SUMMARY OF PH.D. THESIS

Analysis of the transcriptional regulation of the matrilin-1 gene in transgenic mice

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Introduction

Most of the vertebrate skeleton develops through endochondral ossification. This process requires the formation of a cartilaginous template of the future bones. The first step of endochondral bone formation is the condensation of mesenchymal cells. In the center of the condensations, cells differentiate into prechondrocytes and subsequently to chondroblasts, which start to deposit an extracellular matrix characteristic of cartilaginous tissues, comprising mainly of type II, IX., XI. collagen, aggrecan, chondromodulin-1. A few cell layers at the periphery develop into fibroblast-like perichondrial cells. Chondroblasts sequentially differentiate into proliferating, prehypertrophic and hypertrophic chondrocytes. Later capillaries invade carrying osteoclasts which degrade the hypertrophic cartilage matrix. Osteoblasts then produce bone-specific matrix. The layer of epiphyseal chondroblasts does not develop into bone but functions as articular cartilage.

Developing chondrocytes establish growth plates in which the cells are arranged in layers according to their developmental stages. The zone further from the bone diaphysis is the source zone, comprising of small, rapidly dividing cells. In the columnar proliferating zone, cells are dividing slower, arrange into columns. Bone growth is determined by the number of cells proliferating in the columnar zone and progress through hypertrophy. After the prehypertrophic stage, larger and irregularly shaped cells form the hypertrophic zone. Each step of the endochondral bone formation is characterized by a distinctive set of marker gene expression.

The master transcription factor governing chondrocyte differentiation is Sox9. Together with L-Sox5 and Sox6 they form the Sox trio, which is necessary and

sufficient to induce chondrogenesis and cartilage-specific gene expression.

Members of the matrilin protein family are non-collagenous, oligomeric extracellular adaptor proteins. They form both collagen-dependent and -independent networks in the cartilage extracellular matrix. They are present in a great variety of tissue types highlighting their importance in matrix assembly.

The first identified member of the matrilin family is matrilin-1. It functions as an adaptor protein specifically in cartilage, where by binding to other matrix components (decorin, biglycan, COMP) it plays role in the formation of the fibrillar network between aggrecan, type II and VI collagens. Its coding gene (*Matn1*) has the unique feature among other cartilage matrix genes that it is expressed in later stages of chondrogenesis. *In vivo* its expression is restricted to early proliferative and prehypertrophic chondrocytes of the growth plate, therefore it can be considered as a marker gene of this stage. The long promoter of the chicken matrilin-1 gene directs the gene expression to the columnar proliferative and prehypertrophic zones of the growth plate in transgenic mice.

Aims of the study

In our studies we wanted to identify the contribution of previously characterized *Matn1* regulatory elements to the tissue- and developmental stage-specific regulation of the gene. We also wanted to investigate the similarities and differences between the regulatory mechanism of *Matn1* and other cartilage protein genes.

Experimental strategy

To gain insight into the transcriptional regulation of the gene, we used the following experimental strategy. We generated luciferase reporter constructs with different combinations of the regulatory elements and studied their activity in transient expression assays in different cell cultures. Among the reporter constructs, we identified those which affected the tissue- and developmental stage-specific expression of the gene. With these combinations of DNA elements, we made LacZ reporter constructs and generated transgenic mice to study their *in vivo* effect. Histological methods were used to study the spatial and temporal expression pattern of the transgenes in founder embryos and embryos from transgenic lines. Based on these experiments we defined the regulatory regions responsible for this unique expression pattern. We performed *in vitro* EMSA experiments to reveal the binding characteristics of purified Sox factors to the regulatory elements. We also introduced point mutations into the regulatory elements and reporter constructs to study their effects on factor binding and promoter activity in EMSA and in transgenic mice, respectively.

Results

1. We showed that the matrilin-1 short promoter alone exhibits low activity in the proliferative and prehypertrophic zones of the growth plate. Its activity was significantly increased by homologous and heterologous cartilage-specific enhancer elements. Despite of its low activity the short promoter plays pivotal role in the restricted tissue- and developmental stage-specific regulation of the gene. Moreover it can even inhibit the *Col2a1* general cartilage-

specific enhancer in proximal structures and at early differentiation stages.

2. We showed that the paired Sox motifs of the Ine element are capable of binding purified, GST-fused Sox proteins in EMSA experiments. The 5' motif prefers to form nucleoprotein complex with Sox9, while the 3' motif rather binds L-Sox5/Sox6.

3. We demonstrated that mutations in the paired Sox motifs of the Pe1 element dramatically decrease the activation of the long promoter in transient expression assays. In transgenic mouse experiments we also observed the dramatic effect of Sox mutation in the Ine element, but it does not alter the zonal and distal structure-specific activity of the promoter.

Point mutation in the spacer region of the Pe1 element and in the binding site of a yet unidentified factor within the Ine element eliminate the inhibitory effect of the short promoter in the proximal skeletal structures, but does not alter the developmental stage-specific expression.

The evolutionarily conserved DNA elements of the short promoter have crucial role in the promoter activity. The paired Sox9 binding sites of the Pe1 element and the Sox motifs of the Ine element are essential for driving the characteristic expression pattern.

4. The proximal promoter region which contains the conserved Dpe1 and Dpe2 elements largely increased the activity of the short promoter. It also directed the activity of a heterologous promoter to certain zones of the growth plate.

We proved the important role of Dpe1 element in the activation of the short promoter in transient expression experiments. Eight copies of this element together with the short promoter direct zonal expression with extremely high level in distal structures in transgenic mice.

Deletion of the Dpe2 element from the long promoter erased the inhibitory effect in proximal structures, but did not affect the zonal expression pattern.

From these results we concluded that the proximal elements also have zone-specific activity, but short promoter elements are also needed for generating proximo-distal differences.

5. We also studied the role of intronic elements containing Sox and Nfi binding sites. These regions had no or mild effect on the activity of the short promoter, driving strong expression mainly in the head accompanied by high level ectopic expression. Thus these elements play minor role in the transcriptional regulation of the matrilin-1 gene.

6. Accumulation of conserved, tissue-specific regulatory elements in the proximal promoter has key role in the transcriptional regulation of the gene. This arrangement of the regulatory elements is a unique feature of *Matn1*, as it has not been described for other genes encoding cartilage matrix proteins (*Col9a1, Col9a2, Col11a2, Agc1, CD-Rap*).

Discussion

Our results confirm that the transcriptional regulatory mechanisms of the matrilin-1 gene differ from other cartilage-specific genes (*Col2a1, Agc*). The evolutionarily conserved regulatory elements of the short promoter (Pe1, Ine), which can bind the master transcription factors of chondrogenesis (Sox9, L-Sox5/Sox6) *in vitro*, play the key role in the restricted expression pattern of the gene. The modular arrangement of regulatory elements around TATA is a unique feature of the matrilin-1 gene.

Binding of Sox9 to the Sox motifs of Pe1 and Ine is required but not sufficient for the proper expression level, differing from the *Col2a1, Agc* and *Crtl* genes. Based on recent experiments, L-Sox5 and Sox6 can modulate the effect of Sox9 in a dose-dependent and sinergistic manner.

The distal promoter region harbours binding sites for Sox, Nfi and other yet unidentified factors. Their effect is likely mediated by the short promoter elements. Presence of tissue-specific regulatory elements in the proximal promoter region is also a characteristic of the matrilin-1 gene.

Based on the results discussed in this thesis together with pervious and parallel works in our group, we proposed a model for the unique regulatory mechanism of the matrilin-1 gene. According to this, at the early stage of chondrogenesis Sox9 binds to the Pe1 element. L-Sox5/Sox6, which is expressed at lower molar excess relative to Sox9, secures this binding. Binding of Sox factors near TATA may bend the DNA and facilitate the binding of components of the PIC (preinitiation complex). The promoter activity is highest in late proliferative chondroblasts when the occupancy of Sox motifs on Pe1 and Ine are optimal. At later stage or at large molar excess of L-Sox5/Sox6 to Sox9, L-Sox5/Sox6 may decrease the transactivation by Sox9, possibly by competing for its binding sites in Pe1 and other elements. Large excess of Nfi may also decrease the transcriptional activity possibly due to steric reasons by binding close to TATA or due to competition by binding repressor Nfi isoforms to the regulatory elements.

Describing of the unique regulatory mechanism of the matrilin-1 gene facilitated the construction of growth plate zone-specific vectors and the development of biotechnological therapies for skeletal diseases.

The thesis is based on the following publications

Rentsendorj, O., Nagy, A., Sinkó, I., Daraba, A., Barta, E., Kiss, I. (2005) Highly conserved proximal promoter element harbouring paired Sox9-binding sites contributes to the tissue- and developmental stage-specific activity of the matrilin-1 gene. Biochem J **389**, 705-716. IF (2005): 4,224

Nagy, A., Kénesi, E., Rentsendorj, O., Molnár, A., Szénási, T., Sinkó, I., Zvara, A., Thottathil Oommen, S., Barta, E., Puskás, L. G., Lefebvre, V., Kiss, I. (2011) Evolutionarily Conserved, Growth Plate Zone-Specific Regulation of the Matrilin-1 Promoter: L-Sox5/Sox6 and Nfi Factors Bound near TATA Finely Tune Activation by Sox9. Mol Cell Biol **31(4)**, 686-699. IF (2010): 6,057

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Related publication

Karcagi, I., Rauch, T., Hiripi, L., Rentsendorj, O., Nagy, A., Bősze, Zs. and Kiss, I. (2004) Functional analysis of the regulatory regions of the matrilin-1 gene in transgenic mice reveals modular arrangement of tissue-specific control elements. Matrix Biol. **22**, 605-618. IF (2004): 4,104

Conference presentations and posters

Nagy, A., Karcagi, I., Rauch, T., Hiripi, L., Rentsendorj, O., Bősze, Z. and Kiss, I.: Functional analysis of the regulatory regions of the matrilin-1 gene in transgenic mice reveals modular arrangement of tissue-specific control elements. **FEBS J.** 272 (s1), D1-034P, 2005, IF(2004): 3.260

Nagy A., Sinkó I., Molnár A., Kénesi E., Kiss I.: A matrilin-1 szabályozóelemek működésének vizsgálata transzgenikus egerekben. Absztrakt #P-58, **Biokémia**, XXX.évf., 3. szám, szeptember, 2006

Nagy A., Sinkó I., Molnár A., Kénesi E., Kiss I.: Functional analysis of the matrilin-1 control elements in transgenic mice. Absztrakt #F3217, **FECTS XXth and ISMB meeting**, Oulu, Finnország, 2006

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Nagy, A., Sinkó, I., Rentsendorj, O., Karcagi, I., and *Kiss, I.*: Functional analysis of the matrilin-1 control regions directing the zonal expression of the transgene in growth plate cartilage. Poszter, **Gordon Research Conference „Biology and Pathology of Cartilage”**, Il Ciocco, Olaszország, Június 5-9. 2005

Nagy A., Sinkó I., Molnár A., Kénesi E., Kiss I.: Functional analysis of the matrilin-1 control elements in transgenic mice. Absztrakt #F3217, **FECTS XXth and ISMB meeting**, Oulu, Finnország, 2006

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A. Nagy, A. Molnár, I. Sinkó, E. Kénesi and I. Kiss: Functional analysis of Matn-1 promoter regulatory elements in transgenic mice. **Straub Napok**, Szeged, November 15-17. 2006

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