EXPRESSION OF MATRILINS IN MOUSE SKELETAL DEVELOPMENT AND IN A DEER ANTLER MODEL OF BONE REGENERATION

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Summary of Ph.D. Thesis

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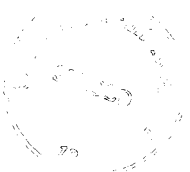

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Introduction

The extracellular matrix (ECM) is a biologically active highly structured network of macromolecules secreted by the cells. Interactions between different matrix proteins are essential for proper ECM assembly and, hence play crucial role in defining the structural integrity and physical properties of connective tissues. Eukaryotic cell adhesion, migration, proliferation and differentiation are examples of biological processes influenced by the composition and structural organization of matrix molecules.

Cartilage, bone, tendon and ligaments that build up the skeletal system have a vast ECM, which occupies much greater space in the tissues than the cells themselves. Bone formation of the skeleton occurs by either intramembranous ossification or by endochondral ossification. Both processes begin with mesenchyme cell condensation. During intramembranous ossification the cells of these condensations directly differentiate into bone forming osteoblasts that secrete collagen I-rich matrix. Endochondral ossification is characterized by the formation of a cartilage template that is later replaced by bone. The chondrocytes, the primary cell type of cartilage secrete a matrix rich in collagen type II, express a characteristic genetic program driven by Sox9 and other transcription factors. The cartilage enlarges through chondrocyte proliferation. Indian hedgehog directly and through the parathyroid hormone related peptide stimulates chondrocyte proliferation; however signaling via fibroblast growth factor receptor-3 (FGFR3) is a negative regulator of cartilage cell division. Chondrocytes at the center of the cartilaginous template of bones stop proliferating, enlarge, become hypertrophic and start to secrete collagen X. Hypertrophic chondrocytes direct mineralization, attract blood vessels and undergo apoptotic death. The cartilage matrix left behind provides a scaffold for osteoblasts that invade the cartilage mould along with blood vessels and lay down a true bone matrix within it.

The matrilins comprise a family of ECM molecules with a modular structure, consisting of one or two von Willebrand factor A-like modules, a various number of epidermal growth factor-like

motifs, and a coiled-coil oligomerization domain. Matrilins are able to form homo-oligomers and the natural occurrence of hetero-oligomers was also shown in case of matrilin-1 and -3. Matrilins have been shown to interact with both collagenous and non-collagenous matrix proteins. They incorporate into extracellular filamentous network.

Regarding to their expression profile, matrilins can be divided into two subgroups. Matrilin-1 and -3 are abundant ECM components of the cartilage. Matrilin-3 was found in bone as well. Matrilin-2 and -4 form the other subgroup with a much broader tissue distribution. Expression of the mouse matrilin-2 was detected by immunostaining in loose and dense connective tissues, and around muscle cells and certain epithelial cell types. Similarly to matrilin-2, matrilin-4 also shows a broad expression in dense and loose connective tissues, nervous tissue.

Aim of the study

Determining the function of matrilins is a permanent challenge and the study is yet to be completed. When the present work had been started, our knowledge on matrilins was even more limited. Only matrilin-1 knock out mice were available with no abnormal phenotype and a few studies on matrilin-2 expression were done in newborn animal only, but a detailed analysis of tissue distribution during ontogenesis was still missing. In order to get closer to the potential function of matrilins we investigated the following aspects:

- The comparison of our cDNA clones to database sequences let us to assume that the gene for Matn2 is transcribed from two promoters. It was our aim to verify the existence of the second promoter in the human *MATN2* gene and compare the promoter usage in various human cell lines.
- As a detailed analysis of matrilin-2 expression during ontogenesis was missing, we aimed its investigation at RNA and protein level as well.

- Because all four matrilins were observed in cartilage, we aimed at describing in detail the expression domains of matrilins during limb skeleton development and in the skull of mice.
- As our work progressed we found matrilins to be useful markers in distinguishing developmental stages of chondrocytes. They were used, in completion with some other extracellular matrix markers, to characterize the cell differentiation events during antler development, a unique osseo-chondrogenic developmental process.
- The molecular mechanism of long bone development is quite well characterized. We aimed to use the deer antler as a model to determine whether some of these molecules may also have a function in regulating cartilage and bone regeneration in an adult mammal.

Materials and Methods

In order to accomplish our aims we applied the following experimental methods on samples originated from mouse embryos and newborn animals, red deer velvet antlers and human cell cultures:

Histology: haematoxylin-eosin staining, alcian blue and von Kossa staining;
Immunohistochemistry and immunofluorescence;
Protein extraction and Western blot analysis;
RNA purification and Northern blot analysis;
RT-PCR and PCR-based cloning;
In situ hybridization.

Results and Discussion

Two promoters of the *MATN2* gene

Comparison of our cDNA clones to database sequences let us to assume that the gene for matrilin-2 is either transcribed from two

promoters or alternative splicing within the 5'-untranslated region is responsible for the heterogeneity of cDNA sequences observed. Sequence analysis did not reveal an alternative acceptor splice site in the corresponding region. To map a potential alternative downstream promoter (P_d) functional only in certain cell types, we carried out RT-PCR analysis on RNA samples isolated from various human cell lines. Oligonucleotide primers specific to one or other mRNA isoform, or both, were designed to investigate how far into the 5' direction the transcribed region protrudes and what proportion of the total matrilin-2 transcript they represent. We have proven that *MATN2* is transcribed from two alternative promoters. We have mapped the second promoter and revealed that it is utilized in WI-26 lung fibroblast and HEp-2 epithelial cell lines, but its activity was undetectable in a primary culture of skin fibroblasts.

Expression of matrilin-2 during mouse embryonic development

We followed matrilin-2 expression during mouse embryonic development by RT-PCR. We showed matrilin-2 expression already in the extraembryonic tissues of the 7.5 dpc mouse embryos and the amount of the transcript increased steadily by age in embryonic tissues.

Expression of matrilins during ossification

All matrilins were observed during endochondral and intramembranous ossification, although showed characteristic differences in distribution. Matrilin-2 and -3 were abundant in occipital bone formed by intramembranous ossification. Matrilin-1 and -4 were found at low level, whereas cartilage link protein was completely missing.

Matrilin-2 differed the most from the matrilin-1 and -3 during endochondral ossification. It was abundant at the articular surface, in perichondria and ligaments, and relatively enriched in cartilage in the hypertrophic zone. The other matrilins were deposited in growth

plate cartilage, but were not visible in articular cartilage. Matrilin-1 was slightly further from the articular surface, than matrilin-3. Furthermore, matrilin-3 was found to be present in the territorial matrix of the cartilage and in the interterritorial matrix both of them were observed.

Antlerogenesis: new findings

The previous study has proven that matrilins are useful markers in identifying the various zones of differentiating cartilage. Therefore, we used matrilins and other ECM markers to characterize in detail the osseo-chondrogenic development of the antler of red deer, *Cervus elaphus*. Antlerogenesis is a rare example of mammalian epimorphic regeneration and implies a modified endochondral ossification for longitudinal growth and intramembranous ossification for appositional growth. We performed a detailed analysis of the velvet antler with histochemistry and immunohistochemistry methods and *in situ* hybridization. The specificity of antisera was tested by immunoblot of protein extracts form various antler zones and the reactivity was also confirmed by immunohistochemistry of fetal growth plate sections. Cell morphology was monitored by haematoxylin-eosin staining, cartilaginous matrix was detected by alcian blue staining and mineral deposition was visualized by von Kossa staining.

During the study differences were observed in the apical and lateral mesenchyme of the antler regarding to the expression of link protein and hyaluronan.

We detected matrilin gene activity both by *in situ* hybridization and by immunohistochemistry in osteoprogenitor cells and osteoblasts. We believe that expression of all matrilins in bone is not a specialty of antler development, because immunohistochemistry signal was also observed in trabecular bone of the fetal humerus. Elevated expression level of matrilin-2 was observed in cells with high differentiation potential such as mesenchyme cells, prechondroblasts and preosteoblasts. Matrilin-2 can be considered as a marker of these cells.

We found all matrilins associated to collagen I-rich structures in the antler. The borderline between the cartilage trabeculae and the perivascular tissue showed deposition of collagen I and all four matrilins, but was devoid of cartilage link protein and collagen II. This colocalization makes possible the interaction between collagen I and the matrilins.

To get a deeper insight into this interesting model of bone regeneration, we have started to investigate the molecular mechanisms, which stay behind this phenomenon. At present we provided expression data about FGFR3 and Sox9, two main regulators of chondrogenesis. We found FGFR3 in the proliferating cells, in prechondrocytes and chondrocytes, however the hypertrophic chondrocytes were not expressing it. Traces of FGFR3 were found in the matrix of hypertophic chondrocytes because of proteolysis. We observed similar expression pattern of FGFR3 in the developing antler to the epiphyseal growth plate and we hypothesize the same function.

Sox9 also showed a similar expression pattern in antler to that one in epiphyseal growth plate. High amount of Sox9 mRNA was found in mesenchyme cells, prechondrocytes, chondrocytes, whereas the hypertophic chondrocytes were lacking Sox9. For the first time we found Sox9 expressed by osteoblasts.

In conclusion, matrilins and other ECM markers served in characterization of limb skeleton formation and antler development. Similarity of the major differentiation events was observed. Differences between the two processes are attributed primarily to the high growing rate and the presence of blood vessels and perivascular tissue in the velvet antler. Further investigations are needed to identify molecular mechanisms that underlie the sterically and temporally regulated gene expression pattern during both processes and reveal the exact role of matrilins in skeletal tissue organisation and development.

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List of Publications

1. Daniela Segat, Christian Frie, Patrick D. Nitsche, Andreas Klatt, Dorothea Piecha, **Éva Korpos,** Ferenc Deák, Raimund Wagener, Mats Paulsson and Neil Smyth (2000) Expression of matrilin1, -2 and -3 in developing mouse limbs and heart. Matrix Biol. 19, 649-655. IF 3.664

2. Lajos Mátés, **Éva Korpos,** Ferenc Deák, Zhangin Liu, David R. Beier, Attila Aszódi, and Ibolya Kiss (2002) Comparative analysis of the mouse and human genes (*Matn2* and *MATN2*) for matrilin-2, a filament-forming protein widely distributed in extracellular matrices. Matrix Biol. 21, 163-174. IF: 4.167

3. **Éva Korpos,** Andrea Molnár, Péter Papp, Ibolya Kiss, László Orosz and Ferenc Deák (2005) Expression pattern of matrilins and other extracellular matrix proteins characterize distinct stages of cell differentiation during antler development. Matrix Biol. 24, 124-135. IF (2005): 4.104

Presentations and Posters

Éva Korpos, Hua Sheng, Ildikó Karcagi, Ibolya Kiss and Ferenc Deák, A matrilin gének kifejeződése az egér egyedfejlődése során. 5th Meeting of the Hungarian Biochemical Society, Sopron, Hungary, 2000.

Éva Korpos, Hua Sheng, Ildikó Karcagi, Ibolya Kiss and Ferenc Deák, Expression of the matrilin genes during mouse development. XVIIth FECTS Meeting, Patras, Greece, 2000.

Éva Korpos, Hua Sheng, Ibolya Kiss and Ferenc Deák, Matrilin-2 expression during mouse development. European Research Conference on "Developmental Biology", Granada, Spain, 2002.

Éva Korpos, Andrea Molnár, Péter Papp, Ibolya Kiss, László Orosz and Ferenc Deák, The expression of matrilins during antler development. BRC-Straub Days, Szeged, Hungary, 2003.

Éva Korpos, Andrea Molnár, Péter Papp, Ibolya Kiss, László Orosz and Ferenc Deák, Regeneration of bony appendage: Monitoring the changes of matrix molecules during antler development. European Research Conference on "Cellular and Molecular Basis of Regeneration", San Feliu de Guixols, Spain, 2004.

Éva Korpos, Andrea Molnár, Péter Papp, Ibolya Kiss, László Orosz and Ferenc Deák, Csontregeneráció követése a fejlődő szarvasagancsban a sejtközötti állomány molekuláinak változásán keresztül. 5th Hungarian Genetic Congress, XIIIth Cell and Developmental Biology Days, Eger, Hungary, 2005.

Éva Korpos, Andrea Molnár, Péter Papp, Ibolya Kiss, László Orosz and Ferenc Deák, Expression pattern of extracellular matrix proteins characterize distinct stages of cell differentiation during antler development. 30th FEBS Congress, Budapest, Hungary, 2005.

Lajos Mátés, **Éva Korpos**, Luca Mendler, Mónika Kiricsi, László Dux, Ferenc Deák and Ibolya Kiss, Activation of matrilin-2 and Prx1 genes during skeletal muscle regeneration. EMBO-FEBS Workshop on "The molecular and cellular mechanisms underlying skeletal muscle formation and repair" Fontevraud l'Abbaye, France, 2005.