JANNE SAHLMAN

Chondrodysplasias Caused by Defects in the \textit{Col2a1} Gene

Doctoral dissertation

To be presented by permission of the Faculty of Medicine of the University of Kuopio for public examination in Auditorium L22, Snellman building, University of Kuopio, on Wednesday 27\textsuperscript{th} June 2007, at 12 noon

Department of Biomedicine, Anatomy
University of Kuopio
ABSTRACT

Mutations in \textit{COL2A1} gene encoding for type II collagen have been shown to cause a variety of chondrodysplasias and osteoarthritis. They also play a role in the pathogenesis of disc degeneration. In this study, we investigated mice with defects in the \textit{Col2a1} gene. Due to the great homology of the human and murine genomes, the hypothesis was that the mice might manifest a similar phenotype as the human patients. It has been shown previously that substitution of arginine at position 519 to cysteine (Arg519Cys) in human type II procollagen \(\alpha_1(II)\) gene causes an early generalized OA with mild chondrodysplasia. Del1\((+/-)\) mice, heterozygous for a transgene harboring a 150 bp deletion mutation in \textit{Col2a1} gene for type II collagen, have also been shown to suffer from progressive osteoarthrosis. The heterozygous knockout of \textit{COL2A1} gene causes Stickler’s syndrome in human patients.

In this study, three lines of mouse models were investigated. First, male mice with heterozygous inactivation of \textit{Col2a1} gene were compared with the control mice. In addition, voluntary running activity was monitored in half of these mice. Secondly, the mice harboring an arginine to cysteine substitution at position 519 of \textit{COL2A1} gene were compared with the control mice. Finally, Del1\((+/-)\) mice were investigated for the skeletal abnormalities.

Heterozygous inactivation of the \textit{Col2a1} caused a growth delay in young mice. They also had irregular vertebral endplates, which calcified prematurely. The proteoglycan concentration in the spinal tissues was decreased. These features were compensated by the age of 15 months. The bone mineral density of the mice with the gene defect did not increase when the mice used running wheels as it did in normal mice. Arg519Cys mutation caused dysplastic changes in the long bones of young mice, flattened vertebral bodies, and osteoarthritic changes in joints. The intervertebral discs of the transgenic animals were degenerated, and their histological structure was disturbed. The changes were more severe in mice with no murine \textit{Col2a1} allele. The 150 bp deletion mutation in \textit{Col2a1} gene caused a decrease in the breaking strength and ultimate stress of femoral bone, despite the fact that femoral size, morphology and mineral density of the 15-month-old transgenic mice were the same as in controls. No differences in hydroxyproline or calcium content, or dry mass of the humeri could be observed between the groups. In polarized light microscopic analysis, the parallelism of the femoral bone collagen network was 4% lower in the transgenic mice. Also in the vertebral bone, the birefringence was 33% and in the annulus fibrosus of the intervertebral disc 57% lower in the transgenic 15-month-old female mice than in the age-matched controls. Femoral cortical bone had islets positive for type II collagen both in the control and transgenic mice.

Mice with heterozygous knockout of \textit{Col2a1} gene show phenotype similar to that seen in patients with Stickler’s syndrome. Also, the Arg519Cys mutation in \textit{COL2A1} gene produced phenotype typical for patients suffering from progressive osteoarthritis and mild chondrodysplasia. The transgene in \textit{Col2a1} gene harboring a 150 bp deletion mutation led to decreased collagen fibril organization in bone and lower bone breaking strength.

We conclude that the defects or alterations in the \textit{Col2a1} gene or genetic background result in a phenotype in mice bearing resemblance to alterations in humans. In future, specific treatments of the disorders caused by defects in the human \textit{COL2A1} gene may be tested in mice.

Medical Subject Headings: Arginine; Bone Density; \textit{Col2a1} protein, mouse; Collagen Type II; Cysteine; Disease Models, Animal; Mice, Transgenic; Mutation; Osteochondrodysplasias; Osteoarthritis; Phenotype; Spinal Diseases
To Johanna and Onni
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Kuopio, June 2007

Janne Sahlman
ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AIR</td>
<td>Area-integrated retardation</td>
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<tr>
<td>Arg519Cys</td>
<td>Substitution of arginine at position 519 to cysteine in type II procollagen</td>
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<tr>
<td>BMD</td>
<td>Bone mineral density</td>
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<td>BMC</td>
<td>Bone mineral content</td>
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<tr>
<td>COL2A1</td>
<td>Human gene encoding for type II procollagen</td>
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<tr>
<td>Col2a1</td>
<td>Murine gene encoding for type II procollagen</td>
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<tr>
<td>Del1(+/-) mouse</td>
<td>Mouse harboring six copies of a 39 kb transgene with 150 bp deletion mutation</td>
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<tr>
<td>Dmm</td>
<td>Disproportionate micromelia</td>
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<tr>
<td>dpi</td>
<td>Dots per inch</td>
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<tr>
<td>DXA</td>
<td>Dual X-ray absorptiometry</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
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<tr>
<td>Gly</td>
<td>Glycine, amino acid</td>
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<td>M&lt;sup&gt;+&lt;/sup&gt;HMut&lt;sup&gt;-&lt;/sup&gt; mouse</td>
<td>Mouse harboring two wild-type murine alleles of Col2a1 gene and no alleles of mutated human COL2A1 gene</td>
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<td>M&lt;sup&gt;+&lt;/sup&gt;HMut&lt;sup&gt;+&lt;/sup&gt; mouse</td>
<td>Mouse harboring two wild-type murine alleles of Col2a1 gene and one allele of mutated human COL2A1 gene</td>
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<td>M&lt;sup&gt;+&lt;/sup&gt;HMut&lt;sup&gt;-&lt;/sup&gt; mouse</td>
<td>Mouse harboring one wild-type murine allele of Col2a1 gene and one allele of mutated human COL2A1 gene</td>
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<td>Mouse harboring one wild-type murine allele of Col2a1 gene and no alleles of mutated human COL2A1 gene</td>
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<tr>
<td>OA</td>
<td>Osteoarthrosis, Osteoarthritis</td>
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<tr>
<td>PG</td>
<td>Proteoglycan</td>
</tr>
<tr>
<td>pQCT</td>
<td>Peripheral quantitative computer tomography</td>
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<tr>
<td>vBMD</td>
<td>Volumetric bone mineral density</td>
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LIST OF THE ORIGINAL PUBLICATIONS

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


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CONTENTS

1. INTRODUCTION

2. REVIEW OF THE LITERATURE
   2.1 Development and structure of the spine
   2.2 Bone and articular cartilage structure
   2.3 Synthesis of collagen
   2.4 Mechanical properties of bone
   2.5 Effect of exercise on bone and cartilage
   2.6 Osteoporosis
   2.7 Osteoarthrosis
   2.8 Disc degeneration
   2.9 Chondrodysplasias
      2.9.1 General aspects
      2.9.2 Stickler’s syndrome
      2.9.3 Primary generalized OA associated with a mild chondrodysplasia

3. AIMS OF THE STUDY

4. MATERIALS AND METHODS
   4.1 Mice
      4.1.1 Mouse strains
      4.1.2 Voluntary exercise
   4.2 Radiological measurements
      4.2.1 X-ray imaging
      4.2.2 Dual X-ray absorptiometry
      4.2.3 Peripheral quantitative computer tomography
   4.3 Evaluation of disc degeneration
4.4 Measurement of proteoglycan concentration

4.5 Evaluation of collagen network
   4.5.1 Polarized light microscopy
   4.5.2 A new method for more accurate measurement of parallelism of fibrils
   4.5.3 Transmission electron microscopy

4.6 Immunohistochemical analysis

4.7 Biomechanical testing of bone

4.8 Statistical analysis

5 RESULTS

   5.1 Running distances
   5.2 Morphological measurements
   5.3 Intervertebral disc degeneration
   5.4 Proteoglycan concentration
   5.5 Collagen orientation
   5.6 Collagen and proteoglycan localization
   5.7 Biomechanical properties of bone

6 DISCUSSION

   6.1 Heterozygous inactivation of Col2a1 gene (I, IV)
   6.2 The Arg519Cys mutation in mice (II)
   6.3 Type II collagen and bone (III, IV)
   6.4 Limitations of the study

7 SUMMARY AND CONCLUSIONS

8 REFERENCES
1 INTRODUCTION

This study investigates the pathogenesis of the most common degenerative diseases of the skeleton – osteoporosis, osteoarthrosis (OA) and intervertebral disc degeneration.

Osteoporosis is a disease of bone characterized by compromised bone strength, which causes an increased risk of fracture (Osteoporosis prevention, diagnosis, and therapy 2001). The psychosocial and financial costs of osteoporosis are high. There are about 7000 hip fractures per year in Finland (Kannus et al. 1999). Approximately two out of five women and every seventh men get a wrist, vertebral or hip fracture after the age of 50 (Riggs and Melton 1995). Especially the hip fracture is often a personal catastrophe, usually requiring surgical treatment and extensive rehabilitation. Furthermore, the life expectancy is reduced after the hip fracture by 1.8 years (Braithwaite et al. 2003). In Finland, the costs for first post-fracture year are around 14 410 euros, increasing to 35 700 euros if the patient is permanently institutionalized (Nurmi et al. 2003).

Osteoarthrosis is a progressive degenerative disease of a joint leading to damage of the articular cartilage and pathological changes in the underlying bone. The primary symptoms include pain, stiffness, and finally, contractures of the affected joints. In Denmark, on the basis of radiological findings, the prevalence of the OA (joint space < 3 mm) for those over 40 years is 4.7% (Jorring 1980). On the other hand, in Iceland the prevalence of hip OA – using the same criteria for OA - is 25.7% in population aged 35 or more (Ingvarsson et al. 1999).

Spinal disorders and low back pain are the leading cause for disability and sick leaves from work in the western countries (Andersson 1999; Bressler et al. 1999; Leino et al. 1994; Nachemson 1992; Seferlis et al. 1999). The prevalence of back pain is from 12-35%, and as many as 10% of the patients become chronically disabled (Maniadakis and Gray 2000). The most common cause of the back pain is regarded to be spinal degeneration, which is common already in the adolescence (Bailey et al. 1999; Tertti et al. 1991).

The pathogenesis of osteoporosis, OA and disc degeneration are to great extent unknown. Genetic factors in addition to environmental factors are stated to be the major contributing factors to degeneration of connective tissue.
The genetic background of osteochondrodysplasias, which resemble alterations in the tissues in aging and degeneration, has been examined and identified. Over 150 osteochondrodysplasias have been characterized, and they cause a variety of symptoms ranging from mild OA to lethal chondrodysplasia (Horton and Hecht 1993). Collagen types II, IX and XI are the predominant molecules building up the collagen fibril network in cartilage. Collagens have a major role in the maintenance of normal cartilage structure. Mutations in collagen genes are prone to lead to connective tissue, and also cartilage diseases. The most common autosomal dominant connective tissue dysplasia is Stickler’s syndrome, a hereditary progressive arthro-opthalmopathy. Its estimated incidence is 1/10 000 in North America (Pyeritz 1989). Mutations in COL2A1 gene encoding for type II procollagen have been linked to Stickler’s disease in half of the cases. Also mutations in COL11A1 and COL11A2 genes coding for type XI collagen have been found in patients suffering from the disease. Families with early progressive OA and mild osteochondrodysplasia have been found to carry substitution mutation Arg519Cys in type II procollagen α1(II). Recently a mouse model carrying the same mutation has been generated (Arita et al. 2002).

The advance in genome studies has revealed a great homology between human and murine genomes (Waterston et al. 2002). Murine models allow studies on the molecular mechanisms of disease pathogenesis in more detail than it is possible in humans. If we were able to generate mouse models with a phenotype similar to that of human disease, it might be possible to find a cure to these disorders, and even prevent them. Also testing therapeutic methods on animal models might turn out invaluable.

The object of this study was to investigate the tissue properties of mice having gene alterations in Col2a1 gene and whether the expressed phenotypes in mice are similar to those in humans. In addition, we wanted to study the molecular and biochemical mechanisms leading to chondrodysplasias.
2 REVIEW OF LITERATURE

2.1 Development and structure of spine

During early development, all the structures of the spine consist of continuous mesenchymal column that is formed around the cylindrical notochord at about 3 weeks of gestation in humans. In the second phase, the mesenchymal column is segmented into hypercellular bands, which later form the intervertebral discs and hypocellular light bands forming the vertebrae. At about 6 weeks of gestation, the differentiation of mesenchyme into cartilage begins in both the dark and light bands. Later the chondrocyte hypertrophy and ossification begins within the ossification centers of the vertebral anlagen. Classically, the mature intervertebral disc is described to consist of a peripheral annulus fibrosus and a central nucleus pulposus (Figure 1) (Moore and Persaud 2003).

![Figure 1. Normal murine intervertebral disc in histological section. NP = nucleus pulposus, AF = annulus fibrosus, EP = endplate, GP = vertebral growth plate, and VB = trabecular vertebral bone. Bar 100 μm.](image)
The nucleus pulposus is notochordal prenatally and in infancy. During growth and development, the notochordal tissue is replaced by fibrocartilaginous tissue. The vascular annulus becomes almost avascular at adolescence. The organized and lamellar outer part of the annulus fibrosus fuses with the longitudinal ligaments and attaches to the vertebral bone. The fibrocartilaginous inner part of the annulus is continuous with the hyaline cartilaginous endplates and encapsulates the nucleus pulposus. The content of type I collagen is high in the outer parts of the disc, decreasing towards the center of the disc. On the contrary, the content of type II collagen is high in the center of the disc and decreases towards the outer parts of the disc (Eyre and Muir 1976). Small amounts of type VI, IX, X, XI, and XII are found in the disc (Cremer et al. 1998).

In humans at the age of about 8 years, the outer 2 to 3 mm broad ring of the cartilaginous endplate calcifies and forms a bony ring called apophysis which fuses with the rim of vertebral bone at the age of 18 to 20 years (Adams and Muir 1976). The central region of the endplate consists of hyaline cartilage and remains cartilaginous. The relatively weak cartilaginous part is liable to herniations from the nucleus into the vertebral bone (Taylor 1986). It has been suggested that the endplate lesions arising in the adolescence may predispose the lumbar spine to disc degeneration in later life (Hilton et al. 1976).

2.2 Bone and articular cartilage structure

At molecular level, bone is formed out of organic and inorganic matrix. The inorganic matrix consists primarily of hydroxyapatite \((\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2)\), which is covered by a layer of calcium carbonate (Glimcher 1992). The mineral deposit of the bone is responsible for the compressional strength of the bone. The organic matrix
consists of collagen (90%), fat, glycoproteins and proteoglycans (PGs) (Robey 1988; Tracy 1987). The collagen of mature bone is almost entirely of type I with small amounts of type II, III, V, IX and X collagens (Burgeson and Nimni 1992). The collagens are thought to contribute to the tensional strength of the bone. In cartilaginous phase of the endochondral ossification, the cartilage collagens types II, IX and XI are strongly expressed. Thus, defects in the genes encoding for these collagens cause a variety of osteochondrodysplasias, in which growth of bones is disturbed, probably due to defects in cartilaginous framework in endochondral ossification and/or dysfunction of cartilaginous growth plates (Olsen 1995; Prockop and Kivirikko 1995; Williams and Jimenez 1995).

There are two types of bone tissue: compact and cancellous bone. The compact bone is made of Haversian system of osteons, which are formed by concentric rings of matrix lamellae around the Haversian canals (Figure 2, A). The bone cells, osteocytes, osteoblasts and osteoclasts are located in lacunae between the lamellae (Figure 2, A). In bone remodeling, invasion of the osteoclast cutting cone is followed by osteoblast activation, leading to the formation of new lamellar bone, i.e. osteon (Figure 2, B). Small canaliculi radiate from the lacunae to the Haversian canals to provide a passageway through the dense bone matrix. Blood vessels are located in the Haversian canals, which interconnect through Volkmann’s canals anastomosing with the vessels of the bone surface. Compact bone forms cortex of the long bones that surrounds the blood marrow (Figure 2, C). (Currey 2002)

The cancellous bone is primary lamellar bone consisting of fragments of Haversian bone. In growth, it can be made of woven or parallel-fibered bone. In lamellar bone, the collagen fibrils and associated mineral are arranged in lamellae, which alternate in thickness. Usually, the collagen fibrils are oriented in one direction
within one lamella. In the cancellous bone, the bone trabeculae surround irregular cavities that contain the blood marrow. Cavities are connected by canaliculi. (Currey 2002)

**Figure 2.** A) Structure of mature cortical bone showing lamellar Haversian canals interconnected with small canaliculi. B) Osteoclast (Ocl) resorbs a pit, which is later filled with lamellar bone formed by osteoblasts (Obl). Osteoblast-derived osteocytes (Ocy) are connected with neighbouring osteocytes and bone lining cells (Blc) through canaliculi. C) Macroscopical image of murine cortical bone (CB) surrounding blood marrow (BM).

Bone is developed either by intramembranous or endochondral ossification. Former means direct ossification of embryonic connective tissue and is responsible for formation of initial bone tissue in the flat bones of the skull and face, mandible and clavicle. Endochondral ossification is characteristic for the vertebrae and long bones. Both the intramembranous and endochondral ossification begin with the proliferation
and aggregation of mesenchymal cells at the site of the future bone. The only distinction between these two processes is the differentiation of mesenchymal cells into chondroblasts before calcification in the endochondral ossification. Later, in bone remodeling, the replacement of the bone is established by appositional growth, i.e. formation of new lamellae on the outer surface of the bone. (Currey 2002)

There are three types of cartilage. The most abundant type, the hyaline cartilage covers the bone ends in the diarthroidal joints, where it functions as a gliding surface and forms a resilient covering protecting bone ends from wearing. In addition, hyaline cartilage forms the growth plates of growing bones. Elastic cartilage is found in the ear and epiglottis, where it provides rigid but elastic framework. Fibrocartilage is found in the symphyseal joints, intervertebral discs and in articular discs, where it provides tough support and great tensile strength.

During skeletal development cartilage provides a framework for endochondral ossification. The process is sensitive to mutations in the genes encoding for the proteins found in cartilage.

The extracellular matrix of cartilage is formed mainly of collagens and PGs synthesized by the chondrocytes. The collagen network is oriented tangentially in the superficial zone of the articular cartilage (Figure 3, A). The superficial zone has the highest collagen content, about 85% of the dry weight, and it shows the highest degree of collagen organization (Figure 3, B). The main function of the superficial zone is to resist shear stress. The collagen concentration decreases and the orientation of the collagen fibrils changes from tangential to radial when the fibrils arch into the depth of articular cartilage. The middle and deep zones comprise about 80% of the articular cartilage depth and they distribute loads and resist compression. In the deepest
calcified zone of articular cartilage the tissue interfaces with the underlying bone. 

(Poole et al. 2001)

**Figure 3.** A) Collagen fibrils are oriented tangentially in the superficial zone and radially in the deep zone of the articular cartilage. B) Polarized light microscopic image of articular cartilage showing a high collagen organization in the superficial and deep zone and lower organization in the middle zone. C) Aggrecan attached to hyaluronan and link-protein within type the II collagen network of articular cartilage. D) An electron micrograph of hyaluronan molecule (arrow) with proteoglycans attached.

The PGs of cartilage have strong negative electric charge. The molecules are constrained within the collagen matrix (Figure 3, C). PGs are large molecules consisting of a core protein and glycosaminoglycan (GAG) side chains (Figure 3, C
and D). The main PG of the articular cartilage is aggrecan. The terminal end of aggrecan links to hyaluronic acid (HA), a large GAG molecule able to form PG aggregates. The PGs are constrained within the collagen framework. (Hardingham and Bayliss 1990)

2.3 Synthesis of collagen

The collagens are a family of proteins that are present in various connective tissues and have numerous functions depending on the collagen type and organ. At least 27 types of collagens have been characterized so far. All collagen molecules consist of three polypeptide \( \alpha \)-chains, which are wrapped around each other, forming a triple helix. In homotrimeric collagens, such as II, III, VII and X, the three \( \alpha \)-chains are identical. While in heterotrimeric collagens, such as I, IV, V, VI, IX and XI, the \( \alpha \)-chains are of two or three different types.

Types I, II, III, V, XI and XVII collagens form long ordered fibrils with a characteristic banding pattern (Boot-Handford et al. 2003). Types IX, XII, XIV, XVI, XIX are fibril-associated and are located on the surface of the collagen fibrils (Myllyharju and Kivirikko 2001). The most abundant collagen in cartilage is of type II, but there are also types IX, X and XI, and small amounts of types VI, XII, XIII and XIV in the matrix (Cremer et al. 1998). During embryonic development type II collagen is expressed also in non-chondrogenic tissues such as the notochord, neural retina, the corneal and conjunctival epithelia and sclera of the developing eye, heart, epidermis, calvarial mesenchyme, inner ear and the fetal brain (Cheah et al. 1991; Kosher and Solursh 1989; Thorogood et al. 1986; Wood et al. 1991).
Type II collagen is a triple helical protein formed of three helical polypeptides called \( \alpha \) chains. Every third amino acid in the \( \alpha \) chains is glycine, which is essential for triple helix formation of collagen. In the Gly-X-Y arrangement of the polypeptide, X is frequently proline and Y 4-hydroxyproline providing stability for the structure. Hydrogen bonds and water bridges strengthen the triple helix. (Beck and Brodsky 1998; Kramer et al. 1999)

Type II collagen is synthesized in two forms, depending on the alternatively spliced mRNA. The exon 2 domain responsible for the alternative splicing encodes for 69 bp cysteine rich NH\(_2\)-propeptide. Type II collagen molecules including this domain (IIA) are expressed in skeletal progenitor cells and non-cartilaginous embryonic tissues, while type IIB procollagen mRNA excluding the NH\(_2\)-propeptide is synthesized in mature chondrocytes (Sandell et al. 1994).

After transcription of the *Col2a1* procollagen gene in the nucleus, the preprocollagen polypeptides are synthesized on the ribosomes of the endoplasmic reticulum. A number of posttranslational modifications occur both in the endoplasmic reticulum and outside the cell. A signal peptidase removes signal peptides at the aminoterminal ends of the chains after translocation through the membrane of the endoplasmic reticulum. Proline and lysine residues in Y-position and some of the prolines in the X-position are hydroxylated. Some of the hydroxylysine residues are then glycosylated. Addition of mannose-rich oligosaccharide to globular amino- and/or C-terminal propeptides follows as well as an association of carboxy-terminal propeptides. Then, intra- and interchain disulfide bonds are formed. After the carboxyterminals have associated and each pro\( \alpha \)-chain has acquired about 100 4-hydroxyproline residues, the triple helical domains of the chains fold into a left-handed helix. The three left-handed helices wrap around each other to form a triple helical
right-handed superhelix. The glycines are located in the center of the superhelix. (Myllyharju and Kivirikko 2001; Prockop and Kivirikko 1995)

The triple helical procollagen molecules are subsequently transported through the Golgi complex. If the procollagen molecule is mutated, as is the case in mice with Arg519Cys mutation in the COL2A1 gene, the secretion may be disturbed and the Golgi vesicles dilate (Arita et al. 2002). Outside the cell, the soluble procollagen molecules are converted into insoluble by N- and C-proteinases, which cleave the propeptides of the collagen. Finally, collagen molecules form fibrils and lysyl-lysyl cross-links are formed between the molecules (Myllyharju and Kivirikko 2001; Prockop and Kivirikko 1995).

In cartilage, the collagen network provides tensile strength and a supportive framework to the structure. Type II collagen, which constitutes about 80-85% of the total collagen content of cartilage, forms fibrils in association with type IX and XI collagens (Eyre 2002). Type II collagen forms the bulk of fibril, while type IX collagen establishes interactions with surrounding PGs and links the type II collagen fibrils together (Figure 4) (Wu and Eyre 1989). Type XI collagen regulates type II collagen fibrillogenesis in cartilage (Blaschke et al. 2000; Rodriguez et al. 2004).

**Figure 4.** Schematic presentation of a collagen fibril consisting of cartilage specific collagens. Type II collagen (straight lines) forms the bulk of the fibril, type XI (broken lines) is found within the fibril, and type IX (bent line with large globular domain) lies on the surface of the fibril.
The mutations in the *COL2A1* gene encoding for the type II procollagen cause a variety of musculoskeletal diseases. About 2% of early-onset OA is estimated to be caused by mutations in the *COL2A1* gene. However, *COL2A1* mutations are commonly found in quite rare chondrodysplasias. (Myllyharju and Kivirikko 2001; Prockop and Kivirikko 1995). The heterozygous deletion of three nucleotides in Col2a1 in the region encoding the C-propeptide results in the substitution of one amino acid, Asn for two amino acids, Lys-Thr, and causes disproportionate micromelia (Dmm) syndrome. Homozygous Dmm mice die at birth for breathing insufficiency (Fernandes et al. 2003).

### 2.4 Mechanical properties of bone

Mechanical properties of bone are affected by bone quality, geometry, and micro-architecture (Jensen and Mosekilde 1990; Jämsä et al. 1998; Keaveny et al. 1993; Kröger et al. 1994; Van Rietbergen et al. 1998). Bone mineral density (BMD) refers to the amount of mineralized bone tissue in a given area, usually calculated as grams per square centimeter. Volumetric BMD (vBMD) (calculated as grams per cubic centimeter) corrects the BMD for bone size. Thus, the thickness of the bone does not correlate with vBMD as it does with BMD. As the bone grows, the volume increases at a faster rate than the area, so the areal BMD will increase even if the vBMD remains stable.

BMD and bone mineral content (BMC) can be measured with dual X-ray absorptiometry (DXA) in human patients, dogs and even rats (Grier et al. 1996). Peripheral quantitative computed tomography (pQCT) is capable of measuring bone’s volume and avoids the confounding effect of bone size. Quantitative ultrasound has
also been used to evaluate the bone quality and fracture risk. According to the World Health Organization, the criterion for osteoporosis is a BMD lower than 2.5 standard deviations below the average BMD of healthy 30 years old population (T-score <2.5 SD).

Murine femoral bone breaking strength correlates with the cross-sectional moment of inertia (r=0.86) and the volumetric cortical bone mineral density (r=0.79) (Jämsä et al. 1998). Although the BMD parameters are most often considered the major determinants of bone strength, also the collagen content and fibril organization has been shown to correlate positively with the mechanical strength of bone (Bailey et al. 1999; Martin and Ishida 1989; Puustjärvi et al. 1999).

The macro- and microscopic architecture of bone adapts to the physical forces applied to it thus obeying the Wolff’s law (Frost 1990). Also, physical exercise has been shown to increase collagen organization in bone (Puustjärvi et al. 1999). The molecular mechanism of the activation of osteoclasts (bone eating cells) and osteoblasts (bone forming cells), which leads to bone remodeling, is still unclear. It is believed that the pressure is transduced through interstitial fluid to the osteocytes, which are considered to sense the pressure alterations and react by changing their expression levels of matrix proteins, producing nitric oxide, and interacting directly via gap junctions with other cells (Bloomfield 2001; Noble and Reeve 2000). Production of cytokines, growth factors and matrix proteins by osteocytes is thought to regulate the osteoclast and osteoblast activity under mechanical stress (Nomura and Takano-Yamamoto 2000).

Genes account for about 70 - 80% of the BMD variability (Brown et al. 2005; Deng et al. 2000; Ferrari and Rizzoli 2005; Hunter et al. 2001; Mitchell et al. 2003). Genes encoding for the proteins that are responsible for the coordination of the
osteoclast and osteoblast function are obviously suspected to be key factors in the genetic variance of BMD, such as genes encoding for vitamin D receptor (Grundberg et al. 2003; Kim et al. 2003), estrogen receptor alpha (Boot et al. 2004; Ioannidis et al. 2002; Ongphiphadhanakul et al. 2005; van Meurs et al. 2003), calcitonin receptor (Masi et al. 1998), osteoprotegerin (Arko et al. 2005; Yamada et al. 2003), interleukin 6 (Lorentzon et al. 2000; Murray et al. 1997; Nordstrom et al. 2004), interleukin 10 (Chen et al. 2005), CYP19-aromatase (Riancho et al. 2005), and phosphodiesterase 4D (Reneland et al. 2005). Also, there is evidence for the BMD variation associated to combined polymorphism of several genetic loci (Willing et al. 2003).

2.5 Effect of exercise on bone and cartilage

As the bone remodelling and adaptation to mechanical environment continues through life, it is possible to change the bone properties with exercise. The BMD and mechanical strength of the bone is increased in weight-bearing exercise in mice and men (Gordon et al. 1992; Gordon et al. 1989; Hind and Burrows 2007). Also, the collagen organization has been shown to increase in dogs during long term exercise (Puustjärvi et al. 1999). However, the mechanism by which the exercise increases BMD, bone collagen organization and strength, is somewhat unclear (Turner and Robling 2005). On the other hand, strenuous exercise can decrease the BMD in professional athletes due to the hormonal imbalance (Drinkwater et al. 1984).

The effect of exercise on articular cartilage is similar, as it is on bone. Mechanical loading and exercise is vital for normal joint health, but repetitive impact loading or forced heavy loading can lead to degeneration of the articular cartilage (Arokoski et al. 2000; Buckwalter 1995b; Lapveteläinen et al. 1995). Also, lower limb
muscle strengthening training has been shown to slow down the OA progression (Mikesky et al. 2006).

2.6 Osteoporosis

Osteoporosis is a generalized disease of bone. The disease is characterized by reduced bone strength, decreased bone density and structural deterioration of bone tissue (Osteoporosis prevention, diagnosis, and therapy 2001). It leads to increased risk of bone fractures, usually vertebral, hip, and wrist fractures. Bone tissue is remodeled throughout the whole life. The bone mass is increased until the age of 20-30 years, when the peak bone mass is achieved. The bone mass remains constant until it begins to decrease after the age of 40 years.

In type I primary or postmenopausal osteoporosis the decline in estrogen levels at menopause accelerates osteoblast and osteoclast activity. Osteoclast activity increases more than osteoblast activity, and bone loss follows (Ensrud et al. 1995; Jones et al. 1994). Type II primary or senile osteoporosis causes age-related loss in the bone mass. It is usually not apparent before the age of 75 years or later. Type II primary osteoporosis is thought to be caused by sex hormone deficiency and hypocalcemia leading to secondary hyperparathyroidism (Kenny et al. 2003). Secondary osteoporosis is caused by medication, diet, disease or other conditions.

2.7 Osteoarthrosis

OA is a progressive degenerative disease of joints. OA of intervertebral discs and facet joints is usually referred to as degenerative disc disease. OA can affect any
joint of the body, but most often, the weight-bearing hip and knee joints are the joints giving the symptoms. The characteristic symptoms of OA are pain, joint stiffness, and restriction of joint mobility. The disease usually takes place with aging, but it can occur prematurely due to secondary causes, such as congenital joint abnormalities, hormonal disorders, gout, trauma, septic arthritis, inflammatory joint diseases or repetitive joint sprains caused by sports.

In aging, the collagen content of cartilage experiences gradual breakdown allowing the water content of the cartilage to increase. The PG concentration of cartilage decreases and protein composition changes. Later, cartilage degenerates and macroscopic erosion of the cartilage takes place. In severe OA, the cartilaginous surfaces between the adjacent bones are completely eroded.

The clinical diagnosis of OA is usually made by obtaining a detailed history of the disease and conducting a physical examination. X-ray imaging and sometimes laboratory tests support the diagnosis. The X-ray images of the affected joints show typically joint space narrowing, osteophyte formation, pseudocysts in subchondral bone, and increased density of subchondral bone.

The main goals of OA treatment are to relieve pain and improve joint function. The treatment choices include muscle strengthening exercise, painkillers, administration of hyaluronate intra-articularly, and surgery.

2.8 Intervertebral disc degeneration

Intervertebral disc degeneration is defined as deterioration of the chemical composition and physical properties of the disc. Pain is usually experienced during some stage of disc degeneration. In the western world, the prevalence of back pain is
12–35% of the population (Maniadakis and Gray 2000). Formerly, heavy physical loading was suspected to be the main risk factor for disc degeneration. However, recent research shows, that 74% of the disc degeneration is explained by heredity (Chan et al. 2006). In addition, risk factors for cardiovascular disease, i.e. diabetes mellitus, high cholesterol, hypertension and having a parent who had suffered a myocardial event before the age of 60 and smoking, are also predisposing to lumbar disc disease (Jhawar et al. 2006).

During skeletal maturation and aging, fibrosis takes place in the nucleus pulposus, and the boundary between annulus fibrosus and nucleus pulposus becomes less obvious (Buckwalter 1995a). In aging and disc degeneration, the morphology of the disc becomes disorganized: the lamellae of the annulus turn irregular and the collagen and elastin networks become disorganized (Urban and Roberts 2003). The endpoint of these changes is often the formation of clefts in the annulus fibrosus and nucleus pulposus, thinning of the disc, herniation of the disc into the adjacent vertebrae (so called Schmorl’s nodus) and spinal canal, and formation of vertebral osteophytes (Urban and Roberts 2003).

While the intervertebral disc is the largest avascular tissue in the mature human body, diffusion of the nutrients through the cartilaginous vertebral endplates plays a key role in the diseases of the disc. Nutrients and oxygen diffuses from the capillaries of the vertebral bodies, traversing the subchondral bone of the endplate, and reaching the endplate (Crock and Goldwasser 1984). The disc cells are sensitive to a decrease of the glucose and oxygen supply. In vitro, the production of the extracellular matrix molecules decreases rapidly in low pH or low concentration of oxygen (Ishihara and Urban 1999; Ohshima and Urban 1992). The disc cell density is regulated by nutritional constraints (Horner and Urban 2001). Reduced disc PG concentration leads
up to 20% decrease in the water content of the disc (Adams and Muir 1976; Antoniou et al. 1996; Pearce et al. 1987). As the cell density decreases and the ability of the remaining cells to produce adequate extracellular matrix is compromised, the intervertebral disc starts to degenerate (Boos et al. 2002).

Usually the lumbar disc degeneration can be treated successively conservatively with painkillers, applying hot or cold over the painful muscles, and with appropriate exercise. Epidural steroid and analgesic injections can also provide help. Operative treatment, usually extirpation of a disc herniation or lumbar fusion, is rarely needed. In addition, non-conventional treatment modalities, such as intradiscal thermal annuloplasty and intervertebral disc replacement surgery, are being studied.

2.9 Chondrodysplasias

2.9.1 General aspects

Chondrodysplasias are a heterogeneous group of over 150 diseases affecting the development and growth of cartilage and bone (Horton and Hecht 1993). The spectrum of diseases ranges from mild late-onset OA to lethal chondrodysplasias. The growth disturbance of cartilage can be hypoplastic, as in spondyloepiphyseal dysplasia, multiple epiphyseal dysplasia, achondroplasia, and metaphyseal dysostosis. Furthermore, the growth disturbance is hyperplastic for example in hyperchondroplasia, enchondromatosis, and progressive diaphyseal dysplasia. As the abnormal growth of cartilage can affect the bone growth, the term osteochondrodysplasia is often used. A number of mutations in the genes encoding for extracellular matrix proteins and molecules that are responsible for posttranslational
processing of those proteins have been found to cause chondrodysplasias (Myllyharju and Kivirikko 2001). This review on the previous literature focuses on the chondrodysplasias that in this thesis work were studied with mouse models.

2.9.2 Stickler’s syndrome

Stickler’s syndrome is an autosomal dominant connective tissue dysplasia. It is characterized by progressive ocular, orofacial, cardiac and musculoskeletal symptoms (Donoso et al. 2003). The incidence of the disease is estimated to be 1 in 10 000 in the USA (Pyeritz 1989).

The Stickler’s syndrome has three different clinical forms. Types I and II exhibit ocular changes in addition to musculoskeletal abnormalities. In type I syndrome, the retrolental space is occupied by vestigial vitreous gel with a distinct folded membrane. In type II disease, sparse and thickened bundles of fibers can be seen in the vitreous cavity (Snead and Yates 1999). In type III form, no ocular anomalies are seen.

The musculoskeletal deformities in all types of the Stickler’s syndrome include spinal deformities caused by abnormal development of epiphyses of vertebrae. Often, degenerative changes in the spine are seen during skeletal growth (Letts et al. 1999). In addition, articular joint changes include hypermobility, hypomobility, and degenerative changes similar to those of OA.

The most common reason for the Stickler’s syndrome is mutation in the \textit{COL2A1} gene, which encodes type II and XI procollagen polypeptide chains. Type I ocular phenotype is usually caused by a mutation in the \textit{COL2A1} gene and in some cases in the \textit{COL11A1} gene (Parentin et al. 2001). Almost all \textit{COL2A1} mutations found
in the syndrome are nucleotide substitutions, small deletions, or insertions, resulting in premature translation termination (Kuivaniemi et al. 1997). This probably causes haploinsufficiency of the type II collagen. Indeed, recently a heterozygous deletion mutation of the COL2A1 gene was found in a family with Stickler’s syndrome, suggesting that the amount of type II procollagen has an important role in the pathogenesis of the disease (Van Der Hout et al. 2002). The heterozygous inactivation of the murine Col2a1 gene causes ocular features similar to the human Sticler’s Syndrome (Kaarniranta et al. 2006).

Type XI collagen is important for the structure of cartilage collagen meshwork. The patients with type II ocular abnormalities carry mutations in the COL11A1 gene, which encodes for the α1 chain of type XI collagen (Snead and Yates 1999). Mutations in COL11A2 gene, encoding for α2 chain of type XI collagen, lead to Stickler’s syndrome type III without ocular phenotype (Snead and Yates 1999).

In addition to the COL2A1, COL11A1 and COL11A2 genes, linkage analyses have provided evidence for one or more unknown chromosomal locuses for Stickler’s syndrome (Martin et al. 1999; Wilkin et al. 1998).

2.9.3 Primary generalized OA associated with a mild chondrodysplasia

A single arginine to cysteine base mutation at position 519 (Arg519Cys) in type II procollagen α1(II) has been found in families with an early-onset OA with mild chondrodysplasia (Ala-Kokko et al. 1990; Bleasel et al. 1998; Mier et al. 2001; Pun et al. 1994; Williams et al. 1995). Children with ages 5 to 9 years with the disease are suffering from pain in their hips, elbows, and knees. They have stiffness, show limp, and limitation in the range of motion in their joints, too (Mier et al. 2001). Later in
their lives patients exhibit mild flattening and wedging of the vertebrae, and irregularity of vertebral endplates (Pun et al. 1994). Because of the premature OA, they undergo arthroplasty at very young age.

Recently, the Arg519Cys mutation was introduced into the murine genome (Arita et al. 2002). The transgenic mice with two alleles of wild-type murine Col2a1 gene and two copies of human COL2A1 transgene with the Arg519Cys mutation (M^{+/+}HMut^{+/+}) had a decreased density of collagen fibrils in the femoral articular cartilage in uterus at the age of 15 days post coitum. M^{+/+}HMut^{+/+} mice were smaller in size as compared to the mice with two wild-type alleles of Col2a1 gene and two copies of the human COL2A1 transgene with Arg519Cys mutation (M^{+/+}HMut^{++}). The M^{+/+}HMut^{++} mice did not show any skeletal abnormalities at this early age point (Arita et al. 2002).
3 AIMS OF THE STUDY

The aim of this study was to find out and to better understand the molecular mechanisms, which cause tissue changes reminiscent of those seen in human intervertebral disc degeneration, osteoarthrosis, osteoporosis, and chondrodysplasia.

Genetic mutations of Stickler’s syndrome and OA combined with mild chondrodysplasia, have been described in human patients. In this study, mouse models were used to explore the molecular mechanisms similar type of conditions. In the future it might be possible to utilize these models to develop a cure for these genetically determined diseases. The objective in detail of this research were the following:

1. To examine the structural alterations in the skeletons of mice induced by inactivation of one allele of murine Col2a1 gene.

2. To study whether the mice with Arg519Cys mutation of COL2A1 gene exhibit generalized progressive OA combined with mild chondrodysplasia as do the patients carrying the same mutation.

3. To study whether a 150 bp deletion mutation in one allele of Col2a1 gene leads to abnormal bone phenotype, possibly resembling some of the human diseases of bone.
4 MATERIALS AND METHODS

Three different mouse models were obtained for this study (Table 1). At first, all the mouse strains and their controls were gross examined after birth and during the time spent in their cages. Post mortem x-ray images of the mice were taken for evaluation of the gross anatomy of their skeletons. At this point, the more specific methods were chosen for the each experiment. As the study got further, additional analyses were performed as needed (Table 2).

Table 1. Mouse strains

<table>
<thead>
<tr>
<th>Age (number of the mice)</th>
<th>Heterozygous Col2a1 knockout mice (studies I and IV)</th>
<th>Mice with Arg519Cys mutation (study II)</th>
<th>Del1(+/−) mice (study III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control female mice</td>
<td>9 months (n=22), 15 months (n=22)</td>
<td>3 months (n=8), 15 months (n=16)</td>
<td></td>
</tr>
<tr>
<td>Female mice with gene defect</td>
<td>9 months (n=27), 15 months (n=27)</td>
<td></td>
<td>3 months (n=7), 15 months (n=9)</td>
</tr>
<tr>
<td>Control male mice</td>
<td>1 month (n=17), 9 months (n=19), 15 months (n=12)</td>
<td>2 months (n=11)</td>
<td></td>
</tr>
<tr>
<td>Male mice with gene defect</td>
<td>1 month (n=12), 9 months (n=19), 15 months (n=14)</td>
<td>2 months M+ HMut+ (n=10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M− HMut− (n=13)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M+ HMut− (n=9)</td>
<td></td>
</tr>
<tr>
<td>Control male mice with running wheel in their cages</td>
<td>9 months (n=19), 15 months (n=19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male mice with gene defect with running wheel in their cages</td>
<td>9 months (n=19), 15 months (n=19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total number of mice (control, mice with gene defect)</strong></td>
<td>130, 137</td>
<td>11, 32</td>
<td>24, 16</td>
</tr>
<tr>
<td>Methods</td>
<td>Heterozygous Col2α1 knockout mice</td>
<td>Mice with Arg519Cys mutation</td>
<td>ΔH1(+/−) mice</td>
</tr>
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<tr>
<td>Peripheral quantitative computed tomography</td>
<td>Morphology, dimensions and mineral density of femurs.</td>
<td></td>
<td>Morphology, dimensions and mineral density of femurs.</td>
</tr>
<tr>
<td>Three-point bending test</td>
<td>Mechanical properties of femurs.</td>
<td></td>
<td>Mechanical properties of femurs.</td>
</tr>
<tr>
<td>Light microscopy</td>
<td></td>
<td>Histological evaluation of Van Gieson stained spinal sections.</td>
<td>Hematoxylin eosin staining of femoral sections for bone histology.</td>
</tr>
<tr>
<td>Immunostaining</td>
<td>Spinal tissues stained for type II and X collagen, chondroitin 6-sulfate, keratan sulfate and biglycan.</td>
<td>Spinal tissues stained for type II collagen.</td>
<td>Femoral bone stained for type II collagen</td>
</tr>
</tbody>
</table>
4.1 Mice

4.1.1 Mouse strains

In studies I and IV, C57BL knockout mice strain with a targeted inactivation of one allele of the Col2a1 gene for collagen type II were obtained from the Thomas Jefferson University, Philadelphia, USA (Li et al. 1995). The control C57BL/6JOlaHsd breeders were obtained from Harlan CPB (Rijswijk, the Netherlands). At the age of one month the mice were divided into four groups: sedentary control mice, sedentary knockout mice, runner control mice, and runner knockout mice. The tissues were analyzed at the age of one month, nine months, and 15 months.

For study II, the mice were obtained from the Thomas Jefferson University. The mice carried the human COL2A1 gene with a single base mutation that leads to Arg519Cys substitution in the procollagen II chain. This mutated gene was isolated from cultured skin fibroblasts of a patient with primary generalized OA associated with mild chondrodysplasia (Ala-Kokko et al. 1990). The transgene was injected into the pro-nuclei of fertilized eggs of FVB/N mouse strain. The transgenic mice were identified by PCR assay (Arita et al. 2002). Mice with two wild-type murine alleles and one allele of mutated human gene (M<sup>+</sup>HMut<sup>−</sup>) were mated with mice carrying one wild-type murine and one murine null allele of Col2a1 gene (M<sup>−</sup>HMut<sup>+</sup>) (Figure 5, A). Transgenic offspring mice with one murine wild-type Col2a1 allele and one allele of transgene (M<sup>++</sup>HMut<sup>+</sup>) were bred to produce different types of transgenic progeny (Figure 5, B). Four groups of mice were selected for this study: control mice carrying two wild-type murine alleles of Col2a1 gene and no mutated human COL2A1 transgene (M<sup>+</sup>“HMut”), mice with no wild-type murine Col2a1 carrying two alleles of
mutated human COL2A1 transgene (M\textsuperscript{−}HMut\textsuperscript{−}), M\textsuperscript{−}HMut\textsuperscript{−} -mice, and mice with no wild-type murine Col2a1 gene carrying one alle of mutated human COL2A1 transgene and (M\textsuperscript{−}HMut\textsuperscript{−}).

Figure 5. Transgenic mice with two wild-type murine alleles and one allele of mutated human transgene (M\textsuperscript{−}HMut\textsuperscript{−}) were mated with mice carrying one wild-type murine and one murine null allele of Col2a1 gene (M\textsuperscript{−}HMut\textsuperscript{−}) to produce progeny with one murine wild-type Col2a1 allele and one allele of transgene (M\textsuperscript{−}HMut\textsuperscript{−}) (A). M\textsuperscript{−}HMut\textsuperscript{−} mice were subsequently mated to produce different types of transgenic progeny. The genotypes chosen for this study are underlined (B).

The Del1(+/−) mice for the study were created and bred in the University of Turku, Turku, Finland. The transgenic mice were obtained by mating the C57BLxDBA Del1(+/−) mice harboring six copies of a 39 kb transgene with 150 bp deletion mutation.
in *Col2a1* gene with normal C57BLxDBA mice (Metsäranta et al. 1992). The non-transgenic littermates formed the control group.

**4.1.2 Voluntary exercise**

![Figure 6. Cages with the running wheels, monitored by computer. Reprinted with permission of Tuomo Lapveteläinen.](image)

The mice with heterozygous inactivation of *Col2a1* gene were housed and bred in the National Laboratory Animal Center (Kuopio, Finland). The mice were housed in individual stainless steel cages 25 x 50 x 15 cm in size. The cages of runner mice were equipped with running wheels, which were connected to a computer using infrared sensors (Figure 6). The movement of the running wheels was monitored with a custom-made signal processing software (Lapveteläinen et al. 1997). The behavior and well-being of the mice were also continuously followed (Harri et al. 1999). The room temperature was set to 21 ± 2 °C and humidity to 50 ± 20%. Mice were fed with R36 Mouse Food (Lactamin AB, Stockholm, Sweden) and they were allowed to drink water
ad libitum. The Animal Care and Use Committee of the University of Kuopio approved the design of the experiments.

4.2 Radiological measurements

4.2.1 X-ray imaging

Total body X-ray images were taken from the mice at the age of 3 to 15 months. The images were digitized using a computer equipped with a scanner at 600 dpi resolution (Scanner Microtek Scanmaker E6, Microtek Lab, Inc., Düsseldorf, Germany). The length of spine from the lower rim of the occipital bone to the lowest sacral vertebra, and also the length of femurs and tibiae were measured with a Prism program (Analytical Vision, Inc., Raleigh, NC, USA). The cervical lordosis and thoracic kyphosis of the spine were measured using the Cobb technique (Merlino 1973) with image analysis (NIH image Version 1.62, NIH, Bethesda, USA).

High-resolution X-ray images of vertebrae were taken with Feinfocus FXS-160.23 X-Ray microscope (Feinfocus Röntgen-Systeme GmbH, Garbsen, Germany) after making the spine free of muscles (II). Theoretical resolution of the equipment was 10 µm and it gave excellent images of the murine vertebrae of about 2 mm size. The digital images were used to evaluate the structure of the bony skeleton and OA of the major joints (Figure 7).
4.2.2 Dual X-ray absorptiometry

On heterozygous knockout mice and their controls, the BMD of the right femurs were measured using a Lunar Expert bone densitometer (Lunar Corp., Madison, WI, USA). The densitometer was calibrated with a bone phantom for every 4 hours according to the guidelines given by the manufacturer. The femurs were positioned axially in a plastic container, which was filled with 2 cm water bath to simulate soft tissue. The anterior surface of the bone was facing up. Region of interest (ROI) box was selected to contain the entire femur. The densitometer program outlined the soft tissue – bone interface automatically. Measurements were made three times, repositioning the sample between measurements. The mean of the measurements was used for calculations.
4.2.3 Peripheral quantitative computer tomography

pQCT of femoral bones was performed using Stratec XCT Research M device with a software version 5.40 (Stratec Medizintechnik Gmbh, Pforzheim, Germany). The scan line was adjusted to mid-diaphysis of the femur using the scout view given by the pQCT device. The measurements were made using a voxel size of 0.070 x 0.070 x 0.5 mm$^3$. A threshold value of 815 mg/cm$^3$ was used for the measurements of cortical bone and a value of 350 mg/cm$^3$ for trabecular bone. Cortical thickness, volumetric BMD, BMC, and cross sectional area of the femoral diaphysis were measured.

4.3 Evaluation of disc degeneration

The gross anatomy of intervertebral discs and endplates was evaluated using high-resolution X-ray images and van Gieson-stained histological sagittal sections of the lumbar spine. Appearance of the bony ring in the cartilaginous endplates of vertebrae was scored by light microscope from mid sagittal sections of the lumbar and thoracic vertebrae stained with Safranin O. The degree of ossification was graded from one to five. Grades one to four refered to the number of ossification centers in each epiphysis. Grade five denotes that both the cranial and caudal cartilaginous endplates were replaced by bone and transformed into true bony epiphyses (I, Figure 1C). The percentage of completely obliterated intervertebral discs was determined (I, Figure 1D).

The heights of the intervertebral discs and growth plates of mice with heterozygous inactivation of Col2a1 gene and their controls were measured from the anterior, medial, and posterior parts of the disc from Safranin O stained midsagittal
sections using image analysis. The shape of the growth plate was estimated by dividing the length of the growth plate by the direct diameter of the plate (I, Figure 1A).

### 4.4 Measurement of proteoglycan concentration

The PG concentration of the annulus fibrosus and nucleus pulposus of the intervertebral discs, and vertebral growth plates and bone was measured from Safranin O-stained histological sections using digital image analysis quantization (Panula et al. 1998). The cationic dye Safranin O binds stoichiometrically to the GAGs (Kiviranta et al. 1985). Leitz Ortholux II Pol BK (Ernst Leitz, Wetzlar, Germany) microscope was used. The wavelength of the light was limited to 492 nm with a monochromator filter (492 nm ± 1%, Spindler & Hoyer, Goettingen, Germany). Images were digitized with a Photometrics CH-200 (Photometrics, AZ, USA) camera and the analyses were made with a computer equipped with image analysis program (NIH image Version 1.62, NIH, Bethesda, USA). The Safranin O-induced light absorption was determined from the three lowest lumbar discs, vertebral trabecular bone, nucleus pulposus, annulus fibrosus and endplates. Stain density in the anterior and posterior parts of the annulus fibrosus and endplates were determined and the average was taken for calculations.

### 4.5 Evaluation of collagen network

#### 4.5.1 Polarized light microscopy

Evaluation of the collagen organization and content in the spinal tissues was carried out using quantitative polarized light microscopy (Kiraly et al. 1997) with a
Leitz Ortholux II Pol BK (Leitz, Wetzlar, Germany) microscope and a Photometrics CH-200 (Photometrics, Tucson, AZ, USA) camera connected to a computer. The section thickness was 5 µm. For transillumination of the specimens, light with the wavelength 591.4 nm was used and adjusted by a monochromator filter (IL 591.4 nm, t 1/2-width 10.3 nm; Schott, Iserlohn, Germany). The antero-posterior axis of the intervertebral disc was set parallel to the axis of the polarizer. Digital images were taken for the birefringence analyses. Region of interest was outlined with free hand to be as large as possible containing subchondral bone of the vertebrae in the first measurement and the annulus fibrosus of the intervertebral disc in the second measurement. Values for birefringence were determined as mean of the filtered light intensity. The measurements were performed twice from the three lowest lumbar discs, and the mean values were used for calculations.

4.5.2 A new method for more accurate measurement of parallelism of fibrils

As the bone strength of the Del1(+/−) mice was shown to be decreased, polarized light microscopic analyses were performed for mechanically tested femora of the mice. After three-point bending test, sections were cut close to the fracture site and fixed in 10% formalin and decalcified with EDTA (Kiviranta et al. 1985). Samples were blind-coded, and embedded in paraffin. Fifteen cross-sections of the femur were cut on three objective glasses (five consecutive ones per glass), and one section per glass was randomly measured with the newly developed enhanced polarized light microscopic technique (Rieppo et al. 2003). The bone was segmented with computerized algorithm in operator-independent manner, and the average collagen fibril parallelism and birefringence was calculated for the whole section.
The enhanced polarized microscopic technique allows the determination of orientation-independent birefringence and parallelism of collagen fibrils. It is based on the determination of the Stokes parameters, which characterize the polarization phenomenon in detail giving information about the collagen birefringence and parallelism (Collett 1993). Precision grade polarizers positioned into computer-controlled rotation stages allowed 0.01° rotation accuracy. Digital images of the specimens were recorded after six 15° stepwise rotations of the polarizers. Images taken at 0°, 45°, 90° and a 90° image together with the \( \lambda/4 \)-retardation plate were used to determine the Stokes parameters. The measurement of collagen fibril parallelism was performed in the following way: the least square-fit was performed on measured signal intensity vs. rotation angle for determination of the maximum (\( I_{\text{max}} \)) and minimum (\( I_{\text{min}} \)) signal intensities. Calculated \( I_{\text{max}} \) and \( I_{\text{min}} \) values were used for determination of the parallelism for each pixel, which indicates the parallelism, i.e. organization of the collagen fibrils. The parallelism parameter (C) was determined as follows:

\[
C = \frac{I_{\text{max}} - I_{\text{min}}}{I_{\text{max}} + I_{\text{min}}}
\]

Also the ellipticity (ratio of the shortest and the longest axis, values from 0 to 1) of femoral cross section was determined using automatic digital image analysis (IP-Lab, Scananalytics, Fairfax, VA, USA) from the cross-sectional images generated by polarization microscopy.
4.5.3 Transmission electron microscopy

To evaluate collagen network in cartilage of mice carrying Arg519Cys mutation in COL2A1 transgene articular cartilage samples for electron microscopy were taken from tibial medial condyles of control mice (M$^{+/-}$Hmut$^{-/-}$) and of mice harboring two alleles of mutated human COL2A1 gene (M$^{-/-}$Hmut$^{+/-}$). Knee joints were opened, fixed in 2% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.4), and decalcified in 10% EDTA buffered with 0.1 M cacodylate (pH 7.4). Tissue cylinders (diameter 1 mm), which were punched out from uncovered areas of medial condyles of tibiae were postfixed in 1% osmium tetroxide, dehydrated in an ascending series of ethanol solutions and embedded in LX-112 epoxy resin (Ladd Research Industries, Burlington, VT, USA). Ultrathin sections with a silver-gray interference color were cut perpendicular to the articular cartilage surface using vertical section sampling (Nyengaard and Gundersen 1992) and stained with tannic acid, uranyl acetate, and lead citrate (Långsjö et al. 1999). Ultrathin sections were photographed with a JEOL-1200EX transmission electron microscope (JEOL, Tokyo, Japan) at 80 kV. Three micrographs per section were taken using systematic random sampling from the interterritorial compartment of the deep zone articular cartilage extracellular matrix at a magnification of x 100 000 (Långsjö et al. 1999).

4.6 Histological analyses

In mice with heterozygous inactivation of Col2a1 gene, the PG concentration was decreased, and we analyzed the characteristics of PGs further. Midsagittal lumbar spine sections (5 µm thick) were immunostained with murine monoclonal antibodies
recognizing chondroitin 6-sulfate (Caterson et al. 1990), keratan sulfate (EFG-11; Serotec, Oxford, UK), and polyclonal antibody LF106 specific for murine biglycan (Fisher and Termine 1985), as described previously (Lammi et al. 2001). They were also stained for hyaluronan (Tammi et al. 1994). Histological sections (5 µm thick) were also stained with murine monoclonal antibodies recognizing collagen type II (Holmdahl et al. 1986) and X (Girkontaite et al. 1996). The monoclonal antibody X53 was provided by Prof. Klaus von der Mark and the polyclonal antibody LF106 from Prof. Larry Fisher.

In situ hybridization for procollagen \( \alpha_1 \)I and \( \alpha_1 \)II was performed as previously described (Lammi et al. 1998), except that UltraHybTM solution was used for hybridization. UltraHybTM hybridization solution was purchased from Ambion (Austin, TX), and digoxigenin RNA Labeling Kit, 5-bromo-4-chloro-indolyl-phosphate, and nitroblue tetrazolium from Roche Molecular Biology (Mannheim, Germany). A HistosStain Plus kit was obtained from Zymed (San Francisco, CA, USA), and avidin-biotin-peroxidase complex (ABC-kit) from Vector Laboratories (Burlingame, CA, USA). Sigma (St. Louis, MO, USA) provided 3,3'-diaminobenzidine dihydrochloride and bovine serum albumin (BSA). The DNA constructs of collagen types I and II (pM2A1) (Metsäranta et al. 1991) were gifts from Prof. Eero Vuorio.

In 15-month-old Del1(+/-) mice, the mid-sagittal sections of distal femora and cross-sections of femoral diaphysis were immunostained with mouse monoclonal antibody E8 (Holmdahl et al. 1986) recognizing collagen type II by using EnVisionTM staining kit according to manufacturer’s instructions (DakoCytomation, Glostrup, Denmark).
4.7 Biomechanical testing of bone

The biomechanical properties of the femoral bones were measured using the three-point-bending test (Jämsä et al. 1996) after pQCT imaging. The samples were placed in a holder unit, the anterior surface of femur pointing upwards. Separation of the two holder fulcra was 6.5 mm. Each bone was compressed with a constant speed of 0.155 mm/sec until failure. Blunt intender and fulcra were used to minimize shear stress. The load-time curve was recorded, converted to force-deformation curve and analyzed using an image analysis software (UTHCA ImageTools for Windows v.1.28, University of Texas Health Science Center in San Antonio, TX, USA). The maximal breaking force ($F_{\text{max}}$) was defined as the bending load at failure. Ultimate stress and Young’s modulus of the bone were calculated based on the data obtained from three-point-bending test and pQCT data. Stiffness was determined as the slope of the load-deformation curve in elastic region below the yield point (Jämsä et al. 1998).

5.0 Statistical analysis

In the first part of this thesis work, the data distributions for each parameter were expressed as mean ± standard error of the mean (SEM). Results on the degree of endplate ossification were given as the median ± quartile deviation. Nonparametric two-tailed Mann-Whitney’s U-test was used to compare the heterozygous knockout and control mice because normal distribution of the data could not be assumed for this sample size (SPSS for Macintosh 6.1.1, SPSS Inc., Chicago, IL, USA).

In the second, third and fourth part of the thesis work, the mean and standard deviation were calculated for the measurements. Mann-Whitney’s U-test was used to
calculate the statistical significances. In the fourth part of the thesis work, the Pearson’s correlation coefficients were calculated to evaluate the correlation between the animal weights and bone properties. P values less than 0.05 were considered statistically significant.
5 RESULTS

5.1 Running distances

During the first three months the daily running distances of the mice with heterozygous inactivation of Col2a1 gene and their controls increased continuously along with the size of the mice (I, Figure 6). Later, daily running distances began to decrease slowly. The cumulative running distance was 23.6% shorter by the age of 9 months with the heterozygous knockout runners as compared with the normal male runners. The difference was the greatest from the age of 9 to 11 months (p<0.001, I, Figure 6). The average running speed was 36.2 ± 1.3 cm/s in the heterozygous knockout runners and 38.9 ± 0.8 cm/s in the normal runners. The knockout and normal runners spent cumulatively 439 ± 43 and 553 ± 36 hours, respectively (p<0.05), at the running wheel at the age of 9 months. A remarkable decline in daily running distance was seen after the age of 6 months in the group of normal male runners and 2 months earlier with the heterozygous knockout male runners. The overall running activity was the highest at the dark hours of the day agreeing the normal murine daily rhythm.

5.2 Morphological measurements

The control runner mice remained 2 – 10% leaner (p = 0.0011 at 6 months) than the sedentary mice. The runner mice with heterozygous inactivation of Col2a1 gene were 5 – 14% leaner than the sedentary knockouts (p = 0.009 to 0.019 from 6 to 9 months) (I, Figure 5). The weight difference between sedentary and runner mice
reached a peak between 8 to 10 months. The sedentary heterozygous knockout mice were 2 to 7% heavier than the sedentary controls throughout the study.

The two-month-old M⁺HMut⁺⁺ mice were 15% and M⁻HMut⁻⁻ mice 20% lighter than the controls (p < 0.001) (Figure 8).

![Figure 8](image)

**Figure 8.** Weights of control mice, mice carrying one wild-type murine allele of Col2a1 gene and one allele of mutated human transgene (M⁺HMut⁺⁺), mice with two alleles of mutated human COL2A1 transgene (M⁻HMut⁻⁻), and mice with one allele of mutated human COL2A1 transgene (M⁻HMut⁻⁻). Mann Whitney U-test was used to test differences between the groups. p*** < 0.001.

There was no difference in the body weights of the Del1(+/−) mice and control mice between 3 to 4 months of age and at the age of 15 months (Säämänen et al. 2000).

There were no malformations or mineralization defects in the radiographs of mice with heterozygous inactivation of Col2a1 gene in any of the age groups. The spines of the one-month-old sedentary knockout mice were 4% shorter than those of the sedentary controls (I, Table 1). This feature was compensated by the age of 9
months. The tibiae were 5%, and the femurs 3 to 6% shorter in the 9- and 15-month-old sedentary knockout mice than in the controls (I, Table 1). The length of skull was 3-4% shorter in the 9- and 15-month old knockout mice than in the controls (I, Table 1). The cervical lordosis angle was 15º larger in the sedentary knockout mice than in the sedentary controls in the 9-month age group and 8º larger in the 15-month-old knockout runners than in the control runners (I, Table 2). There was no difference in the thoracic kyphosis between the two experimental groups (data not shown).

Mild dysplasia of the long bones was observed in the M+/-HMut+/- mice as early as at the age of two months. The vertebral bone appeared to be trabecular in four out of the ten M+/-HMut+/- mice. The vertebral bodies were wider in all transgenic groups, and flatter in M+/-HMut+/- and M+/-HMut+/- groups than in the controls (II, Figure 1 and Table 1). Twelve out of the thirteen two-month-old M+/- HMut+/- mice had moderate dysplastic changes and one mild one in the long bones. Half of the M+/-HMut+/- had trabecular appearance of the vertebral bone in the X-ray images (Figure 1). Moderate dysplastic changes in the long bones were observed in seven out of the nine M+/-HMut+/- mice at the age of two months (II, Figure 1). One M+/-HMut+/- mouse had dysplasia in the humerus only. Trabecular vertebral bone was clearly osteoporotic (II, Figure 3). The M+/-HMut+/- and M+/-HMut+/- mice had significantly shorter spines, skulls, femora and tibiae than the controls (II, Table 1), whereas the replacement of only one murine allele with the mutated human COL2A1 allele did not affect the length of the long bones or spine (II, Table 1). The femoral thickness of the M+/-HMut+/- mice was 8% smaller as compared with the controls (II, Table 1).

The tibiae of the 3-month-old Del1(+/+) mice were 5% shorter and femora 4% shorter than those of the controls (III, Table 1). In the 15-month-old mice, however, there was no difference in the length of the femoral bone, but the tibiae of the
transgenic mice remained 1% shorter as compared with the controls (III, Table 1). There was no difference in the cortical area or ellipticity of mid-diaphyseal femoral cross-sections. The general morphology of femoral bone, as evaluated from the radiographs, appeared not to be disturbed in Del1(+/−) mice.

5.3 Disc degeneration and osteoarthrosis

The vertebral growth plates were 11% thicker in the 1-month-old heterozygous Col2a1 knockout mice than in the control mice (I, Table 2). The curvature index of the growth plates, indicating the irregularity of growth plate, was greater in the 1- and 9-month-old sedentary heterozygous knockout mice than in the control mice (I, Table 3). Herniations of intervertebral discs through endplates were seen both in the heterozygous knockout mice and the controls (I, Figure 4). This difference was not observed in the 15-month-old mice. The notochordal tissue covered almost entirely the nucleus pulposus of the one-month-old mice. At the age of 9 months, percentage of notochordal tissue area in the nucleus pulposus was still 17±11% and 20±9% in the knockout and control mice, respectively. The difference between the groups in the percentual notochordal area was not statistically significant. There was no difference in the heights of the intervertebral discs between the study groups. The endplates ossified earlier in the mice with the heterozygous knockout of Col2a1 gene (I, Table 3). There was no difference in the percentage of obliterated intervertebral spaces between the knockout and control mice, percentage being 20% in both groups. In situ hybridization showed ongoing ossification in the obliterated intervertebral discs (I, Figure 1D).

In two out of the ten M+/−HMut+− mice, only the femoral heads had irregularities resembling mild OA changes in the X-ray images. Four of the M+−HMut+− mice with
dysplastic changes in their long bones had also OA changes in the knees, elbows, and shoulders, and two of them had OA in the hip joints. In the X-ray images, vertebral bodies and their endplates were normal in eight of the M^+/-HMut^-/- mice, while two had small irregularities in the endplates of the lumbar vertebrae (II, Figure 1). In the M^+/-HMut^+/- mice, premature vertebral endplate ossification was observed in all of the animals (II, Figures 2 and 3). Otherwise, the discs seemed normal with regular nucleus pulposus and lamellar annulus fibrosus. No protrusions of intervertebral disc material into vertebral bone could be observed. Four out of 13 two-month-old M^+/-HMut^-/+ mice had mild OA in the knee joints. One M^+/-HMut^-/+ mouse had mild OA in one of the elbow joints. In the X-ray images, all of the M^+/-HMut^-/+ mice had irregular vertebral endplates. In M^+/-HMut^-/+ mice, the annular structure of the intervertebral disc was evident in van Gieson staining (II, Figure 2). However, the nucleus pulposus structure was abnormal, consisting of cell-rich connective tissue. Usually, the disc material protruded through the endplates into the vertebral bone (II, Figure 2 and 3). X-ray images showed that the majority of M^+/-HMut^-/+ mice (6/9) had irregular vertebral endplates (II, Figure 1), two of them had only minor irregularities in the vertebral endplates and one of them had radiologically normal vertebrae. The intervertebral discs of the M^+/-HMut^-/+ mice were formed of irregular, cell-rich fibrous tissue (II, Figures 2 and 3). No distinct endplate, nucleus pulposus or annulus fibrosus was observed in these animals. Due to poorly structured endplate, growth plate and vertebral bone, the amorphous disc material protruded into vertebral bone (II, Figures 2 and 3). In some of the vertebrae, cartilaginous bridges reaching from one intervertebral disc to another could be seen (II, Figure 3). Also, connective tissue with no type II collagen was growing from the periphery into the intervertebral disc (II, Figure 3).
There were no statistically significant differences in the endplate ossification grades between 3 to 4-month-old control (0, 0 – 5) and Del1(+/−) mice (2, 1 – 2) or 15-month-old control (0.5, 0 – 2) and Del1(+/−) mice (2, 0 – 2) (the numbers in parenthesis indicate the median and quartile ranges).

5.4 Proteoglycan concentration

In the 1-month-old mice with heterozygous inactivation of Col2a1 gene, the GAG concentration was 26% lower in the vertebral bone, 19% lower in the annulus fibrosus, and 10% lower in the vertebral endplate than in the age-matched control mice (I, Figure 3). These differences were compensated by the age of 9 months. However, the GAG concentration in the nucleus pulposus was 14% lower in the knockout mice than in the controls still at the age of 9 months, but at the age of 15 months, also this difference had been compensated.

In Del1(+/−) mice the GAG concentration in vertebral growth plate and bone, and in annulus fibrosus and nucleus pulposus of intervertebral disc was the same as in their controls.

5.5 Collagen orientation

In 9-month-old mice with the heterozygous knockout of Col2a1 gene the area-integrated retardation (AIR) value, representing the level of collagen organization, was decreased in spinal tissues. However, the difference was statistically significant only between the runner mice carrying the heterozygous knockout of Col2a1 gene and control runners in the trabecular bone (I, Table 4).
In M"HMut" mice the organization of the intervertebral disc was estimated from polarized microscopy image. The annulus fibrosus was poorly organized and no distinct nucleus pulposus was seen in the middle (II, Figure 2). The optical retardation value, and thus collagen organization in the vertebral bone was 33% (p = 0.005), and in the annulus fibrosus of the intervertebral disc 57% (p = 0.005) lower in the 15-month-old Del1(+/−) mice than in the age-matched controls (III, Figure 2). This difference could not be seen at the age of three months. The parallelism of the collagen network in transversal bone sections was 4.3% (p=0.017) lower in the 15-month-old Del1(+/−) mice as compared with control mice (III, Figure 3). This was also evident in the pseudocolor images reflecting the parallelism of the collagen fibrils (III, Figure 4).

5.6 Collagen and proteoglycan localization

In one-month-old control mice and mice with heterozygous inactivation of the Col2a1 gene, type II collagen stained intensely in the intervertebral disc and in growth plates of vertebrae (I, Figure 2). The peripheral areas of the growth plates were thick and stained intensely for type II collagen (I, Figures 1A and 2). In heterozygous knockout and control mice, type X collagen was observed mainly in the hypertrophic zone of the growth plate (I, Figure 2). Also, the procollagen Iα1 (II) mRNAs were expressed intensely in the vertebral growth plates, whereas in the intervertebral disc, the signal was present in the annulus fibrosus rather than the notochordal tissue of the disc (I, Figure 2). In situ hybridization failed to reveal any differences in the content or location of procollagen Iα1 (II) mRNAs between the adult control and heterozygous knockout mice. Procollagen Iα1 (I) mRNAs were expressed strongly in the trabecular
osteoblasts (I, Figures 1D and 2), and moderately in the annulus fibrosus of the intervertebral disc (I, Figure 2).

In the 9- and 15-months-old heterozygous knockout and control mice, the staining for type II (in the peripheral areas) and X collagen of the growth plate was less prominent than in the young age group (I, Figure 2).

The observed decrease in the total GAG content of spinal tissues of heterozygous knockout mice led us to investigate distribution of specific GAGs in the spinal tissues. Positive staining for chondroitin 6-sulfate was detected in the lamellar annulus fibrosus, outer edge of the nucleus pulposus, and strongly in the hypertrophic zone of the growth plate (I, Figure 4). Chondroitin 6-sulfate was also stained in the intervertebral disc herniations through endplate (I, Figure 4). As seen previously in bovine growth plates (Deutsch et al. 1995), chondroitin 4-sulfate was stained in the whole area of growth plate the staining pattern differing to some extent from the localization of chondroitin 6-sulfate (I, Figure 4). No differences in the distribution of chondroitin sulfates were seen between the study groups. Hyaluronan was strongly stained in the hypertrophic zone of the growth plate, and in the lamellae of the annulus fibrosus, similar to the staining of human and canine tissues (Inkinen et al. 1999). The strong staining for hyaluronan in the peripheral margin of the murine nucleus pulposus (I, Figure 4) is a new unpublished finding. In one-month-old mice, biglycan was localized mainly on the lamellae of the annulus fibrosus and on the surface of the bone trabeculae (I, Figure 4). No difference was observed in hyaluronan or biglycan staining patterns between knockout mice and controls.

In M^Mut^HMut^ mice, type II collagen stained positively in the cartilaginous bridges reaching from one intervertebral disc to another. Despite the disturbed structure of intervertebral discs, the localization and content of type II collagen
appeared similar in mice carrying the Arg519Cys mutation in COL2A1 gene and the controls.

5.7 Biomechanical properties of bone

The maximal breaking force was 24% (p < 0.05) and ultimate stress 30% (p < 0.01) lower in the 15-month-old Del1(+/−) female mice as compared with the age-matched controls (III, Figure 1). There was one spiral fracture on one of the control mice femurs. The result from the latter was about twice the mean breaking force and was excluded from the analysis.

At the age of 9 months the maximal breaking force was 14.8% (p < 0.05) and at the age of 15 months 23.5% (p < 0.01) lower in the heterozygous Col2a1 knockout male runners as compared to the wild-type male runners (IV, Figure 2). The breaking strength was 12.6% and 12.1% higher in the group of normal runners at the age of 9 and 15 months than in their sedentary controls (p < 0.05 and p < 0.01, respectively) (IV, Figure 2). The sedentary female mice had greater maximum bone breaking force and stiffness than the normal running males (IV, Figure 2). Ageing increased bone mechanical strength in the groups of normal runners and normal controls, as well as in the sedentary heterozygous knockout male mice, but not with the heterozygous knockout runners. Linear correlations of parameters interacting with biomechanical properties are presented in IV, table 2.
6 DISCUSSION

Osteoporosis and OA of joints and spine are common health problems, which lead to pain and loss of ability to work and function in everyday life. The medication, surgery, and absence from work for disability caused by these diseases lead to enormous financial losses for industrialised world. As the pathogenesis for OA and osteoporosis are quite poorly understood, the treatment choices remain more or less palliative. For osteoporosis, this often means five years of treatment with rather expensive bisphosphonates (Black et al. 2006) and lifelong calcium and D-vitamin supplementation. The treatment for OA has traditionally involved pain medication and extensive surgical approaches.

This study was designed to study the pathogenesis of osteoporosis and OA and the role of exercise in the possible prevention of these diseases. Also, as the genetic mutations in the genes coding for cartilage collagens are known to cause a variety of different diseases, the phenotype of mouse models carrying similar type of mutations was in our interest.

6.1 Heterozygous inactivation of Col2a1 gene (I and IV)

In humans heterozygous deletion of one allele of the COL2A1 gene is known to cause arthro-opthalmopathy, i.e. Stickler’s syndrome (Van Der Hout et al. 2002), and previously our research group has reported that the mice with heterozygous knockout of Col2a1 gene showed an increased knee OA prevalence (Lapveteläinen et al. 2001). The hypothesis in this study was that the heterozygous inactivation of Col2a1 gene would also cause chondrodysplasia in mice.
The adult mice with heterozygous knockout of \textit{Col2a1} gene were smaller in size as compared with the controls, which is typical for chondrodysplasias. The observed premature ossification of the vertebral endplates and PG loss in the intervertebral disc are signs of early disc degeneration. The calcification of endplates is known to decrease the effusion of nutrients into the intervertebral disc, and it has been linked with disc degeneration with ageing (Bernick and Cailliet 1982; Nachemson et al. 1970; Roberts et al. 1989; Roberts et al. 1996). Therefore, the changes observed in this mouse model may predispose the genetically modified animals to disc degeneration. However, the loss of PGs in the intervertebral disc was not due to any particular GAG type. The distribution of GAGs and biglycan in the spinal tissues was similar to that of the controls.

The analyses of cervical lordosis and thoracic kyphosis suggested that the heterozygous knockout of \textit{Col2a1} gene caused changes in the spine. However, the value of these measurements remain questionable, because reliable determination of the curves was difficult due to the fact that the muscular tone of the spinal muscles of anaesthetized mice varied with the depth of anesthesia.

The average running distance per day was significantly shorter for the heterozygous \textit{Col2a1} knockout mice than for the controls. This can only partially be explained by the differences in body weights or bone lengths of the mice since these differed only about 2 to 7%. Other factors, which might contribute to the lesser physical activity, are the sensation of pain or discomfort caused by the degenerative changes in the skeletal system of mice with the heterozygous \textit{Col2a1} knockout. Of course, it is impossible to say whether the mice had pain in their spine or joints, based on the present morphological findings. The overall correlation with radiological findings to the experienced symptoms of pain at certain timepoint is poor (Boos et al.
1995; Gautier et al. 1999). On the other hand, there is evidence that the endplate changes are predictive of low back pain (Brown et al. 1997; van Dieen et al. 1999; Weishaupt et al. 1998).

In this study, the running exercise did not retard nor accelerate the progression of spinal disc degeneration. Indeed, there appear to be no studies in the literature indicating that physical exercise would protect from disc degeneration. It has been even suggested that excessive exercise would rather slightly increase the risk of lumbar disc degeneration (Ong et al. 2003). However, lifelong wheel running had actually a preventive effect against the knee OA in mice with heterozygous knockout of Col2a1 gene (Lapveteläinen et al. 2001).

Thus, heterozygous Col2a1 mice exhibited classical skeletal features of Stickler’s syndrome: abnormal development of epiphyseal plates, irregularity of the vertebral bodies, platyspondylia (flat vertebral bodies), midface hypoplasia, cleft palate, and joint arthropathy (Letts et al. 1999).

The amount of type II collagen mRNA in cartilage of mice with heterozygous inactivation of Col2a1 gene is about the half of the normal at birth (Li et al. 1995). Also, intracellular retention of procollagen chains has been observed in the heterozygous knockout mice (Li et al. 1995). Thus, it has been speculated that the disturbed growth of cartilage and thus bone in knockout mice is caused by faulty and insufficient secretion and processing, i.e. haploinsufficiency, of normal type II procollagen molecules. The same phenomenon can be seen in the Dmm mice carrying heterozygous deletion of three nucleotides in Col2a1 in the region coding for C-propeptide (Fernandes et al. 2003). However, it is of special interest that the histological stainings did not show marked differences in type II collagen in the older mice harboring a heterozygously inactivated Col2a1 gene. The genetically-modified
animals were also able to largely restore the normal structure and size of the long bones. This might be due to continuous remodelling of long bones and diminishing role of type II collagen in mature bone.

Irregularity and damage of the growth plates, and defective collagen fibril formation is common also for the Scheuermann juvenile kyphosis (Aufdermaur 1981; Scheuermann 1977). Previously, the linkage between the Scheuermann disease and COL1A1 and COL1A2 genes has been excluded. The results of this study suggest that it might be of value to study the role of COL2A1 gene in pathogenesis of Scheuermann disease.

6.2 The Arg519Cys mutation in mice (II)

In this study, two-month-old transgenic mice carrying human COL2A1 gene leading to Arg519Cys substitution in type II collagen α1 chains exhibited primary OA and osteochondrodysplasia. The severity of chondrodysplastic changes varied with genotype. One normal murine Col2a1 gene appeared to rescue especially the spinal column from the severe phenotype observed in M−/−HMut+/+ and M−/−HMut+/−. M−/−HMut+/− mice showed the most severe phenotype.

The chondrodysplasia observed in the transgenic mice was probably caused by the abnormal cartilage ultrastructure generated during organogenesis. In vitro, human Arg519Cys mutated type II procollagen could form collagen fibrils only in mixtures containing also normal type II procollagen (Adachi et al. 1999; Fertala et al. 1997), and a small number of fibril aggregates were formed (Adachi et al. 1999). However, the morphology of collagen fibrils assembled in the mixtures of normal and mutated type II collagen was abnormal (Adachi et al. 1999; Fertala et al. 1997), possibly due to
formation of intermolecular disulfide bonds on the surface of fibrils. On the other hand, the interactions between type II and type IX collagens were changed due to altered electrostatic properties around the Arg519Cys mutation site (Fertala et al. 2001). This has been suggested to result in disorganization of collagen network, leading to increased degradation of matrix collagen (Fertala et al. 2001).

In this study, transmission electron microscopy revealed that mutated type II procollagens alone could assemble into fibrils in the M⁺HMut⁺/⁻ mice. However, the fibrils were thinner in comparison with those of the wild-type mice. Previously, the density of type II collagen fibrils was shown to decrease in the M⁺⁺HMut⁺/⁺ mice, and the chondrocytes had dilated Golgi vesicles, possibly as a reflection of impaired secretion of the mutated type II procollagen (Arita et al. 2002). A marked reduction in cartilage collagen fibrils was also present in the mice harboring the Arg789Cys mutation (Gaiser et al. 2002). However, the phenotype was more severe, obviously due to the inability of Arg789Cys mutated \( \alpha_1 \) chains to incorporate into stable triple helical molecules (Gaiser et al. 2002). In general, lethal phenotype is evident for mutations, which prevent the assembly of type II procollagen trimers and their secretion, as in the homozygote Dmm mice (Fernandes et al. 2003). The somewhat milder phenotype in M⁺⁺HMut⁺/⁺ mice in comparison to heterozygous Col2a1 knockout mice (Buckwalter 1995a) suggest that Arg519Cys mutated collagens have at least some functionality left in the mice carrying one allele of the mutated human gene. The mechanism of the in vivo fibril formation in the absence of normal type II collagen is unknown. However, an evident difference between in vitro and in vivo conditions is the presence of type IX and XI collagens in the murine tissues in vivo. Therefore, it may be that these two collagen types can partly rescue the in vivo fibril formation in mice harboring the COL2A1 transgene with Arg519Cys mutation.
In a cartilage specimen obtained from a human patient carrying the same mutation, 25% of the \( \alpha_1(II) \) chains in type II collagen fibrils were mutated ones (Eyre et al. 1991), showing that mutated collagen molecules can indeed polymerize into fibrils \textit{in vivo} in the presence of normal type II procollagen. However, the lower content of the mutated form than theoretically calculated suggests that mutated type II collagen molecules may be more susceptible to proteolytic degradation (Eyre et al. 1991). Nevertheless, the chondrodysplasia and OA found in the mice and human patients carrying the Arg519Cys mutation is probably caused by functionally impaired \( \alpha_1(II) \) procollagen chains present in cartilage.

\textbf{6.3 Type II collagen and bone (III and IV)}

We examined the biomechanical and structural properties in the bones of Del1(+/-) mice and mice with heterozygous inactivation of \textit{Col2a1} gene, to find out what kind of role does collagen type II play in bone development and remodeling.

Del1(+/-) mice were earlier shown to exhibit OA and mild chondrodysplasia (Savontaus et al. 1996; Säämänen et al. 2000). Reduced metaphyseal cancellous bone volume during embryonal development and bone trabeculation in fracture callus was also reported in Del1(+/-) mice (Hiltunen et al. 1994; Savontaus et al. 1996). Therefore, it was assumed that the mature bone properties would not be normal, either. Indeed, it was observed that the maximal breaking force of femoral bones in three-point-bending test was lower in the 15-month-old Del1(+/-) female mice as compared with the controls. A variety of analyses was performed to describe the bone mass, quality and geometry, which determine the bone strength.
Significantly, the collagen organization detected with polarized light microscopy in the femoral bone, measured adjacent to the site of fracture, was lower in the 15-month-old Del1(+/−) mice as compared with the controls. In addition to this, the collagen-induced birefringence in the vertebral bone and annulus fibrosus of the intervertebral disc was decreased in the same age group. However, the collagen content of the humeral bone was not affected by the transgene. The BMD or morphology of the femoral shaft was not affected in Del1(+/−) mice. This provides further evidence for the previous hypothesis that collagen fibril organization is a significant contributor to bone strength (Martin and Ishida 1989; Puustjärvi et al. 1999; Zhu et al. 1999).

In endochondral ossification the cartilage model, rich in type II collagen, is replaced by mineralized bone containing mainly type I collagen. Yet, there were islets of extracellular matrix containing type II collagen in the mature cortical bone of the control and Del1(+/−) mice. These islets may originate from growth cartilage, and might have escaped the window of programmed degenerative processing during shaft elongation and they remained entrapped within the mineralized matrix. Mutated type II collagen has been shown to disturb chondrogenesis and endochondral ossification (Savontaus et al. 1996). On the basis of this study it seems likely that if type II positive cartilage areas in bones of Del1(+/−) mice have an effect on bone strength, it is due to some disturbance in the arrangement of the type II collagen, since no apparent change was seen in the amount of these areas in comparison to the controls.

The collagen fibril orientation of bone detected with polarized light microscopy was lower in the mice with heterozygous inactivation of Col2a1 gene at the age of 9 months, as compared to the controls. Furthermore, the GAG concentration of bone was lower in the heterozygous knockout mice than in the controls at the age of one month. However, the organization of collagen fibrils and PG concentration were gradually
compensated with skeletal maturation. Thus, the production of type II collagen was sufficient to maintain the basic remodelling of the skeleton.

The physiological adaptation of bone to increased mechanical load by increasing the BMD was not seen in the mice with heterozygous inactivation of Col2a1 (IV, Figure 2). Since the running exercise appeared to cause an increase in the bone turnover, the production of type II collagen and probably PGs was inadequate to respond into increased requirements in the heterozygous knockout mice. According to Skerry et al. (1988), the PGs may act as strain memory of bone tissue and, thereby, have a guiding influence on bone remodeling and micro-architecture (Skerry et al. 1988). A remarkable amount of type II collagen was found in the mature murine long bones. Therefore mutated collagen may decrease mechanical strength of bone. There is small amount of type II collagen in mature human bone also, but the significance of that is not known. Decreased bone mass and resistance to breakage has been reported also in a line of transgenic mice that expressed an internally deleted COL2A1 gene for type II procollagen (Helminen et al. 1993).

In this study, no significant differences were seen in the biomechanical parameters between the sedentary heterozygous Col2a1 knockout mice and wild-type male mice. However, physical activity induced significant decrease in bone fracture resistance and stiffness in the heterozygous Col2a1 knockout mice, suggesting important guiding role of collagen type II not only in chondrogenesis but also in bone maturation and remodeling.

The results of this work concerning Del1(+/−) mice and mice with heterozygous inactivation of Col2a1 gene supports the hypothesis that the type II collagen is important in bone remodeling and in endochondral ossification. The reduced bone
density with increased risk of fracture is also common in Stickler chondrodysplasia (Sebes and Kasthuri 2000; Stickler 1965).

6.4 Limitations of the study

Obvious limitation in our study was the small number of the animals. Thus, the statistical testing had to be performed using a non-parametric test, and possible small differences between the groups may not have been observed. Also, only male mice with heterozygous inactivation of \textit{Col2a1} gene had a running wheel in their cages. Neither female mice with Arg519Cys mutation nor male Del1(+/−) mice were obtained for this study, either. As it was observed in the mice with heterozygous knockout of \textit{Col2a1} gene, the gender of the mice can make significant difference in the reaction of bone properties to gene defect and/or voluntary exercise.

The minimal size of the murine skeletal system provides challenges in the research of often very minor pathological findings. In our project, especially the spinal structures involving the vertebrae and intervertebral discs proved to be difficult to study. We were not able to characterize the possible pathological anatomy of the facet joints in the spine. The "macroscopical" anatomy of the murine spine had to be studied with microscope and radiological equipment with microscopical resolution.

In this study, the use of the second-generation DXA devices on measuring bone density of mice was validated. However, the mineral content of the femoral bone of mice was on the edge of the device sensitivity. Thus, when the amount of specimens is limited, the use of DXA device is not advisable. The pQCT with higher resolution and accuracy, and also possibility for two- or three-dimensional imaging is preferred in experiments with murine bones.
On the other hand, the classical quantitative light microscopy, immunostaining, and transmission electron microscopy were found to be suitable research techniques for the murine tissues. Especially, the polarized light microscopy appeared to provide accurate and important information on the organization of fibrous structures of both cartilaginous and bony tissues.
7 SUMMARY AND CONCLUSIONS

The gene therapy is on the verge of coming to in vivo testing (Wallach et al. 2003; Yamagiwa et al. 2004). Thus, the need of mouse models for diseases caused by genetic defects is urgent. In this study, three mouse models carrying different types of defects in the Col2a1 or COL2A1 transgenes coding for the type II collagen were examined.

This thesis work demonstrates that:

1. The mice with inactivation of one allele of the murine Col2a1 gene exhibit mild osteochondrodysplasia: abnormal development of the epiphyseal plates of the vertebrae, irregularity of the vertebral bodies, platyspondylia, and midface hypoplasia. The results compare well with the recent finding that the inactivation of one allele of COL2A1 causes Stickler’s syndrome in humans (Van Der Hout et al. 2002).

2. The transgenic mice carrying human COL2A1 transgene with Arg519Cys substitution exhibit primary OA and osteochondrodysplasia of varying degree. The degenerative changes in the intervertebral discs and joints were more severe in the M⁻/⁻HMut⁺/+ and M⁻HMut⁻/⁻ mice.

3. The maximal bone breaking strength of 15–month-old mice with 150 bp deletion mutation in one allele of Col2a1 (Del1(+/-)) is decreased. After thorough examination of the bones, it was shown that collagen organization of the bone is decreased in the Del1(+/-) mice.
Our results suggest that diseases caused by mutations in \textit{COL2A1} gene can indeed be studied using mouse models carrying the same mutations. The results also show a vital role of type II collagen in osteogenesis and remodellation. The phenotypes of the mice with the gene defects show remarkable similarities with the human diseases. The heterozygous knockout of \textit{Col2a1} gene was found in human patients after our findings on the corresponding mouse model (Van Der Hout et al. 2002). In the case of Arg519Cys substitution on \textit{Col2a1} gene, the human disease had already been characterized in detail before the mouse model was generated and studied. In the future, as the knowledge of the genetic background of other diseases of musculoskeletal system increases, the mouse models are indeed invaluable for studying the pathogenesis, therapy, and prevention of these diseases.
8 REFERENCES


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Appendix: original publications I-IV
Kuopio University Publications D. Medical Sciences


D 393. Tuhkanen, Hanna. DNA copy number changes in the stromal and epithelial cells of ovarian and breast tumours. 2006. 112 p. Acad. Diss.


D 401. Andrulionyte, Laura. Transcription factors as candidate genes for type 2 diabetes: studies on peroxisome proliferator-activated receptors, hepatic nuclear factor 4α and PPARγ coactivator 1α. 2007. 112 p. Acad. Diss.


