THE LINK PROTEIN IN THE EXTRACELLULAR MATRIX OF CARTILAGE AND OTHER TISSUES

Ph.D. Dissertation

Mátyás Czipri, MD.

Department of Orthopedic Surgery
Clinical Centre
Faculty of Medicine, University of Pécs

Head of Doctoral School:	Kovács L. Gábor, MD, PhD, DSci
Program Director:	Péter Than, MD, Ph
Theme Leader:	Csaba Vermes, MD, PhD
Mentor:	Tibor T. Glant, MD, PhD, DSci

Pécs, Hungary

2015

TABLE OF CONTENTS

ABBREVIATIONS USED

AGC: aggrecan

Agc1: mouse aggrecan gene

BMP: bone morphogenic protein

cDNA: complement deoxyribonucleic acid

CNS: central nervous system

COC: cummulus-oocyte complex

COL2A1: type II collagen alpha-1 chain gene

COL9A2: human type IX collagen alpha-2 chain gene

Col9a1: mouse type IX collagen alpha-1 chain gene

COL10A1: human type X collagen alpha-1 chain gene

COL11A1: human type XI collagen alpha-1 chain gene

Col11a1: mouse type XI collagen alpha-1 chain gene

COL11A2: human type XI collagen alpha-2 chain gene

COMP: cartilage oligomeric matrix protein

CRTL1: human cartilage link protein gene

Crtl1: mouse cartilage link protein gene

Cspg: chondroitin sulphate proteoglycan

Cspg2: mouse chondroitin sulfate proteoglycan 2 gene, mouse versican gene

DMMB: dimethyl-methylene-blue

DNA: deoxyribonucleic acid

E: embryonic day

ECM: extracellular matrix

GAG: glycosaminoglycan

GAPDH: glyceraldehyde-phosphate-dehydrogenase

HA: hyaluronic acid, hyaluronan

Ihh: indian hedgehog

I-α-I: inter-α-trypsin inhibitor

LP: cartilage link protein

LYVE-1: lymphatic vessel endothelial HA receptor 1

mRNA: messenger ribonucleic acid

PCR: polymerase chain reaction

PG: proteoglycan

PTHrP: parathyroid hormone related protein

RACE: rapid amplification of cDNA ends

RNA: ribonucleic acid

RT-PCR: reverse transcription-polymerase chain reaction

RT-QT-PCR: real time-quantitative-PCR

SED: spondyloepiphyseal dysplasia

Tnfip6: tumor necrosis factor induced protein 6

1. INTRODUCTION

1.1 Cartilage extracellular matrix

Chondrocytes like cells in other tissues, exist within an information-rich extracellular environment, consisting of extracellular matrix (ECM) molecules, a milieu which interacts with and modulates the activity of growth factors, hormones and ECM remodelling enzymes. Cell surface adhesion receptors connect structural information in the ECM to a complex cellular response mechanism in the cell interior. This complex cellular response mechanism will determine the chondrocyte gene expression, and hereby the assembly of the ECM. The highly specific macromolecular organization of cartilage ECM is required for chondrogenesis, chondrocyte differentiation, endochondral ossification and for the maintenance of the weight-bearing function of articular cartilage.

Structural molecules in the supramolecular assembly of the ECM comprising collagens, noncollagenous glycoproteins, proteoglycans, and glycosaminoglycans with the predominating presence of collagens and proteoglycans (Fig.1).

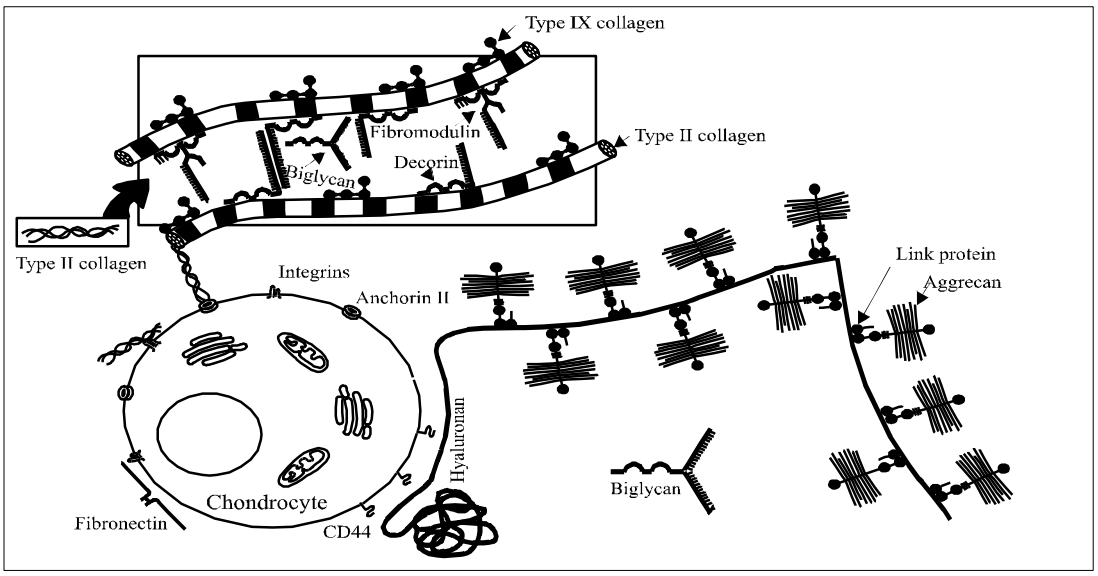

Figure 1. Molecular organization of the extracellular matrix of cartilage. Various matrix components are synthesized by chondrocytes. The hyaluronic acid filament, anchored to cartilage cells via CD 44 receptor, binds high density proteoglycan aggrecan and link protein forming large multimolecular aggregates. Type II collagen interacts with anchorin II and integrins on the surface of chondrocytes. Type IX collagen, decorin, fibromodulin and biglycan are shown on the surface of type II collagen and possibly involved in collagen fibril formation. (Schematic figure is adapted from Kuettner KE, 1994)

1.1.1 Collagens

Collagenes are homo-, or heterotrimeric molecules with one or more collagenous triple helices. Each collagen molecule is consisting of genetically distinct collagen chains, three of which fold into the triple helical molecule. The major collagen in hyaline cartilage is type II collagen representing 80-95% of all collagens in the cartilage [Eyre, 1991], and gives up to 60% of the dry weight of the tissue [Dudhia, 2005]. It is a homotrimeric molecule, with three $\alpha 1$ chains in the triple helix structure, each coded by the COL2A1 gene. Of the less abundant collagen species in cartilage, type IX and XI collagen are cartilage-specific, while type IV and VI are also found in other tissues [van der Rest and Garrone, 1991]. Collagen IX and XI are heterotrimeric molecules composed of $\alpha 1$, $\alpha 2$, and $\alpha 3$ chains, each coded by distinct gene loci for collagen IX (COL9A1, COL9A2, COL9A3) and for collagen XI (COL11A1, COL11A2,

COL11A3). Type X collagen is restricted to the hypertrophic zone of the growth plate and to the articular cartilage [Gannon et al, 1991].

1.1.2 Proteoglycans

Proteoglycans (PG) are the most abundant non-collagenous components of the extracellular matrix of the cartilage. This diverse family of macromolecules is characterized by a core protein to which glycosaminoglycan (GAG) side chains are attached. The GAGs are extended polysaccharides containing repeating disaccharide units. Large PGs (aggrecan and versican) interact with hyaluronic acid (HA) and are the members of the aggregating chondroitin sulphate PGs (Cspg), also termed hyalectins [Walcz et al, 1994; Shinomura et al, 1995]. There are two more members of the hyalectin family (neurocan and brevican) but those are mainly present in the central nervous system (CNS) [Rauch et al, 1995; Yamada et al, 1994]. Small PGs (decorin, biglycan, fibromodulin, and lumnican) interact with collagen. The large HA binding PGs share similarities in the modular domain structure of their core protein, which is coded by one single gene. The N-terminal of these molecules is formed by the globular G1 domain, which is responsible for the binding of HA. The aggrecan molecule has a G2 globular domain as well, but no distinct function has been linked to this domain to date. The N-terminal globular domains are followed by a central portion for the GAG attachment regions, where the chondroitin sulphate and keratan sulphate side chains are located. There is an another globular domain (G3 domain) located on the C-terminal end of these molecules, and consists of three modules. These characteristics indicate that the globular domains likely perform common functions, whereas divergence in the carboxihydrate-bearing region has lead to functional specialisation within this family. [Dudhia, 2005].

Aggrecan (large aggregating proteoglycan, AGC) is the most prominent PG in the cartilage accounting for 90% of the PG content, whileas the absolute amounts of other PGs are relatively low. The molecule has a peculiar brush-like structure and a molecular weight of approximately

2-3 million Da, 90% of which are sulfated GAGs and O- and N-linked oligosaccharides that are attached to a core protein [Hascall, 1988], (Fig. 2). There are approximately 110 chondroitin sulphate and 30 keratan sulphate chains attached in distinct GAG attachment regions. By comparison, versican carries 12-15 chondroitin sulphate chains. AGC binds to HA through the amino-terminal globular domain (G1) of the core protein, and has a specifically long middle part (between G2 and G3 globular domains) that bears the GAG side chains.

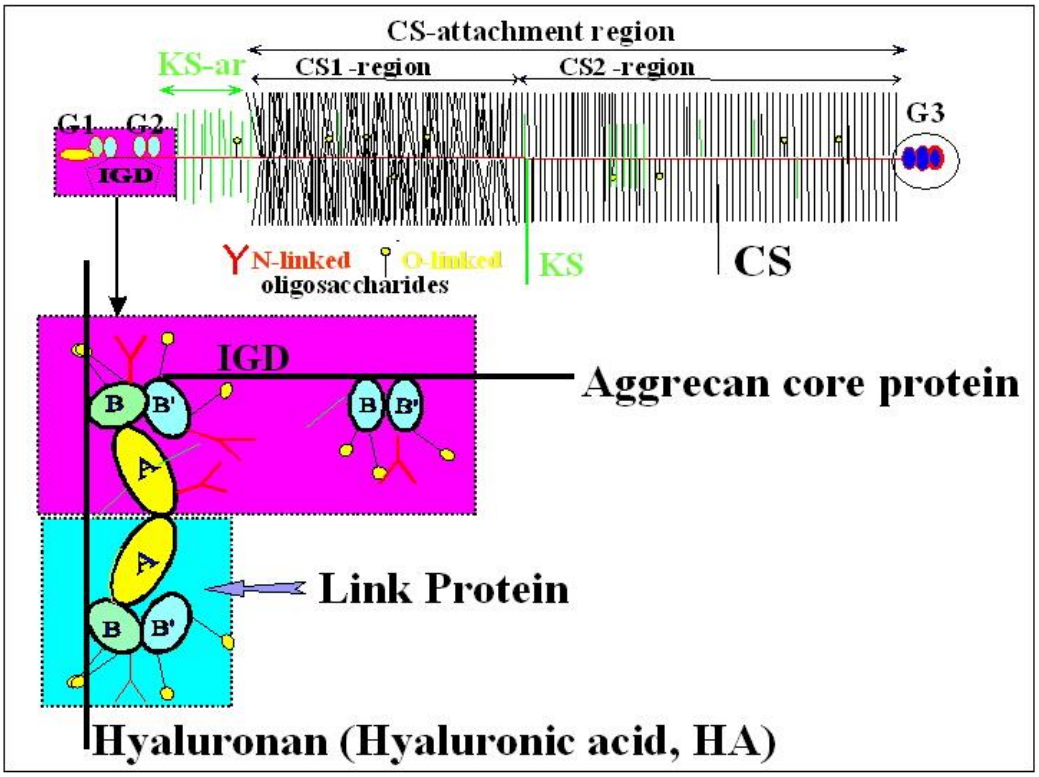

Figure 2. Aggrecan structure and the interaction between aggrecan, link protein and hyaluronan. G1, G2 and G3 are the globular domains; IGD = interglobular domain; KS = keratan sulfate; CS = chondroitin sulfate. The G1 domain contains three loops.The B and B\` loops are responsible for HA binding, and the A loop binds to the A loop of link protein.

The most characteristic function of AGC is that it forms large aggregates in which hundreds of AGC molecules are non-covalently bound to the single central HA filament. This interaction is stabilized by a third component: the link protein [Hadingham, 1979], which is a glycoprotein in the cartilage ECM and will be in the focus of this thesis.

1.1.3 Link protein

The molecule was first isolated from cartilage where it is abundantly present, and was termed as cartilage link protein (LP). LP is a small globular glycoprotein in the ECM of hyalin cartilage, and its most characteristic function is its ability to bind HA. [Hardingham, 1979]. The mouse cartilage LP gene (*Crtl1*) resides on chromosome 13 (chromosome 5 in human) and encodes a primary translation product of 355 amino acids [Czipri et al, 2003]. The LP contains three protein modules, the Immunglobuline-like A domain, B domain, and B` domain. Ten cystein residues, which define five disulfide bonds are thought to be very important to maintain the domain structure, and are well conserved in LPs of different species. The B and B` domains contain homologus amino acid sequences, and are also termed the link modules, which are responsible for the HA binding [Neame et al, 1986, Kohda et al, 1996]. One link module is approximately 100 amino acids in length and has a characteristic consensus sequence with highly conserved functionally important residues for all HA binding proteins [Day, 1999]. These proteins are grouped into the link module superfamily. Some of the members of this superfamily contains one single link module, like Tnfip6 (the protein product of tumor necrosis factor-stimulated gene 6), CD-44 (the major cell surface receptor for HA) and LYVE-1 (lymphatic vessel endothelial HA receptor-1). Other members contain a contiguous pair of link modules, like the cartilage LP and the G1 domain of the chondroitin sulphate PGs: AGC, versican, neurocan and brevican [Lee and Spicer, 2000].

1.1.4 Molecular assembly and function

A highly specific macromolecular organization of ECM is required for chondrogenesis, chondrocyte differentiation, endochondral ossification and for the maintenance of the weight-bearing function of articular cartilage. The major macromolecular components of this ECM are the PG AGC, hyaluronic acid, cartilage LP and type II collagen. AGC forms some 35% (dry weight) of the proteins found in cartilage, although the the most abundant protein is collagen

type II, which is present at up to 60 % by dry weight of the tissue [Dudhia, 2005]. LP on the other hand represents only about 0.05 % of the net weight of cartilage [Neame and Barry, 1993]. Hundreds of AGC molecules associate with a single HA filament to form large PG aggregates with molecular masses of 10^8-10^9 Da. The LP, binding simultaneously to both HA and AGC, stabilizes the aggregate structure [Hardingham, 1979] (Fig.3).

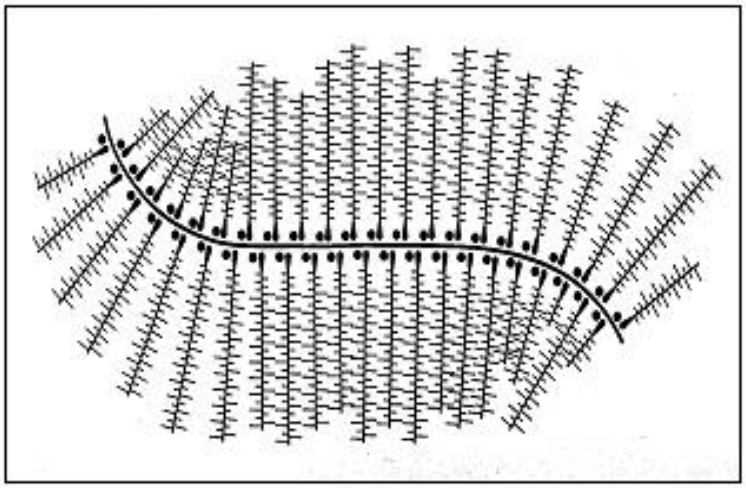

Figure 3. The proteoglycan aggregate structure in cartilage. AGC molecules are attached to a central HA filament. The LP, binding simultaneously to both HA and AGC, stabilizes the aggregate structure.

The essential role of LP in this aggregate structure was shown by rotary shadowing electron microscopy, where aggregates were shown to be smaller and contained less AGC molecules when LP was missing. In addition, in the presence of LP the HA molecules were more packed with AGC molecules with a shorter intermolecular distance in contrast to LP-free conditions [Morgelin et al, 1988]. This is in good concordance with the recent findings about the tertier protein structure of the link modules of LP. It is hypotetized that the repeated array of wedge shaped link modules on HA will introduce a gentle curve into it, giving rise to higher ordered helical structures within HA [Blundell et al, 2005]. The large PG aggregates are entrapped within the mesh-like network of type II collagen fibrils [Neame et al, 1986; Morgelin, 1988; Neame and Barry, 1993]. The negatively charged glycosaminoglycan chains of AGC bind large amounts of water (about 70% of wet weight) and are responsible for the resiliency of cartilage, while the collagen fiber network is responsible for the tensile strength of cartilage [Wight et al,

1991]. These features enable the cartilage to fulfil its physiological functions: the mobile articulation of the neighbouring bones and and resilient weight bearing of the body.

While the proper assembly of the ECM provides the cartilage with its mechanical characteristics, beyond the structural roles of ECM molecules they are also the participants and regulators of the environment in which chondrocytes are existing and differentiating. This way the ECM macromolecules are playing principal roles in chondrogenesis, chondrocyte differentiation and in the process of endochondral ossification.

The process of endochondral ossification commences with aggregation of undifferentiated mesenchyme. Cells within these condensations differentiate to chondrocytes, except for those at the periphery, which become perichondrium. Formation of the cartilaginuos anlage of the skeletal element is accomplished by chondrocyte proliferation and ECM deposition. Ultimately cells in the central region of the anlage stop proliferating and become hypertrophic. The accompanying changes in the ECM in this zone permit vascular invasion. Together with vascularisation is the appearance of bone marrow cells and osteoblasts, which replace the original cartilage with mineralized bone matrix. The zone of hypertrophic cartilage and the ossified region broadens until the bone shaft is nearly completely mineralized except near the ends, where the remaining cartilaginous growth plates containing proliferating and transitional hypertrohic chondrocytes. Premature ossification of growth plate cartilage requires continued proliferation of chondrocytes and precise control of the differentiation process leading to hypertrophy. It is the fine control of this process that determines the dimensions of skeletal elements at maturity [Horton, 1993].

Mutation in genes encoding components of cartilage ECM may result in skeletal disorders, chondrodysplasias in mice [Li Y. et al, 1995; Watanabe et al, 1994; Jacenko and Olsen, 1995; Watanabe and Yamada, 1999] and humans [Horton, 1996; Superti-Furga et al, 2001; Krakow and Rimoin 2010].

2. CHONDRODYSPLASIAS

Chondrocytes and the surrounding ECM exist in a dynamic equilibrium in terms of gene regulation, tissue differentiation, biochemical and biophysical characteristics of cartilage. Extracellular influences like ECM, soluble factors and mechanical stress affect the regulation of chondrocyte biosynthetic and metabolic activity. The balance of these numerous extracellular influences is reqired for normal function of articular cartilage. A defect in, or lack of any component of this highly organized ECM can lead to a chondrodystrophic phenotype and frequently to death. It is also likely that osteoarthritis is the result of an imbalance of regulatory influences that results in changes in gene expression, altered ECM and tissue degradation.

2.1 Human chondrodysplasias

The human chondrodysplasias are a diverse and genetically heterogenous group of disorders of the skeletal development. They are due to mutations that adversely effect endochondral ossification as it occurs in the skeletal growth plate. The target events include the proliferation and differentiation of growth plate chondrocytes and the coincident genesis and modulation of cartilage marix that serves as a template for bone formation and gives rise to the articular surfaces of joints [Horton, 1996].

Although each chondrodysplasia is relatively rare, collectively the birth incidence of these disorders is almost 1/5000 [Krakow and Rimoin, 2010]. The grouping of these genetic skeletal disorders into families was earlier based on clinical and/or radiological characteristics, and is regularly revised by the International Working Group on Constitutional Diseases of Bone [Horton, 1996; Bálint and Szebenyi, 2000]. Explosion of knowledge in the field of molecular genetic background of these disorders has led to a change in their classification by means of involving the affected genes and pathogenetic mechanisms to the classification [Horton, 1994;

Horton, 1996; Superti-Furga et al, 2001; Krakow and Rimoin, 2010]. According to such a classification the molecular defects can be divided into seven groups: defects in extracellular structural proteins; defects in metabolic pathways; defects in folding, processing and degradation of macromolecules; defects in hormones and signal transduction mechanisms; defects in nuclear proteins and transcription factors; defects in oncogenes and tumor-supressor genes; and defects in RNA and DNA processing and metabolism [Superti-Furga et al, 2001].

From our point of view the most important group is of the defects of extracellular structural proteins. This group includes some of the best characterized dysplasia families. Mutations in the principal structural molecule of cartilage, the type II collagen gene locus COL2A1 are responsible for the general clinical phenotype of spondyloepiphyseal dysplasia (SED), characterized by short trunk, short limbs, underdeveloped maxillofacial region, cleft plate, eye and inner ear abnormalities. Radiologically vertebral and epiphyseal flattening and irregularity are present. Specific phenotypes are ranging from achondrogensis type II and hypochondrogenesis at the severe end of the spectrum to late onset SED with precocious osteoarthritis at the other end. Phenotypes of intermediate severity include SED congenita, Kniest dysplasia and Stickler dysplasia. In either case the synthetized collagen chains are truncated and lack the noncollagenous carboxy propeptide necessary for incorporation into triple helical molecules [Horton, 1996]. Other cartilage collagen genes are also harbouring mutations resulting in chondrodysplasias. Mutation in the type IX collagen gene locus COL9A2 was found in some variants of multiple epiphyseal dysplasia. Type IX collagen is a quantitatively minor cartilage collagen thought to participate in the regulation of collagen fibril assembly in cartilage matrix [Horton, 1996; Superti-Furga et al, 2001]. Mutations of COL10A1, the gene encoding the alpha 1 chain of type X collagen were shown in Schmid metaphyseal chondroplasia. Since the distribution of type X collagen is restricted to the hypertrophic zone of the growth plate the mutations adversely affect this stage of endochondral ossification. COL11A1 and COL11A2 gene loci of type XI collagen are harbouring mutations in oto-

spondylo-megaepiphyseal dysplasia and in a Stickler dysplasia-like phenomenon [Horton, 1996; Superti-Furga et al, 2001].

Other disorders in this group are related to noncollagenous extracellular structural proteins. Mutations in the cartilage oligomeric matrix protein (COMP) gene were identified in pseudoachondroplasia and in the Fairbank`s type of multiple epiphyseal dysplasia. COMP is found in the extracellular matrix of cartilage where it exists as a pentomeric molecule. Mutations are mapped mainly to the calmodulin repeats of the molecule, suggesting that a disturbance of calcium binding is involved in the pathogenesis of COMP mutation disorders [Horton, 1996; Superti-Furga et al, 2001]. Genotype to phenotype correlation studies of 300 COMP gene mutations further identified specific regions, where mutations are specifically associated either with pseudoachondroplasia or multiple epiphyseal dysplasia [Briggs at al, 2014]. Perlecan, a large heparan sulfate PG, is expressed in various tissues and is a component of all basement membrane extracellular matrices. It has turned out that perlecan is essential for cartilage development, and mutations in the human perlecan gene are present in dyssegmental dysplasia (Silverman-Handmaker type) and in Schwartz-Jampel syndrome [Superti-Furga et al, 2001; Arikawa-Hirasawa et al, 1999; 2001]. For a long time, perlecan was the only known PG mutation, until two mutations were revealed affecting the AGC gene, the most abundant cartilage PG. Heterozygosity for a null mutation leads to spondyloepiphyseal dysplasia Kimberly-type. The more severe, Aggrecan-type spondyloepimetaphyseal dysplasia is autosomal recessive and is caused by a missense mutation at the G3 domain of the AGC molecule [Krakow and Rimoin, 2010]. Still, several other PGs and glycoproteins remain a candidate. Although earlier the cartilage LP and other cartilage matrix protein genes were excluded as mutant loci in human chondrodysplasias [Loughlin et al, 1994], an experimental animal model of cartilage LP knock-out mice developing dwarfism and chondrodystrophic phenotype characteristic of spondyloepiphyseal dysplasias shed a new light on this cartilage ECM molecule [Watanabe et al, 1999; Czipri et al, 2003].

2.2 Chondrodysplasias in animals

Similarly to human chondrodysplasias, there are naturally occuring mutations in genes coding cartilage extracellular matrix structural proteins in animals. These mutations result in chondrodystrophic phenotype and often in death. In addition to these conditions there are several experimental animal models, mainly in mice, where targeted mutations in different ECM protein genes are created by means of different genetic approaches. These murine models are valuable since they allow studies of molecular mechanisms at a level of detail not possible in humans. Furthermore, the mice may serve as predictors of human diseases, as well as models in which specific questions concerning pathogenesis and novel therapies may be examined.

2.2.1 Naturally occurring mutations

A mutation in the *Col11a1* gene locus of the alpha 1 chain of type XI collagen was found behind the known autosomal recessive chondrodysplasia (*cho*) in mice. Homozygous *cho/cho* mice show a disproportionate shortening of limbs, cleft plate, and underdeveloped rib cage. They die at birth due to the tracheal collapse and lung hypoplasia. Heterozygotes appear to be normal [Li Y. et al, 1995]. The cartilage matrix deficiency (*cmd*) mutation in mice is a lethal autosomal recessive mutation characterized by cleft plate, short limbs, tail and snout, and disproportionate dwarfism. Heterozygous mice show normal phenotype at birth, homozygotes die just after birth due to respiratory failure. The condition is caused by a 7 bp deletion in exon 5 of the AGC gene which is in the G1 globular domain responsible for HA binding and results in a severely truncated molecule [Watatnabe et al, 1994]. In addition it has turned out, that during ageing the *cmd* heterozygous mice show abnormal phenotypic changes. These changes basically include misalignment in the cervical spine, abnormal hyperlordosis in thoracic spine and discus herniations. The spinal abnormalities are accompanied by slight dwarfism and shortened lifetime. On the molecular level these phenotypic changes are explained by reduced amounts of AGC [Watanabe et al, 1997]. Similarly to the *cmd* mutation in mice, *nanomelia*

mutation in the chicken also involves the AGC gene, a single base mutation which leads to the synthesis of a truncated AGC core protein, and results in a lethal condition, where chondrodystrophic phenotype is present [Li H. et al, 1993].

2.2.2 Experimental animal models

There are several animal models in which structural cartilage protein genes are manipulated by means of transgenic or knock-out approaches. These methods result in no or altered protein production, where the synthetized protein is unable to fulfil its physiological function.

One of the early models was the cell lineage ablation of chondrocytes, which involved the placement of the diphteria toxin A chain gene under the control of the collagen II promoter and enhancer. The collagen II promoter and enhancer sequences of the transgenic construct were responsible for the expression of the toxin only in chondrocytes, as these gene regulatory elements were shown to be highly tissue specific. When toxin gene is expressed, it kills the cells and thus eliminates any tissue that would have been derived from that progenitor cell. As there were no viable transgenic offsprings, the physical abnormalities were found in the embryonic life at embryonic day 12 or later, which is coincidental with the onset of chondrogenesis. These embryos had cleft plates, shortened and under-developed limbs, no eyes, kinked tails, they were smaller and generally resembled the phenotype of chondrodystrophic mice strains [Yamada et al, 1990]. Transgenic mice with targeted inactivation of the *Col2a1* gene for collagen type II also resulted in severe chondrodysplasia and perinatal death. The homozygous animals were dwarf with greatly shortened limbs and truncated facial bones as a result of disruption of endochondral ossification [Li SW., 1995]

In an other model aiming to disrupt the function of collagen IX, a transgenic approach was used. The transgene construct was driven by the collagen type II promoter and enhancer, and produced a truncated alpha 1 chain, which competed with the endogenous alpha 1 chain. As a result, the triple helical structure of collagen IX was diminished with the loss of function at the

protein level. Homozygous mice exhibited mild chondrodysplasia and changes in the articular cartilage, which progressed with ageing and resembled human osteoarthritis [Jacenko and Olsen, 1995]. A model of gene knock-out for the collagen IX alpha 1 chain coding gene *Col9a1* also resulted in progressive osteoarthritis-like alterations in articular cartilage. In this case the targeted mutation of the gene has led to an inactive *Col9a1* resulting in no protein production [Fassler et al, 1994].

2.2.3 Link protein knockout mice

Targeted inactivation of the mouse cartilage LP gene (*Crtl1*) also results in death and skeletal abnormalities characteristic of human chondrodysplasias. Gene targeting was performed by disrupting exon 4 of the *Crtl1* gene at the *Bsg*I site upstream of the HA-binding 'link modules' by the pGK-neo^{r}-poly(A) cassette in the pPNT vector [Watanabe and Yamada, 1999].

The homozygous knockout mice (*Crtl1*$^{-/-}$) show dwarfism and flat face, whereas heterozygous mice (*Crtl1*$^{+/-}$) have no apparent phenotype. Most LP knockout homozygotes (about 93 %) die shortly after birth due to respiratory failure, but those who survive develop progressive dwarfism and skeletal abnormalities. These mice have shortened long bones, flattened vertebrae, the antero-posterior axis of the skull is shortened with a dome-like skull and shortened snout (Fig 4).

Figure 4. Wild type and cartilage LP-deficient (*Crtl1*$^{-/-}$) newborn mice. Dwarf *Crtl1*$^{-/-}$ mouse (below) as seen in our breeding, is significantly smaller than the wild type littermate (above) and shows the signs of cyanosis immediately after birth.

The cranial bones formed through cartilage templates are affected, whereas other cranial bones derived from membranous ossification are normal. The structure of the growth plate is changed, as the proliferative and hypertrophic zones are indistinct and the columns of chondrocytes in the growth plate are disorganised with little bone replacement. At the protein level the lack of LP was accompanied by the significant reduction of AGC in cartilage, while the amount of type II collagen was similar to that in wild type mice. This animal model pointed to the important function of cartilage LP in the formation of PG aggregates and chondrocyte differentiation and maturation [Watanabe and Yamada, 1999; 2003]. Although no skeletal disorders have been mapped to the vicinity of the LP gene locus [Hecht et al, 1992; Loughlin et al, 1994], a certain class of chondrodysplasia, likely inherited in a recessive manner, may be due to a defect of the LP gene [Watanabe and Yamada, 1999].

3. GENETIC RESCUE OF CHONDRODYSPLASIA AND PERINATAL LETHAL LETHAL EFFECT OF CARTILAGE LINK PROTEIN DEFICIENCY

To investigate the function of LP in cartilage extracellular matrix and mouse skeletal development first we intended to use a transgenic approach. We expected phenotypic changes due to the transgene-dictated LP overexpression. Unlike in the previously described LP knockout mice [Watanabe and Yamada, 1999] where the rat LP sequences were used as a basis for gene targeting, a transgenic approach necessitated the knowledge of the mouse LP coding sequence. Thus, the first step was to determine the mouse LP gene structure and cDNA sequence. In the second step the coding sequence was inserted into a cartilage-specific expression vector to generate cartilage LP transgenic mice. Finally two independent transgenic lines were established and characterized. These two lines essentially differed in their transgenic protein expression, but none of them showed any apparent phenotypic changes. In the second series of experiments the transgenic mice were used to perform a genetic rescue by the introduction of the LP transgene into the genome of LP knockout homozygous mice, which has previously been proved to be dwarf and to die shortly after birth [Watanabe and Yamada, 1999]. The *Crtl1*-deficient heterozygous founder male was received from the NIH/NIDCR. We hypothesised that the presence of the LP transgene in LP deficient mice will result in adequate protein production, and will lead to the rescue of perinatal lethality and to the „treatment" of chondrodystrophic phenotype. Different overexpressing transgenic lines were used in rescue experiments to determine potential influence of different levels of protein production on the phenotype. After the successful genetic rescue our goal was to investigate the survivals in details.

3.1 Experimental procedures

3.1.1 Isolation and cloning of the murine LP gene

Cartilage from articular joints of neonatal (2-5-days old) BALB/c mice were dissected, total RNA isolated and reverse transcribed (RT) as previously described [Cs-Szabó et al, 1997; Fülöp et al, 1997] Prior to reverse transcription, samples were digested with DNase I (Invitrogen, Carlsbad, CA) to eliminate residual genomic DNA from the RNA sample. First-strand cDNA was synthetized from 1 μg of total RNA primed by oligo-d(T) using SuperScript II reverse transcriptase (Invitrogen) as described [Cs-Szabó et al, 1997]. The strategy for identification of the mouse LP gene was based upon RT-polymerase chain reaction (PCR), first using primers homologous in various species, and then using mouse specific primers. PCR products were directly cloned into the TA-vector (Invitrogen) and sequenced from both directions. Overlapping clones from the 5' and 3' ends were generated, using a 5' RACE system (Clontech, Palo Alto, CA) and 5’ and 3' Genome Walker (GW) systems (Clontech), and cloned into the pT-ADV vector.

In order to identify the 5' transcription start site, primer extension was employed. A reverse primer in exon 1 (LP1) was end-labeled and used to both reverse transcribe mRNA from mouse chondrocytes and sequence a PCR product of genomic DNA corresponding to the LP promoter region. Both the [^{33}P]-ATP labeled RT product and the sequencing reaction were run in parallel on a 7% Long Ranger sequencing gel (FMC, Rockland, ME).

For determination of intron-exon structure, the GW kit was employed using gene-specific nested primers located in the neighbouring exons. Resulting PCR products were sequenced and sequence alignments and analysis were performed using the Omiga^{TM} 2.0 computer package (Oxford Molecular Group, Hunt Valley, MD) or the NIH BLAST Server. Whenever it was possible, a long range PCR using genomic DNA template from BALB/c mice was used to determine the size of introns.

3.1.2 Generation of LP transgenic mice

In order to generate transgenic mice that over-express LP in cartilage, we designed a cartilage- specific transgenic expression vector [Glant et al, 2002] (Fig. 5C). The vector was derived from pSPORT-1. The 5` box contains the type II collagen promoter [Kohno et al, 1986]. At the 3`-end, a 0.5 Kb fragment defined by the *Sma*I and *Xba*I sites consists of the SV40 promoter and poly-A adenylation site. The SV40 also serves a nonspecific enhancer function. At the very 3`-end of the vector, the 1.5 Kb fragment defined by the two *Xba*I sites consists of the type II collagen enhancer [Horton et al, 1987]. Together these regulatory elements provide cartilage-specific expression. A 1758 bp-long cDNA fragment containing the entire LP coding sequence was blunt-end ligated into the polylinker site of the transgenic vector pSP/44-3 (Fig. 5A). After linearization and gel purification, the transgenic construct was used for microinjection (DNX Technologies, Princeton, NJ). Initially, ten founder mice were identified to harbour the transgene. From these mice, four independent transgenic lines were established and then two, a low ($Crtl1^{TgA}$) and a high ($Crtl1^{TgC}$), transgene and protein-expressing lines were used for further experiments.

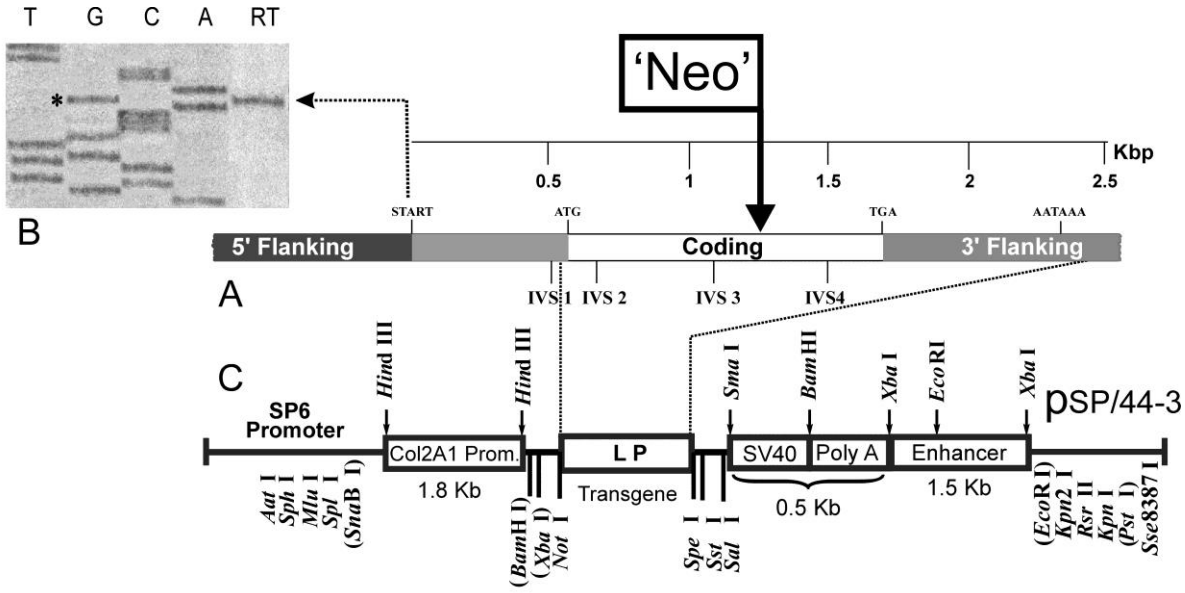

Figure 5. Schematic representation of the murine LP (*Crtl1*) gene (A), the transcription site at -365 bp position (B), and the transgenic construct (C).
Panel A indicates the assembled *Crtl1* gene structure. The LP gene is shown as a continuous box with shaded ends (non-coding sequences) and a white center (coding sequence). The scale in Kbp is given above the sequenced cDNA, whereas the locations of introns are indicated by the designation of IVS1, IVS2, etc. Important landmarks are identified above the gene box. START indicates the transcriptional start site with the corresponding primer extension reaction shown in panel B. The lane labeled RT denotes the reverse transcription reaction, while the arrow indicates the predominant product. Lanes labeled A, C, G, T indicate the corresponding dideoxynucleotide used in sequence analysis. The asterisk indicates the nucleotide that corresponds to the RT product, thus indicating the initiation site for transcription. ATG marks the translational start site, TGA indicates the termination codon, and AATAAA indicates the first polyadenylation signal, which was incorporated into the transgenic construct. '*Neo*' in box shows the position in exon 4 (at the *Bsg*I site) where the Neo^R casette was inserted to disrupt the LP gene [Watanabe and Yamada, 1999], just before the HA-binding motif of the B loop (Table 2). Schematic structure of the LP transgenic construct is shown in panel C. The transgenic construct used for driving cartilage-specific over-expression consists of: (i) a 1.8 Kb type II collagen promoter, followed by (ii) a multiple cloning site for insertion of the LP transgene, (iii) a 0.5 Kb fragment consisting of the SV40 promoter and polyadenylation site, and finally, (iv) a 1.5 kb type II collagen enhancer from the 5' end of intron I.

3.1.3 Determination of genotype of transgenic and knockout mice.

Mice were genotyped by PCR using gene or neomycin (Neo)-specific primers with genomic DNA templates (Fig. 6 and Table I). Genomic DNA was isolated from tails by a standard

method [Sambrook et al, 1989] and a primer pair of LP4 and LP5 was used to identify the presence of the transgene (Fig. 6).

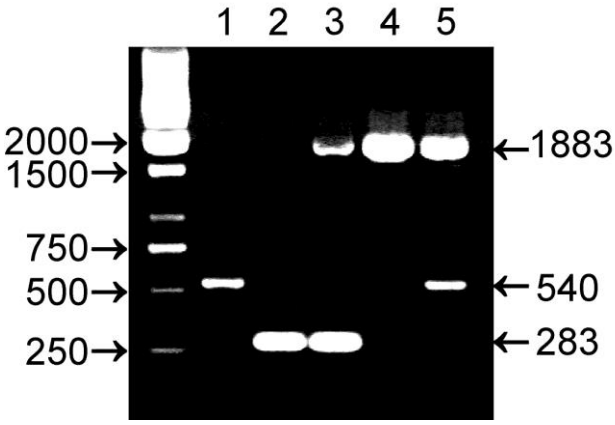

Figure 6. PCR genotyping results. Lane 1 shows the presence of the 540 bp transgenic construct using LP4 and LP5 primers (Table I). Lane 2 is the product of the wild type gene with LP12 (in intron 3) and LP13 (in exon 4) primers (Table I). Using the same primers (LP12/LP13), Lane 3 shows the PCR results of a heterozygous *Crtl1* knockout with (1883 bp) or without (283 bp) the *Neo*-gene. Lane 4 presents a homozygous *Crtl1* knockout. Lane 5 shows a *Crtl1*$^{-/-}$ mouse also carrying the transgene (540 bp).

Crtl1 heterozygosity was demonstrated by the presence of two PCR products (283 bp without and 1.88 Kbp with *Neo*, Fig. 6 lane 3) using gene-specific LP12 forward and LP13 reverse primers (Table I). In addition, homozygosity of the *Crtl1*-deficiency was confirmed by the presence of (i) two Neo-specific (PNeo1 and PNeo2), and (ii) gene- (LP14) and Neo-specific (PNeo3) primer-generated PCR products, and (iii) the absence of the 283 bp PCR product by LP12 and LP13 (Table I).

Table I

Primers Used for Cartilage LP Cloning and Analysis

Primer	Sequence 5' to 3'	Direction	Location	PCR product size (bp)	PCR conditions
LP1	GAGTGCGGTGTGGGTGTG	Rev	Exon 1	Primer Extension.	
Primers for RT-PCR and genotyping transgenic mice					
LP2	CCTTTCAGACAGCTACACTCC	For	Exon 2	292	A
LP3	AAACACTCGACCTTGATAGCC	Rev	Exon 3		
LP4	AGGAATCCACAAAATCC	For	Exon 3	540	B
LP5	CAGAAAACGTCATATCTGC	Rev	Exon 4		
LP6	CTTACCCTGGAGGATTATGG	For	Exon 3	775	C
LP7	TTGCATGAGTTTCATATTAAA	Rev	Exon 5		
Primers for real-time PCR					
LP10	TGCAGTACCCAATCACCAAACC	For	Exon 4	87	D
LP11	CTTGTCCCAAAACCCGTAGTTCC	Rev	Exon 4		
GP1	CTACATGTTCCAGTATGACTCCACTCACG	For	GAPDH	79	E
GP2	GTTGATGACAAGCTTCCCATTCTCG	Rev	GAPDH		
Primers for knockout genotyping					
LP12	TAATGACCTTTCCTGTCTCTCC	For	Intron 3	283 or 1,883	F
LP13	CCCAAAACCCGTAGTTCC	Rev	Exon 4		
PNeo1	GGATCGGCCATTGAACAAG	For	Neomycin	600	B
PNeo2	CACCATGATATTCGGCAAGC	Rev	Neomycin		
LP14	GTCGGGCAGAGGCAGAGCATTTTGAG	For	Intron 3	2,200	G
PNeo3	GCTACCGGTGGATGTGGAATGTGTGC	Rev	Neomycin		

PCR conditions are as follows: A: 94°C for 2 minutes, 30x (94°C for 30 seconds, 59°C for 30 seconds, 72°C for 1 minutes) + final extension at 72 °C for 5 minutes; B: 94°C for 2 minutes, 30x (94°C for 30 seconds, 57°C for 30 seconds, 72°C for 1 minutes) + final extension at 72 °C for 5 minutes; C: 94°C for 5 minutes, 30x (94°C for 30 seconds, 59°C for 1 minutes, 72°C for 1 minutes) + final extension at 72 °C for 5 minutes; D: 94°C for 2 minutes, 35x (94°C for 15 seconds, 58.6°C for 30 seconds, 72°C for 30 seconds); E: 94°C for 2 minutes, 35x (94°C for 15 seconds, 56.0°C for 15 seconds, 72°C for 20 seconds); F: 94°C for 2 minutes, 35x (94°C for 15 seconds, 60.5°C for 30 seconds, 72°C for 5 minutes) + final extension at 72°C for 10 minutes; G: 94°C for 3 minutes, 15x (94°C for 15 seconds, 60°C for 30 seconds, 72°C for 5 minutes), 25x (94°C for 15 seconds, 60°C for 30 seconds, 72°C for 5 minutes + 20 seconds increment at each cycle) + final extension at 72°C for 10 minutes.

3.1.4 Generation of polyclonal antibodies

Polyclonal antibodies were generated against murine cartilage LP by immunizing rabbits with synthetic LP peptides (Research Genetics, Huntsville, AL). Peptides were designed as LPro1 ^{142}VIEGLEDDTGV and LPro2 ^{341}KKHKLYGVYCFRAYN based upon their hydrophilic character [Engelman et al, 1986] and predicted antigenicity [Hopp and Woods, 1981] using methods incorporated in the Omiga 2.0 software package. Peptides were coupled to Keyhole Limpet hemocyanin [Pierschbacher et al, 1983], dissolved in PBS (phosphate buffered saline, pH 7.4) and emulsified in 1 ml complete Freund's adjuvant. Three rabbits were immunized intramuscularly (3-5 mg/injection) with each peptide conjugate. Boosters were given every 2-3 weeks with peptide conjugates emulsified in incomplete Freund's adjuvant. Sera from immunied rabbits were collected weekly after the 5th injection. Antibodies from rabbit sera were purified on corresponding peptides bound to sulfolink columns [Roughley et al, 1996].

While all antibodies detected LP, polyclonal antibodies LPro1-R11 and LPro2-R18 were the most specific, showing essentially no background in Western blotting. Based upon the synthetic peptide sequence (LPro2) used for immunization, the polyclonal antibody LPro2-R18 was not only murine LP specific, but also cross-reacted with LP from human cartilage. Indeed, when LPro2-R18 was first pre-incubated with the synthetic peptide LPro2, or pre-absorbed with either mouse LP or human cartilage extract, it was no longer able to cross-react with either mouse or human cartilages. LPro1-R11 did not react with human cartilage, but could be also neutralised with either synthetic peptide LPro1 or purified mouse LP. These two antibodies were used for both Western blot analysis and immunohistochemistry in this study.

3.1.5 Tissue extraction, protein purification and Western blot analysis

For quantitative analysis, cartilage samples from 4-day-old newborn mice were pulverized under liquid nitrogen and then extracted with 4M guanidinium chloride in the presence of protease inhibitors as described [Glant et al, 1986]. Extracts were dialyzed against water, freeze-dried and normalized for protein content by Bicinchoninic Acid assay (Pierce, Rockford, IL) and for sulfated glycosaminoglycan (GAG) content by dimethyl-methylene-blue (DMMB) assay [Muller and Hanschke, 1996].

Crude extracts from skeletal tissue of wild type, transgenic, knockout and rescued newborn mice were rehydrated in Tris-acetate (0.1M, pH 7.2) buffer containing 0.15 M sodium chloride. Samples normalized to either protein or DMMB content were separated in 10% SDS-polyacrylamide slab gels under reducing conditions. Gels were electrophoretically transferred onto nitrocellulose membranes (BioRad, Hercules CA) and stained with rabbit antibodies to mouse LP (LPro1-R11 or LPro2-R18) (0.05 μg/ml diluted in 1% milk/PBS) followed by affinity purified and peroxidase-conjugated goat anti-rabbit antibody (Accurate Chemical, Westbury, NY) [Cs-Szabo et al, 1997]. Enhanced chemiluminescence (Amersham) at a serial time range was used to detect immune reaction, which was then quantified using a PDI (Huntington Station, NY) gel scanner and an integrated software package program. Relative amounts of antibody-stained proteins were identified on the membrane expressed as pixel densities and normalized to mouse LP (A1A1D6) purified from newborn mouse cartilage by cesium chloride gradient.

3.1.6 Quantitative assesment of gene expression

Crtl1 gene expression in cartilage of newborn mice with different genotypes, was quantified by real-time quantitative RT-PCR using the Smart Cycler System (Cepheid, Sunnyvale, CA), and detection was carried out by measuring the binding of fluorescent SYBR Green-I to double stranded DNA. The PCR reactions were carried out in microtubes in 25 μl volume. The cDNA

template (1 μl) of RT-PCR product was added to a PCR reaction mixture which contained final concentrations of 0.5 μM *Crtl1* specific forward (LP10) and reverse (LP11) primers (Table I), 1:50,000 dilution of SYBR Green-I stock solution (BioWhitaker Mol. Appl. Cambrex, Rockland, ME), 200 μM dNTP, 1.5 mM $MgCl_2$, and 2.5 units of Taq DNA polymerase (Promega, Madison, WI). For normalization, glyceraldehyde-phosphate-dehydrogenase (GAPDH) cDNA was amplified with forward (GP1) and reverse (GP2) primers (Table I).

The fluorescence emitted by the reporter dye was detected online in real-time, and the threshold cycle (*Ct*) of each sample was recorded as a quantitative measure of the amount of PCR product in the sample. The *Ct* is the cycle number at which the fluorescence exceeds a fixed level above baseline. The *Crtl1* signal was normalized against the quantity of GAPDH and expressed as $\Delta Ct = (Ct_{Crtl1}\text{-}Ct_{\text{Gapdh}})$. The differences in *Crtl1* signal were expressed as $\Delta\Delta Ct = \Delta Ct_{\text{other genotypes}} - \Delta Ct_{\text{wild type}}$ in the *Crtl1* expression comparison between different genotypes. Relative gene expressions were than calculated as $2^{-\Delta\Delta Ct}$. The real-time PCR assay included two independent reverse transcribed RNA samples isolated from three newborn mice from three different litters.

3.1.7 Macroscopic and histological staining of skeletal tissues

Bones and cartilage of newborn mice were stained with alizarin red and alcian blue in 70% ethanol as described elsewhere [Kimmel and Trammel, 1981], with the modification that the staining step was extended to 3 days for newborn mice, and 4 days for 4-day-old mice. Specimens for histology were fixed with 10% buffered formalin, decalcified for 1-2 weeks and then embedded in paraffin. Tissue sections (6 μm) were trichrome-stained with safranin O, fast green and iron hematoxylin.

For immunohistochemistry, mouse organs/tissues were embedded in OCT compound (Sakura Finetec, Torrance, CA), and 6-8 μm thick frozen sections on 3-aminopropyltriethoxi-silane-coated (Sigma) slides were fixed in ice-cold acetone for 5 minutes. Sections were washed

with PBS, and pretreated with protease-free chondroitinase ABC (0.5 unit/ml; Seikagaku, Japan) at 37°C for 30 minutes followed by incubation with 10 % normal goat serum in PBS. Sections were immunostained with LPro1-R11 polyclonal antibody (1:100) diluted in PBS containing 1% normal goat serum for 1 hour. All immunostainings were carried out in room temperature. After washing, sections were treated with rhodamine-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100 in PBS for 1 hour. The slides were washed, mounted in Fluoromount (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and analyzed using a Nikon Microphot-FXA microscope or Eclipse TE 200 confocal microscope (Nikon, Garden City, NJ).

3.1.8 Breading protocol for genetic rescue

For rescue experiments, we generated $Crtl1^{-/-}$ animals that also carried the *Crtl1* transgene ($Crtl1^{-/-}Crtl1^{Tg+}$). Initially, homozygous transgenic females of lines A and C ($Crtl1^{TgA}$ and $Crtl1^{TgC}$; F9-F10 generations) were mated with heterozygous LP-null males ($Crtl1^{+/-}$), first to generate heterozygous $Crtl1^{+/-}Crtl1^{Tg+}$ offspring. Progeny (N1 generation) was genotyped by PCR, and $Crtl1^{+/-}$ mice carrying the transgene ($Crtl1^{TgA+}$ or $Crtl1^{TgC+}$) were selected for further breeding. Mice heterozygous for both endogenous and transgene *Crtl1* were intercrossed to generate $Crtl1^{-/-}Crtl1^{TgA+}$ or $Crtl1^{-/-}Crtl1^{TgC+}$ genotypes.

3.2 Results

3.2.1 Coding region and genomic structure of mouse Crtl1 gene

Using a set of primer combinations homologous to human [Osborne-Lawrence et al, 1990], pig [Dudhia and Hardingham, 1989], rat [Doege et al, 1986], bovine [Hering et al, 1995], and chick [Deak et al, 1986] LP sequences, an approximately 650 bp long mouse LP cDNA was PCR-amplified and sequenced from a reverse-transcribed mouse cartilage mRNA template. Subsequently, 3' and 5' RACE systems were used to determine the 2780 bp murine LP sequence, which has been deposited into GeneBank-EMBL (Accession number: AF 098460).

The coding region contained a single open reading frame of 1,065 nucleotides encoding a protein of 355 amino acid residues, which is one residue (His) shorter than described by Deák *et al.* [Deak et al, 1999]. Comparative analysis demonstrated 99% homology with rat [Doege et al, 1986] and 96% homology with human *CRTL1* [Dudhia and Hardingham, 1989]. Most of the amino acid substitutions were identified in the N-terminal region of the protein, prior to the start of the A loop. In order to identify the 5' transcriptional start site, primer extension was employed and a single extension product (5'-CTGGGCACAG…) at -368 bp position relative to the translational start site was identified (Fig. 5B). The size of the 5'-untranslated region in the murine *Crtl1* gene is similar to that identified in human *CRTL1* at -315 bp [Dudhia et al, 1994], but shorter than described for the chick LP gene at -500 bp [Deak et al, 1991], and significantly longer than described for *Crtl1* in rat chondrosarcoma [Rhodes et al, 1991; Dudhia et al, 1994].

In order to determine the intron-exon structure of the murine *Crtl1* gene, exon specific primers and the GW kit were used and the sequencing results are summarized in Table 2. Because of the unusually large size of intron 1 (43 Kbp; Table II), initially we were unable to determine the size of the mouse *Crtl1* gene. Based upon our cDNA sequence, intron-exon borders and the genomic sequences of Celera Discovery System [Kerlavage et al, 2002] (http://www.celera.com/) and the public database Ensembl [Waterston et al, 2002]

(http://www.ensembl.org) the mouse *Crtl1* gene is 68,099 bp-long on chromosome 13 between 90.138 and 90.206 Mb region immediately upstream of the versican gene (*Cspg2*) in a head-to-head orientation. Exon 1 of *Crtl1* was found to be non-coding, while exon 2 contains the translational start site, signal peptide and N-terminal sequence, exon 3 the A loop, exon 4 the B loop and exon 5 the B' loop and 3' untranslated sequences (Fig. 5A and Table II).

Table II

Exon/Intron Structure of Cartilage LP Gene (*Crtl1*)

Exon size (bp)	Coding information	Splice donor.....	Intron size..... (bp)	Splice acceptor
1 (1104)	5' Untranslated	ACAAG**gt**aaaacttccatcg...	43,122....	atgtgatccttac**ag**CAGAG
2 (130)	Leader/N-terminus	TCAAG**gt**aagggaacacaag...	16,787....	cccttctcattat**ag**CAGAA
3 (372)	A Loop	ACAAG**gt**aggtatttataaa....	3,381....	ctctcctttgaat**ag**GTGTG
4 (303)	B Loop	CAACG**gt**aagacagcagctg....	2,360....	tccttctccccac**ag**GCCGA
5 (540)	B' Loop/3' Untranslated			

Capital and lower case letters represent exon and intron sequences respectively. The consensus splice junctions are in boldface and underlined. The *Crtl1* gene spans 68,099 bp and resides on chromosome 13.

3.2.2 Link protein transgenic mice

Four cartilage LP transgenic founder mice showed stable transmission of the transgene to progeny, and were used to establish independent transgenic lines. Based on Southern blot and subsequent densitometric analysis, founder line C was determined to have the highest copy number, greater than 20 copies of the LP transgene. Founders A, B and D were determined to have less than 10 copies of the transgene. Because founder C had the highest copy number of the transgene, and Line A exhibited the best breeding properties, these two lines were

maintained to establish homozygous LP transgenic lines, with different LP expression in cartilage. The LP protein production of cartilage was determined by western blot analysis and is described later. Cartilage-specific overexpression of LP has not resulted in phenotypic abnormalities. Normal tissue and organogenesis, normal endochondral ossification and skeletal development, normal birth ratio and growth were observed.

3.2.3 Genetic rescue from perinatal lethality

Two transgenic lines A and C with different protein production (Fig. 7B,C) offered a possibly different outcome in genetic rescue. It was expected that those *Crtl1*$^{-/-}$ mice which were also carrying the transgene would survive. Indeed, while the *Crtl1*$^{-/-}$ (LP-deficient) mice without the LP transgene died soon after birth, most of the *Crtl1*$^{-/-}$ mice carrying the LP transgene survived and many reached adult age. The *Crtl1*$^{-/-}$ mice were born with severe skeletal deformities and dwarfism and, in contrast to the original study [Watanabe and Yamada, 1999], which described a 7% survival rate of *Crtl1*$^{-/-}$ mice with dwarfism and severe chondrodysplasia, we have never seen any surviving homozygous mice deficient of wild type *Crtl1*.

The survival rates in N2 generations were different in the two rescue groups, and the rates directly correlated with the levels of the transgenic protein product. In the N2 generation, the survival ratio of *Crtl1*$^{-/-}$*Crtl1*$^{TgA+}$ mice was lower (4.2 %) than in the *Crtl1*$^{-/-}$*Crtl1*$^{TgC+}$ mice (14.4 %), both were lower than the expected ratio (Table III). The survival ratio was improved in the N3 generation, which almost reached the expected ratio in the *Crtl1*$^{-/-}$ mice rescued with the higher transgene-expressing line C (Table III).

Table III
Survival Rates of Rescued Mice at Eight Weeks of Age

Parents	Generation	Genotypes	Mendelian ratio (%)	Expected survival ratio (%)	Observed survival ratios line A	Observed survival ratios line C
Crtl1$^{+/-}$ X *Crtl1*$^{Tg+/+}$	**N1**	*All genotypes*	100	100	100 (n=67)	100 (n=45)
		Crtl1$^{+/+}$*Crtl1*$^{Tg+/-}$	50	50	53.7 (n=36)	46.7 (n=21)
		Crtl1$^{+/-}$*Crtl1*$^{Tg+/-}$	50	50	46.3 (n=31)	53.3 (n=24)
Crtl1$^{+/-}$*Crtl1*$^{Tg+/-}$	**N2**	*All genotypes*	100	100	100 (n=71)	100 (n=118)
		Crtl1$^{-/-}$*Crtl1*$^{Tg-}$	6.25	0	0	0
		Crtl1$^{-/-}$*Crtl1*$^{Tg+/- \text{ or } +/+}$	18.75	20	4.2 (n=3)	14.4 (n=17)
		Crtl1$^{+/- \text{ or } +/+}$*Crtl1*$^{Tg-/- \text{ or } +/- \text{ or } +/+}$	75	80	95.8 (n=68)	85.6 (n=101)
Crtl1$^{+/-}$*Crtl1*$^{Tg+/- \text{ or } +/+}$	**N3**	*All genotypes*	100	100	100 (n=77)	100 (n=52)
		Crtl1$^{-/-}$*Crtl1*$^{Tg-}$	2.25	0	0	0
		Crtl1$^{-/-}$*Crtl1*$^{Tg+/- \text{ or } +/+}$	22.75	29.3	12.9 (n=10)	21.1 (n=11)
		Crtl1$^{+/- \text{ or } +/+}$*Crtl1*$^{Tg-/- \text{ or } +/- \text{ or } +/+}$	75	70.7	87.1 (n=67)	78.9 (n=41)

Ratios are expressed in percentage of the total number of mice.

Quite surprisingly, mice with less than 20% of the wild type LP level could survive (Fig. 7C, lane 6), although their growth was significantly retarded and they exhibited severe chondrodysplasia (Figs. 8A-D, 9). In contrast, mice with at least 50% LP level not only survived, but also exhibited normal growth and skeletal structure (Figs. 8E-H, 9, 10).

3.2.4 Gene expression and protein production in wild type and genetically manipulated mice

Because the wild type *Crtl1* gene and the LP-transgene produce indistinguishable transcripts and proteins, overexpression of mRNA was determined by normalization to GAPDH transcripts, and the translated product by normalization to the cartilage protein decorin, since none of these products were expected to affect, or to be affected by, the expression of the transgene. Later, it was observed that decorin content determined by Western blot hybridization and the amount of sulfated glycosaminoglycans measured by DMMB assay correlated well, and the DMMB assay was then routinely used to normalize cartilage samples/extracts.

Although heterozygous (*Crtl1*$^{+/-}$) mice exhibit normal skeletal growth and breeding characteristics both LP mRNA and protein expression was significantly less in these mice (Fig. 7A-C, lane 2). The mRNA expression was less than half, while the protein expression was 54 ± 7% (n=10) when compared to the wild type expression. Neither mRNA nor LP was detectable in homozygous newborn *Crtl1*-deficient mice (Fig. 7A-C, lane 3), or in *Crtl1*$^{-/-}$ fetuses collected at E20.5.

In the earlier transgenic generations (F2-F5), there were significant differences in both mRNA and protein levels measured in offspring (positive litter-mates) of the same line, most likely because mice were not yet homozygous for the transgene, and there was individual variability in the levels of integration sites and copy number seen by Southern blot analysis. Although the Northern dot-blot hybridization (not shown) or real-time RT-PCR (Fig. 7A) could not confirm a higher expression of the transgenic transcript (mRNA) in the skeletal tissue of F6-F8 generations of mice from Line A than wild type mice (Fig. 7A, lanes 1, 4), the translated product (protein) was approximately 50% higher (mean 48 ± 24 %; n = 11) in cartilage collected from 2-4-days old newborn than wild type mice (Fig. 7B,C, lanes 1, 4). In contrast, LP mRNA level was at least 3-times higher in cartilage of transgenic Line C, which was accompanied by 153 ± 21% (n = 9) greater protein expression in newborn cartilage (Fig. 7A-C, lane 1,5).

In both rescue groups we detected decreased mRNA expression which was unexpectedly higher in the rescue group A, than in the rescue group C (Fig. 7A, lanes 1, 6, 7). In contrast the protein expression in rescue group A was only 14 ± 3% (n=10) of that in wild type mice, while in rescue group C it was found to be more than half of the wild type expression (56 ± 6%; n=10) (Fig. 7A-C, lanes 1, 6, 7).

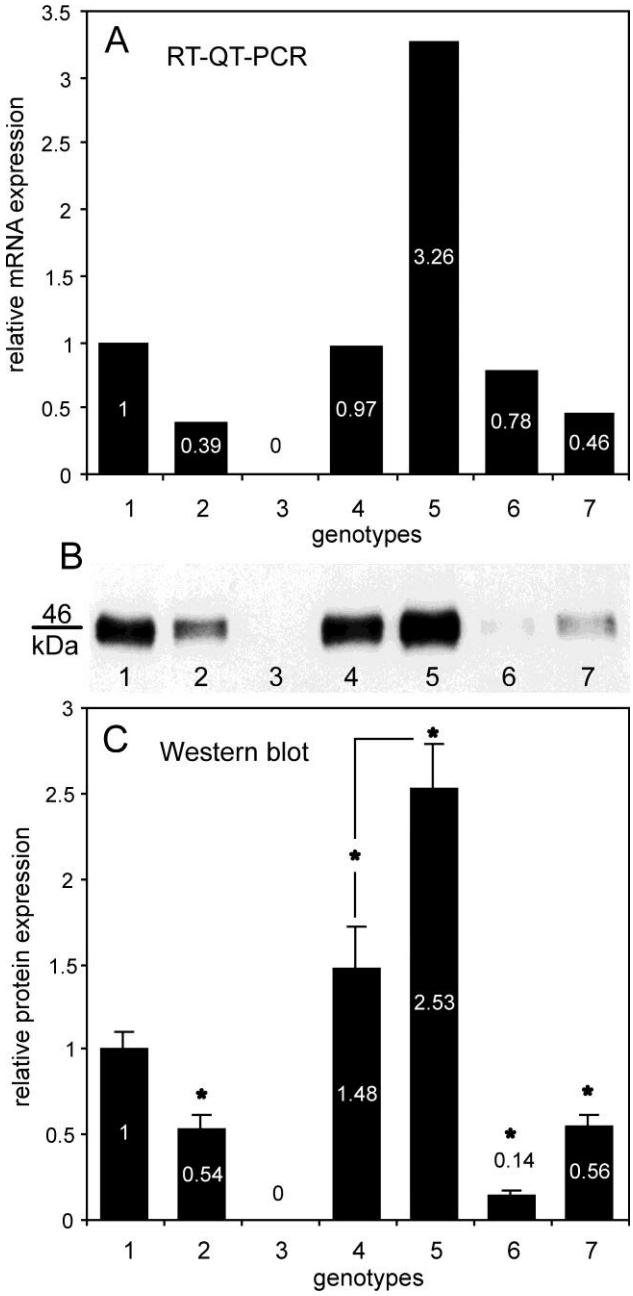

Figure 7. Quantitative analysis of the LP gene-, and protein expression of newborn mice with various genetic combinations used in this study. Panel A summarizes results of Real-time PCR (RT-QT-PCR) (n = 9; 3 mice from 3 different litters), and Panel C shows the quantitative results of Western blot analysis (n = 9-11 mice of each column from 4-5 independent litters). In both cases, expression levels are compared to the mRNA and protein expressions measured in wild type mice (first column). Panel B is a representative Western blot stained with LPro1-R11 rabbit antibody. Columns/lane 1: wild type ($Crtl1^{+/+}$); lane 2: heterozygous LP-knockout ($Crtl1^{+/-}$); lane 3: homozygous LP-knockout ($Crtl1^{-/-}$); lane 4: homozygous LP transgenic A ($Crtl1^{+/+}Crtl1^{TgA+/+}$); lane 5: homozygous LP transgenic C ($Crtl1^{+/+}Crtl1^{TgC+/+}$); lane 6: $Crtl1^{-/-}$ mice rescued with LP transgene A ($Crtl1^{-/-}Crtl1^{TgA+/- \text{ or } +/+}$), and lane 7: $Crtl1^{-/-}$ mice rescued with LP transgene C ($Crtl1^{-/-}Crtl1^{TgC+/- \text{ or } +/+}$).

3.2.5 Phenotype of the link protein-rescued mice

There were evident and significant differences in a number of phenotypic characteristics when *Crtl1*-deficient mice rescued with the low or high transgene-expressing lines were compared. Both bones and cartilage were present in newborn mice of both rescue groups, as demonstrated by the alcian blue/alizarine red staining of newborn skeletons (Fig. 8Aa, Ee). While the $Crtl1^{-/-}Crtl1^{TgA+}$ offspring survived, eventually, all $Crtl1^{TgA+}$-rescued mice developed

more or less severe abnormalities in their skeletal structure (Fig. 8Aa-Dd). These newborn mice were smaller than their litter-mates, their snout was shortened, and the antero-posterior diameter of the head was shorter giving a dome-like appearance to the skull (Fig. 8Aa, Bb). The frontal and parietal bones and occipital squama were normally formed and mineralized, indicating that only cranial bones formed through cartilage templates are affected and bones derived from membranous ossification are normal. Their limbs were significantly shorter due to the 5-15% shorter length of the long bones at the time of birth.

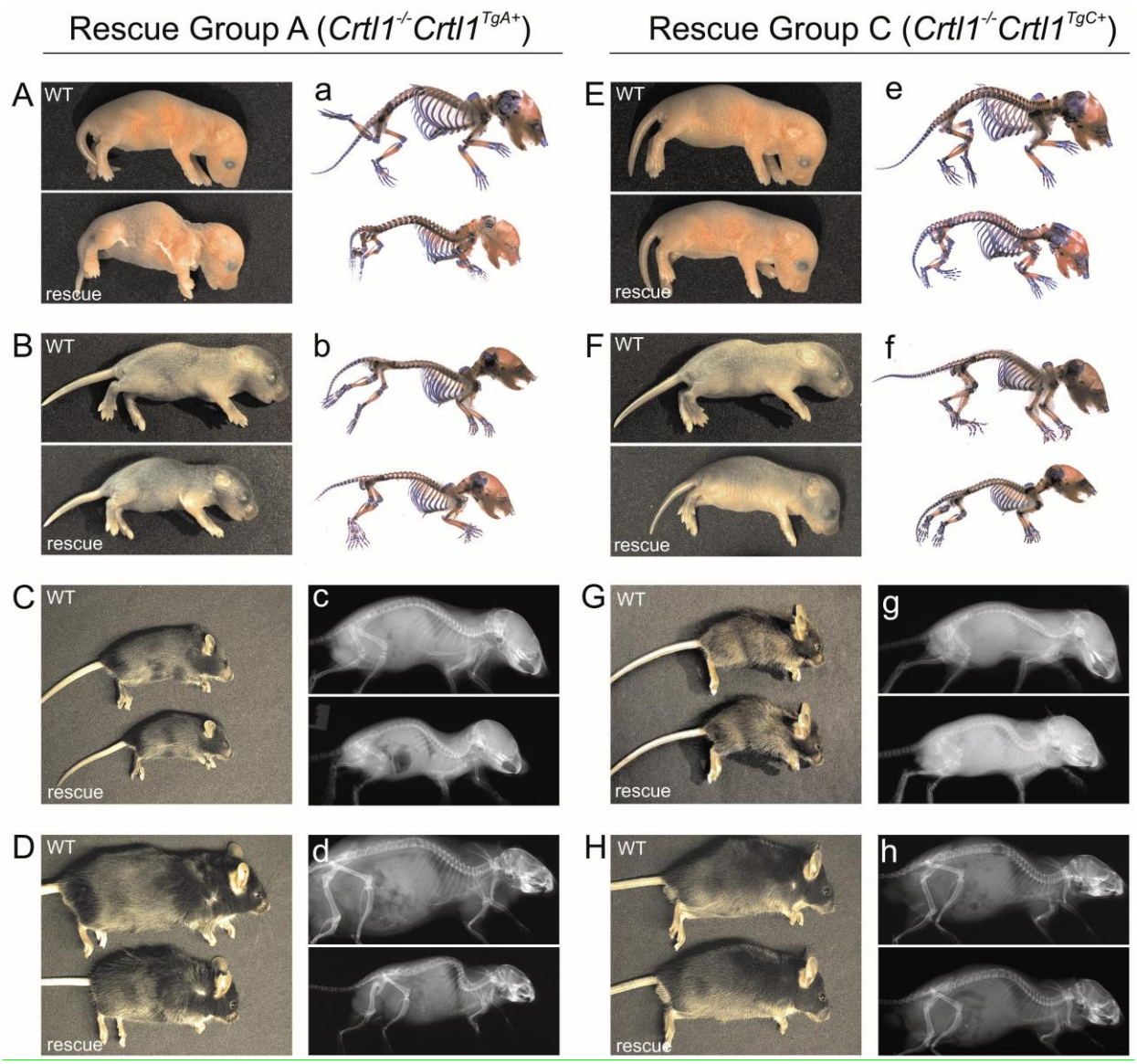

FIG. 8 **Wild type and LP-deficient (*Crtl1*$^{-/-}$) mice rescued with LP-transgene as either 'Rescue Group A': *Crtl1*$^{-/-}$*Crtl*$^{TgA+}$ or 'Rescue Group C': *Crtl1*$^{-/-}$*Crtl1*$^{TgC+}$ of different ages.** In each paired panel, animal in the upper position is a normal wild type compared to the corresponding rescued litter-mate of the F2 generation. Gross phenotype are shown *on the left side panels* of both sets (A-H), whereas skeletons stained with either alcian blue/alizarin red (a, b, e and f) or shown as whole body radiographs (c, d, g and h) are on the right side panels. Newborn (Aa and Ee; less than 1-day old), 4-days old (Bb and Ff), three-weeks old (Cc and Gg), and adult mice (Dd and Hh) are shown.

Delayed appearance of the epiphyseal ossification centers of the long bones was noted (Fig. 9A). Beyond the changes described for the skull and long bones changes in the appearance of the axial skeleton were also present. The height of the vertebral bodies was significantly decreased leading to platyspondylia (Fig. 9B), which was accompanied by spinal deformities in the sagittal plane. A marked hyperlordotic curvature in the upper, and a hyperkyphotic

curvature in the lower thoracic region of the spine created the massive spine deformities, which were consistently present in all mice rescued with the low amount of LP (Fig. 8Cc, Dd).

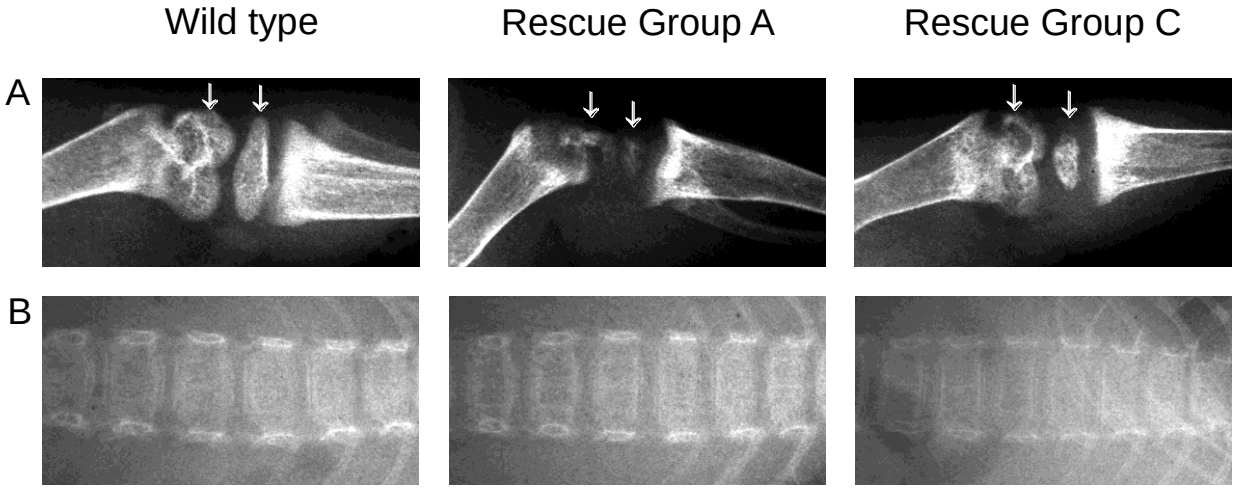

FIG. 9 **Radiographs of knee joints (A) and thoracolumbar junction of the spine (B) of 2-weeks-old wild type and rescued *Crtl1*$^{-/-}$*Crtl1*$^{TgA+}$ and *Crtl*$^{-/-}$*Crtl1*$^{TgC+}$ transgenic mice**. Arrows are pointing to the epiphyseal ossification center of the distal femur and proximal tibia (A and B).

The phenotypic anomalies found in rescue group A were similar to those described in *Crtl1*$^{-/-}$ mice [Watanabe and Yamada, 1999], resembling massive chondrodystrophic abnormalities. The skeletal deformities remained apparent during skeletal maturation, and the dwarfism became even more evident in ageing mice (Fig. 8Aa-Dd).

The growth plate structure of these mice was disorganized, although the columnar organization of hypertrophic chondrocytes was maintained (Fig. 10B). The hypertrophic zone of the growth plate was narrowed due to the reduced number of hypertrophic chondrocytes involved in the chondrocyte columns. A relative decrease in the number of chondrocytes was also present in the prehypertrophic and proliferating zone as well (Fig. 10; rescue group A). The lower number of chondrocytes was associated with an extensive amount of the extracellular matrix, which was as rich in safranin O-stained PG as the growth plate cartilage in wild type animals (Fig. 10A, B). The bony trabecules appeared to be thinner in the calcification zone compared to wild type mice (Fig. 10A, B).

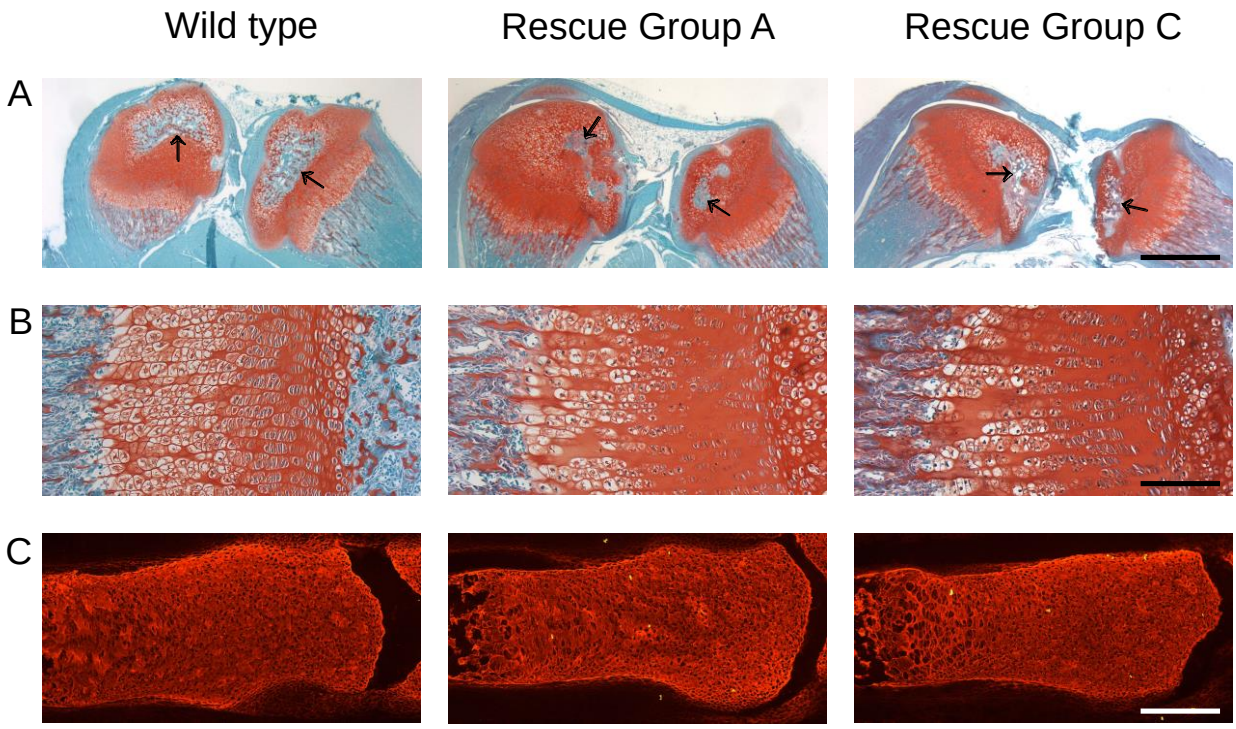

FIG. 10. **Safranin O/fast green-stained micrographs of knee joints of 2-weeks-old (A and B), and LPro1-R11 antibody-immunostained metacarpal bones of newborn (C) wild type and rescued *Crtl1*$^{-/-}$*Crtl1*$^{TgA+}$ and *Crtl*$^{-/-}$*Crtl1*$^{TgC+}$ transgenic mice**. Arrows are pointing to the epiphyseal ossification center of the distal femur and proximal tibia (A). Bars: A, 1 mm; B, 200 µm; C, 500 µm.

In contrast to the skeletal and growth abnormalities present in rescue group A, no skeletal deformities were seen in *Crtl1*$^{-/-}$ mice rescued by LP transgenic line C (Fig. 8Ee-Hh); except for mild phenotypic abnormalities with the smaller epiphyseal ossification centers of long bones and platyspondylia (Fig. 9A, B, rescue group C), and reduced chondrocyte numbers in the growth plate accompanied by increased amount of ECM (Fig. 10A, B, rescue group C). Positive immunostaining of the cartilage in newborn mice proved the presence of the transgenic protein in the ECM of rescued mice (Fig. 10C).

3.3 Discussion

This chapter summarizes the results of a partial to complete rescue of cartilage LP deficiency-induced skeletal abnormalities and the elimination of perinatal mortality. Perinatal

death is believed to be the consequence, at least in part, of insufficient cartilage function of the upper respiratory tract [Watanabe and Yamada, 1999]. The effect of the transgene, i.e. the rescue process, is dose-dependent and the results confirm that approximately 50% reduction of LP in cartilage does not cause significant pathology.

In *Crtl1* null mice the cause of death after birth is directly attributed to the collapse of the upper respiratory tract lacking the functionally sufficient cartilage. The same mechanism is suggested to be responsible for the lethality in other severe murine chondrodysplasas as well, like the *cho* mutation and *cmd* mutation [Li Y. et al, 1993; Watanabe et al, 1994]. In addition, in *Crtl1*$^{-/-}$ mice a spine deformity is present, with decreased antero-posterior diameter of the chest due to the hyperlordosis of the upper thoracic spine. The chest deformity evidently compresses the heart and lungs, which may negatively influence the cardiorespiratory functions in these mice. Even though in rescue group A, the 14±3% of the wild type levels of LP in cartilage appeared to rescue the respiratory problems allowing survival of *Crtl1* null mice in spite of their dwarfism and relatively severe skeletal abnormalities. This finding raises the question of how would this relatively low level of LP be able to restore at least partially but with no doubt very affectively the function of cartilage by only playing a role as a simple structural molecule? Could there be other roles for LP by which it is able to influence the cartilage ECM structure and development?

The growth plate of *Crtl1*$^{-/-}$ mice was described to be highly disoganized in the original study, and the 93% of these mice died shortly after birth [Watanabe and Yamada, 1999]. The proliferative and hypertrophic chondrocytes in these mice were intermingled without distinct zones and the number of hypertrophic chondrocytes was decreased. The conclusion was drawn that the lack of LP primarily affects the differentiation from prehypertrophic to hypertrophic chondrocytes, which correlates well with the expression pattern of LP with the highest level in the prehypertrophic zone. The level of AGC was found to be significantly reduced in the *Crtl1*$^{-/-}$ cartilage confirming an important role of link protein in the deposition of PG aggregates.

Investigation of underlying molecular signalling mechanisms revealed that the expression of Indian hedgehog (Ihh), a marker for prehypertrophic chondrocytes that regulates parathyroid hormone-related protein (PTHrP)-mediated signal transduction and promotes chondrocyte proliferation is reduced in the prehypertrophic zone [Watanabe and Yamada, 1999; 2003]. In contrast to the *Crtl1*$^{-/-}$ mice, the columnar oganization of chondrocytes was well recognizable in both rescue groups with a more significantly reduced number of chondrocytes in all zones of the growt plate in the rescue group A. In addition, the PG content of the cartilage ECM in rescued mice was not different in comparison with wild type mice. Any changes in the structure of the growth plate of rescued mice were mirroring the different levels of expressed protein [Czipri et al, 2003].

As the decreased AGC level in *Crtl1*$^{-/-}$ mice can be attributed to the lack of a stabilizing LP function [Watanabe and Yamada, 1999], one would expect that normal levels of LP are needed to reconstitute the aggregate structure, considering that LP and AGC are present in 1:1 stochiometry in the HA-AGC-LP tertiary complex. In contrast, in our rescue experiments, significantly lower amounts of LP were sufficient to restore the structure of cartilage. Mice from rescue group A having only 14 % of wild type levels of LP in cartilage showed no differences in safranin O staining, which indicates the PG content in tissue sections (Fig. 10A, B), yet these mice developed severe skeletal deformities. Nevertheless, while the skeletal development was retarded, this very low amount of LP seemed to be sufficient to form and maintain the macromolecular organization needed for a certain level of cartilage function, thus preventing the collapse of the respiratory tract for survival. Similarly, while the LP content was only 56% in the skeletal tissues of mice of rescue group C, when compared to wild type litter mates, the intensity of the safranin O staining in cartilage and the DMMB-measured sulfated GAG content were highly comparable in the two groups. These rescued mice with approximately 50% of the wild type level of cartilage LP not only survived but grew normally

without marked skeletal deformities. Thus, the mechanism that leads to skeletal developmental failure is more complex than the loss of a structural element of the ECM.

These findings may be related to the cartilage growth factor characteristics of LP [Martin and Dean, 1993]. A peptide of 16 amino acids, cleaved from the N-terminal end of cartilage LP can function as a growth factor and is able to stimulate the expression of AGC [McKenna et al, 1998, Liu et al, 1999] and type II collagen [Liu et al, 2000]. In addition, the N-terminal peptide (also termed link peptide) stimulated PG [McKenna et al, 1998], and type II collagen [Liu et al, 1999] synthesis in human cartilage showed a dose dependent manner. The link peptide also coordinately upregulates other small glycoproteins in cartilage as decorin and byglican [Liu et al, 1999]. In an experiment with equine cartilage explant it was showed that the link peptide also increased the synthesis of LP mRNA itself. The observation that the addition of link peptide to the culture considerably reduced the secretion of interleukin 1, suggested the mechanism of the reduction of the activity of this catabolic cytokine by which the link peptide exerts its positive effect on the rate of PG synthesis [Dean et al, 2003]. This N-terminal peptide of LP may be involved in the homeostatic regulation and synthesis of matrix components during the normal turnover of cartilage ECM [McKenna et al, 1998, Liu et al, 1999; 2000]. Further evidence of the growth factor-like characteristics of the LP N-terminal peptide is provided by studies on intervertebral disc cells, which are chondrocytes. It was shown to stimulate matrix production including increased accumulation of PG and types II and IX collagens [Mwale et al, 2003]. The same peptide stimulates the expression of type II as well as type I collagen in human intervertebral disc cells [Petit et al, 2011]. A recent study on rabbit intervertebral disc cells detected increased expression of chondrocyte-specific transcription factor SOX9, prompted by the N-terminal peptide. Upstream in this complex regulatory signaling cascade, the link peptide was found to bound to the bone morphogenic protein (BMP) type II receptor [Wang et al, 2013]. Although these functions of the LP were described in human, equine, porcine and rabbit cartilages, the highly conservative sequence of this N-

terminal peptide suggests a potential growth factor-like function in mice as well, which may be critical for skeletal development. This mechanism could, at least partially, explain the dose-dependent recovery of cartilage ECM in mice of rescue group A, where the relatively low LP production controlled by the transgene would provide a sufficient source for N-terminal peptide to be cleaved. This being the case, the growth hormone effect of the N-terminal LP peptide would be restored, resulting in a more physiological composition of the cartilage ECM.

To date, there is no human skeletal disorder connected to the LP gene. In spite of that, from a clinical and orthopaedic point of view it is a very exciting question whether the phenotypic appearance of osteochondral structure in these rescued mice could be compared to any human chondrodyplasia, or not. The phenotypic changes in skeletal structure of the lower LP expressing rescue group A include the head and face, the long bones as well as the spine. These mice are dwarf. The snout is shortened and the antero-posterior diameter of the head is shorter giving a dome-like appearance to the skull. The long bones are shorter, the spine is deformed in the sagittal plane with flattened vertebrae resulting in a short trunk [Czipri et al, 2003]. From the diverse and genetically heterogenous group of human chondrodysplasias, disorders having the most similarities with this mouse model belong to the spondyloepiphyseal dysplasia (SED) group. This group of clinical phenotype is characterized by short trunk, short limbs, underdeveloped maxillofacial region, cleft plate, eye and inner ear abnormalities. Radiologically vertebral and epiphyseal flattening and irregularity are present. Within the SED group the spondyloepiphyseal displasia congenita seems to show the most analogy. This condition lies in the middle of the spectrum of severity, and characterized by flat face, cleft plate and short trunk short stature, although shortening of the limbs is present as well. The spinal involvement includes kyphoscoliosis, dorsolumbar kyphosis and increased lumbar lordosis with platyspondylia and anterior wedging of vertebrae. The ossification is delayed. The joints are contracted, genu varum or genu valgum is present, the femoral neck is shortened and premature osteoarthritis is obligatory [Horton, 1996; Bálint and Szebenyi, 2000]. The general

clinical phenotype of SED is caused by mutations in the gene locus COL2A1, coding for the principal structural molecule of cartilage, the type II collagen. In either case the synthesized collagen chains are truncated and lack the noncollagenous carboxy propeptide necessary for incorporation into triple helical molecules [Horton, 1996].

It is difficult though, to find similarities on the molecular level between the human SEDs and cartilage LP deficiency in mice. A possible explanation could be the connection between the LP and collagen type II production, as the N-terminal peptide of LP shows growth hormone-like characteristics and was proven to increase the production of collagen type II in cartilage [Liu et al, 1999; 2000]. In contrast, the original study of *Crtl1*$^{-/-}$ mice did not detect decreased type II collagen levels in LP deficient mice, while the level of AGC was found to be significantly reduced [Watanabe and Yamada, 1999]. Collagen type II is highly expressed in the growth plate similarly to LP or AGC [Wang et al, 2004]. All are basic structural proteins of the cartilage ECM and are existing in a dynamic equilibrium. The lack of any of these structural proteins can potentially lead to chondrodystrophic phenotype as it is known from animal models. Mice with cartilage matrix deficiency caused by a functional null mutation of the AGC gene [Watanabe et al, 1994], mice with targeted disruption of the LP gene [Watanabe and Yamada, 1999], and mice with targeted inactivation of the gene for collagen type II [Li SW. et al, 1995] all have chondrodystrophic phenotype and die in the perinatal period. These animals all have shortened snout, bulged forehead, shortened limbs and skeletal deformities.

Beyond the findings that the lack of these structural proteins (collagen type II, AGC and LP) causes chondrodysplasia, it is a very interesting question if a missing molecule could have an effect on the other two. Mice lacking cartilage LP have significantly reduced level of AGC in cartilage, but the level of collagen type II seems to be normal [Watanabe and Yamada, 1999]. On the other hand, *cmd* homozygous mice with no AGC production have normal levels of cartilage LP and type II collagen [Watanabe et al, 1994], with the latter molecule showing abnormal fibrillar structure on electron microscopy [Watanabe and Yamada, 2003]. These

observations suggest that these ECM molecules must have a well-balanced interaction in cartilage, with especially important functions for cartilage LP. In addition, genetically rescued mice in the present study have similar but less severe chondrodystrophic characteristics with a LP expression level-dependent manner [Czipri et al, 2003]. Our finding, that only partial replacement of cartilage LP is able to restore cartilage structure and function at least in part by restoring the normal AGC level, also underlines that LP plays a central role in cartilage ECM organisation.

Surprisingly, there is not any human chondrodysplasia to date, which would have been mapped to the LP gene locus. A possible explanation might emerge from those findings, which reveal the importance of LP not only in cartilage but in other tissues and embryogenesis [Colas and Schoenwolf, 2003; Czipri et al, 2003; Lockhart, 2013]. It is possible, that the disfunction of cartilage LP in human has a catastrophic effect and might terminate life early in utero, making the recognition of an osteochondral disorder impossible. Our investigations to explore the presence and possible roles of LP in noncartilaginous tissues will be discussed in the next chapter.

Our results suggest that LP may have at least two functional roles in cartilage. One function is to provide structural stability of the cartilage aggregate by interacting with AGC and HA. Formation of stable cartilage aggregates is essential for the function of the growth plate, but approximately 50% LP expression level in a normal growth plate is sufficient for this stability. The 50% expression level is equivalent to that we could detect in heterozygous LP-null mice where normal phenotype is observed. It is noticeable though, that mice in rescue group C have as much LP protein expressed as the heterozygous knock out mice and still can have some mild chondrodystrophic changes which are never present in *Crtl1*$^{+/-}$ mice. The mRNA expression was also well comparable in the above mentioned two groups. These findings suggest that not only the absolute amount of LP mRNA or protein is important in the regulation and assembly of cartilage ECM, but the gene locus as well with its possible regulatory mechanisms. An

unexpected additional function is to promote cartilage development by increasing levels of PGs and perhaps other cartilage matrix proteins such as type II collagen. The restoration of this function, however, might require less amount of LP, as the 14±3 % of the wild type expression level is able to rescue homozygous LP knockout mice from perinatal death and is able to partially restore cartilage assembly.

4. LINK PROTEIN IN EXTRACARTILAGINOUS TISSUES

Although LP was originally isolated from cartilage, its presence has been reported in numerous non-cartilaginous tissues. This wide distribution of LP suggests that it may have a similar function in various organs to that described for cartilage by stabilizing the interactions between HA and large PGs. Our aim was to define the tissue distribution of LP by systematically investigating the presence of LP in different tissues and organs of developing mice on the level of transcription and protein production as well. In the first step we detected the LP mRNA by RT-PCR, which was than quantified by real-time quantitative PCR. To detect the protein production Western blot analysis and immunohystochemistry were used. We have found *Crtl1* transcripts and corresponding proteins in every organ tested from mouse embryos to ageing animals. The ubiquitous presence of LP suggests a general and systemic function of LP in the organization of ECM in a number of tissues, possibly interacting with other PGs, such as versican, brevican and neurocan.

4.1 Experimental procedures

4.1.1 Identification of transcriptional products

To determine tissue distribution of *Crtl1* transcripts, total RNA was isolated from various mouse organs and tissues at different ages, and reverse transcribed as described in chapter 2 for cartilage. Total RNA was isolated from 10.5 days old whole embryos; from skeletal tissue, brain, liver and heart of 16.5 days old embryos; and from skeletal tissue, brain, liver, lungs, heart, kidney, spleen and intestines of 20.5 days old embryos. The same organs were used for RNA isolation in newborn and adult mice, with the additional four organs of testis, eye, ovarium and uterus in adult age. To identify reverse transcribed LP mRNA in various organs, RT-PCR was employed using LP2 and LP3 or LP4 and LP5 primers (Table I). The same PCR

primers (LP4 and LP5) were used to detect the presence of transgene in genomic DNA samples. PCR products were separated in a 1.5% agarose gel and visualized by ethidium bromide staining. Representative PCR products were isolated from gels (Quiaex II gel extraction kit, Qiagen Inc., Valencia, CA) and sequenced using an ABI 310 genetic analyzer (Perkin Elmer, Branburg, NJ). For Northern dot-blot analysis, total RNA was isolated from 20.5 days old embryos (E20.5) and mRNA purified using Oligotex mRNA kit (Qiagen Inc). Two μg of this oligo-d(T)-purified mRNA in 2 μl final volume was dotted on GeneScreen Plus membrane (New England Nuclear, Boston, MA), baked at 80°C in a vacuum oven, prehybridized [Roebuck et al, 2001], and then hybridized with a ^{32}P-dCTP-labeled 775 bp long PCR product generated by primers LP6 and LP7 (Table I).

4.1.2 Quantitative assessment of gene expression

Crtl1 gene expression in different wild type mouse organs at different ages was quantified by real-time quantitative PCR using the Smart Cycler System (Cepheid, Sunnyvale, CA). Total mouse embryo at 10.5 days; skeletal tissue, brain, liver, heart at E 16.5; skeletal tissue, brain, liver, lungs, heart, kidney, spleen and intestines at newborn and adult age were used. The detection was carried out by measuring the binding of fluorescent SYBR Green-I to double stranded DNA. The PCR reaction mixtures were prepared as described in chapter 2 using the same gene specific primers (Table I).

The *Crtl1* signal was normalized against the quantity of GAPDH and expressed as $2^{-\Delta Ct}$, where $\Delta Ct = (Ct_{Crtl1}-Ct_{\mathrm{Gapdh}})$. The real-time PCR assay included two independent reverse transcribed RNA samples isolated from three embryos, or organs and tissues of three animals.

4.1.3 Tissue extraction, protein purification and Western blot analysis

Crude extracts from various organs of 40 adult mice were prepared in 4M guanidinium chloride containing protease inhibitors as described for cartilaginous tissue in chapter 2, except that tissue samples were homogenized. As the *Crtl1* transcript was detected in many organs, but the translation product was detected only in crude extracts from cartilaginous tissues, subsequent purification was performed. Approximately 600 mg lyophilized tissue/organ extracts were rehydrated in 60 ml of 0.4 M guanidinium chloride in 100 mM acetate buffer (pH 5.8) with enzyme inhibitors. Then 2 mg/ml HA (Sigma, St. Louis, MO) and 2 mg/ml highly purified rat chondrosarcoma AGC (D1D1) [Glant et al, 1986], free of LP, were added to each sample. Samples were gently stirred for 24h at 4°C. The density of each sample was adjusted to 1.5g/ml by cesium chloride and centrifuged at 100,000 *x*g for 48 h at 10°C [Glant et al, 1986]. Guanidine concentration of the bottom 1/3 fraction was increased to 4M, stirred overnight at 4°C and the density adjusted to 1.5 g/ml. Samples were spun as above for 60 h. The top 1/6 fraction of this dissociative gradient (A1A1D6) was collected [Hascall and Heinegard, 1974; Baker and Caterson 1979], dialyzed against water and lyophilized. This procedure resulted in 2-8 mg lyophilized material from the 600 mg tissue extracts, which was used to detect LP in different organs. These purified samples were rehydrated in Tris-acetate (0.1M, pH 7.2) buffer containing 0.15 M sodium chloride and the immunodetection of LP was carried out by Western blot analysis as described in chapter 2 for cartilage.

Immunohistochemistry was carried out on frozen sections and was described in details in chapter 2.

4.2 Results

4.2.1 Tissue distribution and quantitative analysis of link protein transcripts

Total RNA was isolated from E10.5 whole embryos, various organs of E16.5 and E20.5 embryos, newborn (2-4 days old) and adult mice. RT-PCR analysis demonstrated the presence of *Crtl1* transcript in every organ tested in all age group (Fig. 11).

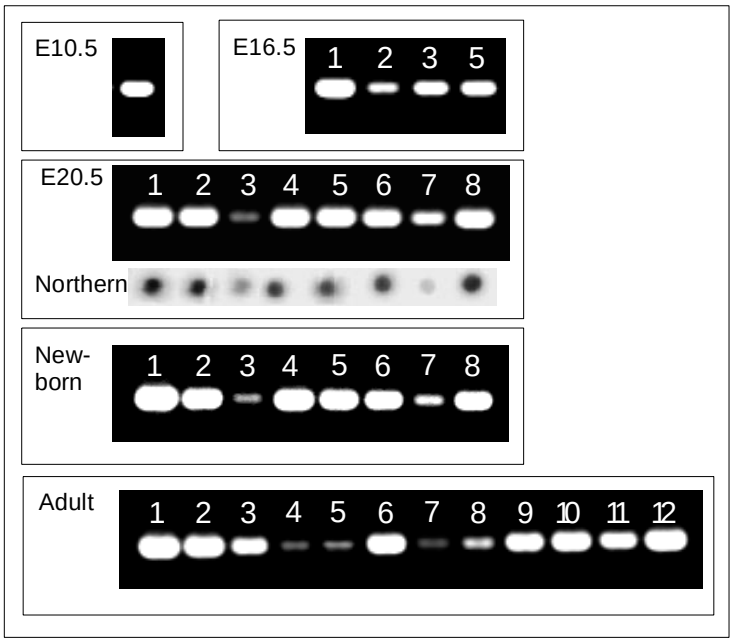

Figure 11. Gene expression of *Crtl1* in wild type mouse organs of different ages. Total RNA was isolated from the whole 10.5-day old embryo E10.5, whereas the numbers above the other RT-PCR products represent the following organs: lane 1: skeletal tissue; lane 2: brain; lane 3: liver; lane 4: lungs; lane 5: heart; lane 6: kidney; lane 7: spleen; lane 8: intestines; lane 9: testis; lane 10: eye; lane 11: ovarium and lane 12: uterus. Under the RT-PCR panel of E20.5, a Northern dot-blot shows the *Crtl1* expression in the corresponding organs.

At the earliest time point, which was set to be prior to the beginning of cartilage formation at E13.5, LP transcripts were well detectable in the E10.5 whole embryos. At E16.5 the size of the mouse embryos allowed us to collect different organs separately. Those organs, which represented reasonable source for tissue samples (skeletal tissue, brain, liver, heart) all had well detectable amounts of *Crtl1* transcripts. At later sages of mouse development (E20), and after birth (newborn, adult) all investigated organs showed *Crtl1* expression. Although RT-PCR does not allow us to draw quantitative conclusions, the mRNA expression in skeletal tissue and brain samples always seemed to be well detectable. Liver samples on the other hand usually gave weak bands on gels, which might be due to the higher risk of enzymatic degradation in these samples. Interestingly, *Crtl1* transcripts were wery well detectable in adult reproductive organs

and in the eye (Fig. 11). Similarly to RT-PCR results, Northern dot-blot hybridization detected the *Crtl1* transcript in all tissues of E20 embryos (Fig. 11).

For quantitative detection of *Crtl1* transcript levels the mRNA samples of different organs were reverse transcribed and amplified by RT-QT-PCR (n= 9-11 samples for each organ). The spatial and temporal differences in the *Crtl1* gene expression were expressed relative to the GAPDH expression and are shown on Fig. 12.

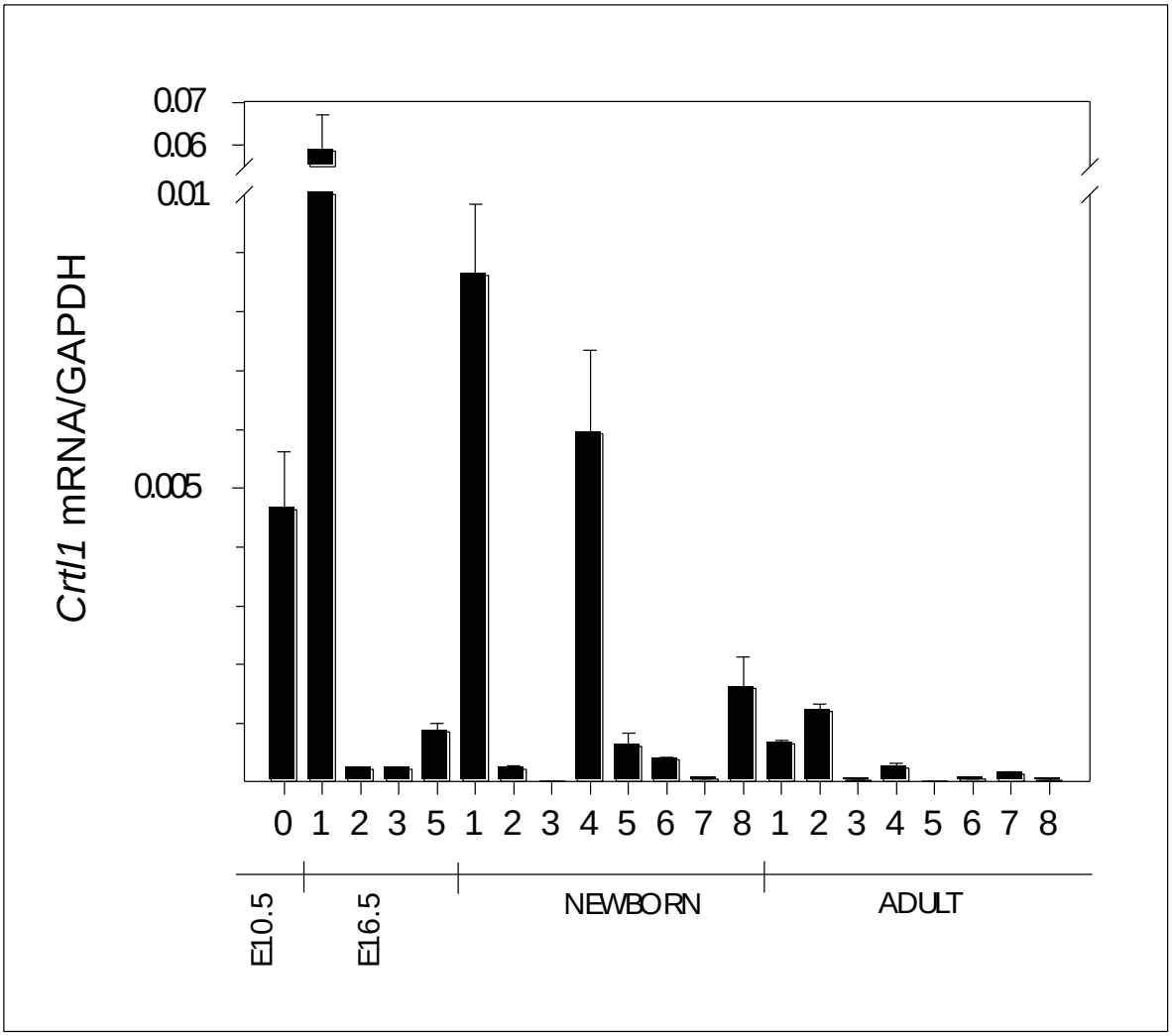

Figure 12. Changes in mRNA expression levels in embryonic, newborn and adult mice as a function of increasing age, measured by RT-QT-PCR analysis. *Crtl1* expression is showed relative to GAPDH expression. Total RNA was isolated from the whole 10.5-day old embryo E10.5, the numbers represent the following organs: lane 1: skeletal tissue; lane 2: brain; lane 3: liver; lane 4: lungs; lane 5: heart; lane 6: kidney; lane 7: spleen; lane 8: intestines.

Relatively high mRNA expression was found in the E10.5 mouse embryo, prior to the commencement of cartilage formation (Fig. 12). The highest expression was present in the

cartilaginous skeletal tissue of the E16.5 mouse embryos at the period of the fetal growth and development which was nearly seven-times higher than at newborn age, and 89-times higher than in adult cartilage (Fig. 12). This declining expression pattern of *Crtl1* in cartilage underlines its essential function in cartilage formation and skeletal development. Similar expression pattern was found in heart, as the *Crtl1* expression decreased by 30% between E16.5 and newborn age, and dropped significantly by 33 times between newborn and adult age. In contrast the levels of *Crtl1* mRNA changed in the opposite direction in mouse brain, with equally low expression at E16.5 and newborn age increasing more than 5 times by adult age, which was eventually found to be higher than the expression in cartilage at that age (Fig. 12). This increasing expression pattern suggests a higher importance for *Crtl1* in the adult CNS than in the developing brain. *Crtl1* mRNA was also highly expressed in newborn lung, reaching more than two third (69%) of the expression in cartilage. This high expression in the respiratory tract at newborn age points to the importance of LP in the cartilage-containing upper respiratory tract and in the respiratory adaptation at that age. It is noteworthy, that the cause of death in LP deficient mice was attributed to the impaired upper respiratory tract and deficient respiratory adaptation [Watanabe and Yamada, 1999]. In adult mice the *Crtl1* expression in the lungs remained relative high and represented 35% of the expression in cartilage. Intermediate levels of expression were seen in intestines and kidney (18.5% and 4.5% of skeletal tissue, respectively) at newborn age, which was higher than the expression of these organs in adult age and may be attributed to the increasing function of these organs after birth (Fig. 12). Although the *Crtl1* expression in the liver was well detectable at E16.5 (0.3% of skeletal tissue), the expression level remained very low in newborn and adult age. Interestingly, spleen was found to be expressing *Crtl1* on a relatively high level in adult mice (21% of skeletal tissue), which was 3 times higher than in newborn, and exceeded the gene expression of the adult kidney and intestines. The different temporal expression patterns suggest different functions and different biological significance for the same gene in different stages of mouse development.

4.2.2 Tissue distribution of link protein translational products

The presence of LP protein was also confirmed by Western blot analysis in protein extracts from different tissues and organs of adult mice. The abundant presence of LP in cartilaginuos tissues allowed the immunodetection of the protein even in crude extracts by Western blot, while other tissues needed to be further purified for adequate detection. Accordingly, presence of LP protein was confirmed in purified protein extracts from all the tested tissues and organs of adult mice (Fig. 13).

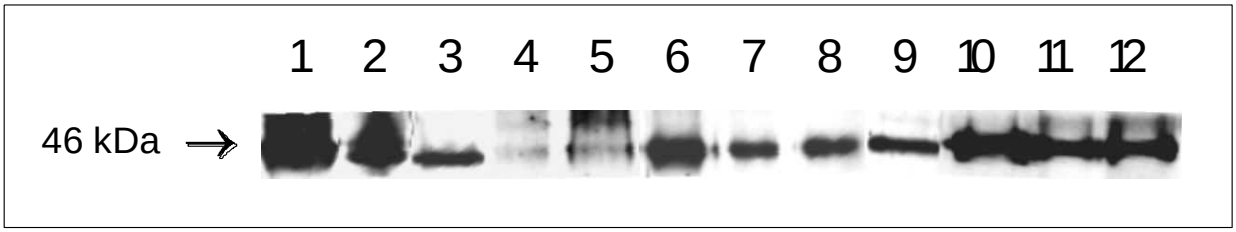

Figure 13. Wesern blot analysis of protein extracts from different mouse organs. The numbers represent the following organs: lane 1: skeletal tissue; lane 2: brain; lane 3: liver; lane 4: lungs; lane 5: heart; lane 6: kidney; lane 7: spleen; lane 8: intestines; lane 9: testis; lane 10: eye; lane 11: ovarium and lane 12: uterus.

To confirm the spatial tissue distribution of the translated *Crtl1* product, frozen sections from different organs were prepared and stained with rabbit polyclonal antibody LPro1-R11. Although at different levels, the LP was detectable in every organ tested by immunohistochemistry. At the earliest time point of immunohistochemical detection of LP in an E13.5 mouse embryo it was shown to be present in the primordial cartilaginous vertebrae and baso-occipital bone. Extracartilaginous detection of LP at the same age included the dermis of the skin, and the aorta. The immunopositivity of the E19.5 fetal kidney was also well demonstrated (Fig. 14).

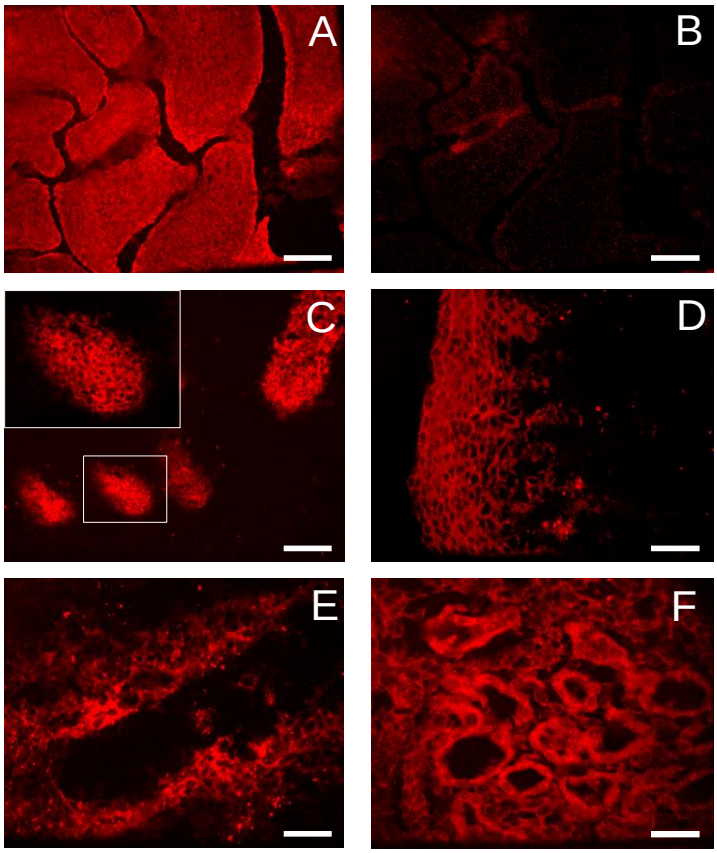

Figure 14. Immunohistological detection of LP in mouse tissue sections. The E13.5 mouse embryo (C-E), the E19.5 fetal kidney (F) and the wrist of a newborn mouse (A and B) were stained with rabbit polyclonal LPro1-R11 antibody, except for panel B (wrist) where the preimmune serum of the same rabbit was used as a negative control. Panels show LP accumulation in the cartilaginous carpal bones (A), primordial cartilaginous vertebrae and baso-occipital bone (C), the dermis of the skin (D), the aorta (E) and fetal kidney (F). Bars: A-C, 200 μm; D-F, 50 μm.

4.3 Discussion

Although LP is abundant in cartilaginous ECM, where it simultaneously interacts with AGC and HA [Hardingham, 1979], the *Crtl1* gene encoded LP was found in a number of tissues other than cartilage. In this study, which was the first systematic search for LP transcripts and translational products in a mammalian system, we systematically tested many organs for the expression of *Crtl1* mRNA and protein during embryonic development and in ageing animals. The presence of *Crtl1* mRNA expression in mouse embryos before the cartilage formation commences, as well as in a large number of non-cartilaginous tissues clearly demonstrates its ubiquitous presence, that has been known from several previous observations [Gardell et al, 1980; Poole et al, 1982; Ripellino et al, 1989; Stripe et al, 1990, Cassiede et al, 1991; Binette et al, 1994; Camaioni et al, 1996; Sun et al, 1999; Spicer, et al, 2003]. The most exciting question

arising from these findings is to find out the possible functions of LP in different ECMs. The HA binding characteristics and possible interaction with different Cspgs could be the key to uncover the roles of LP.

Earlier reports on the presence of LP in non-cartilaginous tissues has been based on electrophoretic and immunological analyses [Gardell et al, 1980; Poole et al, 1982; Fife et al, 1985]. One of the first solid evidences of the extracartilaginous existence of LP was provided by the detection of LP transcripts and LP protein in the chicken embryonic mesonephros by in situ hybridisation, Northern blotting and western blotting [Stripe et al, 1990]. The level of LP transcripts in the mesonephros was estimated to be 5% of that seen in cartilage, and the expression occurs at the same time when the mesonephros is believed to initiate function. In addition, the expression of LP was not followed by other cartilage specific gene expression like *Agc1* known to be coordinately regulated with *Crtl1* at cartilage formation commencement [Stripe and Goetnick, 1989]. In our experiments the *Crtl1* expression in the newborn mouse kidney was shown to be 4.5% of that seen in cartilage, indicating a well detectable intermedier expression level. This level of expression was significantly higher than in adult age, similarly to the gene expression measured in the lungs and intestines. As all these organs are expected to initiate or increase their function immediately after birth, and the upregulated *Crtl1* expression may play an important role in this.

Further systematic search for the LP in the chick embryonic non-cartilaginous tissues revealed its ubiquitous presence. LP transcripts and proteins were detected in the embryonic skin, brain, heart, aorta, proventriculus, gizzard, intestine, muscle and kidney. The investigations on the interactions between LP and PGs extracted from non-cartilaginous tissues demonstrated that LP can enhance the binding of these PGs to HA. These findings expanded the biological significance of LP to non-cartilaginous tissues that contain HA binding PGs [Binette et al; 1994]. An in vivo biodistribution study with monoclonal anti-LP antibody in rat revealed fixation not only in cartilage, but also in skin, aorta and lung [Cassiede et al, 1991].

Immunohistological detection of LP in skin and aorta was also well demonstrated in our study with mice [Czipri et al, 2003]. Extracartilaginous human CRTL1 expression was reported in a restricted manner with primary expression in small intestine and placenta [Spicer et al, 2003].

Our systematic search for LP in tissues other than cartilage in a mammalian system provided similar results and conclusions. In our series LP transcripts and protein were detected in skeletal tissue, brain, liver, lungs, heart, kidney, spleen, intestines, testis, eye, ovarium and in uterus in four ages including adult and newborn mice and mouse embryos [Czipri et al, 2003]. Our results regarding the quantitative detection of *Crtl1* gene expression revealed different temporal expression patterns, which could indicate increasing or decreasing importance for LP at different time points in different tissues. Higher gene expression can indicate a more important role either in developmental processes or in the function of an organ or tissue. For example, the increasing expression pattern of *Crtl1* in mouse brain suggests higher importance for LP in the adult CNS, than in the developing brain. Cartilage LP is known to be present in brain and in spinal cord grey matter [Jager et al, 2013]. It is exclusively produced by neurons and serves as an important building block in the assembly of perineuronal net, a condensation of ECM around neurons and dendrites [Cerulli et al, 2007]. Cartilage LP also seems to trigger the perineuronal net formation in the second week of postnatal development, which leads to the ending of neural plasticity [Galtrey at all, 2008; Cerulli et al, 2010]. This increasing role of LP in the postnatal brain development explains well the 5-times increase in *Crtl1* expression measured between newborn and adult age. In contrast, the declining expression pattern of *Crtl1* in heart between the intrauterine life and adult age suggests more involvement in early heart development for LP. This is in line with the reported *Crtl1* expression by endocardial and endocardium-derived cells in the developing murine heart [Lockhart et al, 2013].

An another functional role was addressed to LP in the mouse follicular development. LP was shown to be produced by cumulus cells, oocytes and granulosa cells. The expansion of the cumulus-oocyte complex (COC) is regulated by gonadotropins and this process necessitates the

increasing presence of HA and it's natural ligand inter-α-trypsin inhibitor (I-α-I). As LP was shown to bind to I-α-I it has been speculated that LP can take part in HA-LP- I-α-I ternary complexes. The presence of LP in such complexes can lead to the stabilisation of ECM, thus facilitating the COC expansion [Sun et al, 1999; 2002; Camaioni et al, 1996]. Indeed, in our series *Crtl1* protein and mRNA was well detectable in mouse reproductive organs including ovarium, uterus and testis as well.

Crtl1 was found to be an excellent candidate neurulation gene in chicken embryos [Colas and Schoenwolf, 2003]. Prominent expression of LP transcripts and protein was found to be present in the epidermal ectoderm just lateral to the neural plate, a tissue known to generate extrinsic forces for banding of the neural plate. The LP gene can be a regulator of cell behaviour which results in the formation, elevation and convergence of the epidermal ectoderm during neurulation. LP may act by stabilizing the ECM in the elevating and converging neural folds, as well as by participating in the generation of the neural fold boundary. Indeed, during our investigations we have tried to identify major developmental failures in mice in utero based on the hypothesis that the lack of the LP might cause extra-skeletal abnormalities as well. Although a large number of embryos was investigated we could not find any consistent disorder, but in some of the *Crtl1*$^{-/-}$ embryos we have found signs of a possible brain developmental failure resembling incomplete neurulation process (Fig. 15). These mutants displayed an exencephalic-like phenotype, which is very similar to the appearance of the *Twist* knockout homozgous mice. The lack of the *Twist* gene, coding a DNA-binding transcriptional factor, was shown to cause a failure of the closure of the cephalic neural tube [Soo, 2002]. Interestingly, this condition is accompanied by defects in the maxillofacial region and fore limb buds, and is lethal in utero at E11.5 [Chen and Behringer, 1995].

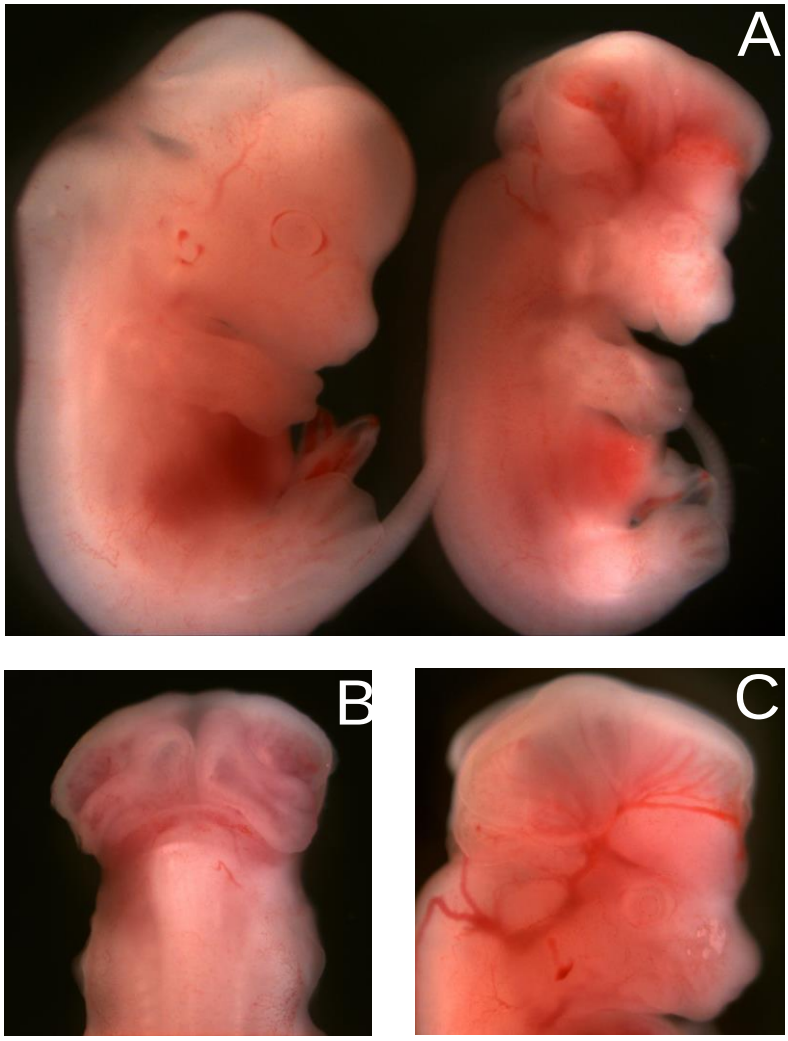

Figure 15. Gross appearance of wild type and *Crtl1*$^{-/-}$ mice in utero. Wild type (on the left) and knockout homozygous (on the right) mice were isolated from the same pregnancy (A). The cranial neural tissues of the *Crtl1*$^{-/-}$ embryo are more exposed and the surface appears to be irregular compared to the wild type control. Note that the shortness of the trunk and limbs and the maxillofacial differences are well detectable at that age. Posterior (B) and lateral (C) view of the cranial region of the knockout mouse embryo.

In finding possible functions of LP in noncartilaginous tissues it is without doubt, that molecular partners have to be searched for. Based on the HA binding characteristics and the well known function of LP in cartilage, the best group of candidate molecules possibly interacting with LP in a ternary complex structure are the chondroitin sulphate PGs (LP-Cspg-HA).

AGC, previously thought to be exclusively present in cartilage where it`s HA binding is facilitated by LP, has been found in several noncartilaginous tissues. AGC has been shown to

be present in the extracellular matrix of developing and adult brain, where it is mainly synthesized by neurons [Milev et al, 1998]. Similarily to other Cspgs in the CNS it shows particular spatiotemporal expression [Oohira et al, 2000], and the concentration of AGC seems to increase steadily during brain development [Milev et al, 1998]. It is noteworthy, that our quantitative detection of *Crtl1* gene expression in brain also showed increasing pattern, suggesting a possible interaction between these molecules in CNS as well. Different putative functions as regulation of the developing brain ECM by defining impenetrable embryonic areas in neural crest cell migration [Perissinotto et al, 2000], regulation and inhibition of neuronal cell adhesion and cell outgrowth in the perineuronal net ECM [Condic et al, 1999; Matthews et al, 2002] has been proposed for the molecule. AGC, similarly to cartilage LP, is present in the perineuronal nets and is upregulated at the time of perineuronal net formation [Galtrey et al, 2008]. The molecule is binding to HA, stabilized by LP and cross-linked by tenascin-R is one of the main components of the ECM in the CNS and is almost the exclusive PG in the perineuronal nets [Morawski et al, 2012]. The first demonstration of AGC in embryonic chick heart revealed unique expression pattern including all the major sites, where mesencymal cells contribute to the future separation of the primary heart tube [Zanin et al, 1999]. AGC is also present in other noncartilaginous tissues, like tendon [Rees et al, 2000], microvascular pericytes [Diefenderfer and Brighton, 2000], and in human sclera [Rada et al, 1997].

Versican, the other large HA binding chondroitin sulphate PG known to be abundantly present in the ECM of a variety of tissues was originally isolated from human fibroblasts [Zimmermann and Rouslahti, 1989]. There are several tissues to date, where versican was found to be present. It has been detected in human arterial smooth muscle cells [Yao et al, 1994] in dermis and proliferating zone of epidermis [Zimmermann et al, 1994], rat brain and precartilaginous mesenchyme [Bignami et al, 1993] and in rat kidney [Pyke et al, 1997]. Systematic search for the versican gene (*Cspg2)* expression in mice revealed high expression levels in embryonic development with a declining tendency from day E13. It was detected in

adult mouse tissues as well: brain, lung, spleen, heart, skeletal muscle, skin, testis, kidney. [Naso et al, 1995]. In the CNS versican is synthetized by astrocytes and oligodendrocytes [Oohira et al, 2000] and the different splice variants have characteristic developmental patterns with the predominant presece of V1 isoform in late embryonic and early postnatal period, whereas V2 isoform is present in the adult brain [Milev et al, 1998; Yamaguchi, 2000]. These observations support the theory that cartilage LP may interact with these large PGs in a number of tissues in different time points.

The interaction between LP-AGC-HA is well known and has been investigated throughly. It is also well documented, that LP has interactions with different Cspgs and is able to facilitate their HA binding. Investigations provided solid evidences [Matsumoto et al, 2003; Seyfried et al, 2005] for the previous hypothesis of molecular interaction [Hardingham and Fosang, 1992] in which link modules of both LP and AGC are engaged in HA binding and the interaction between the lectican and LP happens via the Ig modules. The same series of experiments with recombinant proteins (LP, G1 domain of versican and AGC) also support that versican is able to form stable ternary complexes with LP and HA, but the molecular interaction differs from that of the AGC-LP-HA. As direct interaction between versican G1 domain and LP in the absence of HA is not present it is likely that the interaction between the Ig modules is missing. Two different models for ternary complex formation was defined in which AGC binds to HA at the tandem HA binding domain (B-B`) and to LP at the Ig-like module (A loop), whereas versican binds to both HA and LP at the B-B` stretch [Matsumoto et al, 2003]. Others speculate that LP would be designed to interact preferentially with versican, based on those results which indicate the presence of interaction between LP and versican, and that the affinity of LP to versican is higher than to AGC [Shi et al, 2004].

Neurocan, another member of the large aggregating Cspg family is mainly present in the CNS [Rauch et al, 1995; Yamada et al, 1994]. Experimental evidence seems to support that HA, neurocan and cartilage LP aggregates are likely to form in developing brain. Fusion

proteins containing the HA binding domains of neurocan showed strongly increased HA binding ability in the presence of LP. The link module independent interaction of the neurocan immunglobulin module with LP suggests an HA binding model similar to the AGC-LP-HA complex, in which link modules of both proteins are engaged in HA binding and the interaction between the lectican and LP happens via the immunglobuline modules [Rauch et al, 2004]. Thus, it seems likely, that in the presence of HA, cartilage LP is able to form ternary complexes with at least three different Cspgs (i.e. AGC, versican and neurocan).

The startpoint of our investigations was a well characterized interaction with distinct function of the HA binding molecules (AGC and link LP), both of which was originally thought to be cartilage specific. The result of this work is in concordance with other observers and offers a new and extended way of thinking about these ECM molecules. HA binding PGs as well as LP are represented in many tissues and in many ECMs. These molecules may have pivotal roles in developing embryonic tissues, in the CNS and in many other areas. Similarly to the AGC-LP interaction in cartilage, some special molecular interactions between these molecules is already characterized, but a number of new roles and interactions need to be elucidated offering new opportunities for research.

5. NOVEL FINDINGS

5.1 Genetic rescue of chondrodysplasia and perinatal lethal effect of cartilage link protein deficiency

- We determined the coding region and genomic structure of mouse cartilage LP gene. The *Crtl1* resides on chromosome 13, and the coding region contains a single open reading frame of 1065 nucleotides encoding a protein of 355 amino acid residues. *Crtl1* shows 96% homology with the human CRTL1 gene.
- LP transgenic mice were created with a cartilage specific expression vector, and two LP transgenic mouse lines were established with stabilized LP overexpression. The transgenic lines served as the base for the animal model of genetic rescue, in which the role of LP was addressed. The protein production of LP transgenic mice was quantitatively measured and expressed relative to the wild type protein production. It was shown that the two transgenic lines differed in the LP protein expression. The protein production was approximately 50% and 150% higher relative to the wild type production in the transgenic line A and C respectively.
- The LP transgenic mice (line A and C) were successfully used in a genetic rescue experiment by substituting the missing LP in knockout mice. The lethal effect of the lack of LP was rescued by the LP transgene, the LP knockout homozygous mice carrying the transgene survived and reached adult age. Beyond the survival of these mice, the chondrodystrophic changes and dwarfism was reduced or eliminated by the LP transgene, modelling a successful gene therapy.
- Survival rates and phenotypic appearance were shown to be different in rescued mice depending on the transgene used for the genetic rescue. Mice rescued by the transgenic

line A showed lower survival rates, while as in rescue group C the survival rates almost reached the expected Mendelian ratio.

- Animals in rescue group A were smaller, with shorter limbs, the antero-posterior diameter of their head was shorter, they exhibited platyspondylia and had massive thoracic spine deformities in the sagittal plane. In contrast, the chondrodystrophic phenotype was not present in rescue group C.
- The measurement of LP protein expression in rescued mice revealed, that the phenotypic appearance and changes in osteochondrogenesis were dependent on the amount of the expressed cartilage LP. 14% of the wild type LP expression was sufficient to rescue LP deficient mice from perinatal death, although these mice exhibited severe chondrodysplasia. On the other hand, mice with 56% of the wild type level of cartilage LP not only survived but grew normally without skeletal deformities.

5.2 Link protein in extracartilaginous tissues

- Systematic search for cartilage LP transcripts and protein in various noncartilaginous tissues in different time points of mouse development revealed its ubiquitous presence in tissues other than cartilage. This was the first systematic search for cartilage LP in a mammalian system.
- Cartilage LP is expressed during embryogenesis, even before cartilage formation commences.
- Quantitative analysis of cartilage LP gene expression revealed potentially different importance of LP in different organs in different time points. Some expression patterns suggest increasing importance with upregulation at initiation of the function of an organ.
- LP expression remains dominating in cartilage containing organs at all ages of mouse development with significant increase in the the lungs at birth.

- LP expression in brain remains limited during mouse development, but reaches high levels at adult age and is overcoming the expression even in the cartilaginous tissues.
- LP expression in the developing heart is the second highest in utero after cartilage during organogenesis, and the declining expression after birth suggests less importance at adult age.
- Protein products of the mouse cartilage LP were identified in tissue extracts and in tissue sections with polyclonal antibodies. The LP was found to be present in skeletal tissue, brain, liver, lungs, heart, kidney, spleen, intestines, testis, eye, ovarium, uterus, aorta and skin.

6. ACKNOWLEDGEMENTS

My highest appreciation belongs to Professor Tibor T. Glant for his continuous lead, strong support and mentorship. It would have been impossible to accomplish this work without his guidance and help. He and his entire team at the lab at the Department of Orthopedics, Section of Biochemistry and Molecular Biology at Rush University (Chicago, IL) provided me with the professional atmosphere, that was essential to conduct my research. I would like to thank to Professor Katalin Mikecz, Csaba Vermes, Sonja Velins, Jeffrey Otto, Reinout Stoop, Rajesh V. Kamath, István Gál, Andrew Nesterovitch, Yanal Murad, Kevin Kolman and Jian Zhang for giving me a hand to finish my work. Special thanks to my good friend Tamás Bárdos for his continuous help, advice and support, which made those years spent in Chicago so valuable.

I am thankful to all of my former colleague from my mother institution, the Department of Orthopedic Surgery at the University of Pécs. I would like to express my gratitude to Professor Árpád Bellyei, my progressive-thinking chief at the beginning of my carrier, for giving me the unique opportunity to conduct scientific research in a professional environment, and for his continuous encouragement towads academic achievements.

I am grateful for my program director, Professor Péter Than and my theme leader, Csaba Vermes for their professional support and guidance. Their help was invaluable to me at the finishing stages of my work.

Finally, I express my most sincere thank to my family, my wife, my mother and my brother for their love, sacrifice and unconditional support.

7. REFERENCES

Arikawa-Hirasawa E., Watanabe H., Takami H., Hassell JR., Yamada Y.: Perlecan is essential for cartilage and cephalic development. Nature Genet. 1999; 23:354-358.

Arikawa-Hirasawa E., Wilcox WR., Yamada Y.: Dyssemental displasia, Silverman-Handmaker type: unexpected role of perlecan in cartilage development. Am. J. Med. Genet. 2001; 106:254-257.

Baker JR., Caterson B.: The isolation and characterization of the link proteins from proteoglycan aggregates of bovine nasal cartilage. J. Biol. Chem. 1979; 254: 2387-2393.

Bálint G., Szebenyi B.: Bailliere`s Clinical Rheumatology. 2000; 14:219-250.

Bignami A., Perides G., Rahemtula F.: Versican, a hyaluronate-binding proteoglycan of embryonal precartilaginous mesencyma, is mainly expressed postnatally in rat brain. J. Neurosci. Res. 1993; 34:97-106.

Binette F., Cravens J., Kahoussi B., Haudenschild DR., Goetinck PF.: Link protein is ubiquitously expressed in non-cartilaginous tissues where it enhances and stabilizes the interaction of proteoglycans with hyaluronic acid. J. Biol. Chem. 1994; 269: 19116-19122.

Blundell CD., Almond A., Mahoney DJ., DeAngelis PL., Campbell ID., Day A.J.: Towards a structure for a TSG-6 hyaluronan complex by modeling and NMR spectroscopy. J. Biol. Chem. 2005; 280:18189-18201.

Briggs MD., Brock J., Ramsden SC., Bell PA.: Genotype to phenotype correlations in cartilage oligomeric matrix protein associated chondrodysplasias. Eur. J. Hum. Genet. 2014; 22:1278-1282.

Brunner HG., van Beersum SEC., Warman ML., Olsen BR., Popers HH., Mariman MC.: A second Stickler syndrome gene is linked to the region of chromosome 6 that contains COL11A2. Hum. Mol. Genet. 1994; 9:1561-4.

Camaioni A., Salustri A., Yanagishita M., Hascall VC.: Proteoglycans and proteins in the extracellular matrix of mouse cumulus cell-oocyte complexes. Arch. Biochem. Biophys. 1996; 325:190-198.

Carulli D., Rhodes KE., Fawcett JW.: Uppregulation of aggrecan, link protein 1, and hyaluronan synthases during formation of perineuronal nets in the rat cerebellum. J. Comp. Neurol. 2007; 501:83-94.

Carulli D., Pizzorusso T., Kwok JC., Putignano E., Poli A., Forostyak S., Andrews MR., Deepa SS., Glant TT., Fawcett JW.: Animals lacking link protein have attenuated perineuronal nets and persistent plasticity. Brain. 2010; 133:2331-47.

Cassiede P., Vilamitjjana-Amedee J., Bareille R., Vuillemin L., Harmand MF.: Biodistribution study of a monoclonal antibody specific to link protein from human cartilage. Int. J. Rad. Appl. Instrum. B. 1991; 18:883-90.

Chen Z-F., Behringer RR.: Twist is required in head mesenchyme for cranial neural tube morphogenesis. Genes & Dev. 1995; 9:686-699.

Colas JF., Schoenwolf GC.: Localization of carilage linking protein 1 during primary neurulation in the chick embryo. Dev. Brain Res. 2003; 141:141-148.

Condic ML., Snow DM., Letourneau PC: Embryonic neurons adapt to the inhibitory proteoglycan aggrecan by increasing integrin expression. J. Neurosci. 1999; 19:10036-43.

Cs-Szabó G., Melching, LI., Roughley PJ., Glant TT.: Changes in messenger RNA and protein levels of proteoglycans and link protein in human osteoarthritic cartilage samples. Arthritis Rheum. 1997; 40: 1037-1045.

Czipri M., Otto JM., Cs-Szabo G., Kamath RV., Vermes C., Firneisz G., Kolman KJ., Waanabe H., Li Y., Roughley PJ., Yamada Y., Olsen BR., Glant TT.: Genetic rescue of chondrodysplasia and the perinatal lethal effect of cartilage link protein deficiency. J. Biol. Chem. 2003; 278:39214-39233.

Day, AJ.: The structure and regulation of hyaluronan-binding proteins. Biochem. Soc.Trans. 1999; 27: 115-121.

Deák F., Kiss I., Sparks KJ., Argraves WS., Hampikian G., Goetinck PF.: Complete amino acid sequence of chicken cartilage link protein deducted from cDNA clones. Proc.Natl.Acad.Sci.USA 1986; 83: 3766-3770.

Deák F., Barta E., Mestric S., Biesold M., Kiss I.: Complex pattern of alternative splicing generates unusual diversity in the leader sequence of the chicken link protein mRNA. Nucleic Acids Res. 1991; 19: 4983-4990.

DeákF., Mates L., Krysan K., Liu Z., Szabo PE., Mann JR., Beier DR., Kiss I.: Characterization and chromosome location of the mouse link protein gene (Crtl1). Cytogenet. Cell. Genet. 1999; 87: 75-79.

Dean MF., Lee YW., Dastjerdi, AM., Lees P.: The effect of link peptide on proteoglycan synthesis in equine articular cartilage. Biochim. Biophys. Acta. 2003, 1622:161-8.

Diefenderfer DL., Brighton CT.: Microvascular pericytes express aggrecan message which is regulated by BMP-2. Biochem. Biophys. Res. Comm. 2000; 269:172-178.

Doege K., Hassell JR., Caterson B., Yamada Y.: Link protein cDNA sequence reveals a tandemly repeated protein structure. Proc. Natl. Acad. Sci. USA 1986; 83: 3761-3765.

Dudhia J., Hardingham TE.: Isolation and sequence of cDNA clones for pig and human cartilage link protein. J. Mol. Biol. 1989; 206:749-753.
Dudhia J., Bayliss MT., Hardingham TE.: Human link protein gene: structure and transcription pattern in chondrocytes. Biochem. J. 1994; 303:329-333.

Dudhia J.: Aggrecan, aging and assembly in articular cartilage. Cell. Mol. Life Sci. 2005; 62:2241-2256

Engelman DM., Steitz TA., Goldman A.: Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. Annu. Rev. Biophys. Biophys. Chem. 1986; 15:321-353.

Eyre D.: The collagens of articular cartilage. Semin. Arthritis Rheum. 1991; 21:2-11.

Fassler R., Schnegelsberg PNJ., Dausman J., Shinya T., Muragaki Y., McCarthy MT., Olsen BR., Jaenisch R.: Mice lacking alpha 1 (IX) develop noninflammatory degeneative joint disease. Proc. Natl. Acad. Sci. USA. 1994; 91:5070-4.

Fife RS., Caterson B., Myers SL.: Identification of link proteins in canine synovial cell cultures and canine articular cartilage. J. Cell Biol. 1985; 100:1050-1055.

Fülöp C., Kamath RV., Li Y., Otto JM., Salustri A., Olsen BR., Glant TT., Hascall VC.: Coding sequence, exon-intron structure and chromosomal localization of murine TNF-stimulated gene 6 that is specifically expressed by expanding cumulus cell-oocyte complexes. Gene 1997; 202:95-102.

Galtrey CM., Kwok JC., Carulli D., Rhodes KE., Fawcett JW.: Distribution and synthesis of extracellular matrix proteoglycans, hyaluronan, link proteins and tenascin-R in the rat spinal cord. Eur. J. Neurosci. 2008; 27:1373-90.

Gannon J., Walker G., Fischer M., Carpenter R., Thompson Jr. RC., Oegema Jr. TR.: Localization of type X collagen in canine growth plate and adult canine articular cartilage. J. Orthop. Res. 1991; 1:485-494.

Gardell S., Baker J., Caterson B., Heinegård D., Rodén L.: Link protein and a hyaluronic acid-binding region as components of aorta proteoglycan. Biochem. Biophys. Res. Commun. 1980; 95:1823-1831.

Glant TT., Mikecz K., Roughley PJ., Buzás E., Poole AR.: Age-related changes in protein-related epitopes of human articular-cartilage proteoglycans. Biochem. J. 1986; 236:71-75.

Glant TT., Kamath RV., Bárdos T., Gál I., Szántó S., Murad Y. M., Sandy JD., Mort, JS., Roughley PJ., Mikecz K.: Cartilage-specific constitutive expression of TSG-6 protein (product of tumor necrosis factor α-stimulated gene 6) provides a chondroprotective, but not anti-inflammatory, effect in antigen-induced arthritis. Arthritis Rheum. 2002; 46:2207-2218.

Hardingham TE.: The role of link-protein in the structure of cartilage proteoglycan aggregates. Biochem. J. 1979; 177:237-247.

Hardingham, TE, Fosang AJ.: Proteoglycans: many forms and many functions. FASEB J. 1992; 6:861-870.

Hascall VC., Heinegård D.: Aggregation of cartilage proteoglycans. I. The role of hyaluronic acid. J. Biol. Chem. 1974; 249: 4232-4241.

Hascall VC.: Proteoglycans: the chondroitin sulfate/keratan sulfate proteoglycan of cartilage. ISI Atlas Sci. Biochem. 1988; 1:189-198.

Hecht JT., Blanton SH., Wang Y., Daiger SP., Horton WA., Rhodes C., Yamada Y., Francomano CA.: Exclusion of human proteoglycan link protein (CRTL1) and type II collagen (COL2A1) genes in pseudoachondroplasia. Am. J. Med. Genet. 1992; 44:420-424.

Hering TM., Kollar J., Huynh TD., Sandell LJ.: Bovine chondrocyte link protein cDNA sequence: interspecies conservation of primary structure and mRNA untranslated regions. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 1995; 112:197-203.

Hopp TP., Woods KR.: Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci. USA. 1981; 78:3824-3828.

Horton W., Miyashita T., Kohno K., Hassel JR., Yamada Y.: Identification of a phenotype-specific enhancer in the first intron of the rat collagen II gene. Proc. Natl. Acad. Sci. USA. 1987; 84:8864-8868.

Horton WA.: In vitro chondrogenesis in human chondrodysplasias. Am. J. Med. Genet. 1993; 45:179-182.

Horton WA.: Extending the nosology of the chondrodysplasias to the cellular and molecular levels. Pediatr. Radiol. 1994; 24:410-412.

Horton WA.: Evolution of the bone dysplasia family. Am. J. Med. Genet. 1996; 63: 4-6.

Horton WA.: Progress in human chondrodysplasias: molecular genetics. Ann. N.Y. Acad. Sci. 1996; 785:150-159.

Jacenko O., and Olsen BR.: Transgenic mouse models in studies of skeletal disorders. J.Rheumatol. 1995; 22:39-41.

Jager C., Lendvai D., Seeger G., Bruckner G., Matthews RT., Arendt T., Alpar A., Morawski M.: Perineuronal and perisynaptic extracellular matrix in the human spinal cord. Neuroscience. 2013; 238:168-84.

Kerlavage A., Bonazzi V., di Tommaso,M., Lawrence C., Li P., Mayberry F., Mural R., Nodell M., Yandell M., Zhang J., Thomas P.: The Celera Discovery System. Nucleic Acids Res. 2002; 30:129-136.

Kimmel CA., Trammell C.: A rapid procedure for routine double staining of cartilage and bone in fetal and adult animals. Stain Technol. 1981; 56:271-273.
Kohda D., Morton CJ., Parkar AA., Hatanaka H., Inagaka FM., Campbell ID., Day AJ.: Solution structure of the link module: a hyaluronan-binding domain involved in extracellular matrix stability and cell migration. Cell. 1996; 86:767-775.

Kohno K., Sullivan M., Yamada Y.: Structure of the promoter of the rat type II procollage gene. J. Biol. Chem. 1985; 260:4441-4447.

Krakow D., Rimoin DL.: The skeletal dysplasias. Genet. Med. 2010; 12:327-341.

Kuettner KE.: Osteoarthritis: cartilage integrity and homeostasis. Rheumatology. Edited by Klippel JH and Diepe PA. St.Louis, MO, Mosby Year Book, 1994; pp. 1-16.

Lee JY, Spicer AP.:Hyaluronan: a multifunctional, megaDalton, stealth molecule. Current Opinion in Cell Biology. 2000; 12:581-586.

Li H., Schwartz NB., Vertel BM.: cDNA cloning of chick cartilage chondroitin sulfate (aggrecan) core protein and identification of a stop codon in the aggrecan gene associated with the chondrodystrophy, nanomelia. J. Biol. Chem. 1993; 268:23504-23511.

Li SW., Prockop DJ., Helminen H., Fassler R., Lapvetelainen T., Kiraly K., Peltarri A., Arokoski J., Liu H., Arita M.: Transgenic mice with targeted inactivation of the Col2a1gene for collagen II develop a skeleton with membranous and periosteal bone but no endochondral bone. Genes Dev. 1995; 9:2821-2830.

Li Y., Lacerda DA., Warman ML., Beier DR., Yoshioka H., Ninomiya Y., Oxford JT, Morris NT., Andrikopoluos K, Ramirez F.: A fibrillar collegene gene, Col11a1, is essential for skeletal morphogenesis. Cell. 1995; 80:423-430.

Liu H., McKenna LA., Dean MF.: The macromolecular characteristics of cartilage proteoglycans do not change when synthesis is up-regulated by link protein peptide. Biochem. Biophys. Aspects 1999; 1428:191-200.

Liu H., McKenna LA., Dean, MF.: An N-terminal peptide from link protein can stimulate biosynthesis of collagen by human articular cartilage. Arch. Biochem. Biophys. 2000; 378: 116-122.

Lockhart MM., Wirring EE., Phelps AL., Ghattnekar AV., Barth JL., Norris RA., Wessels A.: Mef2c regulates transcription of the extracellular matrix protein cartilage link protein 1 in the developing murine heart. PLOS One. 2013; 8:e57073.

Loughlin J., Irven C., Sykes, B.: Exclusion of the cartilage link protein and the cartilage matrix protein genes as the mutant loci in several heritable chondrodysplasias. Hum. Genet. 1994; 94:698-700.

Martin H., Dean M.: An N-terminal peptide from link protein is rapidly degraded by chondrocytes, monocytes and B cells. Eur. J. Biochem. 1993; 212:87-94.

Matsumoto K., Shionyu M., Go M., Shimizu K., Shinomura T., Kimata K., Watanabe H.: Distinct interaction of versican/PG-M with hyaluronan and link protein. J. Biol. Chem. 2003; 278(42):41205-41212.

Matthews RT., Kelly GM., Zerillo CA., Gay G., Tiemeyer M., Hockfield S.: Aggrecan glycoforms contribute to the molecular heterogenity of perineuronal nets. J. Neurosci. 2002; 22:7536-47.

McKenna LA., Liu HX., Sansom PA., Dean MF.: An N-terminal peptide from link protein stimulates proteoglycan biosynthesis in human articular cartilage in vitro. Arthritis Rheum. 1998; 41:157-162.

Milev P., Maurel P., Chiba A., Mevissen M., Popp S., Yamaguchi Y., Margolis RK., Margolis RU.: Differential regulation of expression of hyaluronan-binding proteoglycans in developing brain: aggrecan, versican, neurocan, brevican. Biochem. Biophys. Res. Comm. 1998; 247:207-212.

Morawski M., Bruckner G., Arendt T., Matthews RT.: Aggrecan: beyond cartilage and into the brain. Int. J. Biochem. Cell. Biol. 2012; 44:690-3.

Morgelin M., Paulsson M., Hardingham TE., Heinegård D, Engel J.: Cartilage proteoglycans. Assembly with hyaluronate and link protein as studied by electron microscopy. Biochem. J. 1988; 253:175-185.

Muller G., Hanschke M.: Quantitative and qualitative analyses of proteoglycans in cartilage extracts by precipitation with 1,9-dimethylmethylene blue. Connect.Tissue Res. 1996; 33:243-248.

Mwale F., Demers CN., Petit A., Roughley P., Poole AR., Steffen T., Aebi M., Antoniou J.: A synthetic peptide of link protein stimulates the biosynthesis of collagens II, IX and proteoglycan by cells of the intervertebral disc. J. Cell Biochem. 2003; 88:1202-13.

Naso MF, Morgan JL., Buchberg AM., Siracusa LD., Iozzo RV.: Expression pattern and mapping of the murine versican gene (Cspg2) to chromosome 13. Genomics. 1995; 29:297-300.

Neame PJ., Christner JE., and Baker JR.: The primary structure of link protein from rat chondrosarcoma proteoglycan aggregate. J. Biol. Chem. 1986; 261: 3519-3535.

Neame PJ., Barry FP.: The link proteins. J.Cell Biol. 1993; 49:393-402.

Oohira A., Matsui F., Tokita Y., Yamauchi S., Aono S.: Molecular interactions of neural chondroitin sulfate proteoglycans in the brain development. Arch. Biochem. Biophys. 2000; 374:24-34.

Osborne-Lawrence SL., Sinclair AK., Hicks RC., Lacey SW., Eddy Jr. RL., Byers MG., Shows TB., Duby AD.: Complete amino acid sequence of human cartilage link protein (CRTL1) deduced from cDNA clones and chromosomlal assignment of the gene. Genomics. 1990; 8:562-567.

Petit A., Yao G., Rowas SA., Gawri R., Epure L., Antoniou J., Mwale F.: Effect of synthetic link N peptide on the expression of type I and type II collagens in human intervertebral disc cells. Tissue Eng. Part. A. 2011; 17:899-904.
Perissinotto D., Iacopetti P., Bellina I., Doliana R., Colombatti A., Pettway Z., Bronner-Fraser M., Shinomura T., Kimata K., Morgelin M., Lofbeg J., Perris R.: Avian neural crest cell migration is diversely regulated by the two major hyaluronan-binding proteoglycans PG-M/versican and aggrecan. Development . 2000; 127:2823-42.

Pierschbacher M., Hayman EG., Ruoshlati E.: Synthetic peptide with cell attachment activity of fibronectin. Proc. Natl. Acad. Sci. USA. 1983; 80:1224-1227.

Poole AR., Pidoux I., Reiner A., Coster L., Hassell JR:. Mammalian eyes and associated tissue contain molecules that are immunologically related to cartilage proteoglycans and link proteins. J. Cell Biol. 1982; 93:910-920.

Pyke C., Kristensen P., Ostergaard PB., Oturai PS., Romer J.: Proteoglycan expression in the normal rat kidney. Nephron. 1997; 77:461-70.

Rada JA., Achen VR., Perry CA., Fox PW.: Proteoglycans in the human sclera. Evidence for the presence of aggrecan. Invest. Ophthalmol. Vis. Sci. 1997; 38:1740-51.

Rauch U., Grimpe B., Kulbe G., Arnold-Ammer I., Beier DR., Fässler R.: Structure and chromosomal localization of the mouse neurocan gene. Genomics 1995; 28:405-410.

Rauch U., Hirakawa S., Oohashi T., Kappler J., Roos G.: Cartilage link protein interacts with neurocan, which shows hyaluronan binding characeristics different from CD44 and TSG-6. Marix Biol. 2004; 22:629-639.

Rees SG., Lannery CR.: Little CB., Hughes CE., Caterson B., Dent CM.: Catabolism of aggrecan, decorin and biglycan in tendon. Biochem. J. 2000; 350:181-8.

Rhodes C., Savagner P., Line S., Sasaki M., Chirigos M., Doege K., Yamada Y.: Characterization of the promoter for the rat and human link protein gene. Nucleic Acids Res. 1991; 19:1933-1939.

Ripellino JA., Margolis RU., Margolis RK.: Immunoelectron microscopic localization of hyauronic acid- binding region and link protein epitopes in brain. J. Cell Biol. 1989; 108:1899-1907.

Roebuck KA., Vermes C., Carpenter LR., Fritz EA., Ramesh N., Glant TT.: Downregulation of procollagen a1[I] mRNA expression by titanium particles correlates with NF-κB activation and increased Rel A and NF-κB1 binding to the collagen promoter. J.Bone Miner.Res. 2001; 16:501-510.

Roughley PJ., White RJ., Mort JS.: Presence of pro-forms of decorin and biglycan in human articular cartilage. Biochem. J. 1996; 318:779-784.

Sambrook J., Fritsch EF., Maniatis T.: Molecular cloning: a laboratory manual. 1989; Cold Spring Harbor Laboratory Press, New York, NY.

Seyfried NT., McVey GF., Almond M., Mahoney DJ., Dudhia J., Day J.: Expression and purification of functionally active hyaluronan-binding domains from human cartilage link protein, aggrecan and versican. J. Biol. Chem. 2005; 280: 5435-5448.
Shi S., Grothe S., Zhang Y., O`Connor MD., Poole AR., Roughley PJ., Mort JS.: Link protein has greater affinity for versican than aggrecan. J. Biol. Chem. 2004; 279:12060-12066.

Spicer AP., Joo A., Bowling Jr. RA.: A hyaluronan binding link protein gene family whose members are physically linked adjacent to chondroitin sulfate proteoglycan core protein genes. J. Biol. Chem. 2003; 278:21083-91.

Stripe NS., Goetinck PF.: Gene regulation during cartilage differentiation: temporal and spatial expression of link protein and cartilage matrix protein in the developing limb. Development 1989; 107:22-23.

Stirpe NS., Dickerson KT., Goetinck PF.: The chicken embryonic mesonephros synthesizes link protein, an extracellular matrix molecule usually found in cartilage. Dev. Biol. 1990; 137:419-424.

Shinomura T., Zako M., Ito K., Ujita M., Kimata K.: The gene structure and organization of mouse PG-M, a large chondroitin sulfate proteoglycan. Genomic background for the generation of multiple PG-M transcripts. J. Biol. Chem. 1995; 270:10328-10333.

Soo K., O`Rourke MP., Khoo PL., Steiner KA., Wong N., Behringer RB., Tam PPL.: Twist function is required for the morphogenesis of the cephalic neural tube and the differentiation of the cranial neural crest cells in the mouse embryo. Dev. Biol. 2002; 247:251-270.

Sun GW., Kobayashi H., Terao T.: Expression of link protein during mouse follicular development. J. Hystochem. Biochem. 1999; 47:1433-1442.

Sun GW., Kobayashi H., Suzuki M., Kanayama N., Terao T.: Link protein as an enhancer of cumulus cell-oocyte complex expansion. Mol. Reprod. Dev. 2002; 63:223-231.

Superti-Furga A., Bonafe L., Rimoin DL.: Molecular genetic classification of genetic disorders of the skeleton. Am. J. Med. Genet. 2001; 106:282-293.

van der Rest M., Garrone R.: Collagen family of proteins. FASEB J 1991, 5:2814-2823.

Walcz E., Deák F., Erhardt P., Coulter SN., Fülöp C., Horváth P., Doege KJ., Glant TT.: Complete coding sequence, deduced primary structure, chromosomal localization and structural analysis of murine aggrecan. Genomics 1994; 22:364-371.

Wang Y., Middleton F., Horton JA., Reichel L., Farnum CE., Damron TA.: Bone, 2004; 35(6):1273-93.

Wang Z., Weitzmann MN., Sangadala S., Hutton WC., Yoon ST.: Link protein N-terminal peptide binds to bone morphogenic protein (BMP) type II receptor and drives matrix protein expression in rabbit intervertebral disc cells. J. Biol.Chem. 2013; 288:28243-53.

Watanabe H., Kimata K., Line S., Strong D., Gao L.-Y., Kozak CA., and Yamada Y.: Mouse cartilage matrix deficiency (cmd) caused by a 7 bp deletion in the aggrecan gene. Nature Genet. 1994; 7:154-157.

Watanabe H., Nakata K., Kimata K., Nakanishi I., Yamada Y.: Dwarfism and age-associated spinal degeneration of heterozygote cmd mice defective in aggrecan. Proc. Natl. Acad. Sci. 1997; 94:6943-6947.

Watanabe H., Yamada Y.: Mice lacking link protein develop dwarfism and craniofacial abnormalities. Nature Genet. 1999; 21:225-229.

Watanabe H., Yamada Y.: Chondrodysplasia of gene knockout mice for aggrecan and link protein. Glycoconjugate Journal. 2003; 19:269-273.

Waterston R H., Lindblad-Toh K., Birney E., Rogers J., Abril JF., Agarwal P., Agarwala R., Ainscough R.: Initial sequencing and comparative analysis of the mouse genome. Nature 2002; 420:520-562.

Wight TN., Heinegård DK., Hascall VC.: Proteoglycans: structure and function. in Cell Biology of Extracellular Matrix 1991; (ed. E.D.Hay), pp. 45-78. Plenum Press, New York, London.

Yamada Y., Miyashita T., Savagner P., Horton W., Brown KS., Abramczuk J., Hou-Xiang X., Kohno K., Bolander M., and Bruggeman L.: Regulation of the collagen II gene in vitro and in transgenic mice. Ann. N.Y. Acad. Sci. 1990; 580:81-87.

Yamada H., Watanabe K., Shimonaka M., Yamaguchi Y.: Molecular cloning of brevican, a novel brain proteoglycan of the aggrecan/versican family. J. Biol. Chem. 1994; 269:10119-10126.

Yamaguchi Y.: Lecticans: organizers of the brain exptracellular matrix. Cell Mol. Life Sci. 2000; 57:276-289.

Yao LY., Moody C., Schoenherr E., Wight N., Sandell LJ.: Identfication of the proteoglycan versican in aorta and smooth muscle cells by DNA sequence analysis, in situ hybridisation and immunohistochemistry. Marix Biol. 1994; 14:213-225.

Zanin MKB., Bundy J., Ernst H., Wessels A., Conway SJ., Hoffman S.: Distinct spatial and temporal distributions of aggrecan and versican in the embryonic chick heart. Anat. Rec. 1999; 256:366-80.

Zimmermann DR., Rouslahti E.: Multiple domains of the large fibroblast proeoglycan, versican. EMBO J. 1989; 8:2975-2981.

Zimmermann DR., Dours-Zimmermann MT., Schubert M., Bruckner-Tuderman L.: Versican is expressed in the proliferative zone in the epidermis and in association with the elastic network of the dermis. J. Cell Biol. 1994; 124:817-25.

8. BIBLIOGRAPHY

Cumulative impact factor: 34.807

Papers related to this thesis (Impact factor: 15.743)

1. **Czipri M.,** Than P., Vermes C.:
The role of link protein in osteochondrogenesis: an experimental animal model of osteochondrodysplasia with genetic rescue.
Hungarian Journal of Orthopedic and Trauma Surgery.
Accepted for publication.

2. Bárdos T., Szabó Z., **Czipri M.,** Vermes C., Tunyogi-Csapó M., Urban RM., Mikecz K., Glant TT.:
A longitudinal study on an autoimmune murine model of ankylosing spondylitis.
Ann Rheum Dis. 2005; 64:981-7.
IF: 6.956

3. **Czipri M.,** Otto JM., Cs-Szabo G., Kamath RV., Vermes C., Firneisz G., Kolman KJ., Watanabe H., Li FY., Rouhghley PJ., Yamada Y., Olsen BR., Glant TT.:
Genetic rescue of chondrodysplasia and the perinatal lethal effect of cartilage link protein deficiency.
J. Biol. Chem. 2003; 278: 39214-39223.
IF: 6.482

4. Bárdos T., **Czipri M**., Vermes C., Zhang J., Mikecz K., Glant TT.:
Continuous nasal administration of antigen is critical to maintain tolerance in adoptively transferred autoimmune arthritis in SCID mice.
Clin. Exp. Immunol. 2002; 129:224-31.
IF: 2.305

Peer reviewed and published abstracts related to this thesis

1. Murad Y, **Czipri M**., Glant TT.:
Accumulation of link protein and G1 domain of aggrecan in transgenic mice leads to destruction of articular cartilage.
Arthritis Rheum. 2005; 52:S54-S55.

2. **Czipri M.,** Vermes C., Bárdos T., Lovász G., Bellyei Á., Glant TT.:
Genetic repair of a lethal osteochondrodystrophy.
Hungarian Journal of Orthopedic and Trauma Surgery. 2002; 45:S12.

3. **Czipri M.**, Bárdos T., Vermes C., Gál I., Mikecz K., Watanabe H., Yamada Y., Glant TT.:
Genetic rescue of an otherwise perinatal lethal defect in skeletal development.
Orthop. Trans. 2002; 27:0311.

4. Bárdos T., **Czipri M.**, Vermes C., Lovász G., Finnegan A., Mikecz K., Glant TT.:
Progressive spondylarthropathy with ankylosis in murine models of arthritis.
Arthritis Rheum. 2001; 44:S240.

5. **Czipri M.**, Bárdos T., Stoop R., Vermes C., Gál I., Hanyecz A., Mikecz K., Watanabe H., Yamada Y., Glant TT.:
A Novel Approach to gene therapy: Genetic rescue of an otherwise perinatal lethal defect in skeletal development.
Arthritis Rheum. 2001; 44:S149.

Podium and poster presentations related to this thesis

1. Szász K., Morava É., Halmai V., **Czipri M**., Illés T.
Genetic background and clinical aspects of frontometaphyseal dysplasia in spine surgery
49th Congress of Hungarian Orthopaedic Association, 2006, Budapest, Hungary

2. Szász K. Czibula A, Raskó J., Halmai V., **Czipri M**., Illés T.
Investigation of genetic factors in multiple developmental disorder-releted scoliosis and in adolescent idiopahtic scoliosis
49th Congress of Hungarian Orthopaedic Association, 2006, Budapest, Hungary

3. **Czipri M,** Lovász Gy, Bellyei Á, Glant TT.
The roles of link protein in the cartilage extracellular matrix and in osteogenesis
Forum of Young Hungarian Orthopaedic Surgeons, 2004, Kaposvar, Hungary
(Best Paper Award winner)

4. **Czipri M.**
Link proteins: In and out of cartilage
Invited presentation at the Dept. of Biochemistry at Rush Presbyterian St`Lukes Medical Centre 2003, Chicago, USA

5. **Czipri M,** Vermes C, Bárdos T, Lovász G, Bellyei Á, Glant T.
Genetic repair of a lethal osteochondrodystrophy.
45th Congress of Hungarian Orthopaedic Association, 2002, Pecs, Hungary

6. **Czipri M**, Bárdos T, Vermes C, Gál I, Mikecz K, Watanabe H, Yamada Y, Glant TT.
Genetic rescue of an otherwise perinatal lethal defect in skeletal development. (poster)
Midwest Connective Tissue Workshop 2002, Chicago, USA

7. **Czipri M**, Bárdos T, Vermes C, Gál I, Mikecz K, Watanabe H, Yamada Y, Glant TT.
Genetic rescue of an otherwise perinatal lethal defect in skeletal development. (poster)
Rush University Forum for Research and Clinical Investigation 2002, Chicago, USA

8. **Czipri M**, Bárdos T, Vermes C, Gál I, Mikecz K, Watanabe H, Yamada Y, Glant TT.
Genetic rescue of an otherwise perinatal lethal defect in skeletal development. (poster)
69th Annual Meeting of the American Academy of Orthopaedic Surgeons 2002, Dallas, USA

9. **Czipri M**, Bárdos T, Vermes C, Gál I, Mikecz K, Watanabe H, Yamada Y, Glant TT.
Genetic rescue of an otherwise perinatal lethal defect in skeletal development. (poster)
48th Annual Meeting of the Orthopaedic Research Society 2002, Dallas, USA
(Awarded with New Investigator Recognition Award)

10. **Czipri M**, Glant TT.
The "missing link" protein
Midwest Connective Tissue Workshop 2001, Chicago, USA

11. Bárdos T, **Czipri M**, Zhang J, Gál I, Finnegan A, Mikecz K, Glant TT.
Nasal tolerance to proteoglycan (aggrecan)-induced arthritis in BALB/c mice can adoptively transferred and the tolerance reconstituted in SCID mice. (poster)
American College of Rheumatology 65th Scientific Meeting, 2001, San Francisco, USA

12. Bárdos T, **Czipri M**, Vermes C, Lovász G, Finnegan A, Mikecz K, Glant TT.
Progressive spondylarthropathy with ankylosis in murine models of arthritis.
American College of Rheumatology 65th Scientific Meeting, 2001, San Francisco, USA

13. **Czipri M**, Bárdos T, Stoop R, Vermes C, Gál I, Hanyecz A, Mikecz K, Watanabe H, Yamada Y, Glant TT.
A Novel Approach to gene therapy: Genetic rescue of an otherwise perinatal lethal defect in skeletal development.
American College of Rheumatology 65th Scientific Meeting, 2001, San Francisco, USA

14. **Czipri M**, Halmai V, Kránicz J.
Differential diagnostic aspects of the bilateral Perthes disease
Forum of Young Hungarian Orthopaedic Surgeons, 1999, Bekescsaba, Hungary

Other papers published in periodicals (Impact factor: 19.064)

1. Kuzsner J, Tunyogi Csapo M, **Czipri M**, Than P, Vermes C.:
Early diagnostics of aseptic loosening of hip implants with the help of biomarkers.
Hungarian Journal of Orthopedic and Trauma Surgery. 2012; 55:197-202.

2. Vermes Cs., **Czipri M**., Horváth G., Dömös P., Gazsó I.:
Invetsigation of human intervertebral discs with calorimetry.
Hungarian Journal of Orthopedic and Trauma Surgery. 2005; 48:330-339.

3. Szabó I., **Czipri M**., Halmai V., Potó L., Costenoble V., Docquier J.:
Middle term outcomes of the Scarf osteotomy in the treatment of hallux valgus deformity.
Hungarian Journal of Orthopedic and Trauma Surgery. 2005; 48:33-42.

4. Kontrohr T., **Czipri M.**, Than P., Kránicz J.:
Results of operative treatment of the condition after Schlatter-Osgood disease.
Hungarian Journal of Orthopedic and Trauma Surgery. 2004; 47:13-17.

5. Bárdos T., **Czipri M.**, Vermes C., Finnegan A., Mikecz K., Zhang J.:
CD4+CD25+ immunoregultaroy T cells may not be involved in controlling autoimmune arthritis.
Arthritis Research and Therapy. 2003; 5:106-13.
IF: 5.036

6. Kaplan CD., O'Neill SK., Koreny T., **Czipri M.**, Finnegan A.:
Development of inflammation in proteoglycan-induced arthritis is dependent on Fc gamma R regulation of the cytokine/chemokine environment.
J. Immunol. 2002; 169:5851-9.
IF: 7.014

7. Finnegan A., Grusby MJ., Kaplan CD., O'Neill SK., Eibel H., Koreny T., **Czipri M.**, Mikecz K., Zhang J.:
IL-4 and IL-12 regulate proteoglycan-induced arthritis through Stat-dependent mechanisms.
J. Immunol. 2002; 169:3345-52.
IF: 7.014

8. Hübler J., Sükösd F., **Czipri M.**:
Tibia metastasis without prostate specific antigen (PSA) increase following radical vesiculo-prostatectomy.
Int. Urol. Nephrol. 2000; 32:281-4.

9. Kránicz J., **Czipri M.**:
Early experiences with Calcaneo-stop method in the operative treatment of the flatfoot in children.
Hungarian Journal of Orthopedic and Trauma Surgery. 2000; 43:177-182.

10. Domán I., **Czipri M.:**
Report on the First Instructional Course of the European Foot and Ankle Society
Hungarian Journal of Orthopedic and Trauma Surgery. 1999; 42:362-3.

Other peer reviewed and published abstracts

1. Halmai V., Schmidt E., Domán I., **Czipri M.**, Illés T.:
Bone mineral density measurement in idiopathic scoliosis. Report of 120 cases.
Acta Chirurgiae Orthopaedicae et Traumatologiae Cechoslovaca. 2004; Supplement 1, 27.

2. **Czipri M.**, Szabó I., Halmai V., Costenoble V., Docquier J.:
The scarf osteotomy for the correction of hallux valgus deformity: a review of 62 cases.
Acta Chirurgiae Orthopaedicae et Traumatologiae Cechoslovaca. 2004; Supplement 1, 32.

3. Vermes C., Bárdos T., **Czipri M.**, Koreny T., Lovász G., Bellyei Á., Glant TT.:
Periprosthetic osteolysis: multiple cell response for wear debris particles.
Hungarian Journal of Orthopedic and Trauma Surgery 2002, 45:S73.

4. Bárdos T., **Czipri M.**, Vermes C., Lovász G., Bellyei Á., Glant T.:
Arthritis psoriatica in humanised mice offers a new insight to the immunological background of the disease.
Hungarian Journal of Orthopedic and Trauma Surgery. 2002; 45:S6.

5. Vermes C., Bárdos T., **Czipri M.**, Hanyecz A., Lovász G., Bellyei Á., Fritz E., Roebuck K., Jacobs J., Galante J., Andersson GBJ., Glant TT.:
Differential effects of bacterial lipopolysaccharide (LPS) and tumor necrosis factor-apha on the functions of human osteoblast cells.
Orthop. Trans. 2002; 27:0537.

6. Bárdos T., **Czipri M.**, Vermes C., Mikecz K., Glant TT.:
Suppression of autoimmunity in experimental arthritis by nasal tolerance.
Orthop. Trans. 2002; 27:0692.

7. Bálint L., Kránicz J., **Czipri M**.:
Long-term results of more than 700 operatively treated clubfeet.
J. Bone Joint Surg [Br] 2002; 84-B:Supplement 2:117.

8. Bárdos T., **Czipri M.**, Zhang J., Gál I., Finnegan A., Mikecz K., Glant TT.:
Nasal tolerance to proteoglycan (aggrecan)-induced arthritis in BALB/c mice can adoptively transferred and the tolerance reconstituted in SCID mice.
Arthritis Rheum. 2001; 44:S87.

9. de Jonge T., **Czipri M.**, Lovász G.:
Unicompartmental knee arthroplasty: 5-11 years follow-up.
J. Bone Joint Surg [Br] 1999; 81-B:Supplement 2:O431.

10. **Czipri M.**, Bellyei Á.:
One-stage operation of CDH in children aged between 1-5 years.
J Bone Joint Surg [Br] 1999; 81-B:Supplement 2:O461.

Other podium and poster presentations

1. **Czipri M.**
Complex treatment of the diabetic foot.
Annual Meeting of the Hungarian Society for Podiatry and Foot Surgery
2014, Kecskemet, Hungary

2. **Czipri M.**
Orthopaedic problems in diabetic foot
Integrated Diabetic Foot Care Seminar
2013, Exeter, UK

3. **Czipri M.**
Surgical treatment for 2nd metatarsophalangeal joint instability with plantar plate reconstruction: early results
Annual Meeting of the Hungarian Society for Podiatry and Foot Surgery
2013, Siofok, Hungary

4. **Czipri M.,** Sharpe IT.
Reconstructive surgery on neuropathic feet
Annual Meeting of the Hungarian Society for Podiatry and Foot Surgery
2013, Siofok, Hungary

5. **Czipri M.,** Guyver P., Taylor J., Knox R., Talbot NJ., Sharpe IT.
Tibiotalocalcaneal arthrodesis with a retrograde nail: results of 55 cases
Annual Meeting of the Hungarian Society for Podiatry and Foot Surgery
2013, Siofok, Hungary

6. **Czipri M.**
Diabetic foot disorders
3M National Symposium
2013, Staverton Park, Daventry, UK

7. **Czipri M.,** Sharpe IT., Talbot NJ.
Hallux metatarsophalangeal joint hemiprosthesis: short term results
Annual Meeting of the Hungarian Society for Podiatry and Foot Surgery
2012, Cegled, Hungary

8. **Czipri M.,** Sharpe IT., Talbot NJ.
Experiences of surgical treatment of the rheumatoid forefoot deformities
Annual Meeting of the Hungarian Society for Podiatry and Foot Surgery
2012, Cegled, Hungary

9. **Czipri M**., Talbot NJ., Sharpe IT.
Experiences of arthroscopic ankle arthrodesis
Annual Meeting of the Hungarian Society for Podiatry and Foot Surgery
2010, Budapest, Hungary

10**. Czipri M**., Sharpe IT., Talbot NJ.
Surgical treatment of rheumatoid forefoot deformities
52nd Congress of Hungarian Orthopaedic Association, 2009, Szolnok, Hungary

11**. Czipri M**., Sharpe IT., Talbot NJ.
Radiological analysis of the bony healing in 1st metatarsophalangeal joint arthrodesis
52nd Congress of Hungarian Orthopaedic Association, 2009, Szolnok, Hungary

12**. Czipri M.**, Herron M.
Experiences of arthroscopic ankle arthrodesis
51st Congress of Hungarian Orthopaedic Association, 2008, Szekesfehervar, Hungary

13. **Czipri M**.
Hemophilic arthropathy: staging and treatment options.
Hemophilia Symposium, 2006, Pécs, Hungary

14. Halmai V., **Czipri M**., Szász K., Illés T.
The role of the EDF plaster corsette in the conservative treatment of spinal deformities
49th Congress of Hungarian Orthopaedic Association, 2006, Budapest, Hungary

15. Halmai V., **Czipri M**., Szász K., Illés T.
Comparative analysis of classification systems for scoliosis
49th Congress of Hungarian Orthopaedic Association, 2006, Budapest, Hungary

16. **Czipri M**., Halmai V., Szász K., Illés T.
Results of spinal instrumentation of idiopathic scoliosis
4th Congress of the Hungarian Society of Pediatric Spinal Disorders, 2006, Budapest, Hungary

17. **Czipri M**., Halmai V., Szász K., Illés T.
Results of surgical treatment of idiopathic scoliosis
49th Congress of Hungarian Orthopaedic Association, 2006, Budapest, Hungary
18. **Czipri M**., Dömös P., Kiss I.P., Than P., Kránicz J.
Long term results of high tibial osteotomy in varus gonarthrosis
48th Congress of Hungarian Orthopaedic Association, 2005, Galyateto, Hungary

19. **Czipri M.,** Bárdos T., Bálint L., Than P., Kustos T.
Primary Anterior Cruciate Ligament Reconstruction Using Patellar Bone Tendon Bone Autograft or Allograft: a Comparative Study
International Knee Surgery Symposium, 2004, Pécs, Hungary

20. **Czipri M.**, Szabó I., Halmai V., Costenoble V., Docquier J.
Middle Term Outcomes of the Scarf Osteotomy in the Correction of Hallux Valgus Deformity
Forum of Young Hungarian Orthopaedic Surgeons, 2004, Kaposvar, Hungary

21. Halmai V., Schmidt E., Domán I., **Czipri M.**, Illés T.
Bone mineral density measurement in idiopathic scoliosis. Report of 120 cases.
5th Central European Orthopaedic Congress, 2004, Prague, Czech Republic

22. **Czipri M.**, Szabó I., Halmai V., Costenoble V., Docquier J.
The scarf osteotomy for the correction of hallux valgus deformity: a review of 62 cases
5th Central European Orthopaedic Congress, 2004, Prague, Czech Republic

23. Vermes C, Bárdos T, **Czipri M**, Koreny T, Lovász G, Bellyei Á, Glant T.
Periprosthetic osteolysis: multiple cell response for wear debris particles
45th Congress of Hungarian Orthopaedic Association, 2002, Pecs, Hungary

24. Bardos T., **Czipri M**., Vermes C., Lovasz G., Bellyei A., Glant TT.
Arthritis psoriatica in humanized mice offers a new insight to the immunological background of the disease.
45th Congress of Hungarian Orthopaedic Association, 2002, Pecs, Hungary

25. Vermes C, Bárdos T, **Czipri M**, Hanyecz A, Lovász G, Bellyei Á, Fritz E, Roebuck K, Jacobs J, Galante J, Andersson GBJ, Glant TT.
Differential effects of bacterial lipoplysaccharide (LPS) and tumor necrosis factor-apha on the functions of human osteoblast cells. (poster)
48th Annual Meeting of the Orthopaedic Research Society 2002, Dallas, USA

26. Bárdos T, **Czipri M**, Vermes C, Mikecz K, Glant TT.
Suppression of autoimmunity in experimental arthritis by nasal tolerance.
48th Annual Meeting of the Orthopaedic Research Society 2002, Dallas, USA

27. **Czipri M**, de Jonge T, Lovász Gy, Kránicz J, Bellyei Á.
Long term results of unicompartmental knee arthroplasty
43rd Congress of Hungarian Orthopaedic Association, 2000, Debrecen, Hungary

28. Bálint L, Kránicz J, **Czipri M.**
Long term results of 700 operatively treated clubfeet.
8th Instructional Course Lectures of the European Federation of National Associations of Orthopaedics and Traumatology 2000, Prague, Czech Republic

29. **Czipri M**, Kránicz J, Bárdos T.
Experiences with more than 900 operatively treated clubfeet.
3rd Central European Orthopaedic Congress 2000, Prtoroz, Slovenia

30. **Czipri M**, Kránicz J.
Introduction of the Calcaneo-stop surgery
2nd Annual Meeting of Hungarian Foot annd Ankle Society, 1999, Lakitelek, Hungary

31. de Jonge T, **Czipri M**, Lovász G.
Unicompartmental knee arthroplasty: 5-11 years follow-up.
4th Congress of the European Federation of National Associations of Orthopaedics and Traumatology 1999, Brussels, Belgium

32. **Czipri M**, Bellyei Á.
One-stage operation of DDH in children aged between 1-5 years.
4th Congress of the European Federation of National Associations of Orthopaedics and Traumatology 1999, Brussels, Belgium

33. Bellyei Á, **Czipri M.**
Long-term results of one-stage operation of DDH.
SICOT 21st World Conference 1999, Sidney, Australia

34. Bárdos T, Kránicz J, **Czipri M.**
Experience with 900 operatively treated clubfeet
42nd Congress of Hungarian Orthopaedic Association, 1999, Kaposvar, Hungary

35. **Czipri M**, Kránicz J, Bárdos T.
Results of the treatment of the valgus foot deformities in children
42nd Congress of Hungarian Orthopaedic Association, 1999, Kaposvar, Hungary

35. **Czipri M,** Kránicz J.
Introduction of the Calcaneo-stop surgery
Forum of Young Hungarian Orthopaedic Surgeons, 1998, Tata, Hungary
(Best Paper Award winning paper)

37. Bellyei Á**, Czipri M.**
One-stage operation of CDH between 1-5 years
2nd Central European Orthopaedic Congress 1998, Budapest, Hungary