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REVIEW ARTICLE

Protection and selection for gene therapy in the hematopoietic system

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Summary

Hematopoietic stem cell gene therapy is potentially curative for a number of inherited and acquired disorders. However, poor gene transfer and expression in repopulating hematopoietic stem cells attenuate this potential. Here we review potential means of conferring a selective advantage to hematopoietic stem cells and their progeny, and discuss the issues that surround the use of selective advantages *in vivo*. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords gene therapy; hematopoiesis; selection; drug resistance; chemical inducers of dimerisation; HoxB4

Introduction

For a number of inherited and acquired diseases, transfer and expression of therapeutic transgenes in hematopoietic stem cells (HSCs) and their progeny has the potential to provide effective and life-long correction of the disease state. Central to this potential is the ability to achieve efficient gene transfer to repopulating human HSCs and to maintain appropriate levels of expression *in vivo* in these cells and their progeny. In most clinical trials of HSC gene therapy conducted thus far, the transduction efficiencies achieved have been low and the resultant clinical benefits negligible. The most striking exception to this has been the success of HSC gene therapy in treating the inherited disorder, X-linked severe combined immune deficiency (X-SCID). A number of patients have been treated and have shown clear signs of clinical improvement, with increased levels and repertoire of immune effector cells and reduced episodes of infection leading to an increased quality of life [1–3].

The success of this clinical trial owes much to recent advances in the *ex vivo* manipulation of HSCs for gene transfer. However, the biology of the disease is likely to have also contributed, by conferring a survival and proliferative advantage to gene-corrected cells. The genetic defect that underpins X-SCID is in the gamma (signalling) chain that is common to a number of cytokine receptors. Lack of functional activity of these receptors is responsible for a lack of response to cytokines and a reduced survival and maturation of lymphoid cells. Restoration of the common gamma-chain allows lymphoid progenitors to respond appropriately to their cytokines and thus to increased survival and proliferation.

Correction of some other genetic defects, for example, adenosine deaminase deficiency [4] or Fanconi anemia [5], may also confer a selective advantage. Unfortunately, this scenario is likely to be the exception rather than the rule. Indeed, it may be that some otherwise advantageous transgenes could confer a selection disadvantage (perhaps through immune recognition, effects on cell cycle, or even just due to an increased metabolic load) when expressed in HSCs. In such cases it may be important to provide an artificial selection

advantage by incorporating a selectable marker in addition to a therapeutic transgene.

Ex vivo selection

Perhaps the simplest form of selection that could be applied to HSCs is *ex vivo* selection of transduced cells prior to transplantation. This can be used to enrich for gene-modified HSCs prior to reinfusion to a patient and thus remove competition from untransduced HSCs during the engraftment and expansion process post-transplantation. A number of selectable markers have been used in this way and fall broadly into three categories, drug-resistance genes, membrane markers and fluorescent proteins.

The most commonly used of the drug-resistance genes is the *neomycin* resistance (*neo*) gene. This encodes a phosphotransferase protein that detoxifies the drug G418 and thus leads to increased cellular resistance to this agent. Most studies have used resistance to G418 as a means to assess gene-transduction efficiency *in vitro* [6]. However, *in vitro* selection with G418 has also been used prior to transplantation to increase the percentage of engrafting HSCs post-transduction [7].

Membrane antigens have also been investigated as potential *in vitro* selectable markers. Thus CD24 [8,9], murine heat shock antigen [10,11] and a truncated version of the nerve growth factor receptor [12] have all been used, along with either FACS or magnetic bead isolation to provide enriched populations of gene-modified cells. In a similar way, vectors encoding the green fluorescent protein (GFP) or one of its derivatives may also be used. For example, pre-selection of GFP^+ cells improved long-term engraftment and expression of a linked transgene in a mouse transplantation model [13]. Moreover, in studies in the baboon, *ex vivo* selection of GFP^+, $CD34^+$ cells led to around five-fold higher levels of gene-modified cells in the peripheral blood of baboons at 4 weeks post-transplant. However, the levels of transduced cells declined at later time points [14].

In vitro selection, whilst of potential use in enrichment of a graft pre-transplant, suffers from a number of disadvantages. None the least of these is that any protocol relying on *in vitro* selection of gene-modified cells will necessitate further *ex vivo* manipulation of HSCs. This includes extra time in culture to facilitate transgene expression and, where drug selection is used, exposure to cytotoxic compounds. Such increased time in culture is known to increase commitment of HSCs to differentiation and to compromise engraftment capability [15,16]. Thus the benefits of selection may be attenuated due to reduced HSC function *in vivo*. A related issue is that *in vitro* selection will not provide an advantage of engrafting cells over endogenously recovering hematopoiesis (a likely complication in some clinical applications, where ablative pre-transplant conditioning may be inappropriate). Some selective transgenes may also prove detrimental to long-term expression of therapeutic genes. For example, the *neo* gene has been shown to incorporate silencer regions that compromise therapeutic gene expression in HSCs and their progeny [17]. Similarly, there are suggestions that the GFP gene product may compromise long-term hematopoiesis [18] or represent an immunological target [19]. Many of the genes used for *in vitro* selection are non-human in origin. In one clinical trial, an immunological reaction to a non-human transgene (HSV-tk) was associated with T-cell-mediated rejection of gene-modified cells [20]. Similarly, in a study in baboons, cytotoxic T-lymphocyte responses to GFP and YFP were associated with deletion of gene-modified cells [21]. Thus, the generalised use of non-human selectable markers may prove problematic, both for *in vitro* and *in vivo* selection approaches.

In all, whilst *in vitro* selection may have some applications, most likely in the production of gene-modified mature hematopoietic cells such as dendritic [22] or cytotoxic T-cells [23], for most therapeutic strategies the provision of an *in vivo* advantage may be required.

In vivo selection

The *in vivo* selective advantage conferred by correction of the X-SCID phenotype probably represents both a survival and proliferative advantage. Exogenously applied selective advantages fall broadly into two categories: (1) Resistance to conditions (e.g. cytotoxic drug treatment) that lead to elimination of untransduced cells – i.e. a survival advantage; and (2) selective expansion of transduced cells – i.e. a proliferative advantage. Strategies to artificially incorporate a selective advantage into gene-transfer protocols may utilise survival or proliferative advantages singly or in combinations.

Provision of a survival advantage

Elimination of untransduced cells may be most easily accomplished with exposure to a cytotoxic agent in combination with provision of drug resistance to transduced cells. Most of the impetus for development of drug-resistance gene transfer has come from strategies designed to overcome normal tissue toxicity during anti-tumour chemotherapy. Such collateral toxicity is often dose-limiting, and the primary dose-limiting toxicity for many anti-tumour agents is bone marrow toxicity, particularly myelosuppression. Thus a number of groups have been developing vectors capable of transferring drug-resistance factors to HSCs with a view to protecting the bone marrow compartment against collateral toxicity [24].

There is a wide range of different drug-resistance genes that may be used (summarised in Table 1), and these confer resistance to a variety of different drugs. Below we consider some of the major examples of chemoprotective/selective genes under investigation.

Table 1. Drug-resistance genes that are used in gene therapy

Resistance type	Examples	Drugs	Refs
ABC transporters	MDR-1 MRP ABCG2/BRCP1	Anthracyclins Epipodophylotoxins Vinca alkaloids	[25–29,35–42,47–49,52,55,95,158,180–187]
DNA repair	MGMT Various glycosylases	Alkylating agents	[52,79,81–84,86–91,188–195]
Folate metabolism	DHFR TS CDA	Methotrexate/trimetrexate 5-FU Ara-C	[101,152,196–200]
Glutathione levels	GST-pi	Alkylating agents Anthracyclins	[199,201–204]
Redox	SOD	Radiation	[205,206]
Other	ALDH	Cyclophosphamide	[152,207–210]

ABC protein family members

ATP-binding cassette (ABC) family members confer resistance to a wide range of unrelated drugs such as anthracyclins, vinca alkaloids and podophylotoxins. They act as ATP-dependant membrane pumps, causing efflux of drugs from the cytoplasm of cells and thus a lowered intracellular drug concentration and reduced toxicity. The prototypical example of this family is MDR-1 (p-glycoprotein), although protection by MRP-1 and ABCG2 has also been described [25–30].

The first proof of principle for MDR-1-mediated chemoprotection came from studies with transgenic mice expressing high levels of p-glycoprotein in their bone marrow [31]. These mice showed apparently normal haematopoiesis that was resistant to levels of chemotherapeutic agents that caused significant, life-threatening toxicity in control animals. Treatment of these animals with a known p-glycoprotein inhibitor restored the sensitivity of the bone marrow to chemotherapeutic agents, thus implicating MDR-1 in the protective effect [32]. Subsequent studies showed that transplantation of control animals with bone marrow from transgenic donors led to inhibitor-sensitive chemoprotection of hematopoiesis in the recipient animals [33].

In early studies, the MDR-1 gene product was shown to confer resistance to a range of agents, including doxorubicin, etoposide and taxol, following retroviral transfer and expression in hematopoietic cell lines and primary bone marrow cells *in vitro* [34–36]. Subsequent studies showed clear evidence of protection of murine hematopoiesis against the cytotoxicity of various drugs *in vivo* following transplantation of gene-modified cells [37–40]. Evidence for selection *in vivo* was also seen in these early murine studies, with increased numbers of MDR-1-expressing cells detected following drug treatment of transplanted animals. Further studies indicated that protection of hematopoiesis in mice carrying tumours provided an opportunity for dose escalation and led to improved survival of those animals [41].

On the basis of these studies, and others showing transduction and protection of primitive human hematopoietic cells *in vitro* [40,42], clinical trials of MDR-1 gene therapy were initiated [43–46]. These trials were mostly characterised by low transduction frequencies in repopulating cells and with short-term engraftment of MDR-1-positive cells. In a few patients, however, evidence suggestive of a (limited) selective effect has been reported, with the appearance of or an increase in MDR-1 positive cells post-chemotherapy and maintenance of transduced cells for up to a year post-transplant in the face of chemotherapeutic challenge. These studies, whilst providing proof of principle of MDR-1 gene transfer and the potential for selection, highlight the need for robust gene-transfer technology to underpin the chemoprotective effect of MDR-1 (or indeed any drug-resistance gene).

Work continues with MDR-1 and includes improvements such as sequence modification to eliminate cryptic splice sites that compromise therapeutic virus production and MDR-1 expression levels [47]. Recent studies have demonstrated efficient transfer of MDR-1 into Nod/SCID repopulating human HSCs and subsequent protection and selection of transduced cells in this mouse model [48,49]. A few studies have also examined the utility of MDR-1 as a selective marker to achieve efficient co-expression of therapeutic genes [50–55]. In one canine study, selection with paclitaxel was associated with a rise in MDR-1 transgene-positive cells in peripheral blood and bone marrow [56]. Increased expression of a linked therapeutic transgene was also seen. However, it should be stressed that this result was obtained in a single animal and in this study (as with the human studies discussed above) the contribution of natural clonal fluctuations to the observed results was not fully determined. Two further animals in the canine study, selected with higher levels of paclitaxel, died from hematopoietic toxicity. Clearly, the stringency of any selective pressure to be applied clinically will have to be carefully controlled, particularly if starting levels of gene-modified cells are low.

O^6-Methylguanine-DNA-methyltransferase

Another drug-resistance gene that has received much attention in terms of its chemoprotective/selective potential is that encoding the DNA repair protein O^6-methylguanine-DNA-methyltransferase (MGMT, also known as ATase or AGT) [57]. This protein repairs specifically alkylation damage at the O^6-position in guanine, and does so by transferring the alkyl group to a cysteine residue in its active site in a stoichiometric (one

MGMT molecule to one lesion) and auto-inactivating manner. That is, the MGMT molecule becomes permanently alkyl-modified, is then ubiquinated and targeted for degradation. Thus the repair process depletes cellular MGMT and further repair requires *de novo* MGMT synthesis. During this time, cells show increased vulnerability to O^6-alkylating agents [58].

This mechanism has been exploited in clinical trials using O^6-alkylating agents, where pre-treatment of patients with a methylating agent has been used prior to choloroethylating agent treatment to reduce tumour MGMT levels and thus enhance tumour sensitivity to drug-treatment [59,60]. The collateral toxicity of this approach (profound myelosuppression) was unacceptable, however, and a more refined approach has been taken that makes use of small molecular mimics of O^6-alkylguanine in DNA to achieve MGMT inactivation prior to treatment with an O^6-alkylating agent. Pre-clinical studies have shown the efficacy of this approach in sensitising human tumour xenografts to treatment [61–66], and two inactivators, O^6-benzylguanine (O^6-BeG) and O^6-(4-bromothenyl)guanine (PaTrin2), have entered clinical trial. However, a number of pre-clinical studies have also predicted enhanced collateral toxicity (especially myelotoxicity) as a result of inactivator pre-treatment of patients [67–69]. This has been borne out in those clinical trials performed to date, with a decrease in the maximum tolerated dose of O^6-alkylating agents in patients that received inactivator [70,71].

The wild-type human MGMT protein is very sensitive to inactivation by either of the two compounds in clinical use and by a range of related compounds [72]. Based largely on comparisons with bacterial MGMTs, which are resistant to these inactivators [73], point-mutated versions of human MGMT have been derived [74–78] that show partial or complete resistance to inactivation by either O^6-BeG or PaTrin2. Such mutant MGMTs (muMGMTs) confer inactivator-insensitive protection against O^6-alkylating-agent-induced toxicity both *in vitro* [79–81] and *in vivo* [82–88]. The extent of inactivator resistance of these mutants is such that a large selective advantage accrues to cells expressing muMGMT compared with the exquisitely inactivator-sensitive wild-type protein. Thus, even in the face of high levels of wild-type MGMT expression, inactivator/O^6-alkylating agent combinations provide a powerful selective tool. In mouse experiments, clear evidence has been seen for selection of muMGMT-expressing cells following combination treatment with an inactivator and an O^6-alkylating agent [82,86–89]. This has led to enhanced protection against myelotoxicity during anti-tumour treatment in mouse models, facilitating increased tumour kill [90] and improved survival of experimental animals [82,86].

Importantly, a survival advantage and selection of transduced cells has been seen even where limited numbers of gene-modified cells have engrafted or when non-myeloablative conditioning has been given [91]. In one study, following non-myeloablative conditioning, muMGMT-expressing normal bone marrow cells were infused into β-thallasemic mice. In the absence of selection, donor cells contributed minimally to hematopoiesis and haemoglobin levels were low. When selection with an inactivator/O^6-alkylating agent combination was applied, this led to increased donor cell and haemoglobin levels in recipient mice [92]. Clinical trials of muMGMT-mediated chemoprotection are planned and the data from these will be of great importance in determining the potential for genetic chemoprotection/selection in patients. On the basis of the pre-clinical data, however, it might be argued that muMGMT is currently the most powerful tool available for drug selection within the hematopoietic system.

Dihydrofolate reductase

One potential advantage that selectable markers such as MDR and muMGMT may have is that some of the drugs used for selection may be capable of killing primitive (perhaps stem) cells. Moreover, muMGMT may also have an advantage over MDR-1 in that endogenous, wild-type MGMT levels in untransduced cells can be ablated using inactivators, whilst endogenous MDR-1 or other ABC transporters may pose a selective complication, since some HSCs express relatively high levels of such proteins [30,93–96].

Where the drugs to be used are not toxic to the stem cell compartment, this may not be a problem in terms of chemoprotection, where short-term protection of hematopoiesis may be clinically useful. However, in scenarios where long-term selection is the aim, a lack of killing of unmodified HSCs will lead to dilution of the gene-modified cells as the surviving unmodified HSCs will be able to contribute to hematopoietic reconstitution. Such an outcome will reduce clinical efficacy. This problem is exemplified by the use of mutant dihydrofolate reductase (mDHFR) to protect against methotrexate toxicity in bone marrow. From the studies carried out to date it is evident that expression of mDHFR can protect cells and hematopoiesis from the cytotoxic effects of methotrexate and trimetrexate [97,98]. However, the utility of antifolates as selective agents is compromised by their inability to kill primitive hematopoietic cells [99].

One reason for the ineffectiveness of MTX or TMTX in killing more primitive cells is that they express transport proteins that allow uptake of extracellular nucleosides. This permits cells to bypass the need for *de novo* nucleotide synthesis and thus circumvents antifolate toxicity [100]. In a system analogous to that used with muMGMT, an inhibitor of this salvage pathway has been used in combination with mDHFR gene-transfer and TMTX [101]. This has led to improved selection, although a gradual fall off in gene-modified cells with time may suggest that there is still more to be done to achieve complete selection.

Specific expansion of transduced cells

An alternative to the elimination of untransduced cells is to provide a survival growth or self-renewal advantage to

HSCs and their progeny as a result of gene transfer. This might be a constitutive advantage (such as that conferred by HoxB4, see below) or one that relies on addition of an external agent. This latter approach has been adapted for potential use in gene therapy by using chemical inducers of dimerisation (CIDs) to achieve activation of modified cytokine or growth factor receptors [102].

Chemical inducers of dimerisation

Three similar approaches to chemical dimerisation have been taken: a domain from *E. coli* Gyrase B protein that binds the dimeric antibiotic coumermycin; the estrogen receptor and a tamoxifen-responsive mutant of this; and a mutant FK506-binding domain of the immunolipophilin FKBP12.

Fusion of the coumermycin-binding domain of *E. coli* Gyrase B to the granulocyte colony stimulating factor receptor (GCSF-R) facilitated antibiotic-induced stimulation of proliferation in a cell line model [103] as a result of dimerisation of the receptor. Similarly, coumermycin-induced dimerisation of JAK kinases has been used to provide a mitogenic signal to cells *in vitro* [104,105]. Thus far this system has not been tested *in vivo*.

The estrogen receptor has also been used to achieve drug-inducible dimerisation and signalling from the GCSF-R in hematopoietic cell lines and murine bone marrow cells *in vitro* [106–108]. In the absence of hormone, cytoplasmic proteins, including HSP90, sequester the estrogen receptor. Binding of estrogen (or tamoxifen in the case of the tamoxifen-responsive mutant of estrogen receptor) facilitates release from sequestration and subsequent dimerisation. Selection was demonstrated in primary and secondary bone marrow recipients in mouse transplantation studies [109]. In non-human primates, selection for gene-modified cells was achieved *in vivo* in one of two animals, following administration of estrogen [110]. In this animal, selection was transient, with a drop in gene-modified cells following removal of selection, suggesting that selection was operating at the committed progenitor level and not at the stem cell level. Also, worryingly, the level of gene-marked cells in bone marrow (5%, rising to 30% on selection) far outweighed that in peripheral blood (0.1% rising to 1% on selection). Whether this points to a maturation defect in gene-marked cells or simply reflects deletion of GFP^+ cells (perhaps due to immunological surveillance) remains to be established. Similar results were obtained with a tamoxifen-inducible GCSF-R signalling domain. In this case two animals showed transient selection of gene-modified cells post-treatment with toremifine. Two additional animals, treated with 4-hydoxytamoxifen, however, did not show any evidence of selection, although the levels of drug achieved in serum may have been suboptimal. Recent studies have shown tamoxifen-inducible, *in vitro* expansion of hematopoietic cells following transduction with a chimeric version of the thrombopoietin receptor, (mpl) [111].

The system based on the immunolipohilin FKBP12, which binds the immunosuppressive agent FK506, has been most extensively characterised. Early work, using an FK506-binding domain (FKBD) fused to the signalling domain of the eythropoeitin receptor, showed that the growth-factor dependency of a hematopoietic cell line could be overcome by addition of FK1012, a dimeric derivative of FK506, to cells that expressed the chimeric receptor [112]. More recent work utilises a mutant version of the FKBD (F36V) and further derivatives of FK1012 that do not bind to endogenous sequences. In these studies, F36V has been fused to the signalling domain of mpl, which has been shown to be superior to G-CSFR and flt-3 in promoting drug-induced proliferation of hematopoietic progenitors [113].

In an *in vitro* study, Jin *et al*. [114] showed that engagement of mpl signalling led to a marked proliferative expansion of gene-modified cells under conditions where control cells died. An *in vitro* competition assay showed that a minority of gene-modified cells could be selected against the background of a majority of control cells by addition of drug. *Ex vivo* expansion of d12 CFU-S (a multipotent progenitor cell type) has also been seen, although no maintenance of radioprotective (i.e. short-term repopulating) capability was found. The effects of selection were maintained only when the drug was present and there was an early indication of lineage bias in this study, with the best and most sustained amplification occurring in the megakaryocyte lineage.

In vivo murine studies have shown clear evidence of selection using this system [115]. In serial samples of bone marrow taken before and after drug treatment of animals, an increased number of gene-modified GM-CFC was seen post-selection. Sequential analysis of peripheral blood cells for the presence of the GFP marker showed a transient increase in gene-marked cells suggesting that selection was occurring not at the stem-cell level, but at a more committed phase of hematopoiesis. As with the *in vitro* studies, there was evidence to suggest a lineage bias, with the response in the erythroid and platelet compartments (where mpl signalling plays an important role) being more pronounced than that in the myeloid lineages.

The response in human studies has been less marked [116]. *In vitro* studies with cord blood CD34 cells showed a transient response to drug treatment, with increased numbers of late erythroid progenitors produced in expansion cultures, although this was at the expense of more primitive erythroid progenitors. Colony forming assays indicated a supportive effect on erythroid and megakaryocte colony forming cells, but not on myeloid or multipotent progenitors. Further work, looking at the effects of growth factor administration in conjunction with drug treatment, suggests that a judicious choice of cytokine conditions may allow drug-mediated *ex vivo* expansion [117].

Experiments in a canine model indicate that selection can also be achieved in large animals [118]. Clear evidence for selection of (GFP^+) gene-modified cells

was seen, although again this was transient and mostly restricted to the erythroid and platelet compartments. Effects on granulocyte numbers were sporadic and unpredictable. Of note, however, was the transient increase in gene-modified bone marrow $CD34^+$ cells post-drug treatment. These exhibited a mainly pro-B-cell phenotype and their disappearance from bone marrow coincided with a dramatic rise in $CD21^+$ cells in peripheral blood, that subsequently homed to the lymph nodes.

Thus, whilst there are encouraging signs for drug-mediated selection, a number of issues remain to be resolved. For the F36V-based systems, the reduced or negligible effect in the myeloid compartments and the altogether poorer response in human cells are concerns. For some applications (e.g. treatment of thalassemia) bias towards the erythroid lineage might not be undesirable. Clearly, however, for treatment of disorders of myeloid cells (e.g. chronic granulomatous disease, Gaucher's disease), expression and expansion in the appropriate compartments will be essential. With regard to human cell selection/expansion, it is worth noting that the studies to date have all used the murine mpl signalling domain. Primitive murine and human cells differ in their responsiveness to cytokines and growth factors, and there is a lack of cross-reactivity of some factors between these species. It might be the case that the murine mpl signalling domain proves to be sub-optimal for use in human cells and studies with the human equivalent are certainly warranted. Studies with steroid-inducible GCSF-R signalling raise the possibility of poor maturation of gene-marked bone marrow progenitors and this will have to be addressed.

The transient nature of the selection suggests that CID-mediated effects are restricted to committed progenitors and that selection at the stem-cell level does not occur to any significant degree. The reasons for this are not obvious. It seems unlikely that no stem cells were transduced in the *in vivo* studies reported, since in both the canine and monkey studies, gene-modified cells are present over a year post-transplantation. It may be that expression levels in more primitive cells are too low to facilitate selection, or alternatively that the mpl signalling domain transmits an ineffectual signal in these cells. It is worth noting that alternative signalling domains have shown activation of proliferation in other cell types [119–121]. It may be that selection in the stem cell compartment will require use of an alternative receptor, or even engagement of the self-renewal signalling pathway further downstream [104,105].

It is probable that multiple or chronic administration of a selective agent could be used to overcome the current transient nature of the selective effect. With life-long usage there are cost implications and of course the long-term effects of drug administration are of great importance. Tamoxifen has been used in a number of patients and the possible adverse effects of this agent are well documented [122]. One FK1012 analogue has been tested in a limited phase I clinical trial with no significant acute toxicity although this agent was not administered chronically or repeatedly [123]. These potential complications should, however, be weighed against the likely benefits to patients and the availability of alternative treatment strategies.

HoxB4

One molecule that has received much attention as a potential facilitator of engraftment and expansion of gene-modified cells is HoxB4, a member of the homeobox (Hox) family of transcription factors that play an important role in the development of the embryo [124]. A number of these factors are also important in control of differentiation and proliferation in the hematopoietic system. Distinct expression patterns are seen in different stages of hematopoietic development and aberrant expression of some Hox genes is seen in a number of human leukaemias [125]. HoxB4 is found expressed at high levels in primitive hematopoietic cells and is downregulated in more mature cells [126]. Significantly, HoxB4 has not thus been associated with any hematopoietic malignancies.

Constitutive overexpression of HoxB4 in embryonic stem (ES) cells leads to enhanced production of definitive, but not primitive, erythroid colony forming cells derived from differentiating embryoid bodies [127]. Transient expression of HoxB4 in mouse ES and yolk sac cells conferred definitive hematopoietic potential and allowed stable, long-term hematopoietic engraftment of adult mice [128].

In adult hematopoiesis, retroviral-mediated transfer and expression of HoxB4 in murine bone marrow cells leads to increased proliferation of primitive hematopoietic cells *in vitro* and to enhanced hematopoietic repopulation *in vivo* [129,130]. In competitive repopulation experiments, HoxB4-transduced cells outperformed control cells [131]. Furthermore, significant *ex vivo* expansion of murine repopulating stem cells has been documented following HoxB4 gene transfer and expression [132]. In those studies, it appeared that enhanced proliferation and engraftment of HoxB4-expressing HSCs was achieved without apparent adverse effects on differentiation or homeostasis in the murine hematopoietic system, although studies with human cells (see below) suggest otherwise.

In human hematopoiesis, overexpression of HoxB4 led to an increase in human hematopoietic progenitor cells in culture as evidenced by increased secondary plating capacity of colony forming cells and increased numbers of LTCIC [133]. Furthermore, HoxB4 overexpression led to increased numbers of Nod/SCID repopulating cells. These authors reported no evidence of any alteration in the differentiation potential of the engrafting human HSCs, although an increased production of B-cells was seen *in vitro*. A similar study also showed an *in vivo* competitive advantage as a result of high-level expression of HoxB4. This study, however, provided some data suggesting impaired myeloerythroid differentiation and reduced B-cell output [134]. Clearly, as with CID-mediated

expansion (see above), any suggestion of lineage bias or impaired differentiation may have profound consequences for the utility of this selection system. Consequently, more careful studies in mouse and large animal models will be necessary to fully address this issue. Central to those studies will be questions surrounding the window of opportunity for HoxB4 expression (i.e. constitutive or temporally restricted) as well as the levels of expression required.

Achieving co-expression

As described above, there are a number of ways to confer a selective advantage in hematopoietic stem and progenitor cells and to specifically achieve gene-modified hematopoiesis. However, in the context of genetic therapy, selected cells must not only express the selectable marker, but usually an additional therapeutic transgene as well. Thus strategies and vectors must be used that permit expression of two or more genes in transduced cells.

Early dual expression retroviral vectors made use of internal promoters to achieve expression of a selectable marker, with expression of a second gene from the retroviral LTR. However, it soon became clear that a certain amount of promoter interference and competition existed in some such vectors. Consequently, selection on the basis of a marker gene (e.g. a drug-resistance gene) led to downregulation of expression of the second gene [135]. Clearly, in a clinical context this would be problematic and vectors have been improved to ensure that second gene expression is consistently achieved.

One of the strategies most commonly adopted to enable dual expression of genes from a single vector is the use of internal ribosome entry sites (IRESs). In vectors containing such elements, the translation of a cistron occupying the position at the 5′ end of the transcript is initiated through the normal cap-dependent mechanism. However, IRESs form a structural motif in the mRNA that is recognised by the translational apparatus, which positions the initiation complex in such a way as to facilitate the translation of an additional downstream cistron (reviewed in [136,137]). IRES motifs were originally identified in a number of the picornaviral family of viruses and several of these have been subsequently used in dual expression vectors. One general problem with the use of picornovirus-derived IRES sequences, however, is that translation from the IRES tends to be at a much lower level than cap-dependent translation [138]. Thus expression levels of the IRES-driven gene can be very low. To circumvent this, the cDNA encoding the therapeutic trans-gene may be placed in the upstream position in order to maximise its translation. However, this may mean that IRES-driven expression of the selectable marker may be low. For a number of selectable markers, including some drug-resistance genes, HoxB4, and perhaps chemically inducible receptors, the required level of expression to achieve effective selection is likely to be high. Nonetheless, effective selection of cells co-expressing a therapeutic trans-gene and a downstream selectable marker courtesy of an IRES motif has been achieved in several cases [13,53–55]. A number of IRESs have been identified within the genomes of various viral families and within a variety of transcripts derived from an array of multicellular organisms. A comprehensive list of viral and cellular IRES sequences identified so far is available from the Internal Ribosome Entry Site Database [139]. The ongoing characterisation of these IRES motifs has led to the intriguing observation that a number of these show differential activity, dependent on cell type or cell cycle status, or in response to stimuli such as hypoxia, glucose availability, genotoxic stress, heat shock, hypothermia or apoptosis [140–149]. This suggests that, in addition to facilitating constitutive expression of a second gene, IRES motifs may be employed to achieve tissue-specific or stimulus-dependent expression of therapeutic sequences. Indeed, initial progress has been made in the creation of an inducible picornaviral IRES [150].

One potential answer to the problem of low marker-gene expression would be to produce a fusion protein. Thus the level of the therapeutic and selectable gene products would be identical. Such an approach has been used to fuse MDR-1 to adenosine deaminase [51], and to produce dual function chemoprotective genes [151,152]. This approach will only work of course if the fusion does not disrupt the activity of either of the gene products, and if the two products exert their activity in the same cellular compartment. One answer to this has come from the use of a self-processing activity from the 2A region of the foot-and-mouth disease virus polyprotein. This moiety allows the production of two separate protein products from a monocistronic vector, *via* co-translational processing of the elongating 2A protein, resulting in intramolecular separation, with release of the upstream product from the ribosome and continued translation of the downstream product [153,154]. The 2A system has been used to successfully co-express putative selectable markers and therapeutic genes, with evidence of good levels of cleavage [155,156]. In the latter case the two proteins were also appropriately targeted to their respective compartments (nuclear for HoxB4 and cytoplasmic for GFP). One question surrounds whether targeting to other compartments, such as the plasma membrane or to lysosomes, will be affected by this co-expression strategy. Experiments in yeast have shown ER/cytosol compartmentalisation is not affected by the co-expression strategy [157]. Confirmation of this in mammalian cells is still awaited. Another concern stems from the addition of 2A-derived sequences to the protein products. Thus 19 amino acids are added to the C-terminus of the upstream product and an N-terminal proline to the downstream protein product. These may interfere with function or may present new epitopes that could be subject to immunological surveillance.

Other tissues

This review has concentrated on the hematopoietic system since it is here that most work is ongoing in defining selectable markers and where the drive to select has been necessitated by the poor levels of transduction of human hematopoietic stem cells. However, the potential to use selection in other tissues is also under investigation. Thus MDR-1 gene transfer to skin keratinocytes has been combined with topical administration of colchicine to achieve *in vivo* selection of gene-modified cells [158]. Similarly, MGMT-mediated protection of epithelial and mesenchymal cells *in vitro* has been demonstrated [159,160].

CID-mediated selection has also been demonstrated in tissues other than the hematopoietic system. Coumermycin-dependent activation of vascular endothelial growth factor receptor signalling in endothelial cells has been demonstrated [121]. CID-dependent skeletal myoblast proliferation has been driven by an F36V–basic fibroblast growth factor receptor fusion protein [119]. Similarly, fusion of F36V with an interleukin 6 signalling domain allowed CID-mediated proliferation of primary murine hepatocytes and facilitated *in vivo* selection of these cells [120].

Finally, with the apparent potential for HSCs to contribute to non-hematopoietic tissues (particularly in response to tissue injury), gene transfer and selection of HSCs themselves might provide a means to genetic therapy in multiple tissues [161].

Selection – the double-edged sword?

The usual premise in trying to achieve selection of transduced cells is that selection is a useful and necessary part of the therapeutic process. Certainly the selective advantage conferred to T-cells by the corrected gamma-chain in X-SCID patients has contributed to the success of that treatment. However, it has also probably contributed to a serious adverse effect in two of the patients [162]. Both patients have developed a lymphoproliferative disorder and have required treatment to overcome their lymphoid leukaemias. On analysis, both leukaemias seem to derive from an insertional mutagenesis event that has caused activation of the same oncogene (LMO2). LMO2 overexpression alone is not frankly leukaemic [163]. It is possible that an interaction between the transgene and LMO2 is one factor contributing to the expansion of the leukaemic clones.

Only a few murine studies of gene therapy have reported leukaemic induction. In one study, using what was presumed to be an inert marker gene (truncated nerve growth factor receptor), placing gene-modified cells under proliferative stress by secondary transplantation led to the development of a leukaemic clone in the recipient animals [164]. Evidence of oncogene activation by retroviral insertion was found, although the oncogene that was activated (*Evi*-1) is not acutely leukaemic in transgenic mice [165], leading the authors to suggest interaction between *Evi*-1 and the marker gene product. In another study, retroviral gene transfer of MDR-1 was associated with production of a myeloproliferative syndrome [166]. However, a number of groups have failed to replicate this effect of MDR-1, most strikingly in non-human primates using the same vector and analogous transduction conditions to those in the murine study [167]. However, the observation that MDR-1-deficient $APC^{min/+}$ mice show reduced levels of intestinal polyposis, compared with their MDR-1-sufficient counterparts, suggests MDR-1 may have an as-yet occult role in tumourigenesis [168]. With novel membrane receptors such as those fused to F36V, the data thus far suggest no effect of the signalling domains in the absence of dimerising drug.

Similarly, HoxB4 has not thus been associated with malignant hematopoietic disease, although it has demonstrated transforming properties in a fibroblast cell line [169], and enhanced HoxB4 expression was seen in psioratic human skin and basal cell carcinomas [170]. The demonstration that HoxB4, in the context of reduced expression of PBX1, confers even greater proliferative and engraftment potential to HSC should provide a warning [171]. The combination of HoxB4 and PBX1 knockdown proved 40 times more effective than hoxB4 alone in a competitive repopulation assay. However, absolute levels of hematopoietic cells in recipient animals did not exceed the normal range. This strongly suggests a cell-extrinsic control on unrestrained proliferation. Escape from such control as a result of insertional inactivation or some other mutational event(s) could lead to malignant expansion. Obviously, it will be important to examine all of these potentially selective genes for the possibility that they might also interact with an insertionally upregulated oncogene or other pre-leukaemic event to produce adverse effects.

Where resistance to chemotherapeutic agents is used as a selective mechanism, another level of potential adversity is reached. Thus the use of DNA-damaging drugs carries the potential to induce mutations that could lead to leukaemia. The argument is that, since it takes fewer DNA lesions per cell to acquire a mutation than it does to engage the cell-death pathway, chemoprotective gene transfer may lead to a preponderance of cells that have acquire non-lethal, but mutagenic levels of genome damage. Thus the frequency of mutation (and potentially transformation) in the hematopoietic system would be increased. Such fears have not thus far been borne out in transduction studies with MGMT, where overexpression has led to significant decreases in mutagenic and clastogenic events [83–85,172,173]. Furthermore, in transgenic mouse studies, overexpression of MGMT led to a decrease in malignant transformation on carcinogenic challenge [174–176], even in the context of p53 insufficiency [177].

Even if gene-modified cells do not contribute to leukaemogenesis, commonly used chemotherapeutic agents may convert normal, untransduced cells to leukaemic cells.

This is a well-documented sequel to anti-tumour therapy with such agents. Crucial to reducing the levels of leukaemic induction from non-transduced cells might be the ability to efficiently kill such cells with the selective agent. In this regard it is interesting to note that pre-treatment of animals with cytokines leads to increased killing of unmodified bone marrow stem cells by chemotherapeutic agents and to improved selective effects [178,179].

Leukaemia is not the only potential adverse effect that may accrue as a result of selection. Selective pressures that result in skewing of the lineage profile may be equally detrimental. The potential for compromising lineage choice has been raised by experiments with dimerising growth factor receptors and molecules such as HoxB4. Clearly, careful analysis will be required to ascertain whether these fears are justified. Furthermore, experience with growth-factor-induced expansion of hematopoietic cells *ex vivo* has shown that the engagement of some signalling pathways, at least *in vitro*, can lead stem cells to undergo commitment to differentiation. Were this to occur with *in vivo* selective pressures, stem cell exhaustion may occur. Again this requires careful analysis, employing secondary and tertiary transplantation experiments to determine the response of gene-modified and selected stem cells to proliferative stress.

Summary and conclusions

There are a number of alternative and potentially powerful approaches to providing a selective advantage to transduced cells. For most clinical applications, an *in vivo* advantage will be necessary. Selection may occur at the level of the stem cell or at the level of committed hematopoietic progenitors and different clinical applications may require different levels of selection. The potential for adverse effects due to provision of a selective advantage, particularly a proliferative one, means that great care will have to be taken to ensure that appropriate levels of control of gene-modified cells is achieved.

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