRC^{AA}_{TT}GYYY$ (where R is purine and Y pyrimidine) as the binding and recognition site of wtp53 recognition (El-Deiry et al., 1992); two such 10 bp motifs are required for p53 binding separated by up to 13 bp of random sequence. Since the 10 bp motif is a palindrome, the binding site of p53 comprises 4 copies of the half binding sites $^{A}_{T}GYYY$ oriented in opposite directions, which suggested that p53 binds either as a dimer to two cruciforms or as a tetramer with each subunit interacting with one half site. The second possibility is favored since

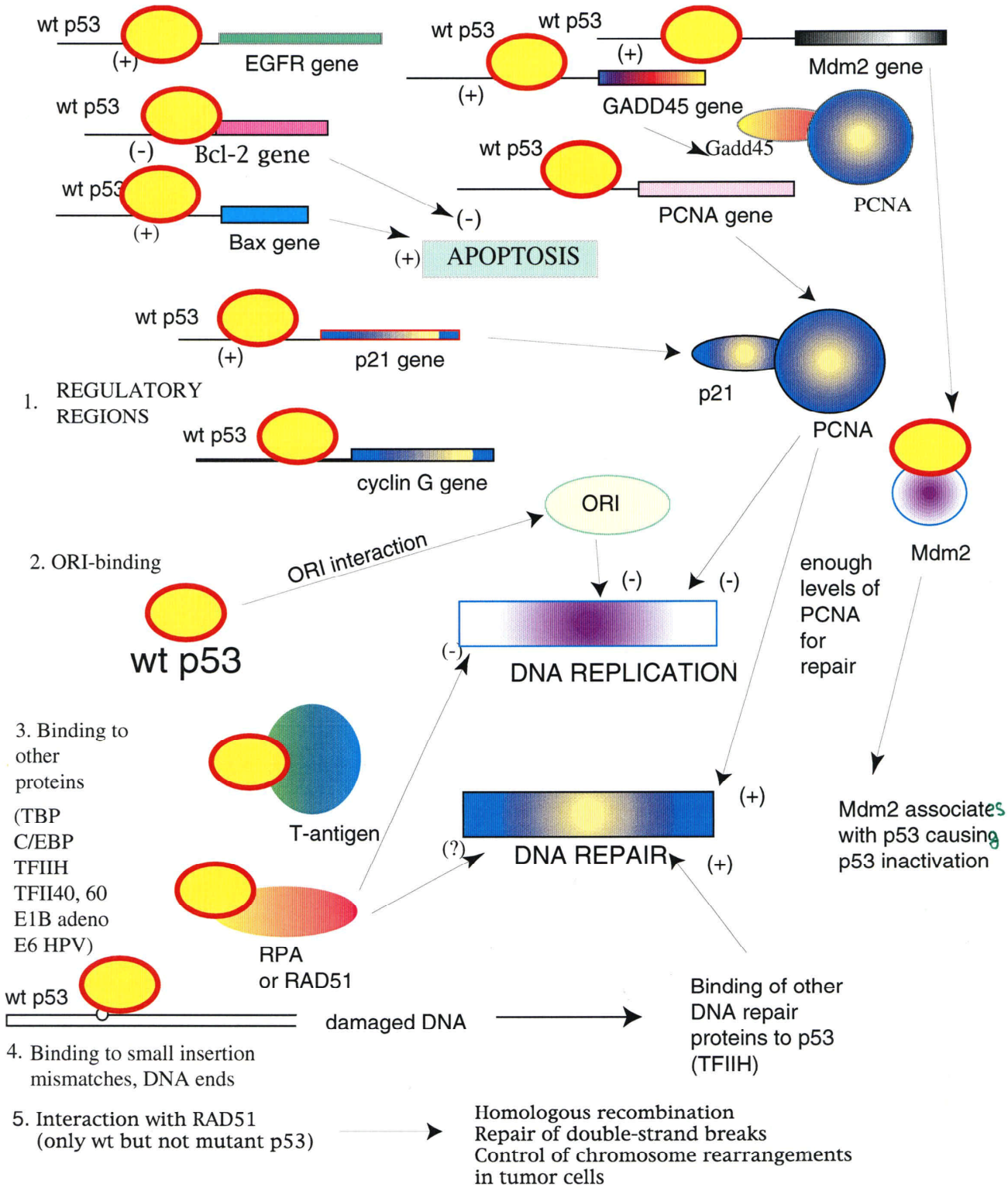


Figure 19. Regulatory circuits involving p53. From Boulikas T (**1997**) Gene therapy of prostate cancer: p53, suicidal genes, and other targets. **Anticancer Res** 17, 1471-1506. With the kind permission of Anticancer Research.

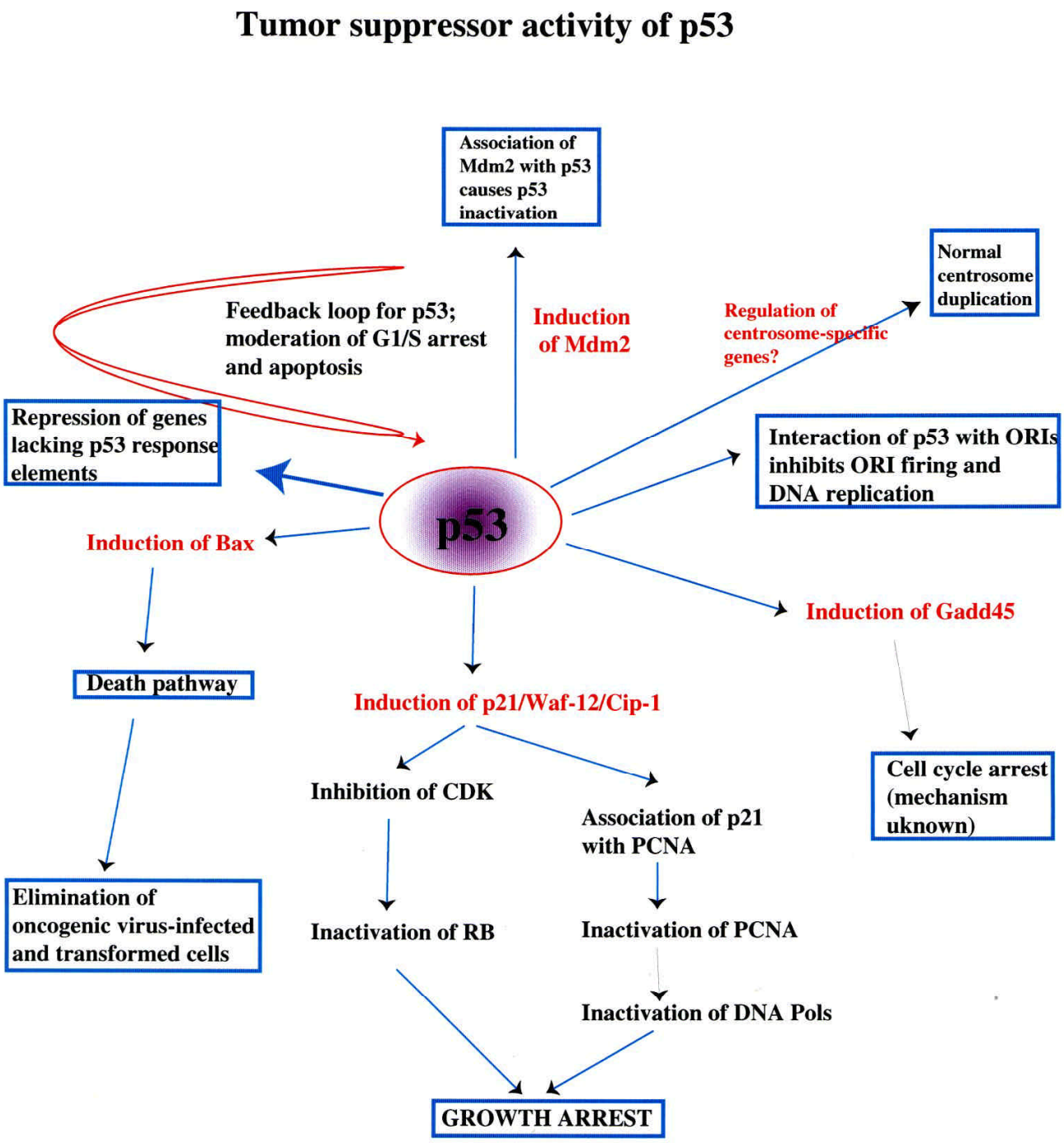


Figure 20. A summary of the apoptotic and cell cycle restrictive activities of p53. From Boulikas T (**1997**) Gene therapy of prostate cancer: p53, suicidal genes, and other targets. **Anticancer Res** 17, 1471-1506. With the kind permission of Anticancer Research.

biophysical studies indicate that p53 exists as a tetramer in solution (Stenger et al., 1992).

Increased levels of p53 upregulate the expression of specific genes including *Cip-1/Waf-1/p21* (El-Deiry et al, 1993), *GADD45* (Kastan et al, 1992), cyclin G (Okamoto and Beach, 1994), and *mdm2* (Perry et al, 1993; Barak et al, 1993; Momand et al, 1992) which is induced by UV damage in a p53-dependent pathway (Perry et al, 1993). Gadd45 inhibits cell cycle progression (Papathanasiou et al, 1991).

Mdm2 acts as a feedback loop for the biological functions of p53 apparently to moderate the G1/S arrest or apoptosis triggered by p53 following severe damage to DNA. Mdm2 protein associates with p53 causing p53 inactivation by preventing its sequence-specific binding to regulatory targets in DNA (Momand et al, 1992; Oliner et al, 1992). Elevated levels of Mdm2 mimic the effect of T antigen, E1B of adenovirus, E6 of HPV, which also inactivate p53 in a similar manner; overexpression of Mdm2 can block the induction of apoptosis by p53 (Chen et al, 1994).

Additional genes up-regulated by p53 include human *PCNA* (Shivakumar et al, 1995), mouse muscle creatine kinase *MCK* (Zambetti et al, 1992), *EGFR* (Deb et al, 1994), the potent promoter of the death pathway *Bax* (Miyashita and Reed, 1995), and thrombospondin-1 (Dameron et al, 1994). Other cellular regulatory regions that interact with p53 include the RGC repeats in the ribosomal gene cluster (Farmer et al, 1992; Kern et al, 1992).

The PCNA promoter is up-regulated in the presence of moderate amounts of wt p53; however, at higher levels of wt p53 the PCNA promoter is inhibited whereas tumor-derived p53 mutants activate the PCNA promoter (Shivakumar et al, 1995); it has been suggested that the moderate elevation in wt p53 seen after DNA damage induces PCNA to cope with its DNA repair activities (Shivakumar et al, 1995); this inhibition in DNA replication but stimulation in repair by p53 might be accomplished by an independent pathway involving induction of p21 (El-Deiry et al, 1993) which interacts with PCNA protein auxiliary to DNA polymerase δ to inhibit the replication but not the repair functions of PCNA (Li et al, 1994).

The *bax* gene which induces apoptosis (**Figure 21**) is upregulated by p53 whereas the *bcl-2* gene which inhibits apoptosis in B cells is down-regulated by p53 (Miyashita et al, 1994a,b; Miyashita and Reed, 1995). Initiated cancer cells may lead to tumor development only when a dysfunction in their apoptotic pathway takes place; some of the mechanisms leading to inactivation of the apoptotic pathway in cancer cells may result from an up-regulation in the *bcl-2* gene (a Bcl-2 chimeric factor is produced in leukemias as a result of a translocation) or down-regulation of the *bax* gene. Gene therapy for cancer could involve restoration of the apoptotic pathway in cancer cells leading to their suicidal death (see below).

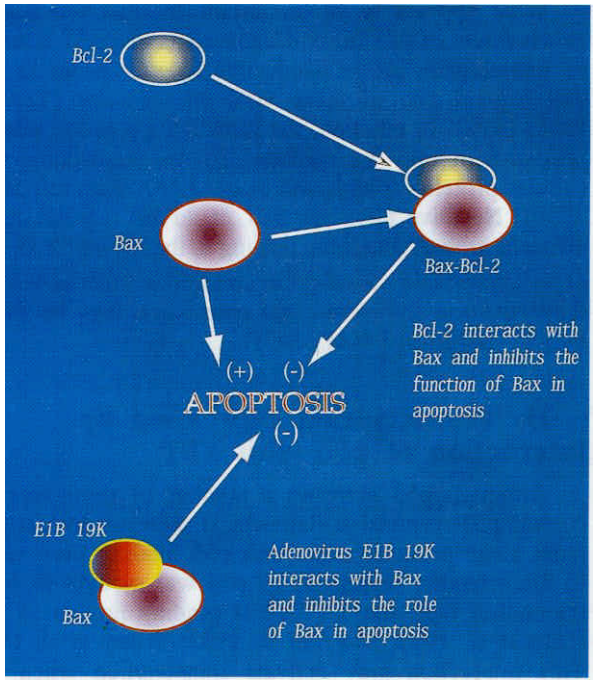


Figure 21. Involvement of Bax and Bcl-2 proteins in apoptosis. Bax is a potent inducer of apoptosis; binding of Bcl-2 to Bax (also binding of the E1B 19 kDa protein of adenovirus to Bax) prevents Bax from its apoptotic functions. From Boulikas T (**1997**) Gene therapy of prostate cancer: p53, suicidal genes, and other targets. **Anticancer Res** 17, 1471-1506. With the kind permission of Anticancer Research.

Binding sites for p53 have been found at the origin of replication of polyomavirus with an inhibitory effect on virus replication in vitro (Miller et al, 1995) and at the SV40 ORI (Bargonetti et al, 1991) as well as in putative cellular origins of replication (Kern et al, 1991).

A number of genes not containing p53 response elements may be repressed by p53 (Ginsberg et al, 1991; Mercer et al, 1991; Shiio et al, 1992; Seto et al, 1992).

C. Binding of p53 to viral oncoproteins

p53 was first detected in rodent cells transformed with SV40 in a complex with T antigen (Lane and Crawford, 1979; Linzer and Levine, 1987). Subsequent studies have shown that p53 can be complexed with adenovirus E1B (Sarnow et al, 1982; van den Heuvel et al., 1990) and the E6 oncoprotein of human papilloma virus (Werness et al., 1990). SV40 T antigen was unable to act as an initiator of SV40 DNA replication in vitro when complexed with wt murine p53 (Wang et al, 1989) thought to act by blocking the interaction of T antigen with DNA polymerase α (Gannon and Lane, 1987; Braithwaite et al, 1987).

What appears to be important in understanding the involvement of p53 in tumorigenesis is that p53 is unable to transactivate the p53-inducible reporter genes in cells

that express one of these viral oncoproteins (Yew and Berk, 1992). In addition, the growth suppressive effect of p53 protein may be mediated by its association with cellular proteins (Fields and Jang, 1990; Raycroft et al., 1990). Negative elements that could be required for an efficient growth shutdown leading to the reversible G_0 state or to irreversible out-of-cycle conditions such as terminal differentiation, apoptosis, and senescence, may be affected by p53 (Bargonetti et al., 1991).

D. Transcription repression by interaction of p53 with TBP

Although p53 activates a number of promoters that contain p53-responsive elements, it represses transcription from many promoters that lack p53 binding sites; central to the promoter repression by p53 was thought to be its interaction with the TATA-box binding protein or TBP (Seto et al, 1992; Mack et al, 1993; Truant et al, 1993). This interaction may activate transcription when TBP interacts with a preformed p53-DNA complex or may repress transcription when p53 interacts with DNA-bound TBP (Deb et al, 1994). However, p53 acts as a repressor only in cells undergoing apoptosis and p53-mediated transcriptional repression is released by adenovirus E1B or cellular Bcl-2 (Shen and Shenk, 1994; Sabbatini et al, 1995).

Both wild-type and mutant p53 interact with C/EBP on the human hsp70 promoter (Agoff, 1993), with TFIIH (Xiao et al, 1994), holo-TFIID (Chen et al, 1993; Liu et al, 1993) and the TAFII40 and TAFII60 subunits of TFIID (Thut et al, 1995).

E. Inhibition of DNA replication by wild-type p53

Several lines of evidence suggested inhibition in DNA replication by wild-type p53 but not by tumor-derived mutant forms of p53. Indeed, SV40 T antigen was unable to act as an initiator of SV40 DNA replication *in vitro* when complexed with p53 (Wang et al, 1989); mutant p53 was unable to cause inhibition in the initiating functions of T antigen *in vitro* (Friedman et al, 1990). Inhibition in DNA replication *in vivo* by p53 (Braithwaite et al, 1987) suggested that p53 might interact with cellular DNA replication initiator proteins or other components of the replication fork. p53 also interacts with replication protein A (RPA) implicated in DNA replication and in repair; interaction of p53 inhibits the replication functions of RPA (Dutta et al, 1993) although interaction of p53 with RPA via its acidic domains stimulates BPV-1 DNA replication in vitro (Li and Botchan, 1993). Immunolocalization of p53 (also of RB and host replication proteins) at foci of viral replication in HSV-infected cells (Wilcock and Lane, 1991) provided further evidence for a direct interaction of p53 with proteins (or DNA sequences) at the replication fork.

According to a second model, p53 can cause inhibition in DNA replication by a direct interaction with origins of replication at the DNA sequence level rather than via its interaction with replication initiator proteins. The potential role of p53 as a down-regulator of DNA replication in a DNA-binding-dependent manner has been suggested from replication assays of polyoma virus *in vitro* (Miller et al, 1995) and from the inhibition in nuclear DNA replication by a form of p53, truncated at its C-terminus, which is constitutively active for DNA binding in transcription incompetent extracts from *Xenopus* eggs (Cox et al, 1995). In the experiments of Miller and coworkers (1995) wild-type p53 suppressed DNA replication in vitro when the p53 binding site $(RGC)_{16}$ from the ribosomal gene cluster was cloned on the late side of the polyomavirus (Py) core origin; when mutated p53-binding sites were used, the inhibition in Py replication was not observed. In addition, RPA (able to interact directly with p53) was unable to relieve the p53-mediated repression in Py replication. Furthermore, tumor-derived mutants of p53 that had lost their sequence-specific DNA-binding capacity were unable to inhibit Py replication of the construct with the wild-type oligomerized RGC sites in vitro.

F. Differences in biological functions between wild-type p53 and tumor-derived p53 mutants

Tumor-derived mutant forms of p53 have lost their DNA sequence-specific binding capacities. For example the Trp-248 and His-273 mutants of p53 have poor DNA-binding abilities and are unable to activate transcription from constructs containing p53 binding sites (Farmer et al, 1992).

Wild-type (wt) p53 tumor suppressor protein negatively regulates cell growth (Hollstein et al, 1991; Prives, 1994). Whereas the wild-type p53 acts as a tumor suppressor, several of the mutant forms display oncogenic activities (Levine, 1993; Prives and Manfredi, 1993; Deppert, 1994). Although the wt p53 has been postulated to repress growth by activating genes that repress growth (*p21*), many of the mutant forms have lost their DNA sequence-specific binding and transcriptional activation capacities (reviewed by Zambetti and Levine, 1993).

According to one model (see Vogelstein and Kinzler, 1992), wt p53 is a positive regulator for the transcription of genes that by themselves are negative regulators of growth control and/or invasion. Indeed, p53 upregulates the genes of p21/CIP1/WAF1 (ElDeiry et al, 1993) and GADD45 (Kastan et al, 1992) whose products interact with PCNA to inhibit its association with DNA polymerase δ thus causing arrest in DNA replication (Smith et al, 1994). This feature of p53 that is central to its ability to suppress neoplastic growth is lost by mutations on p53 that result in loss of its ability to bind to DNA or to interact with other transcription protein factors.

Mutant p53 can transactivate genes that up-regulate cellular growth (Deb et al, 1992; Dittmer et al, 1993) such as *PCNA* (Shivakumar et al, 1995), *EGFR* (Deb et al, 1994), multiple drug resistance (*MDR1*) (Chin et al, 1992; Zastawny et al, 1993), and human *HSP70* in vivo (Tsutsumi-Ishi et al, 1995). These studies support the idea for an oncogene function of the mutant p53 protein compared with the tumor suppressor function of wt p53; mutation in the p53 gene may, thus, cause gain of new functions such as transforming activation and binding to a distinct class of promoters which are not normally regulated by wt p53 (Zambetti and Levine, 1993; Tsutsumi-Ishi et al, 1995). At the same time appearance of mutations in the p53 gene result in the loss of function of the wt p53 (Zambetti and Levine, 1993).

The wild-type but not mutant p53 at low levels transactivates the human *PCNA* promoter in a number of different cell lines; the wild-type p53-response element from the PCNA promoter functions in either orientation when placed on a heterologous synthetic promoter; thus moderate elevation of p53 can induce PCNA, enhancing the nucleotide excision repair functions of PCNA (Shivakumar et al, 1995). Whereas low levels of wild-type p53 activate the PCNA promoter, higher concentrations of wt p53 inhibit the PCNA promoter, and tumor-derived p53 mutants activate the promoter (Shivakumar et al, 1995).

While the wtp53 is endowed with a 3'-to-5' exonuclease activity, associated with the central DNA-binding domain, and thought to function during repair, replication, and recombination, the 273^{His} mutant of p53 has lost the exonuclease activity (Mummenbrauer et al, 1996).

G. Involvement of p53 in repair and control of the cell cycle

p53 controls the level of expression of the *p21* gene, encoding a protein that inhibits the activity of cyclin-dependent kinases (CDKs); CDK activity is essential for the phosphorylation of RB at the G1/S checkpoint of the cell cycle resulting in the release of E2F transcription factor from RB-E2F complexes and in the up-regulation by the released E2F of genes required for DNA synthesis. p21 levels are reduced considerably in tumor cells that have lost the p53 protein or contain a nonfunctional mutated form of p53 (El-Deiry et al, 1993). In addition, the p21 inhibitor of cyclin-dependent kinases associates with PCNA thus blocking its ability to activate DNA polymerase δ; this could give rise to the abnormal control in DNA replication or to the loss of coordination between DNA replication and cell cycle progression seen in tumor cells. Thus the upregulation of the *p21* gene by p53 acts in two different ways causing a cascade of events.

p53 is linked directly to homologous recombination processes via its interaction with the RAD51/RecA protein (Stürzbecher et al, 1996).

H. A proposal for an efficient killing of cancer cells using *p53/PAX5* expression vectors

Introduction of a null p53 mutation by homologous recombination in murine embryonic stem cells gave mice which appeared normal but were susceptible to a variety of neoplasms by 6 months of age (Donehower et al, 1992). Relevant to the issue that p53 is dispensable for embryonic development are the studies of Stuart and coworkers (1995) suggesting that during early embryo development *p53* is not expressed because of the suppression of its gene by Pax5; at later stages of development *Pax5* inactivation allows *p53* to be expressed and exert its control on cell growth (**Figure 22**).

A significant factor to be considered in approaches aimed at transferring the wt *p53* gene to tumor cells is the impairment of the wt p53 functions by the endogenous mutant p53 expressed in tumor cells which is able to tetramerize with wt p53; optimal results will be expected if the endogenous mutant *p53* gene is inhibited concurrently with overexpression of the wt p53 gene. It has been proposed (Boulikas, 1997) that effective suppression of tumor growth with p53 vectors could be achieved by the simultaneous transfer of wt p53 plus Pax5 to cancer cells; Pax5 is a well established supressor of the p53 gene; its effect is exerted via a direct interaction of Pax5 with a control element in the first exon of the *p53* gene (Stuart et al, 1995). Pax5 is an homeotic protein, controlling the formation of body structures during development; Pax5 is expressed in early embryo stages to keep the levels of p53 low and allow rapid proliferation of embryonic tissues. Simultaneous transfer to solid tumors of a PAX5 and p53 genes in the same expression vector but with the wt p53 mutagenized at 2-3 nucleotides to abort the PAX5 suppressive site was proposed as a strategy to effectively suppress tumor cell proliferation (Boulikas, 1997).

I. p53 gene bombs that explode in cancer cells

Exogenous genes encoding "weapons" (suicide genes) and "triggers" have been devised whose delivery to somatic cells will affect only cancer cells. The production of mutated forms of p53 at high levels by cancer cells (normal cells do not have adequate amounts of wt p53 protein) is being exploited to pull a molecular trigger resulting in the transcriptional activation of a toxic gene and in the death of cancer cells (da Costa et al, 1996). This invention is based on the fact that **(i)** powerful chimeric transcription factors can be engineered consisting of a DNA-binding domain (**DBD**) and a transactivation domain (**TAD**) and **(ii)** prokaryotic or viral enzymes are able to convert nontoxic prodrugs into toxic derivatives (suicide genes, see HSV-tk, CD and PNP further below); the toxic derivative produced in tumor cells which are transfected can diffuse to surrounding cells causing their killing even in

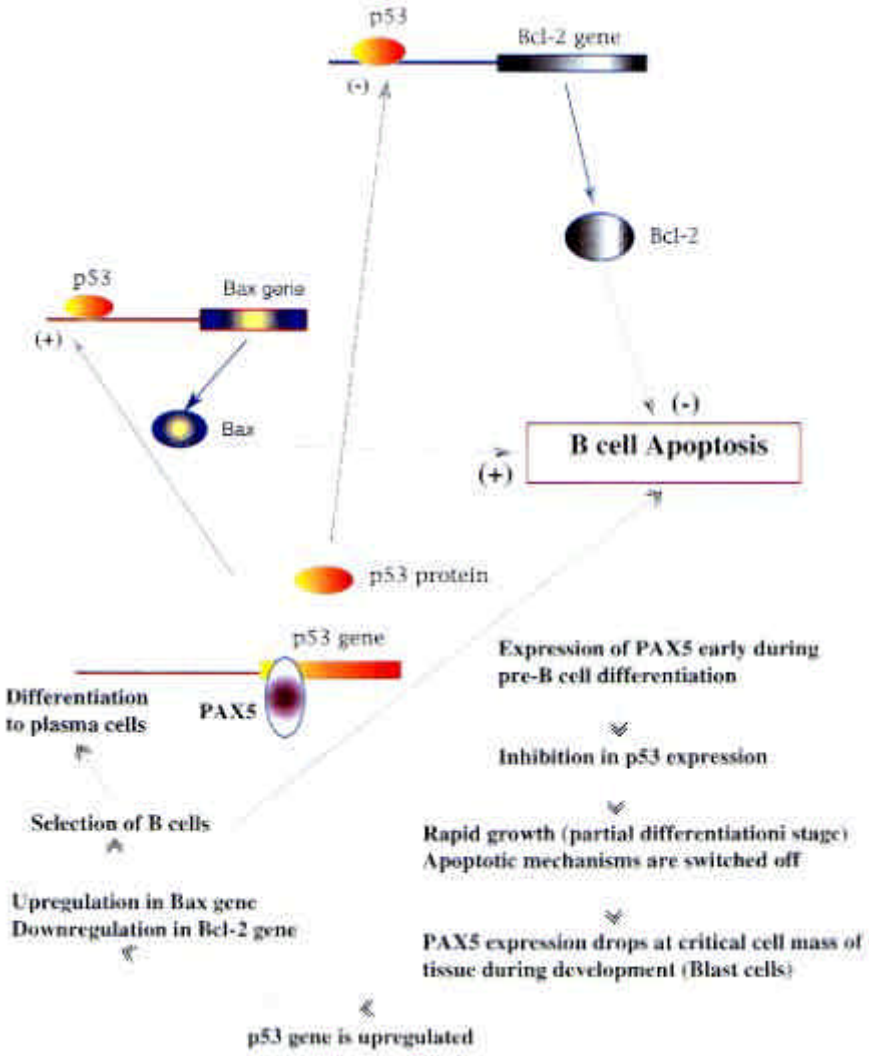


Figure 22. Involvement of p53 and Pax5 in B cell apoptosis. Adapted from Stuart et al (1995).

the absence of transfection of these cells, a phenomenon known as "bystander effect".

Trigger genes in plasmids were made up of the DNA-binding domain of GAL4 (aa 1-147) fused in frame to a protein domain that could interact with p53; the p53-binding domain was the 84-708 aa region of SV40 T antigen or of the 305-393 aa TAD domain of p53 (which acts as a tetramer) and which is similar between wt and mutant p53 (mutations on p53 are within the DBD). The constructs included the *E. coli* (*DeoD*) gene which encodes the purine nucleoside phosphorylase (PNP) under control of the GAL4 response element (known as upstream activating sequence or UAS); the PNP gene can convert the 6-methylpurine deoxyribose (MeP-dR) prodrug into the diffusible, toxic 6-methylpurine (see page 69) and can become a powerful suicide gene under these conditions (Sorscher et al, 1994). Transfection of cells in culture with these constructs followed by treatment of the cells with MeP-dR resulted in the death of the cells (da Costa et al, 1996). The mechanism was based on the fact that most normal cells do not express the p53 gene and those who do express the wt p53 are destined for programmed cell death; therefore, cancer cells containing elevated amounts of mutant forms of p53 were amenable to this strategy.

The 55- kDa E1B protein of adenovirus binds to and inactivates the p53 gene; ONYX-015 is an E1B, 55-kDa gene-attenuated adenovirus unable to replicate and show cytopathogenicity in tumor cell lines which express a wt p53 such as in RKO and U20S carcinoma lines but can cause cytolysis in cell lines expressing a mutated form of p53; a wide range of human tumor cells, including numerous carcinoma lines with either mutant or normal p53 gene sequences (exons 5-9), were efficiently destroyed following intratumoral or intravenous administration of ONYX-015 to nude mouse-human tumor xenografts; furthermore, combination therapy with ONYX-015 plus chemotherapy (cisplatin, 5-fluorouracil) was significantly greater than with either agent alone. On the contrary, normal human cells were highly resistant to cytolysis by the adenovirus (Heise et al, 1997).

J. Transfer of the p53 gene in cell culture

Preclinical studies have shown that both viral and plasmid vectors able to mediate high efficiency delivery and expression of wild-type tumor suppressor p53 gene can cause regression in established human tumors, prevent the growth of human cancer cells in culture, or render malignant cells from human biopsies non-tumorigenic in nude mice. Inhibition in cell proliferation was observed in cell culture and in tumors after induction of *p53* expression with adenovirus vectors (Bacchetti and Graham, 1993; Wills et al, 1994; Zhang et al, 1994).

Transfer of the wild-type p53 gene using a defective HSV vector into a human medulloblastoma cell line containing a mutant copy of p53 resulted in p53 expression, increased the levels of mdm2 proteins and induced cell cycle arrest of the majority of transduced cells (Rosenfeld et al, 1995).

Apoptosis can be induced in cultured NCI-H 596 human non-small cell lung cancer cells, which have a wild-type p53 gene, by EGF signaling in a p53-dependent manner; whereas treatment of these cells with EGF plus p53 sense oligonucleotides induced EGF-dependent and p53-dependent apoptosis within 8 hours, antisense p53 gene therapy suppressed the induction of apoptosis. A new nucleic acid drug was developed based on a mutated p53 antisense with a mutation at three bases immediately 5' and 3' from the CG dinucleotides which potentiated the induction of apoptosis and failed to suppress the induction of EGF-dependent apoptosis (Murayama and Horiuchi, 1997).

Infection of the androgen-independent human prostate Tsu-pr1 cell line lacking functional p53 alleles with recombinant adenovirus vectors (replication-deficient) carrying the p53 gene under control of the CMV promoter resulted in expression of p53 and induced striking morphological changes: the cells were detached from the substratum, condensed, and exhibited breakdown of the nuclear DNA into nucleosome-size fragments characteristic of apoptosis; whereas control cells were able to elicit tumors in nude mice, the AdCMV/p53-infected cells failed to form tumors (Yang et al, 1995).

K. Animal studies using p53 gene transfer

Intratracheal injection of a recombinant retrovirus containing the wt *p53* gene was shown to inhibit the growth of lung tumors in mice nu/nu models inoculated intratracheally with human lung cancer H226Br cells whose p53 gene has a homozygous mutation at codon 254 (Fujiwara et al, 1994). A number of other studies have shown suppression in tumor cell growth and metastasis after delivery and expression of the wt *p53* gene (Diller et al, 1990; Chen et al, 1991; Isaacs et al, 1991; Wang et al, 1993).

The safety of the adenovirus-mediated p53 transduction of the liver in normal rats and in 50%-hepatectomized animals subjected to asanguineous portal perfusion was examined by Drazan et al (1994). The gene transfer rate in whole liver and after hepatectomy ranged from 20% to 40%; liver regeneration and hepatocyte function were unaffected by overexpression of p53.

Delivery of the p53 gene to malignant human breast cancer cells in nude mice using DOTMA:DOPE 1:1 cationic liposomes (400 nmoles liposomes/35 μg DNA) resulted in regression (60% reduction in tumor cell volume) in 8 out of 15 animals treated; animals were receiving one injection every 10 days (Lesoon-Wood et al, 1995). It was thought that wild-type p53 expression (tumor cells were expressing mutant forms of p53) upregulated p21 gene to inhibit cell growth by inhibition in cyclin-dependent kinases but also via induction of apoptosis preferentially in cancer cells.

When a recombinant adenovirus encoding wild-type p53 under the control of the human CMV promoter was introduced into SK-OV-3 human ovarian carcinoma cells it increased by more than 50% the life span of nude mice injected with these cells; control animals in this highly aggressive ovarian xenograft model died between 25-45 days from injection time (Mujoo et al, 1996). Adenoviral transfer of a functional p53 gene into a radiation-resistant SCCHN cell line that harbors mutant p53 restored the G1 block and apoptosis in these cells in vitro and sensitized SCCHN-induced mouse xenografts to radiotherapy in vivo (Chang et al, 1997).

The efficacy of a replication-deficient p53 adenovirus construct was tested against three human breast cancer cell lines expressing mutant p53, MDA-MB-231, -468, and -435 and was found to be highly effective against 231 and 468 cells as well as their tumor xenografts in nude mice but not against 435 cells probably due to their low adenovirus transduction. 37% of growth inhibition of 231 cells was due to p53, while 49% was adenovirus-specific (Nielsen et al, 1997).

Cytotoxic T lymphocytes (CTLs) recognizing a murine wild-type p53 were able to discriminate between p53-overexpressing tumor cells and normal tissue and caused complete and permanent tumor eradication without damage to normal tissue after adoptive transfer into tumor-bearing $p53^{+/+}$ nude mice. CTLs, presented by the MHC class I molecule H-2Kb, were generated by immunizing p53 gene deficient ($p53^{-/-}$) C57BL/6 mice with syngeneic p53-overexpressing tumor cells (Vierboom et al, 1997).

L. Transfer of the p53 tumor suppressor gene to prostate cancer cells

Although primary prostate tumors have few mutations in the *p53* gene (Voeller et al, 1994; Isaacs et al, 1994), specimens from advanced stages of the disease and metastases as well as their cell lines frequently display mutations or deletions at both alleles of the p53 gene (Chi et al, 1994; Dinjens et al, 1994). Three of five prostate cancer cell lines examined (TSUPr-1, PC3, DU145) and one out of two primary prostate cancer specimens were

found to harbor mutations altering the amino acid sequence of the conserved exons 5-8 of the p53 gene; transduction of the p53-defective cell lines with the wt p53 gene using lipofectin showed reduction in tumorigenicity assayed from reduced colony formation and the cells became growth arrested (Isaacs et al, 1991).

Endocrine therapy is ineffective once the prostate cancer becomes androgen-independent; these cancers remain unresponsive to conventional chemotherapy. Androgen-independent and metastatic prostate cancers were established in athymic male mice by co-inoculation with the LNCaP human prostate cancer cell line and the MS human bone stromal cell line; these tumors became necrotic and were successfully eradicated by intratumoral injection of a recombinant p53/adenovirus; the *p53* gene was driven by the CMV promoter and the SV40 poly(A) signal placed in the E1 region of Ad5 (Ko et al, 1996). It was suggested that in addition to the tumor suppressor, apoptotic, and antiangiogenesis function of p53, tumor necrosis was induced by a bystander effect or a general immune response which attracted immune cells to cause tumor cell killing (Ko et al, 1996).

M. Clinical trials using p53 gene transfer

A human clinical trial at M.D. Anderson Cancer Center uses transfer of the wild-type *p53* gene, in patients suffering with non-small cell lung cancer and shown to have p53 mutations in their tumors, using local injection of an Ad5/CMV/p53 recombinant adenovirus at the site of tumor in combination with cisplatin (Roth, 1996; Roth et al, 1996; protocols #29 and 124, **Appendix 1**). A retroviral vector containing the wild-type p53 gene under control of a β-actin promoter was used for multiple percutaneous injections or direct thoracoscopic injections at the site of the tumor into nine patients, all in advanced stages, with non-small cell lung cancers. Patients whose conventional treatments failed were selected for a p53 mutation in the lung tumor. Reduction in tumor volume was achieved via apoptosis (assayed in posttreatment biopsies) in three patients, and arrest in tumor growth in three other patients (Roth et al, 1996).

RAC-approved clinical trials (**Appendix 1**) using p53 cDNA transfer are #29 (treatment of non-small cell lung cancer with p53 and antisense K-ras), #124 (intratumoral delivery of adenoviral p53 cDNA plus cisplatin), #130 (intratumoral injection of adenoviral p53 to treat head and neck squamous cell carcinoma), #131 (primary and metastatic malignant tumors of the liver), #147 (percutaneous injections of adenovirus p53 for hepatocellular carcinoma), #148 (advanced or recurrent adenocarcinoma of the prostate), #152 (intra-tumoral injections of ad5cmv-p53 to patients with recurrent squamous cell carcinoma of the head and neck), #153 (intralesional delivery of adenovirus p53 in combination with chemotherapy in breast cancer), #154 (intratumoral injection of adeno p53 to patients with advanced prostate cancer), #155 (intratumoral injection of adeno p53 to patients with advanced and metastatic bladder cancer), and #156 (adeno p53 for non-small cell lung cancer).

XVIII. p21 and p16 in cancer gene therapy

A. Molecular action of p21

p53 upregulates the p21/CIP1/WAF1 gene (simply called p21) (ElDeiry et al, 1993). Induction of the *p21/Waf-1/Cip-1* gene causes growth arrest via inhibition of cyclin-dependent kinases (CDKs). CDKs are upregulated by cyclins which act as positive regulators of cell cycle progression. Cdk2, also called $p33^{cdk2}$, is the master regulator of the cell cycle at the G1/S transition point. Whereas cdk2 is expressed at constant levels throughout the cell cycle, its activation by phosphorylation is first detected a few hours before the onset of DNA synthesis; furthermore, antibodies directed against Cdk2 blocked mammalian cells from entering S phase. D1 cyclin associates with Cdk2, Cdk4, and Cdk5 to control the G1→S transition point; the genes of cyclins D1 and E are overexpressed or rearranged in malignancies and conditional overexpression of human cyclins D1 and E in Rat-1 fibroblasts causes a decrease in the length of G1 and an acceleration of the G1/S phase transition. D1 appears to be specialized in the emergence of cells from quiescence (Go→G1 transition) whereas cyclin E is more oriented toward control of the G1/S transition. Cdc2, a close relative of Cdk2 and whose pattern of phosphorylation is cell cycle-regulated, becomes associated with cyclin B to regulate the G2→M transition (see Boulikas, 1995a).

CDK activity is essential for the phosphorylation of RB at the G1/S checkpoint of the cell cycle resulting in the release of E2F transcription factor from RB-E2F complexes and in the up-regulation of genes required for DNA synthesis by the released E2F. p21 levels are reduced considerably in tumor cells that have lost the p53 protein or contain a nonfunctional mutated form of p53 (El-Deiry et al, 1993).

Induction of the *p21/Waf-1/Cip-1* gene also causes growth arrest via inactivation of PCNA; indeed, the p21 inhibitor of cyclin-dependent kinases associates with PCNA, the accessory of DNA polymerases α and δ, thus blocking its ability to activate these DNA polymerases; this could give rise to the abnormal control in DNA replication or to the loss of coordination between DNA replication and cell cycle progression seen in tumor cells (Li et al, 1994).

B. p21 and p16 gene transfer

Introduction of the wt p53 or of the p21 downstream mediator of p53-induced growth suppression into a mouse prostate cancer cell line, deficient in p53, led to an association of p21 with Cdk2; this interaction was sufficient to downregulate Cdk2 by 65% (Eastham et al, 1995). The p21 gene, driven by CMV promoter into an

Adenovirus 5 vector, was more effective than the AD5CMV-p53 vector, (harboring the p53 gene under control of the same elements as p21), in reducing tumor volume in syngeneic male mice with established s.c. prostate tumors; tumors were induced by injection of 2 million cells in each animal. These studies suggested that *p21* expression might have more potent growth suppressive effect than p53 in this tumor model and that p21 may be seriously be included in the constellation of anticancer arsenals.

Transfer of p21 is an effective tool to lead carcinoma cells with inactivated p53 into less malignant phenotypes. p53 is frequently inactivated by papilloma viruses in carcinomas of the uterine cervix. Transfer of the p21 gene to HeLa cells, a widely used uterine cervix cell line, resulted in a significant growth retardation by blockage of G1 to S transition, reduced anchorage-independent growth and attenuated telomerase activity (Yokoyama et al, 1997). Introduction of p21 with adenoviral vectors into malignant cells completely suppressed their growth in vivo and also reduced the growth of established pre-existing tumours (Yang et al, 1997).

Transfer of p21 was used to suppress neointimal formation in the balloon-injured porcine or rat carotid arteries in vivo (Yang et al, 1996; Ueno H et al, 1997a). A combination therapy in mice with simultaneous transfer of the p21 gene and of the murine MHC class I H-2Kb gene, which induces an immune response that stimulates tumor regression, was more effective than treatment with either gene alone (Ohno et al, 1997).

Malignant gliomas extensively infiltrate the surrounding normal brain and their diffuse invasion is one of the most important barriers to successful therapy; one of the most frequent abnormalities in the progression of gliomas is the inactivation of the tumor-suppressor gene p16, suggesting that loss of p16 is associated with acquisition of malignant characteristics. Restoring wild-type p16 activity into p16-null malignant glioma cells modified their phenotype. Adenoviral transfer of the p16/CDKN2 cDNA in p16-null SNB19 glioma cells significantly reduced invasion into fetal rat-brain aggregates and reduced expression of matrix metalloproteinase-2 (MMP-2), an enzyme involved in tumor-cell invasion (Chintala et al, 1997).

XIX. Gene therapies based on transfer of the retinoblastoma (RB) gene

A. RB and E2F proteins in the control of the cell cycle and apoptosis

Retinoblastoma protein is a transcription factor (Lee et al, 1987) involved in the regulation of cell cycle progression genes (reviewed by White, 1998, this volume). The role of RB on cell proliferation and tumor suppression arises **(i)** from its association with E2F, an association disrupted by RB phosphorylation at the G1/S checkpoint resulting in release of E2F and in the upregulation of a number of genes required for DNA replication; **(ii)** from the direct association of RB protein with a number of viral oncoproteins or key regulatory proteins including E1A of adenovirus (Whyte et al., 1988), SV40 large T (Ludlow et al., 1990) and the human papilloma virus E7 protein (Dyson et al., 1989). Normal cellular targets of RB, such as the transcription factor E2F (Bagchi et al., 1991; Chellapan et al., 1991) become dissociated from the RB protein in the presence of these viral proteins in the cell (E1A, T antigen, E7), leading to cell cycle progression. This constitutes a mechanism (also the interaction of viral proteins with p53, see above) viruses use to render infected cells continuously cycling.

(iii) RB is able to repress directly c-*fos* gene expression (Robbins et al., 1990) and has been proposed to have a similar effect on c-*myc* expression (Pietenpol et al., 1990). **(iv)** RB also suppresses cell growth by directly repressing transcription of the rRNA and tRNA genes by blocking the activity of RNA polymerase I transcription factor UBF (Cavanaugh et al, 1995; reviewed by White, 1998).

Hypophosphorylated RB, but not mutant RB, was associated with the nuclear matrix, particularly concentrated at the nuclear periphery and in nucleolar remnants, only during early G1; the peripheral matrix proteins lamin A and C bound RB in vitro. This association was thought to be important for the ability of RB to regulate cell cycle progression (Mancini et al, 1994). It is interesting that mutated p53 but not wtp53 interacts with specific types of MARs (Will et al, 1998); nuclear matrix is an essential structure for replication transcription recombination and repair processes intimately connected to mechanisms of carcinogenesis.

The tumor suppressor function of RB is believed to occur by complex formation between E2F and RB or the RB-related proteins p107 and p130, a complex that down-regulates the DNA-binding activities of E2F; the transcription activating capacity of E2F on the genes it regulates can be repressed by interaction with RB (Nevins, 1992). Cyclin A, believed to facilitate DNA replication, also associates with E2F; both types of complexes, E2F-RB and E2F-cyclin A, can be dissociated by the adenovirus E1A protein. The release of E2F by E1A results in cell cycling and this constitutes an additional mechanism of interference of adenoviruses with the proliferation of the infected cells; release of E2F from RB induced by E1A is critical for transformation of cells by E1A (for references see Hiebert et al, 1995).

The p107 protein with similarities in structure and DNA-binding properties to RB also binds cyclin A; whereas RB is complexed to E2F during G1 the p107-cyclin A complex interacted with E2F as cells entered S phase (Shirodkar et al., 1992).

E2F is a transcription factor that activates the adenovirus E2 gene and a number of cellular genes that respond to proliferation signals and that control the passage of the cell cycle through S phase such as *myc* and *DHFR* genes and contributes to the uncontrolled

proliferation of adenovirus-transformed cells (Mudryj et al., 1991; see White, 1998, this volume). It has been speculated that the physiological function of RB (and also of its similar protein p107) in negatively-regulating cell growth and in acting as a tumor suppressor protein are exerted via its ability to down-regulate the activity of E2F (Shirodkar et al., 1992); this has been subsequently confirmed by numerous studies. RNA ligands that bind to E2F1 were selected from RNA libraries and were used to inhibit the induction of S phase in cultured cells (Ishizaki et al, 1996). Such molecules might find applications in cancer therapy because of the important role of E2F proteins in the regulation of cell cycling.

Retinoblastoma protein has a functional domain (the pocket) for binding to transcription factor E2F implicated in cell growth control. The same domain is responsible for the association of RB with the adenovirus E1A, the SV40 large T, and the human papilloma virus E7 proteins (Kaelin et al., 1992). Using an approach for screening λgt11 expression libraries, clones encoding for RB-binding proteins were identified; among those are RBAP-1 and 2, or retinoblastoma-associated proteins 1 and 2 (Kaelin et al., 1992) and RBP3 (Helin et al., 1992). RBAP-1 binds to the RB pocket, copurifies with E2F, contains a functional transactivation domain, and binds to E2F cognate sequences (Kaelin et al., 1992).

E2F contains a RB-binding domain in its C-terminus (Helin et al., 1992; Shan et al., 1992). RB binds directly to the activation domain of E2F1 and silences it, thereby preventing cells from entering S phase. To induce complete G1 arrest, RB requires the presence of the hbrm/BRG-1 proteins, which are components of the coactivator SWI/SNF complex. This cooperation was mediated through a physical interaction between RB and hbrm/BRG-1. RB can contact both E2F1 and hbrm at the same time, thereby targeting hbrm to E2F1 (Trouche et al, 1997).

E2F cooperates with p53 to induce apoptosis (Wu and Levine, 1994) and high levels of wild-type p53 potentiate E2F-induced apoptosis in fibroblasts (Qin et al, 1994). The physiological relevance of E2F in the apoptotic mechanism was thought to arise from the ability of E2F to act as a functional link between p53 and RB; p53 levels increase in response to high levels of E2F (DP is required for the association of E2F with RB); overexpression of both E2F-1 and DP-1 led to a rapid death of (IL-3)-dependent 32D.3 myeloid cells even in the presence of survival factors (Hiebert et al, 1995). Overexpression of exogenous E2F-1 using a tetracycline-controlled expression system in Rat-2 fibroblasts promoted S-phase entry and subsequently led to apoptosis (Shan and Lee, 1994).

B. Phosphorylation of RB: the TGF-β1, IL-1, and IL-6 connection

Work from several groups has shown that RB is un- or under-phosphorylated in G0/G1 and becomes phosphorylated in its N-terminal domain during S and G2/M (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989; Mihara et al., 1989). Only under-phosphorylated RB interacts with E2F (Chellappan et al., 1991). Treatment with TGF-β1 maintained RB protein in its active dephosphorylated form, thus providing a link between RB growth suppression and growth inhibition by TGF-β1.

Interleukin-6 (IL-6), known to mediate autocrine and paracrine growth of multiple myeloma (**MM**) cells and to inhibit tumor cell apoptosis was determined to exert this function via phosphorylation of RB protein; this finding could explain the abnormalities of RB protein and mutations of RB gene associated with up to 70% of MM patients and 80% of MM-derived cell lines. Culture of MM cells with RB antisense, but not RB sense, oligonucleotide triggered IL-6 secretion and proliferation in MM cells; phosphorylated pRB was constitutively expressed in MM cells and IL-6 shifted pRB from its dephosphorylated to its phosphorylated form (Urashima et al, 1996).

Interleukin-1 (IL-1) causes G0/G1 phase growth arrest in human melanoma cells, A375-C6 via hypophosphorylation of RB protein. Exposure to IL-1 caused a time-dependent increase in hypo-phosphorylated RB that correlated with an accumulation of cells arrested in the G0/G1 phase; this was abrogated by the SV40 large T antigen which binds preferentially to hypo-phosphorylated RB, but not by the K1 mutant of the T antigen, which is defective in binding to RB (Muthukkumar et al, 1996).

C. Genes regulated by RB protein

RB represses a number of genes by sequestering or inactivating the positive transcription factor E2F and seems to activate some other genes by interacting with factors like Sp1 or ATF-2 (Rohde et al, 1996). RB protein is a master regulator of a complex network of gene activities defining the difference between dividing and resting or differentiated cells. Using the method of differential display Rohde et al (1996) detected a number of genes which were upregulated by ectopic expression of the RB gene in RB-deficient mammary carcinoma cells including the endothelial growth regulator endothelin-1 and the proteoglycans versican and PG40.

Introduction of the wild-type RB gene via retrovirus-mediated gene transfer has provided several RB-reconstituted retinoblastoma cell lines (Huang et al., 1988; Chen et al., 1992). These RB^+ cell lines showed little difference in their growth rates in culture when compared to the parental or revertant RB^- cells; however, RB^+ cells invariably lost their tumorigenicity in nude mice assays (Chen et al., 1992). RB protein down-regulates its own gene and this negative autoregulation is mediated by the transcription factor E2F; this was shown by inserting the promoter of the RB gene 5' of the bacterial CAT reporter

gene followed by its transfection into RB^+ and RB^- retinoblastoma cells: RB promoter activity was significantly decreased in RB^+ cells (Shan et al., 1994).

D. Transcription factors (TFs) that regulate the RB gene

Several mutations have been found in the promoter region of the RB gene, suggesting that inappropriate transcriptional regulation of this gene contributes to tumorigenesis. The presence of E2F recognition sites in promoters of a number of growth-related genes suggested that expression of these genes might be affected by RB. Understanding the nature and availability of TFs which regulate the RB gene in particular cell types is instructive for a successful gene therapy application involving transfer of *RB*.

An E2F recognition site lies within a region critical for RB gene transcription; binding of E2F-1 at this site transactivates the RB promoter; striking back, the resulting overexpression of RB suppresses E2F-1-mediated stimulation of RB promoter activity and, thus, the expression of RB is negatively autoregulated through E2F-1 (Shan et al, 1994). Up-regulation of the RB gene by E2F was shown by co-transfection of RB^- osteosarcoma Saos2 cells in culture with a plasmid expressing E2F-1 under the control of the CMV immediate-early gene promoter-CAT construct: expression of E2F-1 stimulated RB promoter activity 10-fold under conditions where E2F-1 had little effect on c-*jun*, c-*myc*, and EGR-1 gene expression (Shan et al., 1994). The autoregulation of RB gene by RB may be accomplished via a direct protein-DNA complex formation, via protein-protein interaction regulating the activity of other transcription factors on the promoter of the RB gene, or both.

Two distinct DNA-binding factors, RBF-1 and ATF, play an important part in the transcription of the human RB gene. The promoter of the human RB gene and of the mouse RB1 gene (Zacksenhaus et al., 1993) contain binding sites for ATF, and a Sp1-like transcription factor (Mitchell and Tijan, 1989) where the RBF-1 (retinoblastoma binding factor 1) may bind (Sakai et al., 1991). Human RB gene is also regulated by AP-1 (Linardopoulos et al, 1993), as well by the early response transcription factor, nerve growth factor inducible A gene (NGFI-A) which is expressed in prostate cells and binds to the site GCGGGGGAG at -152 to -144 within the RB gene promoter (Day et al, 1993). The ATF site of the RB promoter is a responsive element during myogenic differentiation; RB promoter activity increased about 4-fold during differentiation and was reduced when a point mutation was designed in the ATF site (Okuyama et al, 1996).

pRB activates expression of the human transforming growth factor-β2 gene through ATF-2; the human RB gene promoter is autoregulated by RB protein via an ATF-2-like binding site at the carboxyl-terminal domain of pRB; overexpression of RB stimulates RB promoter activity through the ATF binding site in a variety of different cell types (Park et al, 1994).

The candidate oncoprotein Bcl-3, previously characterized as a member of the IκB family, activated transcription of the RB gene, whose promoter has no typical NF-κB sites, via binding to a DNA element identical to E4TF1/GABP site; Bcl-3 promoted tetramerization of E4TF1. Expression of the antisense bcl-3 RNA in myoblasts suppressed induction of RB and myogenic differentiation whereas transient expression of bcl-3 in myoblasts was shown to induce expression of the endogenous RB (Shiio et al, 1996).

Two oncogenic point mutations at the Sp1 and ATF sites of the RB gene promoter were identified in two separate hereditary RB families. The Sp1 consensus site mutation was blocking the action of RBF-1, recently identified as the human GABP/E4TF1, a transactivator from the adenovirus early-region 4 promoter. The human GABP/E4TF1 protein enhanced the core RB promoter activity, whereas it did not stimulate a mutant RBF-1 site and was proposed to be the most essential transcription factor for human RB gene activation (Clark et al, 1997).

Whereas binding of the Sp1 transcription factor is not significantly affected by methylation of the CpG dinucleotide within its binding site, 5'-GGGCGG (lower strand, 5'-CCGCCC) methylation of the outer C is inhibitory (mammalian cells also have the capacity to methylate cytosines at CpNpG sites) and in particular methylation of both cytosines $^mCp^mCpG$ inhibited binding by 95%; endogenous $^mCp^mCpG$ methylation of an Sp1 site in the CpG island promoter of the RB gene was identified by genomic sequencing in a proportion of retinoblastoma tumors which were extensively CpG methylated in the RB promoter (Clark et al, 1997).

E. RB gene transfer

Functional loss of the RB gene has been implicated in the initiation or progression of several human tumor types including cancer of the eye, bone, bladder, and prostate. The cancer suppressor activity of RB was directly demonstrated by the introduction of a normal RB gene into retinoblastoma cells that have lost the RB function (inability to be phosphorylated because of mutations at the appropriate sites) by mutation at both alleles; this led to the suppression of the neoplastic phenotype and loss of the tumorigenicity of RB cells in nude mice (Huang et al, 1988). Expression of the normal RB gene into the human prostate carcinoma cell line DU145, mediated by recombinant retrovirus integration, also resulted in loss of its tumorigenic ability in nude mice (Bookstein et al, 1990). Studies with tumor cells reconstituted with RB ex vivo and implanted into immunodeficient mice, as well as with germline transmission of a human RB transgene into tumor-prone $Rb^{+/-}$ mice have demonstrated cancer suppression (see Riley et al, 1996).

DU145 cells express a shorter protein lacking 35 amino acids from exon 21 due to a 105 nucleotide in-frame

deletion (Bookstein et al, 1990). The human bladder carcinoma cell line J82 contains a mutated RB protein with exactly these features (Horowitz et al, 1989); this 35 amino acid stretch is required for complexation with T antigen and E1A. However, the two cell lines have lost exon 21 of RB because of a different type of mutation: J82 cells have a point AG to GG mutation in the intron 20-splice acceptor site but the type of mutation in DU145 leading to exon 21 loss is different (Bookstein et al, 1990).

Intratumoral infection of spontaneous pituitary melanotroph tumors arising in immunocompetent $Rb^{+/-}$ mice with a recombinant adenovirus carrying the RB cDNA inhibited the growth of tumors, re-established innervation by growth-regulatory dopaminergic neurons, and prolonged the life spans of treated animals (Riley et al, 1996).

Retrovirus-mediated gene transfer of RB to the breast carcinoma cell lines MDA-MB468 and BT549, both of which harbor partial RB gene deletions as well as point mutations of their p53 genes, restored its expression in cells, reduced their ability to grow in soft agar, and their tumorigenicity in nude mice, although it did not significantly altered growth rate in culture (Wang et al, 1993).

Future therapeutic approaches using the RB gene are directed toward inhibition in cell proliferation (such as to inhibit neointima formation and smooth muscle cell proliferation in arterial diseases, see Arterial injury below and Chang et al, 1995) rather that aggressive suppression and apoptosis of solid tumors; p53 is a better gene than RB for tumor eradication.

XX. Induction of apoptosis for cancer gene therapy

A. Apoptosis as an essential process

Apoptosis has become a basic tool in developing cancer research in establishing new anticancer strategies. The health of a multicellular organism depends both on the ability of the body to produce new cells but also on the ability of certain type of cells to perish, self-destruct, when they become superfluous or severely damaged. Apoptosis, or programmed cell death, is a biological process associated with pronounced morphological changes, chromatin condensation, drop in pH, and intranucleosomal DNA degradation by which a cell actively commits suicide. Virtually all tissues have apoptotic cells; salient examples in the adult are: the eye lenses which consist of apoptotic cells that replaced their cytoplasm with crystallin; intestinal wall cells which migrate to the tip of the finger-like projections over several days where they die; ineffectual T cells which mature in thymus and which would attack the body's own tissues are eliminated by apoptosis before entering the bloodstream; skin cells migrate from the deepest layers to the surface where they commit suicide forming the outer layer of the skin. Apoptosis is an essential process during embryogenesis: mammals eliminate neuron cells as the nervous system is formed; tadpoles delete their tails by apoptosis (reviewed by Duke et al, 1996).

Virus-transformed as well as severely X-ray-damaged or UV-damaged cells are similarly eliminated from the tissue via apoptosis; if they are left they can form malignant cells. Initiated cancer cells may lead to tumor development only when a dysfunction in their apoptotic pathway takes place. Although the biochemical aspects of cell death are fraught with the problem of cause versus effect, the role of apoptosis in neoplasia and its regulation by a number of oncogenes and p53 has emerged. Apoptosis is essential for normal development and homeostasis; deregulation in the positive control of apoptosis is associated with cancer and autoimmune disease whereas deregulation in the negative control of apoptosis is associated with degenerative diseases (reviewed by White, 1993; Duke et al, 1996).

B. Molecular mechanisms for apoptosis: p53, Bax, Bcl-2, c-Myc and other proteins

Apoptosis is of special interest in gene therapy not only of cancer but of other diseases such as arterial disease. Apoptosis is a complex process involving a significant number of apoptotic and antiapoptotic mechanisms. The cytotoxic (killer) T lymphocytes of the immune system of the infected organism bind to virus-infected cells inflicting their eradication with two different type of proteins: Perforin is a transmembrane molecule transferred from the killer T cell to the membrane of the infected cells forming holes on the membrane of the target cell allowing uptake of proteases called granzymes that activate ICE-like proteases to induce apoptosis. A number of antiviral drug development strategies are based on blockage of the activity of antiapoptotic viral proteins.

Expression of a number of genes induce apoptosis; their protein products include adenovirus E1A (Debbas and White, 1993; Lowe and Rudley, 1993) and c-Myc (Hermeking and Eick, 1994; Wagner et al, 1994). A number of proteins when expressed at sufficient amounts block apoptosis; these include Bcl-2 and E1B 19 kDa protein of adenovirus (Debbas and White, 1993; Chiou et al, 1994). Exposure of cells to a variety of growth factors including IL-3, IL-6, and erythropoietin, acting as survival factors, inhibit induction of apoptosis (Johnson et al, 1993; Yonish-Rouach et al, 1993; Canman et al, 1995).

The role of p53 in these molecular processes has been discussed in previous pages in this review. The involvement of p53 in apoptosis is thought to occur via upregulation of *bax* and downregulation of *bcl-2* genes by wt p53 but not by mutated p53 proteins; Bax protein induces apoptosis and its upregulation triggers the apoptotic mechanism in cells which display elevated levels of p53 as a result, for example, of DNA damage. Down-regulation of Bcl-2 has a similar effect on the induction of apoptosis. p53 may induce apoptosis independently of transcription, although the G1 arrest by p53 requires transcription of p53 targets (reviewed by Ko and Prives, 1996). Induction of the apoptotic pathway by p53 was

proposed to involve: (**i**) transcriptional induction of redox-related genes; (**ii**) formation of reactive oxygen species; and (**iii**) the oxidative degradation of mitochondrial components (Polyak et al, 1997). The potential of p53 in cancer gene therapy is discussed above.

While p53 and E1A activate apoptosis, Bcl-2 and E1B 19k proteins inhibit apoptosis. All four protein molecules act upstream of Bax which is a potent inducer of apoptosis: both the cellular Bcl-2 and the 19 kDa protein E1B of adenovirus are able to interact with Bax inhibiting its involvement in induction of apoptosis (Han et al, 1996; **Figure 1** on page 9). E1A acts upstream of p53 by increasing the half-life of p53 resulting in an accumulation of p53 molecules in the nucleus (Lowe and Ruley, 1993); increased levels of p53 are then believed to upregulate the *bax* gene (**Figure 1**). The survival factors IL-3 and IL-6 appear to prevent p53-dependent apoptosis (see White, 1993).

p53 induces apoptosis after exposure to UV irradiation (Ziegler et al, 1994) and hypoxia (Graeber et al, 1996); this acts as a protective mechanism for the removal of severely damaged cells from the body which could become initiated cancer cells and progress to tumors. Spontaneous or radiation-induced apoptosis mediated by p53 has been shown to act for the removal of cells from the gastrointestinal tract in mice (Merritt et al, 1994) and the skin after sunburn (Ziegler et al, 1994). Epidermal growth factor (EGF) has induced apoptosis in various cancer cell lines via a novel signal transduction pathway of EGF mediated through p53 (Murayama and Horiuchi, 1997).

c-*myc* expression, normally induced in proliferating hematopoietic cells by mitogens, drops dramatically by mitogen withdrawal leading to cell arrest in G1. During deregulated c-myc expression, c-*myc* levels were not down-regulated upon mitogen withdrawal; instead, DNA synthesis continued resulting in apoptosis but not in growth arrest. The transforming segment of c-Myc was responsible for induction of apoptosis (see White, 1993).

Pax5 is a repressor of expression of the p53 gene interacting directly with a regulatory region within exon 1 of the p53 gene. At early stages during pre-B cell development the levels of Pax5 are high and p53 is down-regulated; however, later in development Pax5 levels drop and the p53 gene is activated; this process was proposed to lead to the decision of B cells to enter apoptosis or differentiate into plasma cells (Stuart et al, 1995).

Down regulation of the Cu^{2+}/Zn^{2+} superoxide dismutase (*SOD1*) induced oxidative stress and apoptosis (Troy et al, 1996). A great deal of oxidative damage during the procedures for ex vivo-modification of cells induces their apoptosis; transfer of the Cu^{2+}/Zn^{2+} superoxide dismutase to ex vivo modified cells increased their survival after implantation (see Nakao et al, 1995). This demonstrates the importance of blocking apoptotic pathways during cell manipulation for successful ex vivo gene therapy.

Gene therapy for cancer could involve restoration of the apoptotic pathway in cancer cells leading to their suicidal death; this could be effected by overexpression of the *bax* gene, by suppression of the endogenous *bcl-2* gene (see below), or by transfer of the wt p53 gene.

C. Role of tumor necrosis factor (TNF)

The tumor necrosis factor-α (TNF-α) is a cytokine produced by macrophages, monocytes, lymphoid cells, fibroblasts and other cell types in response to inflammation and infection. TNF-α is produced by lipopolysaccharide (LPS)-stimulated macrophages; the molecular pathways leading to TNF-α production in these specialized cells involves activation by LPS of several kinases including the extracellular-signal-regulated kinases 1 and 2 (ERK1 and ERK2), p38, Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), as well as activation of the immediate upstream MAPK activators MAPK/ERK kinases 1 and 4 (MEK1 and MEK4) and of MEK2, MEK3, and MEK6 (Swantek et al, 1997).

TNF-α binds to two type of specific receptors, TNFR1 and TNFR2, causing their trimerization and leading to activation of a number of kinases (ceramide-activated kinase, IκB kinase, Raf-1, Jun N-terminal kinases or JNKs, p38/Mpk2). Activation of Raf-1, JNK, and p38/Mpk2 contribute to the induction of AP-1 whereas activation of IκB kinase is leading to the activation of the transcription factors NF-κB. This activation leads further to upregulation of genes and induction of other cytokines, metalloproteinases, and immunoregulatory proteins (see Liu et al, 1996 and the references cited therein).

TNF can induce apoptotic death or necrosis in some tumor cells; this effect of TNF could be mediated by activation of sphingomyelinases and phospholipases, synthesis of metabolites of arachidonic acid, generation of free radicals, changes in intracellular calcium, generation of DNA strand breaks and activation of poly(ADP-ribosyl)ation, or activation of ICE-like proteases.

TNF-α, IL-1β, IFN-γ, and vitamin D3 after binding to their transmembrane receptors stimulate the production of the second messager ceramide from sphingomyelin in the plasma membrane by activating sphingomyelinase; this results in a cascade of signal transduction events that result in down regulation of c-myc and induction of apoptosis, to terminal differentiation, or to RB-mediated cell cycle arrest (**Figure 23**).

IL-1 signaling leads to NF-κB activation and to protection against TNF-induced apoptosis. The IL-1R-associated kinase (IRAK) is homologous to Pelle of Drosophila. Two additional proximal mediators, both associating with the IL-1R signaling complex, were required for IL-1R-induced NF-κB activation: IRAK-2, a Pelle family member, and MyD88, an adaptor molecule containing a death domain (Muzio et al, 1997).

Treatment of different cell types with TNF-α results in the activation of the MEKK1 pathway of protein kinases ultimately resulting in AP-1 transcription factor activation and in the upregulation of several cytokine genes. TNF-α-

stimulation also results in the activation of NF-κB and inhibition of apoptosis (**Figure 24**). A TNF-responsive

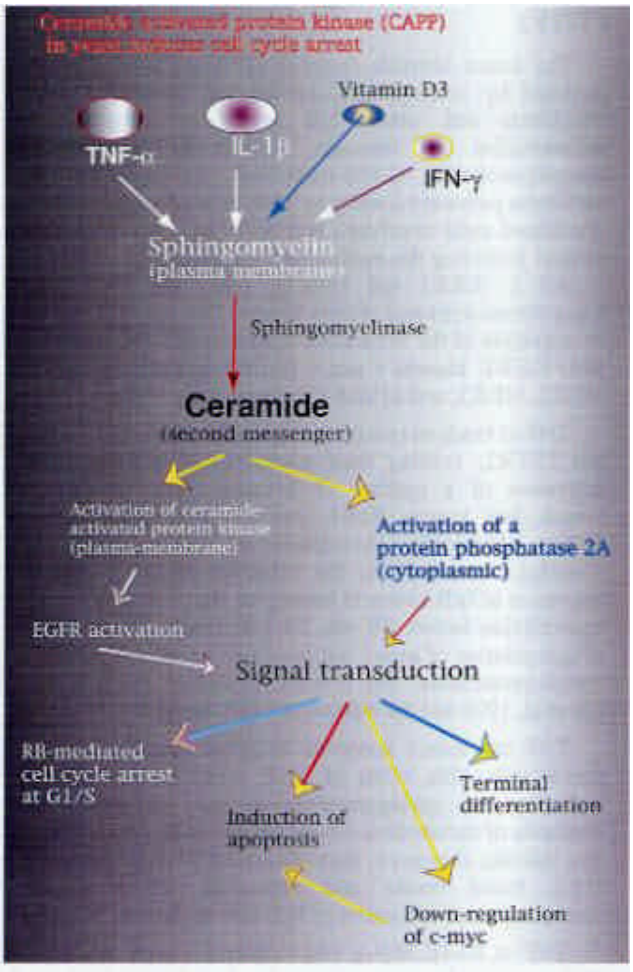


Figure 23. A pathway leading to the induction of growth arrest and apoptosis by the cytokines TNF-α, IL-1β, and IFN-γ. The pathway is conserved between mammalian cells and yeast. Adapted from Nickels and Broach (1996). From Boulikas T (**1997**) Gene therapy of prostate cancer: p53, suicidal genes, and other targets. **Anticancer Res** 17, 1471-1506. Reproduced with the kind permission from Anticancer Research.

serine/threonine protein kinase termed GCK-related (GCKR) most likely signals via mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase kinase 1 (MEKK1) to activate the SAPK pathway (Shi and Kehrl, 1997).

D. NF-κB as anti-apoptotic molecule and TNF-α signaling

Activation of NF-κB is believed to lead to the activation of antiapoptotic genes that have not been fully identified. The antiapoptotic role of NF-κB at the molecular level and the TNF-α connection consists of the following events; signaling by TNF-α induces trimerization of its receptors, an event causing three different cascades: **(i)** Activation of IκB kinase and activation of NF-κB, a pathway which prevents cell death. A key step for NF-κB activation leading to the activation of the stress-activated protein kinase (SAPK, also called c-Jun N-terminal kinase or JNK) is the recruitment to the TNF receptor of TNF receptor-associated factor 2 (TRAF2). **(ii)** induction of apoptosis via a different pathway involving activation of sphingomyelinase in plasma membrane and generation of ceramide leading to EGFR activation and induction of apoptosis; **(iii)** activation of MEKK1 and JNK protein kinases which is not linked to apoptotic death but to AP-1 activation (**Figure 24**). The antiapoptotic function of NF-κB may involve activation of the manganese superoxide dismutase and of the zinc finger protein A20; expression of these genes is induced by TNF and each of them provides protection against apoptosis (Liu et al, 1996).

bcl-2 upregulation during progression of prostate cancer was implicated in the acquisition of the androgen-independent growth; a strong antioxidant that interferes with activation of NF-κB in prostate carcinoma cells, potentiated TNF-α-stimulated apoptosis signaling through a bcl-2-regulated mechanism; based on these studies, modulation of the NF-κB survival signaling was proposed to be used to clinical advantage in the treatment of prostate cancer patients (Herrmann et al, 1997).

Transgenic mice lacking the p65 (RelA) subunit of NF-κB displayed increased apoptosis and degeneration in the liver providing further support to an apoptotic function of NF-κB (Beg et al, 1995). The TNF-induced death of mouse primary fibroblasts expressing deregulated c-Myc was inhibited by transient overexpression of the p65 subunit of NF-κB, which increased NF-κB activity in the cells (Klefstrom et al, 1997). Rel (a protooncogene, member of the NF-κB family) is implicated in both positive and negative regulation of GM-CSF expression in a variety of cell types (Gerontakis et al, 1996).

The elucidation of IL-1, TNF, IFN and other signaling pathways would lead to the discovery of new drugs causing specific inhibition; for example, members of the IL-1 signaling cascade may provide therapeutic targets for inhibiting IL-1-induced inflammation (Muzio et al, 1997).

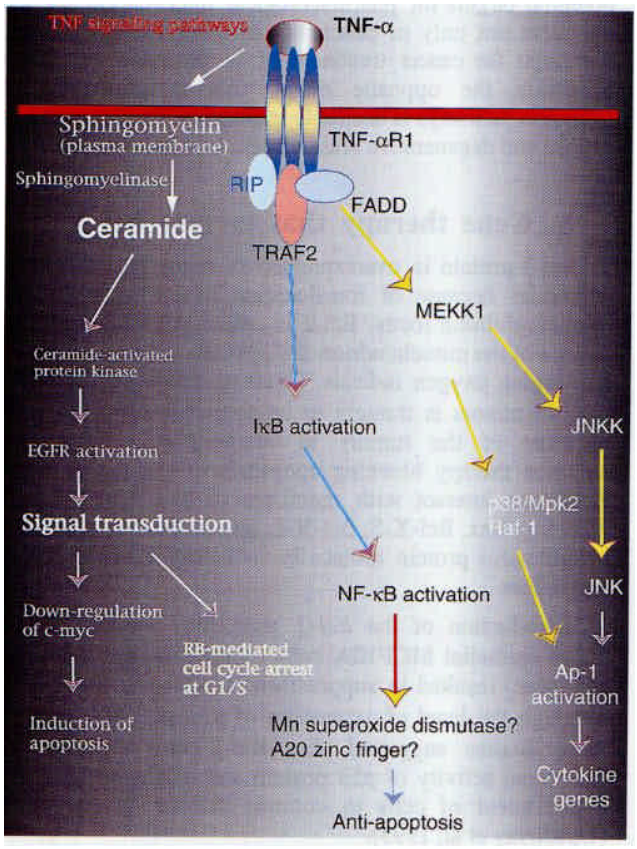


Figure 24. TNF-α signaling via trimerization of its receptors (TNF-αR1), is causing: **(i)** activation of IκB kinase and activation of NF-κB, a pathway which prevents cell death via activation of the manganese superoxide dismutase and of the zinc finger protein A20. **(ii)** induction of apoptosis via second message ceramide (see **Figure 23**) and **(iii)** activation of JNK leading to AP-1 activation and up-regulation of cytokine genes.

E. Interleukin-1β converting enzyme (ICE) and apoptosis

The human interleukin-1β converting enzyme (ICE) is a cysteine-rich protease that can cleave the inactive 31 kDa precursor of IL-1β to generate the active cytokine; it has similarities to the *C. elegans* CED-3 protein. This protease plays a central role in apoptosis; the exact role and the involvement of IL-1β have not been elucidated; it is believed that signals from IL-1β, TNF-α, vitamin D3, and interferon-γ, which induce an antiproliferative response, converge on sphingomyelin of plasma membrane activating a sphingomyelinase which generates a ceramide second messenger (**Figure 23**); in *S. cerevisiae*, this leads to signal transduction via activation both of a cytoplasmic protein phosphatase 2A and a protein kinase leading to down-regulation in c-*myc* expression and induction of apoptosis as well as RB-mediated cell cycle arrest via EGFR activation (Nickels and Broach, 1996).

At least 10 ICE-like proteases have been identified which mediate apoptotic death after their induction by a number of stimuli (see Martin and Green, 1995); these are divided into three families: **(i)** the ICE/CED3 family, including ICE itself; **(ii)** the CPP32/Yama family; and **(iii)** the Ich-1/Nedd2 family; they all contain the conserved QA**C**RG pentapeptide in which the central cysteine participates in proteolytic catalysis (see Jänicke et al, 1996). Activation of these proteases by induction of apoptosis results in the cleavage of a large number of key regulatory proteins including among others poly(ADP-ribose) polymerase or PARP (Lazebnik et al, 1994), RB (Jänicke et al, 1996), PKCd (Emoto et al, 1995), Gas2 affecting microfilament reorganization (Brancolini et al, 1995), the DNA-dependent protein kinase (Casciola-Rosen et al, 1995), and the sterol regulatory element binding proteins (SREBPs) catalyzed by CPP32 ICE-like protease (PARP et al, 1996). Since cleavage of a single protein has not been shown to cause cell death it is not clear how many substrate protein molecules need to be cleaved. In addition different apoptotic pathways may exist and may operate in different cell types.

Expression of the murine *ICE* cDNA in Rat-1 cells induced programmed cell death and this phenomenon could be reversed by overexpression of the *bcl-2* oncogene (Miura et al, 1993). Expression of members of the family of cysteine proteases related to ICE have been shown to be necessary for programmed cell death in a number of organisms (Yuan et al, 1993). Overexpression of murine *ICE* or of the ICE-like proteases NEDD-2/ICH-1 and Yama/apopain induced apoptosis (Miura et al, 1993). Mice lacking ICE were resistant to apoptosis induced by Fas antibody (Kuida et al, 1995).

F. Role of poly(ADP-ribose) polymerase (PARP)

PARP is a central mediator of genome integrity and transmits signals from DNA damage to recruit locally DNA repair activities (Zardo et al, 1998; Quesada, 1998, this volume). An additional role of PARP is its involvement in apoptosis causing suppression of an apoptotic endonuclease; PARP is cleaved by an ICE-like protease during TNF-induced apoptosis (Lazebnik et al, 1994). Cleavage of PARP would abort these pathways resulting in loss of recruitment of DNA repair enzymes at damaged sites but also in loss in the inhibitory function of poly(ADP-ribose) groups on key regulatory enzymes (DNA ligase, topoisomerase). It is unlikely that PARP proteolysis by an ICE-like protease is a primary event since PARP-deficient mice show normal resistance to DNA damaging agents (Wang et al, 1995).

G. Apoptosis in autoimmune disease and ischemic heart disease

T cells are produced by bone marrow and then migrate to the thymus gland where they mature. The cytotoxic or killer T cells directed against foreign bodies are released in

the bloodstream; a specific apoptotic mechanism eliminates T cells directed against specific antigens on healthy cells. However, the body allows some mildly self-reactive lymphocytes to circulate; although harmless, exposure to a microbe or food antigen can stimulate them causing an expansion in their proliferation and resulting in a mild autoimmune disease. Such mild autoimmune reactions usually disappear when the stimulating antigen is cleared away; in more severe autoimmune disease, however, these lymphocytes survive longer inducing apoptosis and self-destruction in healthy cells in various tissues.

A number of classical diseases may originate by autoimmune mechanisms including initiation of atherosclerosis by apoptotic death of the epithelial cells in the arterial wall, diabetes by destruction of the pancreatic cells, lupus erythematosus, rheumatoid arthritis, and others. The mechanism via which T lymphocytes directed against self antigens defy apoptosis is not known; the mechanism might involve overexpression of the Bcl-2 gene in these lymphocytes or down-regulation of a gene encoding for the Fas ligand that sends a death message to the lymphocyte (Weih et al, 1996; reviewed by Duke et al, 1996).

Excessive necrotic death in cells of the coronary artery wall results by oxygen and glucose deprivation after blockage of a blood vessel feeding a segment of the heart (also the brain in stroke). Destructive free radicals are then produced during inflammation of the area which can cause apoptotic or necrotic death in cells in the surroundings. Since both brain and heart cells in the adult are not regenerated, Biotech Companies (for example Genentech) are focusing in developing drugs that block free radical formation, inhibit ICE-like proteases, or inhibit apoptosis via other mechanisms.

The progressive loss of neuron cells in senile or other brain diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophical lateral sclerosis may ensue by apoptosis. Etiologic factors may include excessive levels of neurotransmitters, low levels of NGF, free radical-mediated damage, and deregulation in the expression of genes encoding apoptotic regulators during aging. Deregulation in apoptosis may also have a share in the induction of osteoporosis.

XXI. Genes involved in the regulation of apoptosis as targets for gene therapy

Many of the molecular controllers of apoptosis including cytokine signaling pathways (TNF-α, IL-1β), tumor suppressor proteins (p53), viral proteins (E1A of adenovirus), cellular oncoproteins (Myc), proteins that control the cell cycle (E2F), apoptosis inducers (Bax) and antiapoptotic molecules (Bcl-2, NF-κB) could constitute potential targets for pharmacological intervention for the treatment not only of cancer but of other human disease. Although for cancer treatment it is desirable to induce apoptosis, the opposite effect, that is inhibition of apoptotic pathways is desirable in the gene therapy of heart disease and degenerative brain disease (see below).

A. Gene therapy that targets bcl-2

Bcl-2 protein is overexpressed in a variety of human leukemias because of translocation of its gene to the immunoglobulin locus; Bcl-2 is associated with the outer surface of the mitochondrion and appears to be involved in scavenging oxygen radicals. Overexpression of the *bcl-2* gene in tumors is thought to be responsible for the poor response of the tumors to antineoplastic drugs and radiation therapy blocking apoptosis of the tumor cells. Bcl-2 can interact with members of the Bcl-2 family including Bax, Bcl-X-S, Bcl-X-L, and Mcl-1 but also with heterologous protein molecules including BAG-1, Raf-1, and R-Ras.

Introduction of the *bcl-2* gene into human diploid breast epithelial MCF10A cells (containing the wild-type *p53* gene) resulted in suppression in *p21* gene expression although the level of expression of *p53* was not affected; these studies suggested that Bcl-2 may inhibit the functional activity of p53 protein and might regulate the commitment of cells to commit suicide or proliferate (Upadhyay et al, 1995).

Overexpression of the *bcl-2* gene in tumors is thought to be responsible for the poor response of the tumors to antineoplastic drugs and radiation therapy blocking apoptosis of the tumor cells; therefore, down-regulation of the *bcl-2* gene specifically in tumor cells could induce apoptosis. Primary untreated human prostate cancers were found to express significant levels of this apoptosis-suppressing oncoprotein; this is a striking difference with normal prostate secretory epithelial tissue not expressing Bcl-2 (Raffo et al, 1995). Transfection of LNCaP human prostate cancer cells with a plasmid expressing *bcl-2* rendered these cells highly resistant to a variety of apoptotic stimuli (serum starvation or treatment with phorbol ester) and induced earlier and larger tumors in nude mice. The ability of Bcl-2 to protect prostate cancer cells from apoptotic stimuli correlated with the ability of the cells to form hormone-refractory prostate tumors in nude mice (Raffo et al, 1995).

The Bcl-2 oncoprotein suppresses apoptosis and, when overexpressed in prostate cancer cells, makes these cells resistant to a variety of therapeutic agents, including hormonal ablation. Overexpression of BCL-2 is common in non-Hodgkin lymphoma leading to resistance to apoptosis and promoting tumorigenesis. Therefore, bcl-2 provides a strategic target for the development of gene knockout therapies to treat human prostate cancers (Dorai et al, 1997) and non-Hodgkin lymphomas (Webb et al, 1997).

Down-regulation of Bcl-2 can be accomplished with antisense. In patients with relapsing non-Hodgkin lymphoma, BCL-2 antisense therapy led to an improvement in symptoms; antisense oligonucleotides targeted at the open reading frame of the BCL-2 mRNA

showed effectiveness against lymphoma grown in laboratory animals and has entered human clinical trials. The first study was conducted on nine patients with BCL-2-positive relapsed non-Hodgkin lymphoma using a daily subcutaneous infusion of 18-base, fully phosporothioated antisense oligonucleotide administered for 2 weeks (Webb et al, 1997). A local inflammation at the infusion site was noted. A reduction in tumour size was observed in two patients (one minor, one complete response) using computed tomography scans; in two other patients, the number of circulating lymphoma cells decreased during treatment. In four patients, serum concentrations of lactate dehydrogenase fell, and in two of these patients symptoms improved (Webb et al, 1997).

A divalent hammerhead ribozyme, constructed by recombining two catalytic RNA domains into an antisense segment of the coding region for human bcl-2 mRNA was able to rapidly degrade bcl-2 mRNA in vitro; it was then tested for its ability to eliminate bcl-2 expression from hormone-refractory prostate cancer cells. When this hammerhead ribozyme was directly transfected into cultured prostate cancer cells (LNCaP derivatives), it significantly reduced bcl-2 mRNA and protein levels within 18 hr of treatment and induced apoptosis in a low-bcl-2-expressing variant of LNCaP, but not in a high-bcl-2-expressing LNCaP line (Dorai et al, 1997).

B. Bcl-xs

Many cancers overexpress a member of the Bcl-2 family of inhibitors of apoptosis, such as Bcl-2 and Bcl-xL. Members of the Bcl-2 family were found to be essential for survival of cancer cells derived from solid tissues including breast, colon, stomach, and neuroblasts (Clarke et al, 1995). On the contrary, Bcl-xs is a dominant negative repressor of Bcl-2 and Bcl-xL; thus, Bcl-xs induces apoptosis. Transient overexpression of Bcl-xs in MCF-7 human breast cancer cells, which overexpress Bcl-xL, with a replication-deficient adenoviral vector induced apoptosis in vitro; intratumoral injection of the bcl-xs adenovirus on solid MCF-7 tumors in nude mice showed a 50% reduction in size with evident apoptotic cells at sites of injection (Ealovega et al, 1996).

An adenovirus vector expressing bcl-xs specifically and efficiently killed carcinoma cells arising from multiple organs including breast, colon, stomach, and neuroblasts even in the absence of an exogenous apoptotic signal such as x-irradiation. In contrast, normal hematopoietic progenitor cells and primitive cells capable of repopulating SCID mice were not killed by the bcl-xs adenovirus. Thus, transfer of the bcl-xs gene could be used in killing cancer cells contaminating the bone marrow of patients undergoing autologous bone marrow transplantation (Clarke et al, 1995).

C. E2F-1 and TNF-α gene transfer

E2F cooperates with p53 to induce apoptosis; high levels of wild-type p53 potentiate E2F-induced apoptosis in fibroblasts. The physiological relevance of E2F in the apoptotic mechanism is thought to arise from the ability of E2F to act as a functional link between p53 and RB; p53 levels increased in response to high levels of E2F. Targeted disruption of the E2F-1 gene yields transgenic animals with an excess of mature T cells due to a defect in lymphocyte apoptosis (Field et al, 1996).

Overexpression of the transcription factor E2F-1 could induce apoptosis in quiescent rat embryo fibroblasts in a p53-dependent manner; however, Hunt et al (1997) have shown that overexpression of the E2F-1 gene after adenoviral transfer can mediate apoptosis in the absence of wild-type p53: adenovirus-mediated transfer of the E2F-1 gene under control of the CMV promoter to human breast and ovarian carcinoma cell lines resulted in the induction of significant morphological changes in four of the five cell lines that had mutations in the p53 gene within 48 h of transduction characteristic of apoptosis.

Retroviral vector-mediated transfer of the TNF-α gene into the DNA of human tumor cells induced apoptosis in high- TNF-α-producing clones generated from a human lymphoma T-cell line (ST4); the apoptotic death of the cells was associated with a downregulation of the apoptosis-preventing gene, bcl-2, while the expression of bax and p53 genes persisted (Gillio et al, 1996).

D. E6, E7 of human papillomavirus (HPV)

E6 and E7 of HPV possess transforming ability, have been shown to interact with the cellular tumor suppressors p53 and RB (Werness et al, 1990; Dyson et al., 1989) and are believed to play a central role in HPV-induction of cervical carcinogenesis as well as in the maintenance of the malignant phenotype. Viruses have developed strategies to shut down protein synthesis in the host and subdue its protein synthesizing machinery to produce progeny virus when infecting cells. Because virus-infected cells commit suicide to protect the organism from further infection viruses have evolved mechanisms to prevent apoptosis of the host cell ensuring their propagation; E6 protein interacts with p53 to exclude p53 molecules from their apoptotic functions and to inhibit apoptosis in HPV-infected cells thus giving to HPV a proliferation advantage.

27-mer phosphorothioate oligodeoxynucleotides (oligos) targeting the ATG translational start region of HPV-16 E6 and E7 sequences showed antiproliferative effects in all HPV-16-positive cell lines tested and in primary cervical tumor explants while the endometrial and two ovarian primary tumors as well as the HPV-negative C33-A cell line and HPV-18-positive cell line HeLa were relatively insensitive to the HPV-16 oligos (Madrigal et al, 1997).

E. Prevention of apoptosis for gene therapy of heart disease and for ex vivo manipulations of therapeutic cells

As induction of apoptosis is the desired effect for the gene therapy of cancer, prevention of apoptosis by gene therapy can fight heart disease. Cardiomyocyte death results from heart ischemia proceeding via necrosis and from reperfusion which induces additional cardiomyocyte death by apoptosis; prevention of apoptosis would constitute an important target for fighting heart disease. Prevention of apoptosis should also solve a major problem in cell culture cells which are subject to oxidation damage during their manipulation for *ex vivo* gene transfer and most important during the step of reimplantation, encapsulation in biopolymer membranes for surgical implantation, and similar processes. Prevention of apoptosis could be effected by transfer and overexpression of the *bcl-2* gene. Also prevention of oxidative damage during reimplantation of *ex vivo*-modified cells could be reduced by transfer and overexpression of the Cu/Zn superoxide dismutase gene (Nakao et al, 1995).

Overexpression of bcl-2 delayed onset of motor neuron disease and prolonged survival in a transgenic mouse model of familial amyotrophic lateral sclerosis (Kostic et al, 1997).

XXII. E1A and HER-2/neu (c-erbB-2) in cancer gene therapy

A. HER-2/neu

The human epidermal growth factor receptor-2 (HER2), a membrane tyrosine kinase highly expressed in many epithelial tumors, could be a target for cancer gene therapy. The HER-2/neu (also called c-erbB-2) proto-oncogene is overexpressed in many human cancer cells, including those of breast cancer and ovarian cancer correlating with lower survival rate in ovarian cancer patients; amplification or overexpression of HER-2/neu has also been observed in human lung cancer and has been correlated with poor prognosis and chemoresistance.

A reversible transformation of NIH3T3 fibroblasts by overexpression of the HER2/c-erbB2 receptor tyrosine kinase under control of a tetracycline-responsive promoter has been demonstrated in tissue culture; induction of HER2 expression resulted in cellular transformation in vitro and treatment of transformed cells with the effector anhydrotetracyline switched-off HER2 expression and induced morphological and functional changes characteristic for non-transformed cells (Baasner et al, 1996).

B. E1A-based gene therapy

E1A-based gene therapy approaches are now in clinical trials (see below); the molecular mechanism behind this approach is that the E1A protein of Adenovirus 5 represses HER-2/neu transcription and functions as a tumor suppressor gene in HER-2/neu-overexpressing cancer cells. Breast cancer cells that overexpress HER-2/neu are more resistant to chemotherapeutic agents such as paclitaxel (Taxol) and docetaxel (Taxotere) than those that do not overexpress HER-2/neu; paclitaxel sensitivity correlated with HER-2/neu expression level in a panel of mouse fibroblasts expressing different levels of HER-2/neu; downregulation of HER-2/neu expression by E1A sensitized the cells to paclitaxel. Transfer the E1A gene into two human breast cancer cell lines that overexpress HER-2/neu and E1A gene transfer sensitized these cells to the drug by repressing HER-2/neu expression (Ueno NT et al, 1997).

Increased HER-2/neu expression led to more severe ovarian malignancy and increased metastatic potential in animal models; the adenovirus 5 E1A gene repressed HER-2/neu gene expression and suppressed growth of human ovarian cancer SKOV-3 cells, which overexpress HER-2/neu, in cell culture (Yu et al, 1995). Intraperitoneal injection of SKOV-3 cells into female nu/nu mice elicited tumors and the animals died within 160 days of severe tumor symptoms; cationic liposome-mediated delivery of the E1A gene into adenocarcinomas that developed in the peritoneal cavity and on the mesentery of the mice significantly inhibited growth and dissemination of ovarian cancer cells; about 70% of the treated mice survived at least for 365 days (Yu et al, 1995).

Regulatory regions derived from the 5' flank of the human prostate-specific antigen (PSA) gene were inserted into adenovirus type 5 DNA to drive the expression of the E1A gene; infection of cells in culture with this recombinant adenovirus was able to drive the expression of the E1A gene only in cell lines which expressed PSA such as the human LNCaP cells but not in human DU145 cells which do not express PSA; the recombinant adenovirus destroyed large LNCaP tumors ($1x10^9$ cells) and abolished PSA production in nu/nu mouse xenograft models after a single intratumoral injection (Rodriguez et al, 1997).

A replication-deficient adenovirus containing the E1A gene, Ad.E1A$^+$, was used to transduce E1A into HER-2/neu-overexpressing and low expressing human lung cancer cell lines and shown a better therapeutic efficacy in HER-2/neu-overexpressing cells. The cell culture studies were then extended to animal studies: tumor-bearing mice established by intratracheal injection of lung cancer cells overexpressing HER-2/neu and treated by i.v. tail injections of Ad.E1A$^+$ showed suppression of the intratracheal lung cancer growth. However, no significant tumor suppression effect was observed in mice bearing a low HER-2/neu−expressing cell line with the same regimen (Chang et al, 1996).

C. Clinical trials with E1A and c-Erb-B2

Liposome-mediated E1A gene transfer suppressed tumor development and prolonged survival of mice bearing human breast cancer cells overexpressing HER-2/neu.

These studies resulted in the initiation of a phase I clinical trial using an E1A-liposome complex administered to patients with HER-2/neu-overexpressing breast or ovarian cancer (Protocol 205 in **Table 4** of following article, pages 203-206). The principal investigators are Drs. Hortobagyi, Lopez-Berstein, and Hung at MD Anderson Cancer Center, Houston, Texas). The safety of this regimen was shown by intraperitoneal injection of E1A/liposomes in normal mice and at cumulative doses 5 to 40 times the DNA-lipid starting dose proposed for the phase I clinical trial (Xing et al, 1997). A Phase I multicenter study of intratumoral E1A gene therapy using cationic liposome gene transfer is also in course for patients with unresectable or metastatic solid tumors that overexpress HER-2 /neu (protocol 209, see page 205).

Delivery of an anti-erbB-2 single chain (sFv) antibody gene for previously treated ovarian and extraovarian cancer patients is in clinical trials using adenoviral gene delivery (protocol #133). A clinical trial for tumor vaccination with **HER-2 /Neu** using a B7 expressing tumor cell line prior to treatment with HSV-tk gene-modified cells is in phase I for ovarian cancer (protocol #96, page 165).

XXIII. Suicidal genes for cancer therapy (prodrug gene therapy)

A. Molecular mechanism of cell killing with HSV-tk gene and ganciclovir (GCV)

Expression of genes encoding prodrug-activating enzymes can increase the susceptibility of tumor cells to prodrugs, and may ultimately achieve a better therapeutic index than conventional chemotherapy (**Table 3**). Direct suppression of tumor growth by cytotoxic gene therapy is a successful gene transfer approach. This approach has promise for a variety of other applications where excess cell proliferation is detrimental and has also been used to restrict intimal hyperplasia of the arterial wall and smooth muscle cell growth to limit restenosis after artery angioplasty (see below).

Cancer cells can be induced to be conditionally sensitive to the antiviral drug ganciclovir after their transduction with the thymidine kinase (*tk*) gene from the herpes simplex virus (HSV); ganciclovir (GCV) is the 9-{[2-hydroxy-1-(hydroxymethyl)-ethoxy]methyl}guanine (Field et al, 1983); it is converted by HSV-*tk* into its monophosphate form which is then converted into its triphosphate form by cellular enzymes and is then incorporated into the DNA of replicating mammalian cells leading to inhibition in DNA replication and cell death (Moolten, 1986; Borrelli et al, 1988; Moolten and Wells, 1990). It is only viral TK, not the mammalian enzyme, that can use efficiently ganciclovir as a substrate and this drug has been synthesized to selectively inhibit herpes virus replication (Field et al, 1983); indeed, the mammalian TK has a very low affinity for this guanosine analog. The toxicity of ganciclovir is manifested only when cells undergo DNA replication and it is not harmful to normal nondividing cells. This treatment strategy has been used for hepatocellular carcinoma (Huber et al, 1991; Su et al, 1996), fibrosarcoma, glioma (Culver et al, 1992, see below), adenocarcinoma (Osaki et al, 1994), prostate cancer (Eastham et al, 1996) and many other cancers.

B. Treatment gliomas in rats with HSV-*tk* plus ganciclovir

Brain tumors have the privilege of escaping immunologic rejection; therefore brain tumors are inaccessible to cancer immunotherapy. Culver and cowor-

Table 3. Prodrugs and enzymes used for their activation

Prodrug-activating enzyme	Prodrug	Toxic substance it is converted to
Thymidine kinase from HSV	9-{[2-hydroxy-1-(hydroxymethyl)-ethoxy]methyl}guanine or ganciclovir (GCV)	GCV monophosphate
Cytosine deaminase (CD) from *E. coli*	5-fluorocytosine (5FC)	5-fluorouracil (5FU)
Purine nucleoside phosphorylase (PNP) from *E. coli*	6-methylpurine-2'-deoxyriboside (MeP-dR)	6-methylpurine (a very toxic adenine analog)
Purine nucleoside phosphorylase (PNP) from *E. coli*	Arabinofuranosyl-2-fluoroadenine monophosphate (F-araAMP) commercially known as fludarabine	A very toxic adenine analog
Human deoxycytidine kinase (dCK)	Cytosine arabinoside (ara-C)	A toxic drug inducing lethal strand breaks in DNA
Nitroreductase from *E. coli*	5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954)	A potent dysfunctional alkylating agent which crosslinks DNA

kers (1992) took advantage of the fact that retroviral vectors require DNA synthesis for stable integration into the host genome to target gliomas in rats. Murine fibroblasts were transduced with a retroviral vector expressing the HSV-*tk* gene (see above); the tumor cell mass was then infiltrated by intratumoral injection of the

HSV-*tk*–producing fibroblasts. This treatment gave a continuous local infusion of retroviral vector from the injected fibroblasts, integrating into the dividing cells of the growing brain tumor but not into the nondividing normal cells in the surroundings. Treatment of rats at day 5 after transplantation with ganciclovir (GCV) resulted in the complete regression of the tumor cell mass; this was thought to be induced by killing of cells that respond to signals promoting angiogenesis in the immediate vicinity of the tumor; vascular endothelial cells in the normal brain tissue, exhibiting cycling at a low rate, apparently were not affected. Other proliferating tissues, such as intestinal epithelium, thymus, and bone marrow, which might also uptake the retroviral HSV-*tk* vector and then be destroyed during GCV treatment were not affected by this approach over a 30 day period of treatment with GCV (Culver et al, 1992).

A replication-defective, highly purified retroviral vector at titers of 10^8 colony forming units/mL was used to treat 9L gliosarcoma cells in rat brain. Animals with established 9L tumors treated with intralesional injection of the HSV-*tk* retrovirus followed by GCV treatment showed at day 26 that 29% (4/14) had no tumor and 50% (7/14) of the animals had < 1% tumor volume; substantial numbers of $CD4^+$ and $CD8^+$ lymphocytes infiltrated the tumors of animals treated with HSV-*tk* and GCV; the former tumor bed in cured animals contained cell debris, immune cells, and fibroblasts without signs of damage to the adjacent brain tissue (Kruse et al, 1997).

C. The bystander effect of HSV-tk/GCV

During HSV-tk/GCV treatment of brain tumors products from the dying cells in the brain tumor killed nearby non-HSVtk-transduced cancer cells without affecting normal cells, an effect described as "bystander" antitumor effect (Culver et al, 1992). The bystander effect of the HSV-*tk* plus GCV system appears to be powerful and significant, circumventing the low efficiency of transduction *in vivo* with recombinant retroviruses. Because of this effect, the low-level percentage of cells that can be transduced with a retrovirus can cause the elimination of a much larger percentage of proliferating cells in their surroundings (Kimura et al, 1996).

In vitro, the “bystander” effect works by transfer of cytotoxic small molecules between cells via gap junctions. In order to understand the “bystander effect” mechanism during which adjacent nontransduced tumor cells are killed, Yamamoto et al (1997) used Renca cells from a renal carcinoma cell line transduced with a retroviral vector bearing the HSV-*tk* gene to inoculate BALB/c mice. After complete regression of inoculated tumors with GCV treatment, the animals were challenged with nontransduced tumor cells. In these animals, tumor-specific cytotoxic $CD8^+$ T cells were efficiently induced which promoted the rejection or significant growth inhibition of challenged tumor cells.

In a similar experiment, set to assess the “bystander effect” in vivo, mixtures of HSV-*tk*–transduced and nontransduced oral squamous carcinoma cells were implanted subcutaneously in the left flank of nude mice, and naive HSV tk^- cells were implanted subcutaneously in the right flank. Treatment with GCV eradicated the tumors in the left flank consistent with a predicted bystander effect but also resolved or arrested the growth of the naive tumors in the right flank. The histology of regressing tumors from the right flank showed an infiltration of lymphoid cells suggesting that an immune-related antitumor response accounted for the distant bystander effect (Bi et al, 1997; see also Ramesh et al, 1998 this volume).

The induction of higher levels of HSV-*tk* expression does not augment the sensitivity to GCV: adenoviral vectors that expressed HSV-tk at different efficiencies from CMV versus RSV promoters did not display a significant difference in antitumor effects; thus, increasing the HSV-TK enzyme levels per cell above a minimal threshold level will not be effective in cell killing with GCV. To enhance the therapeutic responses of the HSV-tk/GCV system one needs to improve other parameters such as to use higher doses of GCV, to enhance the "bystander effect," to engineer mutant HSV-tk genes with higher substrate affinities, or to discover vectors with increased transduction efficiencies (Elshami et al, 1997).

Suicide gene therapy may be useful not only for short-term tumor regression mediated by direct cell killing and bystander effect, but may also exert a therapeutic vaccination effect resulting in long-term tumor regression and prevention of recurrence (Yamamoto et al, 1997).

D. Additional examples of tumor eradication with HSV-tk/GCV

Chen et al (1996) used a recombinant adenoviral vector containing the HSV-tk gene for the treatment of metastatic colon carcinoma in the mouse liver; the HSV-tk alone exhibited substantial regression, although all treated animals suffered from subsequent relapses. Delivery of the HSV-tk + mouse IL-2 genes in adenoviral vectors to the hepatic tumors induced an effective antitumor immune response which nevertheless waned with time, and the treated animals eventually succumbed to hepatic tumor relapse; however, after combination treatment with HSV-tk, mouse IL-2, and mouse GM-CSF a fraction of the animals developed long-term antitumor immunity and survived for more than 4 months without tumor recurrence (Chen et al, 1996).

Microinjection of the HSV-*tk* gene, under control of α-fetoprotein enhancer and albumin promoter, in a linear form flanked by the adeno-associated virus ITRs into pronuclei of mouse embryos led to transgenic animals expressing preferentially HSV-*tk* into adult liver cells; this led to an approach for the treatment of hepatocellular carcinomas (Su et al, 1996). Subcutaneous tumors induced

by injection of RM-1 (mouse prostate cancer) cells in mice followed by injection of HSV *tk* in an adenovirus vector and treatment with ganciclovir for 6 days showed reduction in tumor volume (16% of control) and higher apoptotic index in tumor cells (Eastham et al, 1996). Recombinant adenoviruses carrying the HSV-tk gene under control of the CMV promoter displayed a significant cell killing efficiency for the eradication of brain tumors and leptomeningeal metastases in rats (Vincent et al, 1997).

Pancreatic cancer is the fifth leading cause of cancer death in the United States. In order to treat peritoneal dissemination, one of the most common complications of the malignancies of the digestive system such as gastric or pancreatic cancers, mice were intraperitoneally (i.p.) inoculated with the human pancreatic cancer cell line PSN-1; i.p. transfer of the HSV-tk suicidal gene under control of the potent hybrid CAG promoter was achieved with a DNA-lipopolyamine complex given eight days from the injection of cancer cells; animals were treated with GCV for 8 days; 8 out of 14 mice treated with HSV-*tk* and GCV were free of tumors on day 24. The gene transfer method resulted in the transduction of tumor nodule cells and not in normal organs as shown by reverse transcription polymerase chain reaction (RT-PCR) analysis as well as by transfer of the lacZ gene under similar conditions and localization of the blue staining; HSV-tk was expressed in about 10% of tumor cells but not in the normal pancreas or in the small intestine (Aoki et al, 1997).

A murine pancreatic ductal adenocarcinoma cell line was used to induce intrahepatic solid tumors into the left lateral liver lobe; intratumoral injection of an adenovirus vector carrying the HSV-tk gene under control of the RSV promoter in combination with intraperitoneal administration of ganciclovir caused a significant reduction in tumor volume and necrosis; because pancreatic cancer patients have an overall low survival since metastases have already taken place at the time of diagnosis and because surgical resection of pancreatic cancers does not significantly change the clinical outcome even in combination with chemotherapy, gene therapy might offer an effective approach in the near future (Block et al, 1997).

HSV-tk gene transfer was successfully used to eradicate adenocarcinoma-derived peritoneal carcinomatosis, a common clinical situation which, in most cases cannot be controlled by surgery or chemotherapy. DHD/K12 colon carcinoma cells stably expressing the HSV-*tk* gene were injected intraperitoneally to rats leading to the development of peritoneal carcinomatosis within 2-3 weeks from injection (**Figure 25A**). Treatment of these animals with GCV (**Figure 25C**) resulted in the eradication of the peritoneal tumor nodes. It ought to be emphasized, however, that the same spectacular results are not expected when treating tumors in patients; tumor cells in patients need first to be transduced with the HSV-*tk* gene whereas the cells used to elicit these tumors in animals were already transduced with the HSV-*tk* gene in cell culture and most or all cells were expressing the viral thymidine kinase.

Retrovirus-mediated transfer of HSV-*tk* was used to kill proliferating cells in rabbit models of **proliferative vitreoretinopathy** (PVR); traction retinal detachment results from proliferation of retinal pigment epithelial, glial, macrophages, and fibroblast cells in the vitreous cavity of the eye forming contractile membranes on both surfaces of the retina; PVR may ensue after retinal surgery or trauma and can be induced in rabbit models by surgical vitrectomy to facilitate cell attachment to the retina. Injection, into the vitreous cavity, of rabbit dermal fibroblasts transduced i*n vitro* with retroviral vectors carrying the HSV-*tk* gene was used to preferentially kill proliferating cells for PVR in rabbit models; all eyes received 0.2 mg GCV on the following day and on day 4; significant inhibition of PVR was observed thus providing a novel therapeutic strategy for this disease (Kimura et al, 1996).

E. Expression of cytosine deaminase (CD) gene from *E. coli* and treatment with 5-fluorocytosine

Another suicide gene approach has been the expression of the cytosine deaminase (CD) from *E. coli*; mammalian cells, unlike certain bacteria and fungi, do not posses this enzyme. The CD protein normally catalyzes the conversion of cytosine to uracil but has been exploited for the conversion of the prodrug 5-fluorocytosine (5FC) into the toxic 5-fluorouracil (5FU); treatment of cells, transfected with this construct, with 5FC resulted in the conversion of the 5FC into the antitumor drug 5FU into CD-expressing tumor cells (Mullen et al, 1992; Austin and Huber, 1993; Huber et al, 1993; 1994; Richards et al, 1995).

This approach has been used for the treatment of primary and metastatic hepatic tumors based on the overexpression of the suicidal *CD* gene under control of the regulatory regions of the tumor marker gene carcinoembryonic antigen (Richards et al, 1995, see below).

Szary et al (1997) have developed a model for tumor radiosensitization using the CD gene/5FC system; when melanoma cells were transfected with the CD gene, subsequent treatment with 5FC sensitized the cells to radiation damage; 5FC did not change the radiosensitivity of parental, nontransfected cells; increased toxicity to radiation damage was thought to arise from 5-fluorouracil generated by CD.

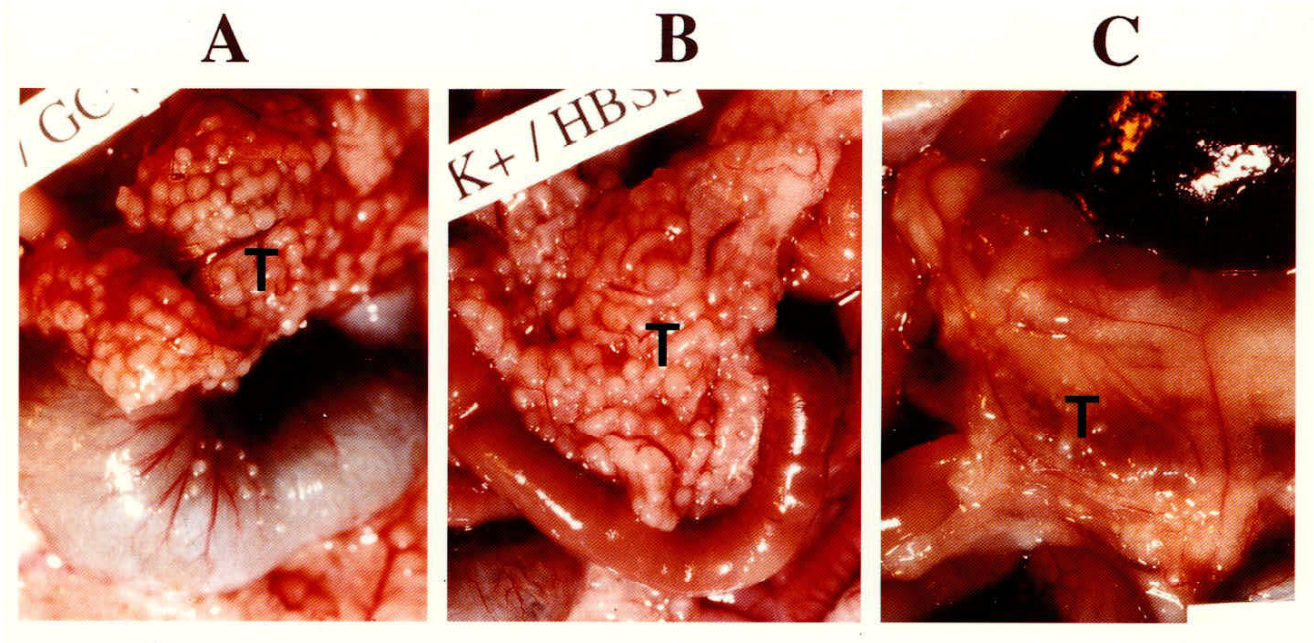


Figure 25. Eradication of peritoneal carcinomatosis with HSV-tk plus GCV. Intraperitoneal injection to rats of DHD/K12 colon carcinoma cells stably expressing the HSV-*tk* gene caused peritoneal carcinomatosis at day 21 (**A**). The animal whose intraperitoneal cavity is shown in (**B**) was treated with HBSS buffer alone and the animal shown in (**C**) was treated with GCV for 5 days at 150mg/Kg. The letter "T" indicates the peritoneal tumor nodes. From Lechanteur C, Princen F, Bue SL, Detroz B, Fillet G, Gielen J, Bours V, and Merville M-P (**1997**) *HSV-1* thymidine kinase gene therapy for colorectal adenocarcinoma-derived peritoneal carcinomatosis. **Gene Ther** 4, 1189-1194. Reproduced with the kind permission of the authors (Vincent Bours, University of Liège, Belgium) and of Stockton Press.

Infection of the human breast cancer cell line, MDA-MB-231, with a recombinant adenovirus expressing the *Escherichia coli* CD resulted in high levels of cytosine deaminase enzyme activity and infected cells became 1000-fold more sensitive to 5-FC than cells infected with a control adenovirus; only 10% of infected cells in a population were needed to induce complete cytotoxicity of noninfectious cells exposed to 5-FC via bystander effects. Direct injection of the CD-adenovirus into human breast tumor xenografts in nude mice, followed by daily intraperitoneal injection of 5-FC was sufficient to inhibit tumor growth (Li et al, 1997).

F. Bacterial purine nucleoside phosphorylase (PNP) gene

Another suicide gene/prodrug couple is the *E. coli DeoD* gene which encodes the purine nucleoside phosphorylase (PNP). The *E. coli* PNP, unlike the mammalian endogenous PNP, can utilize certain adenosine analogs as substrates including nontoxic purine nucleosides converting them to very toxic adenine analogs; these substrates include 6-methylpurine-2'-deoxyriboside (MeP-dR) and arabinofuranosyl-2-fluoroadenine monophosphate (F-araAMP) commercially known as fludarabine. This enzyme converts the 6-methylpurine deoxyribose (MeP-dR) prodrug into the diffusible, toxic 6-methylpurine and can become a powerful suicide gene under these conditions (Sorscher et al, 1994).

The significant advantages in eradicating experimentally-induced human tumors in nude mice with this system were: (**i**) the bystander effect was 2-3 orders of magnitude higher than with HSV-tk/GCV and tumor eradication could be seen only after 3 doses of PNP/MeP-dR treatment, (**ii**) the MeP-dR and F-araAMP crossed readily the cell membrane unlike GCV, and (**iii**) PNP/MeP-dR could kill both proliferating and nonproliferating tumor cells as has been demonstrated by eradication of the slowly-growing D54MG glioma tumors expressing the bacterial PNP gene in nude mice after treatment with MeP-dR (**Figure 26**; Parker et al, 1997).

G. Deoxycytidine kinase/ara-C and nitroreductase/5-(aziridin-1-yl)-2,4-dinitrobenzamide

The human deoxycytidine kinase (dCK) can phosphorylate the prodrug cytosine arabinoside (ara-C), a cytidine analog, and catalyze its conversion into a toxic drug inducing lethal strand breaks in DNA. Although ara-C is a potent antitumor agent for hematologic malignancies it is ineffective against solid tumors; transduction of the *dCK* cDNA with adenovirus and retrovirus into the 9L gliosarcoma cell line followed by establishing intradermal and intracerebral gliomas in syngeneic rats demonstrated

the efficacy of systemic ara-C treatment of the animals in eradicating these tumors (Manome et al, 1996).

The prodrug 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB-1954) is a weak, monofunctional alkylating agent which can be activated by *Escherichia coli* nitroreductase to a potent dysfunctional alkylating agent which crosslinks DNA. Transduction of colorectal and pancreatic cancer cell lines with the nitroreductase gene using a retroviral vector rendered them 50 to 500-fold more sensitive than parental cells to CB1954; concentrations of CB1954 which were minimally toxic to nontransduced cells achieved 100% cell death in a 50:50 mix of parental cells with transduced cells expressing nitroreductase due to "bystander" cell killing (Green et al, 1997).

H. Preferential expression of suicidal genes in cancer cells using promoters/ enhancers from tumor-specific genes

The principle of VDEPT (virus-directed enzyme /prodrug therapy) was used to target hepatocellular carcinoma using the regulatory region from the tumor-specific α-fetoprotein gene to drive the Varicella zoster thymidine kinases gene (Huber et al, 1991).

A similar gene therapy approach has been developed for the treatment of primary and metastatic hepatic tumors based on the overexpression of the suicidal gene cytosine deaminase (*CD*) from *E. coli* under control of the regulatory regions of the tumor marker gene carcinoembryonic antigen (*CEA*) (Richards et al, 1995); this created a chimeric gene that was specifically expressed in neoplastic cells. Development of this strategy has necessitated the identification of the regulatory regions of

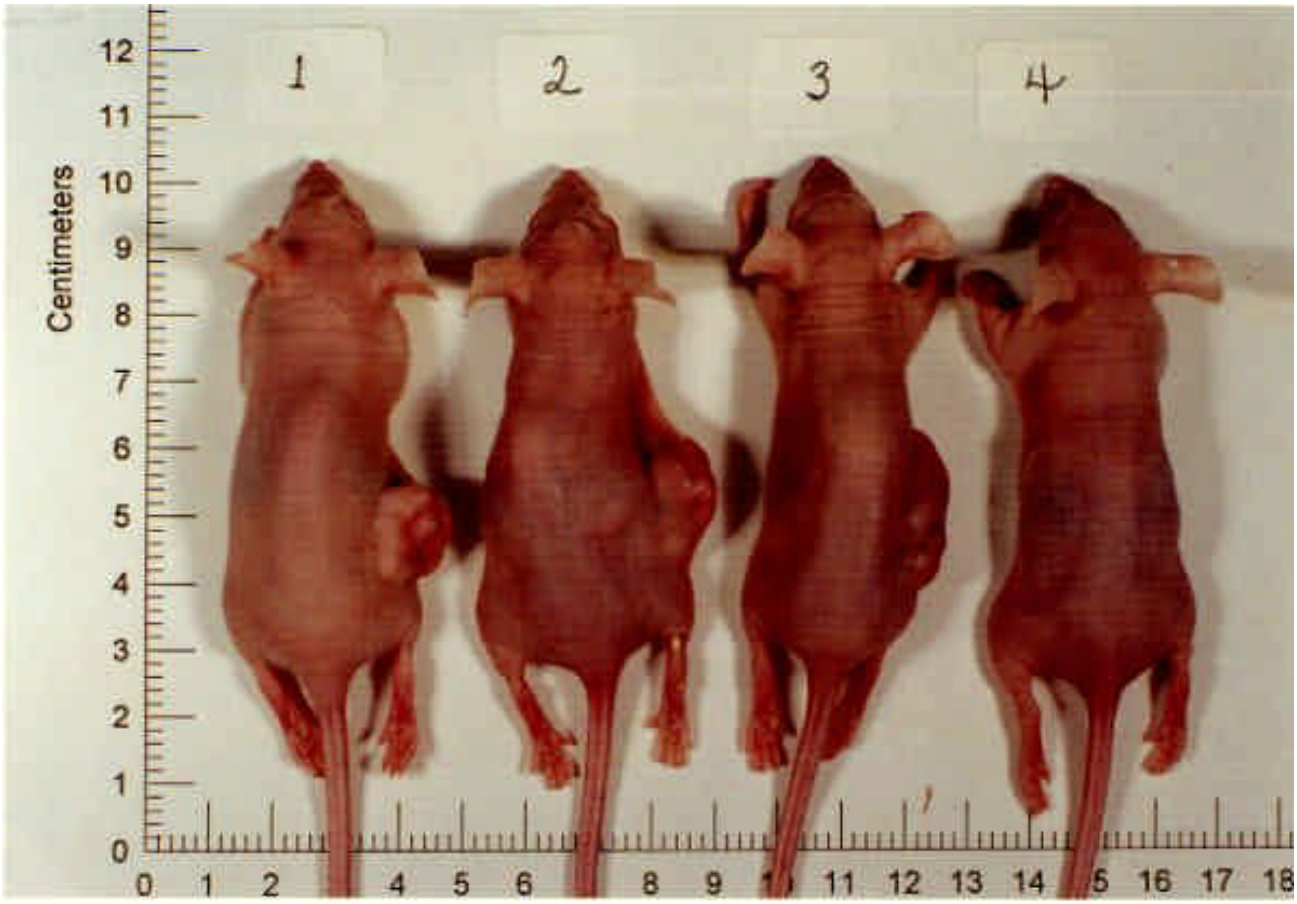


Figure 26. A nude mouse xenograft model was developed bearing malignant gliomas by s.c. injection of D54MG human cells or D54MG human cells transduced and expressing *E. coli* PNP which are called D54-PNP cells; tumors were successfully eradicated with MeP-dR treatment. Representative animals from each of 4 groups at completion of the study (62 days) are shown: **Group 1**: nude mice were injected with D54MG cells, vehicle treated. **Group 2**: nude mice were injected with D54MG cells, MeP-dR treated. **Group 3**: nude mice were injected with D54-PNP cells, vehicle treated. **Group 4**: nude mice were injected with D54-PNP cells, MeP-dR treated. From Parker WB, King SA, Allan PW, Bennett LLJr, Secrist JAIII, Montgomery JA, Gilbert KS, Waud WR, Wells AH, Gillespie GY, and Sorscher EJ (**1997**) *In vivo* gene therapy of cancer with *E. coli* purine nucleoside phosphorylase. **Hum Gene Ther** 8, 1637-1644. With the kind permission from the corresponding author (Eric Sorscher, University of Alabama at Birmingham) and Mary Ann Liebert, Inc.

the *CEA* gene; isolation of 14.5 kb of 5' flanking sequences for this gene followed by subcloning into luciferase pGL2 basic vectors and testing for luciferase activity in transfected LoVo, SW1463, Hep3B, and HuH7 cell lines (the first two express *CEA* whereas the other two do not) has identified the *CEA* promoter between bases -90 and +69, and two enhancers one at -13.6 to -10.7 and the other at -6.1 to -4.0 kb (Richards et al, 1995); these sequences were able to sustain high levels of expression of the *CD* gene into *CEA*-expressing cell lines.

Regulatory sequences from the *CEA* gene (-322 to +111 bp) were also used to express the HSV thymidine kinase gene in pancreatic and lung neoplasms (Dimaio et al, 1994; Osaki et al, 1994).

XXIV. Transfer of drug resistance genes

A. Principles and genes used

An attractive approach to circumvent chemotherapy-induced myelosuppression is the use of gene-transfer technology to introduce new genetic material into hematopoietic cells. Protection of bone marrow progenitor cells by introduction of a drug resistance gene allows larger and curative doses of chemotherapy to be administered to the patient as was shown in several pre-clinical studies. Drug resistance genes under experimental consideration are shown on **Table 4**. Clinical trials are now under way to evaluate the potential use of two gene sequences: MDR1 (protocols #43, 44, 59, 89, and 100) and O^6-methylguanine DNA methyltransferase (#101 see **Appendix 1**) (see also Lee et al, 1998, this volume).

Dose-limiting hematopoietic toxicity produced by the cytosine nucleoside analogue cytosine arabinoside (Ara-C) is one of the major factors that limit its use in the treatment of neoplastic diseases. Deamination of Ara-C by cytidine deaminase results in a loss of its antineoplastic activity. Transfer of human cytidine deaminase into murine fibroblast and hematopoietic cells conferred drug resistance to Ara-C protecting them from drug toxicity (Momparler et al, 1996). It is worth mentioning that apolipoprotein B mRNA editing involves the deamination of cytidine by the cytidine deaminase catalytic subunit that creates a new termination codon and produces a truncated version of apo-B (apo-B48); the cytidine deaminase catalytic subunit (apo-B mRNA-editing enzyme catalytic polypeptide 1) of the multiprotein editing complex has been identified (Yamanaka et al, 1995).

B. Mechanism of MDR1 resistance

A great deal of our knowledge of basic insights on drug uptake and molecular mechanisms of drug action were elucidated from the study of resistance of tumor cells to chemotherapeutic agents. The P-glycoprotein or p170 encoded by the multidrug resistance *MDR1* gene uses the energy of ATP to extrude a variety of drugs apparently unrelated; the only chemical similarity is that they contain condensed aromatic rings and have a positive charge at neutral pH; these drugs, most of which are effective against a variety of human tumors, include molecules found in nature such as colchicine, doxorubicin (also called adriamycin, member of the anthracycline family), actinomycin D, vinblastine, etoposide, taxol, vinca alcaloids, and epipodophyllotoxins collectively called MDR-type of drugs (reviewed by Gottesman and Pastan, 1988; see Lee et al, 1998 this volume).

Cell lines resistant to drugs accumulate far less amounts of drug compared with parental cells because of overexpression of the *MDR1* gene; development of multidrug resistance by tumor cells poses a major impediment to successful cancer chemotherapy. A number of cell lines with multidrug resistance have been derived like KB and K562 cells (Marie et al, 1991; Fardel et al, 1995). The P-glycoprotein is a 1280 amino acid molecule in human cells (Chen et al, 1986) or 1276 amino acid molecule in mouse cells with 80% sequence similarity to the human protein (Gros et al, 1986). P-glycoprotein has 12 hydrophobic domains grouped into pairs representing transmembrane domains. The molecule has a 500 amino acid duplication; each duplicated segment possesses an ATP-binding site on the cytoplasmic side; it also has several site of glycosylation near the N-terminus to the exterior side. Its gene is amplified in multidrug resistant

Table 4. Drug resistance gene designs

Drug resistance gene	Confers resistance to	Reference
MDR1 (multidrug resistance)	Daunomycin, doxorubicin, taxol	Galski et al, 1989; Podda et al, 1992; Sorrentino et al, 1992 (see below)
Mutant dihydrofolate reductase	Methotrexate (MTX)	Williams et al, 1987; Corey et al, 1990; Li et al, 1994; Zhao et al, 1997
Glutathione transferase	DNA alkylating agents	reviewed by Maze et al, 1997
O^6-methyl guanine transferase	Nitrosoureas	Allay et al, 1995
Cytidine deaminase	Cytosine arabinoside (Ara-C)	Momparler et al, 1996
Aldehyde dehydrogenase	Cyclophosphamide	reviewed by Koc et al, 1996

cell lines accompanied by an increased expression of the 4,500 to 5,000-nt in size mRNA for P-glycoprotein (Chen et al, 1986).

Rates of drug influx for lipid-soluble drugs are proportional to drug concentrations in the medium; P-glycoprotein alone or in conjunction with other cellular components seems to transport drugs to the exterior of the cell, a mechanism pronounced in drug-resistant cell lines. Consistent with the presence of a membrane-bound, exchangeable pool of drug and a cytoplasmic, non exchangeable pool, P-glycoprotein was proposed to directly interact via its hydrophobic transmembrane domains with the membrane-associated drug molecules (anthracyclins, vinca alcaloids) to mediate their efflux to the extracellular milieu (Gros et al, 1986). Doxorubicin, an inhibitor of topoisomerase II which is a major nuclear matrix component, has been shown to interact with hydrophobic regions in calmodulin; calmodulin is also a nuclear matrix protein. Photoaffinity-labeled analogs of vinblastine showed direct binding of this drug to P-glycoprotein (Safa et al, 1986).

Expression of P-glycoprotein is consistently low in bone marrow cells rendering them particularly sensitive to certain MDR-type of anticancer drugs; chemotherapy with these drugs largely depletes or wipes off bone marrow pluripotent stem cells from patients (myelosuppression). One approach to this problem has been removal and deep-freezing of bone marrow samples from cancer patients prior to chemotherapy; in a second phase $CD34^+$ cells are isolated from the frozen bone marrow specimen using negative selection on soybean agglutinin plates followed by a positive selection on plates coated with anti-$CD34^+$ antibody (Ward et al, 1994) which are then reimplanted to the patient or are simply injected intravenously and find their way to the bone marrow where they implant; this is a costly undertaking.

Gene therapy approaches are being aimed at transferring the *MDR1* gene under the control of a strong promoter/enhancer into bone marrow stem cells; transfected stem cells, from which all B and T cells are derived, would be rendered resistant to chemotherapeutic drugs used to treat cancer patients and allow administration of higher doses of these drugs. Furthermore, even if a small percentage of cells are successfully transfected, these cells could be expanded by selection with MDR-drug. The same approach could be used to express a nonselectable gene such as the β-globin gene to treat sickle cell anemias and thalassemias inserted in the same construct with the *MDR1* gene as has been suggested by Ward and coworkers (1994).

C. Transfer of the *MDR1* gene into bone marrow cells

The purpose of this approach is to overexpress the *MDR1* gene in bone marrow cells in *ex vivo* or *in vivo* protocols in order to render stem cells resistant to cancer chemotherapy; this will prevent destruction of the bone marrow stem cells during treatment of cancer patients with antineoplastic drugs for killing tumor cells. Transfer of the MDR1 cDNA into primary human hematopoietic progenitor cells of cancer patients undergoing high-dose chemotherapy will protect the bone marrow from the dose-limiting cytotoxicity of cytostatic agents.

Transgenic mice expressing the human *MDR* cDNA in their bone marrow cells were resistant to doxorubicin (Galski et al, 1989; Mickisch et al, 1991). Retroviral transfer of *MDR1* resulted in high level expression of both RNA and P-glycoprotein; taxol-treatment of mouse bone marrow cells killed those that had not been transfected and resulted in an enrichment of the cells containing the human gene (Sorrentino et al, 1992; Podda et al, 1992). Transfer of the MDR1 gene via a retrovirus into human $CD34^+$ cells, isolated from bone marrow and stimulated with IL-3, IL-6, and stem cell factor, showed that 20-70% of the CFU-GM or BFU-E cells contained the transferred *MDR1* gene by PCR analysis (Ward et al, 1994).

AAV and cationic liposomes have been used for the transfer of the human MDR1 cDNA to NIH-3T3 cells followed by selection of successfully transfected cells based on the drug-resistant phenotype conferred by the P-glycoprotein efflux pump; a single intravenous injection of the bicistronic vector complexed to cationic liposomes into recipient mice, achieved delivery of MDR1 and human glucocerebrosidase cDNAs in all the organs tested (Baudard et al, 1996).

Eckert et al (1996) have designed novel retroviral vectors termed SF-MDR and MP-MDR which significantly elevated survival of transduced primary human hematopoietic progenitor cells under moderate doses of colchicine and paclitaxel in vitro when compared with a conventional MoMuLV-based vector; the novel vectors were based on the spleen focus-forming virus or the myeloproliferative sarcoma virus for the enhancer DNA sequence and the murine embryonic stem cell virus for the leader.

A bicistronic retroviral vector (HaMID) containing a modified human MDR-1 cDNA and a mutant human dihydrofolate reductase cDNA bearing a leucine to tyrosine substitution at codon 22 was constructed and used to transduce the human CEM T lymphoblastic cell line as well as primary murine myeloid progenitors; HaMID-transduced cells were highly resistant in the presence of 25 nM taxol and 100 nM trimetrexate simultaneously while control cells were entirely growth inhibited (**Figures 27**, **28**; Galipeau et al, 1997).

Several human clinical trials, approved by RAC and FDA, are under way with the long-term goal of transferring the *MDR1* gene into bone marrow cells of advanced cancer patients using retroviral infection. A human gene therapy protocol (#100) for chemoprotection of patients treated for testicular cancer with high doses of carboplatin and etoposide proposes to use transplantation of these patients with autologous peripheral blood stem cells (drawn, purified and cryopreserved prior to chemotherapy treatment) and transduced with the MDR1

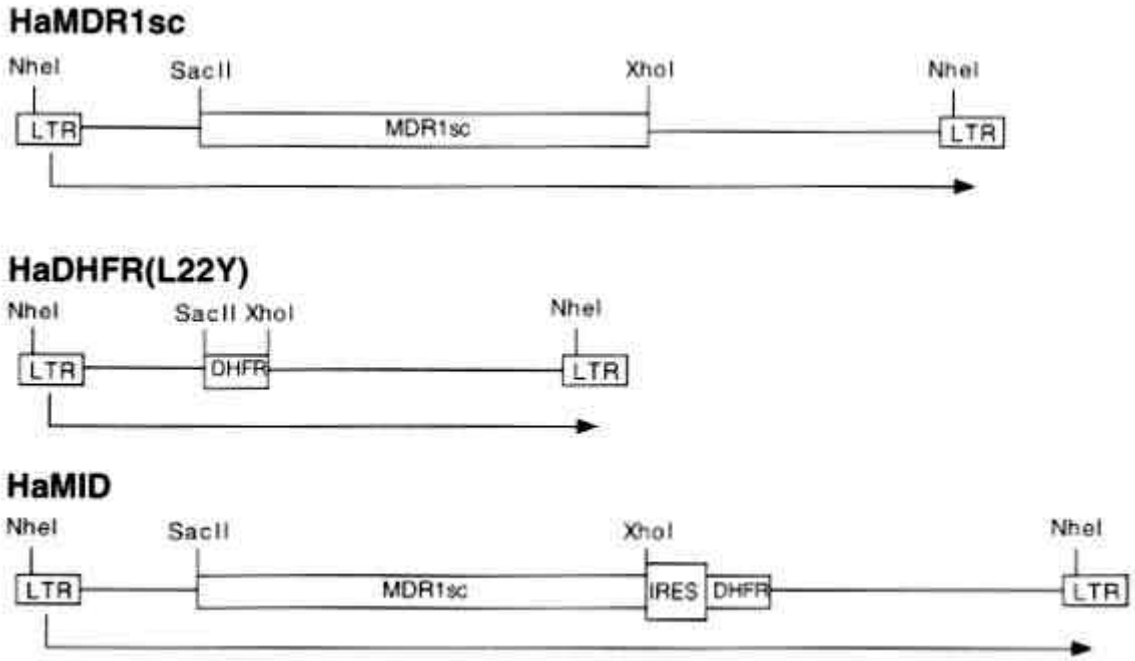


Figure 27. Structure of the retroviral vectors used to deliver the MDR1 and DHFR genes. The vectors are based on the Harvey murine sarcoma virus. A single transcript (arrow) is initiated in the retroviral 5' LTR promoter. HaMDR1sc (top) contains the MDR1sc cDNA and HaDHFR(L22Y) (middle) contains a mutant DHFR cDNA. The bicistronic (two-gene) vector HaMID (bottom) contains both MDR1 and DHFR genes. From Galipeau J, Benaim E, Spencer HT, Blakley RL, Sorrentino BP (**1997**) A bicistronic retroviral vector for protecting hematopoietic cells against antifolates and P-glycoprotein effluxed drugs. **Hum Gene Ther** 8, 1773-1783. Reproduced with kind permission from the authors and Mary Ann Liebert, Inc.

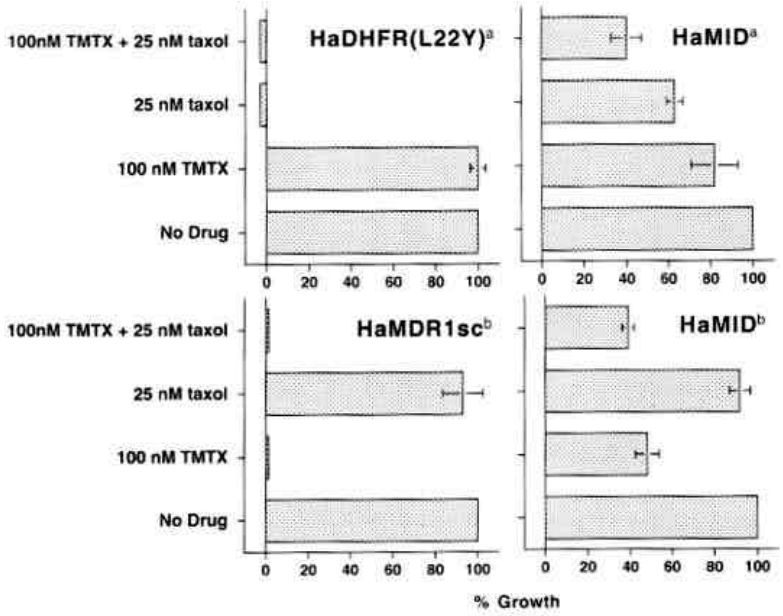


Figure 28. Growth inhibition assays comparing the effect of 25 nM taxol, 100 nM trimetrexate (TMTX) alone and in combination on CEM cells transduced with HaDHFR(L22Y), HaMDR1sc, or HaMID. Drug-selected CEM cells were washed and seeded at $1x10^5$ cells/ml in 2 ml of media containing the indicated concentrations of drugs. After 72 hr, the percentage of growth was calculated by dividing the number of cells at each drug concentration by the number of cells present in control medium (100% growth). Quadruplicate experiments are shown. [a]Cells preselected in 100 nM trimetrexate. [b]Cells preselected in 25 nM taxol. From Galipeau J, Benaim E, Spencer HT, Blakley RL, Sorrentino BP (**1997**) A bicistronic retroviral vector for protecting hematopoietic cells against antifolates and P-glycoprotein effluxed drugs. **Hum Gene Ther** 8, 1773-1783. Reproduced with kind permission from the authors and Mary Ann Liebert, Inc.

cDNA. Similar protocols (#43, 44, 59, 89) use $CD34^+$ autologous bone marrow cells retrovirally-transduced with MDR1 cDNA for hemoprotection of patients treated for ovarian, brain, or breast cancers (**Appendix 1**).

XXV. Antisense gene therapy of cancer.

Among a variety of approaches to gene therapy of cancer, antisense oncogene gene therapy is a strategy aiming at correcting genetic disorders of cancer through correction of the abnormal expression of oncogenes implicated in signal transduction and control of proliferation. A number of protocols have been approved using antisense gene or oligonucleotide delivery. Protocol 29 uses a combination of p53 cDNA and K-ras antisense for non-small cell lung cancer. Protocol 41 uses antisense Rev for AIDS, protocol 91 antisense RRE decoy gene and protocol 168 uses antisense TAR and transdominant Rev protein genes for HIV infections. Protocol 64 uses antisense c-fos or antisense c-myc for breast cancer. Protocol 82 uses intraprostate injection of antisense c-myc for advanced prostate cancer. Protocol 162 uses TGF-ß2 antisense gene-modified autologous tumor cells for malignant glioma. And, protocol 189 uses antisense Insulin-like Growth Factor I for glioblastoma (see below).

A. Antisense c-fos and c-myc

Because c-fos proto-oncogene has been implicated as a regulator of estrogen-mediated cell proliferation, antisense c-fos has been used to cause an inhibition of s.c. tumor growth and invasiveness of cells the growth of which depends on estrogen. Ex vivo transduction of MCF-7 human breast cancer cells with antisense c-fos, regulated by mouse mammary tumor virus control elements and delivered by a retroviral vector, produced expression of anti-fos RNA, decreased expression of the c-fos target mRNA, induced differentiation, and inhibited s.c. tumor growth and invasiveness in breast cancer xenografts in nude mice; a single injection of anti-fos inhibited i.p. MCF-7 tumor growth in athymic mice with a corresponding inhibition of c-fos and TGF-β1 (Arteaga and Holt, 1996). A phase I clinical study for the treatment of metastatic breast cancer uses in vivo infection with breast-targeted retroviral vectors expressing antisense c-fos or antisense c-myc RNA (Holt et al, 1996; protocol #64, **Appendix 1**, page 163).

B. Antisense insulin-like growth factors I and II and their receptors

Insulin-like growth factors I and II (IGF-I and -II) are expressed preferentially in bone tissue and contribute to bone metastases of cancer cells expressing IGF receptors. Prostate cancer cells express IGF-I receptor; this favors metastasis to bone, the most frequent tissue for prostate metastasis. An antisense IGF-IR construct, under control of the $ZnSO_4$-inducible metallothionein-1 promoter, was engineered by reverse transcription-PCR on total RNA with primers specific for the 0.7 kb cDNA of IGF-IR and subcloned into episomal vectors in the antisense orientation. Transfection of the construct into prostate cancer PA-III cells in culture was able to reduce dramatically the expression of IGF-IR after induction of the cells with $ZnSO_4$ (Burfeind et al, 1996). This inhibition resulted in reduction in expression of both uPA and tPA; whereas PA-III cells were able to induce large tumors in nude mice, PA-III cells transfected with the antisense vector either developed tumors 90% smaller or remained tumor -free for long times postinjection (Burfeind et al, 1996).

Lafarge-Frayssinet et al (1997) have developed a strategy for inducing a protective immunity by tumor cells transfected by the IGF-I antisense vector: the hepatocarcinoma cell line LFCI2-A, expressing both IGF I and II, produces voluminous tumors when injected subcutaneously into syngeneic rats; when LFCI2-A cells were transfected with an episomal vector expressing IGF-I antisense RNA, the cells became poorly tumorigenic exhibited a 4-fold increase of the MHC class I antigen, and, when injected subcutaneously, inhibited the growth of the parental tumoral cells or induced regression of established tumors; this loss of tumorigenicity and protective immunity was not observed after transfection with the IGF-II antisense vector (Lafarge-Frayssinet et al, 1997). Cationic lipid-mediated transfer of antisense cDNA for IGF I is in clinical trial for glioblastomas (protocol #189 in **Table 4** in Martin and Boulikas, 1998, this volume, page 203).

C. Antisense ras gene transfer for pancreatic tumors

K-ras point mutations occurs at a characteristically high incidence in human pancreatic cancers. Stable expression of a plasmid expressing antisense K-ras RNA into pancreatic cancer cells with K-ras point mutations (AsPC-1 and MIAPaCa-2) resulted in a significant suppression of cell growth; the effect of antisense treatment was not found in cells with a wild-type K-ras gene (BxPC-3). When the AsPC-1 cells with the K-ras point mutation were inoculated into the intraperitoneal cavity of nude mice, followed 3 days later by i.p. treatment with the antisense K-ras in a liposome complex, only 2 of 12 mice showed any evidence of tumors on day 28 compared with 9 out of 10 control mice that developed peritoneal dissemination and/or solid tumors on the pancreas (Aoki et al, 1995).

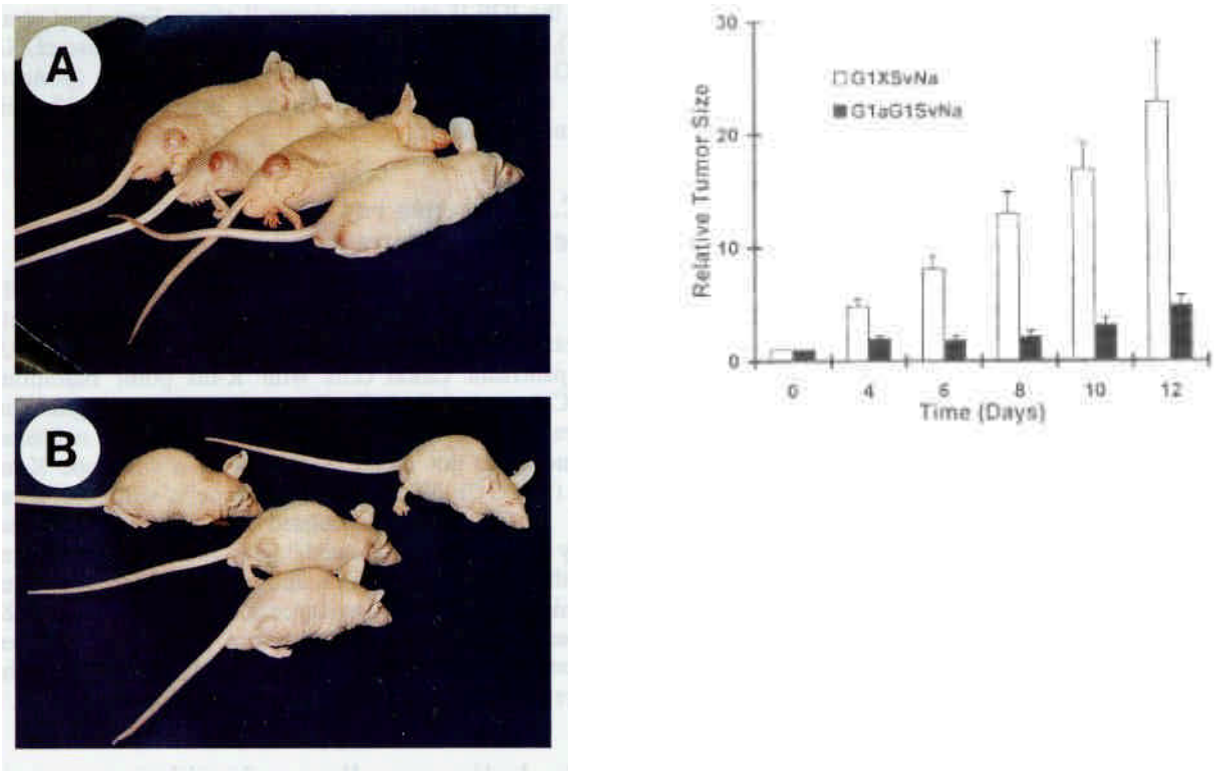
D. Antisense oligonucleotides to metallothionein

Abdel-Mageed and Agrawal (1997) have inhibited the expression of metallothionein (MT) gene using an 18-mer MT antisense phosphorothioate oligomer (complementary to a region 7 bases downstream from the AUG translational start site of the human MT-IIA gene) to elicit antiproliferative effects in breast carcinoma MCF7 cells; indeed, there is an increased MT gene expression in breast cancer which is associated with metastasis and poor prognosis of the disease; overexpression of MT potentiated the growth of MCF7 cells, whereas downregulation of MT elicited antiproliferative effects. Transfection of MCF7 cells with the antisense oligomer inhibited cell growth by 50-60% and induced morphological changes suggestive of apoptotic cell death at 72 hours posttransfection compared to cells transfected with a random 18-mer; the antisense oligomer induced chromatin cleavage into oligonucleosomal fragments, a 2-fold increase in the levels of c-fos and p53 transcripts, a 2.5-fold decrease in c-myc transcripts, and a decrease in Bcl-2 protein levels compared to random oligomer-transfected cells.

On the contrary, the expression of MT was 2.5-fold elevated after transfection of the cells with an expression plasmid encompassing the human MT-IIA cDNA, constitutively driven by β-actin promoter and this was associated with a 2-fold increase in cell multiplication (Abdel-Mageed and Agrawal, 1997)

E. Other antisense approaches

Transfer of an antisense cyclin G1 construct was used to inhibit osteosarcoma tumor growth in nude mice. Overexpression of the cyclin G1 gene is frequently observed in human osteosarcoma cells, and its continued expression is essential for their survival. This modality resulted in a decrease in the number of cells in S and G2/M phases of the cell cycle concomitant with an accumulation of cells in the G1 phase (Chen et al, 1997). **Figure 29** shows that nude mice treated with the antisense cyclin G vector (panel A) have smaller tumors that animal treated with a control vector (panel B). The results of the measurements of the size of the tumor in treated and control animals are shown in C.



F**igure 29**. Photographs of nude mice treated with antisense cyclin G vector (**panel A**) have smaller tumors that animal treated with a control vector (**panel B**). **Panel C**: the relative tumor size (% of day 0 tumor size divided by 100) is plotted, on the vertical axis, as a function of time (days), plotted on the horizontal axis. From Chen DS, Zhu NL, Hung G, Skotzko MJ, Hinton DR, Tolo V, Hall FL, Anderson WF, Gordon EM (**1997**) Retroviral vector-mediated transfer of an antisense cyclin G1 construct inhibits osteosarcoma tumor growth in nude mice. **Hum Gene Ther** 8, 1667-1674. Reproduced with kind permission from the authors and Mary Ann Liebert, Inc.

The replication and expression of hepatitis B virus (HBV) could be inhibited through antisense gene transfer and this could become a new method for clinical gene therapy against HBV; infection of the human hepatoblastoma cell line 2.2.15, which expresses HBV surface antigen and releases HBV particles, with retroviral vectors carrying an antisense preS/S or preC/C genes of HBV inhibited expression of the surface antigen (Ji and St 1997).

Phosphorothioate antisense oligos directed against c-myc and p53 in different cell lines (CAOV-3, SKOV-3, and BG-1) were shown to have both antiproliferative and stimulatory activity, as single agents and in combination; it was concluded that further in vitro studies are needed before considering clinical trials with these agents in gynecologic cancers (Janicek et al, 1995).

Transfection of antisense cDNA constructs encompassing different regions of the c-erbB-2 gene in the lung carcinoma cell line Calu3, which overexpresses the c-erbB-2 oncogene, reduced significantly anchorage-independent growth and tumor size in nude mice (Casalini et al, 1997).

Antisense oligonucleotides against PCNA and cdc2 kinase transferred into injured arterial walls by protein-liposomes greatly reduced mRNA levels for those genes and inhibited neointima formation of the injured artery for 8 weeks; double-stranded oligonucleotides containing the consensus sequence for E2F binding sites also inhibited the growth of smooth muscle cells and prevented neointima formation (Kaneda et al, 1997). Antisense oligonucleotides to angiotensinogen1-receptor mRNA and to angiotensinogen mRNA reduced blood pressure (Tomita et al, 1995; Phillips, 1997; Phillips et al, 1997).

XXVI. Triplex gene therapy

A. Molecular mechanisms for triplex formation

Natural purine·pyrimidine sequences in regulatory regions of genes in eukaryotic cells with a mirror symmetry can form triple-helical structures; in addition, purine-rich segments in DNA unable to form triple helices on their own can be targeted by DNA or RNA oligonucleotides able to form triplex structures with their target DNA and these unusual structures can inhibit transcription factor binding, transcription initiation, and nuclear enzymatic activities. Understanding the advantages, limitations and pitfalls for using oligonucleotides as gene bullets, development of strategies for boosting their therapeutic efficiency, their covalent linkage to DNA damaging molecules to hit a specific genomic DNA sequence, and improvements to the methods for their delivery to cells could make reality their use as tools of micro-targeting specific genomic sites and as pharmacogenomic drugs.

Formation of triple helical DNA was found to take place on AT- and GC-rich stretches. A pyrimidine third strand oligonucleotide, studied by NMR and other approaches, interacts with purine residues in the major groove of the target duplex in a parallel orientation (Moser and Dervan, 1987; Rajagapol and Feigon, 1989; de los Santos et al, 1989) whereas a purine oligonucleotide binds in an antiparallel orientation relative to the purine strand in the duplex (Cooney et al, 1988; Kohwi and Kowhi-Shigematsu, 1988; Beal and Dervan, 1991). In this case G can recognize GC pairs and A or T can recognize AT pairs. Specificity is provided from $T\cdot AT$ and $C^{+}GC$ base triplets where the bases of the third polypyrimidine strand establish Hoogsteen base pairing with the purine strand of the duplex (Hoogsteen, 1959; Rajagopal and Feigor, 1989).

The H form is the structural basis for S1-nuclease hypersensitivity (Mirkin et al, 1987). A restriction fragment from a human U1 gene containing the sequence $d(C\text{-}T)_{18}.d(A\text{-}G)_{18}$ under supercoiling and pH less than or equal to 6.0 showed S1 hyperreactivity in the center and at one end of the $(C\text{-}T)_n$ tract, and continuously from the center to the same end of the (A-G)n tract providing strong support for a triple-helical model (Johnston, 1988).

Homopyrimidine oligodeoxyribonucleotides with EDTA-Fe attached at a single position bound the corresponding homopyrimidine-homopurine tracts within large double-stranded DNA by triple helix formation and cleaved at that site (Moser and Dervan, 1987). Studies from the group of Claude Hélène have similarly focused on the development of artificial scissor oligonucleotides based on triplex technology (Praseuth et al, 1988; Perrouault et al, 1990). However, the feasibility of employing this exciting in vitro technology to animal studies has not yet been demonstrated.

Intramolecular or intermolecular triple helices could be recognized by specific proteins that stabilize triplex structures and might play a role in gene regulation; a protein from HeLa cell nuclear extracts was identified that binds to a 55 nucleotide-long DNA oligomer that could fold on itself to form an intramolecular triple helix of the Py Pu x Py motif (Guieysse et al, 1997).

Triplex-forming oligophosphoramidates containing thymines and cytosines or 5-methyl cytosines (5' $T_4CT_4C_6T$ 3') bind strongly to a 16 base pair oligopurine·oligopyrimidine sequence of HIV proviral DNA even at neutral pH and are remarkably stable compared to oligonucleotides with natural phosphodiester linkages. The phosphoramidate oligomers induced an efficient arrest of both bacteriophage and eukaryotic transcriptional machineries (SP6, T7 or Pol II) under conditions where the phosphodiester oligos had no inhibitory effect and blocked the RNA polymerases at the triplex site (Giovannangeli et al, 1996).

Oligonucleotide-directed triplex formation has been shown to inhibit binding of transcription factors to their cognate DNA sequences. A 21 bp homopurine element

insert flanking a single Sp1 site in the adenovirus E4 promoter was used to study the effect of oligo targeting on transcriptional efficiency in vitro; assembly of the triple helical complex repressed basal transcription by rendering the triplex target inflexible and by blocking assembly of the promoter into initiation complexes; Sp1 was unable to cause derepression (Maher et al, 1992). Thus DNA triplexes can inhibit transcription initiation not only when directed to a TF binding site occluding its binding but also to a flanking region by other possible repression mechanisms including stiffening of the double helix (Maher et al, 1992).

B. Triplex targeting of IGF-I

Oligonucleotide-directed triple helix formation targeted toward IGF-I to inhibit its expression was studied following stable transfection of C6 rat glioblastoma cells with a plasmid from which an RNA was transcribed that coded for the third strand of a potential triple helix. A plasmid encoding the oligopurine variant of the triple helix but not the oligopyrimidine or a control sequence caused a dramatic reduction of IGF-I RNA and protein levels in cultured cells, morphological changes, and increased expression of protease nexin I and MHC class I molecules; the transfected cells displayed a reduced capacity for tumor growth when injected in nude mice (Shevelev et al, 1997).

XXVII. Gene transfer to some characteristic tissues or cell types

A. Transduction of hematopoietic stem cells (HSCs)

Hematopoietic stem cells (HSCs), which can be isolated with high speed flow-cytometric cell sorting from fetal or adult bone marrow and cytokine-mobilized peripheral blood, have extensive self renewal and multilineage repopulating potential; HSCs are being used as an hematopoietic graft to treat cancer patients undergoing high dose chemotherapy which eradicates HSCs; GM-CSF treatment of the patient can enhance mobilization of true HSCs; furthermore, HSCs can be stably transduced at high efficiency (32-75%) by co-culture with a cell line producing recombinant retroviruses containing the neomycin-resistant gene and are targets for hematopoietic cell-based gene therapy especially for the treatment of patients with multiple myeloma (Chen et al, 1995).

The efficiency of gene transfer into monkey pluripotent hematopoietic stem cells (PHSCs) is at least one order of magnitude lower than what has been achieved in mice because primate PHSCs seem to require quite different culture conditions for their maintenance and transduction than mouse PHSCs. Successful retroviral vector-mediated gene transfer into monkey PHSCs supported maintenance of the long-term repopulating ability of autologous monkey grafts and has closed the gap between gene transfer experiments in mouse models and primates opening the door to the clinical application of bone marrow gene therapy to humans (Van Beusechem and Valerio, 1996).

Retroviral vectors pseudotyped with vesicular stomatitis G glycoprotein (VSV-G) and expressing a murine cell surface protein, B7-1, were used to infect the human T-cell line Jurkat and human peripheral blood lymphocytes (PBLs); the transduction efficiency of PBLs with the pseudotyped vector reached a maximum of 16-32% at an moi of 40 (Sharma et al, 1996). Introduction of a mutant H-ras gene (along with a neomycin resistance gene) into normal human bone marrow progenitor cells with a retrovirus followed by selection in cell culture with G418 suggested that expression of mutant H12-ras resulted in enhanced proliferation of early myeloid cells at the expense of differentiation (Maher et al, 1994).

Dendritic cells (DCs) which are the most potent antigen-presenting cells (APCs) for the initiation of antigen-specific T-cell activation can be highly enriched from peripheral blood-adherent leukocytes by short-term culture in the presence of IL-4 and GM-CSF; adenoviral vectors expressing luciferase, β-galactosidase, IL-2, and IL-7 readily transduced human DCs compared to other methods (Arthur et al, 1997).

Transduction of hematopoietic stem cells with human IL-1Ra cDNA was used to alleviate symptoms of RA; the HSCs were subsequently injected into lethally irradiated mice; all of the mice survived and over 98% of the white blood cells in these mice produced biologically active human IL-1Ra type from 2-13 months after transplantation; the animals had the human IL-1Ra protein in their sera for at least 15 months (Boggs et al, 1995).

B. Gene transfer to the brain

Several molecular approaches, including gene transfer with retroviral, adenoviral and herpes simplex virus vectors, as well as antisense vectors, and antisense oligonucleotides have been shown to have in vitro and in vivo activities against brain tumor cells. These approaches are especially important for the treatment of glioblastomas which remain incurable despite an aggressive combination regimens using surgery, radiation, and chemotherapy (reviewed by Yung, 1994).

Intrathecal transplantation of polymer-encapsulated cell lines genetically engineered to release the human ciliary neurotrophic factor (CNTF) provided a means to deliver CNTF continuously behind the blood-brain barrier and bypass the immunologic rejection of allogeneic cells; for example, transduction of mouse C2C12 myoblasts with human CNTF followed by membrane encapsulation and intrathecal implantation in adult rats could partially rescue motor neurons from axotomy-induced cell death (Deglon et al, 1997).

Since adult brain cells are nonproliferative, they are refractory to retroviral infection that could deliver the tyrosine hydroxylase gene to the brain to alleviate degeneration at the nigrostriatal pathway in Parkinson disease (PD). Implantation of immortalized fibroblasts,

primary fibroblasts, or myoblasts, stably transfected in culture with the TH gene (Jiao et al, 1993) or direct injection of lipofectin-plasmid DNA complexes containing the TH gene under the influence of the SV40 promoter/enhancer (Cao et al, 1995) reduced behavioral abnormalities in PD animal models. A 7 kb region encompassing the TH promoter was able to confer expression of β-galactosidase in catecholaminergic cell types in the substantia nigra pars compacta compared to other regions of the brain after HSV-1-mediated transfer to adult rat brains (Song et al, 1997).

C. Gene transfer to hepatocytes

Hepatocytes are responsible for the production of many therapeutically important proteins such as LDL-R which clears LDL from the serum and the blood clotting Factors VIII and IX which are defective in hemophiliacs. Portal vein, rather than systemic intravenous injection, has been used to transduce preferentially hepatocytes (or liver macrophages, known as Kuppfer cells). For example, the Factor IX gene was delivered to a portal vein cannulated into a splenic vein in animals previously subject to two-third hepatectomy and resulted in the expression of low levels of factor IX for up to about 5 months; 0.3-1% of hepatocytes were found to be transduced (Kay et al, 1993).

An adenovirus LDL-R cDNA, infused into the portal vein of rabbits deficient in LDL receptor, resulted in the expression of human LDL-R protein in the majority of hepatocytes that exceeded the levels found in human liver by at least 10-fold (Kozarsky et al, 1994). According to an ex vivo protocol, cultured hepatocytes from a FH patient were transduced ex vivo with the LDL receptor gene and transplanted by infusion into the portal vein of the patient (Wilson et al, 1992; Grossman et al, 1994).

Delivery of a 5.6 kb genomic clone or of a 834-bp cDNA clone encoding the kallikrein gene into the portal vein or tail vein of spontaneously hypertensive rats resulted in significant reduction of their blood pressure for about 5-6 weeks (Chao et al, 1996). Portal vein injection of the human kallistatin cDNA in an adenoviral vector into spontaneously hypertensive rats resulted in a significant reduction of blood pressure for 4 weeks; this method resulted in the transduction not only of liver but also of spleen, kidney, aorta, and lung (Chen et al, 1997).

Hepatocyte Growth Factor (HGF) is the most potent mitogen of mature hepatocytes; transfer of the human HGF gene into a recombinant retroviral cell line produced HGF in the supernatant which was correctly processed and biologically active; primary mouse and human hepatocytes could be transduced with the supernatant of transfected cells and, thus, this cell line should be useful for in vivo liver gene therapy (Pages et al, 1996a).

D. Gene transfer to the embryo

Introduction of normal genes *in utero* or in the early postnatal period could become a successful approach to correct genetic defects; several studies have shown that adenoviral or retroviral vector-mediated gene transfer during the ebryonic or neonatal period results in prolonged gene expression. Gene transfer (or gene disruption) has been extensively studied in preimplantation embryos giving rise to transgenic animals difficient in a specific protein (e.g. Smith et al, 1995; Fong et al, 1995; Shalaby et al, 1995).

Gene transfer to the embryo has shown the importance of the promoter, large genomic regulatory regions, cell-cell interactions and gene switch taking place during embryogenesis in maintaining transgene expression in different tissues; results obtained in embryos reflect the in vivo patterns of tissue-specific expression which could be useful to direct efforts in promoter choices for somatic gene transfer to the adult (as is the case for most gene therapy applications). Furthermore these studies provide the foundation of a new era where genetic manipulation of the embryo could permanently correct monogenic genetic disorders such as hemophilias, thalassemias and others.

The promoter of the *tie* gene, which encodes a receptor tyrosine kinase that is expressed in the endothelium of blood vessels, was used to drive the expression of a luciferase reporter gene construct; in cultured cells the luciferase activity was not restricted to endothelial cells. In contrast, in transgenic mice expression of the reporter β-galactosidase was restricted to endothelial cells undergoing vasculogenesis and angiogenesis; in adult transgenic mice, *tie* promoter activity in lung and many vessels of the kidney was as high as in the vessels of the corresponding embryonic tissues, whereas in the heart, brain and liver, *tie* promoter activity was downregulated and restricted to coronaries, cusps, capillaries, and arteries (Korhonen et al, 1995).

A retroviral VEGF expression vector was used to infect quail ebryo and to increase the level of VEGF during critical periods of avian limb bud growth and morphogenesis. Overexpression of VEGF in the limb bud exclusively resulted in hypervascularization as reflected by an increase in vascular density from an augmentation of the VEGF signaling mechanism in a permissive environment; vascular permeability was also dramatically increased leading to local edema (Flamme et al, 1995).

An avian leukosis virus (ALV)-based retroviral vector system was used for the efficient delivery of genes into preimplantation mouse embryos; a subset of the integrated proviruses expressed the delivered chloramphenicol acetyltransferase (CAT) reporter gene either from the constitutive viral promoter contained in the long terminal repeat or from the internal nonviral tissue-specific promoter in different sets of experiments. Thus, many of the sites that are accessible to viral DNA insertion in preimplantation embryos were thought to be incompatible with expression in older animals (Federspiel et al, 1996).

Baldwin and coworkers (1997) have found that the expression of lacZ gene under control of CMV or RSV promoter transferred to early, postgastrulation mouse embryos gave tissue-specific patterns of expression which depended on the type of promoter used. Embryos were

injected into the mesoderm of the neural fold (**A** in **Figure 30**) and β-galactosidase activity was detected in the head

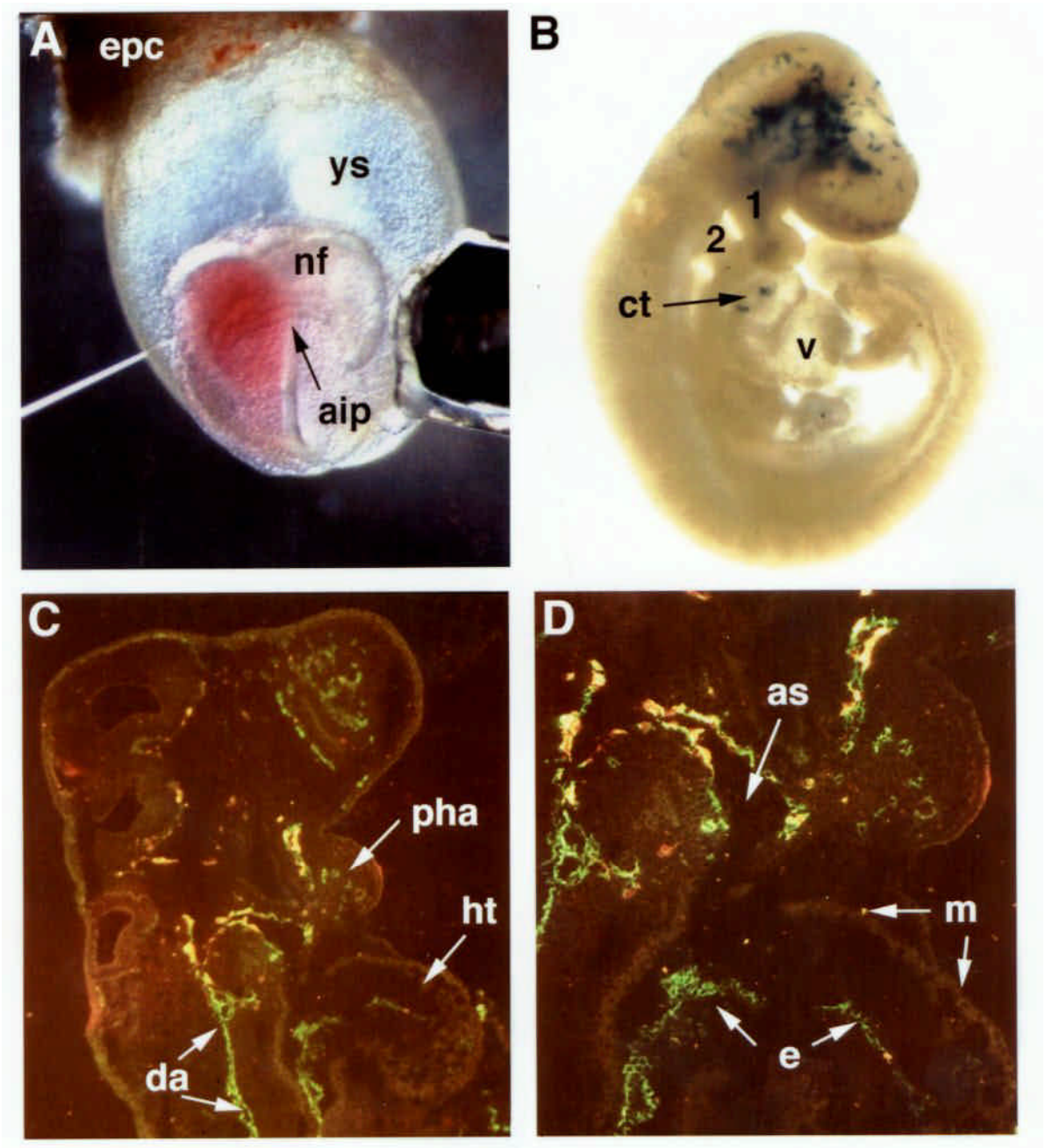


Figure 30.(**A**) E7.25 (1-2 somite) mouse embryos were injected via a micropipette inserted directly into the mesoderm of the neural fold (**nf**). Thus, all head fold mesoderm and neural epithelium were directly exposed to the recombinant adenovirus carrying the LacZ reporter gene (**aip**, anterior intestinal portal; **ys**, yolk sac; **epc**, ectoplacental cone).

(**B**) Following 36 h in culture, β-galactosidase activity was detected in the head process and pharyngeal arches of the embryo; a smaller amount of β-galactosidase activity was detected within the outflow tract (**ct**) of the developing heart (**1** and **2**, first and second pharyngeal arches, **v**, ventricle).

(**C**) Low and (**D**) high magnification dual immunofluorescent photomicrographs of a sagittal section through the head and heart of an embryo stained with a polyclonal antibody to β-galactosidase (**red**) detected by a rhodamine-conjugated second antibody as well as a monoclonal antibody to PECAM-1 (CD-31) (**green**) which is specific for endothelial cells and is detected by a fluorescein-conjugated secondary antibody. Despite exposure of all cell types within the head fold of the embryo β-galactosidase activity is restricted to a subpopulation of endothelial cells within the aortic sac (**as**) and first pharyngeal arch artery (**pha**); (**m**, myocardium; **e**, endocardium). From Baldwin HS, Mickanin C, and Buck C (**1997**) Adenovirus-mediated gene transfer during initial organogenesis in the mammalian embryo is promoter-dependent and tissue-specific. **Gene Ther** 4, 1142-1149. Reproduced with the kind permission of the authors (H Scott Baldwin, Children's Hospital of Philadelphia) and Stockton Press.

process (**B** in **Figure 30**); sagittal sections through the head and heart of the embryos (**c, d**) were stained for β-galactosidase (**red**) and for endothelial cells (**green**); the micrographs show that while not all endothelial cells demonstrated β-galactosidase activity (green only), β-galactosidase was restricted to endothelial cell populations (**yellow**).

DIVISION THREE: GENE THERAPY OF HUMAN DISEASE OTHER THAN CANCER

XXVIII. Ex vivo gene therapy

A. Ex vivo (and in vivo) gene therapy on animal models

A number of experimental approaches for the gene therapy of human disease are first being tested on animal models (preclinical trials) before receiving approval for phase I clinical trials on humans. A number of animal models have been developed such as hemophiliac dogs, rats with high blood pressure, rabbits with coronary heart disease, nude or SCID mice bearing a variety of human cancers, mice with symptoms resembling those of Parkinson's disease patients etc. Then, gene transfer has been used to treat these animals and alleviate the symptoms. The success of these studies is a prelude for their approval as a gene therapy technology on human patients.

Ex vivo techniques although cumbersome are safer, because all genetic manipulations occur outside the body and cells may be extensively screened prior to implantation. According to *ex vivo* protocols cells from the mammalian body are removed, cultured, transduced with therapeutically important genes, and reimplanted into the body of the same individual. A representative number of such studies on animal models are summarized on **Table 5.**

Some examples will be mentioned here. More information can be found in the specialized sections of this review. Ex vivo gene therapy for PD was performed on animal models with TH deficiency using implantation of immortalized rat fibroblasts releasing L-dopa (Wolff et al, 1989), or using primary fibroblasts (Fisher et al, 1991) and myoblasts (Jiao et al, 1993) stably transfected in culture with the *TH* gene. Retroviral vectors have successfully treated mucopolysaccharidosis VII by implantation of ex vivo modified mouse skin fibroblasts to mice (Moullier et al, 1993). Surgical implantation of factor VIII gene-transduced primary mouse fibroblasts into the peritoneal cavity in SCID mice resulted in correction of hemophilia A (Dwarki et al, 1995). Ex vivo transduction of primary myoblasts in mice with the factor IX gene followed by transplantation of the transduced cells led to partial correction of hemophilia B (Dai et al, 1992; Yao et al, 1994).

Intraarticular injection of syngeneic synovial cells transduced with the IL-1R antagonist protein gene alleviated the symptoms of arthritis (Bandara et al, 1993). Similarly, ex vivo retroviral transfer of the secreted human IL-1Ra cDNA to primary synoviocytes followed by engraftment in ankle joints of rats with induced arthritis significantly suppressed the severity of the disease (Makarov et al, 1996; see also Ghivizzani et al, 1997). To demonstrate feasibility of the ex vivo FH therapy, three baboons underwent a partial hepatectomy, their hepatocytes were isolated, cultured, transduced with a retrovirus containing the human LDL-R gene, and infused via a catheter (Grossman et al, 1992).

An important number of studies on cancer immunotherapy have been performed on animal models (For example, see Vieweg et al, 1994; Wiltrout et al, 1995; Caruso et al, 1996; Bramson et al, 1996; Tahara et al, 1996; Zhang et al, 1996; Rakhmilevich et al, 1997; Aruga et al, 1997; Clary et al, 1997; Ju et al, 1997)

Studies with tumor cells reconstituted with RB ex vivo and implanted into immunodeficient mice have demonstrated cancer suppression (see Riley et al, 1996). Transfer of the Cu^{2+}/Zn^{2+} superoxide dismutase into ex vivo modified cells protected the cells from oxidative damage during manipulation and increased their survival after implantation (Nakao et al, 1995). Ex vivo transfer of the *MDR1* gene in bone marrow cells has been used to render stem cells resistant to cancer chemotherapy (Lee et al, 1998, this volume). Ex vivo transduction of MCF-7 human breast cancer cells with antisense c-fos produced expression of antifos RNA, and inhibited s.c. tumor growth and invasiveness in breast cancer xenografts in nude mice (Arteaga and Holt, 1996).

Direct in vivo injection of a gene (intratumoral, intravenous, etc) must be distinguished from ex vivo gene therapy methods. Some representative direct in vivo studies to animals using genes are summarized on **Table 6**.

B. Ex vivo gene therapy on humans

The first person to be treated ex vivo was a 4-year-old suffering with ADA deficiency in 1990 (see ADA deficiency below). The US Patent Office has issued in 1995 a patent covering all *ex vivo* gene therapy to French Anderson, Steven Rosenberg, and Michael Blaese; the technique was developed at NIH in the 1980s and an exclusive license to this work has been awarded to Gene Therapy Inc, (Rockville, Maryland). Of the 220 protocols for Clinical Trials approved by NIH's Recombinant DNA Advisory Committee (RAC), a significant number (over 100) use *ex vivo* gene therapy applications (see Gavaghan, 1995). Ex vivo protocols are marked In Vitro in **Appendicx 1** and **Table 4** in following article (pages 203-206). Also protocols proposing immunotherapy use ex vivo transduction of cells from cancer patients with cytokine genes and immunization of the patient with the transduced cells (**Appendix 1**).

Transduction of cells *in vitro* with adenoviruses makes the patients own cells antigenic leading to their destruction

by T lymphocytes thus eliminating the therapeutic effect after reimplantation (e.g. Yang et al, 1994). It was thought that this antigenicity arises from the adenoviral proteins expressed in transduced cells; however, recent data have demonstrated that antigenicity could also arise from the expression of the therapeutic recombinant protein (see above).

Ex vivo approaches have concentrated on correction of mutated genes involved in purine metabolism including adenosine deaminase (ADA) deficiency in severe combined immunodeficiency (SCID) patients, PNP (purine nucleoside phosphorylase) deficiency, and the therapy of Lesh-Nyhan syndrome caused by a deficiency in hypoxanthine-guanine phosphoribosyltransferase (HG-PRT). The first human trial to be approved for ex vivo gene therapy was for the treatment of ADA deficiency which began in 1990 (Karlsson, 1991; Ferrari et al, 1991). *Ex vivo* studies include transfer of factor IX gene in skin fibroblasts from hemophilia B patients in China followed by subcutaneous injection of the cells to the patient (Wilson et al, 1992; reviewed by Anderson, 1992). From 1990-1992, a clinical trial was initiated using retrovirus mediated transfer of the 1.5 kb ADA gene cDNA to T cells from two children with severe combined immunodeficiency following multiple transplantations of *ex vivo* modified blood cells; the vector was integrated and the ADA gene was expressed for long periods (Blaese et al, 1995; Bordignon et al, 1995).

A clinical protocol for the therapy of amyotrophic lateral sclerosis uses a semipermeable membrane to enclose the *ex vivo* modified xenogenic BKH cells; the membrane is implanted intrathecally to provide human ciliary neurotrophic factor (Deglon et al, 1996; Pochon et al, 1996). An ex vivo clinical trial on humans, homozygous for mutations in the LDL receptor gene, is performed using cultured hepatocytes from the patient which are transduced ex vivo with the LDL receptor gene and transplanted by infusion into the portal vein of the patient (Wilson et al, 1992; Grossman et al, 1994).

Cancer immunotherapy uses transfer of cytokine genes (IL-2, IL-7, IFN-γ, GM-CSF) to autologous (cancer patient's) cells followed by immunization of the patient to elicit activation of tumour-specific T lymphocytes capable of rejecting tumour cells from the patient, especially on immunoresponsive malignancies such as melanomas, colorectal carcinomas, and renal cell carcinomas (Uchiyama et al, 1993; Chang et al, 1996; Finke et al, 1997; Das Gupta et al, 1997; Mahvi et al, 1997).

Table 5. Ex vivo studies on animal models

Gene target	Human disease	Method	Animal model, objective, and method	Results	Reference
ADA	SCID (severe combined immunodeficiency)	Retrovirus	Immunodeficiient mice were injected with peripheral blood lymphocytes from ADA^- patients transduced with a retroviral vector for human *ADA*	Restoration of immune functions (presence of human immunoglobulin and antigen-specific T cells)	Ferrari et al, 1991
bcl-2	prostate cancer		*bcl-2* expressing LNCaP human prostate cancer cells are rendered highly resistant to apoptotic stimuli	LNCaP-*bcl-2* cells induced earlier, larger, and hormone-refractory prostate tumors in nude mice	Rafo et al, 1995
Factor IX	hemophilia B		Injection of transduced primary myoblasts into the muscle	Factor IX was being synthesized and delivered to the circulation for over 6 months	Dai et al, 1992
Factor IX	hemophilia B	retr	Transplantation of retrovirus-transduced keratinocytes	Human factor IX was detected in the bloodstream of nude mice in small quantities for one week	Gerrard et al, 1993,
Factor IX	hemophilia B	retrovirus	Mouse primary myoblasts were infected with retrovirus expressing the canine factor IX under control of mouse muscle creatine kinase and human CMV promoter; myoblasts were injected into the hindlegs of recipient mice; secreted canine factor IX was monitored in the plasma	Sustained expression of factor IX for over six months without any apparent adverse effects on the recipient mice; however, the levels of the factor IX protein secreted into the plasma (10 ng/ml for 10^7 injected cells) were not sufficient to be of therapeutic value; 100 times higher amounts of factor IX were needed	Dai et al, 1992; Yao et al, 1994
Factor VIII	Hemophilia A	transferrin	Transfection of fibroblasts and myoblasts with B-domain-deleted factor VIII gene followed by implantation into mice	Therapeutic levels of factor VIII in the blood of the animals for 24 hours	Zatloukal et al, 1994
Factor VIII	Hemophilia A	Retrovirus	Mouse primary fibroblasts infected with a recombinant retrovirus containing factor VIII gene deleted at the B domain	Therapeutic levels of factor VIII in blood of animals for 1 week after surgical implantation into the peritoneal cavity in SCID mice of 15 million cells in the form of neo-organs	Dwarki et al, 1995

Growth hormone (human)	none	mice electroporation	*Ex vivo* modified C2C12 cells with the *hGH* gene under control of the inducible UAS promoter and a synthetic hybrid steroid receptor (TAXI), activating transcription from the inducible promoter after treatment with the synthetic nontoxic drug inducer RU486; transplanted in mouse muscle	This model allows up to 100-fold induction of the hGH gene and can be finely tuned to lower levels of induction	Delort and Capecchi, 1996
Growth hormone (human, hGH)	general	retr	Injection of genetically engineered myoblasts into mouse muscle	hGH could be detected in serum for 3 months; myoblasts were fused into preexisting multinucleated myofibers that were vascularized and innervated	Dhawan et al, 1991
HSV-tk	glioma	Retr	To directly transfer HSV *TK* gene and kill transduced proliferating brain tumor cells with ganciclovir without affecting nondividing normal cells	Murine fibroblasts transduced ex vivo with HSV TK retroviral vectors caused complete regression of gliomas in rat brain after intratumor injection	Culver et al, 1992
HSV-tk	pancreatic cancer	retrovirus	BXPC3 primary human pancreatic adenocarcinoma cells were transduced with retroviral vector carrying the HSV-tk gene driven by the CEA promoter; engrafted subcutaneously into nude mice eliciting pancreatic tumors	Animals treated with 0.1 mg/Kg ganciclovir exhibited a significant reduction in tumor growth	DiMaio et al, 1994
HSV-tk	proliferative vitreoretinopathy (PVR)	retrovirus-transduced rabbit dermal fibroblasts	Traction retinal detachment results from proliferation of retinal pigment cells in the vitreous cavity of the eye; PVR may ensue after retinal surgery or trauma and can be induced in rabbit models by surgical vitrectomy.	Significant inhibition of PVR (killing of proliferating cells in the retina) was observed in rabbit PVR models after injection into the vitreous cavity of rabbit dermal fibroblasts transduced *in vitro*; all eyes received 0.2 mg GCV on the following day and on day 4;	Kimura et al, 1996
IL-1 -receptor antagonist protein gene	Rheumatoid arthritis (RA)	Retr	Synovial cells were surgically removed from joints of animals with experimental arthritis, cultured, transduced with the IL-1 -receptor antagonist protein gene and reimplanted into the respective donors by intraarticular injection	Improvement in RA symptoms	Bandara et al, 1993
IL-1 receptor antagonist (IL-1Ra)	Rheumatoid arthritis (RA)	Retr	Degradation of cartilage in RA is stimulated by IL-1; to inhibit IL-1; RA synovial fibroblasts transfected with the IL-1Ra gene were coimplanted with normal human cartilage in SCID mice	IL-1Ra expression protected the cartilage from chondrocyte-mediated degradation.	Otani et al, 1996; Makarov et al, 1996; Muller-Ladner et al, 1997b
IL-2; GM-CSF	prostate cancer		Dunning rat R3327-MatLyLu prostate tumor model (an anplastic androgen-dependent, nonimmunogenic tumor that metastasizes to the lymph nodes and the lung); cytokine (IL-2)-secreting human tumor cell preparations (tumor vaccines) were used for the treatment of advanced human prostate cancer in rats	All animals with subcutaneously established tumors were cured; the cancer vaccine induced immunological memory that protected the animals from subsequent tumor challenge; GM-CSF was less effective than IL-2.	Vieweg et al, 1994
LDL receptor	Familial hypercholesterolemia (FH)	Retr	Watanabe heritable hyperlipidemic rabbit (deficient in both alleles of LDL receptor gene); establish hepatocyte culture from animal liver; transduce with LDL receptor gene responsible for LDL internalization into hepatocytes to reduce blood serum cholesterol; transplant hepatocytes into the animal	30-40% decrease in serum cholesterol that persisted for 4 months	Chowdhury et al, 1991
Nerve Growth Factor (NGF)	Alzheimer's disease	rat and primate	Delivery of NGF by ex vivo-modified allogeneic cells surrounded by a semipermeable membrane and implanted intrathecally	Release of NGF by the implant which is not subject to immunologic rejection due to the membrane	Kordower et al, 1994
XPD (ERCC2)	xeroderma pigmentosum (XP)	Retr	To transduce ex vivo human keratinocytes and produce skin grafts on immunodeficient mice; use it on XP patients as reconstructive surgery to alleviate cancers in UV-exposed areas	The retroviral vector carrying the XPD gene and neoR under control of SV40 enhancer fully complemented the DNA repair deficiency of primary skin fibroblasts	Gözükara et al, 1994; Carreau et al, 1995

TH (Tyrosine hydroxylase)	Parkinson's disease (PD)	Retrovirus	Rat fibroblasts transduced with tyrosine hydroxylase (TH) produced and released L-dopa to the culture medium;	When grafted to the striatum of Fischer rats with a prior 6-hydroxydopamine lesion, primary fibroblasts containing the TH transgene survived for 10 weeks, continued to express the transgene, and reduced rotational asymmetry.	Wolff et al, 1989
TH	Parkinson disease (PD)		Overexpress TH that converts tyrosine to L-DOPA to alleviate degeneration of dopaminergic nigrostriatal neurons (DNN) in PD rat models; unilateral destruction of DNN in animals with 6-hydroxydopamine and administration of apomorphine caused PD rats to turn contralaterally (7 or more rotations/min).	Implantation of transgenic immortalized fibroblasts and myoblasts intracerebrally improved rotational behavior	Fischer et al, 1991
TH	Parkinson's disease (PD)	HSV	Infection of 6-hydroxydopamine-lesioned rats, used as a model of PD, with a defective herpes simplex virus type 1 vector expressing TH	Conversion of endogenous striatal cells into L-dopa-producing cells	During et al, 1994
TH	Parkinson's disease (PD)	Lipofectin	To alleviate the symptoms of PD in TH-deficient rats (PD animal models; perform colateral rotations at 15 rounds/min upon administration of apomorphine)	Primary muscle cells were transduced with TH cDNA under control of CMV promoter; 10 million cells were injected into brains of TH-deficient rats; this resulted in 75% decrease in the number of rotations/min for more than 6 months	Jiao et al, 1993

Table 6. In vivo somatic gene transfer strategies to animal models

Gene target or delivered	Human disease	Method	Animal model, objective, and method	Results	Reference
HSV TK and ganciclovir	hepatocellular carcinoma	AAV mice	To preferentially kill hepatocellular carcinoma cells by the suicadal gene HSV TK (driven by the α-fetal protein (AFP) enhancer and albumin promoter) with ganciclovir	Selective killing of AFP-positive cells in culture; transgenic mice were established by injection of AAV ITRs, neo^R, and HSV TK genes as a linear DNA fragment; HSV TK was expressed predominantly in adult liver.	Su et al, 1996
Prostaglandin G/H synthase	acute lung injury	Cat lipid rabbit	Rabbits intravenously transfected with the PGH synthase gene	Increased plasma levels of prostacyclin and PGE2; protection of lungs in rabbits against endotoxin-induced inflammation, pulmonary edema, release of thromboxane B2, and pulmonary hypertension	Conary et al 1994
Ornithine transcarbamylase (OTC)	OTC-deficiency	Adenovirus	iv injection of recombinant adenovirus to *spf-ash* mice (OTC-deficient)	Correction of enzyme deficiency in OTC-deficient mice for over 1 year.	Stratford-Perricaudet et al, 1990
α_1-antitrypsin	α_1-antitrypsin-deficiency in lung	Adenovirus	The adenovirus major late promoter was linked to a human α1-antitrypsin gene for its transfer to lung epithelia of cotton rat respiratory pathway	Both in vitro and in vivo infections have shown production and secretion of α1-antitrypsin by the lung cells for over 1 week	Rosenfeld et al, 1991
α_1-antitrypsin	α_1-antitrypsin-deficiency in liver	Cat lipid mice	Protect connective tissue from the lytic action of the leukocyte neutrophil elastase; plasmid was encapsulated into negatively-charged liposomes containing phoshpatidylcholine	Small liposomes were much more effective in delivering the α_1-antitrypsin gene to mouse hepatocytes in vivo.	Aliño et al, 1996
α_1-antitrypsin (AT, human)	acute and chronic lung diseases.	Cat lipid	Aerosol and intravenous transfection to lungs of rabbits	Human α1AT mRNA and protein were detected for at least 7 days; immunohistochemical staining showed α1AT protein in the pulmonary endothelium following intravenous administration, in alveolar epithelial cells following aerosol administration, and in the airway epithelium by either route	Canonico et al, 1994
CFTR	Cystic fibrosis (CF)	Adenovirus	To alleviate the symptoms of CF	Expression of CFTR after intratracheal instillation into lungs of cotton rats; expression between days 2-10	Rosenfeld et al, 1992

CFTR (cystic fibrosis transmembrane conductance regulator)	Cystic fibrosis (CF)	Cat lipid	To express the normal CFTR gene in lungs of Edinburgh insertional mutant mouse (cf/cf) after delivering CFTR cDNA-liposome complexes into the airways by nebulization.	Full restoration of cAMP related chloride responses in some animals; human CFTR cDNA expression in the same tissues	Alton et al, 1993
CFTR	Cystic fibrosis (CF)	Lipofectin	To express the human CFTR gene in lungs in CFTR-deficient transgenic mice by tracheal instillation of lipofectin-plasmid	Successful transfer of the *CFTR* gene to epithelia and to alveoli deep in the lung leading to correction of the ion conductance defects found in the trachea of transgenic mice	Hyde et al, 1993.
CFTR	CF	Lipofectin	Transduction of airway epithelial cells in normal mice by intratracheal instillation of a plasmid carrying the *CFTR* gene under control of the Rous sarcoma virus promoter	Airway epithelial cells were the major target and site of expression of CFTR	Yoshimura et al, 1992
CFTR cDNA (human)	Cystic fibrosis (CF)	AAV	Intratracheal instillation into neonatal New Zealand white rabbits	Epithelial expression of the human CFTR fusion protein was detected using antisera to both the human CFTR R domain and the amino-terminal epitope at up to 6 weeks after vector inoculation, a time coinciding with the completion of the alveolar phase of lagomorph lung development	Rubenstein et al, 1997
p53	lung cancer	retrovirus	Lung tumors were elicited in nu/nu mice after intratracheal inoculation with human lung cancer H226Br cells whose p53 gene has a homozygous mutation at codon 254	Intratracheal injection of a recombinant retrovirus containing the wt p53 gene was shown to inhibit the growth of the tumor	Fujiwara et al, 1994
MHC class I HLA-B7 heavy chain gene plus β_2-microglobulin		Cat lipid:DNA 1:5 mice	To give a complete Class I molecule with heavy and light chains; MHC class I HLA-B7 heavy chain gene has an internal ribosome entry site; the β_2-microglobulin was driven by RSV promoter	Experiments in mice showed rapid (1 day) destruction of the plasmid in tissues; femtogram amounts only in muscle at 6 months postinjection	Lew et al, 1995
CCK (cholecystokinin)	congenital audiogenic epileptic seizures (AS)	Lipofectin (DOTMA:DOPE)	To suppress audiogenic epileptic seizures by cholecystokinin octapeptide (CCK-8) injected intracerebroventricularly (i.c.v.)	AS in rats was markedly reduced between day 3 and 4.	Zhang et al, 1992
hirudin	restenosis; arterial injury	adenovirus	Virus-injured rat carotid arteries; hirudin (from medicinal leech) is a potent protein inhibitor of thrombin; thrombin converts fibrinogen to fibrin and also stimulates smooth muscle cell proliferation during neointima formation in the arterial walls	35% reduction in neointima formation in hirudin cDNA-transduced arterial wall cells in vivo	Rade et al, 1996
HSV-*tk*	arterial injury; restenosis	Adenovirus	To kill preferentially the smooth muscle cells and reduce neointima formation in the arterial wall	47% reduction in I/M area ratio following local delivery of HSV-tk and systemic ganciclovir therapy	Ohno et al, 1994; Guzman et al, 1994
RB	atherosclerosis, restenosis, arterial injury	Adenovirus	To inhibit vascular smooth muscle cell (VSMC) proliferation after arterial injury	42% reduction in I/M area ratio	Chang et al, 1995a
p21	atherosclerosis, restenosis, arterial injury	Adenovirus	To inhibit vascular smooth muscle cell (VSMC) proliferation after arterial injury; p21 protein functions both by inhibiting cyclin dependent kinases (CDKs) required for the initiation of S phase and by binding to and inhibiting PCNA	Over-expression of human p21 inhibited growth factor-stimulated VSMC proliferation and neointima formation in the rat carotid artery model of balloon angioplasty in vitro by arresting VSMCs in the G1 phase of the cell cycle	Chang et al, 1995b
ras	arterial injury		Transfer of ras transdominant negative mutants to rats in which the common carotid artery was subjected to balloon injury	Reduced neointimal formation	Indolfi et al, 1995
TGF-β	arterial injury		TGF-β accelerates wound healing and inhibits epithelial and smooth cell proliferation; loss of functional TGF-β receptors in cancer cells	Inhibition in epithelial and smooth cell proliferation	Grainger et al, 1995
TGF-β1	Rheumatoid arthritis (RA)	Retr	Mice with induced arthritis	Effective in lowering inflammation of joints with already established arthritis and inhibiting the spreading of the disease to other joints in mice	Chernajovsky et al, 1997

Gene target or delivered	Human disease	Method	Goal/rationale	Results	Reference
LDL receptor	Familial hypercholesterolemia (FH)	Adeno	Infusion of adenovirus human LDL-R cDNA into the portal vein of rabbits deficient in LDL receptor	Human LDL receptor protein was produced in the majority of hepatocytes that exceeded the levels found in human liver by at least 10-fold	Kozarsky et al, 1994
Very low density lipoprotein receptor (VLDL-R)	Familial hypercholesterolemia (FH)	Adeno	LDL-R knockout mice	A single intravenous injection resulted in reduction in total cholesterol by approximately 50% at days 4 and 9; marked reduction in the intermediate density lipoprotein/low density lipoprotein (IDL/LDL)	Kobayashi et al, 1996; Kozarsky et al, 1996
HSV TK+ganciclovir (GCV)	Gliomas	Retr	Treatment of rats with cerebral glioma; intratumoral stereotaxic injection of murine fibroblasts (G418-selected) producing a retroviral vector with the HSV TK gene	Tretament with GCV 5 days postinjection resulted in complete regression of gliomas	Culver et al, 1992
HSV TK plus ganciclovir	prostate cancer	Adenovirus mouse	Ganciclovir is converted by HSV TK into its triphosphate form which is then incorporated into the DNA of replicating mammalian cells leading to inhibition in DNA replication and cell death; it is only viral TK, not the mammalian enzyme, that can use efficiently phosphorylated ganciclovir as a substrate	Subcutaneous tumors induced by injection of RM-1 (mouse prostate cancer) cells followed by injection of HSV TK and treatment with ganciclovir for 6 days showed reduction in tumor volume (16% of control) and higher apoptotic index in tumor cells	Eastham et al, 1996
HSV TK plus ganciclovir	adenocarcinoma		Carcinoembryonic antigen-producing human lung cancer cells	Cell type-specific expression of herpes simplex virus thymidine kinase gene	Osaki et al, 1994
TH	PD	Cat liposrat	Overexpress TH to alleviate degeneration of dopaminergic nigrostriatal neurons (DNN) in PD rat models	Direct injection of lipofectin-TH expression plasmid on nigra-lesioned side generated L-DOPA locally and decreased contralateral rotations.	Cao et al, 1995
rat glial cell line-derived neurotrophic factor (rGDNF),	PD	AAV	To protect nigral dopaminergic neurons in the progressive Sauer and Oertel 6-hydroxydopamine (6-OHDA) lesion model of Parkinson's disease (back-labeled fluorogold-positive neurons in the substantia nigra)	94% cell protection; 85% of tyrosine hydroxylase-positive cells	Mandel et al, 1997
Factor IX	Hemophilia B	Adenovirus mice	Correction of FIX defect by injection of recombinant adenovirus hosting the canine FIX gene into hind leg muscle of mice	Establishment of factor IX levels in the blood in nude mice for 300 days; only for 10 days in normal mice; transduced cells were removed by cell-mediated as well as humoral immune responses; cyclophosphamide or cyclosporin A immunosuppression maintained FIX protein for 5 months	Dai et al, 1995
Factor IX	Hemophilia B	Adenovirus mice in vivo	Correction of the factor IX gene	Therapeutic plasma levels of factor IX in mice	Smith et al, 1993
Factor IX	Hemophilia B	Retrovirus dogs in vivo	Expression of factor IX gene in liver in hemophilia B dogs	1% of hepatocytes were transduced with a retroviral vector carrying the canine factor IX gene injected into the portal vein (2/3 hepatectomy was required); low therapeutic levels of factor IX	Kay et al, 1993
Factor IX	Hemophilia B	Adenovirus dogs in vivo	Establishment of the blood serum factor IX levels in hemophilic B dogs by infection with recombinant adenovirus vectors delivering the Factor IX gene and treatment with cyclosporin A	Treatment with cyclosporin A suppressed T cell activation; T cells attack the adenovirus-transduced cells eliminating them from the body; Cycl A tretment led to prolonged Factor IX levels in the blood of hemophilic dogs	Kay et al, 1994; Fang et al, 1995
Factor IX	Hemophilia B	AAV		Successful transduction of the mouse liver in vivo after a single administration; persistent, curative concentrations of functional human factor IX can be achieved	Snyder et al, 1997

Gene target or delivered	Human disease	Method	Goal/rationale	Results	Reference
Factor IX	Hemophilia B	AAV	Intramuscular injection into hindlimb muscles of C57BL/6 mice and Rag 1 mice	Presence of hF.IX protein by immunofluorescence staining of muscles harvested 3 months after injection in both strains of mice; no plasma FIX in immunocompetent mice; Rag 1 mice which lack functional B and T cells, displayed therapeutic levels (200-350 ng/ml) of F. IX in the plasma in addition to F.IX in muscle cells	Herzog et al, 1997
Factor X	Hemophilia B	Retr	Delivery to rat hepatocytes in vivo during liver regeneration; under control of α1-antitrypsin promoter	10% to 43% of normal human factor X levels in 4 rats; expression remained stable for more than 10 months in two rats	Le et al, 1997
p53	breast carcinoma MDA-MB-435 cells	DOTMA:DOPE	Nude mice inoculated with breast carcinoma cells (have mutated p53)	Iv injection of p53 gene under control of β-actin promoter and intron every 10-12 days resulted in more than 60% reduction in tumor volume	Lesoon-Wood et al, 1995
Cdc2 kinase and PCNA	Restenosis	liposome-Sendai virus	Delivery to rat carotids after balloon injury; inhibition of Cdc2 kinase and PCNA with antisense oligonucleotides using PS:PC:Chol liposomes	Whereas antisense cdc2 kinase or PCNA alone failed to have an effect, combination of the two antisense oligos significantly reduced neointima formation and smooth muscle cell proliferation after balloon injury	Morishita et al, 1993
vascular endothelial growth factor (VEGF)	restenosis	naked plasmid	VEGF promotes endothelial cell proliferation to accelerate re-endothelialization of the artery reducing intimal thickening	VEGF gene expression using ELISA or RT-PCR was detected for 3-14 days after a single transfer using a hydrogel/polymer-coated ballon angioplasty catheter to induce simultaneous injury and delivery of plasmid to the femoral artery in rabbits.	Isner et al, 1996
Kallikrein	hypertension	naked plasmid	Tissue kallikrein is a serine proteinase cleaving the kininogen to produce the vasoactive kinin peptide; kinin causes smooth muscle contraction and relaxation, increase in vascular permeability, and vasodilatation	Significant reduction in blood pressure in spontaneously hypersensitive rats after a single delivery of naked DNA to portal vein which lasted for 5-6 weeks.	Chao et al, 1996
Kallikrein	hypertension	Adeno		Sustained delay in the increase in blood pressure from day 2 to day 41 post injection (iv) into spontaneously hypertensive rats; human tissue kallikrein mRNA was detected in the liver, kidney, spleen, adrenal gland, and aorta.	Jin et al, 1997
Tissue kallikrein-binding protein (HKBP) or kallistatin	hypertension	Adeno	Kallistatin, a serine proteinase inhibitor, may function as a vasodilator in vivo	Delivery of the human kallistatin cDNA/CMV by portal vein injection resulted in a significant reduction of blood pressure of hypertensive rats for 4 weeks.	Chen et al, 1997
Human endothelial NO synthase (eNOS) gene	hypertension		Blood pressure is controled by the endothelium-derived nitric oxide (NO) in peripheral vessels	Significant reduction of systemic blood pressure for 5 to 6 weeks.	Lin et al, 1997
Antisense oligonucleotides to AT1-receptor mRNA and to angiotensinogen mRNA	hypertension	Liposomes	Angiotensinogen, produces angiotensin I in the liver (component of the renin-angiotensin system); mutations in the angiotensinogen (AT) gene are associated with hypertension	Antisense oligonucleotides delivered to rat liver via the portal vein diminished the expression of hepatic angiotensinogen mRNA and reduced blood pressure.	Tomita et al, 1995; Phillips, 1997; Phillips et al, 1997
Endothelial basic FGF (bFGF)	hypertension		Subphysiological amounts in blood vessels of spontaneously hypertensive rats	Restored the physiological levels levels of bFGF in the vascular wall and corrected hypertension	Cuevas et al, 1996
Atrial natriuretic peptide (ANP) gene	hypertension		Chronic infusion of ANP causes natriuresis, diuresis, and hypotension	Significant reduction of systemic blood pressure in young hypertensive rats (4 weeks old); the effect continued for 7 weeks.	Lin et al, 1995

IL-2	prostate cancer	liposomes	Direct transfer of the *IL-2* gene under control of the CMV promoter with or without the AAV inverted terminal repeats	Plasmid DNA containing the AAV inverted terminal repeats showed 3-10 fold higher levels of gene transfer and *IL-2* expression compared with constructs lacking the AAV sequences.	Vieweg et al, 1995
mouse leptin cDNA	obesity	Adeno	ob/ob mouse (which is genetically deficient in leptin and exhibits both an obese and a mild non-insulin-dependent diabetic phenotype)	Dramatic reductions in both food intake and body weight, as well as in normalization of serum insulin levels and glucose tolerance	Muzzin et al, 1996
rat leptin cDNA/CMV	obesity	Adeno	Wistar rats infused with 8 ng/ml adeno/leptin cDNA for 28 days	Animals became hyperleptinemic; 30-50% reduction in food intake; gained only 22 g over the experimental period versus 115-132 gained by control animals	Chen et al, 1996
$VEGF_{165}$	cancer (vascularization)	calcium phosphate	Expression of $VEGF_{165}$ in rat C6 glioma cells and subcutaneous injection of the transduced cells in athymic mice.	Tumors from cells expressing VEGF grew slower than tumors developed from nontransduced C6 cells, were highly vascularized, and contained varying degrees of necrosis and eosinophilic infiltrate	Saleh, 1996
cGMP phosphodiesterase-β (PDE-β) gene	retinal degeneration	AAV	To treat retinal degeneration due to recessive mutation in the endogenous gene	Intraocular injection of AAV-PDE-β cDNA increased retinal expression of immunoreactive PDE protein; treated eyes showed increased numbers of photoreceptors and a two-fold increase in sensitivity to light	Jomary et al, 1997

XXIX. Gene therapy of HIV

A. Mechanism of HIV-1 entry into T cells and macrophages

Targets of human immunodeficiency virus (HIV) are helper T cells and macrophages; macrophage-tropic HIV-1 isolates represent the most prevalent phenotype isolated from individuals shortly after seroconversion during the asymptomatic period of the disease; tropism is determined by specific sequences in the third variable loop (V3 domain) of gp120 coat protein of HIV-1. The CD4 receptor on both macrophages and T cells is the primary receptor mediating HIV-1 entry into the cell; however, HIV-1 was unable to infect $CD4^+$ T cells of mice engineered to express human CD4 (reviewed by D'Souza and Harden, 1996). Thus, a second chemokine receptor was thought to be necessary for HIV entry into immortalized T cell lines.

The second receptor for entry of HIV-1 into T cells and macrophages is CCR-5, a β-chemokine receptor; CCR-5 is a seven transmembrane-domain glycoprotein of the chemokine superfamily of receptors related to the receptor of IL-8 (G protein-coupled proteins that transduce signals from the cell surface to the interior of cells). The β-chemokines RANTES, MIP-1α, and MIP-1β inhibited replication of M-tropic isolates of HIV-1 and were found to be major HIV-suppressive factors produced by $CD8^+$ T cells. The V3 loop of gp120 determines interaction with the chemokine receptor. CD4, in addition to providing a docking surface for the gp120 glycoprotein of HIV-1 promotes exposure of the V3 domain on gp120 that can interact with the chemokine receptor CCR-5 (Scarlatti et al, 1997; reviewed by D'Souza and Harden, 1996).

A small number of individuals (1% in populations of European descent but much lower in non-Caucasian populations) remain uninfected despite multiple high risk exposures; such individuals have a homozygous defect in the *CCR-5* receptor gene which consists of a 32-bp deletion in the region encoding the second extracellular loop of the receptor; the defective protein is not detected at the cell surface; this defect prevents the proper interaction and entry of HIV-1 into their T cells and macrophages (Liu et al, 1996). This defect precludes infection from HIV-1 from all routes. The disease progresses much slower in heterozygotes for the 32-bp deletion (18% in populations of European descent) who are not protected from HIV-1 infections. This finding offers the hope of reconstituting the immune system of HIV-infected individuals with $CD34^+$ stem cells from fetal cord blood or stem cells and lymphoid tissue from individuals who carry the homozygous deletion in the CCR-5 gene, an approach to deal with the immune rejection problems in patients with heart and kidney transplants.

The identification of chemokine receptors and their role in HIV-1 infections has closed a major gap in AIDS research; transgenic animals can now be produced expressing both human CD4 and chemokine receptors to evaluate the efficacy of AIDS therapeutics and testing vaccines; new prophylactic or therapeutic vaccines can be designed by immunization with portions or the entirety of CCR-5 and/or gp120 to generate appropriate antibodies (D'Souza and Harden, 1996). Inactivation of the CCR-5 co-receptor to mimic the natural resistance of the CCR-5-defective individuals, in cultured lymphocytes, rendered them viable and resistant to macrophage-tropic HIV-1 infection (Yang et at, 1997).

B. Therapeutic strategies against HIV

A number of therapy strategies emerged soon after identification of HIV as the etiologic agent of AIDS. Virtually every stage in the viral life cycle and every viral gene product is a potential target. Albeit major efforts for

the combat of HIV have focused on the development of antiviral drugs and preventive vaccines, a number of studies have been aimed at eliminating HIV with gene therapy. The intracellular immunization approach (Baltimore, 1988) has prompted the advent of molecular tactics for inhibiting replication and infection of HIV (Trono et al, 1989; Malim et al, 1989). Four main targets have been defined in HIV therapeutics: **(i)** viral RNAs using ribozymes and antisense RNAs; **(ii)** viral proteins using RNA decoys, trans-dominant viral proteins, intracellular single-chain antibodies, and soluble CD4; **(iii)** infected cells aimed at eliminating those with transfer and expression of suicide genes; and **(iv)** the immune system by in vivo immunization (see Corbeau et al, 1996). Such gene therapeutic approaches can be combined with potent antiretroviral drugs especially the potent reverse transcriptase and protease inhibitors (Junker et al, 1997).

The elucidation of the chemokine receptor mechanisms for entry of HIV-1 into T cells and macrophages provides new tactics for intervention at the level of interaction of gp120 with CCR-5. Inactivation of the *CCR-5* gene might be achieved **(i)** with triplex oligonucleotide technologies once critical transcription factor binding sites in the regulatory region of the gene have been determined; **(ii)** with saturation of the blood of infected individuals with ligands, selected from peptide libraries, that block the extracellular domains of the CCR-5 receptor and preclude interaction with gp120; **(iii)** with antagonists of RANTES (see above) that lack chemotactic activity but can block HIV infections (Arenzana-Seisdedos et al, 1996).

Specificity for the ablation of HIV Tat-expressing cells has been achieved through the use of the promoter element from the long terminal repeat (LTR) of HIV (Venkatesh et al, 1990; Caruso et al, 1992).

C. Gene therapy against HIV in cell culture

Strategies for HIV gene therapy include the inactivation of the CCR-5 coreceptor which is accomplished by targeting a modified CC-chemokine to the endoplasmic reticulum to block the surface expression of newly synthesized CCR-5 (Yang et al, 1997). A different gene therapy strategy proposed is targeting Tat, an early regulatory protein that is critical for viral gene expression and replication and which transactivates the LTR of HIV-1 via its binding to the transactivation response element (TAR); Tat also superactivates the HIV-1 promoter via activation of NF-κB in a pathway involving protein kinase C and TNF-α; combinations of the NF-κB inhibitors, pentoxifylline and Go-6976, with a stably expressed anti-Tat single-chain intracellular antibody suppressed HIV-1 replication and LTR-driven gene expression (Mhashilkar et al, 1997). Production of recombinant retrovirus containing the HSV-*tk* gene coupled to the HIV-2 promoter and Tat responsive region (TAR) has been used on human and mouse cells in culture for the specific elimination of HIV Tat-expressing cells; since the HIV-2 promoter can sustain a considerable level of basal expression in the absence of its activator, Tat, a number of modifications were made to the HIV-2 promoter in order to minimize toxicity to non-infected cells.

Retroviruses export unspliced, intron-containing RNA to the cytoplasm of infected cells despite the fact that intron-containing cellular RNAs cannot be exported; this export pathway is a critical step in the HIV-1 life-cycle. In HIV-1 this is accomplished through an interaction between the viral regulatory Rev protein and the Rev response element (RRE) RNA. In the absence of Rev, these intron-containing HIV-1 RNAs are retained in the nucleus. The nuclear export sequence (NES) LQLPPLERLTL has been identified on Rev that is responsible for its export to the cytoplasm (see Boulikas, 1998, this volume for more details). Targeting of Rev has provided a framework for novel interventions to reduce virus production in the infected host. Because disruption of either Rev or the RRE will completely inhibit HIV-1 replication, an anti-HIV-1 intracellular immunization strategy was developed based on RRE region-specific hammerhead ribozymes and on the intracellular expression of an anti-HIV-1 Rev single chain variable fragment (Sfv), which specifically targets the Rev activation domain. This combination resulted in a potent inhibition of HIV-1 replication in cell culture that holds promise as a future therapeutic regimen (Duan et al, 1997). To disable Rev function, primary T cells or macrophages were transduced with a recombinant AAV carrying an anti-Rev single-chain immunoglobulin (SFv) gene or an RRE decoy gene or with combinations of the two genes to disrupt the interaction between Rev and the RRE; when the transfected cells were then challenged by either clinical or laboratory HIV-1 isolates, this genetic antiviral strategy effectively inhibited infection (Inouye et al, 1997).

A synergic effect of anti-Tat and anti-Rev molecules was found when the RRE sequence was cloned 3' to a tat transdominant negative mutant (tat22/37) gene; for this strategy Jurkat cells were transduced with the recombinant retroviruses containing the tat22/37 gene and an RRE decoy in different positions or the tat22/37 and the RevM10 transdominant negative mutant genes to produce monoclonal and polyclonal cultures expressing the integrated genes; none of these recombinant constructs inhibited virus replication at a high multiplicity of infection (MOI) and combination of tat and rev mutants was ineffective in inhibiting HIV-1 replication at both low and high MOIs; however, at a low MOI, two cell clones containing tat22/37 and the RRE decoy in 3' position showed a long lasting protection against virus replication and in two cell clones, expressing the RevM10 mutant alone, the HIV-1 replication was efficiently blocked (Caputo et al, 1997).

IL-16 is secreted by activated $CD8^+$ T lymphocytes and acts on $CD4^+$ T lymphocytes, monocytes and eosinophils. Recently, the C-terminal 130-amino acid portion of IL-16 was shown to suppress HIV-1 replication in vitro. HIV replication was inhibited by as much as 99% in HIV-1-susceptible $CD4^+$ Jurkat cells following transfection and expression of the C-terminal 130-amino acid portion of IL-

16; the mechanism of HIV-1 inhibition by IL-16 was not at the level of viral entry or reverse transcription, but at the expression of mRNA (Zhou et al, 1997).

The in vitro antiviral efficacy of two gene therapy strategies (trans-dominant RevM10 and Gag antisense RNA) were tested in combination with the clinically relevant reverse transcriptase inhibitors AZT and ddC or the protease inhibitor indinavir by Junker et al (1997). The combination of RevM10 or Gag antisense RNA with antiviral drugs inhibited HIV-1 replication 10-fold more effectively than the single antiviral drug regimen alone in retrovirally transduced human T cell lines after inoculation with high doses of HIV-1HXB3 in the presence or absence of inhibitors. The level of anti-HIV-1 activity of the psi-gag antisense sequence correlated with the length of the antisense transcript and maximal anti-HIV efficacy was observed with complementary sequence more than 1,000 nucleotides long, whereas transcripts less than 400 nucleotides long failed to inhibit HIV-1 replication in a T-cell line and in primary peripheral blood lymphocytes (Veres et al, 1996).

The HSV-1 and HSV-2 virion host shutoff gene (vhs), each of which encodes a protein that accelerates the degradation of mRNA molecules leading to inhibition of protein synthesis, was used as a suicide gene for HIV gene therapy to inhibit replication of HIV; an infectious HIV proviral clone was cotransfected into HeLa cells together with the vhs gene under control of the CMV IE promoter; HSV-1 vhs gene driven by the HIV LTR inhibited HIV replication more than 44,000-fold in comparison to a mutant vhs gene (Hamouda et al, 1997).

The specificity of the Vpr protein for the HIV-1 virus particle was exploited to develop an anti-HIV strategy targeting the events associated with virus maturation; nine cleavage sites of the Gag and Gag-Pol precursors were added to the C terminus of Vpr and the chimeric Vpr genes were introduced into HIV-1 proviral DNA to assess their effect on virus infectivity; the chimeric Vpr containing the cleavage sequences from the junction of p24 and p2 completely abolished virus infectivity (Serio et al, 1997).

D. Gene therapeutic strategies for AIDS

A gene therapy strategy to combat acquired immunodeficiency syndrome (AIDS) in individuals already infected with HIV-1 has been directed toward GM-CSF mobilized peripheral blood $CD34^+$ cells isolated from HIV-1-infected individuals and transduced with retroviral vectors containing three different anti-HIV-1-genes: (**i**) the Rev binding domain of the RRE (RRE decoy) carrying also the Neo^R gene, (**ii**) a double hammerhead ribozyme vector targeted to cleave the tat and rev transcripts (L-TR/TAT-neo), and (**iii**) the RevM10 transdominant negative mutant gene. After selection with G418, transduced cultures displayed up to 1,000-fold inhibition of HIV-1 replication following challenge with HIV-1 (Bauer et al, 1997).

A safe strategy to gene therapy of AIDS aimed at reducing the virus load in HIV-1-infected individuals was developed by Nakaya et al (1997). The Rev protein shifts RNA synthesis to viral transcripts by binding to the RRE within the env gene. Anti-Rev chimeric RNA-DNA oligonucleotides, consisting of 29 or 31 nucleotides, were designed to inhibit the Rev regulatory function and as decoys on HIV-1 replication; anti-Rev oligonucleotides containing an RNA "bubble" structure of 13 oligonucleotides (that bound to Rev with high affinity) were found to reduce more than 90% of the HIV-1 production from infected human T-cell lines and from healthy donor-derived peripheral blood mononuclear cells; control oligonucleotides without the bubble structure, that bound to Rev with considerably less affinity, did not reduce HIV-1 production (Nakaya et al, 1997).

E. Gene therapy of HIV with ribozymes

Antisense, ribozyme, or RNA aptamers, must be efficiently transcribed, stabilized against rapid degradation, folded correctly, and directed to the part of the cell where they can be most effective. Among (i) antisense RNA, (ii) hairpin and hammerhead ribozymes, and (iii) RNA ligands (aptamers) for Tat and Rev RNA binding proteins, Rev-binding RNAs but not the others, efficiently blocked HIV-1 gene expression when tested in expression cassettes based on the human tRNA(met) and U6 snRNA promoters. In situ localization of both tRNA and U6 promoter transcripts revealed primarily punctate nuclear patterns (Good et al, 1997).

Isolation of an RNA aptamer that can bind with high affinity to Tat protein, including two TAR-like RNA motifs for higher-affinity binding to Tat peptides provided a novel therapeutic strategy against HIV (Yamamoto et al, 1998, this volume).

A hairpin ribozyme specific for simian immuno-deficiency virus (SIV) and HIV-2 was used to inhibit viral replication in T lymphocytes derived from transduced $CD34^+$ progenitor cells. Retroviral transduction of rhesus macaque $CD34^+$ progenitor cells with the SIV-specific ribozyme “gene” and the selectable marker neomycin phosphotransferase (Neo^R) gene, followed by expansion and selection with the neomycin analog G418, rendered $CD4^+$ T cells (derived from the transduced $CD34^+$ hematopoietic cells) highly resistant to challenge with SIV; $CD4^+$ T cells exhibited up to a 500-fold decrease in SIV replication, even after high multiplicities of infection (Rosenzweig et al, 1997).

Monomeric (targeting one site) and multimeric (targeting nine highly conserved sites) hammerhead ribozyme genes both directed against the HIV-1 envelope (Env) mRNA were stably expressed in a human $CD4^+$ T lymphocyte cell line; whereas the monomeric ribozyme caused a delay in HIV-1 replication, the multimeric ribozyme caused complete inhibition in HIV-1 replication for up to 60 days after infection (Ramezani et al, 1997).

A multitarget ribozyme of an unusually large size (3.7 kb) had a notable antiviral potential which may lead to a gene therapy approach; this ribozyme, directed against multiple sites within the gp120 coding region of HIV-1 RNA, co-localized with unspliced HIV-1 pre-mRNA and/or genomic HIV-1 RNA in the nucleus catalyzing the reduction of all spliced and unspliced HIV-1 RNAs; the same ribozyme functioned as a mRNA for a chimeric CD4/Env protein in the cytoplasm (Paik et al, 1997).

Antisense oligonucleotides for HIV (anti-TAT) coupled with the influenza HA-2 protein-derived N-terminal fusogenic peptide have improved 5- to 10-fold their antiviral potency (Bongartz et al, 1994).

F. Novel therapies based on HIV vectors

A major drawback in the gene therapy of HIV has been the poor efficiency of gene transfer in vivo; especially important for HIV, most recombinant retroviruses transduce poorly cells harboring HIV, such as monocytes and macrophages, which are nondividing. Transfection of a fibroblast cell line with a HIV vector, bearing a deletion of the major packaging sequence has provided an HIV-1 packaging cell line which produced a large amount of HIV-1 structural proteins and non-infectious mature particles with normal reverse transcriptase activity but lacked RNA. When this cell line was stably transfected with an HIV-1-based retroviral vector virions were produced capable of transducing CD4-positive cells with efficiencies up to 10^5 cells per ml (Corbeau et al, 1996).

For more details see VI. HIV vectors for gene transfer.

E. Clinical trials involving HIV

Gene therapy approaches directed against viral targets have been successful at inhibiting HIV-1 replication in cultured human cells; however, clinical trials involving gene therapy directed at HIV-1 are still in their infancy (reviewed by Gottfredsson and Bohjanen, 1997). Treatment of HIV-1 infections using gene therapy for intracellular immunization strategies is currently being tested in clinical trials. The first RAC-approved phase I study on HIV was to evaluate the safety of cellular adoptive immunotherapy using genetically modified $CD8^+$ HIV-specific T cells in HIV seropositive individuals (protocol #15, **Appendix 1**). The significant number of ongoing clinical trials directed against HIV can be found on protocols 24, 40, 47, 52, 55, 73, 78, 79, 81, 85, 86, 88, 91, 94, 105, 108, 116, 117, and 168 in **Appendix 1**.

XXX. Familial hypercholesterolemia (FH)

A. Molecular mechanisms for FH

FH is an autosomal dominant disorder caused by a defect in the low density lipoprotein (LDL) receptor gene. LDL receptor on hepatocytes clears LDL from the serum; patients with one abnormal LDL receptor allele suffer premature coronary disease and myocardial infarction whereas patients with two abnormal alleles have extraordinarily high levels of cholesterol and accelerated atherosclerosis developing life-threatening coronary artery disease in early childhood. One type of mutation in the LDL receptor gene has incurred by Alu-Alu recombination deleting several exons and thus producing a truncated receptor molecule with loss of function (Lehrman et al, 1987).

Treatment of patients with FH is accomplished through the administration of drugs that stimulate the expression of LDL receptor from the normal allele in order to lower the plasma level of LDL; however this regimen is not effective for the treatment of homozygous deficient patients, especially those that retain less than 2% of residual LDL-R activity. A more direct approach has been to correct the deficiency of hepatic LDL receptor by transplanting a liver that expresses normal levels of LDL receptor; three patients that survived this procedure normalized their serum LDL-cholesterol (see Wilson et al, 1992 for references).

B. Gene therapy of FH: experiments in cell culture

A transmissible retroviral vector containing a full-length human cDNA for the LDL-R was used to infect fibroblasts from the Watanabe heritable hyperlipidemic (WHHL) rabbit which expressed the human receptor efficiently, as indicated by RNA and ligand blotting studies (Miyanohara et al, 1988). The number of hepatocytes that could be transduced by retroviruses bearing the therapeutic gene was one of the limiting steps that could impair the success of this strategy; addition of human hepatocyte growth factor (HGF) to hepatocytes allowed marked increase in the transduction efficiency in mouse (up to 80%) and human (40%) hepatocytes (Pages et al, 1995). Transduction of the human LDL-R cDNA under the transcriptional control of the liver-type pyruvate kinase promoter allowed high and tissue specific expression of the gene in primary hepatocytes; a second vector with a housekeeping promoter corrected the LDL-R deficiency in fibroblasts from a FH patient (Pages et al, 1996b).

C. Gene therapy of FH: experiments on animals

Liver is the preferred target organ for gene transfer-mediated treatment of FH. The presence of unique receptors at the cellular membrane of hepatocytes forms the basis for transfer strategies based on receptor targeting (reviewed by Sandig and Strauss, 1996). An authentic animal model used in FH gene therapy is the Watanabe heritable hyperlipidemic rabbit which is homozygous for FH and has a deletion in a cysteine-rich region of the LDL receptor gene; this renders the receptor completely dysfunctional (Yamamoto et al, 1986); these animals display high levels of serum cholesterol, diffuse atherosclerosis, and die

prematurely. Liver tissue was removed from such animals and the cultured hepatocytes were transduced with retroviruses carrying the rabbit **LDL receptor** gene; the genetically corrected cells were transplanted into the animal from which they were derived. This treatment resulted in a 30-40% reduction in serum cholesterol that lasted for at least 4 months (Chowdhury et al, 1991).

The portal vein has been used for liver targeting in New Zealand White (NZW) rabbits. Expression of lacZ was obtained in virtually all hepatocytes within 3 days (but was undetectable by 3 weeks) after transfer of the lacZ reporter gene under the control of different promoters using recombinant, replication-defective adenoviruses which were infused into the portal circulation. An adenovirus human LDL-R cDNA was then infused into the portal vein of rabbits deficient in LDL receptor and demonstrated human LDL receptor protein in the majority of hepatocytes that exceeded the levels found in human liver by at least 10-fold. Transgene expression diminished to undetectable levels within 3 weeks (Kozarsky et al, 1994).

To demonstrate feasibility of the ex vivo FH therapy, three baboons underwent a partial hepatectomy, their hepatocytes were isolated, cultured, transduced with a retrovirus containing the human LDL-R gene, and infused via a catheter that had been placed into the inferior mesenteric vein at the time of liver resection (Grossman et al, 1992). Gene replacement therapy of human LDL receptor gene into the murine model of FH transiently corrected the dyslipidaemia; long-term expression of the therapeutic gene was extinguished by humoral and cellular immune responses to LDL receptor which developed and possibly contributed to the associated hepatitis (Kozarsky et al, 1996).

As an alternative strategy, expression in the liver of the **very low density lipoprotein (VLDL) receptor**, which is homologous to the LDL receptor but has a different pattern of expression, using recombinant adenoviruses corrected the dyslipidaemia in the FH mouse; transfer of the VLDL receptor gene circumvented immune responses to the transgene leading to a higher duration of metabolic correction (Kozarsky et al, 1996).

Replication-defective adenovirus-mediated gene transfer of the very low density lipoprotein receptor (VLDL-R) driven by a cytomegalovirus promoter in LDL-R knockout mice by a single intravenous injection resulted in reduction in total cholesterol by approximately 50% at days 4 and 9 and returned toward control values on day 21. Lowering in total cholesterol was mediated by a marked reduction in the intermediate density lipoprotein/low density lipoprotein (IDL/LDL) fraction. In treated animals, there was also an approximately 30% reduction in plasma apolipoprotein (apo) E accompanied by a 90% fall in apoB-100 on day 4 of treatment. Thus, adenovirus-mediated transfer of the VLDLR gene induced high-level hepatic expression of the VLDL-R, resulted in a reversal of the hypercholesterolemia, and enhanced the ability of these animals to clear IDL (Kobayashi et al, 1996).

The human apolipoprotein E (**apoE**) gene driven by the cytochrome P450 1A1 promoter produced transgenic mice where robust expression of apoE depended upon injection of the inducer β-naphthoflavone; a transgenic line exhibiting basal expression of apoE in the absence of the inducer upon breeding with hypercholesterolemic apoE-deficient mice produced animals which were as hypercholesterolemic as their nontransgenic apoE-deficient littermates in the basal state. When injected with the inducer, plasma cholesterol levels of the transgenic mice decreased dramatically. The inducer could pass transplacentally and via breast milk from an injected mother to her suckling neonatal pups, giving rise to the induction of human apoE in neonate plasma (Smith et al, 1995).

Other potential approaches for the treatment of FH include transfer of the **apolipoprotein (apo) B mRNA editing protein** which is an essential catalytic component of the apoB mRNA editing enzyme complex. This enzyme deaminates a cytidine residue at nucleotide position 6666 in apoB mRNA, converting it to uridine leading to the production of apoB-48 in place of apoB-100. The editing protein exists as a homodimer and can be used as a therapeutic agent to reduce apoB-100; somatic gene transfer of the editing protein cDNA was highly effective in lowering plasma low density lipoproteins (Chan et al, 1996).

D. Clinical trials on FH

The first clinical trial for gene therapy in the liver, based on ex vivo gene delivery, has shown both the feasibility and the limits of the current technology. According to this protocol, cultured hepatocytes from patients homozygous for mutations in the LDL receptor gene were proposed to be transduced ex vivo with the LDL receptor gene and transplanted by infusion into the portal vein of the patient (Wilson et al, 1992). A 29 year old woman, with homozygous FH, was transplanted with autologous hepatocytes that were genetically corrected with recombinant retroviruses carrying the LDL receptor gene. She tolerated the procedures well and in situ hybridization of liver tissue four months after therapy revealed evidence for engraftment of transgene-expressing cells. The patient's LDL/HDL ratio declined from 10-13 before gene therapy to 5-8 following gene therapy, an improvement which remained stable for the 18-month duration of the treatment (Grossman et al, 1994).

Five patients, ranging in age from 7 to 41 years, with homozygous FH, underwent hepatic resection and placement of a portal venous catheter; primary hepatocyte cultures from the resected liver were transduced with the human LDL-R gene with a retrovirus and the cells were then transplanted into the liver through the portal venous catheter. The liver-directed ex vivo gene therapy was accomplished safely and, in a child patient, normalization of cholesterol levels took place; LDL-R expression was detected in a limited number of hepatocytes of liver tissue

four months after hepatocyte implantation from all five patients whereas significant and prolonged reductions in LDL cholesterol were demonstrated in three of five patients. The major obstacle in this first trial was the level and duration of LDL-R expression which "precluded a broader application of liver-directed gene therapy without modifications supporting substantially greater gene transfer" (Grossman et al, 1995; Raper et al, 1996).

XXXI. Angiogenesis and human disease

A. Formation of new blood vessels (angiogenesis)

Blood vessels, named in anatomy on the basis of their luminar diameter, branching, position and organ supplied, are formed with their proper diameter in the embryo before the heart starts beating. During a complex developmental program leading to formation of the cardiovascular system angioblasts are derived from mesoderm; this process requires the action of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). Angioblasts then differentiate into endothelial cells which undergo proliferation, migration, and morphologic organization in the context of their surrounding tissues to form the blood vessels (reviewed by Folkman and D'Amore, 1996).

Angiogenesis virtually never occurs physiologically in adult tissues except in the ovary, the endometrium and the placenta. During this process, which is also essential in pathological situations such as wound healing and inflammation, formation of new microvessels from parent microvessels takes place. The process involves remodeling of the basement membrane and interstitial extracellular matrix (ECM) using degrading proteases produced by the endothelial cells and other adjacent cells, and the synthesis of ECM. The endothelial cells are able to synthesize and secrete cytokines. The angiogenesis is strictly controlled by a redundancy of pro- and anti-angiogenic paracrine peptide molecules. The tumor suppressor p53 protein has been shown to control the expression and synthesis of two anti-angiogenic factors (reviewed by Norrby, 1997).

Blood vessels in embryogenesis are formed in two stages: **(i)** during vasculogenesis newly differentiated endothelial cells coassemble into tubules that further fuse forming the primary vasculature of the embryo; **(ii)** angiogenesis, involves sprouting of new capillary vessels from preexisitng vasculature, also occurring into initially avascular organs such as the brain and kidney, and remodeling of the primary vascular network into large and small vessels (Davis et al, 1996). During tumor angiogenesis endothelium gives rise to new vessels which requires **(i)** dissolution of the basement membrane, **(ii)** migration and **(iii)** proliferation of endothelial cells, **(iv)** formation of the vascular loop, and **(v)** formation of a new basement membrane; VEGF and PDGF act directly on endothelial cells and may also activate inflammatory cells to synthesize angiogenic factors such as SPARC (Secreted Protein Acidic and Rich in Cysteine) (reviewed by Jendraschak and Sage, 1996).

Additional angiogenic factors include bFGF, angiopoietin-1, and thymidine phosphorylase whereas naturally occurring inhibitors of angiogenesis, other than angiostatin and vasculostatin include thrombospondin (see below, reviewed by Bicknell and Harris, 1996). Tissue factor (TF), which is the principal cellular initiator of coagulation and its deregulated expression has been implicated in cancer and inflammation has a documented role in tumor-associated angiogenesis (reviewed by Carmeliet et al, 1997).

In the adult, angiogenesis accompanies the pathological processes of neovascularization during tumor growth, wound healing, and diabetic retinopathy and the normal processes of ovulation and placental development. There is a difference between vasculogenesis and angiogenesis: vasculogenesis occurs only during early embryogenesis resulting in the formation of the primordia of the heart and large vessels; on the other hand, angiogenesis is required for both the embryonic and postnatal tissues (for references see Davis et al, 1996). These processes involve two families of receptor tyrosine kinases, the Flt-1, Flk-1 receptors which interact with VEGF and the TIE (or Tek) receptor tyrosine kinases which are stimulated by angiopoietin-1 (see below).

B. Vascular endothelial growth factor (VEGF)

VEGF (also known as vascular permeability factor, VPF) is a secreted protein related to PDGF which has been cloned (Keck et al, 1989; Leung et al, 1989; Abraham et al, 1991). VEGF is a mitogen for endothelium required for the differentiation of mesoderm-derived angioblasts into endothelial cells which form *de novo* vessels during embryogenesis, and in pathological states such as cancer and wound healing. VEGF is required for both vasculogenesis and angiogenesis. Targeted disruption of the *VEGF* gene in mice resulted in delayed differentiation of endothelial cells, impairment of vasculogenesis and angiogenesis and death at 8.5 to 9 days of gestation (Carmeliet et al, 1996; Ferrara et al, 1996).

VEGF is a tumor secreted protein but also is synthesized in specialized endothelial and epithelial cells (primarily in alveolar walls of the lung, kidney glomeruli, heart, and adrenal gland, and secondarily in liver, spleen, and in autonomic nerves supplying the smooth muscle layers of the gastrointestinal tract) and in embryonic tissues; placenta expresses a related protein, placenta growth factor (PGF). The expression of VEGF in corpus luteum in primate ovaries is under hormonal control (reviewed by Senger et al, 1993). The positive staining for VEGF in normal corpus luteum is coincidental with the angiogenesis incurring concurrently with development and differentiation in this tissue; on the other hand, the expression of VEGF in lung, kidney, heart, GI tract, and

adrenals, where angiogenesis is not normally occurring, might serve to maintain a certain density of endothelial cells in these tissues as well as for inducing and maintaining the base-line vascular permeability for plasma proteins, including antibodies and lipoproteins (reviewed by Senger et al, 1993).

VEGF is produced in four isoforms by alternative splicing giving polypeptides of 210, 189, 165, and 121 amino acid residues (Leung et al, 1989; Keck et al, 1989; Abraham et al, 1991); VEGF is overexpressed in about 70% of hepatocellular carcinomas and other tumors (Suzuki et al, 1996).

At least three pathophysiological roles for VEGF have been unraveled: **(i)** Primarily, VEGF induces angiogenesis (Plate et al, 1992; Shweiki et al, 1992), i.e. sprouting of capillaries from preexisting blood vessels, a process occurring during embryonic development but also under pathological conditions such as wound healing, eye disease, and tumor growth; VEGF expression is an early event during carcinogenesis and is also involved in metastasis (Liotta et al, 1991). **(ii)** VEGF is a mitogenic factor primarily for vascular endothelial cells stimulating phospholipase C after high affinity binding to the receptor. **(iii)** VEGF also stimulates migration of monocytes across the endothelial cell monolayer. Other endothelial growth factors with angiogenic activity are the platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) (reviewed by Folkman and Klagsbrun, 1987).

C. VEGF receptors

The potential targets of secreted VEGF are proximal cells expressing VEGF receptors. Three VEGF receptors have been identified:

(**i**) A *fms*-like tyrosine kinase, the Flt-1 protein, (also called VEGF-R1), which is a transmembrane receptor with seven immunoglobulin-like domains in the extracellular region, a single transmembrane-spanning region, and a tyrosine kinase sequence (de Vries et al, 1992); Flt-1 is expressed in endothelial cells but is not found in non-endothelial cells (Shweiki et al, 1992). Disruption of the *flt-1* gene permits differentiation of endothelial cells but interferes with a later stage of vasculogenesis causing thin-walled blood vessels with larger diameter and the death of mouse embryos at day 9 (Fong et al, 1995).

(**ii**) The Flk-1, (also called VEGF-R2), also a tyrosine kinase receptor. Flk-1 is endothelial cell-specific, already expressed in the angioblasts of the blood islands in early mouse embryos, progenitors of vascular endothelial cells. The Flk-1, (fetal liver kinase-1) was cloned independently and had been suggested to be involved in hematopoietic stem cell renewal. Flk-1 exhibits a high affinity for VEGF (K_d=10^{-10} M) and is a major regulator of angiogenesis and vasculogenesis (Millauer et al, 1993). Hybridization of a parasagittal section of mouse embryos, at 14.5 days of development, with a single-stranded antisense DNA probe comprising the Flk-1 extracellular domain showed high levels of hybridization in the endothelial lining of the atrium in heart, endothelial cells in the peribronchial capillaries (but not bronchial epithelium) in the lung, the menings, and at the inner surface of the atrium and the aorta; this expression seemed to be restricted to endothelial cells in blood vessels and capillaries. Flk-1 was also expressed in capillaries in brain at postnatal day 4 (Millauer et al, 1993).

(**iii**) The Flt-4 (or VEGF-R3) (for references see Suri et al, 1996).

Transgenic mouse embryos with targeted disruption of the *VEGF-R1* and *R2* genes have unraveled the different roles these receptors undertake during development: Flt-1 regulates normal endothelial cell-cell or cell-matrix interactions during vascular development (Fong et al, 1995) and Flk-1 is required for the formation of blood islands and vascularization of the embryo; disruption of the *flk-1* gene interfered with differentiation of endothelial cells leading to death of embryos at day 8.5 to 9.5 (Shalaby et al, 1995).

The temporal and spatial expression of VEGF exactly correlated with the expression pattern of Flk-1 in all tissues and developmental stages in mice examined suggesting a pivotal role of the growth factor/receptor duet in development and differentiation of the vascular system; their expression was high during development and declined in adulthood (Millauer et al, 1993).

Although VEGF expression showed a moderate increase during tumor development in pancreatic islets of Langerhans, the expression of the *flt-1* and *flk-1* receptor genes remained unchanged during pancreatic carcinogenesis in an animal model (transgenic mice having a targeting expression of the SV40 T antigen gene under control of the insulin gene regulatory regions in β-cells of the pancreatic islets) (Christofori et al, 1995).

D. Angiopoietin and its receptors

Although the TIE1 and TIE2 receptor tyrosine kinases were found to be involved in vasculogenesis already in 1992, angiopoietin-1, the natural ligand of TIE2 was only recognized in 1996 by secretion-trap expression cloning; according to this method entire cDNA libraries were transfected into large numbers of cells and the cell that contained the desired cDNA was uniquely marked on its surface by expression of the desired ligand; this rare cell was isolated within a background of millions of cells and was expanded leading to the isolation of the ligand-encoding cDNA (Davis et al, 1996). This finding represents a significant milestone in the effort to understand the molecular mechanisms that govern formation of blood vessels with important implications for cancer targeting by inhibition of angiogenesis.

Targeted disruption of the angiopoietin-1 gene in mice (Suri et al, 1996) gave defects in the blood vessels reminiscent of those caused by disruption in its receptor TIE2 (Dumont et al, 1994); these defects were mainly in

the endocardium and myocardium but also generalized defects in vascular complexity leading to the death of mice by day 12.5 of gestation. These studies have established the important role of angiopoietin in mediating reciprocal interactions between the endothelium and surrounding matrix and mesenchyme (Suri et al, 1996). However, angiopoietin-1 does not display the mitogenic activities of VEGF on endothelial cells. The efficacy of disrupting the binding of angiopoietin to its ligand in tumor cell growth remains to be established.

A missense mutation in the receptor tyrosine kinase TIE2, resulting in a Arg to Trp substitution at position 849 of the kinase domain, was found in patients from two unrelated families suffering with venous malformations; this disorder is characterized by the presence of veins with large lumens lined by a monolayer of endothelial cells but with thin walls because of a reduction in smooth muscle layers. Expression of the wild-type and mutant TIE2 in insect cells has shown that the mutation caused a 6 to 10-fold increase in autophosphorylation activity of TIE2; thus, this mutation causes a defect in vascular remodeling and the overproliferation of endothelial cells without a complementary increase in smooth muscle layers (Vikkula et al, 1996). This finding demonstrates the significance of the TIE2 signaling pathway for endothelial cell-smooth muscle cell communication in venous morphogenesis.

E. Involvement of VEGF in tumor angiogenesis

Important for tumor growth are alterations in extracellular matrix. Solid tumors are composed of the malignant cells and the supportive vascular and connective tissue stroma whose synthesis is induced by the malignant cells. Fibrin is an essential component of the connective tissue stroma upon which tumor cells depend for their oxygen/nutrient supply and waste disposal. Fibrin gel provides solid tumors with a matrix which favors the ingrowth of macrophages, fibroblasts, and endothelial cells; these compose, along with neoangiogenesis vessels and elements found in normal connective tissue, the mature tumor stroma. Fibrinogen and other plasma proteins are found in increased amounts in tumor stroma and in healing wounds compared with normal stroma of most tissues; deposition of fibrin at the extravascular site requires a previous increase of permeability of the microvasculature thought to be mediated by VEGF (Senger et al, 1993).

VEGF is the factor largely responsible for leakiness and hyperpermeability of tumor blood vessels; leakage of plasminogen (which is converted to plasmin) and of fibrinogen and clotting factors II, V, VII, X, and XIII (responsible for the formation of the extravascular fibrin gel) through postcapillary venules in tumor cells is responsible for generating the supporting matrix for fibroblast migration, angiogenesis, and fibroplasia (reviewed by Senger et al, 1993). Migration of macrophages and fibroblasts in the fibrin gel in tumors is determined by fibrinogen concentration and Factor XIII crosslinking of α and γ chains. Tumor cells as well as inflammatory cells in healing wounds produce a higher concentration of degradative proteases for extracellular matrix but also protease inhibitors which explains the resistance toward degradation of the fibrin gel in solid tumors (reviewed by Senger et al, 1993).

The mechanism of overexpression of *VEGF* in solid tumors might involve its induction by hypoxia (Shweiki et al, 1992); the rapid proliferation of the cells in the center of the tumor induces an increase in the interstitial pressure and may lead to closure of capillaries by compression; inefficient vascular supply, including the compensatory development of collateral blood vessels in ischaemic tissues, leads to neovascularization via production of VEGF. A clustering of capillaries alongside VEGF-producing cells in a subset of glioblastoma cells immediately proximal to necrotic foci have been observed in intracranial brain neoplasms obtained from surgical specimens; this was thought to be the result of a local angiogenic response elicited by VEGF (Shweiki et al, 1992). VEGF expression by hypoxia was also induced in skeletal muscle myoblasts, in the fibroblast mouse L cell line, and in cells from rat heart muscle (Shweiki et al, 1992).

Targeting of *VEGF* gene leading to its transcriptional inactivation (e.g. via triplex-forming oligonucleotides or antisense vectors) is expected to limit growth in solid tumors via inhibition in neo-vascularization; the prolonged sustenance of hypoxia in the center of the tumor is also expected to induce p53. An important concept to understand is that tumor angiogenesis results from a balance between angiogenic and anti-angiogenic factors. Expression of $VEGF_{165}$ in rat C6 glioma cells and subcutaneous injection of the transduced cells in athymic mice has shown that tumors from cells expressing *VEGF* grew slower than tumors developed from nontransduced C6 cells, were highly vascularized, and contained varying degrees of necrosis and eosinophilic infiltrate (Saleh, 1996).

VEGF plays an important role not only in carcinogenesis but also restenosis (see below). VEGF promotes endothelial cell proliferation to accelerate re-endothelialization of the artery reducing intimal thickening; up-regulation of VEGF is the desired effect for treatment of restenosis (Asahara et al, 1996; Isner et al, 1996a). The transfer of the VEGF gene demonstrates a special mission of gene therapy: how to treat one human disease by upregulating the expression of a specific gene while treating a different disease by downregulating the expression of the same gene. Targeting is important. Also, exploring the molecular mechanisms affected by the transfer and overexpression of the cDNA of a gene, in all aspects and at their entire spectrum, is essential for a successful gene therapy application.

F. Transfer of the VEGF gene in ischemia

VEGF gene transfer can improve blood supply to the ischaemic limb and is a promising approach for the treatment of acute limb ischemia. In a gene therapy approach for tissue ischemia, the $VEGF_{165}$ cDNA under the transcriptional control of the HSV immediate-early 4/5 promoter was used to transduce BLK-CL4 fibroblasts resulting in the secretion of high levels of biologically active VEGF; when the transduced cells were resuspended in basement membrane extract (matrigel) and were injected subcutaneously into syngeneic C57BL/6 mice they showed a strong angiogenic response (Mesri et al, 1995). Regional angiogenesis was induced in nonischemic retroperitoneal adipose tissue by adenoviral VEGF gene transfer supporting a 123% increase in vessel number compared to control (Magovern et al, 1997).

Treatment of a 71 year-old patient with an ischaemic leg with 2 mg $phVEGF_{165}$ plasmid applied to the hydrogel polymer coating of an angioplasty balloon and reaching the distal popliteal artery resulted in an increase in collateral vessels at the knee, mid-tibial, and ankle levels, which persisted for 12-weeks (Isner et al, 1996a).

Ischemia, induced in the hindlimb of rats by excision of the femoral artery, was experimentally treated by transfer of the VEGF gene; therapeutic angiogenesis produced morphologically similar, but significantly more extensive, networks of collateral microvessels (Takeshita et al, 1997). Direct i.m. injection of naked $VEGF_{165}$ plasmid DNA into the ischemic thigh muscles in rabbits resulted in more angiographically recognizable collateral vessels at 30 days posttransfection (Tsurumi et al, 1996, 1997).

G. Cancer treatment with angiogenesis inhibitors

On November 20, 1997 the first exciting data on clinical trials using the TNP-470, a drug extracted from fungi which inhibits angiogenesis, were reported in a speech before the National Institutes of Health by Judah Folkman (Harvard University). A woman in Texas with cervical cancer and metastasis to lungs had been tumor-free for months after treated with TNP-470; and a young girl with a slow-growing bone tumor in her jaw was cancer-free after treatment with IFN-α. Both TNP-470 and IFN-α are relatively weak inhibitors of blood vessel formation compared with angiostatin (O'Reilly et al, 1994, 1996), endostatin (O'Reilly et al, 1997), and vasculostatin which eliminated tumors in small animals. A combination therapy with angiostatin and endostatin was even more effective in tumor eradication; furthermore, these drugs have no apparent side effects and there is virtually no resistance of tumors to these drugs (see below). However, the number of human patients treated with angiogenesis inhibitors is too small and a larger number of cases need to be examined.

H. Angiostatin and endostatin

Angiostatin is a potent naturally occurring inhibitor of angiogenesis and growth of tumor metastases, which is generated by cancer-mediated proteolysis of **plasminogen** to a 38 kDa plasminogen fragment; angiostatin selectively instructs endothelium to become refractory to angiogenic stimuli (O'Reilly et al, 1994, 1996). A number of enzymes including metalloelastase, pancreas elastase, plasmin reductase, and plasmin collaborate in the conversion of plasminogen to angiostatin. Systemic administration of angiostatin, but not intact plasminogen, inhibited neovascularization in vitro and in vivo and suppressed the growth of Lewis lung carcinoma metastases (O'Reilly et al, 1994; Gately et al, 1997); human angiostatin inhibited almost completely the growth of three human and three murine primary carcinomas in mice without detectable toxicity or resistance (O'Reilly et al, 1996); these studies have developed the “dormancy therapy” for cancer based on that malignant tumors are regressed by prolonged blockade of angiogenesis to microscopic dormant foci in which tumor cell proliferation is balanced by apoptosis (O'Reilly et al, 1996).

Human angiostatin, administered to mice with s.c. hemangioendothelioma and associated disseminated intravascular coagulopathy (Kasabach-Merritt syndrome), significantly reduced tumor volume and increased survival (Lannutti et al, 1997).

PC-3 human prostate carcinoma cells release uPA and free sulfhydryl donors that converted plasminogen to angiostatin; these two components were sufficient for angiostatin generation (Gately et al, 1997); reduction of one or more disulfide bonds in the serine proteinase, plasmin, by a reductase secreted by Chinese hamster ovary cells triggered proteolysis of plasmin, generating fragments with the domain structure of angiostatin; two reductases (protein disulfide isomerase and thioredoxin) although able to produce biologically active angiostatin from plasmin and to inhibit proliferation of human dermal microvascular endothelial cells, were not the reductases secreted by cultured cells; instead the plasmin reductase factor secreted was a different one requiring reduced glutathione for activity (Stathakis et al, 1997). Two members of the human matrix metalloproteinase (MMP) family, matrilysin (MMP-7) and gelatinase B/type IV collagenase (MMP-9), hydrolyzed human plasminogen to generate angiostatin fragments; 58-, 42- and 38-kDa angiostatin fragments were generated; these studies implicated MMP-7 and MMP-9 in regulation of new blood vessel formation by cleaving plasminogen and generating angiostatin molecules (Patterson and Sang, 1997).

Recent studies exploring the mechanism responsible for the in vivo production of angiostatin that inhibits growth and metastasis in Lewis lung carcinoma have shown that angiostatin is produced by tumor-infiltrating macrophages whose metalloelastase expression is stimulated by tumor cell-derived GM-CSF (Dong et al, 1997).

Endostatin is a 20 kDa C-terminal fragment of collagen XVIII produced by hemangioendotheliomas which

specifically inhibits endothelial cell proliferation, angiogenesis and tumor growth (O'Reilly et al, 1997).

Reports on the transfer of the angiostatin cDNA for the treatment of malignancies are about to appear (Toshihide Tanaka, personal communication).

XXXII. Gene therapy of restenosis

A. Pathophysiology of restenosis

The pathological situation, described as recurrent narrowing of a blood vessel after a successful revascularization procedure, has been termed restenosis (from the Hellenic stenos=narrow); the most frequent revascularization procedure has been the percutaneous transluminal angioplasty (PTA) used to treat atherosclerotic obstructions in the coronary and peripheral vascular circulations; PTA is achieved using a tiny balloon mounted on a catheter which is advanced under x-ray guidance to the site of a blocked artery. The most frequent artery suffering restenosis is the superficial femoral artery (SFA)/popliteal artery of the leg and the iliac arteries.

One of the factors contributing to restenosis is the intimal hyperplasia of the arterial wall; among others, the mechanism for intimal hyperplasia involves increasing the tissue levels of TGF-β following injury; injection of antibodies directed against TGF-β has blocked restenosis in a rat model (reviewed by Border and Noble, 1995). Transfer of the TGF-β gene into porcine arteries caused restenosis (Nabel et al, 1993a,b). Arterial injury has pleiotropic effects at the molecular level; for example, injury of rat arteries led to an increase in FGF receptors in vascular smooth muscle cells.

Atherosclerosis and restenosis following balloon angioplasty are characterized by two steps: during the thrombotic phase in the arterial wall following injury fibrin networks are synthesized with platelet depositions; this phase is followed by smooth muscle cell proliferation. Both the synthesis of fibrin from fibrinogen, as well as the proliferation of platelet and smooth muscle cells are upregulated by the protease thrombin. Inhibition of the action of thrombin in the arterial wall is a potential target against arterial disease. The most potent and specific inhibitor of thrombin known today is the polypeptide hirudin, an anticoagulant derived from the medicinal leech, *Hirudo medicinalis*; especially important is a local delivery of hirudin to prevent thrombosis circumventing the systemic coagulopathy associated with systemic administration of the purified protein (for references see Rade et al, 1996).

The pathogenesis of both atherosclerosis and restenosis involves the migration of medial smooth-muscle cells across the internal elastic lamina to form a neointima; inflammatory reactions involving T cells and other leukocytes maintain smooth-muscle cell migration, proliferation and matrix deposition. The stenotic response involves the expression of HA (hyaluronan) receptors on both the infiltrating white cells and on smooth-muscle cell populations; exposure of injured arteries to high concentrations of HA in vivo resulted in significant inhibition of neointimal formation (Savani and Turley, 1995). Intervening with the HA and receptor genes could provide potential molecular targets for restenosis.

A number of drugs have been developed to inhibit neointima formation such as the drug CVT-313, identified from a purine analog library; CVT-313 is a specific and potent inhibitor of CDK2 reducing hyperphosphorylation of RB (Brooks et al, 1997). The angiotensin-converting enzyme inhibitor, cilazapril, also prevented myointimal proliferation after vascular injury (Powell et al, 1989). However, most of these drugs are toxic and need continuous administration to the artery. Gene therapy could result in stable transfection of the arterial wall cells with the gene of a therapeutic protein circumventing these problems.

Significant progress has been made in the area of coronary restenosis, particularly in identifying target genes to reduce neointima formation in vein grafts used in coronary bypass surgery. Targets of gene therapy include the prevention of postangioplasty restenosis, postbypass atherosclerosis, peripheral atherosclerotic vascular disease and thrombus formation (reviewed by Malosky and Kolansky, 1996; Ylä-Herttuala, 1996).

B. *VEGF* gene transfer for restenosis

Thickening of the arterial intima, composed of smooth muscle cells, is an important area for intervention by gene transfer to alleviate the syptoms of restenosis following balloon treatment. Gene therapy for restenosis may be achieved following transfer of the gene encoding VEGF; this therapy has been applied to animal models and has now entered clinical trials (Isner et al, 1996a). Previous studies using administration of recombinant, 165 amino acid, VEGF protein to rabbits *in vivo* has shown a significant augmentation in collateral vessel development by angiography after excision of the ipsilateral femoral artery in the animal to induce severe hind limb ischemia (Takeshita et al, 1994a). The rationale behind this approach is that VEGF promotes endothelial cell proliferation (Leung et al, 1989) to accelerate re-endothelialization of the artery reducing intimal thickening and thrombogenicity (Asahara et al, 1996); the inner lining of the blood vessels is made up of endothelial cells which are important in preventing the formation of blood clots or atherosclerotic plaques. Animal studies have shown that endothelial cells require a relatively long time for growth after injury. "Naked" plasmid encoding the 165 amino acid VEGF isoform is being delivered using a hydrogel /polymer-coated balloon angioplasty catheter without use of liposomes or recombinant viruses to humans (Isner et al, 1996a).

Ylä-Herttuala and coworkers (Laitinen et al, 1997a) have examined and compared the efficiency of lacZ delivery to the rabbit carotid artery (using a collar method) with (**i**) plasmid/liposome complexes (Lipofectin), (**ii**) replication-deficient Moloney murine leukemia virus (MMLV)-derived

retroviruses, (**iii**) pseudotyped vesicular stomatitis virus protein G (VSV-G)-containing retroviruses and (**iv**) adenoviruses. Transfer of the gene to the adventitia took place with all gene transfer systems tested except MMLV; the adenovirus gave the highest gene transfer efficiency and up to 10% of the cells displayed β-galactosidase activity compared with 0.05% of cells using VSV-G retrovirus, 0.05% with Lipofectin, and less than 0.01% with MMLV retrovirus (**Figure 31**).

During the collar application procedure extravascular gene transfer took place without intravascular manipulation; the model is suited for gene transfer studies involving difussible or secreted gene products (such as VEGF, see **Figure 32**) that act primarily on the endothelium; effects on medial smooth muscle cell (**SMC**) proliferation and even endothelium can be achieved from the adventitial side of the artery (Laitinen et al, 1997a).

Transfer of the VEGF gene was performed on rabbits using a silicone collar inserted around the carotid arteries. The collar acted as an agent that caused intimal SMC growth and as a reservoir for the VEGF gene; the model preserved the integrity of endothelial cells and permitted extravascular gene transfer without intravascular manipulation. A plasmid carrying the mouse $VEGF_{164}$ gene under control of the CMV promoter in a mixture with Lipofectin was injected under anesthesia by gently opening the collar (Laitinen et al, 1997b). As a control, arteries were injected with the lacZ cDNA; animals after 3, 7, or 14 days from the operation were sacrificed and the arteries were examined by electron microscopy using SMC-specific immunostaining (**Figure 32A,B**). One week after VEGF transfer with cationic liposomes there was a significant reduction in intimal thickening (**Figure 32B**) compared to control arteries (**A**). When the nitric oxide synthase inhibitor L-NAME was administered to the animals it abolished the therapeutic efficacy of VEGF and there was no VEGF-induced reduction in intimal thickening of the arteries (Laitinen et al, 1997b).

C. Transfer of other genes against restenosis and arterial injury

A number of vascular disorders can be treated by arterial gene transfer (Nabel et al, 1990, 1993a-c; Ohno et al, 1994; Takeshita et al, 1994b; Chang et al, 1995a,b). One of the drawbacks has been the low percentage of the cells transfected which were in the order of 1% to less than 0.1% using naked plasmid delivery with a balloon catheter (e.g. Isner et al, 1996a), cationic liposomes (Nabel et al, 1990, 1993a-c), or retroviruses (Flugelman et al, 1992). A variety of genes have been transferred into arterial wall cells. Transfer of the human growth hormone gene, a secreted protein, into rabbit arterial organ culture using lipofectin permitted determination of hGH levels in the culture medium using a sensitive immunoassay method (Takeshita et al, 1994b). The *ADA* gene has been transferred efficiently to vascular smooth muscle cells in rats (Lynch et al, 1992). Transfer of the fibroblast growth factor-1 gene (Nabel et al, 1993b), of the platelet-derived growth factor B gene (Nabel et al, 1993c), or of the transforming growth factor-β1 gene (Nabel et al, 1993a,b) into animal arteries promoted intimal hyperplasia and angiogenesis.

The therapeutic induction of angiogenesis in ischemic tissues using recombinant cytokines is also promising for clinical application (Norrby, 1997).

In vivo suppression of injury-induced vascular smooth muscle cell (VSMC) accumulation is a widely used approach (Ohno et al, 1994). Other than *VEGF* gene transfer (Isner et al, 1996a, see above), additional approaches for the treatment of restenosis after injury-induced VSMC accumulation is via delivery of the HSV-tk gene followed by ganciclovir treatment in order to kill preferentially the smooth muscle cells (Guzman et al, 1994; Ohno et al, 1994); by transfer of the cytosine deaminase (CD) gene the product of which is capable of metabolizing 5-fluorocytosine (5-FC) to 5-fluorouracil in a rabbit femoral artery model of balloon-induced injury (Harrell et al, 1997); by transfer of the *RB* (Chang et al, 1995a; Smith et al, 1997), or p21 genes (Chang et al, 1995b); by transfer of *ras* (Indolfi et al, 1995), *TGF*β gene (Grainger et al, 1995); and, by transfer of the nitric oxide synthase gene (von der Leyen et al, 1995).

At the molecular level, arterial injury results in exposure of vascular smooth muscle cells (VSMC) and fibroblasts to multiple growth factors that activate second messengers and induce expression of immediate-early genes within minutes to hours after stimulation resulting in the exit of VSMC from the quiescent G0 state. A series of CDKs are activated and tumor suppressor genes need to be down-regulated for VSMC proliferation including p53, p21, p16, and RB (reviewed by Muller, 1997). Therapeutic strategies to restrict neointima formation in the injured artery include **(i)** inhibition in the expression of proto-oncogenes (c-myc); **(ii)** transfer of suicide genes (HSV-tk, CD); **(iii)** use of molecular decoys or drugs to block specific steps required for cell cycle progression, **(iv)** transfer of tumor suppressor genes (p21, RB); **(v)** treatment with antisense oligonucleotides to down regulate genes required for cell proliferation or DNA synthesis (antisense cyclin G1, PCNA); **(vi)** transfer of a number of unrelated genes such as of gax, TGF-β, hirudin, PKCδ, β-interferon (**Table 7**). It is worth considering that delivery of recombinant adenoviruses themselves causes (i) a pronounced infiltration of T cells throughout the artery wall; (ii) upregulation of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in arterial smooth muscle cells; (iii) neointimal hyperplasia (Newman et al, 1995).

Figure 31. β-galactosidase gene transfer into the collared rabbit carotid arteries using (i) plasmid/liposome complexes, (ii) replication-deficient Moloney murine leukemia virus (MMLV)-derived retroviruses, (iii) pseudotyped vesicular stomatitis virus protein G (VSV-G)-containing retroviruses and (iv) adenoviruses. Gene transfer was done on day 5 after the collar operation. Arteries were analyzed 5 days after the gene transfer for general histology, cell types and β-galactosidase activity using immunocytochemistry and X-gal staining. (**A,B**) Immunostainings of serial sections of an artery transfected with plasmid/liposome complexes. **A:** Endothelium remained anatomically intact during manipulations as shown with endothelial-specific staining using the monoclonal antibody CD31. **B**: The majority of cells in intima and media were smooth muscle cells (SMC) as shown with the SMC-specific antibody HHF-35. **C**: Absence of staining with X-gal in an untransfected control artery. (**D-L**): Arteries transfected with various gene transfer constructs. **D**: Plasmid/liposome complexes (25 μg lacZ plasmid, 25 μg Lipofectin reagent in 600 μl Ringer solution. **E**: MMLV retroviruses (600 μl pLZRNL retrovirus, titer $5x10^5$ cfu/ml). **F**: VSV-G pseudotyped retroviruses (600 μl pLZRNL$^+$G retrovirus, titer $1x10^7$ cfu/ml). **G**: E1−E3-deleted adenoviruses (600 μl nuclear targeted pCMVBA-LACZ Adenovirus 5, titer $1x10^9$ pfu/ml). **H**: Higher magnification of G. **I**: Higher magnification of G showing intense staining of the nucleae (arrow) in the adventitia with the nuclear targeted lacZ construct and X-gal staining of some endothelial cells (arrow-head). (**J,K**): Inflammatory cells were seen in adventitia after the gene transfer with VSV-G retroviruses and adenoviruses: **J**: VSV-G pseudotyped retrovirus-transfected artery (macrophage-specific staining with the monoclonal antibody RAM-11). **K**: Adenovirus-transfected artery (macrophage-specific staining with the monoclonal antibody RAM-11). **L**: Nonimmune control (first antibody omitted). Original magnification X10 (G); X25 (A-C,F,H); X50 (D,J,L); X100 (E,I). From Laitinen M, Pakkanen T, Donetti E, Baetta R, Luoma J, Lehtolainen P, Viita H, Agrawal R, Miyanohara A, Friedmann T, Risau W, Martin JF, Soma M, Ylä-Herttuala S (**1997a**) Gene transfer into the carotid artery using an adventitial collar: comparison of the effectiveness of the plasmid-liposome complexes, retroviruses, pseudotyped retroviruses, and adenoviruses. **Hum Gene Ther** 8, 1645-1650. Reproduced with the kind permission of the authors and Mary Ann Liebert, Inc.

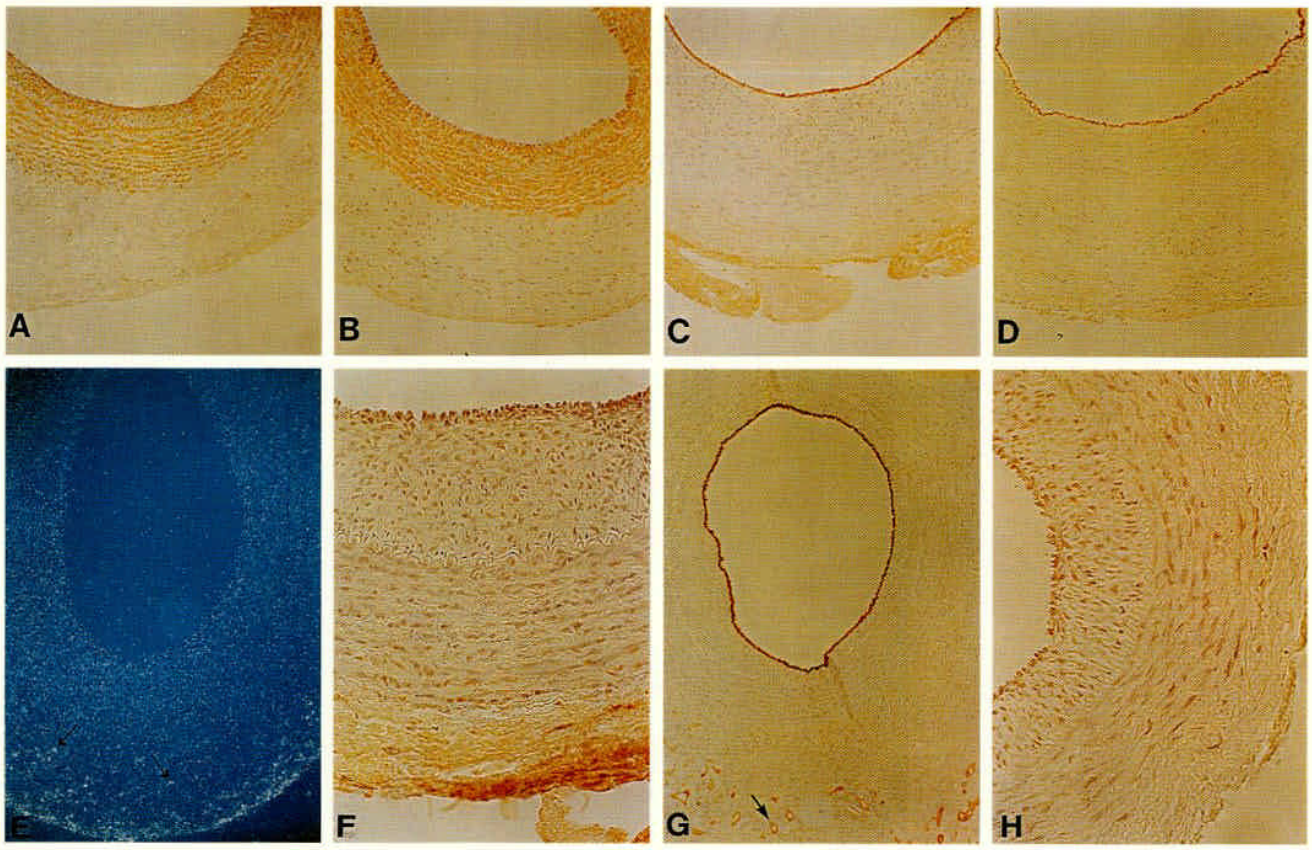


Figure 32. Representative micrographs showing the characteristics of rabbit carotid arteries 7 days after VEGF or lacZ gene transfer. **A**. Control artery transfected with lacZ-plasmid/liposomes using smooth muscle cell (SMC)-specific immunostaining (HHF-35) showing a typical intimal thickening. **B**. Artery transfected with VEGF plasmid/liposomes showing a limited intimal thickening after SMC-specific immunostaining (HHF-35). **C**. Serial section to A, but stained for endothelium with CD31 monoclonal antibodies; it shows the presence of endothelium in all vascular segments examined. **D**. Serial section to B, but stained for endothelium with CD31 monoclonal antibodies, showing an intact endothelium. **E**. In situ hybridization with a VEGF antisense riboprobe labeled with [^{35}S]UTP in VEGF-transfected artery; bright spots (arrows) indicate the expression of VEGF mRNA (dark-field image). Control hybridizations with sense riboprobes were negative (not shown). **F**. The absence of inflammation was shown in VEGF-transfected arteries with macrophage-specific immunostaining (RAM-11). **G**. Neovascularization (arrow) in the adventitia of VEGF-transfected artery 14 days after gene transfer using endothelium-specific immunostaining (CD31). No neovascularization was detectable in lacZ-transfected arteries (not shown). **H**. Nonimmune control for the immunostainings (first antibody omitted); sections were counterstained with hematoxylin. Magnification, 25X in A-E, G and 50X in F,H. From Laitinen M, Zachary I, Breier G, Pakkanen T, Hakkinen T, Luoma J, Abedi H, Risau W, Soma M, Laakso M, Martin JF, Ylä-Herttuala S (**1997b**) VEGF gene transfer reduces intimal thickening via increased production of nitric oxide in carotid arteries. **Hum Gene Ther** 8, 1737-1744. Reproduced with the kind permission of the authors and Mary Ann Liebert, Inc.

Transfer of the bone morphogenetic protein-2 (BMP-2) gene inhibited serum-stimulated increase in DNA synthesis and cell number of cultured rat arterial SMCs as well as injury-induced intimal hyperplasia; the mode of BMP-2 action differed from that mediated by TGF-β; BMP-2 had the ability to inhibit SMC proliferation without stimulating extracellular matrix synthesis (Nakaoka et al, 1997).

cDNA for hirudin has been delivered to smooth muscle cells of injured rat carotid arteries using an adenoviral vector; the coding region for the human growth hormone signal peptide (MATGSRTSLLLAFGLLCLPWLQEGSA) was engineered upstream of the hirudin cDNA in order to achieve secretion of the protein in effectively transduced cells. The therapeutically important levels of hirudin which were secreted in vivo resulted in 35% reduction in neointimal hyperplasia as shown on histologic sections of the carotid arteries (Rade et al, 1996). Significant issues on toxicity and immunogenicity of the vector remain to be resolved for application of the method to humans (Rade et al, 1996).

Table 7. Genes or antisense used to inhibit smooth muscle cell proliferation and neointima formation for the treatment of arterial injury and restenosis

Gene or antisense	Reference
VEGF gene transfer	Isner et al, 1996a; Van Belle et al, 1997; Laitinen et al, 1997a,b
HSV-tk gene /GCV	Guzman et al, 1994; Ohno et al, 1994
Cytosine deaminase (CD) gene /5-fluorocytosine	Harrell et al, 1997
RB gene	Chang et al, 1995a; Smith et al, 1997
p21 gene	Chang et al, 1995b
Hirudin gene	Rade et al, 1996
Dominant-negative mutated form of c-H-ras gene	Indolfi et al, 1995; Ueno H et al, 1997b
*TGF*β gene	Grainger et al, 1995
Nitric oxide synthase gene	von der Leyen et al, 1995
gax gene	Weir et al, 1995; Maillard and Walsh, 1996; Smith et al, 1997
Protein kinase Cδ gene (by suppressing G1 cyclin expression)	Fukumoto et al, 1997
Bone morphogenetic protein-2 (BMP-2) gene	Nakaoka et al, 1997
β-interferon gene	Stephan et al, 1997
Antisense cdk2 oligonucleotides	Morishita et al, 1994
Antisense oligodeoxynucleotides to c-myc	Bennettet al, 1994
Antisense oligonucleotides to PCNA	Simons et al, 1994
Antisense cyclin G1	Zhu et al, 1997

Inhibition in proliferation of the smooth muscle cells has also been achieved by an antibody against basic fibroblast growth factor (Lindner and Reidy, 1991), by antisense oligodeoxynucleotides to c-myc applied in a pluronic gel to the arterial adventitia (Bennettet al, 1994), and by antisense phosphorothioate oligonucleotides to PCNA in a rat carotid artery injury model (Simons et al, 1994). Transfer of the protein kinase Cδ gene to a rat clonal VSMC inhibited the proliferation of vascular smooth muscle cells by suppressing G1 cyclin expression and arrested the cells in the G0/G1 phase of the cell cycle; overexpression of PKCδ caused reduction in the expression of cyclins D1 and E and RB phosphorylation, and increased the protein levels of p27 (Fukumoto et al, 1997).

RB is implicated in the control of the cell cycle via its interaction with E2F (see above); a phosphorylation competent, amino-terminal-truncated RB protein (Rb56) was a more potent inhibitor of E2F-mediated transcription relative to the full-length Rb construct (Rb110); adenoviral transfer of either Rb56 or Rb110 inhibited neointima formation after balloon injury in the rat carotid artery (Smith et al, 1997).

On the other hand, overexpression of the fibroblast growth factor-1 gene (Nabel et al, 1993b), platelet-derived growth factor B gene (Nabel et al, 1993c), and transforming growth factor β1 gene (Nabel et al, 1993a,b) into the arteries induced intimal hyperplasia in vivo.

p21 protein is a negative regulator of mammalian cell cycle progression that functions both by inhibiting cyclin dependent kinases (CDKs) required for the initiation of S phase and by binding to and inhibiting PCNA. p21 gene transfer has been used to inhibit vascular smooth muscle cell (VSMC) proliferation after arterial injury for the treatment of atherosclerosis and restenosis. Over-expression of human p21 inhibited growth factor-stimulated VSMC proliferation and neointima formation in the rat carotid artery (a model of balloon angioplasty) by arresting VSMCs in the G1 phase of the cell cycle. p21-associated cell cycle arrest was associated both with a significant inhibition of the phosphorylation of RB (see above) and with the formation of complexes between p21 and PCNA in VSMCs (Chang et al, 1995b). Transfer of p21 was also used by others to suppress neointimal formation in the balloon-injured porcine or rat carotid arteries in vivo; vascular endothelial and smooth muscle cell growth was arrested through the ability of p21 to inhibit progression through the G1 phase of the cell cycle (Yang et al, 1996; Ueno et al, 1997a).

Gax is a growth arrest gene which regulates proliferation of VSMC; gax is an homeobox gene whose expression in the adult is largely confined to cardiovascular tissues. In contrast to a number of genes which are up-regulated following balloon injury and/or angioplasty, such as the early response genes c-myc and c-fos, gax is down-regulated within hours of balloon injury; gax may be required to maintain the gene expression of proteins in VSMC that are associated with the nonproliferative or

contractile phenotype. Gax is also rapidly down-regulated in cultured VSMC upon stimulation by serum or platelet-derived growth factor (PDGF); like the genes in the gas and gadd families, gax is expressed at its highest levels in quiescent cells and is down-regulated following mitogen activation (Weir et al, 1995). Percutaneous gax adenovirus-mediated gene transfer into normal and atherosclerotic rabbit iliac arteries suggested prevention of neointimal formation (Maillard and Walsh, 1996). The gax-induced growth inhibition correlated with a p53-independent up-regulation of the cyclin-dependent kinase inhibitor p21; gax overexpression also led to an association of p21 with cdk2 complexes and a decrease in cdk2 activity and, thus, gax overexpression inhibited cell proliferation in a p21-dependent manner.

Ras proteins are key transducers of mitogenic signals from the membrane to the nucleus. DNA vectors expressing ras transdominant negative mutants, which interfere with ras function, reduced neointimal formation after injury in rats in which the common carotid artery was subjected to balloon injury (Indolfi et al, 1995). An adenoviral vector, expressing a potent dominant-negative mutated form of c-H-ras, in which tyrosine replaced aspartic acid at residue 57, completely inhibited serum-stimulated activation of mitogen-activated protein kinase, and abolished the DNA synthesis in response to serum mitogens in infected smooth muscle cells in culture. Application of the adenoviral vector into balloon-injured rat carotid arteries from inside the lumen resulted in a significant reduction in neointima formation (Ueno H et al, 1997b).

Nitric oxide-generating vasodilators inhibit vascular smooth muscle cell proliferation. S-nitroso-N-acetylpenicillamine (SNAP), a nitric oxide-releasing agent, inhibited the activity of cyclin-dependent kinase 2 (Cdk2) and the phosphorylation of RB but it did not inhibit the activities of cyclin D-associated kinases Cdk4 and Cdk6 (Ishida et al, 1997). Suppression of injury-induced vascular smooth muscle cell accumulation was also achieved by transfer of the endothelial cell *nitric oxide synthase* gene (von der Leyden et al, 1995). Based on the fact that administration of the nitric oxide (NO) synthase inhibitor L-NAME to rabbit carotids eliminated the difference in intimal thickening between VEGF and mock-transfected (lacZ) arteries it was proposed that VEGF may reduce smooth muscle cell proliferation via VEGF-induced NO production from the endothelium (Laitinen et al, 1997).

D. Atherosclerosis

The atherotic plaque is formed by a complex mechanism initiated by the accumulation of lipid, macrophages and T cells at artery lesions leading to smooth muscle cell proliferation (Ross, 1993). TGF-β is involved in atherosclerosis via its activation by plasmin and via its inhibition by atherogenic lipoprotein deposited on the arterial wall. Inhibition of TGF-β would lead to smooth muscle cell proliferation; patients with advanced coronary disease have decreased serum TGF-β levels; furthermore, transgenic mice overexpressing lipoprotein (a) exhibit decreased levels of TGF-β at sites of lipoprotein (a) accumulation such as in the aortic wall (Grainger et al, 1995). As prolonged overproduction of TGF-β may lead to tissue fibrosis by overproduction of extracellular cell matrix (Border and Noble, 1995) a balanced overexpression of *TGF*-β might be of great therapeutic value for heart disease.

The plasminogen system, via its triggers, t-PA and u-PA and its inhibitor, plasminogen activator inhibitor-1 (PAI-1), has been implicated in thrombosis, arterial neointima formation, and atherosclerosis (reviewed by Carmeliet et al, 1997).

A number of animal models have been used to induce atherosclerotic lesions such as the iliac arteries of New Zealand white rabbits fed with cholesterol. Substantial progress in vector development and the demonstration of efficacy in relevant animal models will be required before gene therapy for atherosclerosis becomes a clinical reality (Rader, 1997).

E. Acidic and basic fibroblast growth factors (aFGF, bFGF)

Acidic and basic fibroblast growth factors and their receptors are involved in many fundamental biological processes but also in pathological processes (Webster and Donoghue, 1998, this volume).

Whereas VEGF is a regulator of vascular permeability and an endothelial cell growth factor the acidic and basic fibroblast growth factor (aFGF, bFGF) and placenta growth factor (PGF) polypeptides are endowed with endothelial cell growth-promoting activity; however, FGFs have not been reported to be expressed in blood vessels in vivo. aFGF and bFGF seem to act as mitogens for a large number of different cell types; in situ hybridization analysis has shown that there is no expression of FGF receptors 1 and 2 (Flg and Bek) in capillary endothelial cells during embryonic development; it is only VEGF and PGF which are known to be specific for endothelial cells (for references see Millauer et al, 1993).

Many of the studies that have demonstrated therapeutic efficacy using fibroblast (aFGF, bFGF, FGF-5), endothelial (VEGF) and other types of factors using the purified peptides can potentially be transferred to the gene level. This will not require repeated administration of the drug, especially whenever the somatic cell targets are transfected efficiently and the expression of the transgene lasts.

Liposome-mediated gene transfer of antisense-oriented bFGF or fibroblast growth factor receptor-1 (FGFR-1) cDNAs in episomal vectors into human melanomas, grown as subcutaneous tumors in nude mice caused complete arrest or regression of the tumors as a result of blocked intratumoral angiogenesis and subsequent necrosis (Wang and Becker, 1997). Inhibition of bFGF synthesis in vivo using an antisense RNA strategy significantly

inhibited intimal thickening after arterial balloon injury (Hanna et al, 1997).

Infection of human umbilical vein endothelial cell cultures with a bFGF-expressing recombinant adenovirus enhanced the proliferation rate and tubular formation of these cells on reconstituted basement membrane (Takahashi et al, 1997). Low level expression of bFGF upregulated Bcl-2 and delayed apoptosis in NIH3T3 cells; on the other hand cells expressing from 8-15 times background levels of bFGF became phenotypically transformed (gave dense foci at confluence, had decreased adherence to tissue culture plates and grew colonies in soft agar) (Wieder et al, 1997). Blood vessels of spontaneously hypertensive rats were shown to be associated with subphysiological amounts of bFGF; transfer of the *bFGF* gene corrected hypertension, restored the physiological levels of bFGF in the vascular wall, and ameliorated endothelial-dependent responses to vasoconstrictors (Cuevas et al, 1996).

F. Wound healing and plasminogen

A number of diseases including cancer, cancer metastasis, atherosclerosis, arthritis, hepatitis, dermatitis, inflammatory bowel disease, sickle cell anemia, and autoimmune disease result in severe tissue damage. Plasminogen has a profound importance in wound healing and might play a central role in many of these diseases (Rømer et al, 1996). Plasminogen is an inactive precursor-protease synthesized and secreted by the liver and converted into plasmin (trypsin-like serine protease) by the action of two different proteases: **(i)** tissue-type plasminogen activator (tPA) and **(ii)** urokinase-type plasminogen activator (uPA); in addition to uPA- and tPA-regulation, the activity of plasminogen is also controlled by plasminogen-specific cell surface receptors, by inhibitors of plasminogen activation (PAI-1 and PAI-2), and by the receptor of uPA (uPAR). Angiostatin is a 38 kDa plasminogen fragment generated by cancer-mediated proteolysis of plasminogen (O'Reilly et al, 1994, 1996, see angiostatin). Endostatin is a 20 kDa C-terminal fragment of collagen XVIII (O'Reilly et al, 1997); both angiostatin and endostatin inhibit angiogenesis and tumor growth.

Plasmin passes to extravascular fluids and stimulates proteolytic activity in the extracellular matrix (such as degradation of fibrin) but also contributes to the activation of growth factors and other proteases (see Rømer et al, 1996). Fibrin is an important component of the wound healing and reepithelization process and is formed from fibrinogen by the action of the protease thrombin (see Rade et al, 1996). These processes take place during wound healing but also during the process of atherosclerosis, restenosis, response to vascular injury, and in tumorigenesis during formation of the tumor stroma.

Plasminogen-deficient mice (transgenic animals produced by targeted disruption in the plasminogen gene) completed embryonic development and survived to adulthood but were predisposed to spontaneous thrombotic lesions in many tissues and displayed fibrin deposition in the liver (Bugge et al, 1995). These animals showed severe impairment in the healing of skin wounds; thus, plasminogen plays a central role in extracellular matrix degradation during wound healing in rodents in vivo and most likely also in humans (Rømer et al, 1996). Fibrin dissolution allows keratinocyte migration from incisional wound edges; detriment in fibrin degradation slows down wound repair by the limited ability of epidermis cells to dissect their way through the extracellular matrix beneath the wound crust (Rømer et al, 1996). Fibrin is a major component of the extracellular matrix in wounds and solid tumors but not in normal embryonic or adult tissue.

G. TGF-β in injury and wound healing

Transforming growth factor-β (TGF-β) is a cytokine implicated in the pathogenesis of impaired wound healing but also in autoimmune disease, malignancy, and atherosclerosis (Grainger et al, 1995). TGF-β has gained interest for the treatment of autoimmune disease, multiple sclerosis (autoimmune encephalomyelitis), and arthritis; however, prolonged overproduction of TGF-β may lead to tissue fibrosis by overproduction of extracellular cell matrix affecting kidney, liver, lung and other organs. TGF-β1 is involved in the pathogenesis of fibrosis by its matrix-inducing effects on stromal cells such as in activation of the pulmonary fibrotic process (Sime et al, 1997). Overexpression of TGF-β1 in the heart is thought to contribute to the development of cardiac hypertrophy and fibrosis (Villarreal et al, 1996). TGF-β can activate and then suppress T cells, macrophages, and leukocytes; transgenic mice with targeted disruption of the *TGF*-β gene die with autoimmune symptoms (reviewed by Border and Noble, 1995).

The preservation and architectural design of the extracellular matrix depends on the action of cytokine polypeptides. TGF-β controls the mitogenic action of platelet-derived growth factor (PDGF); in response to injury or disease, the production of TGF-β and PDGF are increased stimulating extracellular matrix production; this is accomplished by inhibition of proteases and stimulation of synthesis of extracellular matrix proteins by TGF-β. Failure of cells to produce enough TGF-β has been proposed to result in impaired wound healing in the elderly, glucocorticoid-treated individuals, and in diabetics; a single injection of TGF-β has been shown to accelerate wound healing (reviewed by Border and Noble, 1995).

TGF-β inhibits epithelial and smooth cell proliferation; it is believed that one of the factors contributing to the unrestricted growth of cancer cells is their nonresponsiveness to TGF-β because of loss of functional TGF-β receptors; microsatellite instability in colon cancer cells inactivates the type II TGF-β receptors (Markowitz et al, 1995). Tamoxifen, an anticancer drug, stimulates TGF-β thus inhibiting cancer cell proliferation. Restoration of the TGF-β receptor genes might constitute

a strategy for treating human cancers (Border and Noble, 1995).

Transfer of the cDNA of porcine TGF-β1 to rat lung induced prolonged and severe interstitial and pleural fibrosis characterized by extensive deposition of the extracellular matrix (ECM) proteins collagen, fibronectin, and elastin (Sime et al, 1997). Particle-mediated delivery of mutant porcine constitutively active TGF-β1 cDNA to rat skin at the site of skin incisions increased tensile strength up to 80% for 14-21 days (Benn et al, 1996). Neurodegeneration associated with Alzheimer's disease is believed to involve toxicity to β-amyloid and related peptides; this neurotoxicity was significantly attenuated by single treatments with TGF-β1 and was prevented by repetitive treatments, a process associated with a preservation of mitochondrial potential and function (Prehn et al, 1996); this implies a potential avenue of TGF-β1 gene transfer for Alzheimer's disease.

Transfer of TGF-β1 cDNA in vivo suppresses local T cell immunity and prolonged cardiac allograft survival in mice; TGF-β1 gene transfer may become a new type of immunosuppressant avoiding the systemic toxicity of conventional immunosuppression (Qin et al, 1996).

GM-CSF overexpression induced TGF-β1 gene expression and secretion from macrophages purified from bronchoalveolar lavage fluid 7 days after GM-CSF gene transfer; these findings implicate GM-CSF in pulmonary fibrogenesis (Xing et al, 1997).

XXXIII. Cystic fibrosis (CF)

A. Molecular mechanism of CF pathogenesis

CF is a lethal recessive hereditary disorder characterized by abnormalities of the airway epithelium; it affects 1 in 2,000 Caucasians. Inflicted individuals show secretion of thick mucus and chronic colonization of the lung epithelium with pathogens such as *Pseudomonas aeruginosa*. The defect arises from mutations in the 250-kb gene encoding a 12-transmembrane domain glycoprotein (1480 amino acids), called cystic fibrosis transmembrane conductance regulator (CFTR), that modulates the permeability of Cl^- in response to elevation of intracellular cAMP. The defect is caused by deletion of three base pairs eliminating a single phenylalanine residue at the center of the first nucleotide-binding domain of the CFTR protein (Riordan et al, 1989).

Much of the mortality seen in CF is related to chronic infection of the respiratory tract with *Pseudomonas aeruginosa; Pseudomonas* colonization has been attributed to increased numbers of specific cell-surface receptors and to the presence of mucus. Adherence of *P. aeruginosa* directly to the cell surface of CF airway epithelium (from noncultured nasal epithelial cells isolated from CF patients) was significantly increased over that in cells from healthy donors. Liposome-mediated CFTR gene transfer resulted in a significant reduction in the numbers of bacteria bound to ciliated CF epithelial cells (Davies et al, 1997).

The architecture of the lung and the terminal differentiation of the defective cells imposes a serious hurdle for ex vivo gene therapy for CF: the epithelial cells on the airway surface cover the successively branching structures of the lung making impossible their removal and reimplantation (Yoshimura et al, 1992).

Successful introduction of the entire 250 kb human CFTR gene locus and adjacent sequences into Chinese hamster ovary-K1 (CHO) cells which lack endogenous CFTR was achieved using yeast artificial chromosomes (YACs); integration of the human CFTR-containing YACs into the CHO genome took place on the order of one copy per genome; functional human CFTR was expressed from subclones and human CFTR expression in CHO cells was unexpectedly high (Mogayzel et al, 1997). This type of studies are very useful as a number of DNA control elements for CFTR may be “hidden” throughout the gene locus, including enhancers, ORIs, silencers, MARs, that participate in the tissue- and developmental stage-specific CFTR gene expression.

The airway epithelium is in the process of injury and regeneration in CF; regenerating poorly differentiated cells of human airway epithelium in culture were efficiently transfected with CFTR cDNA using adenoviral vectors; CFTR expression and cAMP-regulated stimulation of the cell membrane chloride ion secretion took place on these cells as determined by light fluorescence microscopy and scanning laser confocal microscopy (Dupuit et al, 1997).

B. CFTR gene transfer in animal models

Direct transfer of the human *CFTR* gene was achieved using a replication-defective adenovirus vector by intratracheal instillation into cotton rat lungs; the presence of human CFTR mRNA transcripts was detected by in situ hybridization with a cRNA (antisense) probe as well as by immunohistochemical evaluation using antibodies directed against the CFTR protein (Rosenfeld et al, 1992).

First generation adenovirus-mediated gene transfer of CFTR to the mouse lung resulted in the expression of viral proteins leading to the elimination of the therapeutic cells expressing CFTR by cellular immune responses; second generation E1-deleted viruses displayed substantially longer recombinant gene expression and induced a lower inflammatory response (Yang et al, 1994).

Adenoviral vector constructs with an E1-E3+E4ORF6+ backbone encoding CFTR (or β-galactosidase) produced declining levels of expression while a similar vector with an E1-E3+E4+ backbone gave rise to sustained, long-term reporter gene expression in the lung in nude mice; CTLs directed against either adenoviral proteins or β-galactosidase reduced expression in nude mice stably expressing β-galactosidase from the E4+ vector (Kaplan et al, 1997).

Aerosol delivery of an adenoviral vector encoding CFTR to non-human primates showed human CFTR mRNA in lung tissue from all treated animals on days 3, 7, and 21 post-exposure; other than some rather mild complications on individual animals ranging from an increase in lavage lymphocyte numbers to bronchointerstitial pneumonia, the treatment was rather safe (McDonald et al, 1997).

Adenoviruses elicit am immune response. Effective gene therapy for CF would ideally be accomplished with a vector capable of long-term expression of the CFTR in the absence of a host inflammatory response; in this respect AAV might be better suited. Administration of single doses of AAV-CFTR vector to 10 rhesus macaques by fiberoptic bronchoscopy to the right lower lobe of lungs showed that transfer of the CFTR gene occurred in bronchial epithelial cells of each animal by in situ DNA PCR; vector mRNA was detectable for 180 days after administration as detected by RT-PCR and there was no evidence of inflammation (Conrad et al, 1996).

Transfer of CFTR gene was also achieved using retroviral vectors; sodium butyrate treatment of murine retrovirus packaging cells producing the vector increased the production of the retrovirus vector between 40- and 1,000-fold (Olsen and Sechelski, 1995).

Yoshimura and coworkers (1992) have transduced airway epithelial cells in mice by intratracheal instillation of a plasmid carrying the *CFTR* gene under control of the Rous sarcoma virus promoter with cationic liposomes. Use of liposomes have successfully transferred the *CFTR* gene to epithelia and to alveoli deep in the lung leading to correction of the ion conductance defects found in the trachea of transgenic mice (Hyde et al, 1993).

Complexes of cationic polymers and cationic lipids with adenovirus enhanced gene transfer to the nasal epithelium of cystic fibrosis mice in vivo (Fasbender et al, 1997). The novel cationic lipid EDMPC (1,2-dimyristoylsn-glycero-3-ethylphosphocholine, chloride salt) mediated efficient intralobar DNA delivery of CFTR to rodents; there was no correlation between DNA-EDMPC formulations that delivered DNA most efficiently in vitro and those that worked best in vivo (Gorman et al, 1997). The structures of several novel cationic lipids that were effective for CFTR gene delivery to the lungs of mice were investigated; an amphiphile (lipid #67) consisting of a cholesterol anchor linked to a spermine head-group in a "T-shape" configuration was shown to sustain a 1,000-fold increase in expression above that obtained in animals instilled with naked pDNA alone and was greater than 100-fold more active than other cationic lipids and comparable to that of adenoviral vectors (Lee et al, 1996).

C. Clinical trials on cystic fibrosis patients

A significant number of clinical trials on CF have received RAC approval (**Appendix 1** and **Table 4** in Martin and Boulikas, following article). The clinical protocols approved use adenoviral delivery of CFTR (#118-123, 125, 128, 129) or cationic lipids (#193, 203, 212, and 214). The only two protocols using AAV in clinical trials are for CF (#165, 166) whereas no retroviral protocol has been approved for CF as of December 1997.

Results of clinical trials have been reported and more will become available in the near future. A single dose of 400 μg pCMV-CFTR in complex with 2.4 mg DOTAP, administered to the nasal epithelium of eight CF patients resulted in partial, sustained correction of CFTR-related functional changes toward normal values in two treated patients; transgene DNA was detected in seven of eight treated patients for up to 28 days after treatment; vector derived CFTR mRNA was detected in two of the seven patients at 3 and 7 days from treatment using PCR (Porteous et al, 1997). Complexes of plasmids with DOTAP liposomes rendered their DNA resistant to DNaseI something relevant to clinical trials for gene therapy of CF, in which patients are normally removed from treatment with DNase before receiving administration of DNA (Crook et al, 1997).

A formulation of plasmid encoding CFTR (pCF1-CFTR) was at least as effective as complexes of DNA with lipid in partially correcting the Cl- transport defect in CF patients by administering complexes of DNA-lipid to one nostril and DNA alone to the other nostril in a randomized, double-blind study (Zabner et al, 1997).

The safety of the nebulised cationic lipid formulation (GL-67/DOPE/DMPE-PEG5000) to be used for the transfer of CFTR to CF patients was first tested on 15 healthy volunteers in the absence of plasmid DNA; no adverse clinical events were seen (Chadwick et al, 1997).

XXXIV. Rheumatoid arthritis (RA)

A. Molecular mechanisms for development of RA

RA is a systemic autoimmune disease caused by genetic and environmental factors; chronic inflammation and hyperactivation of synovial cells in the joints is the salient feature of RA resulting in the thickening (hyperplasia) of the synovial membrane lining the interior surface of the joint capsule. The mechanism involves synovial cell proliferation, infiltration by leukocytes, and excessive extracellular cell matrix deposition. The activated synovial cells in the hypertrophied synovium produce inflammatory cytokines and degradative enzymes that invade and erode the articular cartilage leading to partial or complete destruction of cartilage and bones. The inflamed synovium in RA is infiltrated by lymphocytes and monocytes, a process mediated by the enhanced binding of the very late antigen-4 (VLA-4) to vascular cell adhesion molecule-1 (VCAM-1) (Chen et al, 1995).

Several well-characterized murine models of arthritis closely resemble RA immunologically, genetically, and histopathologically and have been developed to study RA. Collagen-induced arthritis in DBA/1 mice is one model of

RA; the animals exhibit marked synovitis and erosions. The disease can be adoptively transferred to SCID mice using arthritogenic splenocytes from DBA/1 mice injected with bovine collagen type II (Chernajovsky et al, 1997).

Bacterial cell wall-induced arthritis in rats is another model (Makarov et al, 1996).

B. Approaches to gene therapy of RA

The current emphasis for RA gene therapy is on transferring genes encoding secreted proteins which possess antiarthritic properties. Genes may be delivered locally to individual diseased joints or systemically to extra-articular sites where the secreted gene products may enter the circulation. Gene transfer to the synovium would ensure local production of anti-inflammatory gene products directly in the articular space where they could exert a down-regulatory effect on the autoimmune process. Although adenoviral delivery appeared best suited for gene delivery to synovium, induction of an inflammatory response resulting in loss of gene expression may take place (Evans and Robbins, 1996).

High efficiency lacZ gene transfer and expression was achieved in both type A and type B synoviocytes throughout the articular and periarticular synovium of the rabbit knee by Roessler et al (1993). Intra-articular administration of an E1a-E3-deleted adenoviral (Ad5) vector expressing the lacZ transgene into mouse joints showed lacZ expression in the articular synovium for at least 14 days. However, a gradual loss of transgene expression was caused by a predominantly neutrophilic, inflammatory response. Pretreatment with the anti-T cell receptor monoclonal antibody (mAb) H57 resulted in a significant reduction in lymphocytic infiltration and in persistence of transgene expression. Thus, anti-T cell mAbs may be useful in inhibiting adenovirus-induced immune responses that lead to the loss of therapeutically transduced cells (Sawchuk et al, 1996).

Many new therapeutic approaches are currently being developed, including the use of soluble receptors to IL-1 or TNF, monoclonal antibodies to TNF-α, and a specific IL-1 receptor antagonist. A number of studies have assessed the impact of gene transfer on inflammatory and chondrodestructive effects during the acute phase of antigen-induced arthritis in RA joints. A promising therapy for RA involves delivery of the TNF-α and IL-1 proteins to the joints to inhibit the activity of proinflammatory cytokines (Bandara et al, 1993; Arend and Dayer, 1995).

Angiogenesis is not only essential for the growth and metastatic spread of solid tumors but in diseases such as rheumatoid arthritis, psoriasis, liver cirrhosis and diabetic retinopathy (Norrby, 1997). Future approaches for the gene therapy of RA may thus include anti-angiogenesis approaches to the inflamed joints.

C. Ex vivo gene therapy of RA using IL-1Ra-transduced cells

Degradation of cartilage in RA in vitro is stimulated by IL-1, a proinflammatory cytokine, which is released from RA synovial fibroblasts (**RA-SF**). Synovial cells were surgically removed from joints of animals with experimental arthritis, cultured and transduced with the naturally occurring inhibitor of IL-1, IL-1-receptor antagonist (**IL-1Ra**) protein gene and reimplanted into the respective donors by intra-articular injection (Bandara et al, 1993). Retroviral transfer of the IL-1Ra gene to RA-SF which were then coimplanted with normal human cartilage in SCID mice protected the cartilage from chondrocyte-mediated degradation; the IL-1Ra-transduced RA-SF continued to secrete IL-1Ra over a 60-day period (Muller-Ladner et al, 1997a,b). Transfer the human IL-1Ra gene to rabbits' knees produced a marked chondroprotective effect although the anti-inflammatory effect was milder (Otani et al, 1996).

Ex vivo retroviral delivery of the secreted human IL-1Ra cDNA to primary synoviocytes followed by engraftment in ankle joints of rats with recurrent bacterial cell wall-induced arthritis significantly suppressed the severity of recurrence of arthritis as assessed by measuring joint swelling and by the gross-observation score; this ex vivo approach attenuated but did not abolish erosion of cartilage and bone; the level of locally expressed IL-1Ra was about four orders of magnitude higher than that attained from systemically administered recombinant IL-1Ra protein (Makarov et al, 1996). These findings provide experimental evidence for the feasibility of antiinflammatory gene therapy for arthritis.

Retroviral transduction of hematopoietic stem cells with human IL-1Ra cDNA was also used for the treatment of RA; HSCs were subsequently injected into lethally irradiated mice; all of the mice survived and over 98% of the white blood cells in these mice were arising from the transduced HSCs (donor type) from 2-13 months after transplantation; the animals had the human IL-1Ra protein in their sera for at least 15 months. These results demonstrated that systemic production of biologically active human IL-1Ra can be obtained by retrovirus-mediated gene transfer to hematopoietic stem cells which could be useful in the treatment of chronic diseases such as rheumatoid arthritis as well as bone degeneration caused by aging (Boggs et al, 1995).

Characterization of the interleukin-1/interleukin-1 receptor antagonist pathways in RA resulted in the first gene therapy trial in animals and humans for RA (Evans et al, 1996; reviewed by Evans and Robbins, 1996; Muller-Ladner et al, 1997a). Protocol #56 (page 162) involves removal of autologous synovial cells from the patient, their retroviral transduction with the IL-1Ra cDNA followed by injection of the transduced cells into the metacarpal phalangeal joints of RA patients.

Infection of arthritogenic splenocytes from DBA/1 mice transferred to SCID mice with a recombinant retrovirus carrying the **TGF-β1** gene was effective in lowering inflammation of joints with already established arthritis and inhibiting the spreading of the disease to other joints in mice (Chernajovsky et al, 1997).

D. Direct gene delivery for RA

A retroviral vector based on a murine leukemia virus was used to deliver the human growth hormone and lacZ genes to the synovium of the rabbit knee to test the efficacy of gene transfer and to develop an approach for the gene therapy of RA (Ghivizzani et al, 1997).

An effective treatment of arthritis is via eliminating most or all of the activated synovial cells. The death factor Fas/Apo-1 and its ligand (**FasL**) play pivotal roles in maintaining self-tolerance and immune privilege; Fas is expressed constitutively in most tissues and is dramatically upregulated at the site of inflammation. Unlike Fas, however, the levels of FasL expressed in the arthritic joints are extremely low, and most activated synovial cells survive despite high levels of Fas expression. Delivery of the FasL gene via a replication-defective adenovirus by injection into inflamed joints conferred high levels of FasL expression, induced apoptosis of synovial cells, and ameliorated collagen-induced arthritis in DBA/1 mice (Zhang et al, 1997).

A strategy was developed for inhibiting T lymphocyte retention and activation within the rheumatoid synovium (Chen et al, 1995). The inflamed synovium in RA is infiltrated by lymphocytes and monocytes, a process mediated by the enhanced binding of the very late antigen-4 (VLA-4) to vascular cell adhesion molecule-1 (**VCAM-1**) expressed on microvascular endothelial cells; VLA-4 binding appears to play a role in T cell retention and activation within the inflamed synovial membrane. Therefore, blocking of VLA-4 binding by utilizing a soluble congener of the VCAM-1 molecule might be of therapeutic efficiency for RA. Adenoviral infection of human synoviocytes carrying the cDNA for a secreted form of VCAM-1 (sVCAM-1) showed secretion of transgenic sVCAM-1 by ELISA of tissue culture supernatants. In vivo, transgenic sVCAM-1 expression was determined by immunohistochemical analysis and in situ hybridization of synovial tissue, and secretion of transgenic sVCAM-1 was demonstrated by ELISA of tidal knee lavage fluid. The results showed that recombinant adenovirus can mediate the expression of a biologically active sVCAM-1 by synoviocytes in vivo and suggested that this strategy may be useful for inhibiting T lymphocyte retention and activation within rheumatoid synovium. Ex vivo studies using this strategy have not been reported.

XXXV. Adenosine deaminase (ADA) deficiency and severe combined immunodeficiency (SCID)

Severe combined immunodeficiency is secondary to the deficiency in adenosine deaminase; the enzyme is involved in purine catabolism. The syndrome is characterized by defective B and T cell function caused by the large amounts of deoxyadenosine which is preferentially converted into the toxic compound deoxyadenosine triphosphate in T cells disabling the immune system (see Blaese et al, 1995 and the references cited therein). Affected individuals experience recurrent infections and the disease is usually fatal unless affected children are kept in isolation. One therapeutic approach has been to partially reconstitute the immune system by bone marrow transplantation from a human leukocyte antigen (HLA)-identical sibling donor (Hirschorn et al, 1981). An enzyme replacement therapy consisted of introducing bovine ADA enzyme conjugated with polyethylene glycol (PEG-ADA) in order to increase the circulation time of the ADA enzyme in the blood and other extracellular fluids (Hershfield et al, 1987).

The first person to be treated ex vivo was a 4-year-old suffering with ADA deficiency in 1990. Protocol #2 treating ADA deficiency with autologous lymphocytes transduced ex vivo with the ADA gene was the second RAC-approved protocol. Because of this innovative work, the US Patent Office has issued in 1995 a patent covering all *ex vivo* gene therapy to French Anderson, Steven Rosenberg, and Michael Blaese.

From 1990-1992, a clinical trial was initiated using retrovirus mediated transfer of the 1.5 kb ADA gene cDNA to T cells from two children with severe combined immunodeficiency following multiple transplantations of *ex vivo* modified blood cells; the integrated ADA gene was expressed for long periods (Blaese et al, 1995; Bordignon et al, 1995). The success of this ex vivo approach probably arose from that the ADA gene-corrected T cells acquired a survival advantage compared with uncorrected cells when transplanted into immunodeficient but ADA normal BNX mice (Ferrari et al, 1991) and humans (Kohn et al, 1995).

Three neonates with ADA deficiency have been successfully treated later with hematopoietic stem $CD34^+$ cells, isolated from their umbilical cord blood, transduced with the ADA gene under control of the LTR of the MoMuLV using retroviral vectors, followed by autologous transplantation (Kohn et al, 1995).

Ex vivo studies have shown correction of the severe combined immunodeficiency in ADA-deficient mice by transfer of human peripheral blood lymphocytes transduced with a retroviral vector in cell culture carrying the *ADA* gene; the injected human cells survived for long times in mice and restored the immune functions (presence of human immunoglobulin and antigen-specific T cells) (Ferrari et al, 1991). *Ex vivo* correction of the defect in T cells from ADA-deficient patients with retroviral vectors gave to these cells an advantage for cell division against a background of slowly dividing uncorrected T cells after their transplantation (Karlsson, 1991).

XXXVI. Gaucher disease, lysosomal storage disease, and mucopolysaccharidosis VII

Many mutations affecting the glucocerebrosidase gene have been defined as causes of the glycolipid storage disorder, **Gaucher disease**; disease symptoms are a result of macrophage engorgement secondary to this enzyme deficiency. The recombinant from of glucocerebrosidase imiglucerase protein is effective in treating the disease as a replacement therapy (reviewed by Beutler, 1997).

An amphotropic producer cell line that synthesized viral particles carrying a fusion of the selectable MDR1 cDNA encoding P-glycoprotein (P-gp) and the human glucocerebrosidase gene was constructed; complete restoration of glucocerebrosidase deficiency in Gaucher fibroblasts was achieved using this retrovirus; selection of the transduced Gaucher fibroblasts in colchicine (MDR1 function) raised their glucocerebrosidase activity from nearly undetectable to normal levels; combination of much lower concentrations of colchicine and inhibitors of the P-gp pump (verapamil) allowed to select for high-level expression of MDR1 and glucocerebrosidase; this regimen, in clinical use for the treatment of multidrug-resistant malignancies, may find application for high level selection of a nonselectable gene such as glucocerebrosidase (Aran et al, 1996; see also Migita et al, 1995).

Allogenic bone marrow transplantation (Parkman, 1986) or intravenous infusion of glucocerebrosidase (enzyme replacement therapy) in a patient with Gaucher's disease (Barton et al, 1990), although has partially corrected deficiencies in lysosomal enzymes, was not amenable to brain cells because of the brain barrier and cannot alleviate symptoms in the central nervous system. Protocols #38, 39, and 51 use glucocerebrosidase cDNA to transduce $CD34^+$ autologous peripheral blood cells followed by intravenous injection of the transduced cells into patients with Gaucher's disease. $CD34^+$ cells obtained from G-CSF mobilized peripheral blood stem cells or from bone marrow (#51) are being transduced ex vivo and reinfused into the patient at the National Institutes of Health and Children's Hospital of Los Angeles (Dunbar and Kohn, 1996).

Type II Glycogen Storage Disease, a deficiency of acid α-glucosidase (GAA), results in the abnormal accumulation of glycogen in skeletal and cardiac muscle lysosomes; this can have devastating effects ultimately leading to death; the wild-type enzyme was produced in deficient myoblasts after gene transfer with a retroviral vector carrying the cDNA for GAA. The transduced cells secreted GAA that was endocytosed via the mannose-6-phosphate receptor into lysosomes of deficient cells and digested glycogen; thus, the transduced cells provided phenotypic correction to distant cells in the culture by secretion. **Figure 33** shows that the GAA-transduced myoblasts (red fluorescence) were able to fuse with deficient myoblasts (green fluorescence) and provide enzyme activity mediating phenotypic correction to neighboring GAA-deficient cells (Zaretsky et al, 1997).

Aspartylglucosaminuria (AGU, appearance of aspartylglucosamine in the urine) is the only known human disease caused by an amidase deficiency, in this case by deficiency of the enzyme aspartylglucosaminidase (AGA) in virtually all cell types of patients; AGA deficiency fails to perform the final breakdown of asparagine-linked glycoproteins leading to the intralysosomal accumulation of uncleaved glycoasparagines and to their abnormal urinary excretion. AGU patients display progressive psychomotor retardation starting in early childhood. The most common mutation in the Finnish population responsible for AGA deficiency is a point mutation resulting in the amino acid substitution Cys-163 to Ser in the *AGA* gene (Ikonen et al, 1991).

Retrovirus-mediated gene transfer was successfully used to correct the *AGA* gene in cultured human primary fibroblasts and lymphoblasts from AGU patients as a prelude to ex vivo human gene therapy; enzyme correction

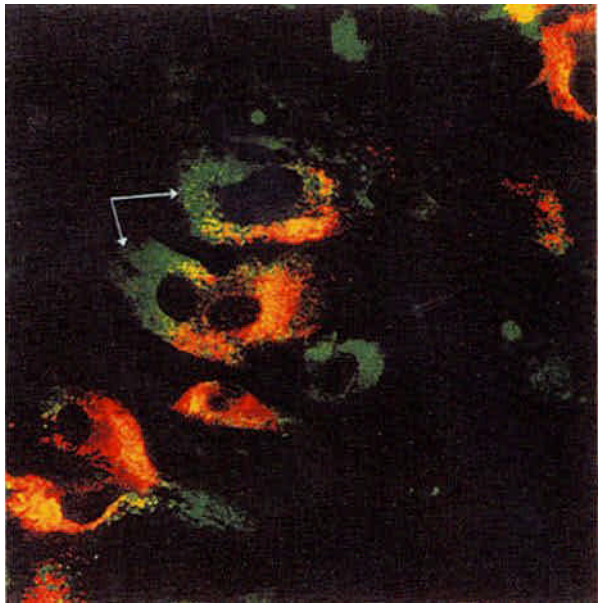


Figure 33. Immunofluorescent detection of in vitro fusion of α-glucosidase (GAA)-deficient muscle cells with myoblasts transduced with the GAA gene using a retroviral vector. The GAA-transduced myoblasts (red fluorescence) were able to fuse (arrows) with deficient myoblasts (green fluorescence) and provide enzyme activity. From Zaretsky JZ, Candotti F, Boerkoel C, Adams EM, Yewdell JW, Blaese RM, Plotz PH (**1997**) Retroviral transfer of acid α-glucosidase cDNA to enzyme-deficient myoblasts results in phenotypic spread of the genotypic correction by both secretion and fusion. **Hum Gene Ther** 8, 1555-1563. Reproduced with the kind permission of the authors and Mary Ann Liebert, Inc.

further took place by cell-to-cell interaction between transduced and nontransduced cells in culture suggesting that only partial cell transduction might be sufficient to correct AGA deficiency in vivo (Enomaa et al, 1995).

Mucopolysaccharidosis type VII (Sly syndrome or MPS VII) is a result of an inherited deficiency of β-glucuronidase in humans, mice, and dogs (see Wolfe et al, 1992 for references). The symptoms are progressive degeneration in several tissues resulting from storage in lysosomes of undegraded glycosaminoglycans affecting the spleen, liver, brain, cornea, kidney, and skeletal muscle. Affected individuals display a reduced life-span which is 5 months in mice. Retroviral vectors have successfully treated mucopolysaccharidosis VII by somatic cell gene transfer in mouse models (Wolfe et al, 1992) or by implantation of ex vivo modified mouse skin fibroblasts (Moullier et al, 1993).

Retrovirus-mediated transfer of the iduronate-2-sulfatase cDNA into lymphocytes is being applied for the clinical treatment of mild Hunter syndrome (mucopolysaccharidosis type II, protocol #65 on page 163).

XXXVII. Hereditary α1-antitrypsin (α1-AT) deficiency

Destruction of components of the extracellular matrix of the lung by neutrophil elastase (**NE**) is believed to be a critical event in the development of obstructive lung disease. α1-antitrypsin (also known as α1-proteinase inhibitor) is an antiprotease that protects the lung from destruction by the powerful protease NE. The hereditary α1-antitrypsin deficiency is caused by mutations in the coding region of the 12.2 kb-gene resulting in decreased serum and lung levels of α1-antitrypsin; affected individuals develop emphysema at age 30-40 (Crystal, 1990). Lung-derived epithelial cells have the capacity to synthesize functional α1-antitrypsin but also to increase the rate of its production when stimulated by specific inflammatory mediators, including oncostatin M, IL-1, and dexamethasone (Cichy et al, 1997).

The respiratory epithelium has been a potential site for somatic gene therapy because of the possibility of direct delivery of a functional gene by tracheal instillation. A drawback for retrovirus-mediated gene transfer arises from that the majority of alveolar and airway epithelial cells are terminally differentiated and only a small fraction of these cells are proliferating and amenable to recombinant retrovirus infection; however, lung epithelial cells are prone to adenovirus infections because host cell replication is not required for expression of adenoviral proteins (see Rosenfeld et al, 1991).

The adenovirus major late promoter was linked to a human α1-antitrypsin gene for its transfer to lung epithelia of cotton rat respiratory pathway as a model for the treatment of α1-antitrypsin deficiency; the cotton rat is an animal commonly used to evaluate the pathogenesis of adenoviral respiratory tract infections. Both i*n vitro* and *in vivo* infections have shown production and secretion of α1-antitrypsin by the lung cells: cells, removed by brushing the epithelial surface of the tracheobronchial tree from the lungs of cotton rats demonstrated the possibility of their infection in culture and secretion of the therapeutic protein; following direct infection of the animals *in vivo*, demonstrated that the protein was synthesized and secreted in the epithelial lining fluid of the lung for over 1 week (Rosenfeld et al, 1991).

i.v. administration of a full-length cDNA encoding human α1-antitrypsin (100 ng/mouse) encapsulated in small liposomes resulted in expression in liver parenchymal cells as shown on immunohistochemical liver sections; this effect remained for at least 2 weeks and some enzyme could be detected in plasma (Aliño et al, 1996). The human α1-antitrypsin gene was transferred to lungs of rabbits; immunohistochemical staining showed α1-AT protein in the pulmonary endothelium following intravenous administration, in alveolar epithelial cells following aerosol administration, and in the airway epithelium by either route (Canonico et al, 1994). A recombinant adenovirus vector was also used for α1-AT cDNA transfer (Gilardi et al, 1990). α1-AT cDNA in an adenoviral vector was administered by retrograde ductal instillation to the submandibular glands of male rats; transient expression took place in salivary glands (Kagami et al, 1996).

Guo et al (1996) have evaluated the transcriptional activities of 5 viral and cellular enhancer/promoter elements, showing either high-level or hepatocyte-specific expression following transient transfection into hepatoma cells using recombinant adenoviruses expressing human α1-antitrypsin; the human elongation factor 1α gene promoter produced 2 μg/ml serum level of human α1-antitrypsin, which is physiologic in humans and will be therapeutic for patients with α1-antitrypsin deficiency.

XXXVIII. Approaches to the gene therapy of Parkinson's disease (PD)

A. Etiology and mechanisms of destruction of neurons in PD

First described by James Parkinson in 1817 this neurodegenerative disorder is characterized by resting tremor, postural instability and bradykinesia (slow movement); surviving neurons display intracytoplasmic inclusions known as Lewy bodies. PD symptoms ensue when the pars compacta region of the substantia nigra (black substance) at the base of the brain loses neurons that normally issue motion-controlling signals (dopamine) to the striatum (divided into caudate nucleus and putamen). The death of neurons is believed to be caused by oxygen free radical damage; brain contains unusually low levels of antioxidants. This damage might be caused by a decline in the activity of the mitochondrial complex I.

PD can be induced in experimental animals by selective destruction of the dopaminergic neurons of the substantia nigra by the neurotoxic drug 1-methyl-4-phenyl-

1,2,3,6-tetrahydropyridine (MPTP) through inhibition of complex I of the mitochondrial respiratory chain (see Polymeropoulos et al, 1996 and the references cited therein). MPTP was found as an impurity in heroin and explained some earlier observations of addicts who became almost completely immobile after making use of the drug, a symptom characteristic of severe PD. MPTP crosses the brain-blood barrier and is converted by mitochondrial monoamine oxidase B into a reactive molecule that inhibits the complex I enzyme resulting in energy deficit and increase in free radicals in the cell (reviewed by Youdim and Riederer, 1997).

There is substantial evidence which implicates immune mechanisms in the destruction of neurons. The substantia nigra of Parkinson's patients contains active microglia which, after stimulation by cytokines, could produce the free radical nitric oxide which can penetrate the cell membrane of vicinal neurons, inhibit the complex I mitochondrial enzyme and activate signal transduction pathways. Furthermore, NO with superoxide, emitted by hyperactive microglia, can free iron ions from intracellular stores which can oxidize dopamine into neuromelanin, a molecule that acts as an oxidant when complexed with transition metals. These oxidative stress mechanisms could trigger apoptosis in neurons. Excessive release of the neurotransmitter glutamate (known to occur in stroke) into the striatum and substantia nigra could induce a similar cascade of NO and free radical damage. These mechanisms suggest that excessive stressful conditions in predisposed individuals might precipitate the onset of PD symptoms.

B. Drug treatment of PD

The first medicament in the mid-1900s included extracts of the deadly nightshade plant which inhibited the activity of acetylcholine in the striatum; acetylcholine overexcites striatal neurons that projected to higher motor regions of the brain, an effect normally counteracted by dopamine. Later in 1960s L-DOPA, which is converted into dopamine, proved valuable for treatment of PD patients; dopamine itself cannot cross the blood-brain barrier (a network of specialized blood vessels that control which substances are allowed to pass from the blood into the central nervous system). Drugs that mimic the actions of dopamine (agonists) have also been used. Selegiline (also called deprenyl), an inhibitor of monoamine oxidase B, the enzyme that breaks down dopamine in the astrocytes and microglia, is of therapeutic potential (reviewed by Youdim and Riederer, 1997).

Amantadine is used to block the effects of glutamate in substantia nigra. Antioxidants able to cross the brain-blood barrier could have a protective effect on the destruction of neurons. Unfortunately, the first indications show that vitamin E in the low doses tested, which can cross to some extent the brain-blood barrier, is ineffective; however, the effect of higher doses of vitamin E need to be investigated. The efficacy of glial-derived neurotrophic factor (**GDNF**) injected into the brain of PD patients is in trials. Rasagiline, which could activate neuronal growth factors in the brain is under investigation on humans. Also in clinical trials are strategies of direct implantation of dopamine-producing cells into the brain of patients (Youdim and Riederer, 1997).

Ex vivo and in vivo gene therapy strategies for PD have a promising future (see below).

C. Candidate genes for PD

A susceptibility gene for PD has been mapped to chromosome 4q21-q23 by genotyping genomic DNA from a large family in Contursi in the Salemo province of Southern Italy where 60 individuals out of 592 members are affected by PD at an average age of 46. A total of 140 genetic markers were typed in the pedigree and only those associated with this chromosomal region were altered showing recombination events in PD patients in this family; this type of recombination does not involve expansions of the CAG trinucleotide repeat (Polymeropoulos et al, 1996).

The neurologic abnormalities associated with PD were thought to result from a severe reduction in L-DOPA as a consequence of degeneration of dopaminergic neurons of the nigrostriatal pathway. L-DOPA is synthesized from tyrosine by the enzyme tyrosine hydroxylase (**TH**); L-DOPA is then converted into the neurotransmitter dopamine by a decarboxylase. Besides TH, other genes whose malfunction has been linked to PD include glutathione peroxidase, a brain-derived neurotrophic factor, catalase, amyloid precursor protein, Cu/Zn superoxide dismutase, and debrisoquine 4-hydroxylase; however, none of these candidate genes are found in the 4q21-q23 region to be linked as etiologic agents of PD; instead, candidate genes in the 4q21-q23 region include alcohol dehydrogenase, formaldehyde dehydrogenase, synuclein, and UDP-N-acetylglycosamine phosphotransferase (Polymeropoulos et al, 1996).

A mutation was identified in the α-synuclein gene, which codes for a presynaptic protein thought to be involved in neuronal plasticity, in the Italian kindred with autosomal dominant inheritance for the PD phenotype (Polymeropoulos et al, 1997). The missense mutation in the α-synuclein gene suggested that at least some fraction of familial PD with diffuse Lewy bodies is the result of an abnormal protein that interferes with normal protein degradation leading to the development of inclusions and ultimately neuronal cell death. Furthermore, a peptide fragment of α-synuclein is known to be a constituent of Alzheimer's disease plaques; there may be common pathogenetic mechanisms involved in α-synuclein mutations in PD and β-amyloid and presenilin gene mutations in Alzheimer's disease (Nussbaum and Polymeropoulos, 1997).

D. Gene and cell therapy for PD

1. Grafting of dopamine neurons

Transplantation of human embryonic dopamine neurons have been performed on patients with Parkinson's disease but the amelioration of the symptoms is transient; death of therapeutic cells was thought to arise from hypoxia, oxidative stress, and trauma during preparation and grafting of the cells. Grafting of dopamine neurons into transgenic mice overexpressing the Cu/Zn superoxide dismutase increased 4-fold the survival of the transplanted cells providing a direct support to the free radical-mediated death of dopaminergic neurons in brain tissue grafts (Nakao et al, 1995).

Cells transduced with tyrosine hydroxylase and GTP cyclohydrolase I were grafted alone or in combination with cells transduced with aromatic L-amino acid decarboxylase into the 6-hydroxydopamine-denervated rat striatum; it was concluded that there is sufficient aromatic L-amino acid decarboxylase near striatal grafts producing L-DOPA and that the close proximity of L-amino acid decarboxylase to TH-producing cells is detrimental for optimal dopamine production (Wachtel et al, 1997).

2. Tyrosine hydroxylase (TH)

Since adult brain cells are nonproliferative, they are refractory to retroviral infection that could deliver the *TH* gene to the brain to alleviate degeneration at the nigrostriatal pathway. Gene therapy of PD has been approached ex vivo using PD animal models with TH deficiency. Unilateral destruction of dopaminergic nigrostriatal neurons in PD animal models with 6-hydroxydopamine and administration of apomorphine causes PD rats to turn contralaterally (7-15 rotations/min).

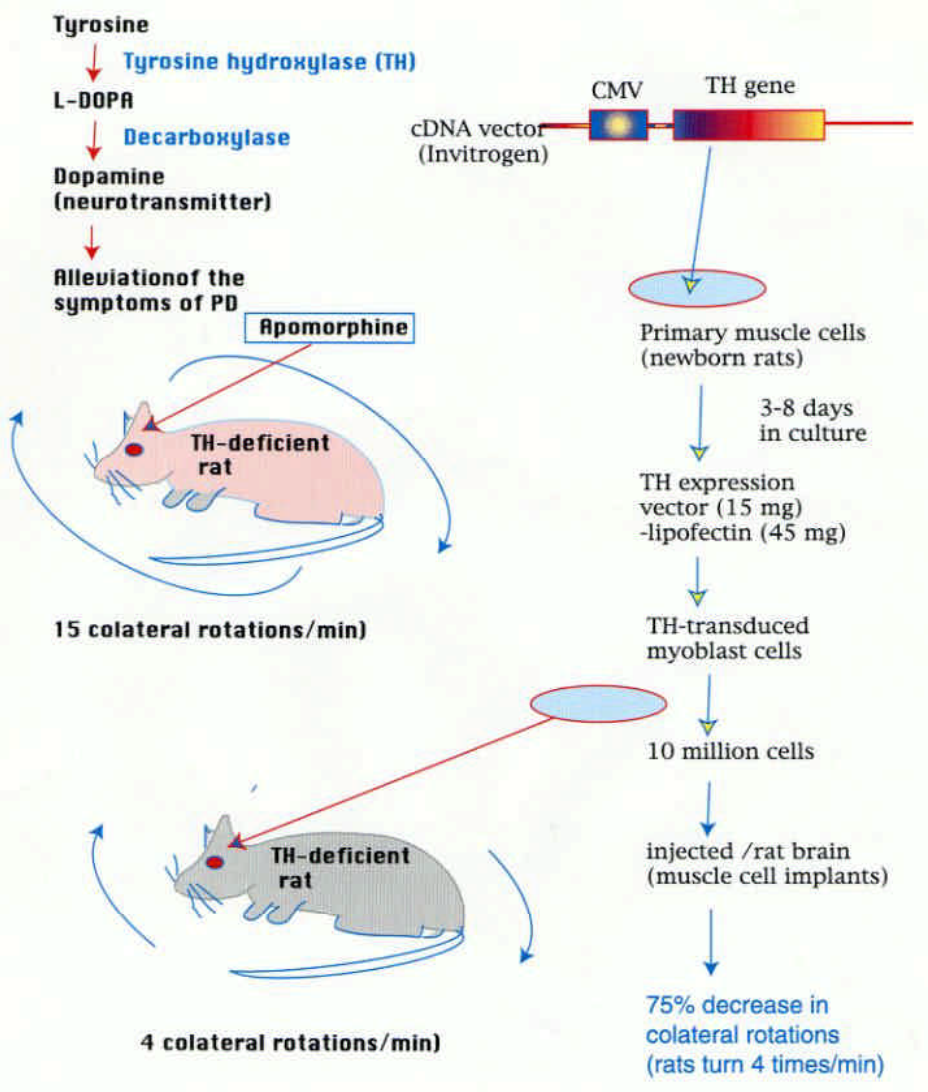


Figure 34. Transfer of the tyrosine hydroxylase gene to primary muscle cells followed by transplantation of these cells to brains of TH-deficient rats has alleviated the number of collateral rotations of the animals which are models for Parkinson's disease. Adapted from Jiao et al, 1993. Reproduced from Boulikas T (**1996b**) Gene therapy to human diseases: ex vivo and in vivo studies. **Int J Oncol** 9, 1239-1251**.** With the kind permission from the International Journal of Oncology.

Implantation of immortalized rat fibroblasts releasing L-dopa into the cell culture medium (Wolff et al, 1989), of primary fibroblasts (Fisher et al, 1991) and myoblasts (Jiao et al, 1993), stably transfected in culture with the *TH* gene, reduced behavioral abnormalities in PD animal models and the number of contralateral rotation dropped to 4 rotations/min (**Figure 34)**.

Direct injection of lipofectin-plasmid DNA complexes containing the *TH* gene under the influence of the SV40 promoter/enhancer (pSVK3 plasmid of Pharmacia) has also shown expression of *TH* into striatal cells compensating for the loss of the intrinsic striatal dopaminergic input reducing quickly and significantly the rotational abnormalities in rat models (Cao et al, 1995).

A different approach has been aimed at converting endogenous striatal cells into L-dopa-producing cells; this was obtained by infection of 6-hydroxydopamine-lesioned rats, used as a model of PD, with a defective herpes simplex virus type 1 vector expressing TH (During et al, 1994).

Recombinant adenovirus are attractive delivery vehicles of genes to alleviate PD symptoms because they can transduce both quiescent and actively dividing cells, thereby allowing both direct in vivo gene transfer and ex vivo gene transfer to neural cells; because the brain is partially protected from the immune system, the expression of adenoviral vectors can persist for several months with little inflammation (reviewed by Horellou and Mallet, 1997).

3. Glial cell line-derived neurotrophic factor

The rat glial cell line-derived neurotrophic factor (rGDNF), a putative central nervous system dopaminergic survival factor, was evaluated for its ability to protect nigral dopaminergic neurons in the progressive Sauer and Oertel 6-hydroxydopamine (6-OHDA) lesion model of Parkinson's disease. Perinigral injections to rats of rGDNF protected a significant number of cells when compared with cell counts of rats injected with a recombinant AAV carrying the lacZ gene (94% vs. 51%, respectively); this treatment gave 85% of tyrosine hydroxylase-positive cells (vs. only 49% in the lacZ group) (Mandel et al, 1997; see also Bohn and Choi-Lundberg, 1998, this volume).

XXXIX. Gene therapy of hemophilia A and B

A. Gene therapy of hemophilia A

Hemophilia A, characterized by hemorrhagic episodes of which the spontaneous intracranial bleeding could result in crippling or death, affects 1 in 10,000 males. It is caused by a deficiency in Factor VIII (FVIII), crucial in blood coagulation, responsible for accelerating activation of factor X by factor IXa in the presence of calcium and phospholipids. Human FVIII is synthesized as a 2351-amino acid precursor protein with a 19- amino acid signal peptide; the 256 kDa single-chain protein composed of the homologous domains A1-A2-B-A3-C1-C2 is processed by proteolysis to a heterodimer composed of a heavy chain (90-200 kDa) and a light chain (80 kDa) which circulates in plasma.

Both the 186 kb gene encoding human FVIII and the 7.2 kb cDNA sequences are known; recombinant FVIII has been expressed from both intact cDNA and a cDNA lacking the B domain; however, the expression of the protein from cell culture was 100-1000 times lower than the expression of other recombinant proteins. This is due to the large size of the protein, its required proteolytic processing, instability of mRNA, abnormal secretion from inefficient transport from the endoplasmic reticulum to the Golgi, and the required N- and O-linked glycosylation for biological activity. Recombinant FVIII is being administered to approximately 50% of the patients. Biologically active FVIII has been produced recently in the milk of transgenic pigs by targeting expression of human FVIII cDNA to the mammary gland of the animals; the expression of the transgene was driven by regulatory sequences from the mouse whey acidic protein gene (Paleyanda et al, 1997).

Infusion of purified factor VIII is the most widely used therapy; however, protein replacement suffers from transfusion-associated complications (AIDS and hepatitis B and C infections); over 50% of the patients treated from 1977 to 1985 were infected with HIV. Improved manufacturing procedures and production of recombinant factor VIII have reduced infectious complications.

Hemophilia A is particularly amenable to gene therapy because even a slight increase in blood plasma of factor VIII can convert a severe form of the disease to a mild form. Application of retrovirus-mediated transfer of factor VIII gene has been hampered by a 100- to 1000-fold reduction in mRNA accumulation and protein production in cells, as well as in retrovirus titer, because of the presence of a secondary structure (arising from inverted repeats) within the DNA coding region of clotting *factor VIII* gene (Lynch et al, 1993; Chuah et al, 1995). Partial solution to this problem has been provided by Chuag and coworkers (1995) who determined that insertion of a 5' intron in the retrovirus vector increased 20-fold gene expression and 40-fold virus titer after transfection of the human T cell line SupT1, human Raji Burkitt B lymphoblastoma and other cell lines.

An more exciting approach has been the use of a B-domain-deleted form of factor VIII; at the protein level, this domain is not required for pro-coagulant activity and retains the thrombin-cleavage sites. Transferrin-mediated transfection of fibroblasts and myoblasts with B-domain-deleted factor VIII gene followed by implantation into mice gave therapeutic levels of factor VIII in the blood of the animals for 24 hours (Zatloukal et al, 1994).

A similar *ex vivo* transfection procedure used retroviral vectors for the introduction of B-domain−deleted factor VIII gene into primary mouse fibroblasts in culture; use of the MFG vector, which utilizes authentic viral splicing

signals and lacks a selectable marker gene was crucial in producing high titer viral stocks (Dwarki et al, 1995). This procedure was followed by surgical implantation into the peritoneal cavity in SCID mice of 15 million cells in the form of neo-organs formed on expanded poly(tetra fluoroethylene) (PTFE) fibers after they were coated with type I collagen from rat tail. Coated fibers were arranged to the bottom of tissue culture dishes and genetically modified cells were allowed to solidify on the PTFE fibers for 1-2 days; the levels of factor VIII obtained were 50-1000 ng/ml which are 10-fold higher than those required for correction of hemophilia A (Dwarki et al, 1995). Efficient delivery of factor VIII was also observed after direct i.v. or i.p. injection but not after i.m. or s.c. injection of genetically-modified cells; this difference might arise from protease levels in the extracellular space of muscle and skin (Dwarki et al, 1995).

The subject is being reviewed in depth by Connelly and Kaleko (1998) and Hoeben (1998) in this volume.

B. Gene therapy of hemophilia B

Hemophilia B is caused by a defect in the blood clotting factor IX (FIX) affecting about 1 in 30,000 males. The therapy consists on administration of factor IX concentrates prepared from human plasma, a fact that led to the infection of hemophiliacs with HIV and hepatitis B virus in the 80s. Current research efforts are focused on the delivery of factor IX gene using ex vivo transduction of primary myoblasts in mice with factor IX gene followed by transplantation of the transduced cells (Dai et al, 1992; Yao et al, 1994). Mouse primary myoblasts were infected with retrovirus expressing the canine factor IX under control of mouse muscle creatine kinase and human CMV promoter; successfully infected myoblasts, selected in the presence of G418, were injected into the hindlegs of recipient mice; secreted canine factor IX was monitored in the plasma. Sustained expression of factor IX for over six months without any apparent adverse effects on the recipient mice was obtained; however, the levels of the factor IX protein secreted into the plasma (10 ng/ml for 10^7 injected cells) were not sufficient to be of therapeutic value but 100 times below the desired levels (Dai et al, 1992).

Dogs, lacking a functional factor IX gene, have been used as animal models for hemophilia B. The liver of the animals is the organ responsible for the production of factor IX; direct infusion of recombinant retroviral vectors, carrying the canine factor IX gene, into the portal vein cannulated into a splenic vein in animals previously subject to two-thirds hepatectomy resulted in the expression of low levels of factor IX for up to about 5 months; about 0.3-1% of hepatocytes were found to be transduced and stained blue with X-Gal in liver sections when the β-galactosidase gene of *E. coli* was delivered with the same retroviral vector (Kay et al, 1993).

A sustained partial correction of the defect was succeeded in hemophilia B dogs by directly delivering the factor IX gene in adenovirus vectors by injection into the splenic veins of 1.6 to 2.2 pfu/Kg adenovirus (Kay et al, 1994). The therapy, however, obtained was transient and although these animals displayed 300% factor IX levels in their blood at day 2 from treatment, the levels dropped to 1% by three weeks and to 0.1% by 2 months (Kay et al, 1994). Since T cell immunity was responsible for attacking and eliminating virally-transduced cells from the body, clearing the corrected cells from the liver of animals, daily administration of 19.5 mg/Kg cyclosporin A led to prolongation of the therapeutic effect and to the persistence of adenovirus-transduced cells (10% of factor IX levels by two months (Fang et al, 1995; **Figure 35**).

Cyclosporin A (CsA) is a cyclic peptide, fungal metabolite, displaying low myelotoxicity but toxic to T cell lymphocytes; it is widely used for immuno-suppression of individuals receiving renal and other organ transplants but also for the therapy of autoimmune diseases. CsA inhibits activation of IL-2 gene, an early event required for T cell activation and this mechanism is thought to govern its immunosuppressive effects. However, CsA also inhibits the activity of TBP required for transcription from the adenovirus major late promoter. Recombinant adenovirus-transduced cells were able to elute host immune surveillance in dogs by cyclosporin A treatment (Fang et al, 1995). Similar conclusions were reached by Dai and coworkers (1995) using adenoviral vectors for delivering the canine factor IX gene into the hind leg muscle of mice: whereas in nude mice a high level of expression of FIX protein was detectable for 300 days, expression of FIX protein lasted for 7-10 days in normal mice. $CD8^+$ lymphocytes were localized in the site of injection; both cell-mediated and humoral immune responses were found to be responsible for eliminating the adenovirus-infected cells from the organism.

Recently, successful transduction of the mouse liver in vivo after a single hepatic gene transfer of F.IX cDNA in an AAV vector was achieved; persistent and curative concentrations of functional human factor IX were detected in the blood of the animals (Snyder et al, 1997). Intramuscular injection of a recombinant AAV vector expressing human factor IX (hF.IX) into hindlimb muscles of C57BL/6 mice and Rag 1 mice demonstrated the presence of hF.IX protein by immunofluorescence staining of muscles harvested 3 months after injection; however, no hF.IX was detected in the plasma of immunocompetent C57BL/6 mice because these animals had developed circulating antibodies to hF.IX. Rag 1 mice on the other hand, which carry a mutation in the recombinase activating gene-1 and thus lack functional B and T cells, displayed therapeutic levels (200-350 ng/ml) of F.IX in the plasma in addition to muscle cells; F.IX levels gradually increased over a period of several weeks before reaching a plateau that was stable 6 months after injection. Furthermore, these studies have demonstrated colocalization of hF.IX and collagen IV in interstitial spaces between muscle fibers; this was explained following identification of collagen IV as a F.IX-binding protein (Herzog et al, 1997).

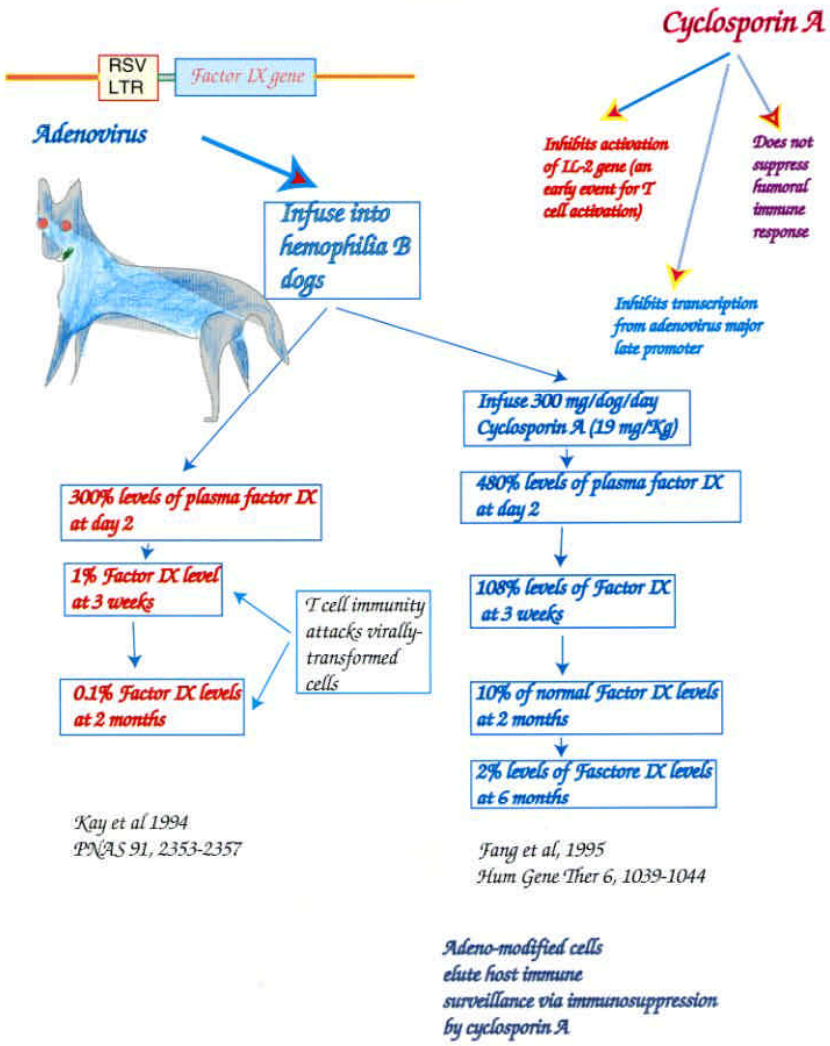


Figure 35. Transfer of the Factor IX gene for the treatment of hemophilia B. The FIX gene under control of RSV LTR in a recombinant adenovirus was infused into the portal vein of hemophiliac dogs (see text for details). Reproduced from Boulikas T (**1996b**) Gene therapy to human diseases: ex vivo and in vivo studies. **Int J Oncol** 9, 1239-1251. With the kind permission from the International Journal of Oncology.

A mouse model for hemophilia B was generated by homologous recombination-mediated disruption of the clotting factor IX gene; the factor IX coagulant activities for wild-type (+/+), heterozygous (+/-), and homozygous (-/-) mice were 92%, 53%, and <5%, respectively. Plasma factor IX activity in the deficient mice (-/-) was restored by introducing wild-type murine FIX gene via adenoviral vectors (Wang L et al, 1997).

Primary skeletal myoblast-mediated gene transfer was tested for achieving a long-term stable systemic production of human factor IX in SCID mice; a hFIX minigene under the control of a β-actin promoter with the muscle creatine kinase enhancers was used; myotubes derived from the myoblasts produced 1,750 ng hFIX/10^6 cells/24 hours in culture; intramuscular injection of 5-20x 10^6 myoblasts to SCID mice stably produced hFIX into the systemic circulation for at least up to 10 months (Wang JM et al, 1997).

XL. Gene therapy of hypertension

A. Molecular mechanisms of high blood pressure

Blood pressure can be altered by mutations in at least 10 genes which alter blood pressure through a common pathway, affecting salt and water reabsorption in the kidney. Probably the most promising lead has involved the genes governing the structure of angiotensinogen, the substrate in the renin reaction. Disorders associated with hypertension are the glucocorticoid-remediable aldosteronism, the syndrome of apparent mineralocorticoid

excess, and Liddle's syndrome. Syndromes linked with hypotension are the pseudohypoaldosteronism type 1, and Bartter's, and Gitelman's syndromes (reviewed by Hollenberg, 1996; Karet and Lifton, 1997).

Identification of mutations in the gene encoding a subunit of the renal sodium channel in the Liddle syndrome has unraveled the mechanisms involved in this form of hereditary hypertension. Salt retention and secondary high blood pressure are the result of constitutive activation of the renal sodium channel by mutations in its gene.

The pathophysiological basis of Liddle's syndrome (pseudoaldosteronism), a rare autosomal dominant form of arterial hypertension, has been found to rest on missense mutations or truncations of the β- and γ-subunits of the epithelial sodium channel (ENaC) which controls sodium reabsorption in the distal nephron; these mutations result in constitutive activation of the amiloride-sensitive distal renal epithelial sodium channel (Shimkets et al, 1994); its activity is under the control of aldosterone. The genes encoding ENaC have been identified and revealed an heteromultimeric structure of the protein composed of three homologous α β γ subunits. Most of the mutations on ENaC reported are either nonsense mutations or frame shift mutations which would truncate the cytoplasmic carboxyl terminus of the β or γ subunits of the channel.

The original report was that Liddle's syndrome is caused by a premature stop codon that truncates the cytoplasmic carboxyl terminus of the encoded β subunit (Shimkets et al, 1994). Sequencing of the ENaC in a family with Liddle syndrome has also revealed a missense mutation in the β subunit which predicted substitution of Tyr by His at codon 618 (Tamura et al, 1996; reviewed by Schild, 1996); molecular variants of these genes might also contribute to the common polygenic forms of hypertension. Liddle's syndrome can also result from a mutation truncating the carboxy terminus of the γ subunit of this channel; this truncated subunit also activates channel activity. These findings indicate independent roles of β and γ subunits in the negative regulation of channel activity (Hansson et al, 1995).

Expression of the mineralocorticoid receptor (MR) is restricted to some sodium-transporting epithelia and a few nonepithelial target tissues. The genomic structure of the human MR (hMR) revealed two different untranslated exons (1α and 1β), which splice alternatively into the common exon 2, giving rise to two hMR mRNA isoforms (hMR-α and hMR-β). Expression of the human MR transcripts in renal, cardiac, skin, and colon tissue samples was examined by in situ hybridization. Functional hypermineralocorticism was associated with reduced expression of hMR β in sweat glands of two patients affected by Conn's and Liddle's syndrom (Zennaro et al, 1997).

Blood vessels and other tissues of hypertensive patients may have abnormal levels of several factors such as **(i)** the peptide kinin (produced from kininogen) and its specific receptor, **(ii)** kallikrein which processes kininogen into kinin, **(iii)** tissue kallikrein-binding protein (kallistatin), **(iv)** endothelial basic FGF, **(v)** the endothelium-derived nitric oxide (NO) and endothelial NO synthase, and **(vi)** the renin-angiotensin system (angiotensinogen, AT) and its receptor (AT1 receptor).

B. Gene therapy for hypertension

The peptide **kinin**, after binding to its specific receptor, triggers a broad spectrum of biological effects such as vasodilatation, increase in vascular permeability, smooth muscle contraction and relaxation, and electrolyte and glucose transport; it plays an important role in homeostasis of blood pressure, sodium excretion in the kidney, and inflammatory disorders. It is produced from a larger oligopeptide precursor, **kininogen**, after cleavage by a specific protease called **kallikrein**. Low levels of this protease in urine have been associated with hypertension. Repeated oral administration of swine pancreatic kallikrein can lower the blood pressure of hypertensive patients albeit in a temporal manner.

Delivery of a 5.6 kb genomic clone or of a 834-bp cDNA clone encoding the kallikrein gene under control of the albumin promoter, CMV, RSV, or metallothionein promoters into the portal vein or tail vein of spontaneously hypertensive rats resulted in significant reduction of their blood pressure for about 5-6 weeks (Chao et al, 1996). Intravenous injection of an adenoviral vector containing the human tissue kallikrein gene under the control of a CMV promoter, into spontaneously hypertensive rats caused a sustained delay in the increase in blood pressure from day 2 to day 41 post-injection. The therapeutic effect was a result of transfection of the human gene into several rat tissues and human tissue kallikrein mRNA was detected in the liver, kidney, spleen, adrenal gland, and aorta (Jin et al, 1997).

Treatment of hypertension with gene therapy has also been attained by transfer of the human tissue kallikrein-binding protein (HKBP) or **kallistatin**, a serine proteinase inhibitor (serpin). Transgenic mice overexpressing rat kallikrein-binding protein are hypotensive; kallistatin may function as a vasodilator in vivo. Delivery of the human kallistatin cDNA under control of the RSV 3' LTR in an adenoviral vector into spontaneously hypertensive rats by portal vein injection resulted in a significant reduction of blood pressure for 4 weeks; human kallistatin mRNA was detected in liver, spleen, kidney, aorta, and lung (Chen et al, 1997).

Blood pressure is also controlled by other factors such as by the endothelium-derived nitric oxide (NO) in peripheral vessels. Transfer of the **human endothelial NO synthase** (eNOS) gene to spontaneously hypertensive rats gave a continuous supply of eNOS which caused a significant reduction of systemic blood pressure for 5 to 6 weeks; the effect continued for up to 10 weeks after a second injection (Lin et al, 1997).

Angiotensinogen, a substrate for angiotensin I generation, is mainly produced in the liver, and is a unique

component of the renin-angiotensin system. Mutations in the angiotensinogen gene are associated with hypertension. It is unclear whether circulating angiotensinogen is a rate-limiting step in blood pressure regulation. Transfer of antisense oligonucleotides against rat angiotensinogen into the rat liver via the portal vein diminished the expression of hepatic angiotensinogen mRNA and resulted in a transient decrease in plasma angiotensinogen levels in spontaneously hypertensive rats from day 1 to day 7 after the injection. Liposomes were used for the transfer of oligonucleotides containing viral agglutinins to promote fusion with target cells. This treatment resulted in a decrease in plasma angiotensin II concentration; transfection of sense and scrambled oligonucleotides did not show any changes in plasma angiotensinogen level, blood pressure, or angiotensinogen mRNA level (Tomita et al, 1995).

The **renin-angiotensin** system plays an important role in blood pressure regulation; Phillips and coworkers (1997) have targeted the renin-angiotensin system at the level of synthesis (angiotensinogen, AT) and the receptor (AT1 receptor). Antisense oligonucleotides to AT1-receptor mRNA and to angiotensinogen mRNA reduced blood pressure. The cDNA for the AT1 receptor was inserted in the antisense direction under control of CMV promoter in AAV (which was the system of choice among adeno, retrovirus, naked DNA and liposomes tested) and injected either directly in the hypothalamus (1 µL) or in the lateral ventricles (5 µL). A prolonged decrease in blood pressure in spontaneously hypertensive rats was achieved via delivery of antisense DNA for AT1-R causing a significant reduction in AT1 receptors. After a single injection there was a significant decrease of blood pressure (approximately 23 +/- 2 mm Hg) for up to 9 weeks (Phillips, 1997; Phillips et al, 1997).

Blood vessels of spontaneously hypertensive rats were shown to be associated with sub-physiological amounts of **endothelial basic FGF** (bFGF); this decrease correlated both with hypertension and with a decrease in the endothelial content of nitric oxide synthase. As a consequence, transfer of the *bFGF* gene corrected hypertension, restored the physiological levels of bFGF in the vascular wall, significantly enhanced the number of endothelial cells with positive immunostaining for nitric oxide synthase, and ameliorated endothelial-dependent responses to vasoconstrictors (Cuevas et al, 1996).

Gene therapy of hypertension has been achieved via transfer of the **atrial natriuretic peptide** (ANP) gene to genetically hypertensive rats; chronic infusion of ANP has been shown to cause natriuresis, diuresis, and hypotension in rats and humans. Intravenous delivery of the human *ANP* gene fused to the RSV 3'-LTR (shown to be expressed in heart, lung, and kidney) caused a significant reduction of systemic blood pressure in young hypertensive rats (4 weeks old), and the effect continued for 7 weeks; a maximal blood pressure reduction of 21 mm Hg in young hypertensive rats was observed 5 weeks after injection along with significant increases in urinary volume and urinary potassium output (Lin et al, 1995).

XLI. Gene therapy for obesity

A. Molecular mechanisms of obesity

Obesity results from an imbalance in the mechanisms which control storage of energy as triglycerides in adipose cells versus energy expenditure. The identification of the *ob* gene, and its encoded protein leptin, as subfunctional in obesity (Zhang et al, 1994) has advanced our understanding on the mechanisms of receival and integration of a feedback signaling reflecting the amount of adipose energy stores (reviewed by Spiegelman and Flier, 1996). Further advancement was the identification of the *db* gene (also known as *OB-R* gene) on mouse chromosome 4 encoding the receptor of leptin which is expressed primarily in the hypothalamus and choroid plexus; OB-R is a single membrane-spanning receptor most related to the gp130 signal-transducing component of the IL-6 receptor, the G-CSF receptor, and the LIF receptor (Tartaglia et al, 1995).

The leptin is a hormone which is secreted from the white adipose tissue as a plasma protein, that acts in the hypothalamus to regulate the size of the body fat depot; the leptin with its receptor constitute a hormone-receptor pair that signals the status and magnitude of energy (fat) stores to the brain serving as an adipostatic signal to reduce food intake and body weight. Leptin might have evolved to inform the brain that energy stores in adipose tissue are sufficient but also to trigger a neuroendocrine response to fasting and limitation of food intake (Ahima et al, 1996). Leptin also acts acutely to increase glucose metabolism after intravenous and intracerebroventricular administrations; both intravenous or intracerebroventricular infusion of leptin into wild-type mice increased glucose turnover and glucose uptake (the plasma levels of insulin and glucose did not change), but decreased hepatic glycogen content; thus, the effects of leptin on glucose metabolism are mediated by the central nervous system (Kamohara et al, 1997).

Plasma leptin was found to be highly correlated with body mass index (BMI) in rodents and in 87 lean and obese humans. In humans, there was variability in plasma leptin at each BMI group suggesting that there are differences in its secretion rate from fat. Weight loss due to food restriction was associated with a decrease in plasma leptin in samples from mice and obese humans (Maffei et al, 1995).

B. Animal models for obesity

A number of rodent models for obesity are being used in the laboratories including db/db, fa/fa, yellow (Ay/a) VMH-lesioned, and those induced by gold thioglucose, monosodium glutamate, and by transgenic ablation of brown adipose tissue. The ob/ob mouse is genetically deficient in leptin. The expression of leptin mRNA and the level of circulating leptin are increased in these animal models, suggesting resistance to one or more of the actions of leptin. High-fat diet was found to evoke a

sustained increase in circulating leptin in normal FVB mice and FVB mice with transgene-induced ablation of brown adipose tissue; leptin levels were found to accurately reflect the amount of body lipid across a broad range of body fat. However, despite increased leptin levels, animals fed a high-fat diet became obese without decreasing their caloric intake, suggesting that a high content of dietary fat limits the action of leptin (Frederich et al, 1995). Peripheral and central administration of microgram doses of OB (leptin) protein reduced food intake and body weight of ob/ob and diet-induced obese mice but not in db/db obese mice (Campfield et al, 1995).

Body weight and adiposity appear to play a critical role in the timing of puberty in humans and rodents. Leptin is the signal that informs the brain that energy stores are sufficient to support the high energy demands of reproduction, and may be a major determinant of the timing of puberty. Indeed, injections of recombinant leptin (once daily) in female mice showed an earlier onset of three classic pubertal parameters (i.e., vaginal opening, estrus, and cycling) compared with saline-injected controls. In addition to its effects on body weight, chronic leptin treatment restored puberty and fertility to ob/ob mice with total leptin deficiency, and acute treatment with leptin substantially corrected hypogonadism in mice starved for 2 days without affecting body weight (Ahima et al, 1997). In a different study leptin was found to play a significant role in sustaining the male mouse reproductive pathways: all leptin-treated ob/ob males fertilized normal females mice that carried out normal pregnancies and deliveries, demonstrating that the reproductive capacity of sterile ob/ob males was corrected only with leptin treatment (Mounzih et al, 1997).

C. Glucocorticoids and obesity

The crucial role of glucocorticoids in obesity and insulin resistance and the actions of the OB protein leptin on the hypothalamic-pituitary-adrenal axis suggest that there is an important interaction of leptin with the glucocorticoid system. Leptin inhibits cortisol production in adrenocortical cells and therefore appears to be a metabolic signal that directly acts on the adrenal gland (Bornstein et al, 1997). Glucocorticoids play a key inhibitory role in the action of leptin: the permissive role of glucocorticoids in the establishment and maintenance of obesity syndromes in rodents arises from that glucocorticoids restrain the effect of leptin. Leptin injected intracerebroventricularly in normal rats induced modest reductions in body weight and food intake. In marked contrast, the same dose of leptin had very potent and long-lasting effects in decreasing both body weight and food intake when administered to adrenalectomized rats (Zakrzewska et al, 1997).

D. Therapy of obesity with leptin infusion

High leptin levels are observed in obese humans and rodents, suggesting that, in some cases, obesity is the result of leptin insensitivity. To test this hypothesis Halaas et al (1997) have used subcutaneous infusion of leptin to lean mice; this resulted in a dose-dependent loss of body weight at physiologic plasma levels. Chronic infusions of leptin intracerebroventricularly (i.c.v.) at doses of 3 ng/hr or greater resulted in complete depletion of visible adipose tissue, which was maintained throughout 30 days of continuous i.c.v. infusion. Direct measurement of energy balance indicated that leptin treatment prevented the energy decrease that follows reduced food intake but did not increase total energy expenditure (Halaas et al, 1997). In New Zealand Obese (NZO) mice, which were unresponsive to peripheral leptin but were responsive to i.c.v. leptin, obesity was the result of leptin resistance most likely arising from a decreased transport of leptin into the cerebrospinal fluid(Halaas et al, 1997).

Leptin administration reduced obesity in leptin-deficient ob/ob mice. Van Heek et al (1997) examined whether diet-induced obesity in mice produces resistance to peripheral and/or central leptin treatment. In a diet-induced obesity model, mice exhibited resistance to peripherally administered leptin, while retaining sensitivity to centrally administered leptin (by a single intracerebroventricular infusion). Whereas C57BL/6 mice initially responded to peripherally-administered leptin with a marked decrease in food intake, leptin resistance developed after 16 days on high fat diet; however, central administration of leptin to peripherally leptin-resistant mice resulted in a robust response to leptin. Thus, the effects of additional leptin administration in obese humans who have high circulating leptin levels, especially after intravenous injection (peripheral) versus intracerebroventricular infusion, remain to be determined. This study also implies the importance of the tissue target for the delivery of the leptin gene for treatment of obesity in humans.

E. The leptin and leptin receptor genes

Cloning of the gene encoding leptin (*ob* gene) and its receptor (*db* gene) has provided spectacular insights in elucidating the mechanisms involved in the control of food intake and body weight maintenance in obese and lean individuals. Transgenic mice lacking both alleles of either *ob* or *db* genes showed early onset obesity from excessive food intake and decreased energy expenditure, and in addition showed severe insulin resistance, diabetes, and sterility; administration of recombinant leptin had weight reducing effects (Campfield et al, 1995; Halaas et al, 1995; Pellymounter et al, 1995).

However, the vast majority of obese humans appear to have excessively high levels of leptin and absence of mutations in the *OB* gene (Considine et al, 1995; Maffei et al, 1996). The nonsense mutation in the ob mouse which results in the conversion of arginine 105 to a stop codon of leptin gene was not present in human obesity. The defect in humans is localized in the signaling pathway in the brain which might involve: **(i)** the leptin receptor; **(ii)** the Tub protein, expressed in the hypothalamus, that

mediates the signaling to the interior of the hypothalamus cell; **(iii)** the Agouti protein which is expressed in all tissues in obese mice but in the skin in normal mice, supposed to antagonize melanocortin signal to the CNS; and **(iv)** carboxypeptidase E (product of the *fat* gene) expressed in endocrine and neuroendocrine tissues (reviewed by Spiegelman and Flier, 1996).

Using a reverse transcription PCR product of the coding region of the Obese (ob) gene from five lean and five obese subjects it was determined that there was 72% more ob gene expression in eight obese subjects compared to eight lean controls; thus, ob gene expression is increased in human obesity (Considine et al, 1995). Limitation in food intake reduced the reproductive competence, reduced the levels of thyroid hormone, and activated the adrenal-pituitary stress axis; these starvation adaptations were reversed with administration of recombinant leptin (Ahima et al, 1996).

Obesity can result from a promotion in adipocyte differentiation. A number of mitogens (PDGF, EGF, FGF, tumor promoters), cytokines (TNF-α, IL-1, IL-6, TGF-β, IFN-γ) and oncogenes inhibit adipocyte differentiation and, therefore, inhibited adipogenesis and obesity. Insulin plays a positive role in the differentiation of adipocyte precursors and stimulates lipogenesis in adipose cells but exerts a negative lipogenic response in fibroblasts; preadipocytes which express small amounts of insulin receptors require insulin or insulin-like growth factor-1 for optimal differentiation.

The mechanism of inhibition of adipogenesis by mitogens involves activation of the mitogen-induced MAP kinase which phosphorylates at serine-112 the adipogenic transcription factor PPARγ (peroxisome proliferator-activated receptor γ). PPARγ acts as a dimer with RXRα to regulate adipocyte differentiation and sensitivity of the adipose cells to insulin. PPARγ is the high affinity receptor for the thiazolidinedione class of insulin-sensitizing drugs and the PPARγ-drug binding results in a powerful adipogenic response; thus, factors which stimulate the MAP kinase phosphorylation of PPARγ, could cause resistance to insulin (Hu et al, 1996).

F. Gene transfer of the leptin gene

This field is in its infancy; however, animal studies showed that treatment of obese persons is one of the future prospects of gene therapy. Adenovirus-mediated transfer of the mouse leptin cDNA in the ob/ob mouse (which is genetically deficient in leptin and exhibits both an obese and a mild non-insulin-dependent diabetic phenotype) resulted in dramatic reductions in both food intake and body weight, as well as the normalization of serum insulin levels and glucose tolerance (Muzzin et al, 1996). Thus, transfer of the leptin gene can correct the obese and diabetic phenotypes in the adult ob/ob mice; these studies also provided confirming evidence that the control in body weight may be critical in the long-term management of non-insulin-dependent diabetes mellitus in obese patients.

Infusion of a recombinant adenovirus containing the rat leptin cDNA under control of the CMV promoter to Wistar rats (8 ng/ml for 28 days) made them hyperleptinemic; these animals exhibited a 30-50% reduction in food intake and gained only 22 g over the experimental period versus 115-132 gained by control animals. Body fat was absent and plasma triglycerides and insulin levels were significantly lower in hyperleptinemic compared to control rats (Chen et al, 1996).

On the contrary, delivery of the rat neuropeptide Y (NPY) cDNA with AAV and Sendai virosomes into the rat hypothalamic para-ventricular nucleus increased body weight and food intake for 21 days (Wu et al, 1996).

XLII. Profile of Biotech companies: the big race

Research and drug development in Biotech Industry have played and continue to play an important role in advancing molecular medicine. A number of Biotechnology Companies sponsor ongoing gene therapy Clinical Trials led by Genetic Therapy Incorporated (part of Novartis, Gaithersburg, MD) with the phase III trial in glioblastoma.

Biotech Companies have the expertise and resources in large scale production, regulatory affairs, and clinical development to take off the basic science of gene therapy into the skies of commercialization and clinical trials. This is more evident in USA where strong bridges between the academia and industry have been established and numerous biotechnology companies dedicated to gene therapy have been founded. As of 1997 Europe lags some 3 to 5 years behind USA in translating basic science into commercial technology; however, European Pharmaceutical Companies have invested $1.4 billion in the American gene and cell therapy industry (compared with a modest $140 million investment from USA biotech firms to European partners). It is certain that both USA and Europe will ultimately benefit from the successful gene therapy development (Martin, 1997).

Table 8 gives a glimpse of the role of Biotech Industry in drug discovery related to gene therapy and the clinical trials sponsored.

Table 8. Biotech companies and drug development related to genes or cancer targeting

Company	**Drug/Gene type**	**Goal/Disease**	**Status of drug development** (end of 1997)
Calydon (San Francisco, CA)	Prostate cancer gene therapy		Preclinical trials
Canji/Schering-Plough (San Diego, CA)	p53 to restore p53-mediated tumor suppression or apoptosis	Colon with hepatic metastases(#131); prostate (#148)	Phase I
Canji/Schering-Plough (San Diego, CA)	RB to restore RB-mediated cell cycle arrest	Bladder(#140), non-small cell lung cancer (#156), lung, ovarian, and liver cancers	Early clinical trials
Cell Genesys, Inc	CC49-Zeta T cell receptor	Colorectal carcinomas expressing the tumor-associated antigen, TAG-72 (#110)	Phase I /II
Cell Genesys, Inc	CD4-zeta Chimeric Receptor	HIV (#85, 116)	Phase I-II
Chiron Corporation (Emerville & San Diego CA)	HIV-1IIIB envelope protein (#108)	HIV	Phase I
IDUN Pharmaceutical (San Diego, CA)	*bcl-2* inhibition		Preclinical trials
Incyte Pharmaceuticals (Palo Alto, CA)	Gene sequence databases		License access to database for drug discovery to different companies
Introgen Therapeutics (Austin and Houston, TX)	*p53* gene therapies to restore p53-mediated tumor suppression or apoptosis	Lung, head and neck cancers, prostate cancer (#154)	Phase I-II
Gene Medicine, Inc. (The Woodlands, Texas)	α1-Antitrypsin cDNA/cationic lipids	α1-antitrypsin deficiency (#194)	Phase I
Gene Medicine, Inc.	Human insulin-like growth factor-1(hIGF-1)	Cubital Tunnel syndrome (#164)	Phase I
Genetic Therapy, Inc. /Novartis (Gaithersburg, MD)	HSV-tk	brain tumors (#17), recurrent pediatric brain tumors (#45), recurrent glioblastoma (#32, 97, 99), astrocytoma (#42, 68), leptomeningeal carcinomatosis (#49), multiple myeloma (#75)	Phase I
Genetic Therapy, Inc. /Novartis	CFTR/adenovirus vector	Cystic fibrosis (#121)	Phase I
Genetic Therapy, Inc. /Novartis	Glucocerebrosidase cDNA	Gaucher disease (#39)	Phase I
Genset		Isolation of regulatory regions	
Genta (La Jolla, CA)	*bcl-2* inhibition with antisense	cancer	Early clinical trials
Genzyme Corporation (Framingham, MA)	CFTR/adenovirus vector/cationic lipids	Cystic fibrosis (#120, 128, 129, 203)	Phase I
GenVec, Inc.	$VEGF_{121}$ cDNA to the ischemic myocardium	Coronary artery disease (#157)	Phase I
Glaxo Wellcome Inc.	CFTR/Cationic Liposome Complex	Cystic fibrosis	Phase I
LXR (Richmond, CA)	Antiapoptotics		Advanced clinical trials
Mitotix (Cambridge, MA)	Cell cycle inhibitors		Preclinical trials
Myriad Genetics (Salt Lake City, UT)	Diagnostic genetic testing	BRAC analysis kit for breast cancer	In the market
Onyx (Richmond, CA)	*p53* gene therapies killing cells that lack p53		Early clinical trials
Ribozyme Pharmaceuticals, Inc (Boulder, CO)	Tat and Rev Hammerhead Ribozyme	HIV replication inhibition (#117)	Phase II
Ribozyme Pharmaceuticals, Inc (Boulder, CO)	Growth factor inhibition		Preclinical trials
Rhône-Poulenc Rorer Gencell (Vitry-sur-Seine, France &	p53	Head and neck squamous cell carcinoma (#152)	Phase II

Santa Clara, California)			
SEQUUS Pharmaceuticals (Menlo Park, CA)	Doxorubicin encapsulated into "stealth" liposomes	Kaposis sarcoma	In the market (Doxil); clinical trials for other cancers
SUGEN (Redwood City, CA)	Tyrosine kinase inhibitor to inhibit PDGF receptor	Gliomas	Clinical trials
Targeted Genetics Corporation	HSV-tk	HIV (#81)	Phase I/II
Targeted Genetics Corporation	E1A/ DC-Chol-DOPE	Metastatic breast or ovarian cancer, metastatic solid tumors that overexpress HER-2 /neu	Phase I
Targeted Genetics Corporation	CFTR/AAV	Cystic fibrosis	Phase I
Vical, Inc.	Tumor idiotype	Non-Hodgkin's B-cell lymphoma (#161)	Phase I/II
Vical, Inc. (San Diego, California)	β-2 Microglobulin cDNA/cationic lipids	Immunotherapy of advanced colorectal carcinoma, renal cancer (#195), melanoma (#196), metastatic malignancies (#201)	Phase I/II
Vical, Inc.	Interleukin-2 cDNA	Lymphoma (#198), solid tumor immunotherapy (#211)	Phase I
Vical, Inc.	HLA B7 cDNA	Renal cancer immunotherapy	Phase I

XLIII. Prospects

A. Gene discovery: novel horizons in gene therapy

By the year 2005 the human genome project will be completed and by the end of 1998 the entire cDNA repertoire of the 125,000 human genes will be sequenced completely (Incyte Pharmaceuticals, Palo Alto, CA). Every single open reading frame of the human genome will become known.

Genes implicated in human disease are being identified by mapping the mutation to a chromosomal locus after examining the DNA from a number of patients; candidate genes residing in this locus are then examined in a large number of patients for inactivating mutations (e.g. Polymeropoulos et al, 1996, 1997). A number of other classical techniques are aimed at identifying novel tumor suppressor genes or genes involved in metastasis, adding new weapons to the fight against cancer. The elucidation of many of the pathways implicated in the regulation of the cell cycle, signaling pathways with cytokines, and activation and action of transcription factors on the regulatory regions of genes lead to the discovery of new drugs interfering with those pathways. The elucidation of a number of players in apoptosis provides also targets not only for cancer treatment but for a number of neurodegenerative diseases. All these studies will ultimately provide new targets and genes for gene therapy.

B. Mutations in DNA and human disease

A number of human disorders have been linked to mutations in specific genes that result in loss of function of a specific protein in all somatic cells of the body. In the majority of cases known today the mutation is at the coding region of the gene resulting in one amino acid substitution at an important domain of the encoded protein, in amino acid deletions, or in protein truncation. For example, deletion of three nucleotides resulting in deletion of a single phenylalanine at the protein level of the CFTR molecule is responsible for cystic fibrosis (Riordan et al, 1989); an A to T transversion leading to a premature stop at amino acid 337 in one allele and a C to T transition triggering an erroneous splice event and to frameshift in the other allele are associated with mutations in the ERCC6 helicase in Cockayne's syndrome (Troelstra et al, 1992).

Mutations could result in failure of the protein to interact with DNA (mutated p53), with other regulatory proteins, or in enzymatic dysfunction of the molecule. Defects at the nuclear localization signal of a nuclear protein resulting in its cytoplasmic retention have been identified in cancer cells (Chen et al, 1995). Mutations in regulatory regions (promoters, enhancers) of genes, poorly understood but expected to play an important role in human disease, could result in down-regulation of the gene they dictate; mutation in the DNA-binding or transactivation domains of transcription factors are expected to down-regulate the expression of their target genes. Most important, mutations in genes involved in DNA repair are expected to have a domino effect on the appearance of mutations in other regions of the genome, since it is these genes that are responsible for removal of premutagenic lesions incurring by a number of xenobiotics by patrolling the human genome (Boulikas, 1996c).

C. Regulatory regions and the MAR project

The identification of the regulatory regions from the human genome should also become a first priority. Regulatory regions will provide new DNA control

elements for the tissue-specific expression, episomal replication, insulation, and silencing of genes in gene therapy protocols but also targets, using small oligonucleotides (e.g. triplex) to abort transcription of specific genes. Regulatory regions include enhancers (ENHs, at least two for each gene), promoters (about 125,000 total, a significant fraction of which might be known because of their proximity to the 5' end), origins of replication (ORIs, about 50,000 have been estimated), silencers, locus control regions (LCRs, perhaps several thousand), and matrix-attached regions (seem to coincide with enhancers and ORIs). 445,000 is a modest estimate of the total number of regulatory regions in the human genome. Their size ranges from 100-500 bp but much more for LCRs and some ORIs. Identification of strong regulatory regions from the human genome is expected to provide strong promoter and enhancer sequences for the universal and cell type-specific expression of transgenes in gene therapy but also strong human ORIs able to sustain extrachromosomal replication of plasmids loaded with therapeutic genes in human and animal model tissues.

Transcription factor recognition sequence databases can be used in conjuction with other software methods (DNA curvature, inverted repeats, triplex DNA, Z-DNA, phased nucleosomes) to predict regulatory regions from the large DNA sequence information arising from the human genome project (Boulikas, 1995b; Bode et al, 1998, this volume).

A technology developed in our laboratory based on isolation and cloning of matrix-attached regions, shown to harbor a large fraction of regulatory regions from the human and other genomes, is being applied for identifying human regulatory regions (MAR project). MAR libraries include tissue-specific and tumor-specific regulatory regions. One particular MAR clone that has been extensively characterized (Boulikas et al, in preparation) represents the ORI, enhancer, and MAR of the human choline acetyltransferase gene, of crucial importance in neurological disorders including Alzheimer's disease. When a subfragment of only 513 bp of this MAR/ORI/ENH was placed at the flanks of the luciferase gene it was able to sustain episomal replication in human culture cells (K562 erythroleukemia) for more than 4 months. The actual 3.6 kb ChAT ORI region comprises a 1.2 kb silencer whose presence inhibits the ORI function; thus, mammalian origins of replication are much more sophisticated than viral ORIs and contain a number of control elements, including silencers, for the cell type and developmental stage-specific regulation. Identification and elimination of silencers from human ORIs is of importance in the exploitation of ORI fragments in the episomal replication of therapeutic genes.

MAR sequences sorted out into MAR/ORI, MAR/enhancer and MAR/insulators can be used to promote extrachromosomal replication, to enhance the transcription of genes or to insulate genes from position effects from chromatin surroundings after integration. A number of studies show that MARs act as insulators of genes shielding them from position effect variegation from neighboring chromatin domains in transgenic studies; this shielding results in a 2 to 1000-fold increase in the expression level of transgenes when MARs are included on both sides of the foreign gene (see Boulikas 1995b).

Identification of tumor-specific MARs, such as identification of the MARs of the carcinoembryonic antigen (CEA) gene, the breast cancer/ovarian cancer BRCA1 gene, and others can lead to the development of plasmid vectors able to drive the expression of therapeutic genes in specific tumor cell types. In the postgenomic era, identification of a reasonable fraction of regulatory regions will revolutionarize our approaches to human disease.

D. What is next on gene therapy?

Theoretically, most human disorders could constitute targets for gene therapy, aimed at correcting the defect either by transferring the wild-type gene in all somatic cells of the body or to those specific cell types responsible mainly for the synthesis of the particular protein (e.g. factor IX gene in liver cells of hemophilia B patients).

Nuclear localization signal (NLS) peptides hooked to triplex-oligonucleotides or to plasmids, or complexation of plasmids with nuclear proteins possessing multiple NLSs are expected to increase nuclear localization and enhanced expression of foreign genes.

A significant number of discoveries in molecular biology of human diseases have opened doors to the development of strategies for gene therapy. New genes whose mutations are responsible for human disease, from mild to life threatening, are being discovered and the molecular mechanisms are being unraveled. Many pieces of the puzzle aimed at elucidating mechanisms leading to human disease and the genes implicated have been solved and lie as scattered pieces of knowledge in various publications, lab notebooks, or patent applications. Preexisting Biotech Companies redefine their missions and new Biotech Companies are being founded to explore new discoveries and develop new drugs; to win the race in the fight against human disease, especially cancer and AIDS, we need to gather the right components into a successful assemble.

Retroviruses, adenoviruses, AAV, HSV, naked plasmid delivery, and liposomes all have a good share as delivery vehicles for genes and it seems that they will be developed independently, each with its own strengths and limitations for particular gene therapy protocols. For example, liposomes have a distinct advantage over other systems for the delivery of oligonucleotides, stealth liposomes could prove their strength in the systemic delivery of genes by intravenous injection, retroviruses and adenoviruses for their high transfection efficiency, AAV for not stimulating inflammation, HSV as a vehicle for gene therapy to the nervous system, HIV and HSV vectors for their high payload capacity. Furthermore, adenoviruses, AAV, HSV-1, HIV-1 vectors can transduce nondividing cells (**Table 1** on page 29).

A lot has been learned about the involvement of the tumor suppressor p53 protein in cancer etiology. The current view is that an initiated tumor cell in the body, having mutations in one or more oncogenes needs to acquire loss in function in both alleles of p53 or other tumor suppressor gene in order to expand into the tumor cell mass. Expression of the wild-type (non-mutated form) of p53 arrests the proliferation in tumor cells and induces apoptosis (suicidal programmed death) by boosting the expression of the genes of p21, bax, and Gadd45 and by repressing the bcl-2 gene. Transfer of the p53 gene with adenovirus or retrovirus after intratumoral injection has successfully led to eradication of tumors in animal models and in human patients at advanced stages of non small cell lung cancer. Intratumoral injection, however, is not expected to be applicable to metastases very frequently associated with advanced stages of cancer. Stealth liposomes might offer a solution to this problem.

Anti-angiogenesis therapy, both drug-mediated and gene therapy, would bring important ammunition in the fight against cancer.

Improvements in oligonucleotide delivery in vivo, a very promising field that is in its infancy at the delivery level, will advance the field of pharmacogenomics by providing triplex-forming oligonucleotide drugs to inhibit the transcription of specific genes or ribozyme drugs to lower the mRNA level of a specific target protein.

We expect the final victory of the human race on cancer to be accomplished over the next 10 years. Gene therapy would, no doubt, have an important role to play. It is likely that a combination of gene therapy (p53, HSV-tk, angiostatin) along with the already existing antineoplastic drugs (doxorubicin, cisplatin) but at subtoxic doses and at much lower concentrations than those used today, as well as less severe doses of radiation, would become routine regimens in hospitals for the eradication of all cancer types. In this respect, the emerging priority in gene therapy is to improve the efficiency of tissue targeting and gene delivery.

Acknowledgements

I am grateful to all authors who have kindly provided their permission and original photos to use in this article. Special thanks to François Meyer, Max Birnstiel, Brian Johnston, Marc Vasseur, Claude Hélène, Frank Martin, Joe Vallner, Douglas Jolly, Jeff Seilhamer, Demetrios Spandidos, Kaz Taira, Ernst Wagner, Peter Dehlinger, Ko Kurachi, Dan Maneval, Jerzy Jurkat, Ed Trifonov, and Emile Zuckerkandl for stimulating discussions. I am also grateful to past and present members of my group for their contributions to the ongoing projects related to the subjects discussed here in particular Linda Hsie, CF Kong, Dawn Brooks, John Costouros, and Jie Hu, as well as to my collaborators Maria Zannis-Hadjopoulos, Jürgen Bode, and Wolfgang Deppert.

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Appendix 1. RAC-approved human gene therapy protocols

Using retrovirus, protocols: 1-117
Using adenovirus: 118-157
With naked plasmid: 158-164
Adeno-Associated virus: 165, 166
Immunotherapy or other: 167-186
Cationic lipids: 187-220 on page 203-206 of following article (Martin and Boulikas, 1998)
(Last updated: 21 November 1997)

RETROVIRAL GENE DELIVERY

	Disease	Protocol title	Procedures	Principa investigator
1.	Gene Marking /Cancer	The Treatment of Patients with Advanced Cancer Using Cyclophosphamide, Interleukin-2 and Tumor Infiltrating Lymphocytes.	In Vitro /**Tumor Infiltrating Lymphocytes** /Retrovirus /Neomycin Phosphotransferase cDNA /Intravenous	Rosenberg, Steven A
2.	Gene Therapy /Phase I /Monogenic Disease /Severe Combined Immune Deficiency due to **Adenosine Deaminase Deficiency**	Treatment of Severe Combined Immune Deficiency (SCID) due to Adenosine Deaminase (ADA) Deficiency with Autologous Lymphocytes Transduced with the Human ADA Gene: An Experimental Study.	In Vitro /Autologous Peripheral Blood Cells /$CD34^+$ Autologous Peripheral Blood Cells /Cord Blood /Placenta Cells /Retrovirus /Adenosine Deaminase cDNA /Neomycin Phosphotransferase cDNA /Intravenous	Blaese, R. Michael
3.	Gene Therapy /Phase I /Cancer /Melanoma /**Immunotherapy**	Gene Therapy of Patients with Advanced Cancer Using **Tumor Infiltrating Lymphocytes** Transduced with the Gene Coding for Tumor Necrosis Factor.	In Vitro /Tumor Infiltrating Lymphocytes /Retrovirus /Cytokine /**Tumor Necrosis Factor** cDNA /Neomycin Phosphotransferase cDNA /Intravenous	Rosenberg, Steven A
4.	Gene Marking /Cancer /Acute Myelogenous Leukemia	Autologous Bone Marrow Transplant for Children with Acute Myelogenous Leukemia in First Complete Remission: Use of Marker Genes to Investigate the Biology of Marrow Reconstitution and the Mechanism of Relapse.	In Vitro /Autologous Bone Marrow Cells /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Brenner, Malcolm K.
5.	Gene Marking /Cancer /Neuroblastoma	A Phase I /II Trial of High Dose Carboplatin and Etoposide with Autologous Marrow Support for Treatment of Stage D Neuroblastoma in First Remission: Use of Marker Genes to Investigate the Biology of Marrow Reconstitution and the Mechanism of Relapse.	In Vitro /Autologous Bone Marrow Cells /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Brenner, Malcolm K.
6.	Gene Marking /Cancer /Neuroblastoma	A Phase II Trial of High-Dose Carboplatin and Etoposide with Autologous Marrow Support for Treatment of Relapse /Refractory Neuroblastoma Without Apparent Bone Marrow Involvement.	In Vitro /Autologous Bone Marrow Cells /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Brenner, Malcolm K
7.	Gene Marking /Cancer /Chronic Myelogenous Leukemia	Autologous Bone Marrow Transplantation for Chronic Myelogenous Leukemia in which Retroviral Markers are Used to Discriminate between Relapse which Arises from Systemic Disease Remaining after PreparativeTherapy Versus Relapse due to Residual Leukemic Cells in Autologous Marrow: A Pilot Trial.	In Vitro /Autologous Bone Marrow Cells /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Deisseroth, Albert B
8.	Gene Marking /Acute Hepatic Failure	Hepatocellular Transplantation in Acute Hepatic Failure and Targeting Genetic Markers to Hepatic Cells.	In Vitro /Autologous Hepatocytes /Retrovirus /Neomycin Phosphotransferase cDNA /Intrahepatic	Ledley, Frec
9.	Gene Marking /Cancer /Melanoma	The Administration of Interleukin-2 and **Tumor Infiltrating Lymphocytes** to Patients with Melanoma.	In Vitro /Tumor Infiltrating Lymphocytes /Retrovirus /Neomycin Phosphotransferase cDNA /Intravenous	Lotze, Mich T
10.	Gene Therapy /Phase I /Cancer /Melanoma /Renal Cell /Colon /Breast /**Immunotherapy**	Immunization of Cancer Patients Using Autologous Cancer Cells Modified by Insertion of the Gene for **Tumor Necrosis Factor** (TNF).	In Vitro /Autologous Tumor Cells /Lethally Irradiated /Retrovirus /Cytokine /Tumor Necrosis Factor cDNA /Neomycin Phosphotransferase cDNA /Subcutaneous Injection	Rosenberg, Steven A
11.	Gene Therapy /Phase I /Cancer /Melanoma /Renal Cell /Colon /**Immunotherapy**	Immunization of Cancer Patients Using Autologous Cancer Cells Modified by Insertion of the Gene for Interleukin-2 (IL-2).	In Vitro /Autologous Tumor Cells /Lethally Irradiated /Retrovirus /Cytokine /**Interleukin-2 cDNA** /Subcutaneous Injection	Rosenberg, Steven A

12.	Gene Marking /Cancer /Acute Myelogenous Leukemia /Acute Lymphocytic Leukemia	Retroviral-Mediated Gene Transfer of Bone Marrow Cells during Autologous Bone Marrow Transplantation for Acute Leukemia.	In Vitro /Autologous Bone Marrow Cells /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Cornetta, Kenneth
13.	Gene Marking /Cancer /Melanoma /Renal Cell	The Treatment of Patients with Metastatic Melanoma and Renal Cell Cancer Using In Vitro Expanded and Genetically-Engineered (Neomycin Phosphotransferase) Bulk, $CD8^+$ and /or $CD4^+$ **Tumor Infiltrating Lymphocytes** and Bulk, $CD8^+$ and /or $CD4^+$ Peripheral Blood Leukocytes in Combination with Recombinant Interleukin-2 Alone, or with Recombinant Interleukin-2 and Recombinant α Interferon.	In Vitro /$CD4^+$ Autologous Peripheral Blood Lymphocytes /$CD8^+$ Autologous Peripheral Blood Lymphocytes /$CD4^+$ Autologous Tumor Infiltrating Lymphocytes /$CD8^+$ Autologous Tumor Infiltrating Lymphocytes /Retrovirus /Neomycin Phosphotransferase cDNA /Intravenous	Economou, James S. and Belldegrun, Arie
14.	Gene Therapy /Phase I /Cancer /Ovarian /**Pro-Drug**	Gene Transfer for the Treatment of Cancer.	In Vitro /Allogeneic Tumor Cells /Lethally Irradiated /PA317 /Retrovirus /Herpes Simplex Virus Thymidine Kinase cDNA /Ganciclovir /Intraperitoneal Administration	Freeman, Sc M
15.	Gene Therapy /Infectious Disease /Human Immunodeficiency Virus	Phase I Study to Evaluate the Safety of Cellular Adoptive Immunotherapy Using Genetically Modified $CD8^+$ **HIV**-Specific T Cells in HIV Seropositive Individuals.	In Vitro /$CD8^+$ Allogeneic Cytotoxic T Lymphocytes /$CD8^+$ Syngeneic Cytotoxic T Lymphocytes /Retrovirus /Hygromycin Phosphotransferase /Herpes Simplex Virus Thymidine Kinase cDNA /Intravenous	Greenberg, Philip D. and Riddell, Star
16.	Gene Therapy /Phase I /Cancer /Relapsed-Refractory Neuroblastoma /**Immunotherapy**	Phase I Study of Cytokine-Gene Modified Autologous Neuroblastoma Cells for Treatment of Relapsed /Refractory Neuroblastoma.	In Vitro /Autologous Neuroblastoma Cells /Allogeneic Partially HLA-Matched /Retrovirus /Cytokine /**Interleukin-2 cDNA** /Subcutaneous Injection	Brenner, Malcolm K.
17.	Gene Therapy /Phase I /Cancer /Brain /**Pro-Drug**	Gene Therapy for the Treatment of Brain Tumors Using Intra-Tumoral Transduction with the Thymidine Kinase Gene and Intravenous Ganciclovir. **Sponsor**: Genetic Therapy, Inc. /Novartis	In Vivo /Autologous Tumor Cells /PA317 /Retrovirus /Herpes Simplex Virus Thymidine Kinase cDNA /Ganciclovir /Intratumoral /Stereotactic Injection	Oldfield, Edward
18.	Gene Marking /Cancer /Chronic Myelogenous Leukemia	Use of Two Retroviral Markers to Test Relative Contribution of Marrow and Peripheral Blood Autologous Cells to Recovery After Preparative Therapy.	In Vitro /Autologous Bone Marrow Cells /Autologous Peripheral Blood Cells /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Deisseroth, Albert B
19.	Gene Therapy /Phase I /Cancer /Melanoma /**Immunotherapy**	Immunization with HLA-A2 matched Allogeneic Melanoma Cells that Secrete Interleukin-2 in Patients with Metastatic Melanoma.	In Vitro /Allogeneic Partially HLA-Matched /Retrovirus /Cytokine /**Interleukin-2 cDNA** /Subcutaneous Injection	Gansbacher Bernd
20.	Gene Therapy /Phase I /Cancer /Renal Cell /**Immunotherapy**	Immunization with Interleukin-2 Secreting Allogeneic HLA-A2 Matched Renal Cell Carcinoma Cells in Patients with Advanced Renal Cell Carcinoma.	In Vitro /Allogeneic Partially HLA-Matched /Retrovirus /Cytokine /**Interleukin-2 cDNA** /Subcutaneous Injection	Gansbacher Bernd
21.	Gene Marking /Cancer /Multiple Myeloma	Retroviral-Mediated Gene Transfer of Bone Marrow and Peripheral Blood Stem Cells During Autologous Bone Marrow Transplantation for Multiple Myeloma.	In Vitro /$CD34^+$ Autologous Peripheral Blood Cells /Intravenous /Autologous Bone Marrow Cells /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Dunbar, Cyr
22.	Gene Marking /Cancer /Breast	Retroviral-Mediated Gene Transfer of Bone Marrow and Peripheral Blood Stem Cells During Autologous Bone Marrow Transplantation for Metastatic Breast Cancer.	In Vitro /$CD34^+$ Autologous Peripheral Blood Cells /Intravenous /Autologous Bone Marrow Cells /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Dunbar, Cyr
23.	Gene Marking /Cancer /Chronic Myelogenous Leukemia	Retroviral-Mediated Gene Transfer of Bone Marrow and Peripheral Blood Stem Cells During Autologous Bone Marrow Transplantation for Chronic Myelogenous Leukemia.	In Vitro /$CD34^+$ Autologous Peripheral Blood Cells /Intravenous /Autologous Bone Marrow Cells /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Dunbar, Cyr
24.	Gene Marking /Infectious Disease /Human Immunodeficiency Virus	A Study of the Safety and Survival of the Adoptive Transfer of Genetically Marked Syngeneic Lymphocytes in **HIV** Infected Identical Twins.	In Vitro /Syngeneic Peripheral Blood Lymphocytes /Retrovirus /Neomycin Phosphotransferase cDNA /Intravenous	Walker, Rol E
25.	Gene Marking /Cancer	Study on Contribution of Genetically Marked Peripheral Blood Repopulating Cells to Hematopoietic Reconstitution after Transplantation.	In Vitro /G-CSF Mobilized $CD34^+$ Autologous Peripheral Blood Cells /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Schuening, Friedrich G.

26.	Gene Marking /Cancer /Lymphoid Malignancies	Evaluation of the Use of Recombinant Human G-CSF Stimulated Peripheral Blood Progenitor Cell Supplementation in Autologous Bone Marrow Transplantation in Patients with Lymphoid Malignancies.	In Vitro /G-CSF Mobilized Autologous Peripheral Blood Cells /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Schuening, Friedrich G
27.	Gene Marking /Cancer	A Trial of G-CSF Stimulated Peripheral Blood Stem Cells for Engraftment in Identical Twins.	In Vitro /G-CSF Mobilized $CD34^+$ Autologous Peripheral Blood Cells /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Schuening, Friedrich G
28.	Gene Marking /Cancer /Chronic Lymphocytic Leukemia /Follicular Non-hodgkins Lymphoma	Use of Retroviral Markers to Identify Efficacy of Purging and Origin of Relapse Following Autologous Bone Marrow and Peripheral Blood Cell Transplantation in Indolent B Cell Neoplasms (Follicular Non-Hodgkin's Lymphoma or Chronic Lymphocytic Leukemia) Patients.	In Vitro /Autologous Bone Marrow Cells /Autologous Peripheral Blood Cells /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Deisseroth, Albert B
29.	Gene Therapy /Phase I /Cancer /Non-small Cell Lung Cancer /Antisense /Tumor Suppressor Gene	Clinical Protocol for Modification of Oncogene and Tumor Suppressor Gene Expression in Non-Small Cell Lung Cancer (NSCLC).	In Vivo /Autologous Tumor Cells /Retrovirus /**p53 cDNA** /**K-ras Antisense** /Intratumoral /Bronchoscope	Roth, Jack A
30.	Gene Marking /Cancer /Neuroblastoma	A Phase II Trial of the Baxter Neuroblastoma Bone Marrow Purging System Using Gene Marking to Assess Efficacy.	In Vitro /Autologous Bone Marrow Cells /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Brenner, Malcolm K
31.	Gene Therapy /Phase I /Cancer /Renal Cell /**Immunotherapy**	A Pilot Study of IL-4 Gene Modified Antitumor Vaccines.	In Vitro /Autologous Fibroblasts /Lethally Irradiated /In Combination with Untransduced Autologous Tumor Cells /Retrovirus /Cytokine /Interleukin-4 cDNA /Subcutaneous Injection	Lotze, Mich T. and Rubi Joshua T
32.	Gene Therapy /Phase I /Cancer /Glioblastoma /**Pro-Drug**	Gene Therapy for the Treatment of Recurrent Glioblastoma Multiforme with In Vivo Tumor Transduction with the Herpes Simplex Thymidine Kinase Gene /Ganciclovir System. **Sponsor**: Genetic Therapy, Inc. /Novartis	In Vivo /Autologous Tumor Cells /PA317 /Retrovirus /Herpes Simplex Virus Thymidine Kinase cDNA /Ganciclovir /Intratumoral /Direct Injection	Van Gilder, John C.et al
33.	Gene Marking /Cancer /Leukemia /Non-malignant Disorders	Administration of Neomycin Resistance Gene Marked EBV Specific Cytotoxic T Lymphocytes to Recipients of Mismatched-Related or Phenotypically Similar Unrelated Donor Marrow Grafts.	In Vitro /Epstein-Barr Virus Specific Allogeneic Cytotoxic T Lymphocytes /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Heslop, Hel E.
34.	Gene Marking /Cancer /Acute Myelogenous Leukemia	Assessment of the Efficacy of Purging by Using Gene-Marked Autologous Marrow Transplantation for Children with Acute Myelogenous Leukemia in First Complete Remission.	In Vitro /Autologous Bone Marrow Cells /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Brenner, Malcolm K
35.	Gene Therapy /Phase I /Cancer /Renal Cell /**Immunotherapy**	Phase I Study of Non-Replicating Autologous Tumor Cell Injections Using Cells Prepared With or Without **Granulocyte-Macrophage Colony Stimulating Factor** Gene Transduction in Patients with Metastatic Renal Cell Carcinoma.	In Vitro /Autologous Tumor Cells /Lethally Irradiated /Retrovirus /Cytokine /Granulocyte-Macrophage Colony Stimulating Factor cDNA /Subcutaneous Injection	Simons, Jonathan
36.	Gene Therapy /Phase I /Cancer /Melanoma /**Immunotherapy**	A Phase I Trial of Human γ**Interferon**-Transduced Autologous Tumor Cells in Patients With Disseminated Malignant Melanoma.	In Vitro /Autologous Tumor Cells /Lethally Irradiated /Retrovirus /γ Interferon cDNA /Subcutaneous Injection	Seigler, Hill F and Merrit James A
37.	Gene Therapy /Phase I /Cancer /Ovarian /Chemoprotection	Use of Safety-Modified Retroviruses to Introduce Chemotherapy Resistance Sequences into Normal Hematopoietic Cells for Chemoprotection During the Therapy of Ovarian Cancer: A Pilot Trial.	In Vitro /$CD34^+$ Autologous Bone Marrow Cells /Retrovirus /**Multi-Drug Resistance**-1 cDNA /Bone Marrow Transplant	Deisseroth, Albert B.
38.	Gene Therapy /Phase I /Monogenic Disease /**Gaucher Disease**	Gene Therapy for Gaucher Disease: Ex Vivo Gene Transfer and Autologous Transplantation of $CD34^+$ Cells.	In Vitro /$CD34^+$ Autologous Peripheral Blood Cells /Retrovirus /Glucocerebrosidase cDNA /Bone Marrow Transplant	Barranger, J A
39.	Gene Therapy /Phase I /Monogenic Disease /**Gaucher Disease**	Retroviral Mediated Transfer of the cDNA for Human Glucocerebrosidase into Hematopoietic Stem Cells of Patients with Gaucher Disease. **Sponsor**: Genetic Therapy, Inc. /Novartis	In Vitro /$CD34^+$ Autologous Peripheral Blood Cells /Retrovirus /Glucocerebrosidase cDNA /Bone Marrow Transplant	Karlsson, St
40.	Gene Therapy /Phase I /Infectious Disease /Human Immunodeficiency Virus /**Immunotherapy**	A Preliminary Study to Evaluate the Safety and Biologic Effects of Murine Retroviral Vector Encoding **HIV**-1 Genes [HIV-IT(V)] in Asymptomatic Subjects Infected with HIV-1. **Sponsor**: Chiron Corporation	In Vivo /Autologous Muscle Cells /Retrovirus /HIV-1IIIB Envelope Protein /Intramuscular Injection	Galpin, Jeff E

41.	Gene Therapy /Phase I /Infectious Disease /Human Immunodeficiency Virus /Replication Inhibition /**Antisense**	A Molecular Genetic Intervention for **AIDS** - Effects of a Transdominant Negative Form of Rev.	In Vitro /$CD4^+$ Autologous Peripheral Blood Cells /Retrovirus /Particle Mediated Gene Transfer (Accell®) /RSV-tar /Rev M10 /Intravenous	Nabel, Gary
42.	Gene Therapy /Phase I /Cancer /Astrocytoma /**Pro-Drug**	Gene Therapy for the Treatment of Recurrent Pediatric Malignant Astrocytomas with In Vivo Tumor Transduction with the Herpes Simplex Thymidine Kinase Gene. **Sponsor**: Genetic Therapy, Inc. /Novartis	In Vivo /Autologous Tumor Cells /PA317 /Retrovirus /Herpes Simplex Virus Thymidine Kinase cDNA /Ganciclovir /Intratumoral /Ommaya Injection	Raffel, Core
43.	Gene Therapy /Phase I /Cancer /Ovarian /Brain /Chemoprotection	Human MDR Gene Transfer in Patients with Advanced Cancer.	In Vitro /$CD34^+$ Autologous Bone Marrow Cells /Retrovirus /**Multi-Drug Resistance**-1 cDNA /Bone Marrow Transplant	Hesdorffer, Charles and Antman, Ka
44.	Gene Therapy /Phase I /Cancer /Breast /Chemoprotection	Retroviral Mediated Transfer of the Human Multi-Drug Resistance Gene (MDR-1) into Hematopoietic Stem Cells During Autologous Transplantation after Intensive Chemotherapy for Breast Cancer.	In Vitro /$CD34^+$ Autologous Peripheral Blood Cells /Retrovirus /**Multi-Drug Resistance**-1 cDNA /Intravenous	O'Shaughnes Joyce
45.	Gene Therapy /Phase I /Cancer /Brain Tumors /**Pro-Drug**	Gene Therapy for Recurrent Pediatric Brain Tumors. **Sponsor**: Genetic Therapy, Inc. /Novartis	In Vivo /Autologous Tumor Cells /PA317 /Retrovirus /Herpes Simplex Virus Thymidine Kinase cDNA /Ganciclovir /Intratumoral /Direct Injection	Kun, Larry
46.	Gene Therapy /Phase I /Cancer /Melanoma /**Immunotherapy**	Immunization of Malignant Melanoma Patients with Interleukin 2-Secreting Melanoma Cells Expressing Defined Allogeneic Histocompatibility Antigens.	In Vitro /Allogeneic Tumor Cells /Lethally Irradiated /Retrovirus /**Interleukin-2 cDNA** /Neomycin Phosphotransferase cDNA /Subcutaneous Injection	Das Gupta, Tapas K. an Cohen, Edw P
47.	Gene Therapy /Phase I /Infectious Disease /Human Immunodeficiency Virus-1 /Replication Inhibition /Hairpin Ribozyme	A Phase I Clinical Trial to Evaluate the Safety and Effects in **HIV**-1 Infected Humans of Autologous Lymphocytes Transduced with a Ribozyme that Cleaves HIV-1 RNA.	In Vitro /$CD4^+$ Peripheral Blood Cells /Retrovirus /Hairpin Ribozyme /Intravenous	Wong-Staal, Flossie
48.	Gene Therapy /Phase I /Cancer /Melanoma /**Immunotherapy**	Genetically Engineered Autologous Tumor Vaccines Producing Interleukin-2 for the Treatment of Metastatic Melanoma.	In Vitro /Allogeneic Tumor Cells /Lethally Irradiated /In Combination with Untransduced Autologous Tumor Cells /Retrovirus /**Interleukin-2 cDNA** /Subcutaneous Injection	Economou, James S. and Glasby, John
49.	Gene Therapy /Phase I /Cancer /Leptomeningeal Carcinomatosis /**Pro-Drug**	Intrathecal Gene Therapy for the Treatment of Leptomeningeal Carcinomatosis. **Sponsor**: Genetic Therapy, Inc. /Novartis	In Vivo /Autologous Tumor Cells /PA317 /Retrovirus /Herpes Simplex Virus Thymidine Kinase cDNA /Ganciclovir /Intraventricular Injection /Subarachnoid Injection	Oldfield, Edward H. a Ram, Zvi
50.	Gene Therapy /Phase I /Cancer /Colon /**Immunotherapy**	Injection of Colon Carcinoma Patients with Autologous Irradiated Tumor Cells and Fibroblasts Genetically Modified to Secrete Interleukin-2.	In Vitro /Autologous Fibroblasts /Lethally Irradiated /In Combination with Untransduced Autologous Tumor Cells /Retrovirus /**Interleukin-2 cDNA** /Subcutaneous Injection	Sobol, Robe and Royston Ivor
51.	Gene Therapy /Phase I /Monogenic Disease /**Gaucher Disease**	Retrovirus-Mediated Transfer of the cDNA for Human Glucocerebrosidase into Peripheral Blood Repopulating Cells of Patients with Gaucher's Disease.	In Vitro /G-CSF Mobilized $CD34^+$ Autologous Peripheral Blood Cells /Retrovirus /Glucocerebrosidase cDNA /Intravenous	Schuening, Friedrich
52.	Gene Therapy /Phase I /Infectious Disease /Human Immunodeficiency Virus /**Immunotherapy**	An Open Label, Phase I /II Clinical Trial to Evaluate the Safety and Biological Activity of **HIV**-IT(V) (HIV-1 IIBenv /Retroviral Vector) in HIV-1 Infected Subjects.	In Vivo /Autologous Muscle Cells /Retrovirus /HIV-1IIIB Envelope Protein /Intramuscular Injection	Haubrich, Richard and Merritt, Jam A
53.	Gene Therapy /Phase I /Cancer /Melanoma /**Immunotherapy**	Adoptive Immunotherapy of Cancer with Activated Lymph Node Cells Primed In Vivo with Autologous Tumor Cells Transduced with the **GM-CSF** Gene.	In Vitro /Autologous Tumor Cells /Lethally Irradiated /Used in Combination with Anti-CD3 and Interleukin-2 Primed Autologous Lymph Node Cells to Prime Autologous Peripheral Blood Cells In Vitro /Retrovirus /GM-CSF cDNA /Intravenous	Chang, Alfr
54.	Gene Therapy /Phase I /Cancer /Neuroblastoma /**Immunotherapy**	A Phase I Study of Immunization with γ **Interferon** Transduced Neuroblastoma Cells.	In Vitro /Autologous Tumor Cells /Allogeneic Tumor Cells /Lethally Irradiated /Retrovirus /γ Interferon cDNA /Subcutaneous Injection	Rosenblatt, Joseph

55.	Gene Therapy /Phase I-II /Infectious Disease /Human Immunodeficiency Virus /**Immunotherapy**	A Phase I /II Pilot Study of the Safety of the Adoptive Transfer of Syngeneic Gene-Modified Cytotoxic T-Lymphocytes in **HIV**-Infected Identical Twins. **Sponsor**: NIH /Cell Genesys, Inc.	In Vitro /$CD8^+$ Syngeneic Peripheral Blood Cells /Retrovirus /CD4-zeta Chimeric Receptor /Intravenous /Concurrent Interleukin-2 Therapy	Walker, Rol
56.	Gene Therapy /Phase I /Other /**Rheumatoid Arthritis**	Clinical Trial to Assess the Safety, Feasibility, and Efficacy of Transferring a Potentially Anti-arthritic Cytokine Gene to Human Joints with Rheumatoid Arthritis.	In Vivo /Autologous Synovial Cells /Retrovirus /Interleukin-1 Receptor Antagonist Protein cDNA /Intrajoint /Metacarpal Phalangeal Joints	Evans, C. H. and Robbins Paul
57.	Gene Marking /Cancer /Ovarian	Use of a Retroviral Vector to Study the Trafficking Patterns of Purified Ovarian TIL Populations Used in Intraperitoneal Adoptive Immunotherapy of Ovarian Cancer Patients: A Pilot Study.	In Vitro /Autologous Peripheral Blood Cells /Autologous **Tumor Infiltrating Lymphocytes** /Retrovirus /Neomycin Phosphotransferase cDNA /Intraperitoneal	Freedman, Ralph
58.	Gene Marking /Cancer /Pediatric Malignancies	Use of Double Marking with Retroviral Vectors to Determine the Rate of Reconstitution of Untreated and Cytokine Expanded $CD34^+$ Selected Marrow Cells in Patients Undergoing Autologous Bone Marrow Transplantation.	In Vitro /CD34+ Autologous Bone Marrow Cells /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Heslop, Hel
59.	Gene Therapy /Phase I /Cancer /Breast /Chemoprotection	Use of Safety-Modified Retroviruses to Introduce Chemotherapy Resistance Sequences into Normal Hematopoietic Cells for Chemoprotection During the Therapy of Breast Cancer: A Pilot Trial.	In Vitro /$CD34^+$ Autologous Peripheral Blood Cells /Retrovirus /**Multi-Drug Resistance**-1 cDNA /Intravenous	Deisseroth, Albert
60.	Gene Therapy /Phase I /Monogenic Disease /Fanconi Anemia	Retroviral Mediated Gene Transfer of the Fanconi Anemia Complementation Group C Gene to Hematopoietic Progenitors of Group C Patients.	In Vitro /$CD34^+$ Autologous Peripheral Blood Cells /Retrovirus /Fanconi Anemia Complementation Group C cDNA /Intravenous	Liu, Johnsor and Young, Neal S
61.	Gene Therapy /Phase I /Cancer /Glioblastoma /**Immunotherapy**	Injection of Glioblastoma Patients with Tumor Cells Genetically Modified to Secrete Interleukin-2 (IL-2): A Phase I Study.	In Vitro /Autologous Fibroblasts /Lethally Irradiated /In Combination with Untransduced Autologous Tumor Cells /Lethally Irradiated /Retrovirus /Cytokine /**Interleukin-2 cDNA** /Subcutaneous Injection	Sobol, Robe and Royston Ivor
62.	Gene Therapy /Phase I /Cancer /Melanoma /Lymphoma /Breast /Head and Neck Cancer /**Immunotherapy**	IL-12 Gene Therapy Using Direct Injection of Tumor with Genetically Engineered Autologous Fibroblasts.	In Vitro /Autologous Fibroblasts /Lethally Irradiated /Retrovirus /Cytokine /**Interleukin-12 cDNA** /Neomycin Phosphotransferase cDNA /Intratumoral /Direct Injection	Lotze, Mich T
63.	Gene Therapy /Phase I /Cancer /Prostate /**Immunotherapy**	Phase I /II Study of Autologous Human **GM-CSF** Gene Transduced Prostate Cancer Vaccines in Patients with Metastatic Prostate Carcinoma.	In Vitro /Autologous Tumor Cells /Lethally Irradiated /Retrovirus /Cytokine /Granulocyte-Macrophage Colony Stimulating Factor cDNA /Subcutaneous Injection	Simons, Jonathan
64.	Gene Therapy /Phase I /Cancer /Breast /Antisense	Gene Therapy for the Treatment of Metastatic Breast Cancer by In Vivo Infection with Breast-Targeted Retroviral Vectors Expressing **Antisense c-fos or Antisense c-myc** RNA.	In Vivo /Autologous Tumor Cells /Retrovirus /c-fos Antisense RNA /c-myc Antisense /Intrapleural /Intraperitoneal	Holt, Jeffrey and Arteaga Carlos B
65.	Gene Therapy /Phase I /Monogenic Disease /**Hunter Syndrome**	Retroviral-Mediated Transfer of the Iduronate-2-Sulfatase Gene into Lymphocytes for Treatment of Mild Hunter Syndrome (Mucopolysaccharidosis Type II).	In Vitro /Autologous Peripheral Blood Cells /Retrovirus /Iduronate-2-Sulfatase cDNA /Intravenous	Whitley, Chester B
66.	Gene Marking /Cancer /Lymphoma /Breast	High Dose Chemotherapy and Autologous Bone Marrow plus Peripheral Blood Stem Cell Transplantation for Patients with Lymphoma or Metastatic Breast Cancer: Use of Marker Genes to Investigate the Biology of Hematopoietic Reconstitution in Adults.	In Vitro /$CD34^+$ Autologous Bone Marrow Cells /$CD34^+$ Autologous Peripheral Blood Cells /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Douer, Dan Kenneth No
67.	Gene Therapy /Phase I /Cancer /Melanoma /**Immunotherapy**	A Phase I Study of Vaccination with Autologous, Irradiated Melanoma Cells Engineered to Secrete Human Granulocyte-Macrophage Colony Stimulating Factor.	In Vitro /Autologous Tumor Cells /Lethally Irradiated /Retrovirus /Cytokine /Granulocyte-Macrophage Colony Stimulating FactorcDNA /Subcutaneous Injection	Dranoff, Gl
68.	Gene Therapy /Phase I /Cancer /Astrocytoma /**Pro-Drug**	Stereotaxic Injection of Herpes Simplex Thymidine Kinase Vector Producer Cells (PA317 /G1TkSvNa.7) and Intravenous Ganciclovir for the Treatment of Recurrent Malignant Glioma. **Sponsor**: Genetic Therapy, Inc. /Novartis	In Vivo /Autologous Tumor Cells /PA317 /Retrovirus /Herpes Simplex Virus Thymidine Kinase cDNA /Ganciclovir /Intratumoral /Stereotactic Injection	Fetell, Mich
69.	Gene Therapy /Phase I /Cancer /Ovarian / **Pro-Drug**	A Phase I Trial of In Vivo Gene Therapy with Herpes Simplex Thymidine Kinase /Ganciclovir System for the Treatment of Refractory or Recurrent Ovarian Cancer.	In Vivo /Autologous Tumor Cells /PA317 /Retrovirus /Herpes Simplex Virus Thymidine Kinase cDNA /Ganciclovir /Intraperitoneal /Catheter	Link, Charle and Moorma Donald

70.	Gene Therapy /Phase I /Cancer /Melanoma /**Immunotherapy**	A Phase I Testing of Genetically Engineered Interleukin-7 Melanoma Vaccines.	In Vitro /Allogeneic Tumor Cells /Lethally Irradiated /Retrovirus /Cytokine /**Interleukin-7 cDNA** /Hygromycin Phosphotransferase /Herpes Simplex Virus Thymidine Kinase cDNA /Subcutaneous Injection	Economou, James
71.	Gene Therapy /Phase I /Cancer /Melanoma /**Immunotherapy**	Phase I /II Study of Immunization with MHC Class I Matched Allogeneic Human Prostatic Carcinoma Cells Engineered to Secrete Interleukin-2 and **Interferon**-γ	In Vitro /HLA-Matched Allogeneic Tumor Cells /Lethally Irradiated /Retrovirus /Cytokine /**Interleukin-2 cDNA** /γ Interferon cDNA /Subcutaneous Injection	Gansbacher Bernd
72.	Gene Therapy /Phase I /Monogenic Disease /**Chronic Granulomatous Disease**	Gene Therapy Approach for Chronic Granulomatous Disease.	In Vitro /G-CSF Mobilized $CD34^+$ Autologous Peripheral Blood Cells /Retrovirus /p47phox /Intravenous	Malech, Ha
73.	Gene Therapy /Phase II /Infectious Disease /Human Immunodeficiency Virus /**Immunotherapy**	A Repeat Dose Safety and Efficacy Study of **HIV**-IT(V) in HIV-1 Infected Subjects with Greater Than or Equal to 100 $CD4^+$ T Cells and No **AIDS** Defining Symptoms.	In Vivo /Autologous Muscle Cells /Retrovirus /HIV-1IIIB Envelope Protein /Intramuscular Injection	Parenti, Dav
74.	Gene Marking /Cancer /Chronic Myelogenous Leukemia	Autologous Marrow Transplantation for Chronic Myelogenous Leukemia Using Stem Cells Obtained After In Vivo Chemotherapy Cytokine Priming.	In Vitro /Autologous G-CSF and ATA-C Mobilized Bone Marrow Cells /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Verfaillie, Catherine
75.	Gene Therapy /Phase I /Cancer /Multiple Myeloma /**Pro-Drug**	Thymidine Kinase (TK) Transduced Donor Leukocyte Infusions as a Treatment for Patients with Relapsed or Persistent Multiple Myeloma after T-cell Depleted Allogeneic Bone Marrow Transplant. **Sponsor**: Genetic Therapy, Inc. /Novartis	In Vitro /Allogeneic T Lymphocytes /Retrovirus /Herpes Simplex Thymidine Kinase /Ganciclovir /Intravenous	Munshi, Nik C. and Barlo Bart
76.	Gene Therapy /Phase I /Cancer /Ovarian /**Immunotherapy**	Treatment of Patients with Advanced Epithelial Ovarian Cancer using Anti-CD3 Stimulated Peripheral Blood Lymphocytes Transduced with a Gene Encoding a Chimeric T-cell Receptor Reactive with Folate Binding Protein.	In Vitro /Anti-CD3 Stimulated Autologous Peripheral Blood Lymphocytes /Retrovirus /Antibody /MOv-γ (Reactive with Folate Binding Protein) /Intravenous /Intraperitoneal	Hwu, Patricl
77.	Gene Therapy /Phase I /Monogenic Disease /**Purine Nucleoside Phosphorylase Deficiency**	Gene Therapy for Purine Nucleoside Phosphorylase Deficiency.	In Vitro /Autologous Peripheral Blood Lymphocytes /Retrovirus /Purine Nucleoside Phosphorylase cDNA /Intravenous	McIvor, R. S
78.	Gene Therapy /Phase I /Infectious Disease /Human Immunodeficiency Virus / Replication Inhibition /Single Chain Antibody Gene	Intracellular Antibodies Against **HIV**-1 Envelope Protein for **AIDS** Gene Therapy.	In Vitro /$CD4^+$ Autologous Peripheral Blood Lymphocytes /Retrovirus /sFv105 Anti-HIV-1 Envelope Protein(gp160)Gene /Intravenous	Marasco, Wayne A
79.	Human Immunodeficiency Virus-1 /**Immunotherapy**	A Randomized, Double Blinded, Phase I /II Dosing Study to Evaluate the Safety and Optimal CTL Inducing Dose of **HIV**-IT(V) in Pre-Selected HIV-1 Infected Subjects.	In Vivo /Autologous Muscle Cells /Retrovirus /HIV-1IIIB Envelope Protein /Intramuscular Injection	Conant, Mar
80.	Gene Therapy /Phase I /Cancer /Glioma /**Immunotherapy**	Gene Therapy of Malignant Gliomas: A Phase I Study of IL-4 Gene -Modified Autologous Tumor to Elicit an Immune Response.	In Vitro /Autologous Tumor (Glioma) Cells /Non-Irradiated /Retrovirus /Cytokine /Interleukin-4 cDNA /Subcutaneous Injection	Bozik, Mich
81.	Gene Therapy /Phase I /Human Immunodeficiency Virus-1	Phase I Study to Evaluate the Safety of Cellular Adoptive Immunotherapy using Autologous Unmodified and Genetically Modified $CD8^+$ **HIV**-Specific T Cells in HIV Seropositive Individuals. **Sponsor**: Targeted Genetics Corporation	In Vitro /$CD8^+$ Allogeneic Cytotoxic T Lymphocytes /$CD8^+$ Syngeneic Cytotoxic T Lymphocytes /Retrovirus /Neomycin Phosphotransferase /Herpes Simplex Virus Thymidine Kinase cDNA /Retrovirus /Intravenous	Riddell, Star R
82.	Gene Therapy /Phase I /Cancer /Prostate /Antisense	Gene Therapy for the Treatment of Advanced Prostate Cancer by In Vivo Transduction with Prostate-Targeted Retroviral Vectors Expressing **Antisense c-myc** RNA.	In Vivo /Autologous Tumor Cells /Retrovirus /Antisense c-myc RNA /Intraprostate Injection	Steiner, Mitc S
83.	Gene Marking /Cancer /EBV-Positive Hodgkin Disease	Administration of Neomycin Resistance Gene Marked EBV Specific Cytotoxic T Lymphocytes as Therapy for Patients Receiving a Bone Marrow Transplant for Relapsed EBV-Positive Hodgkin Disease.	In Vitro /EBV-Specific Cytotoxic T Lymphocytes /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Roskrow, M
84.	Gene Marking /Cancer	Administration of Neomycin Resistance Gene	In Vitro /EBV-Specific Cytotoxic T	Roskrow, M

	/EBV-Positive Hodgkin Disease	Marked EBV Specific Cytotoxic T Lymphocytes to Patients with Relapsed EBV-Positive Hodgkin Disease.	Lymphocytes /Retrovirus /Neomycin Phosphotranspherase cDNA /Intravenous Administration	
85.	Gene Therapy /Phase II /Infectious Disease /Human Immunodeficiency Virus	A Randomized, Controlled, Phase II Study of the Activity and Safety of Autologous CD4-Zeta Gene-Modified T Cells in **HIV**-Infected Patients. **Sponsor**: Cell Genesys	In Vitro /Autologous $CD8^+$ T Cells /Retrovirus /CD4-Zeta Chimeric Receptor /Intravenous	Connick, Elizabeth
86.	Gene Therapy /Phase I /Infectious Disease /Human Immunodeficiency Virus /Replication Inhibition	Phase I Study to Evaluate the Safety and In Vivo Persistence of Adoptively Transferred Autologous $CD4^+$ T Cells Genetically Modified to Resist **HIV** Replication.	In Vitro /Autologous $CD4^+$ T Cells /Retrovirus /Neomycin Phosphotransferase Gene /PolyTAR Decoy Gene /RRE-polyTAR Decoy Gene	Greenberg, Philip D
87.	Gene Therapy /Phase I /Cancer /Metastatic Melanoma /**Immunotherapy**	Phase I Study to Evaluate the Safety of Cellular Adoptive Immunotherapy Using Autologous Unmodified and Genetically Modified $CD8^+$ Tyrosinase-Specific T Cells in Patients with Metastatic Melanoma.	In Vitro /Autologous $CD8^+$ Tyrosinase-Specific TCells /Retrovirus /Hygromycin Phosphotranspherase /Intravenous Administration	Yee, Cassia and Greenb Philip D
88.	Gene Therapy /Phase I /Infectious Disease /Human Immunodeficiency Virus-1 /Replication Inhibition	Intracellular Immunization Against **HIV**-1 Infection Using an Anti-Rev Single Chain Variable Fragment (SFv).	In Vitro /Autologous $CD4^+$ Peripheral Blood Lymphocytes /Retrovirus /Anti-Rev SFv /Intravenous	Pomerantz, Roger J
89.	Gene Therapy /Phase I /Cancer /Breast /Chemoprotection	Antimetabolite Induction, High-Dose Alkylating Agent Consolidation, and Retroviral Transduction of the MDR1 Gene Into Peripheral Blood Progenitor Cells Followed by Intensification Therapy with Sequential Paclitaxel and Doxorubicin for Stage 4 Breast Cancer.	In Vitro /Autologous $CD34^+$ Peripheral Blood Lymphocytes / /Retrovirus /**Multi-Drug Resistance**-1 cDNA /Neomycin Phosphotransferase cDNA /Intravenous	Cowen, Kenneth H
90.	Gene Therapy /Phase I /Cancer /Hematologic Malignancies Following Allogeneic Bone Marrow Transplant /**Pro-Drug** /Elimination of Graft Versus Host Disease	Adoptive Immunotherapy for Leukemia: Donor Lymphocytes Transduced with the Herpes Simplex Thymidine Kinase Gene for Remission Induction.	In Vitro /Allogeneic Peripheral Blood Lymphocytes /Retrovirus /Herpes Simplex Virus Thymidine Kinase cDNA /Ganciclovir /Intravenous	Link, Charle
91.	Gene Therapy /Phase I /Infectious Disease /Human Immunodeficiency Virus /Replication Inhibition /Antisense	Transduction of $CD34^+$ Cells from the Bone Marrow of **HIV**-1 Infected Children: Comparative Marking by and RRE Decoy.	In Vitro /$CD34^+$ Autologous Bone Marrow Cells /Retrovirus /RRE Decoy Gene, and Retrovirus /Neomycin Phosphotransferase Gene /Intravenous	Kohn, Dona
92.	Gene Therapy /Phase I /Cancer /Ovarian /Tumor Suppressor Gene	Ovarian Cancer Gene Therapy with BRCA-1.	In Vivo /Autologous Tumor Cells /Retrovirus /BRCA-1 Gene /Intraperitoneal Administration (Ultrasound Guided)	Holt, Jeffrey
93.	Gene Therapy /Phase I /Inherited Genetic Disorder /Monogenic Disease /**X-Linked Severe Combined Immune Deficiency** /Correction	Gene Therapy for X-linked Severe Combined Immune Deficiency using Retroviral Mediated Transduction of the c cDNA into $CD34^+$ Cells.	In Vitro /$CD34^+$ Autologous Umbilical Cord Blood or Bone Marrow /Retrovirus /cDNA for Common Chain of Multiple Cytokine Receptors /Intravenous	Weinberg, Kenneth I
94.	Gene Therapy /Phase I /Infectious Disease /Human Immunodeficiency Virus /Replication Inhibition /Hammerhead Ribozyme	Transduction of $CD34^+$ Autologous Peripheral Blood Progenitor Cells from **HIV**-1 Infected Persons: a Phase I Study of Comparative Marking Using a Ribozyme Gene and a Neutral Gene.	In Vitro /$CD34^+$ Autologous Peripheral Blood Cells /Retrovirus /Tat and Rev Hammerhead Ribozyme /Intravenous	Kohn, Dona
95.	Gene Therapy /Phase I /Cancer /Brain Tumors /**Pro-Drug**	Phase I Study of Retroviral-Mediated Incorporation of the HSV Thymidine Kinase Gene and Ganciclovir in Malignant Gliomas.	In Vivo /Autologous Tumor Cells /psiCRIP-MFG-S-TK1-67 Cells /Retrovirus /Herpes Simplex Thymidine Kinase cDNA /Ganciclovir /Intratumoral /Direct Injection	Harsh IV, Griffith R.
96.	Gene Therapy /Phase I /Cancer /Ovarian /**Pro-**	Tumor Vaccination With **HER-2 /Neu** Using a B7 Expressing Tumor Cell Line Prior To Treatment With	In Vitro /Allogeneic Tumor Cells /Cationic Liposome Complex /B7(CD80) cDNA	Freeman, Sc M., and

	Drug /Immunotherapy	HSV-TK Gene-Modified Cells.	/Retrovirus /Herpes Simplex Thymidine Kinase /Ganciclovir /Intraperitoneal	Robinson III William R
97.	Gene Therapy /Phase III of #9303-037 /Cancer /Glioblastoma /**Pro-Drug**	Prospective, Open-Label, Parallel-Group, Randomized Multicenter Trial Comparing the Efficacy of Surgery, Radiation, and Injection of Murine Cells Producing Herpes Simplex Thymidine Kinase Vector Followed by Intravenous Ganciclovir Against the Efficacy of Surgery and Radiation in the Treatment of Newly Diagnosed, Previoulsy Untreated Glioblastoma. **Sponsor**: Genetic Therapy, Inc. /Novartis	In Vivo /Autologous Tumor Cells /PA317 /Retrovirus /Herpes Simplex Virus Thymidine Kinase cDNA /Ganciclovir /Intratumoral /Direct Injection	Maria, Bern
98.	Gene Marking /Cancer /Pediatric Malignancies	A Comparative Evaluation of the Utility of Hemopoietic Progenitor Cells Derived from Peripheral Blood vs Bone Marrow.	In Vitro /$CD34^+$ Autologous Bone Marrow and Peripheral Blood /Retrovirus /Neomycin Phosphotransferase cDNA /Bone Marrow Transplant	Heslop, Hel E.
99.	Gene Therapy /Phase II /Cancer /Glioblastoma /**Pro-Drug**	Multicenter, Extension Trial for the Treatment of Recurrent Glioblastoma Multiforme with Surgery and Injection of Murine Cells Producing Herpes Simplex Thymidine Kinase Vector Followed by Intravenous Ganciclovir for Patients with Disease Progression Following Standard Treatment on Protocol GTI-0115. **Sponsor**: Genetic Therapy, Inc. /Novartis	In Vivo /Autologous Tumor Cells /PA317 /Retrovirus /Herpes Simplex Thymidine Kinase cDNA /Ganciclovir /Intratumoral /Direct Injection	Maria, Bern et.al.
100.	Gene Therapy /Phase I /Cancer /Germ Cell Tumors (Testicular Cancer) /Chemoprotection	High Dose Carboplatin and Etoposide Followed by Transplantation with Peripheral Blood Stem Cells Transduced with the Multiple Drug Resistance Gene in the Treatment of Germ Cell Tumors - A Pilot Study.	In Vitro /G-CSF Mobilized Autologous $CD34^+$ Peripheral Blood Cells /Retrovirus /**Multi-Drug Resistance**-1 cDNA /Bone Marrow Transplant	Cornetta, Kenneth
101.	Gene Therapy /Phase I /Cancer /Brain Tumors /Chemoprotection	A Pilot Study of Dose Intensified Procarbazine, CCNU, Vincristine(PCV) for Poor Prognosis Pediatric and Adult Brain Tumors Utilizing Fibronectin-Assisted, Retroviral-Mediated Modification of $CD34^+$ Peripheral Blood Cells with **O^6-Methylguanine DNA Methyltransferase**.	In Vitro /Peripheral Blood $CD34^+$ Cells /Retrovirus /O6-Methylguanine DNA Methyltransferase cDNA /Intravenous Infusion	Williams, D A
102.	Gene Therapy /Phase I /Cancer /Melanoma /**Immunotherapy**	A Pilot Study Using Interleukin-2 Transfected Irradiated Allogeneic Melanoma Cells Encapsulated in an Immunoisolation Device In Patients with Metastatic Malignant Melanoma.	In Vitro /Allogeneic Tumor Cells /Lethally Irradiated /Retrovirus /**Interleukin-2 cDNA** /Neomycin Phosphotransferase cDNA /Immunoisolation Device /Subcutaneous Implantation	Das Gupta, Tapas K
103.	Gene Marking /Cancer /Chronic Myelogenous Leukemia	Autologous Marrow Transplantation for Chronic Myelogenous Leukemia Using Retrovirally Marked Peripheral Blood Progenitor Cells Obtained after In Vivo Cyclophosphamide /G-CSF Priming.	In Vitro /Autologous Peripheral Blood Cells Mobilized by Cyclophosphamide and G-CSF /Retrovirus /Neomycin Phosphotransferase cDNA /Autologous Bone Marrow Transplant	Verfaille, Catherine,
104.	Gene Therapy /Phase I /Infectious Disease /Human Immunodeficiency Virus /Replication Inhibition	Phase I Clinical Trial of TREV Gene Therapy for Pediatric **AIDS**.	In Vitro /$CD34^+$ Autologous Cord Blood Cells /Retrovirus /Transdominant Trev /Intravenous	Belmont, Jol W
105.	Gene Therapy /Phase II /Infectious Disease /Human Immunodeficiency Virus	A Phase II Study of the Activity and Safety of Autologous CD4-Zeta Gene-Modified T Cells With or Without Exogenous Interleukin-2 in **HIV** Infected Patients. **Sponsor**: Cell Genesys, Inc.	In Vitro /Autologous $CD8^+$ and $CD4^+$ T Lymphocytes /Retrovirus /CD4-Zeta Chimeric Receptor /Intravenous /Concurrent Interleukin-2 Therapy	Connick, Elizabeth
106.	Gene Marking /Cancer /EBV-Positive Hodgkin Disease	Administration of Neomycin Resistance Gene Marked EBV Specific Cytotoxic T-Lymphocytes To Patients With Relapsed EBV-Positive Hodgkin Disease.	In Vitro /EBV-Specific Hodgkin Disease /In Vitro /EBV-Specific Cytotoxic Lymphocytes /Retrovirus /Neomycin Phosphotransferase /Bone Marrow Transplant	Straus, Stepl E
107.	Gene Therapy /Phase I /Cancer /Chronic Myelogenous Leukemia /Chemoprotection /Tyr-22 Murine Dihydrofolate Reductase Gene /**Antisense** /Anti-b3a2BCR /ABL Gene	Autologous Transplantation for Chronic Myelogenous Leukemia with Stem Cells Transduced with a Methotrexate Resistant DHFR and Anti-BCR /ABL Containing Vector and Post Transplant Methotrexate Administration.	In Vitro /Autologous Peripheral Blood $CD34^+$ Cells Mobilized by Cyclophosphamide and G-CSF /Retrovirus /Autologous Bone Marrow Transplant	Verfaillie, Catherine
108.	Gene Therapy /Phase II /Infectious Disease /Human	A Phase II, Randomized, Double Blind Placebo Controlled Study of Combination Drug Anti-Retroviral Therapy to Include a Reverse	In Vivo /Autologous Muscle Cells /Retrovirus /HIV-1 IIIB Envelope Protein /Intramuscular Injection	Aboulafia, David

	Immunodeficiency Virus /**Immunotherapy**	Transcriptase Inhibitor and a Protease Inhibitor Plus **HIV**-IT(V) or Placebo in HIV Patients with $CD4^+$ Counts > 100, and HIV RNA > 1K, and < 10K **Sponsor**: Chiron Corporation		
109.	Gene Therapy /Phase I /Monogenic Disease /**Chronic Granulomatous Disease**	Fibronectin-Assisted, Retroviral-Mediated Transduction of $CD34^+$ Peripheral Blood Cells with gp91 phox in Patients with X-Linked Chronic Granulomatous Disease: A Phase I Study	In Vitro /G-CSF Mobilized $CD34^+$ Autologous Peripheral Blood Cells /Retrovirus /gp91phox /Intravenous Infusion	Smith, Frank O., and Dinauer, Ma C
110.	Gene Therapy /Phase I /II /Cancer /Colorectal Carcinoma Expressing TAG-72	A Phase I /II Study of Autologous CC49-Zeta Gene-Modified T Cells and a-Interferon in Patients with Advanced Colorectal Carcinomas Expressing the Tumor-Associated Antigen, TAG-72 **Sponsor**: Cell Genesys, Inc.	In Vitro /Autologous $CD8^+$ and $CD4^+$ T Lymphocytes /Retrovirus /CC49-Zeta T Cell Receptor /Intravenous Infusion	Venook, Ala
111.	Gene Therapy /Phase I /Cancer /Melanoma /Breast /Head and Neck Cancer /Cutaneous T-Cell Lymphoma /Immumotherapy	IL-12 Gene Therapy Using Direct Injection of Tumors with Genetically Engineered Autologous Fibroblasts	In Vitro /Autologous Fibroblasts /Lethally Irradiated /Retrovirus /Cytokine /**Interleukin-12** /Intratumoral Injection	Park, Chan I
112.	Gene Therapy /Phase I /Monogenic Disease /**Leukocyte Adherence Deficiency** (LAD)	Retrovirus-Mediated Transfer of the cDNA for Human CD18 into Perpheral Blood Repopulating Cells of Patients with Leukocyte Adherence Deficiency	In Vitro /G-CSF Mobilized $CD34^+$ Autologous Peripheral Blood Cells /Retrovirus /CD18 /Intravenous Infusion	Hickstein, Dennis
113.	Gene Therapy /Phase I /II /Cancer /Prostate /**Immunotherapy**	Phase I /II Study of Allogeneic Human **GM-CSF** Gene Transduced Irradiated Prostate Cancer Cell Vaccines in Patients with Prostate Cancer	In Vitro /Allogeneic Tumor Cells /Lethally Irradiated /Retrovirus /Cytokine /Granulocyte-Macrophage Colony Stimulating Factor /Subcutaneous Injection	Simons, Jonathan W
114.	Gene Therapy /Phase I /II /Cancer /Chronic Myelogenous Leukemia /Adoptive **Immunotherapy**	Infusion of Polyclonal HyTK (hygromycin phosphotransferase and HSV thymidine kinase gene)-transduced Donor T Cells for Adoptive Immunotherapy in Patients with Relapsed CML after Allogeneic Stem Cell Transplant: Phase I-II Clinical Trial	In Vitro /Donor $CD8^+$ and $CD4^+$ Lymphocytes /Retrovirus /Hygromycin Phosphotransferase-Herpes Simplex Thymidine Kinase Fusion Gene /Intravenous Infusion	Flowers, Ma E. D. and Riddell, Star
115.	Gene Therapy /Phase I /Cancer /Mesothelioma /**Pro-Drug**	The Treatment of Malignant Pleural Mesothelioma with aGene-Modified Cancer Vaccine: A Phase I Study	In Vivo /Allogeneic Tumor Cells /Lethally Irradiated /Retrovirus /Herpes Simplex Virus Thymidine Kinase /Ganciclovir /Intrapleural Administration	Schwarzenb er, Paul
116.	Gene Therapy /Phase II /Infectious Disease /Human Immunodeficiency Virus	A Phase II Study of Autologous CD4-Zeta Gene-Modified T Cells in **HIV**-Infected Patients with Undectable Plasma Viremia on Combination Antiretroviral Drug Therapy **Sponsor**: Cell Genesys, Inc.	In Vitro /Autologous $CD8^+$ T Cells /Retrovirus /CD4-Zeta Chimeric Receptor /Intravenous	Deeks, Stev G
117.	Gene Therapy /Phase II /Infectious Disease /Human Immunodeficiency Virus /Replication Inhibition /Hammerhead Ribozyme	High Dose Chemotherapy and Autologous Peripheral Stem Cell Transplantation for **HIV** Lymphomas: A Phase IIa Study of Comparative Marking Using a Ribozyme Gene and a Neutral Gene **Sponsor**: Ribozyme Pharmaceuticals, Inc.	In Vitro /$CD34^+$ Autologous Peripheral Blood Cells /Retrovirus /Tat and Rev Hammerhead Ribozyme /Intravenous	Krishnan, Amrita and Zaia, John, A
118.	**ADENOVIRUS** Gene Therapy /Phase I /Monogenic Disease /**Cystic Fibrosis**	A Phase I Study, in Cystic Fibrosis Patients, of the Safety, Toxicity, and Biological Efficacy of a Single Administration of a Replication Deficient, Recombinant Adenovirus Carrying the cDNA of the Normal Human Cystic Fibrosis Transmembrane Conductance Regulator Gene in the Lung.	In Vivo /Nasal Epithelial Cells /Respiratory Epithelial Cells /Adenovirus /Serotype 5 /Cystic Fibrosis Transmembrane Conductance Regulator cDNA /Intranasal /Respiratory Tract Administration (Bronchoscope)	Crystal, Ron G
119.	Gene Therapy /Phase I /Monogenic Disease /**Cystic Fibrosis**	Gene Therapy of Cystic Fibrosis Lung Diseases Using E1 Deleted Adenoviruses: A Phase I Trial.	In Vivo /Nasal Epithelial Cells /Respiratory Epithelial Cells /Adenovirus /Serotype 5 /E2a Temperature Sensitive Mutant /Cystic Fibrosis Transmembrane Conductance Regulator cDNA /Intranasal /Respiratory Tract Administration (Bronchoscope)	Wilson, Jam M
120.	Gene Therapy /Phase I /Monogenic Disease /**Cystic Fibrosis**	Cystic Fibrosis Gene Therapy Using an Adenovirus Vector: In Vivo Safety and Efficacy in Nasal Epithelium.	In Vivo /Nasal Epithelial Cells /Adenovirus /Serotype 2 /Cystic Fibrosis Transmembrane Conductance Regulator cDNA /Intranasal	Welsh, Mich J and Smith, Alan E

		Sponsor: Genzyme Corporation		
121.	Gene Therapy /Phase I /Monogenic Disease /**Cystic Fibrosis**	A Phase I Study of Gene Therapy of Cystic Fibrosis Utilizing a Replication Deficient Recombinant Adenovirus Vector to Deliver the Human Cystic Fibrosis Transmembrane Conductance Regulator cDNA to the Airways.	In Vivo /Nasal Epithelial Cells /Respiratory Epithelial Cells /Adenovirus /Serotype 5 /Cystic Fibrosis Transmembrane Conductance Regulator cDNA /Intranasal /Respiratory Tract Administration (Bronchoscope)	Wilmott, Ro W
		Sponsor: Genetic Therapy, Inc. /Novartis		
122.	Gene Therapy /Phase I /Monogenic Disease /**Cystic Fibrosis**	Gene Therapy for Cystic Fibrosis Using E1 Deleted Adenovirus: A Phase I Trial in the Nasal Cavity.	In Vivo /Nasal Epithelial Cells /Adenovirus /Serotype 5 /Cystic Fibrosis Transmembrane Conductance Regulator CDNA /Intranasal	Boucher, Richard C. a Knowles, Michael R
123.	Gene Therapy /Phase I /Monogenic Disease /**Cystic Fibrosis**	Adenovirus-Mediated Gene Transfer of CFTR to the Nasal Epithelium and Maxillary Sinus of Patients with Cystic Fibrosis.	In Vivo /Nasal Epithelial Cells /Maxillary Sinus Epithelial Cells /Adenovirus /Serotype 2 /Cystic Fibrosis Transmembrane Conductance Regulator cDNA /Intranasal /Maxillary Sinus Administration	Welsh, Micl J
		Sponsor: Genzyme Corporation		
124.	Gene Therapy /Phase I /Cancer /Non-small Cell Lung Cancer /Tumor Suppressor Gene	Clinical Protocol for Modification of Tumor Suppressor Gene Expression and Induction of Apoptosis in Non-Small Cell Lung Cancer (NSCLC) with an Adenovirus Vector Expressing Wildtype p53 and Cisplatin.	In Vivo /Autologous Tumor Cells /Adenovirus /Serotype 5 /**p53 cDNA** /Intratumoral /Bronchoscope	Roth, Jack A
125.	Gene Therapy /Phase I /Monogenic Disease /**Cystic Fibrosis**	Evaluation of Repeat Administration of a Replication Deficient, Recombinant Adenovirus Containing the Normal Cystic Fibrosis Transmembrane Conductance Regulator cDNA to the Airways of Individuals with Cystic Fibrosis.	In Vivo /Nasal Epithelial Cells /Respiratory Epithelial Cells /Adenovirus /Serotype 5 /Cystic Fibrosis Transmembrane Conductance Regulator cDNA /Intranasal /Respiratory Tract Administration (Bronchoscope) /Multiple Dose	Crystal, Ron G
126.	Gene Therapy /Phase I /Cancer /Central Nervous System /**Pro-Drug**	Treatment of Advanced CNS Malignancy with the Recombinant Adenovirus H5.020RSVTK: A Phase I Trial.	In Vivo /Autologous Tumor Cells /Adenovirus /Serotype 5 /Herpes Simplex Virus Thymidine Kinase cDNA /Ganciclovir /Stereotactic Injection	Eck, Stephei and Alavi, J B
127.	Gene Therapy /Phase I /Cancer /n /**Pro-Drug**	Treatment of Advanced Mesothelioma with the Recombinant Adenovirus H5.010RSVTK: A Phase I Trial.	In Vivo /Autologous Tumor Cells /Adenovirus /Serotype 5 /Herpes Simplex Virus Thymidine Kinase cDNA /Ganciclovir /Intrapleural	Albelda, Ste M
128.	Gene Therapy /Phase I /Monogenic Disease /**Cystic Fibrosis**	Adenovirus Mediated Gene Transfer for Cystic Fibrosis: Safety of Single Administration in the Lung (lobar instillation).	In Vivo /Respiratory Epithelial Cells /Adenovirus /Serotype 2 /Cystic Fibrosis Transmembrane Conductance Regulator cDNA /Respiratory Epithelial Cells /Bronchoscope	Dorkin, Her L and Lapey Allen
		Sponsor: Genzyme Corporation		
129.	Gene Therapy /Phase I /Monogenic Disease /**Cystic Fibrosis**	Adenovirus Mediated Gene Transfer for Cystic Fibrosis: Safety of a Single Administration in the Lung (aerosol administration).	In Vivo /Respiratory Epithelial Cells /Adenovirus /Serotype 2 /Cystic Fibrosis Transmembrane Conductance Regulator cDNA /Respiratory Epithelial Cells /Aerosol Administration	Dorkin, Her L. and Lape Allen
		Sponsor: Genzyme Corporation		
130.	Gene Therapy /Phase I /Cancer /Head and Neck Squamous Cell /Tumor Suppressor Gene	Clinical Protocol for Modification of Tumor Suppressor Gene Expression in Head and Neck Squamous Cell Carcinoma (HNSCC) with an Adenovirus Vector Expressing Wild-type p53.	In Vivo /Autologous Tumor Cells /Adenovirus /Serotype 5 /**p53 cDNA** /Intratumoral /Bronchoscope	Clayman, G
131.	Gene Therapy /Phase I /Cancer /Colon /Hepatic Metastases /Tumor Suppressor Gene	Gene Therapy of Primary and Metastatic Malignant Tumors of the Liver Using ACN53 Via Hepatic Artery Infusion: A Phase I Study.	In Vivo /Autologous Tumor Cells /Adenovirus /Serotype 5 /**p53 cDNA** /Intrahepatic /Hepatic Artery /Bolus Infusion	Venook, Ala and Warren Robert
		Sponsor: Schering Plough Corporation (formerly Canji)		
132.	Gene Therapy /Phase I /Cancer /Central Nervous System Malignancies /**Pro-Drug**	Phase I Study of Adenoviral Vector Delivery of the HSV-TK Gene and the Intravenous Administration of Ganciclovir in Adults with Malignant Tumors of the Central Nervous System.	In Vivo /Autologous Tumor Cells /Adenovirus /Serotype 5 /Herpes Simplex Virus Thymidine Kinase cDNA /Ganciclovir /Intra- tumoral /Stereotactic Injection	Grossman, Robert and Woo, Savio
133.	Gene Therapy /Phase I /Cancer /Ovarian and Extraovarian /**Anti-erbB-2 Single Chain Antibody Gene**	A Phase I Study of Recombinant Adenovirus Vector-Mediated Delivery of an Anti-erbB-2 Single Chain (sFv) Antibody Gene for Previously Treated Ovarian and Extraovarian Cancer Patients.	In Vivo /Autologous Tumor Cells /Adenovirus /Anti-erbB-2 (oncoprotein /extracellular domain) Single-chain Antibody Gene /Intraperitoneal Injection	Curiel, Davi and Alvarez Ronald D
134.	Gene Therapy /Phase I /Cancer /Colon Carcinoma (Hepatic	A Phase I Study of Direct Administration of a Replication-Deficient Adenovirus Vector Containing the E. coli Cytosine Deaminase Gene to Metastatic	In Vivo /Autologous Tumor Cells /Adenovirus /E. coli **Cytosine Deaminase cDNA** /Intratumoral (Hepatic) Injection /Combined with	Crystal, Ron G

	Metastases) /**Pro-Drug**	Colon Carcinoma of the Liver in Association with the Oral Administration of the Pro-Drug 5-Fluorocytosine.	Oral 5-Fluorocytosine	
135.	Gene Therapy /Phase I /Cancer /Neuroblastoma /**Immunotherapy**	Phase I Study of Cytokine Gene Modified Autologous Neuroblastoma Cells for Treatment of Relapsed /Refractory Neuroblastoma Using an Adenoviral Vector.	In Vitro /Autologous Tumor Cells (Non-irradiated) /Type 5 Adenovirus /Cytokine /**Interleukin-2 cDNA** /Subcutaneous Injection	Brenner, Malcolm K
136.	Gene Therapy /Phase I /Cancer /Ovarian and Extraovarian Cancer /Single Chain Antibody	A Phase I Study of Recombinant Adenovirus Vector-Mediated Intraperitoneal Delivery of Herpes Simplex Virus Thymidine Kinase (HSV-TK) Gene and Intravenous Ganciclovir for Previously Treated Ovarian and Extraovarian Cancer Patients.	In Vivo /Autologous Tumor Cells /Adenovirus /**Herpes Simplex Thymidine Kinase Gene** /Intraperitoneal Injection /Combined with Intravenous Ganciclovir Administration	Alvarez, Ro D. and Curie David T
137.	Gene Therapy /Phase I /Monogenic Disease /**Partial Ornithine Transcarbamylase (OTC) Deficiency**	A Phase I Study of Adenoviral Vector Mediated Gene Transfer to Liver in Adults with Partial Ornithine Transcarbamylase Deficiency.	In Vivo /Autologous Peripheral Blood Cells /Adenovirus /Type 5 (E2a Temperature-Sensitive Mutant) /Ornithine Transcarbamylase cDNA /Intravenous	Batshaw, M
138.	Gene Therapy /Phase I /Cancer /Melanoma /**Immunotherapy**	Phase I Trial in Patients with Metastatic Melanoma of Immunization with a Recombinant Adenovirus Encoding the MART-1 Melanoma Antigen.	In Vivo /Adenovirus /Type 2 /MART-1 Melanoma Antigen /Subcutaneous Injection /Immunization	Rosenberg, Steven A
139.	Gene Therapy /Phase I /Cancer /Prostate /**Pro-Drug**	Phase I Study of Adenoviral Vector Delivery of the HSV-tk Gene and the Intravenous Administration of Ganciclovir in Men with Local Recurrence of Prostate Cancer after Radiation Therapy.	In Vivo /Autologous Tumor Cells /Adenovirus /Serotype 5 /Herpes Simplex Virus Thymidine Kinase cDNA /Ganciclovir /Intratumoral /Intra-prostatic Tumor Injection	Scardino, Pe T
140.	Gene Therapy /Phase I /Cancer /Bladder /Tumor Suppressor Gene	Gene Therapy of Bladder Cancer Using Recombinant Adenovirus Containing the Retinoblastoma Gene (ACNRB): A Phase IA Study. **Sponsor**: Schering Plough Corporation (formerly Canji)	In Vivo /Autologous Tumor Cells /Adenovirus /Serotype 5 /**Retinoblastoma** cDNA /Intravesical Catheter Administration	Small, Eric J and Carroll, Peter R
141.	Gene Therapy /Phase I /Cancer /Head and Neck Squamous Cell Carcinoma /**Pro-Drug**	Phase I Study of Adenoviral Vector Delivery of the HSV-tk Gene and the Intravenous Administration of Ganciclovir in Adults with Recurrent or Persistent Head and Neck Cancer.	In Vivo /Autologous Tumor Cells /Adenovirus /Serotype 5 /Herpes Simplex Virus Thymidine Kinase cDNA /Ganciclovir /Intratumoral Injection	O'Malley, B W
142.	Gene Therapy /Phase I / Cancer /Melanoma /**Immunotherapy**	Phase I Trial in Patients with Metastatic Melanoma of Immunization with a Recombinant Adenovirus Encoding the GP100 Melanoma Antigen.	In Vivo /AutologousTumor Cells /Adenovirus /Serotype 2 /GP100 Melanoma Antigen /Subcutaneous or Intramuscular Injection /Concurrent Interleukin-2 Therapy	Rosenberg, Steven A
143.	Gene Therapy /Phase I /Cancer /Liver(Hepatic)Metastases /**Pro-Drug**	Phase I Trial of Adenoviral Vector Delivery of the Herpes Simplex Thymidine Kinase Gene by Intratumoral Injection Followed by Intravenous Ganciclovir in Patients with Hepatic Metastases.	In Vivo /Autologous Tumor Cells /Adenovirus /Serotype 5 /Herpes Simplex Thymidine Kinase Gene /Ganciclovir /Intratumoral Injection	Sung, Max V and Woo, Sa L.C
144.	Non-Therapeutic	Immune Response to Intradermal Administration of an Adenovirus Type 5 Gene Transfer Vector (AdGVCD.10) in Normal Individuals.	In Vivo /Intradermal Cells /Adenovirus /Serotype 5 /E.coli Cytosine Deaminase /Intradermal Injection	Harvey, Ber Gary, and Crystal, Ron G
145.	Gene Therapy /Phase I /Cancer /Glioblastoma /**Pro-Drug**	Gene Therapy for Recurrent Glioblastoma Multiforme: Phase I Trial of Intraparenchymal Adenoviral Vector Delibvery of the HSV-TK Gene and Intravenous Administration of Ganciclovir.	In Vivo /Autologous Tumor Cells /Adenovirus /Serotype 5 /Herpes Simplex Thymidine Kinase cDNA /Ganciclovir /Intratumoral /Stereotactic Injection	Lieberman, Frank
146.	Gene Therapy /Phase I /Cancer /Prostate /**Pro-Drug**	Phase I Trial of Adenoviral-Mediated Herpes Simplex Thymidine Kinase Gene Transduction in Conjuction with Ganciclovir Therapy as Neo-adjuvant Treatment for Patients with Clinically Localized (Stage T1c and T2b&c)Prostate Cancer Prior to Radical Prostatectomy.	In Vivo /AutologousTumor Cells /Adenovirus /Serotype 5 /Herpes Simplex Thymidine Kinase Gene /Ganciclovir /Intratumoral Injection	Hall, Simon and Woo, Sa L.C
147.	Gene Therapy /Phase I /Cancer /Hepatocellular Carcinoma /Tumor Supressor Gene	Phase I Study of Percutaneous Injections of Adenovirus p53 Construct (Adeno-p53) for Hepatocellular Carcinoma.	In Vivo /AutologousTumor Cells /Adenovirus /Serotype 5 /**p53 cDNA** /Intratumoral Injection	Belani, Char P
148.	Gene Therapy /Phase I /Cancer /Prostate /Tumor suppressor Gene	A Phase I Study in Patients with Locally Advanced or Recurrent Adenocarcinoma of the Prostate Using SCH58500 (rAd /p53) Administered by Intratumoral Injection **Sponsor**: Schering-Plough Corporation	In Vivo /Autologous Tumor Cells /Adenovirus /Serotype 5 /**p53 cDNA** /Intratumoral Injection	Belldegrun, Arie, and Fig Robert
149.	Gene Therapy /Phase I /**Immunotherapy** /Cancer /Melanoma	A Phase I Study of Vaccination with Autologous, Lethally Irradiated Melanoma Cells Engineered by Adenoviral Mediated Gene Transfer to Secrete Human Granulocyte-Macrophage Colony Stimulating	In Vitro /Autologous Tumor Cells /Lethally Irradiated /Adenovirus /Serotype 5 /Cytokine /**Granulocyte-Macrophage Colony Stimulating Factor** (**GM-CSF**) /Subcutaneous Injection	Dranoff, Gl and Soiffer, Robert

		Factor		
150.	Gene Therapy /Phase I /**Immunotherapy** /Cancer /Non-Small Cell Lung Carcinoma (NSCLC)	A Phase I Study of Vaccination with Autologous, Lethally Irradiated Non-Small Cell Lung Carcinoma Cells Engineered by Adenoviral Mediated Gene Transfer to Secrete Human Granulocyte-Macrophage Colony Stimulating Factor	In Vitro /Autologous Tumor Cells /Lethally Irradiated /Adenovirus /Serotype 5 /Cytokine /Granulocyte-Macrophage Colony Stimulating Factor (**GM-CSF**) /Subcutaneous Injection	Dranoff, Gl and Salgia, I
151.	Non-Therapeutic (under review)	Systemic and Respiratory Immune Response to Administration of an Adenovirus Type 5 Gene Transfer Vector (AdGVCD.10)	In Vivo /Bronchial Epithelial Cells /Adenovirus /Serotype 5 /E. coli Cytosine Deaminase /Intrabronchial Administration	Harvey, Ber Gary and Crystal, Ron G
152.	Gene Therapy /Phase II /Cancer /Head and Neck Squamous Cell Carcinoma /Tumor Suppressor Gene	A Phase II Multi-Center, Open Label, Randomized Study to Evaluate Effectiveness and Safety of Two Treatment Regimens of Ad5CMV-p53 Administered by Intra-Tumoral Injections in 78 Patients with Recurrent Squamous Cell Carcinoma of the Head and Neck (SCCHN) **Sponsor**: Gencell (Division of Rhone-Poulenc Rorer Pharmaceuticals)	In Vivo /Autologous Tumor Cells /Adenovirus /Serotype 5 /**p53**	Breau, Rand L
153.	Gene Therapy /Phase I /Cancer /Breast /Tumor Suppressor Gene	Phase I /Pilot Study of p53 Intralesional Gene Therapy with Chemotherapy in Breast Cancer **Sponsor**: National Cancer Institue - Cancer Therapy Evaluation Program (NCI-CTEP)	In Vivo /Autologous Tumor Cells /Adenovirus /Serotype 5 /**p53 cDNA** /Cutaneous or Subcutaneous	von Mehren Margaret
154.	Gene Therapy /Phase I-II /Cancer /Prostate /Tumor Suppressor Gene	A Tolerance and Efficacy Study of Intraprostatic INGN 201 Followed by Pathological Staging and Possible Radical Prostatectomy in Patients with Locally Advanced Prostate Cancer **Sponsor**: Introgen Therapeutics, Inc.	In Vivo /Autologous Tumor Cells /Adenovirus /Serotype 5 /**p53 cDNA** /Intratumoral Injection	Logothetis, Christopher
155.	Gene Therapy /Phase I /Cancer /Bladder /Tumor Suppressor Gene	A Phase I Trial of Intravesical Ad-p53 Treatment in Locally Advanced and Metastatic Bladder Cancer	In Vivo /Autologous Tumor Cells /Adenovirus /Serotype 5 /**p53 cDNA** /Intravesical Administration	Pagliaro, La C
156.	Gene Therapy /Phase II /Cancer /Non-Small Cell Lung Cancer /Tumor Suppressor Gene (under review)	A Phase II Gene Therapy Study in Patients wigh Non-Small Cell Lung Cancer Using SCH 58500 (rAd /p53) in Combination with Chemotherapy for Multiple Cycles **Sponsor**: Schering Plough Research Institute	In Vivo /Autologous Tumor Cells /Adenovirus /Serotype 5 /**p53 cDNA** /Bronchoscopy or Percutaneous Intratumoral Injection	Dobbs, Trac W
157.	Gene Therapy /Phase I /Other / Coronary Artery Disease (under review)	Phase I Study of Direct Administration of a Replication-Deficient Adenovirus Vector (AdGVVEGF121.10) Containing the VEGF121 cDNA to the Ischemic Myocardium of Individuals with Life Threatening Diffuse Coronary Artery Disease **Sponsor**: GenVec, Inc.	In Vivo /Ischemic Myocardium /Adenovirus /Serotype 5 /Vascular Endothelial Growth Factor (**VEGF**) cDNA /Cardiac Administration	Crystal, Ron G
158.	**Naked Plasmid DNA** Gene Therapy /Phase I /Colon /**Immunotherapy**	Phase I Trial of a Polynucleotide Augmented Anti-Tumor Immunization to Human Carcinoembryonic Antigen in Patients with Metastatic Colorectal Cancer.	In Vivo /Autologous Tumor Cells /Plasmid DNA /Carcinoembryonic Antigen Plasmid Expression Vector /Kanamycin Resistance cDNA /Intratumoral /Direct Injection	Curiel, Davi
159.	Gene Therapy /Phase I /Other /**Peripheral Artery Disease**	Arterial Gene Transfer for Therapeutic Angiogenesis in Patients with Peripheral Artery Disease.	In Vivo /Vascular Endothelial Cells /Plasmid DNA /**Vascular Endothelial Growth Factor cDNA** /Intraarterial /Angioplasty Catheter /Hydrogel Coated Balloon	Isner, Jeffre M. and Wals Kenneth
160.	Gene Therapy /Phase I /Other /**Restenosis**	Accelerated Re-endothelialization and Reduced Neointimal Thickening Following Catheter Transfer of phVEGF165.	In Vivo /Vascular Endothelial Cells /Plasmid DNA /**Vascular Endothelial Growth Factor cDNA** /Intraarterial /Angioplasty Catheter /Hydrogel Coated Balloon	Isner, Jeffre M
161.	Gene Therapy /Phase I /II /Cancer /Non-Hodgkin's B-Cell Lymphoma /Mantle Cell Lymphoma /Immumotherapy	A Phase I /II Study of Vaccine Therapy for B-Cell Lymphoma Utilizing Plasmid DNA Coding for Tumor Idiotype. **Sponsor**: Vical, Inc.	In Vivo /Naked Plasmid DNA /Tumor Idiotype /Intramuscular Injection	Levy, Ronal
162.	Gene Therapy /Phase I /Cancer /Malignant Glioma /Antisense	A Phase I Study of the Safety of Injecting Malignant Glioma Patients with Irradiated **TGF-ß2 Antisense** Gene Modified Autologous Tumor Cells.	In Vitro /Autologous Tumor Cells /Lethally Irradiated /Plasmid DNA--Electroporation /TGF-ß2 /Subcutaneous Injection	Black, Keith and Fakhrai Habib
163.	Gene Therapy /Phase I-	Phase I /IB Study of Immunization with Autologous	In Vitro /Autologous Tumor Cells /Lethally	Mahvi, Davi

	IB /Cancer /Melanoma or Sarcoma /**Immunotherapy**	Tumor Cells Transfected with the **GM-CSF** Gene by Particle-Mediated Transfer in Patients with Melanoma or Sarcoma.	Irradiated /Plasmid DNA /Particle Mediated Gene Transfer (Accell®) /Cytokine /GM-CSF cDNA /Subcutaneous Injection	
164.	Gene Therapy /Phase I /Other /**Cubital Tunnel Syndrome**	Phase I Single Dose-Ranging Study Of Formulated hIGF-I Plasmid In Subjects With Cubital Tunnel Syndrome. **Sponsor**: Gene Medicine, Inc.	In Vivo /Autologous Muscle Cells /Plasmid DNA /Polyvinylpyrrolidone (PVP) /Human Insulin-Like Growth Factor-1(hIGF-1) /Intramuscular Injection	Netscher, D
165.	**Adeno-Associated Virus** Gene Therapy /Phase I /Monogenic Disease /**Cystic Fibrosis**	A Phase I Study of an Adeno-associated Virus-CFTR Gene Vector in Adult CF Patients with Mild Lung Disease. **Sponsor**: Targeted Genetics Corporation	In Vivo /Nasal Epithelial Cells /Respiratory Epithelial Cells /**Adeno-Associated Virus** /Cystic Fibrosis Transmembrane Conductance Regulator cDNA /Intranasal /Respiratory Tract Administration (Bronchoscope)	Flotte, Tere R and Zeitli Pamela L
166.	Gene Therapy /Phase II /Monogenic Inherited Disorder /**Cystic Fibrosis** /Sinusitis /Correction	A Phase I /II Study of tgAAVCF for the Treatment of Chronic Sinusitis With Cystic Fibrosis. **Sponsor**: Targeted Genetics Corporation	In Vivo /Maxillary Sinus Epithelial Cells /**Adeno-Associated Virus** /Cystic Fibrosis Transmembrane Conductance Regulator cDNA /Maxillary Sinus Administration	Gardner, Phy
167.	**IMMUNOTHERAPY or OTHER** Gene Therapy /Phase I /Monogenic Disease /**Familial Hypercholesterolemia**	Ex Vivo Gene Therapy of Familial Hypercholesterolemia.	In Vitro /Low Density Lipoprotein Receptor cDNA /Intrahepatic /Portal Vein Catheter	Wilson, Jam M
168.	Gene Therapy /Phase I /Infectious Disease /Human Immunodeficiency Virus /Replication Inhibition /Antisense	Gene Therapy for **AIDS** using Retroviral Mediated Gene Transfer to deliver **HIV**-1 Antisense TAR and Transdominant Rev Protein Genes to Syngeneic Lymphocytes in HIV Infected Identical Twins.	In Vitro /Antisense TAR /Transdominant Rev /Intravenous	Morgan, Richard and Walker, Rol
169.	Gene Therapy /Phase I /Cancer /Melanoma /**Immunotherapy**	Phase I Study of Tumor-Infiltrating Lymphocytes Derived from In Vivo HLA-B7 Gene Modified Tumors in the Adoptive Immunotherapy of Melanoma.	In Vivo /Autologous Tumor Cells /Used to Derive **Tumor Infiltrating Lymphocytes** /HLA-B7 cDNA /Intravenous	Chang, Alfr E. and Nabe Gary J
170.	Gene Therapy /Phase I /Cancer /CEA-Expressing Malignancies (type of cancer not specified) /**Immunotherapy**	A Study of Recombinant ALVAC Virus that Expresses Carcinoembryonic Antigen in Patients with Advanced Cancers.	In Vivo /Autologous Muscle Cells /**Canarypox Virus** /Carcinoembryonic Antigen cDNA /Intramuscular Injection	Hawkins, Michael J. a Marshall, Jo L
171.	Gene Therapy /Phase I /Cancer /Prostate Adenocarcinoma /**Immunotherapy**	A Phase I Study of Recombinant Vaccinia that Expresses Prostate Specific Antigen in Adult Patients with Adenocarcinoma of the Prostate.	In Vivo /Vaccination /**Vaccinia Virus** /Prostate Specific Antigen /Intradermal Injection	Chen, A.P
172.	Gene Therapy /Phase I /Cancer /Gastrointestinal Tract, Breast, or Lung Adenocarcinoma (CEA-Expressing Malignancies) /**Immunotherapy**	Phase I Study of Recombinant CEA Vaccinia Virus Vaccine with Post Vaccination CEA Peptide Challenge.	In Vivo /Vaccination /**Vaccinia Virus** /Carcinoembryonic Antigen /Intradermal Injection in Combination with Subcutaneous Peptide Challenge	Cole, David
173.	Gene Therapy /Phase I /Cancer /Melanoma /**Immunotherapy**	Evaluation of Intratumoral Gene Therapy with HLA-B7 /DMRIE /DOPE plus Subcutaneous Low Dose Il-2.	In Vivo /Autologous Tumor Cells /HLA B7 cDNA /Intratumoral /Concurrent Interleukin-2 Therapy	Hersh, Evan and Sondak, Vernon K.
174.	Gene Therapy /Phase I /Cancer /Prostate Adenocarcinoma /**Immunotherapy**	A Phase I Trial Of Recombinant Vaccina Virus That Expresses PSA In Patients With Adenocarcinoma Of The Prostate.	In Vivo /Vaccination /**Vaccinia Virus** /Prostate Specific Antigen /Intradermal Injection	Kufe, Donal W., and Ede Joseph Paul
175.	Gene Therapy /Phase I /Cancer /Melanoma /**Immunotherapy**	Phase I Trial In Patients With Metastatic Melanoma Of Immunization With A Recombinant Fowlpox Virus Encoding The MART-1 Melanoma Antigen.	In Vivo /**Fowlpox Virus** /MART-1 Melanoma Antigen /Intramuscular Injection	Rosenberg, Steven A
176.	Gene Therapy /Phase I /Cancer /Melanoma /**Immunotherapy**	Phase I Trial In Patients With Metastatic Melanoma Of Immunization With A Recombinant Fowlpox Virus Encoding the GP100 Melanoma Antigen.	In Vivo /**Fowlpox Virus** /gp100 Melanoma Antigen /Intramuscular Injection	Rosenberg, Steven A
177.	Gene Therapy /Phase I /Cancer /Melanoma /**Immunotherapy**	Phase I Trial In Patients With Metastatic Melanoma Of Immunization With A Recombinant Vaccinia Virus Encoding the MART-1 Melanoma Antigen.	In Vivo /**Vaccinia Virus** /MART-1 Melanoma Antigen /Intramuscular Injection	Rosenberg, Steven A
178.	Gene Therapy /Phase I	A Phase I /II Clinical Trial Evaluating the Safety	In Vivo /Vaccination /**Vaccinia Virus** /Prostate	Sanda, Mart

	/II /Cancer /Prostate Adenocarcinoma /**Immunotherapy**	and Biological Acivity of Recombinant Vaccinia-PSA Vaccine in Patients with Serological Recurrence of Prostate Cancer Following Radical Prostatectomy.	Specific Antigen /Intradermal Injection	
179.	Gene Therapy /Phase I /Cancer /CEA-Expressing Malignancies /**Immunotherapy**	A Phase I Study of Active Immunotherapy With Carcinoembronic Antigen RNA-Pulsed Autologous Human Cultured Dendritic Cells In Patients Wlth Metastatic Malignancies Expressing Carcinoembryonic Antigen.	In Vitro /Autologous Dendritic Cells /RNA Transfer /Carcinoembryonic Antigen /Intravenous	Lyerly, Kim
180.	Gene Therapy /Phase I /Cancer /Melanoma /**Immunotherapy**	Phase Ib Trial of Intratumoral Injection of a Recombinant Canarypox Virus Encoding the Human Interleukin-12 Gene (ALVAC-hIL-12) in Patients with Surgically Incurable Melanoma **Sponsor**: NCI- Cancer Therapy Evaluation Program	In Vivo /Autologous Melanoma Cell /**Canarypox Virus** /Cytokine /**Interleukin-12** cDNA /Intratumoral Injection	Conry, Robe M
181.	Gene Therapy /Phase I /Cancer /**Immunotherapy** /CEA-Expressing Malignancies	A Pilot Study of Sequential Vaccinations with ALVAC-CEA and Vaccina-CEA with the addition of IL-2 and **GM-CSF** in Patients with CEA Expressing Tumors **Sponsor**: National Cancer Institute-Cancer Therapy Evaluation Program (NCI-CTEP)	In Vivo /Autologous Muscle Cells /**Canarypox Virus** /Vaccinia Virus /Carcinoembryonic Antigen cDNA /Intramuscular and Percutaneous Injection	Marshall, Jo L
182.	Gene Therapy /Phase I /Cancer /**Immunotherapy** /CEA-Expressing Malignancies	A Phase I Trial of a Recombinant Vaccinia-CEA (180 Kd) Vaccine Delivered by Intradermal Needle Injection Versus Subcutaneous Jet Injection in Patients with Metastatic CEA-Expressing Adenocarcinoma **Sponsor**: NCI, NIH	In Vivo /**Vaccinia Virus** /Carcinoembryonic Antigen cDNA /Intradermal and Subcutaneous Injections	Conry, Robe M
183.	Gene Therapy /Phase I /Cancer /Melanoma /**Immunotherapy**	Phase Ib Trial of Intratumoral Injection of a Recombinant Canarypox Virus Encoding Human B7.1 (ALVAC-hB7.1) or a Combination of ALVAC-hB7.1 and a Recombinant Canarypox Virus Encoding Human Interleukin-12 (ALVAC-hIL-12) in Patients with Surgically Incurable Melanoma **Sponsor**: National Cancer Institute-Cancer Therapy Evaluation Program (NCI-CTEP)	InVivo /Autologous Melanoma Cell /**Canarypox Virus** /B7(CD80) /**Interleukin-12** /Cytokine /Intratumoral Injection	Conry, Robe M
184.	Gene Therapy /Phase I / Cancer /Ovarian /**Immunotherapy**	Intraperitoneal (IP) Auatologous Therapeutic Tumor Vaccine (AUT-OV-ALVAC-hB7.1) plus IP rIFN-γ for Patients with Ovarian Cancer. A Pilot Study **Sponsor**: NCI Cancer Therapy Evaluation Program (NCI-CTEP)	In Vitro /Autologous Tumor Cells /**Canarypox Virus** /B7.1 (CD80) /Intraperitoneal Injection	Freedman, Ralph
185.	Gene Therapy /Phase I /Cancer /Colorectal /**Immunotherapy** (under review)	Phase I Clinical Trial of a Recombinant ALVAC-CEA-B7 Vaccine in the Treatment of Advanced Colorectal Carcinoma. **Sponsor**: National Cancer Institute-Cancer Therapy Evaluation Program (NCI-CTEP)	In Vivo /Autologous Tumor Cells /**Canarypox Virus** /Carcinoembryonic Antigen /B7.1 (CD80) /Intradermal Scarification	Kaufman, Howard L
186.	Gene Therapy /Phase I /Cancer /CEA-Expressing Malignancies /**Immunotherapy** (under review)	Phase I /Pilot Study of ALVAC-CEA-B7.1 Immunization in Patients with Advanced Adenocarcinoma Expressing CEA **Sponsor**: National Cancer Institue - Cancer Therapy Evaluation Program (NCI-CTEP)	In Vivo /Autologous Tumor Cells /**Canarypox Virus** /Carcinoembryonic Antigen /B7.1 (CD80) /Intramuscular and Intradermal Injections	von Mehren Margaret