CHARACTERIZATION OF THE FUNCTION OF TYPE XIII COLLAGEN IN MICE; SPECIFIC ROLES DURING CARDIOVASCULAR DEVELOPMENT AND POSTNATALLY IN BONE MODELING

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Abstract
Type XIII collagen is a type II transmembrane protein which is expressed in many tissues throughout development and adult life. It is located in focal adhesions of cultured cells and in the adhesive structures of tissues such as the myotendinous junctions in muscle, intercalated discs in the heart and the cell-basement membrane interphases. To further characterize the function of this protein, we generated transgenic mice overexpressing it in normal and mutant forms.

A large in-frame deletion in the COL2 domain of type XIII collagen led to synthesis of truncated α1(XIII) chains in transgenic mice, disrupting the assembly of normal type XIII collagen trimers. Fibroblasts derived from the mutant mice expressed shortened α1(XIII) chains, and no intracellular accumulation of the mutant protein was detected, suggesting that the mutant molecules were expressed on the cell surface. Transgene expression led to an embryonally lethal phenotype in offspring from heterozygous mating at two distinct stages of development. The early phenotype fetuses died due to the lack of chorioallantoic fusion and functioning placenta at 10.5 dpc, while the death of the late phenotype fetuses was caused by cardiac and placental defects around 13.5 dpc. The phenotype resembles closely several other cell adhesion molecule mutants, indicating that type XIII collagen has an essential role in certain adhesive interactions that are necessary for normal development.

Mice overexpressing type XIII collagen with or without a point mutation developed postnatally an unexpected skeletal phenotype marked by a massive increase in bone mass. The cortical bone cross-sectional area and volumetric bone mineral density were highly increased, but trabecular bone volume was not significantly altered. The bone formation rate was several times higher in the mutant mice than in their normal littermates, while the osteoclast number and resorption activity were normal. Type XIII collagen was expressed highly in primary osteoblasts derived from the transgenic mice. Overexpression of type XIII collagen in osteoblasts enhanced both cell proliferation and differentiation while lack of it had opposite effects. Furthermore, mutant cells responded to mechanical strain differently than wild-type cells. The findings suggest that type XIII collagen has an important role in bone modeling, and it may in particular have a function in coupling the regulation of bone mass to mechanical usage.

Keywords: bone formation, cardiogenesis, collagen, development, mechanical strain, osteoblast, placentation, transgenic mice, vascularization
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Abbreviations

αx(y) collagen polypeptide; x: number of chain; y: collagen type
ALP alkaline phosphatase
BM basement membrane
BMC bone mineral content
BMD bone mineral density
BMP bone morphogenetic protein
bp base pair
BSA bovine serum albumin
C- carboxy-
cDNA complementary DNA
COL collagenous
α-MEM minimal essential medium alpha modification
dpc day post coitum
kb kilobase
kDa kilodalton
mRNA messenger RNA
ECM extracellular matrix
EDTA ethylenediaminetetraacetic acid
FBS fetal bovine serum
FGF fibroblast growth factor
HA hemagglutinin
IGF insulin-like growth factor
M-CSF macrophage colony-stimulating factor
N- amino-
NC noncollagenous
OPG osteoprotegerin
PBS phosphate-buffered saline
PCR polymerase chain reaction
pQCT peripheral quantitative computed tomography
RANKL receptor activator of nuclear factor κB ligand
RT-PCR reverse transcriptase polymerase chain reaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Runx2</td>
<td>runt-related transcription factor 2</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TRACP</td>
<td>tartrate resistant acid phosphatase</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridinetriphosphate (dUTP) nick end labeling</td>
</tr>
<tr>
<td>X-</td>
<td>any amino acid in Gly-X-Y</td>
</tr>
<tr>
<td>Y-</td>
<td>any amino acid in Gly-X-Y</td>
</tr>
</tbody>
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List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


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1 Introduction

The extracellular matrix (ECM), composed of collagens, proteoglycans, noncollagenous glycoproteins and elastin, gives mechanical support to tissues and functions as a substrate for cell adhesion, growth, migration and differentiation. The ECM provides information to developing cells from the first stages of embryogenesis via protein interactions and by presenting growth factors and morphogens to cells. Cell-matrix interactions are needed throughout life for tissue homeostasis and remodeling. For example, connective tissue cells are able to adapt their ECM to changes in mechanical load during bone remodeling and wound healing.

Type XIII collagen is a type II transmembrane protein with a short cytosolic domain and a large mainly collagenous ectodomain. It is expressed in low levels in most developing and adult tissues. Cell culture studies have shown that type XIII collagen localizes to focal adhesions, and in tissues it is also found in adhesive structures such as intercalated discs in the heart, myotendinous junctions in the skeletal muscle and at cell-basement membrane interphases. Although the structure and tissue distribution of type XIII collagen have been well characterized its biological function is still largely unknown. Transgenic mouse technologies have become one of the most important tools to study the function of genes. These mouse models have provided new insights into understanding the causes of several diseases and possible platforms on which to test new treatments.

The purpose of this study was to clarify the function of type XIII collagen using different transgenic mouse models. We have generated and analyzed transgenic mice expressing type XIII collagen with a 90 amino acid in-frame deletion in the COL2 domain. Since this mutation led to an embryonally lethal phenotype the effect of milder mutations was tested and two mouse lines were generated, one overexpressing normal type XIII collagen and another with a point mutation changing glycine to tryptophan in COL3 domain of type XIII collagen. Analysis of these three different mouse lines indicated that type XIII collagen has an important role during embryogenesis regulating cardiovascular development, but it also has a function postnatally maintaining homeostasis of skeletal tissues.
2 Review of the literature

2.1 The collagen superfamily

Collagens are the most abundant proteins of the extracellular matrix that surrounds the cells, providing them mechanical support and a substrate for cell adhesion, growth and differentiation. In addition to the well-characterized role of collagens in maintaining the structural integrity of various tissues they have other important biological functions (Myllyharju & Kivirikko 2004). So far 28 collagen types with 42 distinct α chains and over 20 additional proteins with collagen-like domains have been described (Myllyharju & Kivirikko 2004, Koch et al. 2004). Collagenous domains are characterized by Gly-X-Y amino acid repeats enabling the triple helix formation from three polypeptides called α chains. Depending on collagen type, all three α chains can be identical, or the triple helix may consist of two or three different α chains. The X and Y positions can have any other amino acid than glycine, but they are often occupied by proline and hydroxyproline providing stability for the triple helical structure (van der Rest & Garrone 1991, Kivirikko 1993, Brown & Timpl 1995, Brodsky & Shah 1995, Prockop & Kivirikko 1995, Pihlajaniemi & Rehn 1995, Myllyharju & Kivirikko 2001, Gelse et al. 2003, Myllyharju & Kivirikko 2004).

The members of the collagen superfamily can be divided into two major groups, fibril-forming and non-fibrillar collagens according to their supramolecular assemblies, primary structure and other features. The classical fibril-forming collagens including collagen types I, II, III, V, VI, XXIV and XXVII are characterized by their ability to aggregate into highly ordered quarter-staggered fibrils. The collagen types IX, XII, XIV, XVI, XIX, XX, XXI, XXII and XXVI form the largest group among the non-fibrillar collagens, a group of fibril-associated collagens with interrupted triple helices (FACIT and structurally related collagens). FACIT collagens have interruptions in their collagenous domains, and some of them are known to associate with the surfaces of fibrils. Other subfamilies in the heterogeneous group of non-fibrillar collagens as listed are: collagens forming hexagonal networks (collagen types VIII and X), the family of type IV collagens found in basement membranes, type VI collagen forming beaded filaments, type VII collagen forming anchoring fibrils, collagens with transmembrane
domains (collagen types XIII, XVII, XXIII and XXV) and the family of type XV and XVIII collagens characterized by the antiangiogenic endostatin, restin domain. In addition, the collagen superfamily includes a group of proteins that contain triple-helical collagenous domains but are not defined as collagens (Prockop & Kivirikko 1995, Myllyharju & Kivirikko 2001, Gelse et al. 2003, Myllyharju & Kivirikko 2004).

### 2.2 Transmembrane collagens

The subfamily of transmembrane collagens consists of collagen types XIII, XVII, XXIII and XXV. They are all homotrimers and located on cell membranes in type II orientation with an N-terminal intracellular domain, a hydrophobic transmembrane domain and a large extracellular C-terminal domain. The ectodomain of these collagens can be cleaved from plasma membrane to produce a soluble shorter form (Schäcke et al. 1998, Snellman et al. 2000b, Banyard et al. 2003, Väisänen et al. 2004). Some transmembrane proteins with small collagenous triple helical stretches, e.g. macrophage scavenger receptors (MSRs), macrophage receptor with collagenous structures (MARCO), scavenger receptor with C-type lectin (SRCL), ectodysplasin A and colmedins are also included in the group of transmembrane collagens although they are not classified as collagens (Snellman & Pihlajaniemi 2003, Franzke et al. 2005). The actual transmembrane collagens presented in Figure 1 are discussed below in more detail.

![Schematic presentation of the structure of transmembrane collagens](image)

**Fig. 1. Schematic presentation of the structure of transmembrane collagens (Modified from Snellman & Pihlajaniemi 2003 and Franzke et al. 2005).**
2.2.1 Type XIII collagen

2.2.1.1 Structure and chromosomal localization of type XIII collagen gene

The primary structure of the type XIII collagen α chain consists of three collagenous domains (COL1-COL3) separated by four non-collagenous domains (NC1-NC4). In the N-terminal end forming part of the NC1 domain there is a short cytosolic domain and the transmembrane domain, while other domains form the larger, mainly collagenous ectodomain (Pihlajaniemi et al. 1987, Tikka et al. 1988, Pihlajaniemi & Tamminen 1990, Tikka et al. 1991, Hägg et al. 1998, Snellman et al. 2000a). The type XIII collagen gene is located in chromosome 10 both in human and mouse. The complete exon-intron structure of type XIII collagen has been characterized and shown to be approximately 140 kb in size and to contain 41 exons in human, and to be 135 kb in size and to contain 42 exons in mouse (Tikka et al. 1991, Kvist et al. 1999). The type XIII collagen gene is located in chromosome 10 both in human and mouse. The complete exon-intron structure of type XIII collagen has been characterized and shown to be approximately 140 kb in size and to contain 41 exons in human, and to be 135 kb in size and to contain 42 exons in mouse (Tikka et al. 1991, Kvist et al. 1999). The potential promoters for type XIII collagen have been predicted to locate between nucleotides -905 and -605 in human and between nucleotides -864 and -568 in mouse with respect to the initiation ATG (Kvist et al. 1999). An interesting feature for type XI II collagen is that its mRNA transcripts undergo complex alternative splicing affecting the structures of the COL1, NC2, COL3 and NC4 domains. Although the expression of different splice variants in various tissues has been characterized, the functional significance of different type XIII collagen forms is still open for future studies (Pihlajaniemi et al. 1987, Tikka et al. 1988, Pihlajaniemi & Tamminen 1990, Juvonen & Pihlajaniemi 1992, Juvonen et al. 1992, Juvonen et al. 1993, Peltonen et al. 1997).

2.2.1.2 Biosynthesis and biochemical features of type XIII collagen

Type XIII collagen resides on the plasma membrane in a type II orientation with a short intracellular part, a transmembrane domain and a collagenous ectodomain, which is a rod about 150 nm in length with two flexible hinges coinciding with the NC2 and NC3 domains (Hägg et al. 1998, Snellman et al. 2000a, Tu et al. 2002). Although type XIII collagen is found in various tissues its expression level is relatively low. To characterize type XIII collagen on protein level a recombinant protein was produced in insect cells using a baculovirus expression system (Snellman et al. 2000a). The recombinant type XIII collagen α chains formed disulphide-bonded homotrimers (Snellman et al. 2000a). It has been shown that the sequences needed for the association of the three type XIII collagen α-chains reside in their N-terminal portions, and that triple helix formation appears to proceed from the N-terminus to the C-terminus (Snellman et al. 2000b). Recently, it has been reported that type XIII collagen contains two coiled-coil domains, one near the transmembrane domain and another one located in the NC3 domain functioning as independent oligomerization domains (Latvanlehto et al. 2003). Cell culture studies have indicated that the ectodomain of type XIII collagen can be shed from plasma membrane by proteinases of the furin family (Snellman et al. 2000b, Väisänen et al. 2004). The cleavage occurs both in the plasma membrane and trans-Golgi network,
and it has been proposed that shedding is one of the mechanisms needed for maintaining the amount of type XIII collagen molecules on plasma membrane (Väisänen et al. 2004). The resulting ectodomain is a biologically active molecule and has been shown to inhibit the early phase of cell adhesion and spreading as well as cell migration and proliferation on a vitronectin surface (Väisänen et al. 2004). In vitro studies have also revealed that the ectodomain of type XIII collagen interacts with other extracellular matrix components such as fibronectin, heparin, the basement membrane components nidogen-2 and perlecan, and the α1 subunit of integrin (Nykvist et al. 2000, Tu et al. 2002). Moreover, cell culture studies have shown that the type XIII collagen ectodomain can associate with the fibrillar fibronectin matrix, influencing its assembly (Väisänen et al. 2005b).

### 2.2.1.3 Localization of type XIII collagen in tissues and cultured cells

Northern and in situ hybridization analyses have localized type XIII collagen mRNAs in a number of human fetal tissues such as bone, cartilage, intestine, skin and muscle and in the early human placenta (Sandberg et al. 1989, Tamminen et al. 1993). In developing bone and cartilage mRNAs are detected more specifically in the periosteum, in the mesenchymal cells forming the reticulin fibers of the bone marrow and in the proliferative and hypertrophic chondrocytes of the growth plate (Sandberg et al. 1989). In mouse fetuses type XIII collagen expression has been located by in situ hybridization and immunofluorescence stainings to the same tissues as in human, but strong expression is also detected in the heart and the developing nervous system (Sund et al. 2001). During mouse development type XIII collagen is found in the cartilage as soon as mesenchymal cells start to condensate, and an increase of type XIII collagen expression is detected at late stages of organogenesis, the sites of expression including structures undergoing endochondral ossification (Sund et al. 2001).

Immunofluorescence analysis has located type XIII collagen to several adhesive structures of tissues such as the myotendinous junctions in muscle, intercalated discs in the heart and the cell-basement membrane interphases. In cultured cells type XIII collagen is concentrated in focal adhesions at the end of stress fibers co-localizing with vinculin and talin (Peltonen et al. 1999, Hägg et al. 2001, Sund et al. 2001).

### 2.2.1.4 The functional analysis of type XIII collagen

The function of type XIII collagen has been studied using transgenic mouse models. A schematic presentation of the different transgene products is given in Figure 3. A mouse line, Col13a1N/N, expressing modified type XIII collagen that lacks the cytosolic, transmembrane and association domains was generated by homologous gene targeting. In these mice N-terminally truncated type XIII collagen molecules are transported to roughly correct location despite their lack of a transmembrane domain, leading to a mild muscular phenotype, including abnormalities in the sarcolemma-basement membrane interphase. Muscle damage was induced by exercise (Kvist et al. 2001). The findings
suggest that type XIII collagen has a role in maintaining the linkage between muscle fibers and the basement membrane (Kvist et al. 2001).

Recently, two additional mouse lines, type XIII collagen knock-out (Col13a1/−) and knock-in (Col13a1LacZ/LacZ), have been generated by homologous recombination to study the expression pattern and function of type XIII collagen (Latvanlehto 2004). The Col13a1LacZ/LacZ mice synthesize chimeric molecules with the cytosolic and transmembrane domains of type XIII collagen linked in-frame to the enzyme β-galactosidase that replaces the ectodomain. The type XIII collagen promoter-driven LacZ expression was detected widely, but strong signals were found in neuromuscular junctions and in developing and adult bone, more specifically in periosteal osteoblasts. Some phenotypic changes were detected, such as twitching or gasping followed by shivering, suggested to be physiological consequences resulting from impaired structure and function of neuromuscular junctions. Moreover, the Col13a1LacZ/LacZ mice were smaller than wild-type littersmates and their bones were thinner, with reduced mechanical properties (Latvanlehto 2004). The results from transgenic mice showing defects in bone and muscle with altered myotendinous and neuromuscular junctions have further demonstrated the importance of type XIII collagen at the sites of cell-matrix interaction.

Little is known so far about type XIII collagen expression in human diseases. Serum antibodies against type XIII collagen have been detected in the autoimmune-mediated inflammation of Graves’ ophthalmopathy, but their significance is unclear (Mizokami et al. 2004, De Bellis et al. 2005). In recent studies by tissue microarray analysis type XIII collagen expression has been localized in the stromal compartment of epithelial and mesenchymal tumors (Väisänen et al. 2005a). Factors secreted by tumor cells, in particular the growth factor TGF-β1, induced type XIII collagen expression and triggered morphological changes in cultured primary fibroblasts, suggesting that malignant transformation stimulates the expression of type XIII collagen in the cancer stroma contributing to tumor progression and behavior (Väisänen et al. 2005a). Due to its location in tissues and cultured cells and its binding properties, type XIII collagen is thought to be involved in cellular adhesion and migration.

### 2.2.2 Type XVII collagen

Type XVII collagen, initially identified as the 180-kDa bullous pemphigoid antigen (BP180) in the skin, is a distinct member of the transmembrane collagens. It is a structural component of hemidesmosomes, which mediate adhesion of basal epidermal keratinocytes and some epithelial cells to the basement membrane. Type XVII collagen is a homotrimer of three α1(XVII) chains with a globular intracellular N-terminal domain, a transmembrane domain and a large extracellular C-terminus (Giudice et al. 1992). The intracellular domain of type XVII collagen is located in the cytoplasmic plaque of hemidesmosomes interacting with β4 integrin, BP230 and plectin (Koster et al. 2003). The ectodomain is a flexible rod capable of binding to α6 integrin and laminin 5 (Hopkinson et. al 1995, Tasanen et al. 2004). The shedding of type XVII collagen ectodomain in keratinocytes is catalyzed by the members of the ADAM family and has been shown to be regulated by plasma membrane lipid organization (Franzke et al. 2002,
Collagen types XXIII (Banyard et al. 2003) and XXV (Hashimoto et al. 2002), the most recently found members of the transmembrane collagen family, are structurally similar to type XIII collagen. They are both type II transmembrane proteins consisting of a short N-terminal cytosolic domain, a transmembrane region and three collagenous domains flanked by short non-collagenous domains (Snellman & Pihlajaniemi 2003). The ectodomain of type XXIII and XXV collagens can be cleaved from the plasma membrane similarly to type XIII collagen by furin proteases. Recent results also show that the coiled-coil domains of type XIII collagen located in the NC1 and NC3 domains are highly conserved in collagen types XIII, XXIII and XXV (Latvanlehto et al. 2003).

Type XXIII collagen has been identified from rat and human RNA. It is expressed in normal human heart and retina, and overexpression of type XXIII collagen is detected in highly metastatic prostate cancer cells. The ectodomain of type XXIII collagen binds at low affinity to heparin. It has been suggested that due to the increased shedding of the ectodomain in type XXIII collagen overexpressing tumor cells it could be considered as a potential diagnostic or prognostic marker (Banyard et al. 2003).

Type XXV collagen, also called CLAC-P (collagen-like Alzheimer amyloid plaque component precursor), was identified in a search for components of senile plaques (SPs) which are β-amyloid (Aβ) depositions characteristic of Alzheimer’s disease. RNA analysis of mouse and human tissues showed specific expression of type XXV collagen in brain and neurons. In addition, low-level expression was detected in mouse heart, testis and eye. Type XIII and XXV collagens are about 43% homologous at the amino acid level and as with type XIII collagen, the primary transcripts of type XXV collagen undergo alternative splicing. The ectodomain of type XXV collagen (CLAC) is secreted by furin and its N-terminus is pyroglutamate modified. Both secreted and membrane-bound forms of type XXV collagen bind to fibrillized Aβ (Hashimoto et al. 2002). The contribution of CLAC to the pathogenesis of Alzheimer’s disease and plaque formation is unclear. It has been shown to inhibit formation of amyloid fibrils and on the other hand, to assemble fibrils into protease resistant aggregates (Osada et al. 2005, Söderberg et al. 2005).

2.3 Analysis of collagen gene function using transgenic mouse models

The importance of collagens in biology has been demonstrated by the wide spectrum of diseases caused by mutations in collagen genes. The transgenic mouse technology has
provided tools for studying the consequences of disease-causing mutations and testing for potential therapies. Spontaneous and man-made mutations have even led to identification of new diseases caused by mutations in collagen genes. Moreover, the significance and function of the various collagen types has been characterized using transgenic mice (Kivirikko & Prockop 1995, Myllyharju & Kivirikko 2001, Myllyharju & Kivirikko 2004).

There are two strategies that are most commonly used for generation of genetically engineered mice. The first strategy, injection of functional DNA, a transgene, into single-cell embryos has been used for over 20 years. These transgenes typically consist of cDNAs that encode wild-type or mutated gene products driven by a promoter to target the expression to specific cell lineages. The construct DNA is microinjected into the pronucleus of fertilized oocytes. The embryos are then transferred to the oviducts of a pseudopregnant foster mother, and some of them develop to term (Palmiter & Brinster 1985, Hogan et al. 1986). The drawbacks of this technique are random insertion of the transgene into the genome and variations in inserted transgene copy number. The expression of the transgene is unpredictable and can be affected by local factors due to the insertion site. However, the microinjection technique is often used to produce gain-of-function mutations in which the transgene is designed to overexpress the gene product and to interfere with the function of an endogenous gene. For example, transgenic expression of an antisense mRNA can decrease endogenous gene function. Furthermore, synthesis of a mutant protein subunit can be used to disrupt the assembly of multimeric proteins such as trimeric collagens (Hardouin & Nagy 2000). The mutated α-chains usually associate with the endogenous α-chains, leading to defects in the formation of the collagen triple helix. These trimers with mutant α-chains are degraded or they function abnormally, causing a dominant negative effect (Kivirikko 1993, Kivirikko & Prockop 1995).

The second strategy, gene targeting, introduces changes directly into endogenous genes in the chromosomal locus, avoiding the disadvantages of the microinjection technique (Capecchi 1989, Hardouin & Nagy 2000, Capecchi 2005). The targeting is based on homologous recombination between the genome and an exogenous DNA fragment including homologous sequences to the genomic locus. In most cases a targeting vector is constructed so that successful recombination results in replacement of part of a gene with a selectable marker, such as the neomycin resistance gene. Also markers for negative selection against the non-homologous integration, such as thymidine kinase gene, are included in the constructs. The targeting vector is introduced into cultured embryonic stem cells. The clones where homologous recombination has occurred are selected and injected into recipient blastocysts to generate chimeric mice. If chimerism extends to the germ cells, transgenic mice can be generated by breeding the chimeric founder. Gene targeting was originally used for gene inactivation (knock-out). Since the selectable marker may interfere with the expression of the targeted allele, subtle changes can be created in the genome by flanking the selectable marker with loxP sites. Loss or gain of gene function in specific tissues and/or at a restricted time can be studied using a site-specific recombination system such as Cre-loxP. In targeting vector loxP-sites are placed around a functionally essential part of the gene but otherwise leaving a minimal change in the gene structure. Introduction of Cre promotes recombination at the loxP-sites removing the selected area of DNA. Cre is often introduced with a simple
breeding scheme combining the conditional allele and a transgene expressing the Cre recombinase in a spatially or temporally restricted manner (Hardouin & Nagy 2000, Capecchi 2005). The Cre-\textit{loxP} recombination system allows also genome engineering with respect to large deletions and translocations between non-homologous chromosomes (Hardouin & Nagy 2000).

In addition to direct mutagenesis, indirect approaches have been used to establish disease models. Mutations are usually introduced by chemically-induced random mutagenesis using a point mutagen, N-ethyl-N-nitrosourea (ENU mutagenesis), or by large-scale gene trapping (Justice \textit{et al.} 1999, Evans \textit{et al.} 1997). The phenotypes of the transgenic mice are screened for similarities to human diseases, and good candidate genes can then be identified and characterized.

The function of several collagen genes has been studied using transgenic mice generated by random integration transgenesis and/or gene targeting. The phenotypes of mice with naturally occurring and man-made collagen gene mutations are listed in Table 1.

\begin{table}
\caption{Transgenic mouse models for collagen mutations.}
\begin{tabular}{llll}
\hline
Collagen type & Mutation & Phenotype & Reference \\
\hline
\(\alpha_1(\text{I})\) & TG (retroviral insertion in intron 1) & Fetal lethality, circulatory failure, as heterozygous: hearing loss, bone defect & Jaenich \textit{et al.} 1983, Lohler \textit{et al.} 1984, Bonadio \textit{et al.} 1990 \\
\(\alpha_1(\text{I})\) & TG (Gly\textsubscript{859}→Cys) & Perinatal lethality, short, poorly mineralized bones & Stacey \textit{et al.} 1988 \\
\(\alpha_1(\text{I})\) & TG (in-frame deletion of 41 exons) & High expression: perinatal lethality, multiple fractures Moderate expression: fractures, brittle bones & Khillan \textit{et al.} 1991, Pereira \textit{et al.} 1993 \\
\(\alpha_1(\text{I})\) & T (Gly\textsubscript{859}→Cys) & Perinatal lethality (40-60%), bone fragility and deformity, fractures & Forlino \textit{et al.} 1999 \\
\(\alpha_1(\text{I})\) & TG, T (Gln\textsubscript{774}, Ala\textsubscript{777}→Pro and Ile\textsubscript{776}→Met) & TG: Fetal lethality T: Viable, impaired collagen I cleavage, fibrosis osteocyte/osteoblast apoptosis, increased bone deposition & Liu \textit{et al.} 1995 \\
\(\alpha_2(\text{I})\) & N (oim, single base deletion) & Viable, skeletal deformities, osteopenia, fractures & Chipman \textit{et al.} 1993 \\
\(\alpha_1(\text{II})\) & N (dmm, 3 nucleotide deletion) & Perinatal lethality, lung hypoplasia, severe skeletal dysplasia & Brown \textit{et al.} 1981 \\
\(\alpha_1(\text{II})\) & TG (in-frame deletion of 12 exons) & Perinatal lethality, respiratory failure, severe chondrodysplasia & Vandenbarg \textit{et al.} 1991 \\
\(\alpha_1(\text{II})\) & TG (Gly\textsubscript{859}→Cys) & Perinatal lethality, respiratory failure, severe chondrodysplasia & Garofalo \textit{et al.} 1991 \\
\(\alpha_1(\text{II})\) & TG (15 amino acid deletion) & Perinatal lethality, respiratory distress, lethal chondrodysplasia & Metsärand \textit{et al.} 1992 \\
\hline
\end{tabular}
\end{table}
<table>
<thead>
<tr>
<th>Collagen type</th>
<th>Mutation</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1(II)</td>
<td>TG (overexpression)</td>
<td>Perinatal lethality, respiratory distress, cartilage abnormalities</td>
<td>Garofalo et al. 1993</td>
</tr>
<tr>
<td>α1(II)</td>
<td>KO</td>
<td>Perinatal lethality, lack of endochondral bone and bone marrow</td>
<td>Li et al. 1995a</td>
</tr>
<tr>
<td>α1(II)</td>
<td>TG (Gly574→Ser)</td>
<td>Perinatal lethality (25-50%), delayed ossification, abnormal growth plates</td>
<td>Maddox et al. 1997</td>
</tr>
<tr>
<td>α1(II)</td>
<td>TG (Arg789→Cys)</td>
<td>Perinatal lethality, short bones, delayed ossification, abnormal growth plates</td>
<td>Gaiser et al. 2002</td>
</tr>
<tr>
<td>α1(II)</td>
<td>TG (human Arg519→Cys)</td>
<td>Viable, retarded skeletal development, defects in growth plates, articular cartilage and intervertebral discs</td>
<td>Arita et al. 2002</td>
</tr>
<tr>
<td>α1(II)</td>
<td>N (sedc, Arg1417→Cys)</td>
<td>Viable, dysplastic bones, hearing loss</td>
<td>Donahue et al. 2003</td>
</tr>
<tr>
<td>α1(II)</td>
<td>TG (exon 48 deletion)</td>
<td>Viable, retarded skeletal development, short bones, abnormal growth plates, chondrocyte differentiation defect</td>
<td>Barbieri et al. 2003</td>
</tr>
<tr>
<td>α1(III)</td>
<td>KO</td>
<td>Viable (10%), shortened life span, rupture of blood vessels, abnormal collagen I fibrillogenesis</td>
<td>Liu et al. 1997</td>
</tr>
<tr>
<td>α1, α2(IV)</td>
<td>KO</td>
<td>Fetal lethality, structural defects in the basement membranes</td>
<td>Pöschl et al. 2004</td>
</tr>
<tr>
<td>α3(IV)</td>
<td>KO</td>
<td>Postnatal lethality, renal failure</td>
<td>Cosgrove et al. 1996, Miner &amp; Sanes 1996</td>
</tr>
<tr>
<td>α3, α4(IV)</td>
<td>TG (insertion leading to double knock-out)</td>
<td>Postnatal lethality, renal failure</td>
<td>Lu et al. 1999</td>
</tr>
<tr>
<td>α2(V)</td>
<td>T (exon 6 deletion)</td>
<td>Perinatal lethality, respiratory distress due to spinal deformities, skin and eye abnormalities</td>
<td>Andrikopoulos et al. 1995</td>
</tr>
<tr>
<td>α1(VI)</td>
<td>KO</td>
<td>Viable, early onset myopathy</td>
<td>Bonaldo et al. 1998</td>
</tr>
<tr>
<td>α1(VII)</td>
<td>KO</td>
<td>Postnatal lethality, skin blistering</td>
<td>Heinonen et al. 1999</td>
</tr>
<tr>
<td>α1, α2 (VIII)</td>
<td>KO</td>
<td>Viable, eye abnormalities</td>
<td>Hopfer et al. 2005</td>
</tr>
<tr>
<td>α1(IX)</td>
<td>TG (central in-frame deletion)</td>
<td>Viable, ophthalmopathy, mild dwarfism, early onset of osteoarthritis</td>
<td>Nakata et al. 1993</td>
</tr>
<tr>
<td>α1(IX)</td>
<td>KO</td>
<td>Viable, noninflammatory osteoarthritis</td>
<td>Fässler et al. 1994</td>
</tr>
<tr>
<td>α1(X)</td>
<td>KO</td>
<td>Viable, perinatal lethality 11%, subtle growth plate compressions and hematopoietic changes</td>
<td>Rosati et al. 1994, Kwan et al. 1997, Gress &amp; Jacenko 2000</td>
</tr>
<tr>
<td>α1(XI)</td>
<td>N (cho, single base deletion)</td>
<td>Perinatal lethality, disorganized growth plates, thick collagen fibers, dwarfism</td>
<td>Li et al. 1995b</td>
</tr>
</tbody>
</table>
Table 1. Continued.

<table>
<thead>
<tr>
<th>Collagen type</th>
<th>Mutation</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2(XI) KO</td>
<td></td>
<td>Viable, small body size, disorganized growth plates, hearing loss</td>
<td>Li et al. 2001</td>
</tr>
<tr>
<td>α1(XII) TG (in-frame deletion)</td>
<td>Viable, disrupted matrix structure of periodontal ligament and skin</td>
<td>Reichenberger et al. 2000</td>
<td></td>
</tr>
<tr>
<td>α1(XIII) TG (90 amino acid in-frame deletion)</td>
<td>Fetal lethality, cardiovascular and placental defects</td>
<td>Sund et al. (I)</td>
<td></td>
</tr>
<tr>
<td>α1(XIII) T (N-terminus altered)</td>
<td>Viable, progressive myopathy</td>
<td>Kvist et al. 2001</td>
<td></td>
</tr>
<tr>
<td>α1(XIII) TG (overexpression)</td>
<td>Viable, increased bone formation</td>
<td>Ylönen et al. (II)</td>
<td></td>
</tr>
<tr>
<td>α1(XIII) T (lacking ectodomain)</td>
<td>Viable, small body size, altered neuromuscular junctions, bone defects</td>
<td>Latvanlehto 2004</td>
<td></td>
</tr>
<tr>
<td>α1(XV) KO</td>
<td>Viable, mild myopathy, cardiovascular defects</td>
<td>Eklund et al. 2001</td>
<td></td>
</tr>
<tr>
<td>α1(XVIII) KO</td>
<td>Viable, eye abnormalities</td>
<td>Fukai et al. 2002, Ylikärppä et al. 2003a</td>
<td></td>
</tr>
<tr>
<td>α1(XVIII) TG (endostatin overexpression)</td>
<td>Altered BMs, hydrocephalus (20%)</td>
<td>Utriainen et al. 2004</td>
<td></td>
</tr>
<tr>
<td>α1(XV), α1(XVIII) KO</td>
<td>Viable, no major defects compared to single allele KO phenotypes, but altered retinal astrocyte function</td>
<td>Ylikärppä et al. 2003b, Hurskainen et al. 2005</td>
<td></td>
</tr>
<tr>
<td>α1(XIX) KO</td>
<td>Viable, abnormal muscle cell layer in the esophagus</td>
<td>Sumiyoshi et al. 2004</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: KO, knock-out, T, other targeted, TG, transgenic and N, naturally occurring mutation.

2.4 Placental development

The placenta is critical for fetal survival and normal development. The placenta between the fetal and maternal environment is needed for the exchange of gases, nutrients and waste products. In addition, it produces pregnancy-associated hormones and growth factors and participates in immune protection of the fetus. Failures in implantation and placental development caused by genetic or environmental factors can lead to early pregnancy loss and pregnancy complications, such as pre-eclampsia (Cross et al. 1994, Rossant & Cross 2001). Recent studies of transgenic mice with placental defects have provided new insights into the genetic control of placental development (Rossant & Cross 2001). Although the structures of the placenta differ between the species, considerable similarities exist, and there are several features of pregnancy that are similar in humans and mice (Rossant & Cross 2001, Cross et al. 2003). The principle of murine placentation is described in the following paragraph.

The implantation of the mouse embryo takes place around 4.5 dpc. The trophectoderm cells overlying the inner cell mass (ICM) continue to proliferate and form the extra-
embryonic ectoderm and ectoplacental cone as the peripheral trophectoderm cells give rise to trophoblast giant cells. Later the expanding extra-embryonic ectoderm forms the chorionic epithelium which contacts around 8.5 dpc the allantois, which is formed from the mesoderm at the posterior end of the embryo. After chorioallantoic fusion, fetal blood vessels grow out of the allantois and invade the chorion generating the fetal part of the placental vasculature. Villous branching of trophoblasts and associated fetal vessels creates a labyrinth, and simultaneously chorionic trophoblasts begin to differentiate into the labyrinthine trophoblast cells. The labyrinth trophoblast layer and the mature placenta are formed. The maternal blood enters small spaces of the labyrinth where it is in direct contact with the fetal trophoblastic villi, allowing material exchange between the two blood systems (Cross et al. 1994, Rossant & Cross 2001).

Recently, it has been shown that the main signaling pathway involved in the interaction between trophoblasts and ICM is the fibroblast growth factor (FGF) signaling pathway (Rossant & Cross 2001). FGF4 is expressed widely during early preimplantation development and becomes restricted to the ICM at the blastocyst stage. FGF4 deficient mice die at implantation with poor development of all cell lineages, and lack of FGF receptor signaling has been shown to block proliferation of trophoblasts at the blastocyst stage (Feldman et al. 1995, Arman et al. 1998). The transcription factors caudal-type homeobox 2 (Cdx2) and eomesodermin homologue (Eomes) are the likely downstream targets of FGF signaling, and are in turn essential for the maintenance of trophoblast stem cells (Chawengsaksophak et al. 1997, Russ et al. 2000). Mutations that interfere with the development of the allantois will result in failure of chorioallantoic fusion and functioning placenta (Rossant & Cross 2001). Altered function of several embryonic-patterning genes, such as the T-box gene, Brachyury, and the bone morphogenetic protein (BMP) genes, Bmp5 and Bmp7, have been shown to cause allantoic defects and placental failure (Rashbass et al. 1991, Solloway & Robertson 1999). Mice lacking cell adhesion molecules, vascular cell-adhesion molecule 1 (VCAM-1) or its receptor α4 integrin also show defects in chorioallantoic fusion (Gurtner et al. 1995, Kwee et al. 1995, Yang et al. 1995). The transcription factor glial cells missing 1 (Gcm1), expressed in the labyrinth from the beginning of the chorioallantoic fusion, has been shown by gene inactivation to be necessary both for the initiation of branching morphogenesis and syncytiotrophoblast differentiation (Anson-Cartwright et al. 2000). The subsequent labyrinth development is dependent on FGF, hepatocyte growth factor (HGF) and Wnt signaling pathways. For example, mice homozygous for the Fgfr2, c-Met and Wnt2 null alleles show labyrinth defects (Rossant & Cross 2001).

### 2.5 Development of the cardiovascular system

After the formation of a functioning placenta there are still many other circulatory problems that can lead to embryonic lethality. The heart is the first organ to develop in vertebrates, requiring complex interactions involving cells from different embryonic lineages, including the myocytes of the myocardium, the endothelial cells of the endocardium and the neural crest cells. In mice, heart development starts around 7.5 dpc. Cardiogenic cells migrate from two regions of the splanchnic mesoderm to a ventral
midline and fuse forming a tube of contracting muscle cells. The primitive cardiac tube contains an outer myocardium and an inner endocardium separated by extracellular matrix, “the cardiac jelly”. The tubular heart then undergoes rightward looping to form a S-shaped structure with a single atrium and a single ventricle. Following looping the atria, ventricles and outflow tract become separated by endocardial cushions that form as regional swellings of the cardiac jelly. Neural crest cells enter the heart participating in the formation of the outflow tract. Eventually the cushions fuse to form the anlage of the septal and valvular structures, and the four-chambered heart is formed by the growth of atrial and ventricular septa towards the cushion (Olson & Srivastava 1996, Gilbert 1997).

Several transcription factors, including GATA4, MADS box transcription Mef2 and Nkx2-5, are expressed in cardiogenic progenitors (Rossant 1996, Bruneau 2003). Nkx2-5 belongs to the family of NK class of homeodomain proteins, related to Drosophila tinman. Tinman is a recessive mutation that leads to the complete absence of cardiac precursors (Harvey 1996). In mice, deficiency of Nkx2-5 does not result in lack of a heart, but instead leads to defects in differentiation and morphogenesis of the heart (Lyons et al. 1995). The external signals that promote cardiac differentiation are related to the BMP, FGF and Wnt families of secreted proteins. It has been proposed that intersection of Wnt antagonism along the embryo’s anterior-posterior axis and BMP and FGF signaling along the dorso-ventral axis creates a region of competence for cardiac differentiation (Bruneau 2003). Transgenic mouse models have provided new insights into the patterning of the heart into its various segments. Deficiency of the basic helix-loop-helix transcription factor, Hand2, results in a heart that lacks a right ventricle (Srivastava et al. 1997). The transcription factor Mef2 is required for the growth of the ventricles, but not the atria (Lin et al. 1997). Similarly, mice lacking the T-box transcription factor, Tbx5, show hypoplasia of the atria and left ventricle, but the right ventricle and outflow tract are formed normally (Bruneau et al. 2001). The specific mechanisms of how the formation of distinct structures of the heart is affected by different transcription factors are not clear. Several ligands and signaling pathways have been linked to heart valve development, including VEGF, NFATc1, Notch, Wnt, BMP/TGF-β, ErbB and NF1/Ras (Armstrong & Bischoff 2004). For example, gene-inactivation of the BMP receptor ALK3 and the transcription factor NFATc1 have led to absence of valve formation (Gaussin et al. 2002, de la Pompa et al. 1998, Ranger et al. 1998). The role of neural crest cells in outflow tract septation is debated. Recent studies in animal models, such as the TGF-β2 and retinoid acid receptor, RxRα, knock-out mice, have shown that in the case of severe outflow tract malformations neural crest cells can still be present in the outflow tract (Gruber et al. 1996, Bartram et al. 2001). Furthermore, the T-box transcription factor Tbx1, a candidate gene for the 22q11 syndrome, characterized by neural crest defects including cardiac, thymic and craniofacial abnormalities, is expressed in the embryonic mesoderm surrounding the pharyngeal arch arteries, not in the neural crest cells (Jerome et al. 2001, Lindsay et al. 2001, Liao et al. 2004). It has been suggested that the outflow tract and perhaps the right ventricle arise from a secondary population of cardiac cells, “the secondary heart field”, instead of the cardiac crescent derived myocardium (Waldo et al. 2002, Bruneau 2003).

The mature vascular system is formed by co-ordinated vasculogenesis, where new blood vessels are formed from aggregated angioblasts in the mesoderm, and by angiogenesis, where new vessels are formed from pre-existing ones (Risau et al. 1988,
Pardanaud et al. 1989, Cleaver & Krieg 1999). Vascular endothelial growth factor (VEGF) and its cellular receptors (VEGFR) are the most critical drivers of embryonic vessel formation. Mice lacking VEGF or its receptor VEGFR2 die during embryonic development 8.5-9.5 dpc due to failure of vascular development (Yancopoulos et al. 2000). Mutations of genes involved in cell adhesion leading to abnormal placental and/or cardiovascular development are discussed in the following chapter.

2.6 Transgenic mice with placental and/or cardiovascular defects caused by mutated cell adhesion molecules

Several transgenic mouse lines have been described with mutations affecting trophoblast and placental development, defects in chorioallantoic fusion being one of the common causes of mid-gestation embryonic lethality (Rossant & Cross 2001). Since a functioning circulatory system is important for fetal survival, many cases of pregnancy loss must also be linked to cardiovascular defects (Rossant 1996). Gene inactivations of α5 integrin and its ligand fibronectin, N-cadherin, and focal adhesion kinase (FAK) in mice have led to embryoally lethal phenotypes at early stages, mainly due to a general defect of mesoderm development. It is not known whether the lethality is mainly caused by defects in the yolk sac structures or failure of normal heart development and vasculogenesis (Yang et al. 1993, George et al. 1993, Georges-Labouesse et al. 1996, Radice et al. 1997, Furuta et al. 1995). Mice lacking VCAM-1 or its receptor α4 integrin both show defects in chorioallantoic fusion (Gurtner et al. 1995, Kwee et al. 1995, Yang et al. 1995). VCAM-1 is expressed on the tip of the allantois, whereas α4 integrin is expressed on the chorion, suggesting interaction between these molecules in the process of chorioallantoic fusion. Some of the VCAM-1 and α4 integrin deficient embryos survive past this stage of development but die later due to cardiac and perhaps placental problems. In these embryos the epicardium is detached from the myocardium, leading to blood leakage from developing coronary vessels to the pericardial cavity. In addition, in the VCAM-1 knock-out mice the compact layer of the myocardium and intraventricular septum was reduced (Gurtner et al. 1995, Kwee et al. 1995, Yang et al. 1995).

In some mouse models generated for studies of cell adhesion molecules placental defects were detected in the labyrinth in which the fetal and maternal circulations come close to each other for material exchange. β3 integrin, αv integrin and laminin α5 chain knock-out mice have shown placental defects with smaller or thickened labyrinth leading to retarded growth and development of the embryos and increased mortality (Hodivala-Dilke et al. 1999, Bader et al. 1998, Miner et al. 1998). Mice lacking β8 integrin die at midgestation due to insufficient vascularization of the placenta and yolk sac. The only partner identified for β8 integrin is αv integrin. The similarities in phenotypes observed for β8 and αv integrin mutant mice suggest that integrin αvβ8 is required for normal vascular development in the placenta (Zhu et al. 2002).

Mutations of some cell adhesion molecules have mainly affected the developing heart after mid-gestation when the embryo becomes dependent on its own blood circulation. Mice lacking vinculin, a major constituent of cell-cell and cell-matrix junctions, die by day 10.5 of development due to heart defects, characterized by reduced and akinetic
myocardial and endocardial structures (Xu et al. 1998). Deficiency of plakoglobin found in adherens junctions and desmosomes also leads to defects in heart function. In these mice absence of desmosomes and altered adherens junctions is detected in the heart, leading to the rupture of heart ventricles and bleeding to the pericardial cavity (Bierkamp et al. 1996, Ruiz et al. 1996). Mice lacking connexin43, a gap junction protein needed in cell-cell interaction, die after birth due to cardiac malformations (Reaume et al. 1995).

Extracellular matrix and cell-adhesion proteins are also needed in the development and maturation of the vasculature. It is known that inactivation of type I collagen function by insertional mutagenesis leads to embryonally lethal phenotype caused by rupture of blood vessels (Jaenisch et al. 1983, Schnieke et al. 1983, Harbers et al. 1984, Lohler et al. 1984). Mice lacking VE-cadherin, a cadherin mediating adhesion between endothelial cells, die at 9.5 dpc due to vascular insufficiency caused by increased endothelial apoptosis (Carmeliet et al. 1999). Although mice lacking β-catenin die at gastrulation (8-9 dpc), mice with a conditional knock-out of this gene in endothelial cells survive to around 11-13 dpc and die due to alterations of vascular morphogenesis (Cattelino et al. 2003). It has been shown by the same methods that FAK also has a function in vascular development in late embryogenesis (Shen et al. 2005). Although mice lacking β-catenin die at gastrulation (8-9 dpc), mice with a conditional knock-out of this gene in endothelial cells survive to around 11-13 dpc and die due to alterations of vascular morphogenesis (Cattelino et al. 2003). It has been shown by the same methods that FAK also has a function in vascular development in late embryogenesis (Shen et al. 2005). Although mice lacking β-catenin die at gastrulation (8-9 dpc), mice with a conditional knock-out of this gene in endothelial cells survive to around 11-13 dpc and die due to alterations of vascular morphogenesis (Cattelino et al. 2003). It has been shown by the same methods that FAK also has a function in vascular development in late embryogenesis (Shen et al. 2005).}

### 2.7 Bone

Bone is a specialized form of connective tissue consisting of osteoblasts, osteocytes and osteoclasts surrounded by mineralized extracellular matrix. Osteoblasts synthesize the organic matrix of bone which contains collagens, proteoglycans and glycoproteins, and they also regulate the mineralization of the tissue. Osteocytes are terminally differentiated osteoblasts, which have become trapped within the newly laid-down osteoid. They are the most abundant cell type within the bone matrix. Osteocytes act as mechanosensors and are responsible for the maintenance of bone integrity. Bone resorption is carried out by osteoclasts. The primary component of bone matrix is type I collagen, comprising nearly 90% of organic matrix. The mineral is composed of calcium and phosphate in the form of hydroxyapatite. With respect to other collagens small amounts of type V and very low quantities of type III and FACIT collagens can be detected. The most abundant proteoglycans in bone matrix are decorin and biglycan. Alkaline phosphatase (ALP) and osteonectin are the bone glycoproteins regulating matrix mineralization. Some of the other glycoproteins found in bone including thrombospondin, fibronectin, vitronectin, osteopontin and bone sialoprotein contain the Arg-Gly-Asp (RGD) consensus sequence which is recognized by the integrin class of cell surface receptors. The proteins containing γ-carboxylated glutamic acid (gla) residues, matrix gla protein (MGP) and osteocalcin (bone gla protein, BGP) are also important constituents of bone matrix. The gla residues in these proteins give them high affinity for mineral ions (Marks & Hermey 1996, Robey 1996).
2.7.1 Skeletal development

The vertebrate skeleton is made up of two distinct components, cartilage and bone. The branchial arch and most of the craniofacial bones are formed by cranial neural crest cells. The sclerotome generates the axial skeleton and the lateral plate mesoderm interacts with an overlying apical ridge of ectodermal cells (AER) generating the limb skeleton. Cells in these lineages migrate to the locations where skeletal elements will develop and form densely-packed mesenchymal condensations which can become either osteogenic or chondrogenic. Extracellular matrix molecules, cell surface receptors and cell adhesion molecules, such as fibronectin, tenascin, syndecan and N-CAM, initiate the formation of these condensations and set their boundaries. Transcription factors such as homeobox (Hox) genes modulate cell proliferation within condensations, and indirectly participate in cell adhesion. Cell adhesion is ensured directly through cell adhesion molecules, such as N-CAM and N-cadherin. Growth of the condensation is regulated by BMPs. Eventually growth ceases when noggin inhibits BMP signaling leading to differentiation of cells to chondroblasts or osteoblasts (Olsen et al. 2000, Hall & Miyake 2000).

Beyond the patterning event bone formation occurs through intramembranous or endochondral ossification. The flat bones of the skull, much of the facial skeleton and part of the clavicle are formed by intramembranous ossification. Mesenchymal cells derived from the neural crest proliferate and form condensations prefiguring the future bones. Some of these cells develop into capillaries and others differentiate into osteoblasts which deposit bone matrix (Gilbert 1997). The key regulators of osteoblast differentiation are the two transcription factors, runt-related transcription factor 2 (Runx2) and Osterix (Ducy et al. 1997, Komori et al. 1997, Otto et al. 1997, Nakashima et al. 2002) whose function is described in more detail in the following chapter. In the skull the ossification centers form within the primitive membrane that covers the cranial vault creating bony plates that are separated by un-ossified sutures. The precursor cells that undergo osteoblastic differentiation deposit bone at the outer border of the ossification centers and later at the edge of the bones in the sutures. Several growth factors such as FGFs and BMPs as well as transcription factors such as winged helix transcription factor Foxc1, Twist, msh homeobox homologue Msx and Runx2 are involved in the regulation of this process (Rice et al. 2000, Wilkie & Morriss-Kay 2001). Craniosynostosis syndromes illustrate growth disturbances of the membranous calvarial bones that lead to premature closure of sutures. Dominant, gain-of-function mutations in FGF receptors (FGFR1, FGFR2 and FGFR3) or loss-of-function mutations in the transcription factor TWIST account for approximately 20% of cases of craniosynostosis (Wilkie & Morriss-Kay 2001). The importance of FGF signaling in cranial bone development has been further supported by mouse models harboring mutations in the FGF signaling pathway. For example, mice overexpressing FGF2 develop enlarged occipital bones, and a point mutation in Fgfr1 causes premature fusion of calvarial sutures (Coffin et al. 1995, Zhou et al. 2000). Furthermore, mice lacking FGF18 have delayed calvarial ossification, suggesting a role for FGF18 in intramembranous bone formation (Liu et al. 2002, Ohbayashi et al. 2002). Mice heterozygous for Twist-1 inactivation present a craniosynostosis phenotype with increased bone formation in cranial sutures (Chen & Behringer 1995). It has been shown that Twist acts as an
upstream regulator and as a downstream target of FGFR/FGF signaling (Rice et al. 2000). BMP signaling has been demonstrated to induce expression of Msx transcription factors (Kim et al. 1998). Mutations in MSX2 and RUNX2 have also led to craniosynostosis syndromes (Ma et al. 1996, Mundlos et al. 1997). Mice lacking Msx2 show delayed ossification of the calvarial bones accompanied by a downregulation of Runx2, indicating that Msx2 directly or indirectly regulates Runx2 expression (Satokata et al. 2000, Karsenty & Wagner 2002). Recent results suggest that Foxc1 integrates the BMP and FGF signaling pathways during calvarial development independently of Twist or the BMP antagonist noggin (Rice et al. 2005).

In the remaining skeleton differentiation into chondrocytes produces cartilage models of the future bones that are subsequently replaced by bone and bone marrow through the process of endochondral ossification. Cells of the condensations become chondrocytes. Chondrocytes at the center of these condensations stop proliferating, begin to secrete matrix proteins and become hypertrophic. Changes in the composition and properties of the cartilage matrix in the hypertrophic zone allow the invasion of blood vessels which bring in osteoblasts, osteoclasts and hematopoietic cells. The hypertrophic cartilage matrix is degraded, the hypertrophic cells undergo apoptosis, and osteoblasts begin to secrete bone matrix, replacing cartilage with trabecular bone. Simultaneously, perichondrial cells differentiate into osteoblasts followed by the deposition of calcified bone matrix, “the bone collar” around the middle part of the cartilage. The center of the cartilage model is converted into bone and an ossification front is formed between the newly synthesized bone and cartilage. As the ossification front nears the ends of the cartilage model, the chondrocytes above proliferate pushing out the ends of the bone. These regions are called epiphyseal growth plates. Long bones grow in length until epiphyseal closure after adolescence. The width of the long bones increases by deposition of new matrix on the outer periosteal surface by osteoblasts (Gilbert 1997, Olsen et al. 2000, Karsenty & Wagner 2002). The regulation of bone growth is complex, involving several transcription factors and signaling systems. During endochondral ossification Indian hedgehog (Ihh) is considered a master regulator of both chondrocyte and osteoblast differentiation. It stimulates chondrocyte proliferation directly and indirectly by activating parathyroid hormone related peptide (PTHrP) synthesis. PTHrP primarily keeps chondrocytes in the proliferative stage. The distance from the end of the bone where chondrocytes stop proliferating and undergo hypertrophic differentiation is determined by Ihh. It also acts on perichondrial cells, stimulating the formation of bone collar (Kronenberg 2003). Ihh−/− mice exhibit a disruption of osteoblastic development in endochondral bones. In addition, all their cartilage elements are small due to decreased proliferation of chondrocytes while the number of hypertrophic chondrocytes is increased (St-Jacques et al. 1999). Genetic studies have shown that FGF signaling also has an important role during chondrocyte differentiation and proliferation. All the FGF receptors have their own distinct expression patterns in different types of chondrocytes. Mice lacking FGFR3, which is expressed in proliferating chondrocytes, show an expansion of the length of chondrocyte columns due to increased proliferation of those cells (Colvin et al. 1996, Deng et al. 1996). In these mice the expression of Ihh is increased, suggesting that part of the effects of FGF signaling is mediated by suppression of Ihh expression (Ornitz & Marie 2002, Kronenberg 2003). BMPs have multiple roles during bone formation. They are essential for the growth of mesenchymal condensations. BMPs have
been shown to increase chondrocyte proliferation when added to bone explants, while addition of their antagonist noggin inhibits chondrocyte proliferation. Opposite to FGF, BMP signaling increases the expression of Ihh in prehypertrophic chondrocytes and increases chondrocyte proliferation (Kronenberg 2003). The transcription factor Sox9 is critical for all phases of chondrocyte lineage. It is needed for converting cells of condensation into chondrocytes and also at further stages of chondrocyte differentiation. When Sox9 was deleted from early limb bud mesenchyme, no cartilage condensations formed. Furthermore, lack of Sox9 from later stage chondrocytes resulted in decreased chondrocyte proliferation and decreased expression of matrix genes and elements of the Ihh-PTHrP signaling pathway (Akiyama et al. 2002, Kronenberg 2003).

Several extracellular matrix genes are involved in skeletal development. Mutations in the genes coding for type I collagen α1 and α2 chains cause osteogenesis imperfecta (OI), a skeletal disorder characterized by brittle bones, hearing loss, blue sclerae and dentinogenesis imperfecta. In milder forms of OI osteopenia and fractures characteristic of osteoporosis can be detected. Mutations in collagen types II, IX, X and XI have been shown to primarily affect the cartilage, leading to chondrodysplasias, osteoarthrosis and intervertebral disc disease (Kivirikko & Prockop 1995, Myllyharju & Kivirikko 2001, Myllyharju & Kivirikko 2004). The various mouse models for collagen mutations affecting the skeleton are included in Table 1. Aggrecan is the major proteoglycan in cartilage. Mice with an aggrecan frameshift mutation (cmd mice) have short limbs, tails and snouts, cleft palate and intervertebral disc abnormalities. The aggrecan link protein knock-out mice also show chondrodysplasia and craniofacial bone defects, further revealing the role of aggrecan in skeletal development (Olsen et al. 2000, Watanabe & Yamada 2003). Perlecan is another proteoglycan found in the cartilage. Many of the perlecan null embryos die at mid-gestation due to heart failure, and the surviving ones develop severe chondrodysplasia. Perlecan deficiency interferes with endochondral ossification leading to abnormal development of all endochondral bones (Arikawa-Hirasawa et al. 1999, Costell et al. 1999).

2.7.2 Skeletal homeostasis

After skeletal development the integrity of bone is maintained by bone remodeling. The worn-out bone is removed by bone-resorbing cells, osteoclasts, and filled with new bone by bone-forming cells, osteoblasts. The bone remodeling is regulated by local and endocrine factors. Certain bone diseases are caused by an imbalance between bone formation and resorption. Increased bone mass can be caused by loss of function of the osteoclast (osteopetrosis) or gain of function of the osteoblast (osteosclerosis), and an increase of bone resorption over bone formation results in osteoporosis (Karsenty 1999). The functions of osteoblasts and osteoclasts are coupled together, although they are derived from different cell lineages. The differentiation and function of these cells are discussed in the following chapters.
Fig. 2. Regulation of the osteoclast and osteoblast differentiation. The two main genes required for osteoclast differentiation are M-CSF and RANKL. OPG acts as a decoy receptor for RANKL inhibiting osteoclastogenesis. All these factors are expressed by osteoblasts whose differentiation from mesenchymal progenitor cells is mainly controlled by Runx2 and Osx.

2.7.2.1 Osteoclast differentiation and function

Osteoclasts are multinucleated cells that are formed by fusion of mononuclear precursors of the monocyte/macrophage lineage in the presence of factors released by bone marrow stromal cells. They resorb bone matrix and mineral by releasing proteases and protons from their ruffled border membranes (Suda et al. 1996, Väänänen 1996). Two hematopoietic factors, the TNF-related cytokine RANKL and macrophage colony-stimulating growth factor M-CSF, are needed for osteoclast differentiation (Boyle et al. 2003). RANKL expressed on the surfaces of osteoblasts/stromal cells interacts with its receptor RANK on osteoclasts and their precursors resulting in fusion of pre-osteoclasts into multinucleated bone-resorbing cells. RANKL and M-CSF induce the expression of osteoclast-specific genes such as trartrate-resistant acid phosphatase (TRACP), cathepsin K, calcitonin receptor and β3 integrin (Lacey et al. 1998, Hsu et al. 1999, Boyle et al. 2003). Osteoprotegerin (OPG) is also produced by osteoblasts. It acts as a decoy receptor by blocking the binding of RANKL and thus inhibiting osteoclastogenesis (Simonet et al. 1997, Boyle et al. 2003). RANKL expression is induced by certain hormones, cytokines and humoral factors such as PTH, 1,25(OH)2 vitamin D3, corticosteroids, PGE2 and interleukins. Estrogens, calcitonin and TGF-β, to name a few, have the opposite effect, decreasing bone resorption (Katagiri & Takahashi 2002, Boyle et al. 2003). T cells are also a source of RANKL in the bone, and their activation leads to increased osteoclastogenesis and bone resorption, suggesting that inflammation contributes to bone loss (Kong et al. 1999). During activation osteoclasts undergo changes in polarization and rearrangement of the actin cytoskeleton. They attach tightly to the bone matrix through a specialized cell-extracellular matrix adhesion structure, the sealing zone, and form by fusion of intracellular acidic vesicles another membrane domain, the ruffled border, facing the resorption lacuna. Eventually the mineralized bone matrix is degraded.
in the resorption lacuna by secretion of HCl and proteolytic enzymes (Blair et al. 1989, Väänänen & Horton 1995, Väänänen et al. 2000). αvβ3 integrin has been shown to have a central role in osteoclast/bone recognition. Mice lacking β3 integrin develop osteosclerosis due to dysfunctional osteoclasts (McHugh et al. 2000).

### 2.7.2.2 Osteoblast differentiation and function

Bone forming cells, osteoblasts aligned on the bone surface, produce most of the bone matrix proteins and regulate the formation of hydroxyapatite crystals in the newly formed bone, osteoid. Osteoblasts and chondrocytes are derived from common mesenchymal progenitor cells and their differentiation is regulated by various hormones and local factors. Two transcription factors, Runx2 and Osterix (Osx, SP7), are required for osteoblast differentiation (Ducy et al. 1997, Komori et al. 1997, Otto et al. 1997, Nakashima et al. 2002). The importance of these factors has been demonstrated by gene targeting. Transgenic mice lacking Runx2 or Osx have no bone or osteoblasts. Runx2 is expressed in Osx-null embryos, but Osterix expression cannot be seen in Runx2-null mice, suggesting that Osterix has a function downstream of Runx2 in bone development (Komori et al. 1997, Otto et al. 1997, Nakashima et al. 2002). Studies with transgenic mice have shown that Runx2 controls also bone formation by differentiated osteoblasts (Ducy et al. 1999). It regulates expression of certain genes found in differentiated osteoblasts such as type I collagen, osteocalcin, bone sialoprotein and osteopontin (Ducy et al. 1997). The homeobox-containing transcription factors distal-less homeobox 5 (Dlx5) and Msx2 have a role in early stages of osteoblast differentiation (Harada & Rodan 2003). It has recently been shown that Twist-1 inhibits osteoblast differentiation through the interaction of a novel domain which interacts with the Runx2 DNA binding domain, suggesting that the relief of inhibition by Twist-1 is required for initiation of osteoblast differentiation (Bialek et al. 2004). In addition, the fos family transcription factors have shown to affect both osteoblast and osteoclast differentiation. Overexpression of two fos proteins, Fra-1 and ΔFosB, leads to increased bone formation and osteosclerosis, whereas lack of c-fos leads to decreased bone resorption and osteopetrosis in mice (Johnson et al. 1992, Wang et al. 1992, Jochum et al. 2000, Sabatakos et al. 2000). Members of the TGF-β/BMP superfamily are the most potent local regulators of osteoblast differentiation. They have been shown to increase the expression of osteoblast-specific genes and to induce osteoblast differentiation in vitro (Yamaguchi et al. 2000, Katagiri & Takahashi 2002). It was recently shown that bone formation is also under endocrine control. Deficiency of leptin, a hormone secreted by adipocytes, leads to obesity and sterility by hypogonadism. Mice lacking leptin (ob/ob) or its receptor (dp/dp) develop a high bone mass phenotype. Intracerebroventricular infusion of leptin decreased bone mass in ob/ob and wild-type mice through a hypothalamic relay (Ducy et al. 2000). The anti-osteogenic effect of leptin was shown to be mediated by the sympathetic nervous system via the hypothalamus (Takeda et al. 2002). Quite recently a new gene, LRP5 encoding low-density lipoprotein-receptor-related protein 5, was found to affect postnatal bone formation through the Wnt signaling pathway. Genetic analysis of human disorders as well as studies of transgenic mice have indicated that gain of function
of **LRP5** leads to high bone mass and loss of function causes osteoporosis (Gong et al. 2001, Boyden et al. 2002, Kato et al. 2002).

### 2.7.3 Cell-cell and cell-matrix interactions in bone formation

Bone modeling and remodeling are processes that require regulated mechanisms of cell-cell and cell-matrix interactions. Osteoblasts adhere to each other mainly through cadherin-mediated adherens junctions, cell-cell communication is mediated via connexins, and gap-junctions and integrins are involved in cell-matrix interactions (Damsky 1999, Marie 2002, Stains & Civitelli 2005). Osteoblasts express several cadherins. When differentiation of osteoblast proceeds, R-cadherin/cadherin-4 is rapidly downregulated and cadherin-11 is upregulated, whereas N-cadherin is expressed throughout the osteogenic development (Stains & Civitelli 2005). Mice lacking N-cadherin die at early stages of development, but heterozygous mutants with lowered N-cadherin expression survive and show some abnormalities in osteoblasts (Radice et al. 1997, Lai et al. 2003). Furthermore, transgenic expression of a dominant negative N-cadherin leads to delayed acquisition of peak bone mass due to impaired osteogenic differentiation (Castro et al. 2004). In contrast, cadherin-11 deficient mice are viable and develop modest osteopenia later in life due to defects in osteoblast function (Kawaguchi et al. 2001). Gap junctions in murine and human osteoblasts mediating cell-cell communication are primarily composed of connexin43 (Cx43). Genetic studies have demonstrated the important role of Cx43 in bone formation. Mice lacking Cx43 die after birth due to cardiac malformations (Reaume et al. 1995). The skeletons of the newborn mice are not properly ossified and they have craniofacial abnormalities due to dysfunctional osteoblasts (Lecanda et al. 2000). In vitro studies have shown that inhibition of gap junctional communication reduces osteoblast differentiation potential (Schiller et al. 2001, Stains & Civitelli 2005). It has been suggested that gap junctions may also have a role in transmission of mechanically generated signals among osteoblasts and osteocytes (Stains & Civitelli 2005). Cell adhesion to extracellular matrices is essential to differentiation and survival of primary osteoblasts during development and bone remodeling (Damsky 1999). Integrins are the main cell surface receptors mediating cell-matrix interactions (Hynes 2002). It has been shown that perturbing the interaction between primary osteoblasts and fibronectin or its integrin receptors decreases the formation of mineralized matrix, and blocking interaction between differentiated osteoblasts and fibronectin causes apoptosis (Moursi et al. 1996, Moursi et al. 1997, Globus et al. 1998). Similar findings have been reported for integrin interactions with type I collagen. When the collagen-integrin interaction was disrupted the activity of transcription factor Runx2, expression of osteoblast-specific genes and matrix mineralization were decreased (Xiao et al. 1998, Jikko et al. 1999, Mizuno et al. 2000). The α subunits α1, α2, α10 and α11 contain a special collagen-binding domain and the integrins α1β1 and α2β1 are considered major collagen receptors (Heino 2000). The importance of β1 integrin in osteoblast function has been demonstrated by gene inactivation. Transgenic mice expressing a dominant negative β1 integrin in differentiated osteoblasts and osteocytes showed impaired membranous bone formation (Zimmerman et
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al. 2000). The α1 integrin knock-out mice develop normally without bone defects, but display diminished callus size and cartilage synthesis during bone fracture healing (Gardner et al. 1996, Ekholm et al. 2002).

2.7.4 Mechanical loading and bone formation

Increase of mechanical loading stimulates bone formation and decreases resorption whereas immobilization can cause osteoporosis due to decreased bone formation. It has been suggested that the skeleton has a biological control system, a “mechanostat”, strengthening bone in highly stressed regions (Frost 2003). The factors mediating these processes are still unknown, although it has been suggested that stress-sensitive calcium channels and integrins as well as paracrine mediators such as prostaglandins and nitric oxide may have a role (Ehrlich & Lanyon 2002, Pavalko et al. 2003).

In bone osteocytes are considered primary mechanical sensor cells and osteoblasts are considered effector cells. Mechanical loading of the bone causes deformation of osteocytes and in addition, generates movement of the interstitial fluid exposing osteocytes to fluid shear stress and electric potentials. In response bone cells release signaling molecules and growth factors such as prostaglandins, nitric oxide and insulin-like growth factors that stimulate bone cell proliferation and matrix formation. It has been suggested that mechanotransduction involves signaling through mechanically activated ion channels in the cell membrane, focal adhesions of the cytoskeleton and a G protein-coupled mechanoreceptor. Some hormones including PTH and estrogen may amplify the effects of mechanical loading (Ehrlich & Lanyon 2002, Pavalko et al. 2003).
3 Outlines of the present study

When this thesis work began the primary structure of type XIII collagen had been characterized and its wide tissue distribution and localization to adhesive structures in cells and tissues had been shown. Despite a growing amount of biochemical data the biological function of type XIII collagen was still unclear. Transgenic mouse models have shown their usefulness in the studies of other collagens. Mutations leading to structurally altered α-chains that can still associate with the endogenous α-chains usually cause more severe phenotypes than null alleles interfering with the folding or formation of supramolecular assemblies. Based on the reviewed literature, we hypothesized that type XIII collagen may have multiple functions during embryonic development and also postnatally in tissue homeostasis. The transgenic approach was chosen to study the function of type XIII collagen in mice. The specific aims were:

1. to analyze the effect of the overexpression of type XIII collagen α-chains with a 90 amino acid in-frame deletion mutation of the COL2 domain in transgenic mice,
2. to generate two transgenic mouse lines, one overexpressing normal type XIII collagen and another with a point mutation in the COL3 domain of type XIII collagen, and to analyze the phenotypical consequences of the overexpression of normal and mutated type XIII collagen, and
3. to study the role of type XIII collagen in osteoblast function and bone formation using primary osteoblasts from transgenic mice overexpressing or lacking type XIII collagen.
4 Materials and methods

Detailed descriptions of the materials and methods can be found in the original articles I-III.

4.1 Analysis of mouse type XIII collagen promoter (II)

4.1.1 Constructs for promoter analysis (II)

To analyze the type XIII collagen promoter region, three constructs consisting of different lengths of 5'-flanking sequences of the mouse type XIII collagen gene were prepared and subcloned into the pGL2-basic vector (Promega) upstream from the luciferase gene. Plasmids containing Col13a1 sequences (Sund M, unpublished results) were first cleaved by Bgl II (Luc 1 -construct), Sca I (Luc 1.5 -construct) or Nsi I (Luc 5 -construct) restriction enzymes. A linker primer containing restriction sites Not I, Spe I, Sal I and Bgl II was attached to the 5' end of Luc 1 and a linker with Not I, Spe I, Sal I and Nsi restriction sites was attached to the 5' end of Luc 5. A Bst B1 restriction site was used as a common 3' end for all three constructs. Finally, the ends of the promoter fragments were made blunt and a Sma I site in the pGL2-basic vector was used for subcloning. The constructs used in promoter analysis consisted of the following fragments: Luc1, -984 - -231, Luc1.5, -1754 - -231 and Luc5, -4878 - -231.

4.1.2 Cell culture and transfection assays (II)

NIH/3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum, penicillin and streptomycin at 37°C in a 5% CO₂ atmosphere. NIH/3T3 cells were transiently transfected with the FuGENETM 6 Transfection Reagent (Roche) according to manufacturer’s instructions using 2 μg of various type XIII collagen promoter constructs with 1 μg of the pCMV-β-galactosidase
plasmid (Clontech) to normalize transfection efficiencies. 24 hours after transfection cell extracts were collected and luciferase activity was measured using the Luciferase assay system (Promega). The \( \beta \)-galactosidase activity was measured using the \( \beta \)-galactosidase enzyme assay system (Promega). The pGL2-Basic vector was used as negative control and the pGL2-Control vector as positive control (Promega).

4.2 Cloning of type XIII collagen transgene constructs (I, II)

4.2.1 Site-directed mutagenesis (I, II)

Point mutations were generated in the type XIII collagen cDNA clone P40, which contains exons 2-41 (Peltonen et al. 1997). Two separate mutations changing a glycine to a cysteine in exon 24 of the COL2 domain and a glycine to a tryptophan in exon 32 of the COL3 domain were made in the cDNA using the Pharmacia U.S.E. kit system (Pharmacia Biotech). The oligonucleotides used to generate the point mutation in the COL2 domain were: Hindmut1 5’-CCAAAGGGAGAAGCTT*GTGTTGATGGGC-3’ and Hindmutrev1 5’-GGCCATCAACACACAGTCTTCTCCCCTTTGG-3’ and the oligonucleotides used to generate the point mutation in the COL3 domain were: Hindmut2 5’-AGAAAGGAGAAGCTT*GGGAGAAAGGCGA-3’ and Hindmutrev2 5’-TCGCCCTTCTCCCAAGTCTTCTTCT-3’ (new Hind III restriction sites underlined, with the altered bases marked with an asterisk). The cDNA clones with the point mutations were named Hindmut1 and Hindmut2 and used for the subsequent cloning of the transgene constructs.

4.2.2 Cloning of the type XIII collagen COL2 deletion construct (I)

The Hindmut1 clone was used for the generation of the COL2del construct. A 270 bp deletion was made in the COL2 domain using the new Hind III restriction site in exon 24 and a Bam HI restriction site in exon 18, and the ends were ligated to the oligonucleotides BHlink 5’-GATCGCTATGGAGA-3’ and BHlinkrev 5’-AGCTCTCCATAGCCCTG-3’. A genomic DNA fragment containing the 159 bp extreme 3’ sequences of the first intron and the first 91 bp of the second exon was generated by PCR using the plasmid 3HA (Sund M., unpublished results) and ligated to the 5’ end of the cDNA clone. To drive expression of the transgene, a 2.5-kb Bgl II-digested genomic DNA fragment containing 1 kb of promoter and 5’ flanking sequences, the complete first exon and 0.8 kb of the first intron was further cloned at the 5’ end of the cDNA. Sequences for transcription termination and the SV40 Poly-A DNA fragment were cloned at the 3’ end of the cDNA. The sequences of the entire type XIII collagen COL2del construct were verified by manual sequencing. However, a base insertion was later detected by automated sequencing at the 3’ end of the cDNA causing the shortening of the COL3 domain by six collagenous repeats and a lack of the NC4 domain. The COL2del construct was liberated from the vector by Spe I and Sac I digestion.
4.2.3 Cloning of type XIII collagen overexpression constructs

Col13a1<sup>oe</sup> and Col13a1<sup>oew</sup> (II)

The mouse P40 cDNA clone (Peltonen et al. 1997) was used to generate the Col13a1<sup>oe</sup> construct. A genomic DNA fragment containing the 159 bp extreme 3' sequences of the first intron and the first 91 bp of the second exon was generated by PCR using the plasmid 3HA (Sund M., unpublished results) and ligated to the 5' end of the cDNA clones. To drive expression of the transgene, a 2.5-kb Bgl II-digested genomic DNA fragment containing 1 kb of promoter and 5' flanking sequences, the complete first exon and 0.8 kb of the first intron was further cloned at the 5' end of the cDNA. A hemagglutinin (HA) tag, sequences for transcription termination and the SV40 Poly-A DNA fragment were cloned at the 3' end of the cDNA. The Hindmut2 clone containing a point mutation changing a glycine in exon 32 of the COL3 domain of type XIII collagen to a tryptophan was used to generate a second construct Col13a1<sup>oew</sup>. The mutated cDNA clone was used for subsequent cloning of the transgene construct as described above. The constructs were released from the vector by Spe I digestion.

4.3 Generation and identification of transgenic mice (I, II)

The purified transgene constructs were microinjected into pronuclei of one-cell stage embryos obtained from B6D2F<sub>1</sub> (hybrid of C57BL/6J x DBA/2J) mice and implanted into NMRI pseudopregnant foster mothers. The transgene positive mice were identified by PCR using DNA samples extracted from tails or ear lobes of two-week-old mice, yolk sacs of fetuses (10.5-15.5 dpc) or fetal tissues (8.5-9.5 dpc). The COL2del mice and Col13a1<sup>oe</sup> mice were genotyped using the following primers: MutScreen2 5'-GGTTTAC CGGGGCCTCCTGGACCAAAGGG-3' and MutScreen2rev 5'-GGCCTGCTTGTCCTG TCTCCCTTTCTCC-3'. The DNA samples of the Col13a1<sup>oew</sup> mice were amplified using the following primers: Mut 2 for 5'-GCCAGGGACGCCAGGAACCAAGGG-3' and Mut 2 rev 5'-CCAGGCAATCCCAGAGGCCCCCGG-3'. After amplification, the 740 bp fragment was digested with Hind III restriction enzyme to detect the additional cleavage site caused by the mutation. The genotypes of all founder mice and COL2del mice were also verified by southern blot analysis. Genomic DNA was digested with the Kpn I and Xba I restriction enzymes and southern blots were probed with a 1.2 kb mouse type XIII collagen cDNA fragment generated by Kpn I and Xba I digestion of clone 3VPL6 (Sund M., unpublished data).

The number of COL2del transgene copies integrated in the different transgenic lines was evaluated by southern blot analysis. Southern blots of Sph I-digested genomic DNA from transgenic mice were probed with a 0.85 kb Nar I fragment of the genomic type XIII collagen clone P7 (Sund M., unpublished results) recognizing a 6 kb endogenous type XIII collagen fragment and a 3.5 kb transgene fragment.
4.4 RNA analysis (I, II, III)

4.4.1 RNA extraction (I, II, III)

Total RNA was extracted from several mouse tissues as previously described (Chomczynski & Sacchi 1987). Skeletal tissues were ground in a mortar in liquid nitrogen before proceeding to RNA extraction. The RNeasy mini kit (Qiagen) was used according to manufacturer’s instructions to obtain RNA from cells.

4.4.2 Northern blotting, RT-PCR and quantitative real-time RT-PCR (I, II, III)

Total RNA isolated from skeletal tissues of transgenic mice overexpressing type XIII collagen and wild-type littermates was analyzed by northern blotting. RNA samples were electrophoresed in 1% agarose-5.5% formaldehyde gels and transferred to Hybond-N membranes (Amersham). 32P-labeled cDNA probes for collagen types I, II, X and XIII and for osteocalcin were used for hybridization. Equal loading of the samples was verified by β-actin hybridization.

RT-PCR was used for RNA analysis from tissues of COL2del transgenic mice. RT-reactions were prepared using 150 ng of random hexamers per 2.5 μg of total RNA. After incubation at 70°C for 10 min reactions were chilled on ice and carried out at 42°C for 50 min with 200 units of M-MLV RT enzyme (GibcoBRL). Finally, two units of RNaseH (GibcoBRL) was added to the reactions and they were incubated at 37°C for 20 min. The PCR was performed from two μl of the RT reaction with following primers: RTpcr1 5’-GATGCTGCCATTATAATCCACCATCTC-3’ and RTpcr2 5’-CCTAAAGGGGAACA AAATCAGACTGGC-3’ at 94°C for 1 min, at 67°C for 1 min and at 72°C for 1 min for 30 cycles. The resulting fragments, 567 bp from the endogenous type XIII collagen transcript and 297 bp from the transgene transcript, were verified by southern blot analysis. The blots were probed with a 1.2 kb mouse type XIII collagen fragment generated by Kpn I and Xba I digestion of clone 3VPL6 (Sund M., unpublished data).

RNA samples extracted from the femurs of transgenic mice overexpressing type XIII collagen and wild-type littermates as well as total RNA from primary osteoblasts were analyzed by real-time quantitative RT-PCR. 200 ng of total RNA was used for RT reactions that were performed using the SuperScript First-Strand Synthesis system for RT-PCR kit (Invitrogen) according to the manufacturer’s instructions. The PCR was performed with the ABI 7700 Sequence Detection System using TaqMan chemistry as previously described (Sund et al. 2001). The forward and reverse primers and fluorescent probes used were (a) type XIII collagen, GGGAAAGCCCGAAGATGT, TCTTCCAGTG GGACCAGGAG and 5’-Fam-TCCAGGGATGTAACCTGCCACCAGGA-Tamra-3’, (b) Runx2, ACTGGCCTGCAACCAAGAC, CTGTCATCTCCTCGAGGGG and 5’-Fam- CGGCCGTTGGCCTCAGGTTTG-Tamra-3’, and (c) insulin-like growth factor II (IGF-II), GAGCTTTGTGACACGCTTCAGT, CGGCTTGAGGGCCCTGCT and 5’-Fam-
TGTCGTTCGGACCGCGGCTTCTA-Tamra-3’. The results were normalized to 18S RNA quantified from the same samples using the forward and reverse primers TGGTTGCAAAGCTGAAACTTAAAG and AGTCAAATTAAGCCGCAGGC, respectively. The probe for the 18S amplicon was 5’-Vic-CCTGGTGTTGCCCCCTCCGTCA-Tamra-3’.

4.5 Western blot analysis (I, II, III)

Fibroblasts derived from COL2del transgenic and wild-type mouse fetuses (12.5 dpc) and primary osteoblasts from calvariae and femurs of Col13a1lox transgenic and wild-type mice were extracted in Triton X-100 lysis buffer and prepared for SDS-PAGE and western blotting as previously described (Hägg et al. 1998). Tissues were rapidly frozen in liquid nitrogen and stored at -70°C until used. Skeletal tissues were ground in a mortar in liquid nitrogen before protein extraction. Tissues were first homogenized in sample buffer (0.06M Tris pH 6.8, 0.5M urea, 10% glycerol), and SDS (2%) and bromphenol blue were added. β-Mercaptoethanol (5%) was included in reduced samples. The samples were incubated for 5 minutes at +95-100°C and centrifuged for 30 minutes. The supernatants were analyzed by SDS-PAGE followed by western blotting as previously described (Hägg et al. 1998) with antibodies specific to type XIII collagen (Hägg et al. 1998) and the HA-tag (Santa Cruz Biotechnology).

4.6 Preparation of tissues for histological and immunohistochemical analysis (I, II)

To obtain mouse fetuses heterozygous mice were mated overnight and the appearance of vaginal plug in the morning was designated as day 0.5 post coitum (dpc). For histological and immunohistochemical analysis, mouse fetuses (8.5-17.5 dpc) and tissues from newborn, 1-month-, 2-month- and 6- to 12-month-old mice were dissected and fixed in 10% phosphate-buffered formalin, pH 7.0. Skeletal tissues were either decalcified in 0.5M EDTA, pH 7.4, or embedded in methyl-methacrylate and processed for hard tissue sections. For frozen sections tissues were embedded in Tissue Tek O.C.T compound (Sakura Finetek) and then rapidly frozen in liquid nitrogen and stored at -70°C until used.

4.7 Immunohistochemical analysis of tissues and in situ hybridization (I, II)

For immunofluorescence staining 5 μm cryosections of fetal and placental samples were cut onto Super Frost Plus glass slides (Menzel Gläser, Germany). The samples were fixed in ethanol, methanol or acetone at -20°C for 10 min followed by incubation in 1% BSA-PBS at room temperature for 60 min to reduce non-specific staining. When a mouse monoclonal antibody was used on mouse tissues, 2% goat serum and anti-mouse IgGs
were added to the blocking solution (Vectastain). Primary antibody incubation was carried out overnight at 4°C with antibodies against type XIII collagen (Hägg et al. 1998, Kvist et al. 2001), CD31 (PECAM) and CD34 (Pharmingen), type IV collagen (Chemicon), desmoplakin (Boehringer Mannheim), pannexin and HA-tag (Santa Cruz Biotechnology). After washing in PBS, secondary antibodies conjugated with Cy2 and/or Cy3 or with TRITC and/or FITC were added for 60 min. After incubation the slides were washed in PBS and mounted with Glysergel (Dako A/S) or with Immu-mount (Shandon Inc.). The immunofluorescence stainings were examined under an epifluorescence (Leitz Aristoplan or Olympus BX51 with Olympus DP50 digital camera system) or confocal microscope.

For histological and immunohistochemical analysis tissues were embedded in paraffin and sectioned at 5 μm. The paraffin sections were stained with hematoxylin and eosin using standard methods for basic histology. Antibodies against type XIII collagen (Hägg et al. 1998, Kvist et al. 2001), CD31 (PECAM) and CD34 (Pharmingen), cytokeratin, vimentin (Zymed), desmin (Sigma), desmoplakin (Boehringer Mannheim), PCNA, pannexin and HA-tag (Santa Cruz Biotechnology) were used for immunohistochemical analysis with the Histomouse SP bulk kit (Zymed Laboratories) according to the manufacturer’s instructions. Hard tissue samples were sectioned at 10 μm and analyzed unstained or with Masson Goldner trichrome staining. Apoptosis was studied using the TUNEL (Terminal dUTP-biotin Nick End Labelling) assay kit (Boehringer Mannheim) according to the manufacturer’s instructions.

In situ hybridization was performed as described previously (Sund et al. 2001). In brief, 4 μm paraffin sections from control and COL2del mutant placentas were pretreated with HCL and proteinase K and acetylated. The hybridizations were carried out at 60°C for 24 hours using digoxygenin-11-UTP labeled RNA probes. After washing the immunological detection was performed with alkaline phosphatase (AP)-conjugated antidigoxygenin antibody. Color reaction was performed by Fast Red tablets according to the manufacturer’s protocol and slides were counterstained with methyl green. The probes were generated from plasmid JA-2 containing a 720 bp fragment, corresponding to nucleotides 1419-2139 of the mouse type XIII collagen cDNA (Hägg et al. 1998).

4.8 Electron microscopy (I, II)

For electron microscopy, hearts from 10.5 dpc fetuses were fixed in 2.5% glutaraldehyde and 0.1 M phosphate buffer, pH 7.4, and postfixed in 1% osmium tetroxide, dehydrated in acetone and embedded in Epon LX112. Bone samples were decalcified after fixation in a solution containing 5% EDTA and 1% glutaraldehyde in 0.1 M phosphate buffer. Thin sections were cut with a Reichert Ultracut E-ultramicrotome (Reichert-Jung) and examined on a Philips CM100 transmission electron microscope (Philips Export B.V.) using an accelerating voltage of 80 kV.
4.9 Histomorphometry of mutant fetuses and placentas (I)

For histomorphometric analysis of vascularization, the areas of interest from mutant and control fetuses and placentas were photographed. The number of capillaries or distance between the capillaries and maternal blood lacunae was measured in 15 squares out of a grid containing 25 squares of 6.25 cm² superimposed on the photographs.

4.10 Characterization of the bone phenotype

4.10.1 X-ray analysis and staining of skeletons (II)

Radiographs were taken from anesthetized 6-month-old wild-type and transgenic mice. Alizarin red/alcian blue staining of the skeletons was performed as previously described (McLeod 1980, Selby 1987). First, the skeletons were dissected out and eviscerated. In the case of 16.5 dpc fetuses and newborn mice, skeletons were fixed for 5 days in 95% ethanol and stained for cartilage in alcian blue and for bone in alizarin red. The cleaning of the 1- to 6-month-old mouse skeletons was finished in KOH, followed by staining in alizarin red solution. Stained samples were cleared in KOH of decreasing strengths and stored in glycerine.

4.10.2 Peripheral quantitative computed tomography (pQCT) (II)

Tibias and femurs from 3-month-old wild-type and transgenic mice overexpressing type XIII collagen were analyzed by pQCT (XCT 960A, Stratek, Germany) as shown previously (Jämsä et al. 1998). The bones were inserted into glass tubes and scanned at the mid-diaphysis. Bone mineral content (BMC), bone mineral density (BMD), cortical area (CSA) and cross-sectional moment of inertia (CSMI) were analyzed. The cortical attenuation threshold was 0.700 cm⁻¹. The voxel size for wild-type bones was 0.092 x 0.092 x 1.25 (mm³) and that for transgenic bones 0.148 x 0.148 x 1.25 (mm³).

4.10.3 Bone histomorphometry (II)

Paraffin sections from wild-type and transgenic femurs were analyzed for osteoclast number and trabecular bone volume. The trabecular bone volume was measured from the distal femoral metaphysis of 1-month-old mice. An area located 0.5 to 1 mm from the epiphyseal cartilage and extending across the marrow cavity was measured. A Nikon Optiphot II microscope was used and the images were digitized with a Sony DXP-930P 3CCD camera and analyzed with MCID-M4 image analysis software (Imaging Research Inc., Canada).
To detect tartrate-resistant acid phosphatase (TRACP) positive osteoclasts sections were stained using a leukocyte acid phosphatase kit (Sigma Diagnostics, St. Louis, Missouri, USA). The number of osteoclasts/mm of trabecular bone surface was calculated from the distal metaphyseal area using a light microscope.

4.10.4 Bone formation rate measurement (II)

The bone formation rate of 3-month-old wild-type and transgenic mice overexpressing type XIII collagen was analyzed by tetracycline double labeling. Tetracycline (Terramycin 200 mg/ml; Pfizer Animal Health) was injected (20mg/kg) intraperitoneally 10 days and again 3 days before the mice were sacrificed. After dissection the femurs and tibias were fixed in 70% ethanol and processed for hard tissue sections. The sections were examined under fluorescence microscope.

4.11 Primary cell cultures (I-III)

4.11.1 Mouse fibroblast cultures (I)

Fibroblast cultures were established from 11.5 dpc fetuses that were obtained from heterozygous matings of COL2del mice. The fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, antibiotics and non-essential amino acids.

4.11.2 Mouse osteoclast culture and resorption activity measurements (II)

Primary osteoclast cultures were established from 6-day-old wild-type and transgenic mice overexpressing type XIII collagen as previously described (Lakkakorpi et al. 1989). Briefly, marrow cells were mechanically harvested from the long bones and cultured on bovine bone slices. After 30 min unattached cells were washed away and bone slices were transferred into fresh DMEM containing 10% fetal calf serum. Cells were cultured at 37°C (5% CO₂) for 48h and then fixed with 3% PFA-PBS. Bone slices were stained for TRACP using a leukocyte acid phosphatase kit (Sigma Diagnostics, St. Louis, Missouri, USA). Multinuclear TRACP-positive cells were counted as osteoclasts, and resorption pits were visualized by WGA-Lectin and active osteoclasts by phalloidin staining as previously described (Lakkakorpi et al. 1989, Selander et al. 1994). The osteoclast resorption activity was expressed as resorbed bone area/osteoclast.
Mouse primary osteoblast cultures were prepared from calvariae of newborn and from long bones of 3- to 5-week-old wild-type and transgenic Col13a1<sup>lox/lox</sup> and Col13a1<sup>LacZ/LacZ</sup> mice. Cells were maintained in α-MEM supplemented with 10% FBS and antibiotics. To induce differentiation of osteoblasts ascorbic acid (50μg/ml) and 10mM β-glycerophosphate were added (referred as osteogenic medium).

Mouse calvarial osteoblasts were isolated as previously described (Lecanda et al. 2000). Briefly, calvariae from newborn mice were dissected out, minced and pretreated with a solution containing collagenase type 2 (2mg/ml) in serum-free medium at 37°C for 10 min. The medium was discarded and samples were further digested with a solution containing collagenase (2mg/ml) and Dnase (5μg/ml) in serum-free medium at 37°C for 2 hours. The cells were collected by centrifugation, washed several times and resuspended in α-MEM supplemented with 10% FBS. The experiments were performed using cells from the first passage.

For the isolation of osteoblasts from cortical explants of the long bones, femurs were dissected out, and soft tissue and periosteum were carefully removed. The cartilage ends of the bones were cut off and the bone marrow was removed by aspirating with PBS. Cleaned bones were cut into small pieces and briefly digested with collagenase (2mg/ml in α-MEM) for 1-2 hours at 37°C. The bone chips were washed with PBS and cultured in 6-well plates in α-MEM supplemented with 10% FBS and antibiotics. Cells grown out of the chips were subcultured at confluence. Experiments were performed using cells from passages 2-3.

4.12 Immunofluorescence staining of primary osteoblasts (III)

For immunofluorescence staining primary osteoblasts were grown on glass coverslips. After fixation in methanol at -20°C for 5 min or 3% paraformaldehyde (PFA) at room temperature for 15 min, cells were blocked in 1% BSA-PBS for 60 min to reduce non-specific staining. The cells were incubated overnight at 4°C or 60 min at room temperature with primary antibodies against type XIII collagen (Kvist et al. 2001) and vinculin (Sigma). After washing in PBS, secondary antibody incubation was carried out for 60 min at room temperature using a Cy2-conjugated mouse anti-rabbit (Jackson ImmunoResearch Laboratories, Inc.) or an Alexa 568-conjugated goat anti-mouse (Molecular probes) antibody. The coverslips were washed in PBS and mounted on microscope slides with Immu-mount (Shandon Inc.). A phalloidin probe, Alexa Fluor 488 phalloidin, was used for staining of actin filaments with methods provided by the manufacturer (Molecular Probes).
4.13 Cell proliferation assay (III)

To study cell proliferation primary osteoblasts (1-2 x 10^4 /well) were cultured on 96-well plates for 72h. The DNA content of the wells was assayed using a CyQuant Cell Proliferation kit (Molecular Probes) and quantified at 485/535 nm in a 1420 Victor™ multilabel plate reader (PerkinElmer Life Sciences/Wallac).

4.14 Measurement of cellular alkaline phosphatase activity (III)

Cellular ALP activity was assayed as previously described (Leskelä et al. 2003). Briefly, primary osteoblasts were extracted into 200 μl 0.1% Triton X-100 buffer (PH 7.6). After freezing and thawing cell lysates were analyzed for alkaline phosphatase activity using 0.1mM 4-p-nitrophenylphosphate (Sigma) as substrate at pH 9.7 and quantified at 405 nm in a 1420 Victor™ multilabel plate reader (PerkinElmer Life Sciences/Wallac). In parallel, the protein concentration of the samples was determined using a BCA Protein Assay kit (Pierce). The alkaline phosphatase activity was expressed as units/mg protein.

4.15 Calcium measurement (III)

To measure matrix mineralization cells were washed three times in PBS and incubated in 0.6M HCl overnight to solubilize accumulated calcium. Cell extracts were assayed for calcium content using the o-cresolphthalein reaction (Roche Diagnostics) according to the manufacturer’s instructions. The colorimetric reaction was quantified at 570 nm in a 1420 Victor™ multilabel plate reader (PerkinElmer Life Sciences/Wallac). The calcium concentration of the samples was determined based on standard solutions prepared in parallel.

4.16 Mechanical straining of cells (III)

Primary osteoblasts were plated at a density of 2.5 x 10^5 /well on 6-well culture dishes with type I collagen coated flexible silicone rubber bottoms (Flexcell) and maintained in culture for 21 days in an osteogenic medium. Mechanical strain was applied to cells from 10-15 days of culture continuously at 3 cycles/min (10s on, 10s off) using a Flexercell Strain Unit FX-3000 (Flexcell). The vacuum promoted approximately a 10% elongation of the cells. Unstrained control plates were maintained identically except for the application of strain.
4.17 Statistical analysis (I, II, III)

The statistical evaluation of the differences in the angiogenesis in the type XIII collagen COL2 deletion transgenic mice was performed using the Mann-Whitney u-test. In other measurements data were analyzed for variation with the f-test and for statistical significance with Student’s t-test. Values of $p < 0.05$ were considered statistically significant, (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).
5 Results

5.1 Generation of type XIII collagen COL2 deletion mice (I)

To study the function of type XIII collagen we have generated transgenic mice with a 90 amino acid in-frame deletion in the conserved COL2 domain of type XIII collagen (COL2del). Transgenic mouse lines were obtained by the microinjection technique. Nine of the ten transgene positive founder mice showed germline integration giving rise to nine individual mouse lines. The number of COL2del transgene copies integrated in the genome in the different mouse lines was analyzed by southern blotting. A 6-kb endogenous type XIII collagen gene fragment and a 3.5-kb transgene fragment were identified by a genomic DNA probe recognizing the first exon of the type XIII collagen gene. The number of transgene copies ranged from 1 to 20 in the lines.

5.2 Expression of the mutant type XIII collagen in COL2del mice (I)

Total RNA was extracted from cartilage, skin, brain, lung, intestine, liver, spleen, kidney, heart, skeletal muscle and placenta of heterozygous COL2del mice, and the expression of the transgene was studied by RT-PCR. The PCR primers were chosen from the COL2 domain of type XIII collagen bracketing the deletion to detect the endogenous and transgenic gene products from the same reactions. The expression pattern of the transgene was similar to that of the endogenous type XIII collagen gene. In some lines (17, 18, 23, 26) the expression level resembled the endogenous gene products, while in others (32, 35, 41) it was clearly lower, and in two of the transgenic mouse lines (2, 14) both differences and similarities were detected in the expression level. The number of integrated transgene copies did not correlate with the expression level.

To verify that the in-frame deletion in the COL2 domain of type XIII collagen leads to expression of shortened polypeptides the cell extracts from mutant and control fetal mouse fibroblasts were compared by western blotting with a type XIII collagen antibody. In addition to endogenous type XIII collagen a smaller band of truncated mutant protein
was detected, indicating that transgene was translated. Under non-reduced conditions the mutant polypeptides formed disulphide-bonded homotrimers.

Adult and developing mouse tissues from lines 17, 23 and 26, with a similar COL2del transgene and endogenous type XIII collagen RNA expression pattern, were analyzed for protein localization. Immunofluorescence stainings with a type XIII collagen antibody showed that the transgene expression followed closely that of endogenous type XIII collagen, with the same pattern as previously found for type XIII collagen expression during normal mouse development (Hägg et al. 2001, Sund et al. 2001). Intracellular accumulation of mutant protein could not be found, suggesting that it was correctly transported to the plasma membrane in transgene positive tissues. An unexpected increase in the expression of mutant protein was detected in the proliferating chondrocytes of bones undergoing endochondral ossification, in the osteoblasts of the developing bones and in the capsule of the liver of the COL2del mice. The type XIII collagen promoter sequences used to drive the expression of the transgene may be particularly active in these cells.

5.3 Fetal lethality of COL2del transgenic mice (I)

Heterozygous COL2del transgenic mice were viable and fertile and they were bred further to increase the load of the mutant protein. In heterozygote matings in four (17, 23, 26 and 35) out of nine mouse lines the expected Mendelian ratio of 75% transgene-positive and 25% transgene-negative mice could not be observed, suggesting that homozygous mice are not compatible with life. To analyze the possible developmental defects pregnancy terminations of heterozygote matings were performed at days 9.5, 12.5 and 15.5 of development. At day 9.5 the offspring showed Mendelian distribution of genotypes. At later stages of development an increasing amount of aborted fetuses was detected, and at day 15.5 the genotype distribution was similar to that seen at birth. Further analysis showed that the abortions took place at two different time points; some of the fetuses died at day 10.5 of development, while the surviving fetuses were lost by day 13.5. Some of the heterozygous fetuses were also aborted, since the amount of lost fetuses was more than 25%.

5.3.1 The early phenotype (I)

The fetuses of the early phenotype were small and severely retarded and died by day 10.5 of development. In the dissections a balloon-shaped sac, the allantois membrane, was detected next to the fetus, indicating a failure in the fusion of the chorion and allantois membranes and thus defective formation of a functioning placenta. Histological analysis revealed that at day 10.5 in the control fetuses the labyrinth layer of the placenta had formed and both maternal and fetal blood cells could be detected, whereas in the mutants the fetal portion of the placenta and fetal blood cells were lacking. However, a fetal trophoblast giant cell layer lining the maternal portion of the mutant placenta was detected, indicating successful implantation of the fetus. It was shown by TUNEL assay
that these trophoblast giant cells were undergoing apoptosis. Approximately half of the aborted fetuses represented the early phenotype.

### 5.3.2 Adherence junction defect in the heart of late phenotype fetuses (I)

The late phenotype fetuses survived beyond day 10.5 and developed further, but they were small and pale and died eventually by day 13.5 of development. The heartbeat of the mutant fetuses was irregular and weak compared to wild-type littermates. The retarded growth, paleness and dysfunction of the heart suggested that death of mutant fetuses at a later stage of development could be caused by cardiovascular defects.

Macroscopic and histological analysis of mutant fetuses showed that the four-chambered heart with its inflow and outflow tracts had formed normally. The three layers of the heart, endocardium, myocardium and epicardium, had also developed, but the myocardium was thinner and trabeculation of the ventricles was reduced compared to control heart. The structure of the myocardium was shown by electron microscopy to be altered, with less electron dense adherence junction structures and detachment of myofilaments from these junctions. Interestingly, no differences were detected in other adhesive structures such as desmosomes and gap junctions. This finding was verified by immunohistochemical analysis of mutant and wild-type fetuses. There were no differences in the expression level or pattern of the desmosomal marker desmoplakin, but the expression of the adherence junction component cadherin was decreased with an irregular staining pattern in the mutant myocardium compared to wild-type littermates.

### 5.3.3 Defects in angiogenesis of late phenotype fetuses (I)

The development of vasculature was analyzed by immunohistochemistry using antibodies against endothelial markers CD31 (PECAM) and CD34, and by histomorphometry. The blood vessels formed by vasculogenesis appeared normal, but there were defects in the smaller vessels and microvascularization formed by angiogenesis in mutant fetuses. In certain cranial regions, such as the developing central nervous system and the trigeminal ganglia the number of microvessels was decreased, while in other regions such as the cephalic mesenchyme and the upper limb statistically significant differences could not be detected between mutant and wild-type mice.

Also the vasculature of placentas of late phenotype fetuses was analyzed. In the mutant placenta the labyrinth layer was thicker, less vascularized and there were more apoptotic cells detected by TUNEL staining than in control placenta. The spreading of fetal blood vessels was defective in the labyrinth layer of mutant placenta. Histomorphometric analysis of placentas revealed that the distance between the fetal vessels and maternal blood lacunae was significantly increased in the mutant placenta (24.5 μm, SD 5.75 μm) compared to wild-type placenta (6 μm, SD 1.5 μm). The development of other compartments of the placenta was studied using cell lineage
specific antibodies, cytokeratin for trophoblasts, vimentin and desmin for decidua, but no additional defects could be detected. Also cell proliferation analyzed by PCNA staining was normal in the mutant placenta. The expression of type XIII collagen was localized in the placental stroma by immunofluorescence staining and in the endodermal cells of the visceral yolk sac and in the trophoblastic cells by in situ hybridization. Both at the protein and mRNA levels the expression pattern of type XIII collagen was similar in the mutant and the control placenta.

5.4 Analysis of the mouse Col13a1 promoter and generation of transgenic mice overexpressing type XIII collagen (II)

Since the deletion in the COL2 domain of type XIII collagen led to an embryonally lethal phenotype (article I) we wanted to study the effect of milder mutations and overexpression of normal type XIII collagen in transgenic mice. Based on previous experience with collagen mutations, the substitution of a glycine in the collagenous sequence with a bulky amino acid residue would be likely to impair collagen triple helix formation and lead to phenotypic abnormalities (Prockop & Kivirikko 1995, Myllyharju & Kivirikko 2001). Transgene constructs for misexpression of normal type XIII collagen (Col13a1oe) and for mutant type XIII collagen with a point mutation changing glycine to tryptophan in the COL3 domain (Col13a1owo) were produced.

The promoter of the mouse type XIII collagen gene has been predicted to locate between -834 and -584 bp upstream of the initiation ATG (Kvist et al. 1999). The efficiency of nucleotides -984 - -231, -1754 - -231 and -4878 - -231 of the Col13a15'-flanking sequences was tested to drive the expression of a luciferase reporter gene in transiently transfected NIH/3T3 cells. There were no significant differences in luciferase activity between the different constructs. Thus the shortest 5' fragment that was selected for use in the generation of the COL2del transgenic mice was found to be sufficient to drive gene expression, and it was further utilized in the new transgene constructs.

Transgenic mice were generated by means of microinjections into fertilized oocytes, and altogether 16 founder mice transferring the transgene to their offspring were obtained. Six of these transgenic mouse lines (two for Col13a1oe and four for Col13a1owo) were characterized further. Transgene positive offspring from the heterozygous matings were born in a normal ratio. Identical phenotypes were observed in both types of mutant mouse line, suggesting that the overexpression rather than the point mutation in the COL3 domain was the cause of the phenotype. In the presentation of the phenotypic consequences of the overexpression of normal or mutant type XIII collagen we show alternately pictures of Col13a1oe and Col13a1owo, the data being applicable to both types of mouse line.
5.5 Expression of the transgene and endogenous type XIII collagen in mouse tissues (II)

Protein samples were extracted from several tissues of adult transgenic and wild-type mice and analyzed by western blotting with HA-tag and type XIII collagen antibodies. In transgenic mouse tissues type XIII collagen expression was detected widely, resembling the expression of endogenous type XIII collagen except in skin, cartilage and skeletal tissues, where the expression was markedly higher. Protein samples from calvariae and femurs were also analyzed using an HA-tag antibody which specifically detects the transgene expression. Analysis under reduced and non-reduced conditions showed that mutated type XIII collagen α-chains formed disulphide-bonded trimers. Strong type XIII collagen expression in transgenic bone was also detected at RNA level by northern blotting and quantitative RT-PCR.

Immunofluorescence staining of adult and developing mouse tissues with HA-tag and type XIII collagen antibodies showed a similar expression pattern in transgenic and wild-type tissues as previously described for fetal and adult mice, although in higher levels in the transgenic cartilage and bone (Hägg et al. 2001, Sund et al. 2001). More specifically, high transgene expression was detected with the HA-tag antibody in the proliferative zone of the growth plate and periosteum.

5.6 Expression of bone-specific genes in transgenic skeletal tissues (II)

The expression of selected bone-specific genes in transgenic and wild-type mouse femurs was studied by northern blotting and quantitative RT-PCR. Surprisingly, differences in the expression level of collagen types I, II and X or osteocalcin could not be detected between control and transgenic mice. The expression of the osteoblast-specific transcription-factor Runx2 and one of the most abundant growth factors in bone, insulin-like growth factor II (IGF-II), were upregulated in the transgenic bone.

5.7 Overexpression of type XIII collagen in skeletal tissues leads to abnormally high bone mass (II)

Macroscopical and histological analysis of transgenic and wild-type mice did not reveal any differences at birth. However, at the age of two months the transgenic mice began to walk abnormally and their hind limbs were affected. X-ray analysis and alizarin red/alcian blue stainings of the skeleton showed that the heterozygous mice overexpressing type XIII collagen had abnormally high bone mass, clearly visible in the long bones. The phenotype became apparent in heterozygous mice at the age of 3-4 weeks and progressed with age. This massive increase in bone mass can already be seen in heterozygous mice, and no further alterations were noted in homozygous mice. The
bone overgrowth was most noticeable in the proximal long bones, but was also detectable in calvarial bones.

The femurs and tibias from 3-month-old overexpression and control mice were analyzed by peripheral quantitative computed tomography (pQCT). The cross-sectional cortical area (CSA), bone mineral content (BMC) and bone mineral density (BMD) were significantly elevated in the transgenic bones compared to wild-type littermates. The moment of inertia at mid-diaphysis indicating the mechanical strength of the bone was 100 (tibia) to 1000 (femur) times higher in the mutant mice.

Histological analysis of skeletal tissues from transgenic and wild-type mice showed a massive increase in cortical bone in transgenic long bones. Abnormally high bone mass was also detected in calvariae, clavicles and pelvic bones, indicating that bones formed by both endochondral and intramembranous ossification were affected. The bone marrow space was formed normally, and the trabecular bone volume analyzed by histomorphometry from the distal femoral metaphyses of 1-month-old mice was not significantly altered in transgenic mice. The proportional area of trabecular bone from the marrow space was 7.0 ± 1.3% (n = 3) in wild-type and 4.7 ± 1.1% (n = 3) in transgenic mice (p > 0.05). Histological analysis of growth plates and cartilage did not reveal any differences between wild-type and transgenic mice. The basic histology of other organs also seemed normal.

Immunohistochemical analysis of femurs from 2-month-old wild-type mice showed endogenous type XIII collagen expression in periosteal and endosteal osteoblasts. In transgenic bone both periosteal and endosteal osteoblasts seemed to be active, with high transgene expression, and the long bones exhibited thickenings of the periosteum, with several layers of cells, whereas only one cell layer of bone-lining osteoblasts was detected in the controls. The periosteal osteoblasts were shown by electron microscopy to be larger, with an enlarged endoplasmic reticulum, suggesting increased activity, and they also had more protrusions towards the bone matrix. In addition, an increased amount of osteoid was detected in hard tissue sections of mutant bone indicating enhanced bone deposition.

### 5.8 High bone mass phenotype is caused by increased bone formation rather than impaired bone resorption (II)

Increased bone mass can result from increased bone formation over bone resorption or impaired bone resorption. The bone formation rate was measured from 3-month-old transgenic and wild-type mice by tetracycline double labeling. Mice received injections of tetracycline twice, ten days and again three days before sacrifice, so that the distance between the two labels could be taken to represent the extent of bone formation in seven days. The bone formation rate was found to be several times higher in the overexpression mice than in their wild-type littermates.

Bone resorption was analyzed using immunohistochemical detection of osteoclasts from tissue sections and by culturing osteoclasts from transgenic and wild-type mice. There were no significant differences in the number of TRACP positive cells, calculated as osteoclasts from the distal metaphyseal area of femurs, between transgenic and wild-
type mice. The overexpression mice had 7.0 ± 0.7 osteoclasts/mm of trabecular bone surface and the controls had 7.7 ± 1.6 osteoclasts/mm (p > 0.05). The resorption activity of the osteoclasts was analyzed by culturing bone marrow derived cells from transgenic and wild-type mice on bovine bone slices. The number of osteoclasts and the areas of resorption pits were calculated, and the resorption activity was expressed as the resorbed bone area/osteoclast (μm²), being 498.9 ± 266.4 for the wild-type and 493.6 ± 381.9 for the transgenic mice (mean ± SD, p > 0.05). Neither the number of osteoclasts nor their resorption activity, shown by formation of actin rings and resorbed bone area/osteoclast, were significantly changed between the overexpression mice and their wild-type littermates.

To study bone metabolism serum samples were collected from 6-week-old mice and analyzed for calcium (S-Ca) and phosphate (S-Pi) content and for alkaline phosphatase activity (S-ALP). S-ALP activity was significantly higher in the overexpression mice, indicating increased osteoblast activity and bone formation, but S-Ca and S-Pi levels did not differ. Also serum PTH, known to increase serum calcium levels by inducing bone resorption, was measured, but no significant differences between overexpression and wild-type mice could be detected.

5.9 Primary osteoblast cultures from transgenic mice (III)

Transgenic mice overexpressing type XIII collagen in skeletal tissues showed high bone mass phenotype due to increased bone formation (article II), whereas the Col13a1\textsuperscript{LacZ/LacZ} mice lacking the type XIII collagen ectodomain have thin bones with reduced mechanical strength (Latvanlehto 2004). To investigate the role of type XIII collagen in osteoblasts and bone modeling we have generated primary osteoblast cultures from wild-type and transgenic mice either overexpressing type XIII collagen (Col13a1\textsuperscript{oe}) or lacking normal type XIII collagen expression (Col13a1\textsuperscript{LacZ/LacZ}). Osteoblast cultures were prepared both from calvariae of newborn mice and from long bones of 3- to 5-week-old mice.

Primary osteoblasts extracted from type XIII collagen overexpressing mice showed strong type XIII collagen expression both at RNA and protein level compared to wild-type cells. The transgene product was also shown to be secreted in the cell culture media. By immunofluorescence staining with a type XIII collagen antibody a similar pattern of intracellular expression, although more intense, was detected in osteoblasts derived from transgenic mice compared to control cells. The morphology of the cells, either overexpressing or lacking type XIII collagen, seemed normal, with an organized cytoskeleton and formation of focal adhesions.

5.10 Misexpression of type XIII collagen in primary osteoblasts affects their proliferation and differentiation (III)

A slight but statistically significant increase was detected in the proliferation rate of primary osteoblasts from transgenic mice overexpressing type XIII collagen, whereas the
proliferation of osteoblasts from \textit{Col13a1}\textsuperscript{LacZ/LacZ} mice was not changed compared to controls.

Differentiation of the primary osteoblasts was analyzed using measurements for alkaline phosphatase activity and calcium concentration from cells that were cultured for 21 days in osteogenic medium. Cells grown on plastic cell culture dishes did not show any differences in ALP activity or in calcium secretion when comparing cells overexpressing type XIII collagen and control cells. In contrast, osteoblasts extracted from \textit{Col13a1}\textsuperscript{LacZ/LacZ} mice lacking the type XIII collagen ectodomain showed a markedly decreased ALP activity relative to wild-type cells. The transcription factor Runx2 is required for osteoblast differentiation, and it is expressed with other osteoblast-specific proteins such as type I collagen by differentiating osteoblasts (Ducy \textit{et al.} 1997, Komori \textit{et al.} 1997, Otto \textit{et al.} 1997). Quantitative RT-PCR analysis showed that the Runx2 mRNA level was increased by approximately 20% in osteoblasts overexpressing type XIII collagen. In addition, type I collagen was found to be upregulated in mutant osteoblasts by western blot analysis using procollagen type I antibody.

5.11 Effect of mechanical loading on type XIII collagen overexpressing cells (III)

Since the skeletal phenotype of transgenic mice overexpressing type XIII collagen was seen postnatally affecting bone modeling/remodeling processes (article II), we wanted to test the effect of mechanical loading on differentiating osteoblasts and matrix deposition. Mechanical strain was applied using a Flexercell strain unit system to primary osteoblasts extracted from wild-type and transgenic mice. Cells were exposed to strain for 10-15 days of culture during a 21-day-culture period in osteogenic medium. Interestingly, when cells were grown on plates with type I collagen coated silicone membranes the type XIII collagen overexpressing osteoblasts showed increased ALP activity even without strain-induced deformation. Calcium secretion was not affected. Mechanical straining decreased the ALP activity of the mutant cells to the control level. Calcium secretion was increased in strained osteoblasts overexpressing type XIII collagen, whereas there was no change in calcium secretion in the wild-type cells under these experimental conditions.
6 Discussion

Type XIII collagen is a transmembrane protein expressed at low levels in most developing and adult tissues such as heart, muscle, skin, cartilage and bone. It is located in focal adhesions of cultured cells and in the adhesive structures of tissues. We have previously generated by gene targeting a mouse line, \textit{Col13a1}^NN, expressing modified type XIII collagen that lacks the cytosolic, transmembrane and association domains. In these mice N-terminally truncated type XIII collagen molecules are transported to the roughly correct location despite their lack of a transmembrane domain, leading to a mild muscular phenotype, including abnormalities in the sarcolemma-basement membrane interphase. The findings suggested that type XIII collagen has a role maintaining the linkage between muscle fibers and the basement membrane (Kvist \textit{et al.} 2001). To further characterize the function of this protein, we generated transgenic mice overexpressing it in normal and mutant forms. Based on previous studies, collagen mutations causing synthesis of structurally altered polypeptides are likely to impair collagen triple helix formation and lead to phenotypic abnormalities (Prockop & Kivirikko 1995, Myllyharju \& Kivirikko 2001, Myllyharju \& Kivirikko 2004).

To characterize the function of type XIII collagen \textit{in vivo} a transgenic mouse line overexpressing type XIII collagen $\alpha$-chains with a 90 amino acid in-frame deletion mutation of the conserved COL2 domain was generated. The deleted area was chosen from the central portion of the molecule so as not to affect sequences needed for the association of $\alpha$1(XIII) chains or sequences that undergo alternative splicing (Peltonen \textit{et al.} 1997, Snellman \textit{et al.} 2000b). The deletion was large and thus expected to cause clear phenotypic changes. Shortened $\alpha$1(XIII) chains detected by western blotting from fibroblasts of mutant fetuses and immunohistochemical analysis of mutant tissues showing no intracellular accumulation of type XIII collagen indicated that mutant $\alpha$1(XIII) chains are likely to associate with mutant and endogenous $\alpha$1(XIII) chains and to be transported to the plasma membrane of cells. Probably several types of collagen XIII molecules form in the cells of mutant mice, including heterotrimeric of mutant and normal chains, homotrimers of normal $\alpha$1(XIII) chains and homotrimers of mutant $\alpha$1(XIII) chains, and the functionally abnormal mutant molecules can be expected to interfere with the functions of the wild-type gene product (Fig. 3). Lack of type XIII collagen does not lead to the same effects as seen in the COL2del mice (Latvanlehto
Indeed, the lack of this collagen appears to lead to milder phenotypic consequences, suggesting that the mutant α1(XIII) chains cause functional disturbances of other interacting molecules.

Expression of truncated type XIII collagen α-chains caused an embryonally lethal phenotype. Viable homozygous offspring could not be obtained after 13.5 dpc. Although the mutation was recessively lethal, some of the heterozygous offspring were also lost, probably due to differences in the actual transcription level of the mutant protein. The fetuses died at two stages of development. Approximately 50% of the lost fetuses were aborted by 10.5 dpc due to the failure of chorioallantoic fusion and lack of a functioning placenta. The fetuses that survived beyond this stage and formed a placenta were considered late phenotype fetuses. They were aborted by 13.5 dpc due to cardiovascular defects. Differences in the expression level and/or pattern of the transgene in these fetuses may explain the abortion at two distinct time points.

Once the functional placenta has formed even fetuses with massive abnormalities in major organ systems can survive until birth, with the exception of cardiovascular and hematopoietic defects which often lead to embryonic death (Cross et al. 1994). In the developing heart type XIII collagen expression is detected throughout the myocardium and becoming accentuated to cell-cell contacts (Sund et al. 2001). Hearts of the COL2del late phenotype fetuses were beating poorly, suggesting that the expression of type XIII collagen is needed for normal development of the heart. Histological analysis of the mutant fetuses revealed hypoplasia, and reduced trabeculation of the myocardium and abnormal adherence junctions were detected between cardiomyocytes with detachment of myofilaments by electron microscopy. Desmosomes were not affected. Immunohistochemical analysis of mutant hearts with a pan-cadherin antibody showed disorganized structures of the adherence junctions, while the staining pattern for the desmosomal marker desmoplakin was similar between the mutant and the control heart. These findings suggest that expression of mutant type XIII collagen α1-chains leads to deterioration of the adherence junctions in the heart.

Although type XIII collagen is not normally expressed in the endothelia of blood vessels some defects were detected in the microvascularization of mutant COL2del fetuses. In the CNS and in the trigeminal ganglion of the mutant fetuses the number of capillaries was reduced compared to wild-type littermates, whereas in regions surrounding the internal carotid artery and in the upper limbs of the same fetuses no differences could be detected. During mouse development the CNS and ganglia are the sites for strong type XIII collagen expression, while lower expression is detected in the mesenchyme of the head and the limbs (Sund et al. 2001). The microvascularization defect in mutant fetuses was detected in the areas where type XIII collagen is normally expressed in high levels and likely to show strong expression of mutant molecules as well. The collagen receptor α1β1 integrin may have a role in this process. It is expressed in endothelial cells of blood vessels (Hodivala-Dilke et al. 2003) and has been shown to interact with type XIII collagen (Nykvist et al. 2000). The altered expression of type XIII collagen may affect interaction or signal transduction between endothelial cells and extracellular matrix, interfering with sprouting of endothelial cells and formation of new vessels.

Vascularization defects were also detected in the placentas of late phenotype fetuses of the COL2del mice. The labyrinth layer, which is normally well vascularized, with the
fetal and maternal blood vessels in close contact, was dense and less vascularized in the placentas of COL2del mice. The fetal vessels showed defects similar to those previously detected in the late phenotype fetuses. It has been shown by in situ hybridization that in the human placenta type XIII collagen is expressed in the fibroblastic stromal cells in the placental villi, developing endothelia, cytotrophoblastic cells and decidual cells (Juvonen et al. 1993). In the mouse placenta type XIII collagen was localized by immunohistochemical analysis in the trophoblasts of the labyrinth and the spongiotrophoblastic layer, but no staining was detected in the endothelia (article I). As in the CNS and in the trigeminal ganglia, type XIII collagen is expressed in the placenta, and placental vessels show similar defects as detected in those areas of mutant fetuses, suggesting that mutant type XIII collagen affects angiogenesis similarly in the placenta and in the late phenotype fetuses.

Previous studies have localized type XIII collagen to focal adhesions in cultured cells and adhesive structures in tissues. Cell-cell and cell-matrix interactions are crucial for the development to proceed. Several transgenic mouse lines misexpressing or lacking known cell adhesion molecules show embryonally lethal phenotypes with developmental defects (Hynes 1996). Some of the phenotypes resemble those detected in the COL2del transgenic mice. Mice lacking the cell adhesion molecule VCAM-1 or its receptor α4 integrin show similar two-stage abortion of fetuses as observed in the COL2del mice (Yang et al. 1995, Gurtner et al. 1995, Kwee et al. 1995). In these mice the early phenotype around 10.5 dpc was caused by the lack of chorioallantoic fusion, and fetuses that managed to circumvent this defect died later due to cardiac problems. However, the cardiac phenotype was caused by detachment of the epicardium from the myocardium resulting in the leakage of blood from the heart, which differs from the cardiac phenotype seen in COL2del mice. Targeted disruption of plakoglobin or focal adhesion component vinculin led to embryonally lethal phenotype due to cardiac defects resembling those seen in type XIII collagen mutant mice (Bierkamp et al. 1996, Ruiz et al. 1996, Xu et al. 1998). The deficiency of plakoglobin caused formation of fused desmosomes and adherence junctions in the heart leading to cardiac failure (Ruiz et al. 1996). Mice lacking vinculin died due to dysfunctional heart (Xu et al. 1996). Inactivation of N-cadherin, a component of adherence junctions, caused severe cell adhesion defects in the heart affecting heart muscle development (Radice et al. 1997). The formation of intercalated discs which are important for generating mechanical strength needed in cell-cell adhesions in the myocardium requires the correct sequential formation of the cellular junctions (Angst et al. 1997). Existing mouse models of cell adhesion molecules including type XIII collagen mutant mice indicate that altered structure of the junctions can interfere with this process and lead to a dysfunctional heart. In some mouse models generated for studies of cell adhesion molecules similar placental defects were also detected as in the type XIII collagen mutant mice. β3 integrin and αv integrin knock-out mice show placental defects with an abnormal labyrinth layer (Hoivala-Dilke et al. 1999, Bader et al. 1998). Mice lacking β8 integrin die at midgestation due to insufficient vascularization of the placenta and yolk sac (Zhu et al. 2002).

Cell adhesion is needed in all aspects of development and a given cell adhesion molecule can function at different stages of development in different morphogenetic and differentiation processes (Thiery 2003). Expression of the mutant α1(XIII) chains resulted in embryonic lethality due to a lack of placental formation or cardiovascular
defects. Type XIII collagen is found in adhesive structures and the phenotype resembles closely those detected in mice lacking classical cell adhesion molecules. These findings indicate that type XIII collagen has an important role in cell-cell and cell-matrix interactions that are necessary for normal development. Cell adhesion is essential for initiation of mesenchymal condensations that prefigure the future bones (Hall & Miyake 2000). Also bone modeling and remodeling are processes that require regulated mechanisms of cell-cell and cell-matrix interactions (Damsky 1999, Marie 2002, Stains & Civitelli 2005). This suggests a role for type XIII collagen in bone cell adhesion.

Since the deletion mutation in the type XIII collagen COL2 domain resulted in fetal lethality we wanted to study the effect of milder mutations in order to get new insights into the function of type XIII collagen in postnatal life. Thus transgene constructs for misexpression of normal (Col13a1oe) and mutant (Col13a1owe) type XIII collagen were produced. High transgene expression was detected in bone, cartilage and skin, locations also containing the endogenous protein (Sandberg et al. 1989, Sund et al. 2001). Identical phenotypes were observed in both types of mutant mouse line, so the results suggested that it was the overexpression rather than the point mutation in the COL3 domain that was the cause of the phenotype. The abnormal increase in bone mass was most conspicuous in long bones formed by endochondral ossification, but it was also found in calvarial bones, which are formed by intramembranous ossification. No differences between transgenic mice and their wild-type littermates could be observed at birth, although high transgene expression was already detectable in the developing bone and cartilage. Thus the overexpression of type XIII collagen allows normal skeletal development, but affects bone mass postnatally in a rapidly progressive manner at the age of 3-4 weeks. Macroscopical and histological analysis of the bones showed symmetrical increase in cortical bone throughout the skeleton, suggesting benign bone overgrowth rather than malignant transformation of the bone cells. These findings suggest that type XIII collagen affects bone modeling and/or remodeling processes.

The transgene-derived α1(XIII) chains were expressed in skeletal tissues of transgenic mice and formed disulphide-bonded trimers in the manner previously reported for endogenously produced and recombinant type XIII collagen (Hägg et al. 2001, Snellman et al. 2000a). The expression pattern of the transgenic type XIII collagen in tissues was compared with that of endogenous molecules using antibodies against type XIII collagen and against the HA-tag located at the extreme C-terminal end of the transgene product. The same expression pattern was obtained in the developing cartilage and bone and in various other tissues, such as skin and peripheral nerves, both from transgenic and wild-type mice. Notable differences in staining were observed in the skeletal muscle and heart, which are known to contain type XIII collagen (Hägg et al. 2001), but which lacked the transgene product. The different phenotypes of COL2del and Col13a1oe mice may be partially explained by the different expression levels of the transgenes in the heart, which is the first organ to develop. In situ hybridization analysis of adult bone has localized type XIII collagen in the periosteum (Sandberg et al. 1989). In addition, recent results from targeted knock-in mice expressing the lacZ gene under control of the type XIII collagen promoter have indicated that type XIII collagen is strongly expressed in developing and adult bone, more specifically in periosteal osteoblasts (Latvanlehto 2004). Taken together, western blotting and immunofluorescence staining of tissues suggested that the transgene...
type XIII collagen is properly folded and located in a number of tissues and the observed phenotype reflects overexpression of type XIII collagen in the skeletal tissues.

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Fig. 3. Schematic presentation of different type XIII collagen transgene products. Lack of the cytosolic, transmembrane and association domains of type XIII collagen in transgenic mice (Col13a1<sup>NN</sup>) resulted in progressive myopathy (Kvist et al. 2001). A large deletion in the COL2 domain (COL2del) leads to embryonic lethality. Mutant molecules may have structural abnormalities that interfere with the formation of the supramolecular assemblies and cause functional disturbances of other interacting molecules. Overexpression of normal type XIII collagen (Col13a1<sup>OE</sup>) in fetal and adult bone allows development to proceed but affects bone modeling postnatally. Knock-in mice lacking the type XIII collagen ectodomain (Col13a1<sup>LacZ</sup>) also develop normally, possibly due to compensation of some other cell adhesion proteins. However, opposite bone defects to type XIII collagen overexpression, such as thin bones with reduced mechanical properties, can be detected. Collagenous domains are denoted by helical structures (normal α<sub>1</sub>(XIII) chains, grey, mutant α<sub>1</sub>(XIII) chains, black), non-collagenous domains by white boxes and transmembrane domains by black boxes. A grey box represents the LacZ gene.

After skeletal development bone is continuously remodeled by bone-resorbing osteoclasts and bone-forming osteoblasts. Defects in this process may cause increase in bone mass, as described for osteopetrosis, which is due to a failure of osteoclast formation or function, and osteosclerosis, which is due to increased osteoblast activity. A relative increase of bone resorption over bone formation results in decreased bone mass, osteoporosis (Karsenty 1999). In transgenic mice overexpressing type XIII collagen the amount of cortical bone was notably increased, especially in the proximal long bones, but the trabecular bone volume was not significantly altered and the bone marrow was normally formed. The bone formation rate as detected by tetracycline double labeling was clearly increased, while tissue stainings indicated a normal number of osteoclasts in the transgenic bones. The resorption activity of osteoclasts was also analyzed, but differences between transgenic mice and their wild-type littermates could not be detected, excluding an osteopetrotic phenotype. However, bone resorption and osteoclastogenesis are
regulated by osteoblasts. RANKL expressed on the surfaces of osteoblasts coordinates bone remodeling by binding to its receptor RANK on osteoclasts and their precursors, resulting in fusion of pre-osteoclasts into multinucleated bone-resorbing cells (Boyle et al. 2003). Our recent microarray analysis from femurs of 1-month-old wild-type and transgenic mice did not reveal any differences in the expression level of RANKL or RANK between these two (Ylönen R, unpublished data), suggesting that osteoblasts overexpressing type XIII collagen are capable of supporting normal osteoclastogenesis. Furthermore, the transgenic mice did not have cartilage defects and the growth plates were intact throughout development. The histology of other organs also seemed normal without ectopic bone formation. Thus the phenotype observed in the type XIII collagen overexpression mice resembles osteosclerosis.

Overexpression of fos-proteins Fra-1 and ΔFosB are known to cause osteosclerotic phenotypes in mice (Jochum et al. 2000, Sabatakos et al. 2000). Gene inactivation of the osteoblast-specific marker, osteocalcin, also results in increased cortical bone thickness, and mice lacking leptin or its receptor have similar bone defects (Ducy et al. 1996, Ducy et al. 2000). However, in these mouse models the increase in bone mass is considerably lower compared to that seen in the type XIII collagen overexpression mice, and both the cortical and the trabecular bone were affected, whereas in the transgenic mice overexpressing type XIII collagen the increase was mostly detected in the cortical bone. The radial growth of the long bones is caused by direct apposition of cortical bone by periosteal osteoblasts. The molecular mechanisms that specifically activate periosteal expansion are poorly understood (Allen et al. 2004). In the transgenic bone both endosteal and periosteal osteoblasts showed strong transgene expression, but only the periosteal zones were clearly thicker, suggesting that the periosteal cells may be more sensitive to type XIII collagen leading to enhanced function of those cells.

The essential role of the transcription factor Runx2 in osteoblast differentiation and function has been demonstrated by gene-inactivation in mice resulting in a cartilaginous skeleton with complete absence of osteoblasts (Komori et al. 1997, Otto et al. 1997). In addition to its role in skeletal development, Runx2 is also expressed in osteoblasts postnatally, and it regulates bone matrix deposition by differentiated osteoblasts (Ducy et al. 1997, Ducy et al. 1999). Recently, it has been reported that Runx2 downregulates osteoblast proliferation and may support the transition stage to exit cell cycle for phenotype commitment and osteoblast maturation (Pratap et al. 2003, Galindo et al. 2005). The quantitative RT-PCR analysis of femurs and primary osteoblasts of the type XIII collagen overexpression mice revealed increased Runx2 expression in the transgenic bone and osteoblasts compared to controls. Cbfa-1 sites are known to occur in genes expressed in osteoblasts (Ducy et al. 1997, Harada et al. 1999, Jimenez et al. 1999, Kern et al. 2001). Based on our computer analysis, the type XIII collagen promoter has several possible binding sites for this transcription factor, one of which is included in the promoter sequence of the transgene constructs. These results indicate that type XIII collagen may have a role in bone formation by differentiated osteoblasts after birth, through interaction with other osteoblast-specific bone markers such as Runx2.

Bone mass is maintained locally by coupled mechanisms of bone resorption and formation. Factors stimulating bone formation such as TGF-β and insulin-like growth factors (IGF-I and -II) are released during bone resorption (Harada & Rodan 2003). Interestingly, we detected upregulation of the IGF-II RNA in transgenic femurs compared
to controls. Also immunohistochemical analysis of tissues revealed stronger IGF-II expression in the mutant osteoblasts than in the wild-type ones. It has been shown that IGF-II promotes proliferation and differentiation of human osteoblasts and induces bone formation in rats in vivo (Langdahl et al. 1998, Ishibe et al. 1998). Furthermore, mechanical strain-related proliferation of osteoblast-like cells appears to be mediated by IGF-II (Zaman et al. 1997, Cheng et al. 1999). The upregulation of IGF-II in transgenic bone may affect the normal growth of osteoblasts, leading to increased activity of those in a periosteal position. Surprisingly, increased bone formation in transgenic mice overexpressing type XIII collagen was not associated with increases in mRNAs encoding type I collagen, the major organic constituent of bone, or the cartilage collagen types II, X and XI. Thus it is likely that high bone mass develops because there are more osteoblasts and osteocytes, as witnessed by the expanded osteoblast zones and the thick cortical bone, producing matrix components in the mutant mice.

Recent characterization of mice lacking the ectodomain of type XIII collagen (Col13a1LacZ/LacZ) provides further evidence for the involvement of this collagen in bone biology. Col13a1LacZ/LacZ mice synthesize chimeric molecules with the cytosolic and transmembrane domains of type XIII collagen linked in-frame to the enzyme β-galactosidase, which replaces the ectodomain. LacZ expression was detected in developing and adult bone and osteoblasts. These mice lacking normal type XIII collagen expression display retarded growth, and their bones are thinner with reduced mechanical properties compared to wild-type mice (Latvanlehto 2004). To investigate the effect of type XIII collagen in osteoblast function, we generated primary osteoblast cultures from wild-type and transgenic mice either overexpressing type XIII collagen (Col13a1OEC) or lacking normal type XIII collagen expression (Col13a1LacZ/LacZ).

As described earlier, in situ hybridization and immunohistochemical analysis have shown that the developing and adult bone is a major expression site for type XIII collagen in both mouse and human (Sandberg et al. 1989, Sund et al. 2001, Latvanlehto 2004). Recently, Kalajzic et al. have also showed by microarray analysis that type XIII collagen is highly expressed in mature osteoblasts (Kalajzic et al. 2005). In our study the primary osteoblasts extracted from calvarial bones and femurs of Col13a1OEC mice showed strong type XIII collagen expression compared to wild-type cells both at RNA and protein level, providing a valuable tool for analysis of type XIII collagen in osteoblast function in vitro. The transgene was also secreted into the cell culture medium, apparently reflecting shedding of this molecule previously shown to occur in various cultured cells (Snellman et al. 2000b, Väisänen et al. 2004).

The high bone mass phenotype in the Col13a1OEC mice was caused by increased bone formation, suggesting that type XIII collagen may have a role in osteoblast function. In cell culture studies the proliferation rate of primary osteoblasts overexpressing type XIII collagen was only moderately increased compared to wild-type cells and the cells differentiated normally. However, when the mutant cells were cultured on type I collagen coated plates they showed increased ALP activity compared to control cells, whereas calcium secretion was not effected. Interestingly, osteoblasts extracted from Col13a1LacZ/LacZ mice differentiated more slowly than wild-type cells and the ALP activity was decreased. Although type I collagen RNA was not upregulated in transgenic skeletal tissues, the western blot analysis of primary osteoblasts extracted from femurs of transgenic mice overexpressing type XIII collagen revealed a moderate increase in type I
collagen expression. These results indicate that type XIII collagen may have a role in the early stages of osteoblast differentiation by affecting both cell proliferation and maturation. The proliferation rate of osteoblasts lacking the type XIII collagen ectodomain was not changed. Cell adhesion to extracellular matrices including type I collagen plays an important role in the regulation of cell growth and differentiation (Lynch et al. 1995, Garcia & Reyes 2004). The surprisingly small changes observed in primary cell cultures of Col13a1oe mice compared to bone phenotype in the transgenic mice may be due to a lack of proper matrix and interactions with other osteogenic proteins in the cell culture conditions used here.

Mechanical loading stimulates bone formation and inhibits resorption while immobilization has opposite effects. “Wolff’s law”, formulated over a century ago, proposed that bone thickness and number of trabeculae corresponds to the quantitative distribution of mechanical stresses, forming the basis of the current concepts of bone adaptation (Wolff 1892). It has been suggested that osteocytes have a function as primary mechanical sensor cells and osteoblasts as effector cells. The mechanotransduction may involve signaling through mechanically activated ion channels in the cell membrane, focal adhesions of the cytoskeleton or a G protein-coupled mechanoreceptor (Ehrlich & Lanyon 2002, Pavalko et al. 2003). Increased bone mass in the long bones of the transgenic mice overexpressing type XIII collagen was first detected at the age of 3-4 weeks, coinciding with the age when the mice start to move more actively and their long bones are exposed to increasing mechanical stress. The pQCT analysis of the long bones indicated increased resistance to mechanical loading since the BMC, BMD and the moment of inertia at mid-diaphysis were significantly elevated in the transgenic mice. In contrast, the mechanical properties of the bones from the Col13a1LacZ/LacZ mice were reduced (Latvanlehto 2004). Furthermore, when primary osteoblasts overexpressing type XIII collagen were exposed to mechanical strain, they showed an enhanced differentiation capacity compared to wild-type cells. These results suggest that type XIII collagen may have a role in processes regulating osteoblast function and bone formation by mechanical stress. Overexpression of type XIII collagen in osteoblasts may enhance its interaction with other matrix components or interfere with certain critical signaling events and mechanotransduction.

All in all, type XIII collagen is found in osteoblasts in developing and adult bone, and the studies presented here suggest that changes in its expression can greatly affect bone biology. The skeletal phenotype of the mice overexpressing type XIII collagen is distinct from any other phenotype previously described, and is due to increased bone formation rather than decreased bone resorption. RNA analysis of transgenic mice suggested that the expression of this collagen type may be linked to IGF-II and Runx2. Overexpression of type XIII collagen in primary osteoblasts affects both cell proliferation and differentiation, while lack of it has opposite effects. These findings include type XIII collagen in the group of bone-specific proteins, and it could be considered as a new target molecule with therapeutic potential within complex disorders of bone homeostasis.
7 Future perspectives

In the future, COL2del mice can be used to study the functional consequences of mutant type XIII collagen on the placental and cardiac function and to analyze at molecular level how this mutation affects the ligand binding properties of type XIII collagen and what the subsequent cellular effects are. The observed vascularization defects in the mutant placentas and fetuses were unexpected, suggesting a new role for type XIII collagen during angiogenesis which will be studied further. Previously unidentified functions may also be found. Indeed, the careful characterization of heterozygous COL2del mice has revealed some additional functions for type XIII collagen. Transgenic mice overexpressing type XIII collagen in skeletal tissues developed normally, but showed postnatally an abnormally high bone mass due to increased bone formation. Our results suggested that the expression of type XIII collagen in bone may be linked to IGF-II and Runx2. In future studies, it should be clarified whether type XIII collagen could be a transcriptional target gene of Runx2. Osteoblasts from the recently generated Col13alacZ/lacZ mouse line lacking the type XIII collagen ectodomain can be used in addition to the type XIII collagen overexpressing cells for a more detailed analysis of type XIII collagen in osteoblast function and mechanotransduction. For example, the effect of type XIII collagen misexpression on the expression of other bone-specific proteins, such as Runx2 and IGF-II, and thus on the function of primary osteoblasts, could be studied using function-blocking antibodies in normal and stressed cell culture conditions. The possible signaling pathways involving type XIII collagen may be also addressed using these primary cells. Cell-matrix interactions associated with bone resorption by osteoclasts have been studied quite extensively, but less is known about cell-cell and cell-matrix interactions of osteoblasts. Previous studies have located type XIII collagen to focal adhesions in cultured cells and adhesive structures in tissues, suggesting a possible role in osteoblast adhesion as well, which will be addressed in future studies.

Different mutations of type XIII collagen in transgenic mice have led to a variety of phenotypes increasing our knowledge about its function. The mouse models presented in this thesis have provided new data about type XIII collagen as an important cell adhesion molecule during placental and cardiovascular development and as an osteoblast-specific protein needed for normal bone formation and skeletal homeostasis. Human diseases
caused by mutations in type XIII collagen gene have not been identified. The results presented here propose a role in spontaneous abortions taking place in early human pregnancies and in bone disorders such as osteoporosis and high bone mass. The most widely used approach to treat osteoporosis affects both bone resorption and bone formation, converting the high remodeling state to a lower level. Type XIII collagen could be considered as an anabolic agent that increases bone formation without affecting resorption, and improves bone strength by expanding the periosteal perimeter.
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