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

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1. ABBREVIATIONS

AP	antlerogenic periosteum
Ab-vK	Alcian blue-von Kossa
CMP	cartilage matrix protein
COMP	cartilage oligomeric matrix protein
Crtl1	cartilage link protein
DMEM	Dulbecco's modified Eagle's medium
dpc	days post coitum
EGF	epidermal growth factor
ECM	extracellular matrix
FCS	fetal calf serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
HA	hyaluronic acid
HMG	high-mobility-group
Ihh	Indian hedgehog
Matn1-4	mouse matrilin genes
Matn1-4	matrilin1-4 protein
MATN2	human Matn2 gene
MATN3	human Matn3 gene
MED	multiple epiphyseal dysplasia
NHSF	normal human skin fibroblast
PBS	phosphate buffered saline
PTHrP	parathyroid hormone–related peptide
TBS	Tris-buffered saline
TGF-β1	transforming growth factor-beta1
TRAP	tartrate resistant acid phosphatase
VEGF	vascular endothelial growth factor
vWFA	von Willebrand factor A domain

2. INTRODUCTION

2.1. Extracellular matrix

2.1.1. *Structure and importance of the ECM*

The extracellular matrix (ECM) is a biologically active complex network of macromolecules, that in addition to serving a structural function, also profoundly affects the cellular physiology of an organism. Eukaryotic cell adhesion, migration, proliferation and differentiation are examples of biological processes influenced by the composition and structural organization of the surrounding extracellular matrices. The ECM not only affects the behaviour of cells of the host organism, but can also serve as a substrate for the attachment and colonization of pathogenic microorganisms. The matrix also communicates directly and indirectly with the intracellular signalling pathways that direct a cell to carry out specific functions.

Specific ECM components can directly activate cytosolic signal-transduction pathways by binding to cell-adhesion protein receptors in the plasma membrane. Alternatively, by binding growth factors and other hormones, the matrix can either sequester these signals from cells or, conversely, present them to cells, thereby indirectly inducing or inhibiting signaling pathways. Morphogenesis, the later stage of embryonic development during which form is achieved by cell movements and rearrangements, is also critically dependent on ECM components, which are constantly being remodeled, degraded, and resynthesized locally. Even in adults, degradation and resynthesis of ECM components occur for example in areas of wounding.

The different ECMs mainly consist of three major types of macromolecular components: 1. fibrillar proteins, 2. glycosaminoglycans (hyaluronan) and proteoglycans; and 3. non-collagenous proteins (Aumailley and Gayraud, 1998). Importantly, the distribution and organization of these molecules vary with tissue type: mesenchymal cells are immersed in an interstitial matrix whereas epithelial and endothelial cells contact a basement membrane only through their basal surface. ECM composition is not static during development but rather varies from stage to stage and tissue to tissue. The diversity of composition, organization and distribution of ECM arises not only from the differential expression of genes for the various

molecules in specific tissues, but also from the existence of differential splicing and posttranslational modifications of those molecules.

1. Among *fibrillar proteins* collagens are almost ubiquitous and in some tissues elastin and fibrillin fibers. So far, 26 genetically distinct collagen types have been described (Gelse et al., 2003), all share the collagenous triple helix as the most characteristic molecular attribute. It is made up of (XYGly)n repeats and form triple right α-helices. Collagens are present in all types of connective tissues. They form strong fibrils with large tearing strength, while other collagens assemble into sheets or other supramolecular structures contributing to the formation of basement membranes and different contacts between ECM components. Collagens can be divided into different subgroups: *fibrillar collagens* (collagens I-III, V, XI), *network forming collagens* (collagens IV, VIII, X), *fibril associated collagens* (collagens IX, XII, XIV, XVI, XIX), *transmembrane collagens* (collagens XIII and XVII), etc. The other fibrillar protein, *elastin*, polymerizes to elastic fibers of the skin, lung, blood vessels, ligaments and tendons. *Fibrillin-1* and *-2* are also components of elastic fibers, and of different lamellar structures and ligaments.

2. *Glycosaminoglycans* are also main ingredients of the ECM either existing alone as *hyaluronic acid* or covalently bound to one protein core, as in the case of *proteoglycans (Heinegard et al., 1987). Hyaluronic acid (HA)* is a major component of the extracellular matrix that surrounds migrating and proliferating cells, particularly in embryonic tissues. It is built up of 1000-50000 repeats of disaccharide units and because of a large number of anionic residues on its surface, HA binds a large amount of water and forms, even at low concentrations, a viscous hydrated gel. When bound to specific receptors on certain cells, HA inhibits cell-cell adhesion and facilitates cell migration. *Proteoglycans* contain one or multiple glycosaminoglycan chains that branch from a linear protein core. Because of their high content of charged polysaccharides, proteoglycans are highly hydrated. The swollen, hydrated structure of proteoglycans is largely responsible for the volume of the extracellular matrix and acts to permit diffusion of small molecules between cells and tissues. We distinguish *matrix* and *cell surface proteoglycans.* The first group includes *hyalectans, basement membrane proteoglycans* and *small leucine-rich proteoglycans.* The *hyalectans* are large *proteoglycans,* which are able to form networks with lectins and *HA,* and they can interact with *multiadhesion proteins* (for example in the cartilage with link protein and matrilins) and collagen fibrils as well. Perlecan and agrin are heparan sulphate proteoglycans glycans of various basement membranes.

Among the small leucin-rich proteoglycans decorin and fibromodulin have to be mentioned, that interact with collagens and present a regulatory function in collagen fibril formation. Proteoglycans are also found on the surface of cells, where they function as co-receptors to help cells respond to secreted signal proteins. The *cell surface proteoglycans* are either membrane bound as the glypicans, or true transmembrane molecules as the syndecan family members. Via their heparin sulphate side chains, they accumulate growth hormones and facilitate binding of growth hormones to their true cell surface receptors, thus triggering intracellular signaling pathways (Tkachenko et al., 2005).

3. The *non-collagenous* ECM proteins are very often long, flexible molecules that contain multiple domains responsible for binding to a variety of collagen types, other matrix proteins, polysaccharides, cell-surface proteins, and signaling molecules such as growth factors and hormones. They play important roles in matrix-matrix and cell-matrix connection. For example cartilage link protein connects the proteoglycan aggrecan to hyaluronic acid, while nidogen/entactin binds the basement membrane components laminin and collagen IV. The matrilins belong to this group of ECM proteins. They are able to bind collagens and other matrix components as discussed later (see part 2.1.2.2). The *multiadhesive proteins* involved in cell-matrix contacts, generally contain RGD triplets to which the integrins are able to bind. These molecules, e.g. fibronectin, vitronectin, laminin and tenascin, are able to enhance cell adhesion.

2.1.2. *The matrilin protein family*

2.1.2.1. The structure and evolution of matrilins

With the discovery of matrilin-2 (Matn2) (Deák et al., 1997), matrilin-3 (Matn3) (Belluoccio and Trueb, 1997; Wagener et al., 1997) and matrilin-4 (Matn4) (Wagener et al., 1998a) it turned out that the matrilins form a family with a closely similar domain structure (Fig. 1). The prototype member of this family is matrilin-1 (Matn1), which was earlier referred to as cartilage matrix protein (CMP). It was initially identified as an abundant, proteoglycan-associated protein present in many forms of cartilage (Paulsson and Heinegard, 1979, 1981, 1982). The primary structure of matrilin-1 has been deduced from chicken (Argraves et al., 1987; Kiss et al., 1989), man (Jenkins et al., 1990) and mouse (Aszódi et al., 1996) cDNA. It showed to be a modular protein consisting of two von Willebrand Factor A (vWFA) domains, connected by a single epidermal growth factor like domain (EGF),

followed by a C-terminal α- helical coiled-coil module which allows the oligomerisation of the single subunits in a bouquet-like fashion (Argraves et al., 1987; Kiss et al., 1989; Hauser and Paulsson, 1994). The other matrilins contain the same domain in the same order as in matrilin-1. Only Matn3 lacks the second vWFA domain and here the EGF-like domains are directly connected to the coiled-coil domain. In addition, Matn2 and Matn3 contain a stretch of amino acid residues at the N-terminus with a high frequency of positively charged side chains. Uniquely, Matn2 contains a module between the second vWFA domain and the oligomerisation domain that has no homology to any other known protein sequence.

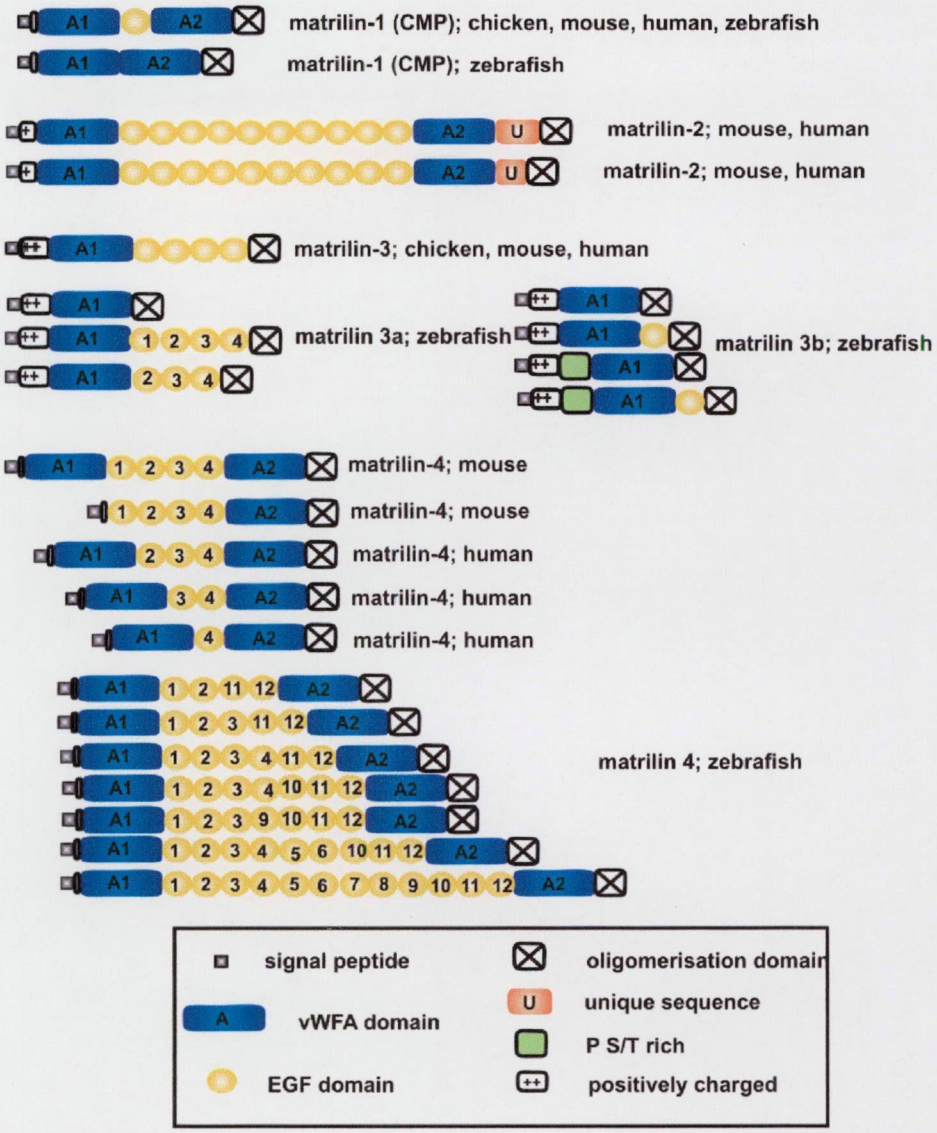

Figure 1. Domain structure of mouse, human, chicken and zebrafish matrilins. For explanation see the box on the bottom.

The vWFA domains fall into two groups with the vWFA1 module always occuring towards the N-terminus and the vWFA2 module closer to the C-terminus. In Matn3 the

vWFA2 module and in one splice variant of mouse Matn4 the vWFA1 module has been deleted. vWFA domains are found not only in the matrilins, but also in a large number of other extracellular proteins such as von Willebrand factor, collagens VI, VII, XII, XIV, etc. These proteins are collectively referred to as the von Willebrand Factor Type A-like module superfamily.

With the exception of splice variants of Matn1 and Matn3 in zebrafish, all other matrilins contain at least one EGF-like domain, the highest number being in a splice variant of zebrafish Matn4 where 12 EGF domains are present (Ko et al., 2004). They are not Ca^{++}-binding type and even though they show an overall structural similarity to epidermal growth factor, there is no evidence that they retain growth factor activity. It is more likely that they serve as spacers between vWFA domains, which in many other proteins show ligand binding activities. The EGF like modules are commonly found in extracellular proteins and when arranged in tandem they give rise to flexible rods as seen, e.g. in the short arms of laminin (Beck et al., 1990).

Matn1 is a homotrimeric glycoprotein of about 50000 Da subunits (Hauser and Paulsson, 1994).

Matn2 is the largest matrilin with a calculated molecular weight of 104 300 Da in mouse and carries N-linked oligosaccharides (Piecha et al., 1999). Similarly to Matn1, in electron microscopy the two vWFA domains appear to bind to each other causing the subunit to form a loop extending from the central coiled-coil. Both recombinant Matn2 and the protein detected by immunoblot in tissue extracts display an unusual extent of structural heterogeneity, most likely due to proteolytic processing.

Matn3 is the smallest family member, mostly coexpressed with Matn1 (Wagener et al., 1997; Klatt et al., 2000). By MALDI-TOF mass spectrometry, the molecular mass of mouse Matn3 was determined to be 49 300 Da. This value closely matches the calculated mass, indicating the lack of glycosylation. Due to the absence of the second vWFA domain, there is no self-interaction between the subunits and in electron microscopy, the tetrameric Matn3 appears to have more extended, flexible arms (Klatt et al., 2000).

Matn4 contains four EGF domains between the two vWFA domains in mouse (Wagener et al., 1998a; 1998b; Klatt et al., 2001). The recombinant protein has a molecular weight of 72 900 Da indicating 7% posttranslational modifcations. Although Matn4 carries two vWFA domains, in electron microscopy these show no obvious interaction (Klat et al., 2001). The images show the three C-terminal vWFA domains at the center, presumably held together by the coiled-coil domain. The subunits extend from this central structure and end in

globular domains representing the N-terminal vWFA domain. The structure of Matn4 is reminescent of Matn3, except for the latter protein forming tetramers.

The matrilins presumably originate from a common ancestor, in which the vWFA module had been already duplicated, because all the vWFA1 modules of matrilins are more closely related to other vWFA1 modules than to the vWFA2 modules. The hypothesis related to the evolution of matrilins is that the primordial matrilin gene duplicated, giving rise to common ancestors of Matn1 and Matn3 or Matn2 and Matn4. Furthermore, after a second gene duplication, the vWFA module of the Matn3 precursor, which could be equivalent to the vWFA2 module in Matn1, was deleted (Deák et al., 1999).

2.1.2.2. Oligomerisation and interaction with other ECM components

By electron microscopy of full-length proteins, it has been shown that Matn1 and -4 form homo-trimers (Hauser and Paulsson., 1994; Klatt et al., 2001), whereas Matn2 and -3 form homo-tetramers (Piecha et al., 1999; Klatt et al., 2000). Hetero-oligomers of Matn1 and Matn3 were isolated from fetal human and calf cartilage (Wu and Eyre, 1998; Klatt et al., 2000; Kleemann-Fischer et al., 2001). Hetereo-oligomer formation was shown between recombinantly expressed coiled-coil domains of Matn1 and Matn2; Matn1 and Matn3; Matn1 and Matn4; and Matn2 and Matn4. Hetero-oligomers containing Matn2 and Matn3 or Matn3 and Matn4 were not found in these experiments (Frank et al., 2002). Further studies are needed to prove the natural occurrence of those hetero-oligomers.

Matn1 was first recognised as a protein tightly bound to aggrecan and copurified with aggrecan in a variety of separation methods (Paulsson and Heinegard, 1979). It is not known if this complex formation is unique for Matn1 and aggrecan (Hauser et al., 1996) or if other matrilins can also bind to proteoglycans, perhaps to other members of the lectican family. Matn1 binds to type II collagen with certain periodicity (Winterbottom et al., 1992). Chondrocyte cell culture experiments showed Matn1 to be present in two kinds of pericellular filaments, where one type was collagen-dependent, as it required ascorbate for formation, and the other not (Chen et al., 1995). Similar staining of filaments in the pericellular matrix of cultured cells has been seen for each of the other matrilins (Wagener et al., 2005) and, in case of Matn2 and Matn4, not only around chondrocytes, but also in cultures of other cell types that express these matrilins (Piecha et al., 1999; Klatt et al., 2000; 2001). It was shown that Matn1, Matn3 and Matn4 connect the collagen VI-containing

microfibrils to aggrecan core proteins or to collagen II-containing fibrils through biglycan or decorin made assemblies (Wiberg et al., 2003).

Matn2 binds to collagen I, the most abundant collagen component in tissues, where the Matn2 gene is expressed. Current data demonstrate that Matn2 interacts with collagen I with preferential binding to sites close to the two termini of the tropocollagen molecule and at some distinct points within it. Therefore, Matn2 can potentially be associated with the surface of collagen I-containing fibrils in a manner analogous with that of Matn1 bound to cartilage collagen fibrils (Piecha et al., 2002). The interaction of Matn2 with collagens II, III, IV and V was also demonstrated by surface plasmon resonance method (Piecha et al., 2002). Matn2 is localised to basement membranes and microfibrils, and its binding to some components of those structures have also been demonstrated. Both fibronectin and laminin-1-nidogen-1 complexes showed binding to a truncated form of the protein in surface plasmon resonance assay (Piecha et al., 2002). All variants of Matn2 tested strongly bind to fibrillin-2, a microfibrillar protein organized in 10-12 nm diameter microfibrils, which often intersect into the dermo-epidermal basement membrane. The role of this anchorage and the underlying molecular interactions are not known (Piecha et al., 2002).

It was shown by ELISA-style ligand binding assay that Matn4 binds to collagen I, II, XI, XII, XIV, XXII (Mann et al., 2004). However, some non-collagenous molecules, in particular cartilage oligomeric matrix protein (COMP) and decorin, bound to matrilins with higher affinity (Mann et al., 2004) and as COMP and decorin are known to interact with collagens, it may well be that matrilins are attached to collagen fibrils as part of a "sandwich" where other components may be the direct and high-affinity collagen binders.

2.1.2.3. Overall tissue distribution of matrilins

Regarding to their expression profile, matrilins can be divided into two subgroups. Matn1 and -3 are abundant ECM components of the skeletal elements. Extraskeletal expression of the Matn1 gene was shown, by a sensitive RT-PCR assay in the eye of adult mouse (Aszódi et al., 1996). By immunohistochemistry, the Matn1 was detected in the cornea, sclera, choroid, lens capsule, and lens epithelium, but not in the retina of chicken embryos (Tsonis and Goetinck, 1988) and by *in situ* hybridization in the retina of human embryos (Mundlos and Zabel, 1994). A transient expression of Matn1 occurs in the embryonal mouse heart between days 9.5 and 14.5 dpc (Segat et al., 2001). Matn3 was found

in every cartilage type studied and in bone as well (Wagener et al., 1997; Klatt et al., 2000), having a wider expression domain than Matn1 and restricted only to the skeletal elements.

Matn2 and Matn4 form the other subgroup with a much broader tissue distribution. Northern analysis revealed the highest level of Matn2 expression in the uterus, heart, calvaria and brain, and lower level of expression in the kidney, skeletal muscle, and skin. Moreover, a lower level of the mRNA can be detected in all of the organs tested. Expression of the mouse Matn2 gene was detected in loose and dense connective tissues, muscle cells and certain epithelial cell types (Deák et al., 1997; Piecha et al., 1999). Similarly to Matn2, Matn4 also shows a broad expression in dense and loose connective tissues, bone, cartilage, nervous system and in a variety of organs (Wagener et al., 1998a; 1998b; Klatt et al., 2001), in a partially overlapping pattern with Matn2.

2.1.2.4. Phenotypical consequences of matrilin genes mutation

To date, Matn3 is the only known member of the matrilin family found to be associated with osteochondrodysplasias. A mild form of the autosomal dominant multiple epiphyseal dysplasia (MED) is caused by missense mutations that affect β-sheet regions in the vWFA domain encoded by exon 2 (Chapman et al., 2001; Jackson et al., 2004; Mabuchi et al., 2004). These observations highlight the importance of the vWFA domain in the function of *MATN3*. The vWFA domains are thought to mediate interactions with other proteins, and their involvement in oligomerization, filamentous network formation, cell adhesion and spreading has been described (Whittaker and Hynes, 2002). The accumulation of mutations in the MATN3 vWFA domain gives *in vivo* evidence about its important role in MATN3 function. It was shown that mutation can affect the first EGF domain of MATN3 causing hand osteoarthritis (Stefansson et al., 2003). Whereas MATN3 carrying the hand osteoarthritis mutation could be secreted by chondrocytes at a similar rate as wild type MATN3, the MATN3 mutants causing MED, respectively, were retained in the endoplasmic reticulum. It is likely that the abnormality is similar to the case of COMP mutation causing MED, where mutant COMP accumulates within the rough endoplasmatic reticulum of the chondrocyte and selectively impairs secretion of other matrix proteins, leading to downstream effects of abnormal matrix and chondrocyte death (Hashimoto et al., 2003). Recently it was shown that mutations in COMP affect not only the secretion of COMP itself but that of collagen IX and Matn3, as all are selectively sequestered, resulting in diminished presence of

all three proteins in the matrix. It is suggested that COMP, collagen IX and Matn3 participate together in the assembly of cartilage matrix (Hecht et al., 2005).

Matn1 shows similar expression pattern in cartilage to Matn3, but not any MATN1 mutation has been detected in MED patients (Jackson et al., 2004). However, autoantibodies against MATN1 were detected in relapsing polychondritis patients (Buckner et al., 2000) and increased MATN1 level was shown in knee osteoarthritic cartilage (Okimura et al., 1997) and sera of reumathoid arthritis patients (Saxne et al., 1989). In spite of the facts described above, null mutants generated for matrilins do not show any obvious phenotype (Aszódi et al., 1999; Ko et al., 2004; Mátés et al., 2004). Only a weak alteration in the collagen II fibrillogenesis was shown in one of the *Matn1* knock out mice (Huang et al., 1999), which has not been found in the independently made *Matn1* null mutant (Aszódi et al., 1999). The absence of relevant phenotype of matrilin knock out mice shows the possible redundant function among of matrilin family.

2.1.2.5. Promoters of matrilin genes

The *Matn1* and *Matn3* genes are transcribed from single promoters (Kiss et al., 1989; Jenkins et al., 1990, Wagener et al., 2000). The *Matn4* gene in mouse is transcribed from two promoters. The proximal promoter is 5-10-fold more active in most tissues than the distal one located 548 bp further upstream (Wagener et al., 2001). Previously we determined the cDNA sequences encoding mouse Matn2 (Deák et al., 1997) and human matrilin-2 (MATN2) (Muratoglu et al., 2000). The two sequences differed in the 5'-untranslated regions. When we analyzed in detail the mouse *Matn2* gene, upstream of exon 2 a similar sequence to the human cDNA 5'-end was identified. Concomitantly, as the Human Genome program progressed, a sequence similar to the 5'-end of the mouse cDNA clones was revealed. Taken the two together we assumed that the two 5'-untranslated regions are transcribed from alternative promoters active in both species. The mouse *Matn2* promoters were characterized by Lajos Mátés and are described in his PhD Thesis.

The upstream promoter of the *Matn2* gene was identified as a housekeeping type promoter, with multiple initiation sites between positions -39 and +56 relative to the most upstream strong start site. The region was not preceded by a TATA motif, but carried several putative Sp1 recognition sites characteristic of housekeeping promoters. The downstream promoter was mapped 10 kb downstream in the mouse genome, with a CACCCT sequence

motif matching to the CANYYY canonical transcription start site of eukaryotic genes preceded by a TATA-like sequence.

Simultaneously, the corresponding sequence in the *MATN2* gene was analyzed and extension of the downstream promoter region was determined in human samples. Results are presented in this Thesis.

2.2. Skeletal development

2.2 1. Skeletal morphogenesis

The skeletal elements provide mechanical support, protection of vital organs, mineral homeostasis for calcium and phosphate ions and are the source of hematopoietic cells. Formation of the skeleton occurs by either intramembranous ossification, whereby mesenchyme-derived cells directly differentiate into osteoblasts, or by endochondral ossification, characterized by the existence of a cartilage intermediate that is later replaced by bone. Once the adult skeleton is formed, homeostasis is maintained by the balanced action of bone-forming osteoblasts and bone-resorbing osteoclasts in mineralized tissues (Parfitt, 1994). In humans, this balance between bone formation and resorption shifts later in life toward a net bone loss, leading to pathological conditions such as osteoporosis. Skeletal tissues that are lost owing not only to age-related degeneration or destruction but also to trauma or the removal of cancerous tissue (*e.g.*, osteosarcoma) are not at all or only incompletely regenerated without intervention.

The formation of the skeleton occurs in three stages:

(a) Patterning/cell migration. From an ontogenetic perspective, mineralized tissues in vertebrates are derived from three distinct embryonic lineages. The axial skeleton is derived from paraxial mesoderm (somites), the appendicular skeleton originates from lateral plate mesodermal cells, and the craniofacial skeleton is a derivative of cranial neural crest (Fig.2). Neural-crest-derived cells migrate to sites of craniofacial skeleton formation, where they differentiate directly or under the influence of epithelial-mesenchymal interactions. Mesoderm-derived cells migrate to regions of future axial and appendicular skeletal development, where they form mesenchymal cell condensations.

(b) Mesenchymal differentiation. Condensations of skeletal mesenchymal cells differentiate into chondrocytes to form a model framework of cartilage (Hall and Miyake, 2000) that is subsequently replaced by bone (endochondral ossification). Cells of the future

craniofacial skeleton and parts of the clavicle migrate and differentiate directly into bone cells without forming a cartilage intermediate (intramembranous ossification).

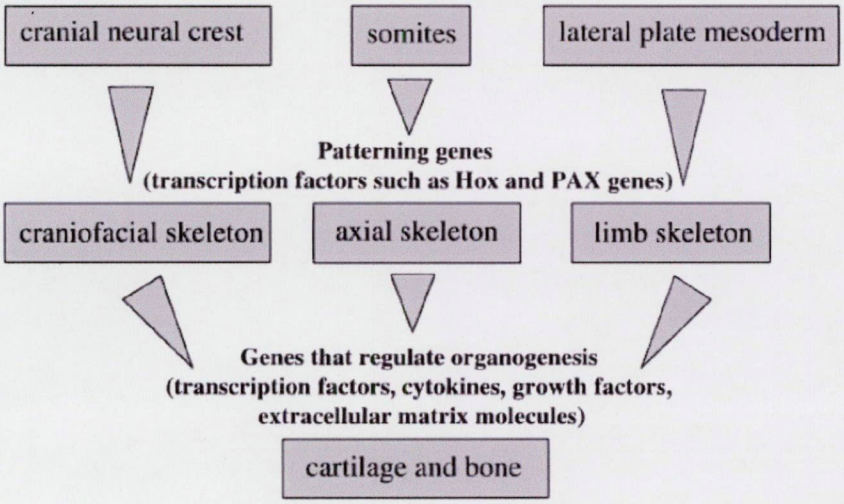

Figure 2. Cell lineages and early steps in development of vertebrate skeleton (Olsen et al., 2000).

c) Organogenesis. Cells in mesenchymal condensations of future long bones express cartilage- specific genes and produce a chondrocyte-specific extracellular matrix, rich in collagen II. Chondrocytes in the centre of the cartilage mould stop proliferating, enlarge (hypertrophy) and change their genetic program to synthesize collagen X (Fig. 3A and B). The hypertrophic chondrocyte, simply through its size, is the principal engine of bone growth, and is a master regulatory cell (Noonan et al., 1998; Kronenberg, 2003). Hypertrophic chondrocytes direct the mineralization of their surrounding matrix, attract blood vessels through the production of vascular endothelial growth factor and other factors, and attract chondroclasts (closely related or identical to osteoclasts, which are cells of the macrophage lineage that digest matrix). During endochondral ossification, an avascular tissue (cartilage) is gradually converted into one of the most highly vascularized tissues in the vertebrate body (bone). This conversion is dependent upon an angiogenic switch along the growth plate. A strong gradient of angiogenic factors, including vascular endothelial growth factor (VEGF), secreted by hypertrophic chondrocytes induce sprouting angiogenesis from the perichondrium. With the vessels come osteoblasts, osteoclasts, and hematopoietic cells. This results in the formation of primary ossification centers. Within this ossification centers hypertrophic chondrocytes undergo apoptothic cell death. At the same time, osteoblasts in the perichondrium form a collar of compact bone around the middle portion (diaphysis) of the cartilage, so that the primary ossification center ends up being located inside a tube of bone (for review see Olsen et al, 2000; Kronenberg, 2003). The perichondrium plays a dual role in

the process of endochondral ossification by providing osteoblast precursors and regulating vascular invasion (Colnot et al., 2004).

As bones enlarge further, so-called secondary ossification centres are established when chondrocytes in characteristic, new locations stop proliferating, hypertrophy, and attract vascular invasion along with osteoblasts. In the long bones of the limb, chondrocytes continue to proliferate between regions of bone of the primary and secondary ossification centres.

Cells of the craniofacial skeleton synthesize an osteoid matrix, which mineralizes under the guidance of non-collagenous extracellular matrix proteins such as bone sialoprotein (reviewed by Ganss et al., 1999), ultimately to form the mature skeleton.

2.2.2. Molecular regulation of endochondral ossification

Systemic hormones and local paracrine and autocrine factors control skeletal development. While the molecular mechanisms of growth regulation by systemic factors are still largely unknown, it is evident that the locally restricted, molecular regulation of skeletal growth is achieved by the activation of signaling cascades *via* secreted molecules and the modulation of gene expression in the target cell through responsive transcription factors. The main transcription factors of endochondral ossification are the members of Sox protein family (Sox5, 6 and 9) and Cbfa1.

Various signaling molecules control these processes. They include Indian hedgehog (Ihh), parathyroid hormone–related peptide (PTHrP), and the receptor of parathyroid hormone and PTHrP (Karaplis et al., 1994; Lanske et al., 1996; Vortkamp et al., 1996; St-Jacques et al., 1999), fibroblast growth factor receptor-3 (FGFR3) and its ligand FGF18 (Colvin et al., 1996; Deng et al., 1996; Liu et al., 2002; Ohbayashi et al., 2002), bone morphogenetic proteins (Zou et al., 1997), and Wnt proteins (Hartmann and Tabin, 2000; Yang et al., 2003).

Sox5, 6, 9, the master transcription factors of endochondral ossification (Fig 3C).

Sox9 is a member of a large family of transcription factors that contain a *SRY*-related (sex-determining region Y gene) high-mobility-group (HMG) DNA binding region (Pevny and Lovell-Badge, 1997). About 20 *Sox* genes have been identified and they all share at least 50% identity within the 79-amino acid residue long HMG domain (Soullier et al 1999). Despite the high homology within the HMG domain, little similarity is found among *Sox* genes outside the DNA-binding motif. Sox9 comes closest to serving the function of a master

regulator of the chondrocyte lineage of any molecule characterized to date (de Crombrugghe et al., 2000). *Sox9* transcripts are detected in all prechondrogenic mesenchymal condensations as early as 8.5 to 9.5 days of mouse embryonic development, and the expression peaks in cartilage primordia at 11.5 to 14.5 days (Ng et al., 1997). Like *Col2a1* encoding collagen II, Sox9 is expressed at high levels in all prechondrocytes and chondrocytes. In the growth plate of long bone, *Sox9* and *Col2a1* levels are high in both resting and proliferating chondrocytes, while *Sox9* is switched off in hypertrophic chondrocytes where *Col2a1* transcripts are still detectable. Sox9 is required for expression of cartilage-specific extracellular matrix components such as collagen II, collagens IX and XI, and the large proteoglycan aggrecan (Lefebvre et al., 1998; Bi et al., 1999) (Fig. 3C). Two other members of the family, L-Sox5 and Sox6, are also involved in *Col2a1* expression. They appear to form a large complex with Sox9 and other nuclear proteins in chondrocytes, and *L-Sox5* and *Sox6* transcripts are coexpressed with *Sox9* at all chondrogenic sites. Therefore, it is believed that the three *Sox* genes cooperatively activate the *Col2a1* gene (Lefebvre et al., 1998).

Sox5 and Sox6 have essential, redundant roles in early chondroblasts (Smits et al., 2001). Although *Sox5-/-* and *Sox6-/-* mice are born with minor cartilage defects, *Sox5-/-*, *Sox6-/-* double mutant embryos develop a severe, generalized chondrodysplasia due to considerable delay and impairment of chondroblast proliferation and expression of cartilage matrix genes. They die around embryonic day 16.5 with rudimentary and matrix-deficient cartilage primordia. Sox5 and Sox6 promote the development of a highly proliferating pool of chondroblasts between the epiphyses and metaphyses of future long bones. This pool is the likely cellular source of growth plates. Sox5 and Sox6 permit formation of growth plate columnar zones by keeping chondroblasts proliferating and by delaying chondrocyte prehypertrophy. They allow induction of chondrocyte hypertrophy and permit formation of prehypertrophic and hypertrophic zones by delaying chondrocyte terminal differentiation induced by ossification fronts (Smits et al., 2004).

Inactivation of *Sox9* in limb buds both before and after mesenchymal condensations abolished the expression of *Sox5* and *Sox6*, indicating that Sox9 was required for expression of these two related *Sox* genes (Fig. 3C). In addition, inactivation of *Sox9* before chondrogenic mesenchymal condensations produced no transcripts for *Cbfa1*, which is ordinarily expressed at late stages of condensations and is required for osteoblast differentiation (Otto et al., 1997). This indicates that Sox9 is needed for *Cbfa1* expression in early limb buds. However, if *Sox9* was inactivated after mesenchymal condensations, *Cbfa1* expression and osteoblast differentiation took place. Thus, in the absence of *Sox9* in early

limb buds, there are no mesenchymal condensations, no chondrocyte differentiation, no establishing of the osteoblast lineage, and no osteoblast differentiation.

Cbfa1, the master transcription factor of osteoblast differentiation and function (Fig. 3C). Osteoblasts, which originate from mesenchymal cells, are responsible for bone matrix deposition in both intramembraneous and endochondral bone formation. A key regulator of osteoblast differentiation and function is Cbfa1 (Fig 3C) (Ducy et al., 1997, Komori et al., 1997, Otto et al., 1997), a member of a small transcription factor family that shares DNA-binding domains of homology with *Drosophila Runt*. No bone tissue is formed in homozygous *Cbfa1*-deficient mice although a relatively normal cartilage skeleton develops. In addition to the role of Cbfa1 in osteoblast differentiation, Cbfa1 activity is also required for bone matrix deposition by mature osteoblasts (Ducy et al 1999). Cbfa1 is targeted to the promoters of several bone proteins, including osteocalcin (an osteoblast-specific marker), bone sialoprotein, alkaline phosphatase, and collagen I (Ducy et al 1997). Cbfa1 is expressed in the late condensation stage of chondrogenesis and then has substantially decreased expression in proliferating chondrocytes, with increased expression again in prehypertrophic and hypertrophic chondrocytes. It is highly expressed in perichondrial cells and in osteoblasts. Cbfa1, in addition to inducing osteoblast differentiation, is required or represents a limiting factor for chondrocyte hypertrophy. It is highly expressed in perichondrial cells and in osteoblasts.

Ihh-PTHrP signaling pathway (Fig 3A). Ihh, secreted by cells in the prehypertrophic zone, stimulates PTHrP expression in periarticular perichondrial cells (Vortkamp et al., 1996). PTHrP appears to prevent chondrocyte hypertrophy in the growth plate and maintains a pool of cells above the hypertrophic zone in a proliferative condition (Karp et al., 2000). The effects of PTHrP are mediated by the PTHrP receptor, a G protein–coupled receptor expressed in the proliferating and prehypertrophic cells (Lanske et al., 1996). Thus, Ihh and periarticular perichondrial PTHrP are thought to be components of a feedback loop that regulates the relative proportions of proliferating and hypertrophic chondrocytes in growth plates. Ihh appears as a coordinator of endochondral ossification, regulating chondrocyte proliferation and differentiation as well as osteoblast differentiation, and coupling chondrogenesis to osteogenesis (St Jacques et al., 1999; Karp et al., 2000).

Fibroblast growth factor (FGF) signaling (Fig. 3A). FGF signalling crucially regulates chondrocyte proliferation and differentiation. Many of the 22 distinct FGF genes and four FGF receptor genes are expressed at every stage of endochondral bone formation (Ornitz and Marie, 2002). FGFR share a basic structure of three glycosylated Ig-like domains,

a single transmembrane domain and a cytoplasmic tyrosine kinase domain. In the earliest stages of endochondral bone development, FGFR2 is expressed in condensing mesenchyme. Multiple FGFs are expressed in condensations and surrounding mesenchyme, although their roles are not established. One possible role of FGF signalling at this early stage is suggested by the finding that FGFs can stimulate SOX9 expression (see below) in a cultured mesenchymal cell line (Murakami et al., 2000). As chondrocytes form, proliferating chondrocytes express FGFR3 and prehypertrophic/hypertrophic chondrocytes express FGFR1. The first demonstration that FGFR3 signalling is crucial in growth-plate physiology came from reports that mutations in *FGFR3* were responsible for several forms of dwarfism in humans (Tavormina et al., 1999).

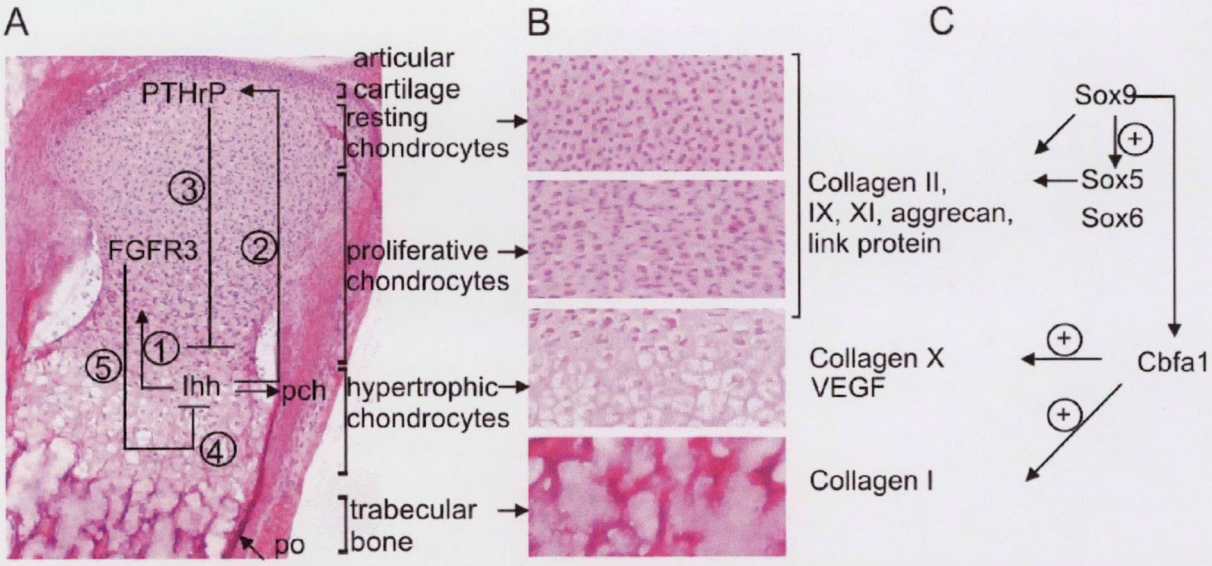

Figure 3. Cellular organization of the growth plate and the main regulators of the endochondral ossification. (A and B) Zones of the growth plate of newborn mouse tibia stained by haematoxylin and eosin. (A) Major signalling molecules and their receptors. Ihh acts on its receptors and stimulates chondrocytes proliferation (1) and PTHrP (2) production. PTHrP keeps the chondrocytes proliferating and inhibits Ihh production of hypertophic chondrocytes (3). Ihh influences the perichondrial cells to convert to osteoblasts (4). FGFR3 has a role in chondrocyte proliferation and inhibits the production of Ihh (5), pch, perichondrium; po, periosteum. (B) Zonal expression of the main ECM components of the growth plate. (C) Transcriptional regulation of chondrocyte and osteoblast differentiation by Sox5, 6, 9 and Cbfa1. Sox9 is the main regulator of cartilage ECM genes and of Sox5, 6 and Cbfa1. Cbfa1 is essential for osteoblast differentiation.

Each of these receptors has distinct and important roles in bone development; FGFR3 is best understood. Knockout of *Fgfr3* in mice leads to an increased rate of proliferation of

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chondrocytes and an expansion of the length of chondrocyte columns (Colvin et al. 1996; Deng et al., 1996). Activating point mutations in *Fgfr3* that are found in human chondrodystrophies decrease the rate of proliferation of chondrocytes and lead to shortened, disorganized columns in transgenic mice (Naski et al., 1998).

Knockout of the *Fgfr3* gene increases Ihh expression and activation of *Fgfr3* decreases Ihh expression (Naski et al., 1998; Ornitz et al., 2002). *In vitro* studies, in which levels of Ihh and PTHrP can be regulated independently, support the idea that part of the effects of FGF signalling is mediated by suppression of Ihh expression (Minina et al., 2002). Thus, FGF signalling shortens proliferative columns both by decreasing chondrocyte proliferation directly and by suppressing Ihh expression (Fig. 3A).

2.2.3. The major ECM components of cartilage and bone

Extracellular matrix components of cartilage and bone (Fig. 3C) have critical roles in skeletal development. Collagens, noncollagenous glycoproteins, and proteoglycans are assembled in a highly organized fashion in the matrix. Collagen II, a homotrimer encoded by *COL2A1*, is the major collagen of cartilage. In addition to collagen II, a number of minor collagens (IX, X, XI, and XII) also contribute to the cartilage matrix. Collagen IX is a heterotrimeric nonfibrillar collagen composed of three different α chains, α1(IX), α2(IX), and α3(IX) that are coexpressed in cartilage and other cartilage-like tissues with collagen II. Collagen IX molecules are localized on the surface of the fibrils where they are cross-linked to residues within the N- and C-telopeptides of collagen II (for review, see Olsen, 1997). Collagen X, a member of the short chain collagen family, is a homotrimeric molecule expressed specifically by hypertrophic chondrocytes during endochondral ossification. Collagen XI, a quantitatively minor collagen in cartilage, copolymerizes with collagen II and appears to regulate the fibril diameter (for review, see Petit et al., 1992; Olsen, 1996). Collagen I is the major collagen triplex in mineralized tissues. It is composed of two α1[I] chains and one α2 chain α2[I].

Aggrecan is the principal proteoglycan molecule in the cartilage matrix. 80-100 chondroitin sulfate side chains are attached to the aggrecan core protein. More than 100 aggrecan molecules bind to HA polymers, leading to formation of complexes over 10^8.

The aggrecan-HA aggregates provide the osmotic resistance necessary for cartilage to resist compressive load (Hascall et al., 1991). Decorin and biglycan, two smaller proteoglycan molecules, may also serve important functions. Decorin, for example, coats the outside of the collagen fibrils and may play a role in the regulation of collagen fibrillogenesis. Decorin binds TGF-ß, may sequester or release it, influencing chondrocyte proliferation (Dallas et al., 2000).

Cartilage link protein (Crtl1), an extracellular matrix glycoprotein in cartilage, stabilizes the interaction of aggrecan and hyaluronan. In the absence of Crtl1, aggregates are smaller and less stable (Hascall et al., 1991; Neame and Barry, 1993; Hardingham et al., 1994). Fibronectin is one of the most abundant non-collagenous glycoproteins in the bone matrix, which may regulate mineralization in bone (Couchourel et al., 1999; Daculsi et al., 1999). Fibronectin and collagen I can bind to integrin receptors via their RGD motif, influencing in this way osteoblast survival, proliferation and differentiation. COMP is an extracellular calcium binding glycoprotein belonging to the thrombospondin family, it has a role in the cartilage matrix assembly (Hecht et al., 2005).

2.3. Antler development, as a unique model of bone regeneration

2.3.1. Antler development

Antlers of a red deer stag are large branched bony structures that are used for display and fighting. The annual re-growth of antlers constitutes the only example of regeneration of a complete bony appendage in mammals (Goss, 1983; Bubenik, 1990). Antlers do not grow directly from the top of deer skull, they are cast and regenerate from the apices of permanent protuberancens of the frontal bones, the pedicles (Li and Suttie, 2001). Timing of the antler cycle is closely linked to seasonal fluctuation in concentration of circulating testosterone (Bubenik, 1990) (Fig. 4).

Antlerogenic periosteum (AP) is that piece of periosteum, which covers the lateral crest of prepubertal deer frontal bone, and gives rise to pedicles and antlers (Li and Suttie, 2001). The importance of AP in the pedicle and antler formation was shown by deletion and transplantation experiments. AP is built up by two layers: an inner cellular layer and outer fibrous layer (Li and Suttie, 2001). These layers are thicker than that of the facial periosteum. AP presents embryonic stem tissue features until deer postnatal life like self-differentiating capability, incredible growth potential and richness in intracellular glycogen. It is considered that AP is a piece of postnatally retained embryonic tissue. Antler development is a unique

example of rapid growth accompanied with mixed type ossification (Banks and Newbrey, 1983). Antler re-growth is initiated at the distal part of the pedicle, where mesenchymal cells of the AP sequentially proliferate and differentiate into chondroblasts and chondrocytes.

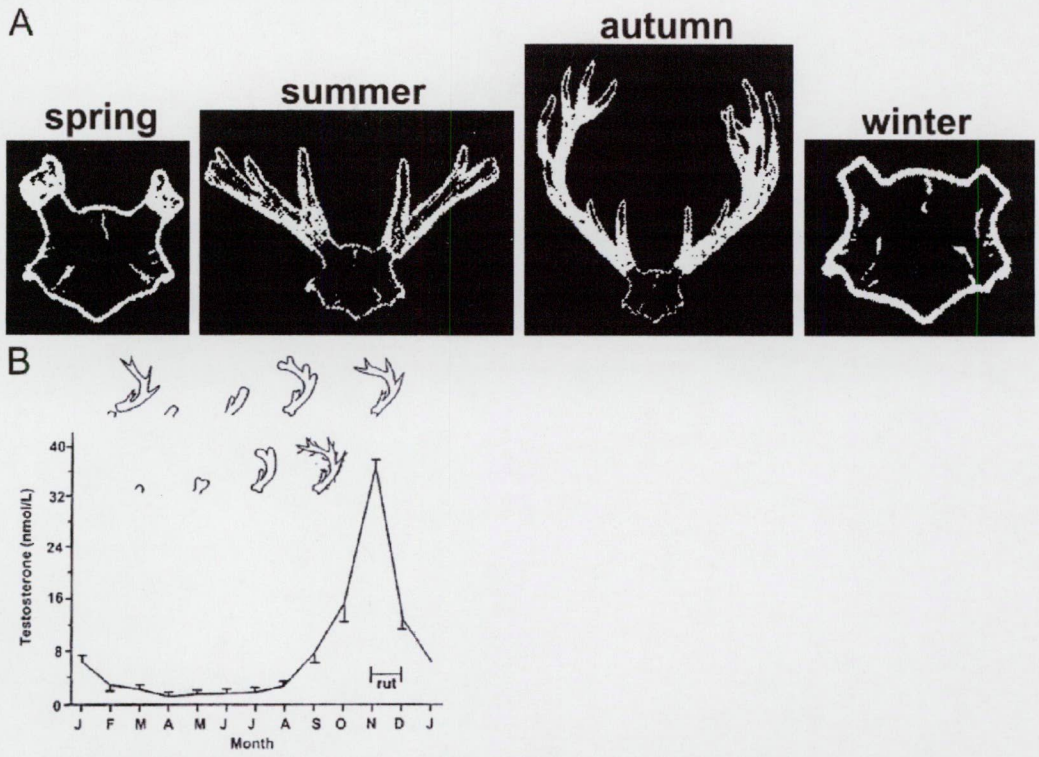

Figure 4. Summary of antler development. (A) Seasonal changes; (B) Regulation of antler development by seasonal level of testosterone (Bubenik, 1990).

Chondrocytes are arranged in longitudinal trabeculae, separated by an extensive vascular network. This abundant blood supply is a source for haematopoetically derived chondro-/osteoclast progenitors. As a result of chondroclastic activity, the cartilaginous columns, in disto-proximal direction lose their distinct, parallel relationship and form anastomotic columns of irregular shape. On the cartilage scaffold, ossified bony trabeculae are formed by appositional mineral deposition. At the periphery of the antler the bone is formed via intramembranous ossification by osteoblasts derived from the osteoprogenitors of the periosteum. Mineralization gradually occludes the abundant blood supply of the antler. Growth slows, and then stops and the spongiform bone increases in density by appositional bone formation. Finally, the antler velvet skin is shed and the "polished" antler is ready for the rutting season.

2.3.2. ECM during antler development

The same structural molecules seem to be involved in antler development as in the long bone growth. Although similarities of antler development and embryonic bone formation, antler tissue show differences regarding to expression pattern of the main matrix proteins, collagens I, II and X implicated in endochondral ossification (Price et al., 1996). In antler collagen I is expressed in perichondrium, in the cells adjacent to perichondrium and the expression goes on until the cells begin to assume columnar arrangement. Mature chondrocytes do not express any more collagen I, the expression is limited to a distinct population of small, flattened cells aligned on the periphery of the cartilage trabeculae.

Collagen IIA is expressed by prechondrocytes, cells below the mesenchyme, not fully differentiated to chondrocytes. The collagen IIB variant is expressed by the mature chondrocytes. Expression of collagen X was observed in hypertrophic chondrocytes of the cartilage columns (Allen et al., 2002). Aggrecan and decorin were also identified by biochemical means (Sunwoo et al., 1998). Most recently deer antler proteome analysis identified biglycan and desmocollin 1 and 3 (Park et al., 2004).

3. 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.

4. MATERIALS AND METHODS

4.1. Specimen handling

Mouse embryos and newborn animals were obtained from mating of C57BL/6xCBA F1 hybrid males and females. Plug recorded matings were isolated at various days through pregnancy. Embryos and fetuses were submitted to RNA isolation followed by RT-PCR analysis. From 14.5 dpc-17.5 dpc embryos and newborn mice the limbs were isolated, washed in PBS and embedded in Tissue tek O.T. C: Compound (Sakura) cryomount, and stored at -80°C until required. Either parasagittal or cross sections, 15μm thick, were cut on a Leitz 1720 Digital Kryostat.

Red deer velvet antlers were collected from the Deer Farm of Pannon Equestrian Academy, Bőszénfa, Hungary, during commercial harvest. Close to the apex, one quarter of the antler tip was sectioned parasagittally. Another quarter was cut horizontally into 5 mm thick blocks denoted consecutively as level a, b, c, d, etc., starting at the apex. 2-month old fetuses were collected from pregnant female hinds shot for selection purposes. One part of tissue specimens was used to prepare cryoblocks, the other part was fixed overnight in 4 % freshly hydrolyzed paraformaldehyde in PBS, dehydrated in ethanol, cleared in xylol and embedded in low-melting-point paraffin (Paraplast, Sigma). Sections were mounted on slides coated with (3-aminopropyl) triethoxy-silane. 10 μm cryosections were used for immunostaining and 5 μm paraffin sections were used for *in situ* hybridization.

Human cell cultures. Permanent human cell lines WI26 and Hep-2 were obtained from the American Type Culture Collection. The primary culture of normal human skin fibroblast was obtained as previously described (Krieg et al., 1980) and was a gift from Dr. M. Aumailley. The cell cultures were maintained in DMEM supplemented with 10% FCS (Gibco Life Sciences) and utilized for RNA isolation.

4.2. Histology and immunohistochemistry

Haematoxylin-eosin staining. The 5 μm paraffin sections were deparaffinized, rehydrated and stained in Mayer's haematoxylin for 10 min and kept under running tap water for nuclei color development. After washing in distilled water, the sections were stained in 1% eosin in distilled water for 5 min. After the final washing step the sections were dehydrated through increasing alcohol series, cleared in xylene, and mounted using DPX mounting media (Fluka).

Alcian blue and von Kossa staining (Ab-vK). 10 µm cryosections were fixed in acetone, rinsed in distilled water and stained in 2% silver nitrate solution for 30 min. at room temperature. After washing in distilled water, sections were placed in 2.5% sodium thiosulphate for 5 minutes. The sections were washed again in distilled water and immersed first in 3% acetic acid for 30 min then in 1% alcian blue dissolved in 3% acetic acid (pH 2.5) for 30 min at room temperature. After rinsing twice each in 3% acetic acid and distilled water, the sections were dehydrated in ethanol series and mounted using DPX mounting media (Fluka).

Immunohistochemistry

Immunohistochemistry was performed on acetone-fixed 10 µm cryosections. To improve antibody penetration, the tissue sections were treated with 0.5 mg/ml collagenase type II (Sigma) in 0.25 M NaCl, 50 mM TrisHCl pH 7.4 and 1 mM EDTA at 37°C for 30 min. For collagen detection we used 40mU/ml chondroitinase ABC (Fluka) in Tris-buffered saline (TBS) containing 0.01 % bovine serum albumin for 1 h at room temperature for optimal antigen retrieval. To avoid nonspecific binding of the antibodies, the sections were incubated for 1 h with 10% (w/v) normal goat serum in TBS. Using primary antisera (Table 1) with overnight incubation at 4°C followed by affinity-purified, peroxidase-conjugated goat anti-rabbit IgG antiserum and color development 3-amino-9-ethylcarbazole as substrate. The sections were incubated 45 min at room temperature with the secondary antibody (Table 1). Antler immunohistochemistry sections were counterstained with Mayer's haematoxylin and mounted with Kaiser's glycerol gelatin. Negative controls not including primary antibody were applied for each experiment.

Immunofluorescence staining

Acetone fixed cryosections were also used for immunofluorescence staining and the antigen retrieval and blocking was done as described above. The secondary antibodies were conjugated with different fluorochromes (Table 1). After rinsing the sections were mounted with fluorescent mounting medium (Dako) and viewed and photographed with a Nikon Eclipse E600 microscope equipped for epifluorescence. For double immunofluorescence, we used primary antibodies raised in rabbit and chicken against Matn1 and -3 (Table 1). Negative controls not including primary antibody were applied for each experiment.

Table 1. Antisera used for immunohistochemsitry

Primary antisera	**Dilution**	**Origin**
Rabbit anti mouse Matn1	1:400	Hauser and Paulsson, 1994
Rabbit anti mouse Matn2	1:100	Piecha et al., 1999
Rabbit anti mouse Matn3	1:100	Klatt et al., 2000
Rabbit anti mouse Matn4	1:100	Klatt et al., 2001
Chicken anti mouse Matn1	1:200	gift from Raimund Wagener
Chicken anti mouse Matn3	1:200	gift from Raimund Wagener
Rabbit anti bovine cartilage link protein	1:400	gift from Dick Heinegard
Rabbit anti mouse type I collagen	1:400	gift from Rupert Timpl and Attila Aszódi
Rabbit anti mouse type II collagen	1:400	gift from Rupert Timpl and Attila Aszódi
Rabbit anti mouse laminin 1	1:800	gift from Rupert Timpl and Attila Aszódi
Rabbit anti mouse FGFR3	1:100	gift from Rupert Timpl and Attila Aszódi
Rabbit anti mouse Ki67	1:800	gift from Rupert Timpl and Attila Aszódi
Secondary antisera		
HRP-conjugated goat anti rabbit IgG	1:100	Sigma
Cy3 conjugated goat anti chicken IgG	1:600	Jackson ImmunoResearch Laboratories
Alexa 488 conjugated anti rabbit IgG (H+L)	1:600	Molecular Probes

4.3. Protein extraction and Western blot analysis

Tissue pieces were homogenized by a Turrax homogenizer in tenfold volume of 0.1 M NaCl, 50 mM Tris-HCl, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride and 2 mM N-ethylmaleimide (pH 7.4) buffer. Debris was removed by centrifugation at 10000 × g for 10 min at 4 °C. Proteins in the supernatant were separated by SDS-polyacrylamide gel electrophoresis, and blotted onto Protran nitrocellulose membranes (Schleicher and Schuell).

Matrix proteins were identified with specific rabbit antisera followed by horseradish peroxidase-conjugated affinity-purified goat anti-rabbit IgG (Sigma) and the enhanced chemiluminescence protocol.

4.4. Cloning of the red deer matrilin and cartilage link protein genes

The velvet skin was pulled off from the developing antler. The soft tissue underneath was scraped off with a scalpel and served as a sample enriched in mesenchyme cells. Immediately below a tissue slice, without apparent cartilage trabeculae, was considered as precartilage sample. Trabecular cartilage sample was gained from the center of a more proximal tissue piece. RNA samples were isolated and gene specific cDNA fragments were amplified as described below (see 4.5.1.) and the RT-PCR products were cloned into pBluescript II SK^+ vector. The identity of deer clones was confirmed by nucleotide sequencing. Gen Bank accession numbers are C.e.Crtl1 AY831770; C.e.Matn1 AY831771; C.e.Matn2 AY831772; C.e.Matn3 AY831773; C.e.Matn4 AY831774.

4.5. Analysis of RNA production

4.5.1. RNA purification and RT-PCR

Total RNA was prepared from guanidinium-thiocyanate extracts of tissue samples and cell cultures using acidic phenol-chloroform extraction (Chomczynsky and Sacchi, 1987). cDNA was generated with SuperScript™ II reverse transcriptase (Invitrogen), using 1 μg of total RNA template and gene-specific primers (Table 2). One tenth of cDNA was subjected to polymerase chain reaction. By using the appropriate annealing temperature according to the primer pair used in a given reaction, all PCRs were performed in the following reaction cycles: one cycle of 95°C for 1 min, annealing temperature for 1 min, 72 °C for 3 min followed by 30 cycles of 95°C for 1 min, annealing temperature for 1 min, 72 °C for 1 min. The reaction was finished by incubation at 72 °C for 7 min. The PCR products were separated on agarose gel and visualized by ethidium bromide staining.

Oligonucleotide primers used for identification of alternative promoter of *MATN2* by RT-PCR are shown in Table 2.

Table 2. Oligonucleotide primer sequences

Name	Sequences	Position
Human primers		
pQn	AGGCCGACGAGGAAGACC	41941–41958 [a]
pRn	CCAACTATGCATGCCCTGCT	60583–60603 [a]
pSn	AAGCTCTCAAAGGCCAAACA	60641–60650 [a]
pTn	TGAGTGCAAGTGGTCTGTTTG	60675–60695 [a]
pVn	GGTCACGTGGGAGGTCCA	202–219 [b]
Mouse primers		
pKr	TCTGCCCGCTTATTCTCACA	430–411 [c]
pMr	CTTGACCGTGCTGCCATATT	578–559 [c]
Primers for RT-PCR of mouse embryos		
CRP2635/19R	CCTACCCCAACGGCATACA	
CRP1001/20N	CACTTCTGCCTCAACACACC	
CRP1250/22R	CGTGTTCACATCCATGATTTC	

Accession Numbers: [a] AP002906; [b]U69263
[c] Positions are given from start of the longest transcript.

Table 3. Probes for *in situ* and blot hybridization

gene	probe origin	fragment (bp)	primer sequence	primer origin	T_a (°C)
Matn1	*Cervus e.*	680	GGCCCACAGACCTGGTGTT GCCACCGTTGCAGACATTG	*Homo s.* *Bos t.*	59
Matn2	*Cervus e.*	383	CATTGACAAGCATCTCTTCT CTGTATCTCAGGCGATTTTC	*Mus m.* *Homo s.*	53
Matn3	*Cervus e.*	440	GGGTGGCAGTGGTGAAC CGGGTCCAGAGCACAAA	*Bos t.* *Bos t.*	57
Matn4	*Cervus e.*	433	TCCCCGGACTTCAGCAC CGCAGTCCGACTTCTGGT	*Bos t.* *Bos t.*	61
Crtl1	*Cervus e.*	520	GACCCTACAGCATTTGGCTC CGTAGTTCCTGACTCCAGGC	*Mus m.* *Mus m.*	56
SOX9	*Homo s.*	633 [a]			
COl2a1	*Homo s.*	905 [b]			
Gapd	*Mus m.*	402 [c]			

T_a, annealing temperature in PCR; [a] Subcloned from full-length cDNA of De Santa Barbara et al., 1998; [b] Elima et al., 1987; [c] gift from P. Szabó, Beckman Research Institute of the City of Hope, Duarte, CA.

4.5.2. Northern blot analysis

For RNA blot analysis 10 μg aliquots were electrophoresed, blotted to Hybond-N filter (Amersham) and hybridized consecutively with ^{32}P-labeled cDNA fragments. Hybridization probes used for RNA-blot hybridization and *in situ* hybridization are summarized in Table 3. The signal intensities of different samples were compared using Phosphorimage Analyzer 445 SI (Molecular Dynamics).

4.5.3. In situ hybridization

5 μm paraffin sections were deparaffinized and rehydrated, then digested with proteinase K (Merck, 1 μg/ml in PBS), postfixed, and acetylated in 0.25 % acetic anhydride. Riboprobes were labeled with digoxygenin-UTP. Prior to hybridization, the sections together with probes were denatured at 94°C for 3 min and then incubated at probe-specific temperature for 12-16 h. After hybridization, the tissue sections were washed at the hybridization temperature in 2 x SSC, 50% formamide, 20 min, treated with 20μg/ml RNAse A in TNE buffer (10mM TrisHCl, pH 7, 0.5 M NaCl, 1mM EDTA) at 37°C 30 min and further washed at 50 °C in 2 x SSC, 0.01% TritonX-100; 0.1 x SSC, 0.01% TritonX-100, 5mM EDTA, 20 min each. Digoxygenin was detected by sheep anti-digoxygenin alkaline phosphatase Fab fragments (Roche), followed by alkaline phosphatase reaction using nitrotetrazolium blue chloride and 5-bromo-4-chloro-3-indolyl phosphate. Sections were mounted using Kaiser's glycerin-gelatin.

5. RESULTS

5.1. Identification of alternative promoters of the *MATN2* gene

The human gene showed a high degree of sequence conservation not only within exon 1, but in the upstream promoter region as well. The *MATN2* promoter also lacked a TATA motif and carried putative Sp1 sites in conserved positions (data not shown). In the presumed downstream promoter region, a potential transcription start site CATTTT was found 150 bp upstream of the translation start site. The motif was preceded by the TATA-like ACTTAA sequence (Fig. 5).

To address the question which of the two promoters is preferred in human cells, we carried out RT-PCR analysis on RNA samples isolated from cell cultures. A set of oligonucleotide primers was designed to investigate how far into the 5' direction the transcribed region protrudes (Table 2 and Fig. 6A). Reverse primers pKr and pMr from exon 3 were combined with forward primers from exon 2B (pVn) or further upstream (pTn, pSn and pRn). We detected downstream promoter activity in RNA samples prepared from HEp-2 epithelial and WI-26 lung fibroblast cell lines but not in a primary culture of adult skin fibroblasts (Fig. 6B).

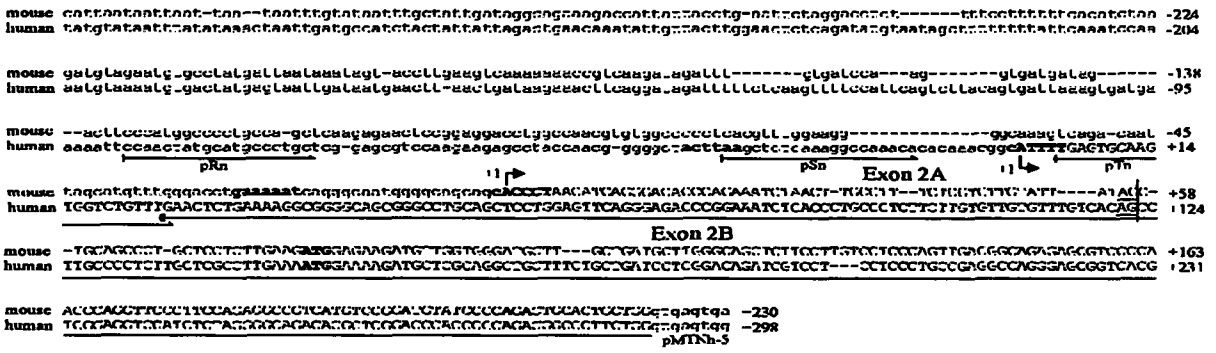

Fig. 5. Comparison of the downstream promoter regions of *Matn2* and *MATN2*. Translation initiation sites, TATA-like motifs and putative transcription start sites are boldfaced. The highlighted letters indicate matching bases. The 5' end of pMTNh-5 cDNA clone and positions of primers used to map the start sites are delineated.

The pQn-pKr primer combination in all samples gave amplified fragments, indicating that the upstream promoter was active in all cell cultures tested. Only the pTn-pKr combination resulted in the amplified fragment, indicating that the transcripts do not protrude

up to the pSn oligonucleotide sequence. We designated A in the CATTTT motif found within the region as the most probable transcription start point. 34 bp further upstream a TATA-like motif (ACTTAA) was found (Fig. 5).

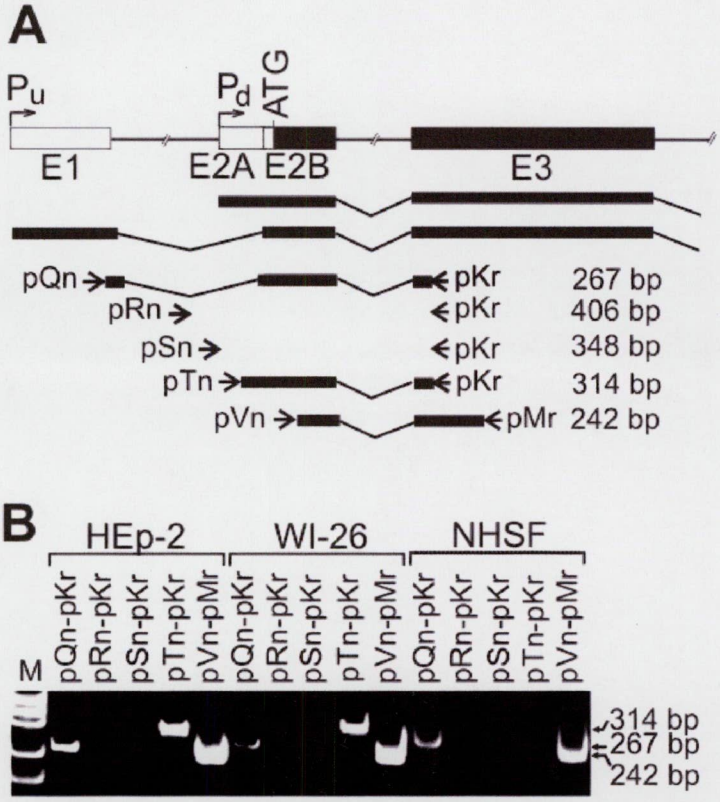

Figure 6. Identification and tissue specificity of the downstream promoter of *MATN2* by RT-PCR analysis. (A) Schematic drawing of the PCR primers (Table 2) and products with respect to the positions of exons and the two promoters. Open bars indicate the 5'-UTR. (B) RT-PCR analysis of RNA samples derived from human permanent cell lines (HEp-2 and WI-26) and primary culture of adult skin fibroblasts (NHSF). The anneling temperature of all PCR reactions was 57°C. RT-PCR products were electrophoresed in polyacrylamide gels. M, *Msp*I digest of pUC12 (B).

5.2. Expression of Matn2 during mouse embryonic development

We performed RT-PCR analysis in order to follow expression of Matn2 during embryonic development. Matn2 mRNA was detectable already at age of 7.5 dpc and the amount of Matn2 transcript increased with fetal age (Fig. 7).

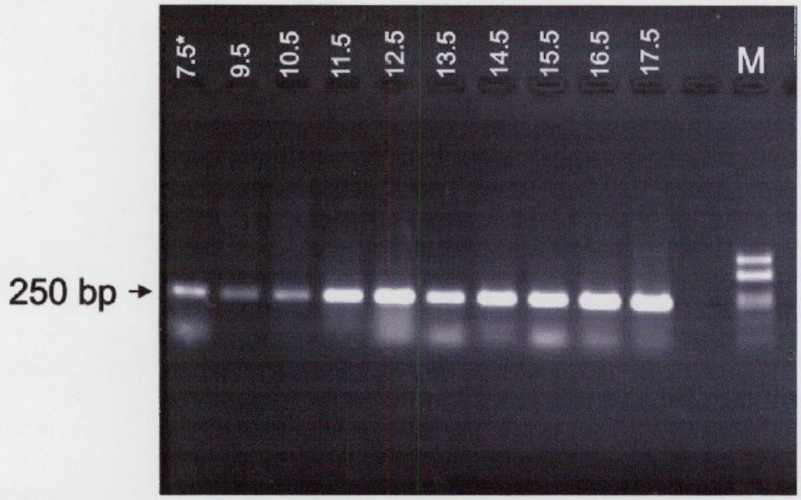

Figure 7. RT-PCR analysis of *Matn2* expression during development from 7.5 dpc to 17.5 dpc mouse embryos. RNA was isolated from 7.5 dpc embryos within the decidua. The amnion was removed from all other embryos before RNA extraction. CRP2635/19R oligonucleotide was used for RT and CRP1001/20N, CRP1250/22R for PCR reaction. Oligonucleotide sequences are shown in Table 2. The anneling temperature of PCR reaction was 51°C. M, *Msp*I digest of pUC12.

Matn2 immunoreactivity was detected in extraembryonic tissues, such as Reichert's membrane, ectoplacental cone and in basement membrane of capillaries from the mesometrial zone of decidua during the peri-implantation period of 7.5 dpc embryo (data not shown). From 9.5 to 17.5 dpc mouse embryos Matn2 immunosignal was observed as soon as the corresponding structures developed: in the developing heart, in the tunica adventitia of bronchioles; in the meninges (dura mater, pia mater, arachnoidea); perineurium, epineurium, endoneurium of the peripherial nerves; in the perimysium, epimysium and endomysium of the skeletal muscle; in the lens capsule, basement membrane of notochord cells and blood vessel endothels (data not shown).

In this thesis I focuse on the skeletal expression of Matn2 during embryonic development by comparing to the localization of the other matrilins.

5.3. Expression of matrilins during ossification

5.3.1. Expression of Matn1, Matn2 and Matn3 during endochondral ossification in the developing limb

To characterize tissue distribution of Matn1, Matn2 and Matn3 at various stages of development limb buds or limbs were removed from embryos and submitted to immunohistochemistry. Matn1 and Matn3 were first detected by immunohistochemistry at 12.5 dpc in the condensing cartilage of the limb bud (data not shown). This was the earliest expression of Matn3 in any part of the embryo. During development, the signal intensity of Matn1 and Matn3 increased as cartilage differentiationprogressed. In the elbow joint at 14.5 dpc (Fig. 8) Matn1 and Matn3 showed the same expression pattern as type II collagen. All matrilins were found in the resting, proliferating and hypertrophic zones of epiphyseal cartilage. Matn2 expression was more restricted to several regions, such as perichondrium, hypertrophic zone of epiphyseal cartilage and articular cartilage surface. Matn1 and Matn3 were missing from the articular cartilage.

This expression pattern is maintained during endochondral ossification of the developing limb, as it was shown in sections from 17.5 dpc embryos and newborn mice. Intense accumulation of Matn2 was seen in the developing joints of fingers at 17.5 dpc (Fig. 9A), newborn knee joint and tendon (Fig. 9B); however, Matn3 was missing from articular cartilage and tendon as well (Fig. 9C and D). In humerus of newborn limb, Matn1 and -3 show uniform expression along the epiphyseal growth plate, whereas Matn2 is restricted to the late proliferative and early hypertrophic zone (data not shown). All three matrilins appear associated to bony matrix (data not shown).

Double immunfluorescence staining revealed tiny differences in the immunoreactivity of Matn1 and Matn3 (Fig. 10). Matn3 is more extended under the articular surface than Matn1 (Fig. 10A-C). Furthermore, Matn3 was found to be present in the territorial matrix of the cartilage and in the interterritorial matrix both of Matn1 and Matn3 observed (Fig 10D). Double immunolocalization, using rabbit anti-mouse Matn1, Matn3, and chicken anti-mouse Matn1 and Matn3 demonstrated this.

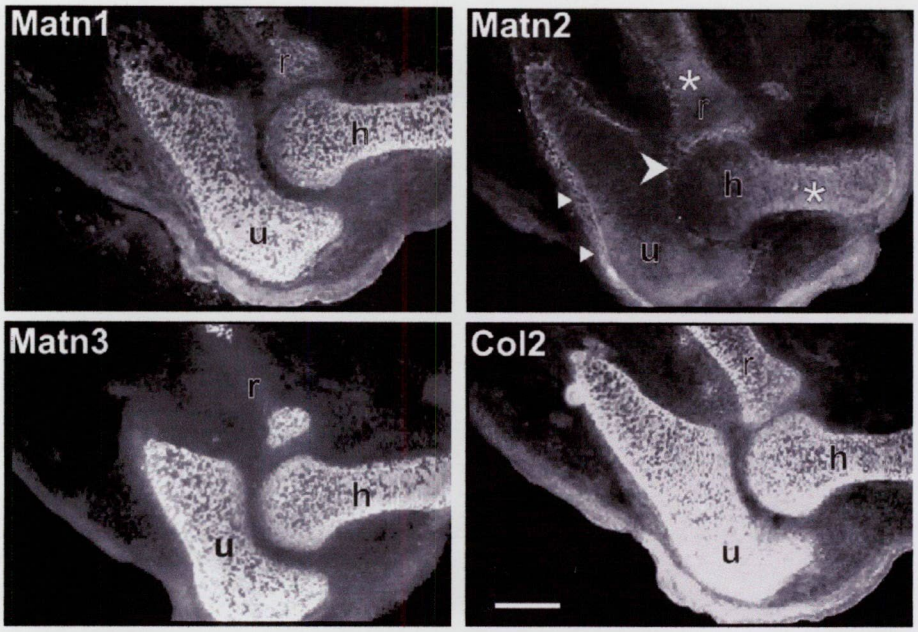

Figure 8. Immunofluorescence detection of Matn1, Matn2 and Matn3 in the elbow of a 14.5-day mouse embryo. The developing humerus (h), radius (r) and ulna (u) are marked. Matn1 and Matn3 as well as collagen II are detectable in the cartilage anlage of the limb skeleton, in the zones of proliferating and early hypertrophic chondrocytes. Matn2 can be observed in hypertrophic cartilage (*), in the perichondrium (►) and at the articular surface (➤). Bar 0.2 mm.

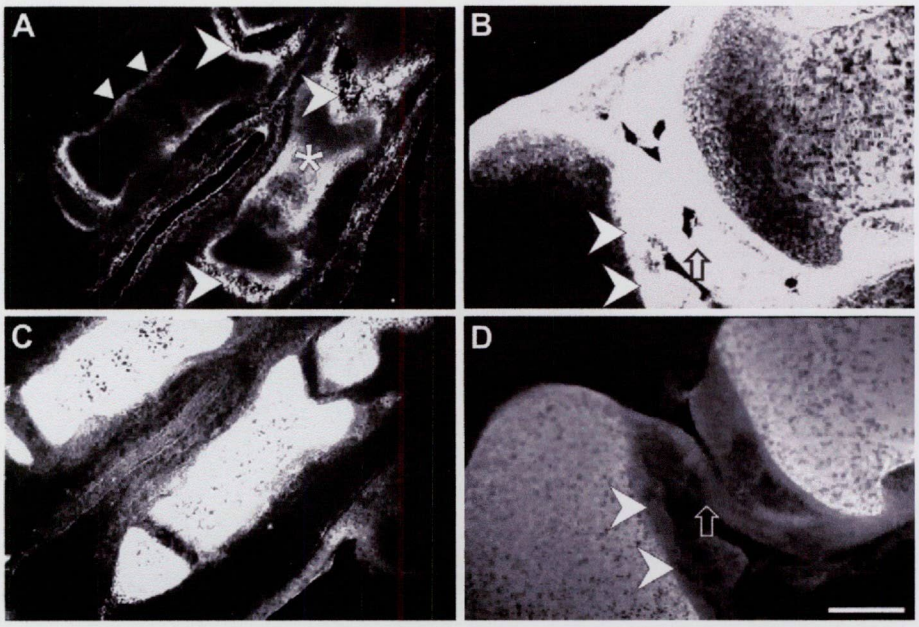

Figure 9. Immunofluorescence detection of Matn2 (A, B) and Matn3 (C, D) in the digits of 17.5 dpc mouse foetus (A, C) and in the knee joint of newborn mouse (B, D). Matn2 is expressed in the perichondrium (►) and developing articular surface (➤), in the hypertrophic zone of epiphyseal cartilage (*) and in ligaments (⇦). Matn3 shows uniform expression in cartilage and it is absent from the articular carilage (➤) and ligaments (➘). Bar 0.2 mm.

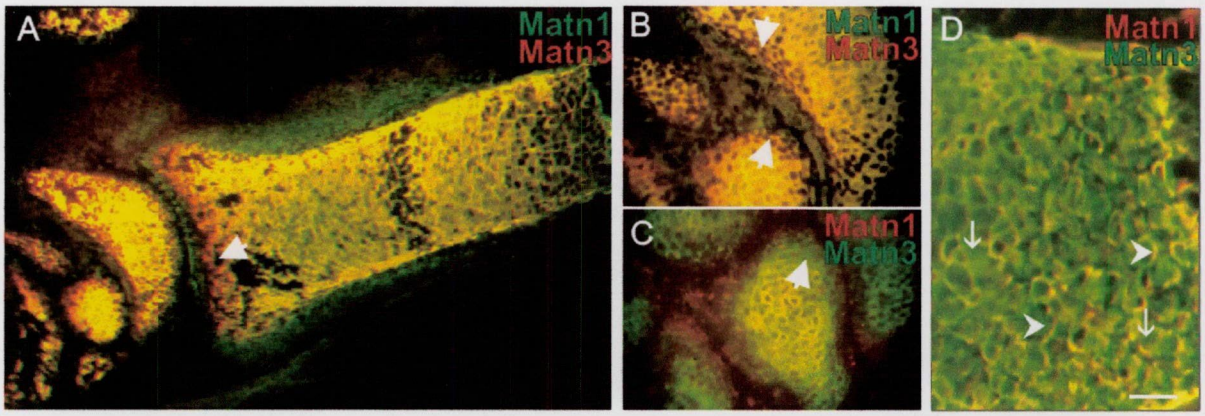

Figure 10. Double immunofluorescence detection of Matn1 and Matn3 in the skeletal structures of the upper limb of 16.5 dpc mouse foetus. Both of the matrilins are strongly expressed in cartilage, although Matn3 is more abundant immediately under the articular cartilage, indicated by the arrowhead (A-C). Matn1 and Matn3 are coexpressed in the interterritorial matrix (➤), but in the territorial matrix mainly Matn3 (↓) is expressed (D). Bar 0.2 mm in A and 0.1 mm in B-D.

5.3.2. Expression of matrilins during intramembranous ossification of newborn mouse skull

In the present study, we performed immunohistochemical studies on cross sections from the occipital region of newborn skull (Fig. 11). The cartilaginous matrix was indicated by alcian-blue staining and von Kossa staining visualized mineral deposition. Matn1, Matn3 and Matn4 were detected in the cartilage primordium of the occipital bone, however, Matn2 was mostly restricted to the perichondrium. Cartilage link protein (Crtl1) was also found in the cartilaginous elements of developing skull. Immunostaining for type I collagen was used to identify bony structures. Matn2 and Matn3 were abundant in occipital bone formed by intramembranous ossification, directly from mesenchyme cells, without cartilage template. Matn1 and Matn4 were found at low level in occipital bone, whereas cartilage link protein was completely missing.

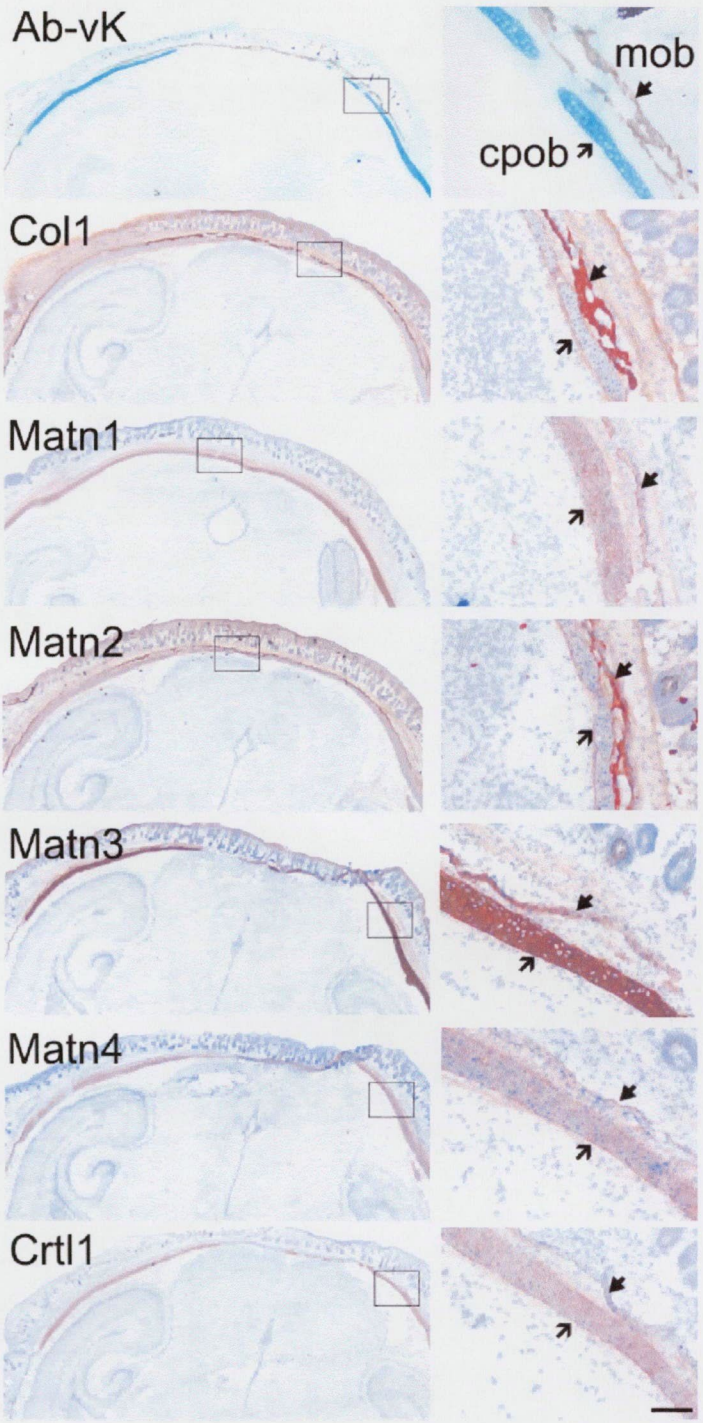

Figure 11. Immunohistochemical detection of matrilins and cartilage link protein in newborn skull. Alcian-blue staining shows the cartilaginous primordium of occipital bone (cpob, ↓), von-Kossa staining indicates the membranous bony part of the occipital bone (mob, ➔). Immunostaining for type I collagen was used as a positive control for bony structures. Matrilins are present both in cartilage primordium and in membranous bony part of occipital bone with different intensity. Crtl1 is present only in the cartilaginous primordium. Bar 1 mm in the left column; 0.1 mm in the right column.

5.4. Changes of the level and distribution of matrix proteins and regulatory molecules during antler development

5.4.1. Distribution of ECM markers in deer growth plate cartilage

Specificity of the antisera and cross-reactivity with deer proteins was tested in immunoblot and immunohistochemistry. Immunoblot analysis of antler samples showed that antisera against both Matn1 and cartilage link protein reacted specifically with the corresponding antigens (Fig. 12). Without sample reduction Matn2 appeared as a set of multiple electrophoretic bands, resulting from proteolytic processing of the homo-oligomer, as observed previously. Crtl1 gave two bands around the size marker of 45 kDa relative electrophoretic mobility. The link protein was abundant in the antler cartilage extract, and little, but clearly detectable in the sample of mesenchyme origin. Matn2 signal, on the contrary, was stronger in the mesenchyme sample, and less intense in the cartilage extract (Fig. 12). Detection of Matn1 and Matn2 oligomers in the cartilage extract was further hampered by the strong proteolysis in the tissue.

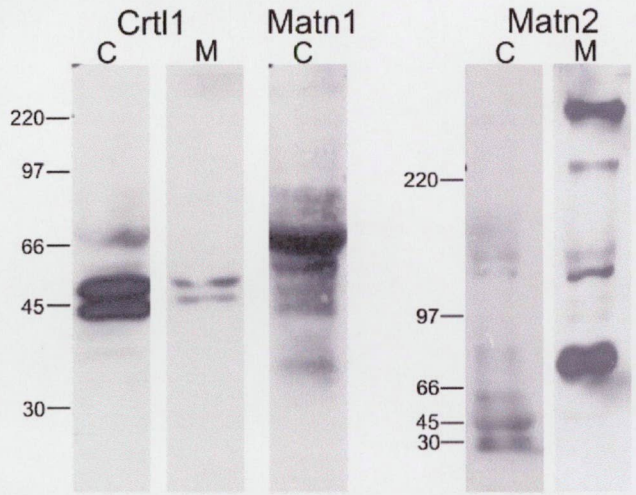

Figure 12. Immunoblot analysis of antler sample extracts. Apical mesenchyme cell region (M) and cartilage tissue (C) samples were extracted and aliquots were submitted to electrophoresis in 12 % (Crtl1, Matn1) or 4-12% (Matn2) SDS-polyacrylamide gels without prior reduction.

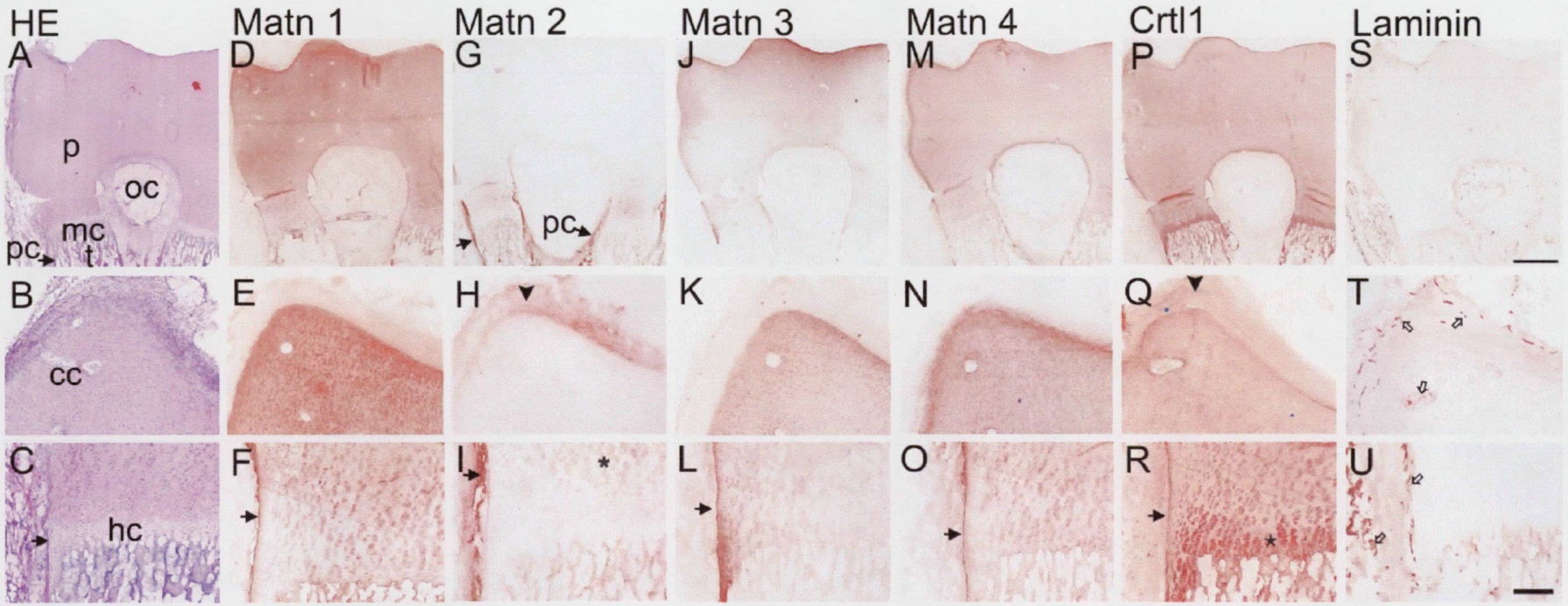

Figure 13. Immunolocalisation of matrilins and cartilage link protein in the distal epiphysis of the developing humerus of red deer fetus. The distal end of humerus is sectioned in a frontal plane, close to the rear side. Therefore, the olecranon fossa has been cut through and the perichondrium bordering the fossa is also visible. (A-C) Hematoxylin and eosin (HE) staining of deer fetus epiphysis. Matn1 (D-F), Matn3 (J-L), Matn4 (M-O) and Crtl1 (P-R) are detectable in the zones of proliferating (p) and hypertrophic chondrocytes (hc). Matn-2 (G-I) is expressed in the prehypertrophic cartilage (asterisk), mineralized cartilage (mc) and subchondral bone. Crtl1 (R) is most abundant in hypertrophic cartilage (asterisk). All five matrix proteins are deposited in the perichondrium (pc, arrow), but Matn2 is the most abundant. The developing articular cartilage is positive for Matn2 (H) and Matn4 (N) and the articular capsule (arrowhead) is stained for Matn2 (H) and Crtl1 (Q). Immunostaining of laminin (S-U) detects the capillaries (empty arrow) in the cartilage canals (cc), as well as in loose connective tissue attached to perichondrium and articular capsule. Bar 1 mm in the top, 0.2 mm in the middle and bottom panels; oc, olecranon fossa; t, trabecular bone.

The antisera were tested in histological sections of the humerus of red deer fetus (Fig. 13). Matn1 (Fig. 13D), Matn3 (Fig. 13J), Matn4 (Fig. 13M) and Crtl1 (Fig. 13P) were detected almost uniformly in the zone of proliferating chondrocytes. Reactivity of the Matn2 antiserum confined to a limited area of prehypertrophic chondrocytes (Fig. 13I). In the zone of hypertrophic chondrocytes, the immunoreactivity persisted for Matn1 (Fig. 13F), Matn3 (Fig. 13L) and Matn4 (Fig. 13O), and was enhanced in the territorial matrix for Crtl1 (Fig. 13R). Both the trabecular bone and the perichondrium adjacent to the hypertrophic zone were immunopositive with all five antisera (Fig. 13F-R). Toward the distal end of the developing humerus, the articular surface showed enhanced positivity for Matn4 (Fig. 13N), whereas the developing articular capsule and fetal connective tissue attached to the lateral perichondrium gave signal for Matn2 (Fig. 13H) and Crtl1 (Fig. 13Q).

5.4.2. Histology of the antler tip

Red deer developing antler tips were harvested and submitted to histology and immunohistochemistry analysis. When viewed in a coronal plane, the antler tip revealed distinct zones (Fig. 14A), the most apical zones being the least differentiated ones. The cover is the velvet skin with large superficial blood vessels. Proximally there follow the perichondrium, apical mesenchyme, precartilage region, which further differentiates into cartilage, then mineralized cartilage. On the cross-section of a more proximal region, cortical bone layers are visible, surrounding the central spongious bone (Fig. 14B). Hematoxylin and eosin staining helped identifying morphological changes during cell differentiation (Fig. 14D-I). The fibrous layer of perichondrium contains very narrow cells with abundant fibrous extracellular matrix. Below there is the cellular layer of perichondrium, a region of small, densely packed, spindle-shaped cells, with numerous small vessels and relatively little matrix produced. As we approach the axis of the developing antler, the cells become more rounded and start to assume a more random orientation (Fig. 14D, E). This mesenchyme cell region is the thickest in the apex of the velvet antler, and becomes narrower toward the basis (Fig. 14A, D). More proximally, the ovoid to spindle-shaped cells appear in a helical pattern around the vascular channels (Fig. 14F). The extracellular matrix increases in proportion, but it is weakly alcian blue positive yet (see next, Fig. 15I). These prechondroblasts then divide, the cells stay together, form the chondrocyte lacunae (Fig. 14G) and deposit cartilaginous alcian blue-positive matrix (Fig. 16H). At level i beneath the perichondrium eosinophil osteoid matrix is obvious, which is deposited by osteoblasts differentiating directly from mesenchyme cells (Fig. 14H). At the same level, inside the antler shaft, the blood vessels

appear wider and the perivascular tissue has narrowed to a few layers of elongated cells (Fig. 14I).

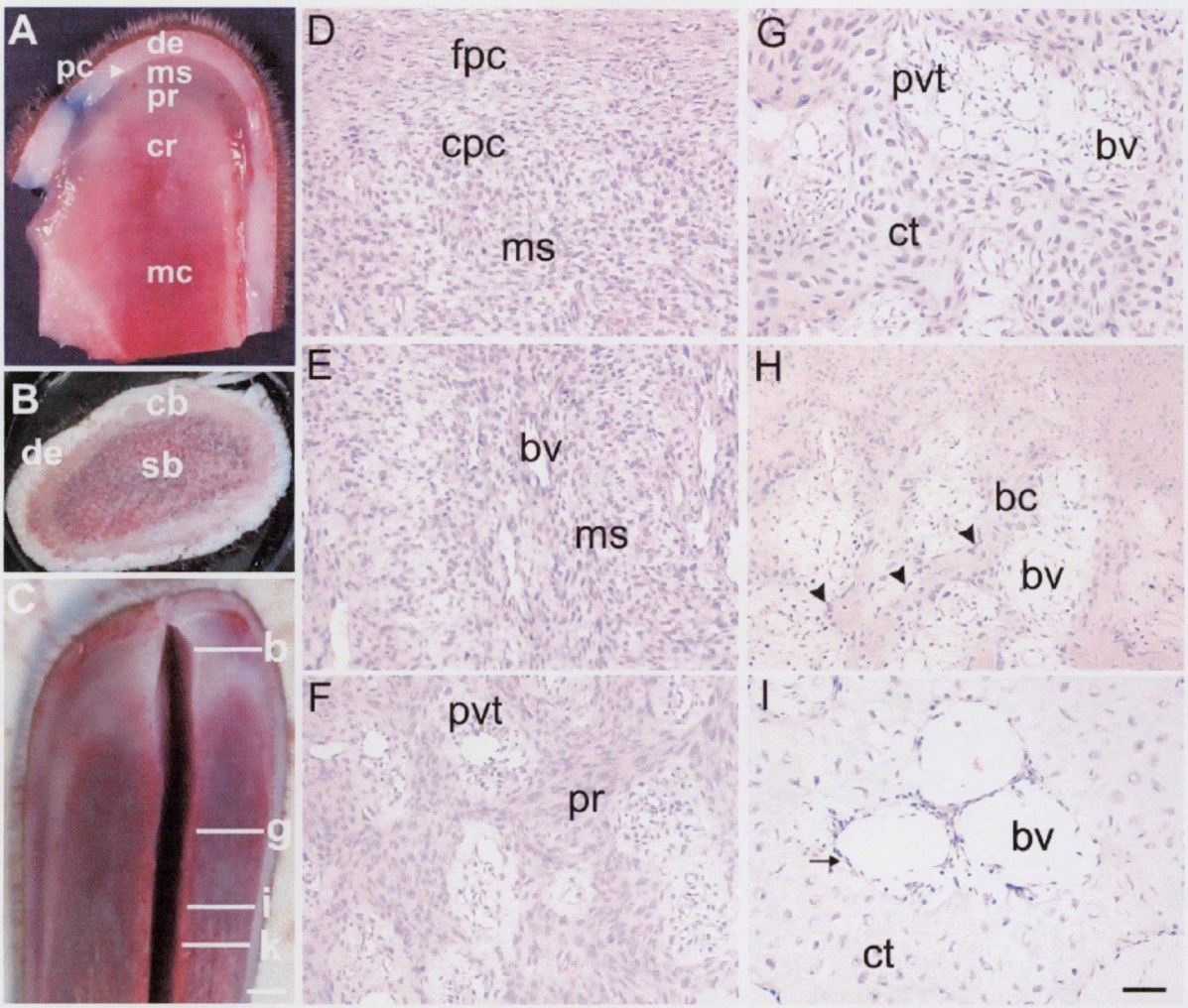

Figure 14. Histology of developing deer antler. (A) Coronal plane section through the growing antler tip shows zonal distribution of different cell types. The antler is covered by the velvet skin, (de, dermis), beneath there is the perichondrium (pc) followed by the mesenchyme (ms), which differentiates to precartilage (pr), cartilage (cr) and with mineral deposition mineralized cartilage is formed (mc). (B) Cross section of antler 30 cm from the tip reveals a compact bone layer (cb) immediately beneath dermis (de) and spongy bone (sb) in the centre. (C) Antler tip cut sagittally at the median plane and cut further into two quarters. Levels b, g, i, k denote horizontal planes from where sections presented in this publication originate. (D-I) Characteristic details of the distinct zones stained with hematoxylin and eosin. Pictures (D-E) are made from section at level b; (F, G) are from level g; (H, I) are photographed from level i. (D) Beneath dermis and the fibrous layer of perichondrium (fpc), the cellular layer of perichondrium (cpc) merges into mesenchyme (ms). (E and F) The mesenchyme cells divide, surround blood vessels (bv), enlarge in volume, and form prechondrobasts in the precartilage area (pr). (G) The chondrocytes deposit

cartilaginous matrix and reside in the lacunae of cartilage trabeculae (ct). Perivascular tissue (pvt) surrounding blood vessels separate the trabeculae. (H) At level i osteoid matrix is deposited beneath the perichondrium, where mesenchyme cells differentiate directly to osteoblasts (arrowhead). bc, bone collar. (I) In mineralized cartilage the blood vessels are wider in diameter; and the perivascular tissue has narrowed to a few cell layers (arrow). Bar 5 mm in A-C; 0.2 mm in D-I.

5.4.3. Immunohistochemical analysis of ECM marker gene expression in the antler

Horizontal sections 0.5-1 cm from the tip were analyzed first for the presence of the four matrilins and cartilage link protein (Fig. 15). The perichondrium was alcian blue negative, but showed strong accumulation of Matn2 and intense staining for Matn3, which faded toward the mesenchyme. The mesenchyme cell zone gave enhanced reaction for Crtl1, but only at level b (Fig 15G), not at level g, farther from the tip (Fig. 15H). Staining was also strong for Matn2. Prechondroblasts were most intensely stained for link protein, and gave moderate immunohistochemistry reaction for Matn2 and -3. Matn4 deposition was observed around particular prechondroblasts and strong Matn2 positivity was seen bordering the vascular channels.

At level g, 3 cm from the tip, the mesenchyme cell layer was thinner than at level b, but it stained faintly for Matn3 and very intensely for Matn2 (not shown). Further changes were also observed (Fig. 16). In the precartilage area the chondrocyte islands have not been merged yet, but they stained all over for Matn4 and link protein (Fig. 16F, G) and started depositing Matn1 and -3 around particular chondroblasts (Fig. 16C, E). The matrix within cartilage trabeculae stained strongly for collagen II and link protein, and not as strongly, but substantially for all four matrilins. The latter ones, however, showed local accumulation at the border of cartilage and perivascular tissue (see arrowheads), where collagen II (Fig. 16I) or link protein (Fig. 16P) were not detectable. Further, Matn2 deposition was observed in the perivascular tissue and blood vessel walls.

At level k, 4.5 cm from the tip, the matrix assembly is combined with mineral deposition at separate locations by independently differentiating cell types (Fig. 17). In the absence of cartilage markers the subperichondrial mineral deposition, visualized by von Kossa staining (Fig. 17A), must have resulted from intramembranous ossification. This view

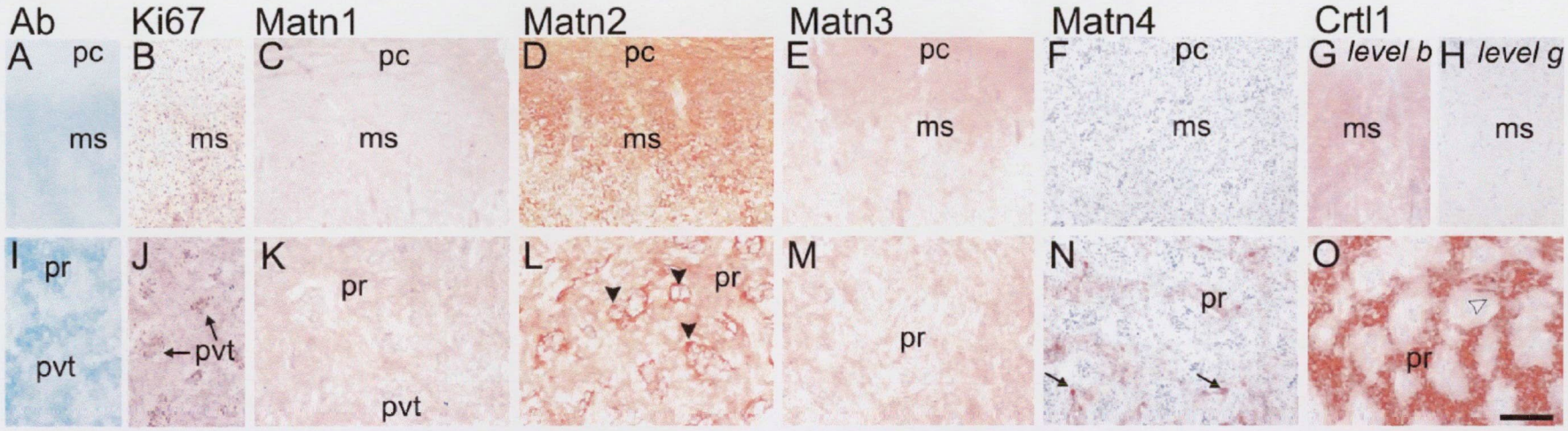

Figure 15. Immunolocalisation of matrilins and link protein in the mesenchyme and precartilage at the transversal section level b. (A, I) Alcian blue staining indicates low level of glycoseaminoglycan in the apical mesenchyme and precartilage. (B, J) Immunostaining with Ki67 antibody shows a high number of proliferating cells in the mesenchyme and in perivascular tissue. (C, K) Faint staining for Matn1 can be seen in the apical mesenchyme and precartilage. (D, L) Strong Matn2 immunoreactivity was detected in the perichondrium, mesenchyme, at the periphery of vascular channels (arrowheads) and in the precartilage matrix. (E, M) Matn3 signal is detected in the perichondrium, and less intensely in the mesenchyme and precartilage matrix. (F, N) Matn4 is absent from the mesenchyme but detectable around particular chondroblasts in the precartilage area (arrows). The Crtl1 immunoreactivity at level b (G), disappears at level g (H). The precartilage matrix is strongly immunopositive for link protein (O). ms, mesenchyme; pc, perichondrium; pr, precartilage; pvt, perivascular tissue. Bar 0.2 mm.

Figure 16. Immunolocalisation of ECM proteins in cartilage trabeculae at the transversal section level g. The appearance of cartilaginous matrix is shown by alcian blue staining (A, H) and collagen II expression (B, I). Mineralization was not detected in cartilage trabeculae by von-Kossa staining (H). The slides were immunostained with antisera for Matn1 (C, J), Matn2 (D, K, L), Matn3 (E, N), Matn4 (F, O), Crtl1 (G, P) and laminin (M). Arrows point at particular chondrocytes showing enhanced pericellular immunoreactivity with Matn1 (C) and Matn3 (E) antisera. Matn4 and Crtl1 are stained more intensely at the periphery (F, G) than closer to the axis of the antler (O, P). The subendothelial basement membrane (diamond), which is detected by laminin staining (M), is also positive for Matn2 (K). The zone around vascular channels is rich in all four matrilins (arrowhead in J, L, N, O), but negative for collagen II and Crtl1 (open arrowheads in I and P). bv, blood vessel; ct, cartilage trabeculae; pr, precartilage; asterisk, vascular channel. Bar 0.1 mm.

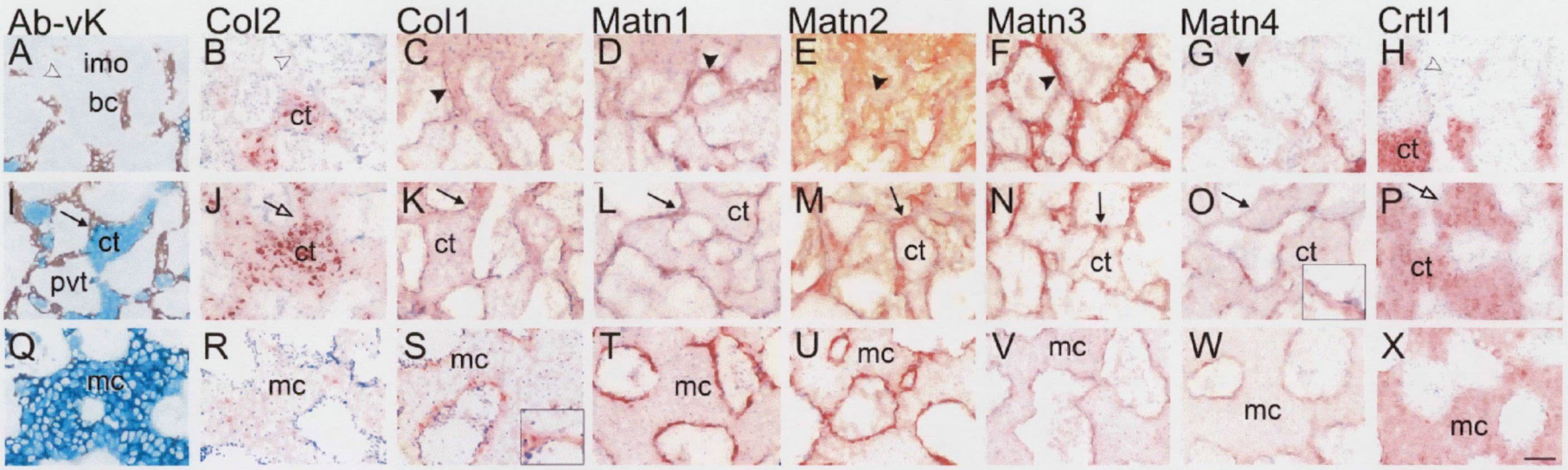

Figure 17. Immunolocalisation of matrilins and link protein during intramembranous ossification and in mineralized cartilage at the transversal section level k. (A-H) Intramembranous ossification is marked by deposition of mineral (A) and collagen I (C), in the absence (open arrowhead) of alcian blue positivity (A), collagen II (B) and cartilage link protein (H). In the bony collar Matn1 (D), Matn2 (E), Matn3 (F) and Matn4 (G) are found associated to bone (solid arrowhead). (I-P) Hypertrophic chondrocytes. Epichondral mineral accumulation at the surface of non-mineralized cartilage trabeculae (I), where collagen II (J) and Crtl1 (P) levels are greatly diminished (open arrow), colocalizes with collagen I positivity (K) and accumulation of all four matrilins (L-O, solid arrow). (Q-X) Mineralized cartilage. As mineralization extends within cartilage trabeculae (Q), collagen II immunostaining disappears (R). All four matrilins are detectable in mineralized cartilage (T-W), albeit at lower level than at the interface with vascular channels. bc, bone collar; ct, cartilage trabeculae; imo, intramembranous ossification; mc, mineralized cartilage; pvt, perivascular tissue. Bar 0.1 mm in all panels, 0.05 mm in inserts.

is confirmed by immunological detection of collagen I, a marker of bone formation (Fig. 17C). Deeper inside the antler shaft (Fig. 17Q-X) hypertrophic chondrocytes deposited mineral within the cartilage trabeculae. On the surface of mineralized cartilage trabeculae intense tartrate resistant acid phosphatase staining occurred in large multinucleated osteoclasts and smaller mononuclear cells, regarded as osteoclast progenitors (not shown). In the intramembranous ossification area, all four matrilins were deposited in the bony matrix. Within the mineralized cartilage matrix, the matrilins and link protein were detectable. The interface between cartilage columns and vascular channels was enriched in all four matrilins and collagen I. Pericellular staining of 1-3 layers of flat, elongated cells was observed.

5.4.4. Comparison of steady-state mRNA levels in differentiating cell types

The steady-state RNA levels in the consecutive zones of velvet antler were assessed by RNA blot-hybridization. First, hybridization probes were generated. Red deer cDNA fragments for the four matrilins and link protein were amplified by RT-PCR, using heterologous primers (Table 3), and cloned. Nucleotide and amino acid sequence identity for the cloned segments with the corresponding human proteins varied between 89% and 93% for matrilins. The identity of deer Crtl1 with the human sequence was 93% at cDNA level and 97% at protein level. RNA aliquots extracted from tissue samples enriched in mesenchyme cells, prechondroblasts and chondrocytes were tested by blot hybridization (Fig. 18).

Figure 18. RNA blot hybridization of antler samples. RNA was extracted from tissue pieces enriched in mesenchyme (1), precartilage (2) and trabecular cartilage (3).

All cDNA fragments hybridized to distinct RNA species. The transcript sizes, 5 kb for Matn1, 4 kb for Matn2, 2.2 kb for Matn4, multiple forms of 6 kb, 3 kb and 1.5 kb for link protein, 6 kb and a minor 8 kb mRNA for collagen II were similar or identical with the length of the corresponding human transcripts. The Matn3 cDNA hybridized to multiple bands, 7 kb, 5.5 kb and 4 kb, all three are longer than the 2.8 kb human Matn3 mRNA identified previously (Belluoccio et al., 1998). Crtl1, Col2a1 and all Matn transcripts were detectable already in the mesenchyme and the steady state mRNA levels increased further toward chondrogenesis. mRNA

levels for Crtl1, Col2a1 and Matn1 raised 32-fold, 17.3-fold, and 8.7-fold, respectively, from the mesenchyme sample to trabecular cartilage.

The steeper increase was seen for Crtl1, Col2a1 between mesenchyme and precartilage, 7-fold and 4-fold, respectively, the level of Matn1 mRNA increased more steeply, 4-fold, from precartilage to cartilage. Matn2 mRNA was already abundant in the mesenchyme sample, henceforth signal intensity of the cartilage sample showed only 1.4-fold increase (Fig. 18), compliant with immunohistochemistry results.

5.4.5. Identification of expressing cells by in situ hybridization

In order to gain information about the cell types, and determine the expression domains more precisely, *in situ* hybridization was performed. First, parasagittal sections were hybridized with Col2a1 and Matn2 antisense RNA probes. Expression of the Col2a1 gene is concentrated to chondroblasts and chondrocytes (Fig. 19A-C). In the precartilage region, only 30-40% of the cells gave positive hybridization signal. In the cartilage trabeculae, the differentiated chondrocytes all expressed the gene. Matn2 expression can be observed in several regions and cell types (Fig. 19D-H). Dermis fibroblasts showed moderate positivity. Hybridization signal in the densely packed cells of the perichondrium was less intense. The overall hybridization signal was the strongest in the mesenchyme cell region, but the precartilage and cartilage cells were also positive, as well as the perivascular tissue cells (Fig. 19D-H).

Thinner sections (5 μm) at level b were also hybridized, facilitating identification of cell types. The small mesenchyme cells were clearly hybridization positive, similarly to chondroblasts in the precartilage area, and chondrocytes in the cartilage trabeculae. Positivity of the endothelial cells and thin, fibroblast-like cells around the vascular channels was also visible (Fig. 19L, M).

Hybridization patterns of the other three matrilins and collagen I are shown in Fig. 20. The cellular layer of perichondrium and the mesenchyme cells gave positive hybridization signal with Matn1, Matn3 and Matn4 probes (Fig. 20A-C). The prechondroblasts produced uniform hybridization signal with the Matn4 probe. Matn1 and Matn3 gene expression showed uneven pattern (Fig. 20D-F). All three Matn genes were expressed in chondrocytes (Fig. 20G-I). In the intramembranous ossification area osteoprogenitor cells and osteoblasts at the surface and within peripheral bony spicules expressed Col1a1, Matn1, Matn3 and Matn4 at high level (Fig. 20J-M). Flat, elongated cells, separating the vascular channels from the trabeculae of hypertrophic chondrocytes that deposited von Kossa-positive matrix within

the trabeculae but have not yet accumulated mineral at the trabecular surface, gave also strong hybridization signal for all four genes (Fig. 20N-Q). These perivascular cells were mostly found in a single layer, adjacent to cartilage trabeculae and not localized between two blood vessels within the vascular channels (Fig. 20O-Q). These flat cells with elongated nuclei resemble in morphology to the cells of the perichondrium-periosteum. The hypertrophic chondrocytes were hybridization positive for Matn1, Matn3 and Matn4, but negative for Col1a1 (Fig. 20N-Q).

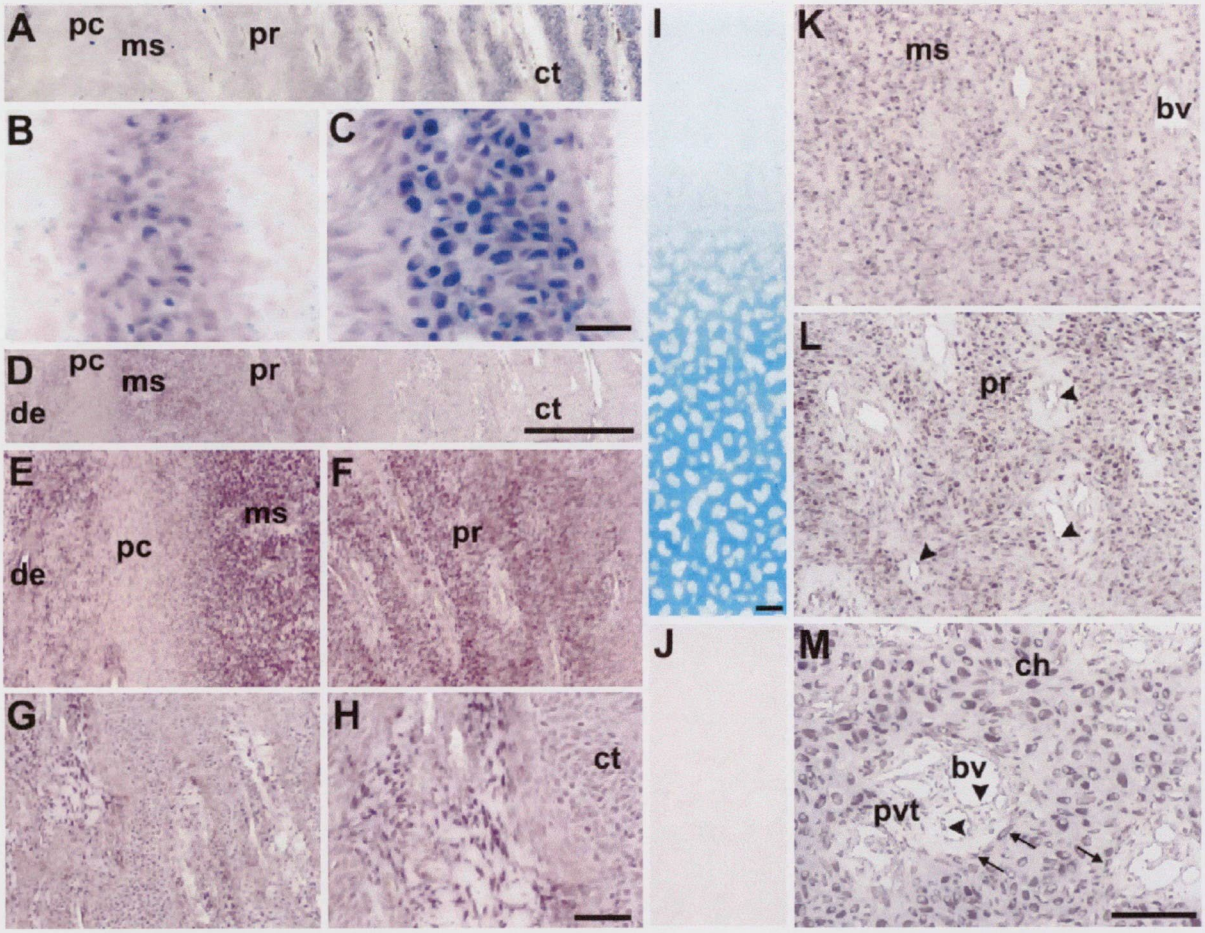

Figure 19. Detection of Col2a1 and Matn2 mRNA by *in situ* hybridization. (A-H) Analysis of 10 µm thick parasagittal sections 1 cm from the tip. (A-C) Col2a1 expression. Observe hybridization signal in the most differentiated cells of the precartilage region (B), and uniform labelling of chondrocytes in cartilage trabeculae (C). (D-H) Matn2 expression. Note the mesenchyme cell region giving the strongest hybridization signal. (I-M) Analysis of 5 µm thick transversal sections at level b. (I) Alcian-blue staining. (J-M) Matn2 mRNA detection. Hybridization signal can be observed in numerous small cells in the mesenchyme region (K); in elongated, fibroblastoid cells around the vascular channels (arrows), and in vascular endothelial cells (arrowheads) (L and M). Chondrocytes (ch) in (M) clearly show cytoplasmic labelling. (J) Sense control. bv, blood vessel; ch, chondrocytes; ct, cartilage

trabeculae; de, dermis; ms, mesenchyme cells; pc, perichondrium; pr, precartilage; pvt, perivascular tissue. Bar 1 mm in A, D, I, 0.1 mm in E-H and J-M, 0.05 mm in B and C.

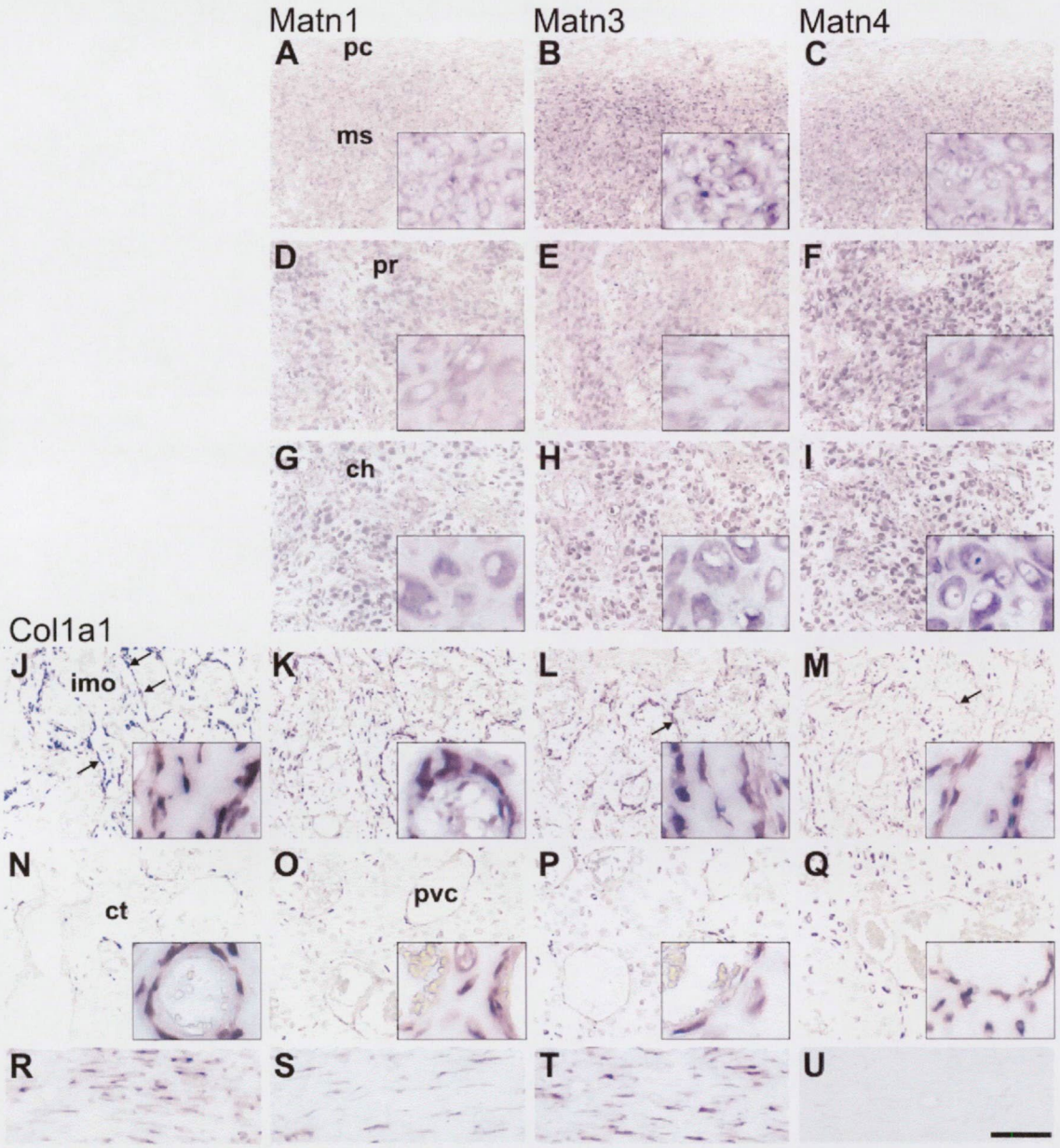

Figure 20. Detection of Col1A1, Matn1, Matn3 and Matn4 mRNA by *in situ* hybridization. *In situ* hybridization of antisense riboprobes to perichondrium and mesenchyme cells (A-C), prechondroblasts (D-F), chondrocytes (G-I), cells of the intramembranous ossification process (J-M), mineralized cartilage (N-Q), periosteum (R-U). Arrows point at osteoblasts in J-M. ch, chondrocytes; ct, cartilage trabeculae; imo, intramembranous ossification; ms, mesenchyme cells; pc, perichondrium; pr, prechondroblasts; pvc, perivascular cells. Bar 0.13 mm in A-Q, 0.044 mm in R-U and in inserts of A-Q.

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5.4.6. Distribution of some regulatory molecules during antler formation

We examined the expression of FGFR3 and Sox9 during antler development. **FGFR3** showed expression pattern during antler development resembling to endochondral ossification. Mesenchyme cells and the perivascular tissue were exempt from FGFR3. The immunosignal for FGFR3 was abundant and uniform in precartilage columns (Fig. 21D) at section level b. FGFR3 was still uniformly localized in early cartilage trabeculae, which were built up by proliferating chondrocytes (Fig. 21B), at section level g. Reduction of FGFR3 immunosignal intensity was observed in the cartilage columns (Fig. 21E). At level k, the number of proliferating chondrocytes was reduced (Fig. 21C) and the number of hypertophic chondrocytes was increased (Fig. 21F). We noticed that FGFR3 presented cellular localization in prechondrocytes (Fig. 21D) and proliferating chondrocytes (Fig. 21B, C and E), but weak immunosignal was also found in the matrix of hypertrophic chondrocytes (Fig. 21E and F).

Sox9 transcript was detected by RNA blot hybridization in the mesenchyme, precartilage and cartilage samples (Fig 22). The level of Sox9 mRNA decreased in the mineralized cartilage sample (Fig. 22). The *in situ* hybridization data corresponded with the RNA blot results (Fig. 22). Mesenchyme cells (Fig. 22A and B), prechondrocytes (Fig. 22E) and chondrocytes (Fig. 22F, I and J) were abundant in Sox9 mRNA. As differentiation proceeded, the number of hypertrophic chondrocytes increased and the proportion of Sox9 expressing cells decreased. Only a few Sox9 positive cells were found in the hypertophic cartilage columns, mostly at the periphery of the columns (Fig. 22K). Mineralized cartilage columns were almost devoid of Sox9 expressing cells (Fig. 22L and M). The lateral mesenchyme cells expressed Sox9 (Fig 22C and D) although their expression level was below that of the apical mesenchyme. Osteoblasts of the cortical bone collar were found to display Sox9 mRNA expression (Fig. 22G and H).

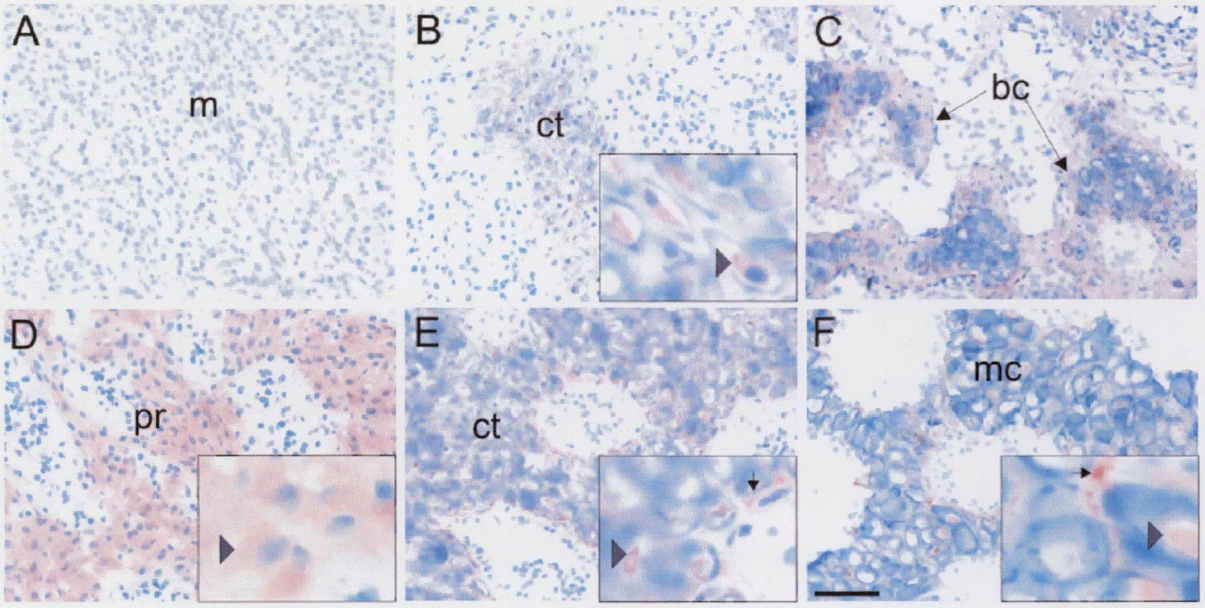

Figure 21. Immunolocalization of FGFR3 at different chondrocyte developmental stages of the red deer antler. (A) FGFR3 is missing from the mesenchyme cells, (D) however, prechondrocytes express it at high level. (B, E) FGFR3 is also localized to the chondrocytes of the cartilage trabeculae (arrowhead). FGFR3 immunosignal is also detectable in the matrix of mineralized cartilage trabeculae (arrow, E and F), but is not present in bone collars (C). bc, bone collar; ct, cartilage trabeculae; m, mesenchyme; mc, mineralized cartilage; pr, precartilage. Bar 0.1 mm in A-G; 0.05 mm in all inserts.

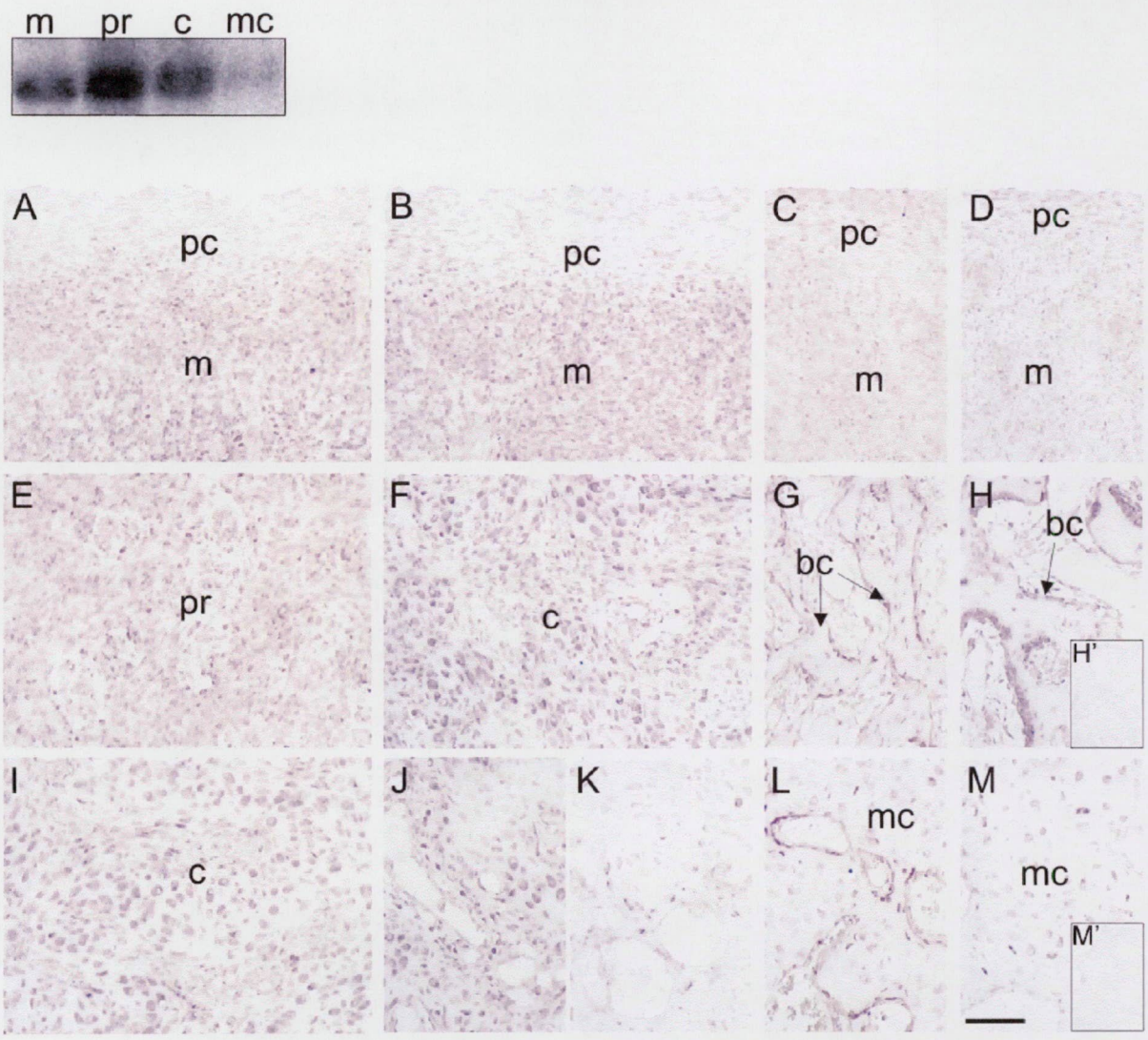

Figure 22. Detection of Sox9 mRNA in antler samples by blot hybridization (top) and in sections by *in situ* hybridization. The mesenchyme cells (A-D), prechondrocytes (E) and chondrocytes (I, F) show strong hybridization signal, however the number of Sox9 positive cells is reduced in the hypertophic (K) and mineralized cartilage columns (L, M). Note the osteoblasts of the cortical bone giving hybridization signal (G, H). Sense control (H', M'). bc, bone collar; c, cartilage; m, mesenchyme cells; mc, mineralized cartilage; pc, perichondrium; pr, precartilage. Bar 0.1 mm in A-M; 0.05 in H` and M`.

6. DISCUSSION

6.1. Two promoters of the *MATN2* gene

While a single promoter drives the transcription from *Matn1* and *Matn3* (Kiss et al., 1989; Wagener et al., 2000), the broad tissue-specificity of *Matn4* is achieved by the usage of alternative promoters (Wagener et al., 2001). Contrary to *Matn4*, however, the upstream promoter of *Matn2* and *MATN2* was identified during this work as a major promoter in most tissues and the downstream promoter showed a limited tissue-specificity. Data presented here indicate that P_u, which exhibits the characteristics of housekeeping promoters, is used much more frequently than P_d. Previously, *MATN2* expression was found by RT-PCR in a variety of human primary and permanent cell lines. There was no RNA sample studied where *MATN2* expression was below the detectability threshold (Muratoglu et al., 2000). Therefore, we may conclude that there is a basic level of transcription of *MATN2* in the various cell types, most probably from the P_u promoter (Mátés et al., 2002). The Sp1-binding motifs found in multiple copies in mouse and human as well may have a role in the ubiquitous activity of P_u, as Sp1 was demonstrated to contribute to the activity of many genes with broad expression pattern (Philipsen and Suske, 1999). On the other hand, P_d seems to be regulated more strictly and have a preference in human for the permanent fibroblast and epithelial cell lines, but not for the primary fibroblast cell line. Similar results were obtained for the tested mouse organs and different cell line samples regarding to the alternative promoter usage of *Matn2* (Mátés et al., 2002). It was shown that the upstream housekeeping-type promoter is functional in fibroblasts and in a great variety of other cell types expressing the gene. However, the downstream TATA-like promoter had a restricted tissue-specificity, being active in mice only in primary embryonic fibroblasts and in NIH-3T3 embryo fibroblast cell line (Mátés et al., 2002).

6.2. Expression of matrilins during ossification

Expression of Matn1, Matn2 and Matn3 during endochondral ossification in the developing limb

Matn1 and Matn3 were expressed in the condensations of cartilage primordia of 12.5 dpc mouse embryo limb. Matn2 showed a complementary expression pattern: it was found in the surroundings of mesenchyme condensations, similarly to Prx1/2 expression (ten Berge et al., 1998). The similar expression pattern was maintained during development regarding to perichondrium and periosteum. It was demonstrated by Lajos Mátés using oligonucleotide overlay and luciferase assays that Prx1/2 binds and activates the alternative promoter of the *Matn2* (Lajos Mátés, Ph.D thesis). It is possible that Matn2 expression in the perichondrium and periosteum is driven by the transcription factors Prx1/2 from the alternative promoter. Additionally, Matn2 was expressed in the late proliferative and hypertophic zone of epiphyseal cartilage and in bone matrix. Matn1 and Matn3 showed broader expression pattern in cartilage, they were abundant in the reserve, proliferative and hypertophic zones of epiphyseal cartilage, however, they were missing from the articular cartilage. In the immediate pericellular areas, Matn3 was relatively more abundant. At the border of chondrons, the amount of proteins was balanced. Matn2 was abundantly expressed in the structures exposed to friction such as articular cartilage and tension such as tendons/ligaments.

Although the matrilins show wide distribution pattern during endochondral ossification, the available single knockuts of *Matn1*, *Matn2* and *Matn3* in mouse gave no obvious phenotype (Aszódi et al., 1999; Huang et al., 1999; Ko et al., 2004; Mátés et al., 2004), possibly due to a redundancy in matrilin function.

Expression of matrilins during intramembranous ossification of newborn mouse skull.

All matrilins were detectable in the developing skull formed by a direct conversion of mesenchymal cells to osteoblasts. In knockout mice of *Matn2*, phenotypical alteration was identified in the calvarial bone (Mátés et al., 2004). The mutant parietal bone contains significantly more cavities as compared to the wild type. The phenotype is prominent at about 4 months of age, which correlates well with the fact that by that time, Matn4 expression is restricted to the periosteum, and Matn2 remains the only highly expressing matrilin in the osteogenic connective tissue (Mátés et al., 2004). The skull of *Matn1* and *Matn3* knockout mice do not show any abnormal phenotype maybe due to their low expression level in bony structures.

6.3. The changes of ECM gene expression during antler ossification

The antlers of cervids are the only mammalian bone structures that regenerate completely every year and may serve as a unique model for osteological studies. To date only a few components of the antler extracellular matrix have been described. No data have been published about noncollagenous matrix proteins, their significance in the organization of the extracellular matrix and their role during histogenesis of antlers. Because from the studies of others and ourselves it became clear that all four matrilins are integrant components of the skeletal elements (Deák et al, 1999; Segat et al., 2000; Klatt et al., 2000; 2001) we monitored their expression during antler development. We successfully used the matrilins in characterizing distinct differentiation stages during antler histogenesis. Furthermore, we learned more about matrilins and their potential role in organizing the extracellular matrix.

Immunoblot and immunohistochemistry results were concordant with RNA blot and *in situ* hybridization data. Cartilage link protein visualized by immunostaining in the apical mesenchyme was also detected by immunoblot in the extract of a parallel tissue sample and the corresponding transcript was also seen in the RNA preparation from mesenchyme cells. The amount of true cartilage transcripts increased steeply when RNA samples extracted from mesenchyme, precartilage and cartilage were compared in blot hybridization. Matn1 and Matn3 transcripts changed in parallel and increased only in the cartilage sample. Matn4 mRNA level showed increase in precartilage, the amount of matn2 mRNA was already high in the mesenchyme sample. *In situ* hybridization confirmed that Col2A1, Matn1, Matn3 and Matn4 transcripts were already produced by mesenchyme cells, but chondrocytes, in the case of Matn4 also prechondroblasts, expressed these genes at higher level. Matn2 *in situ* hybridization signal was highest in the mesenchyme cell zone and less intense in the cartilage trabeculae, due to a lower cell density in the latter area. The proportion of Matn2 mRNA is, however, comparable in the two samples, as the same amount of total RNA applied gave similarly intense hybridizing bands. A slight discrepancy was found between relatively strong *in situ* positivity and weak immunohistochemistry signal for Matn4 in the antler mesenchyme. Low cross-reactivity of the antiserum cannot be responsible for the difference, because the antiserum reacted well with the protein in humerus. Immunoblot (data not shown) and RNA blot results were concordant: both the protein and Matn4 mRNA were detectable but several fold less in the mesenchyme sample than in cartilage extract. Therefore, we concluded that the rapidly dividing mesenchyme cells might transcribe the Matn4 gene but synthesis or deposition of the protein must be hampered by factors we do not

know yet. A similar phenomenon was reported for collagen X. *In situ* hybridization detected the transcript already in chondroprogenitors and mRNA expression was maintained throughout the cartilage trabeculae (Price et al., 1996). A mouse anti-deer collagen X antibody, however, stained the protein exclusively around hypertrophic chondrocytes (Faucheux et al., 2004). Taken together the immunohistochemistry and RNA analysis results, the order of ECM marker expression during antler chondrogenesis can be summarized (Fig. 23). Committed mesenchyme cells predominantly express and deposit Matn2. Prechondroblasts deposit collagen II and link protein, followed by synthesis of Matn4. In chondrocytes synthesis of collagen II and link protein continues, Matn4 is not accumulated further. Matn1 gene expresses the latest. During chondrocyte maturation, all matrilins behave similarly. The focus of gene expression and protein deposition tends to be shifted from mature chondrocytes within cartilage trabeculae toward osteoprogenitor cells at the interface to perivascular tissue.

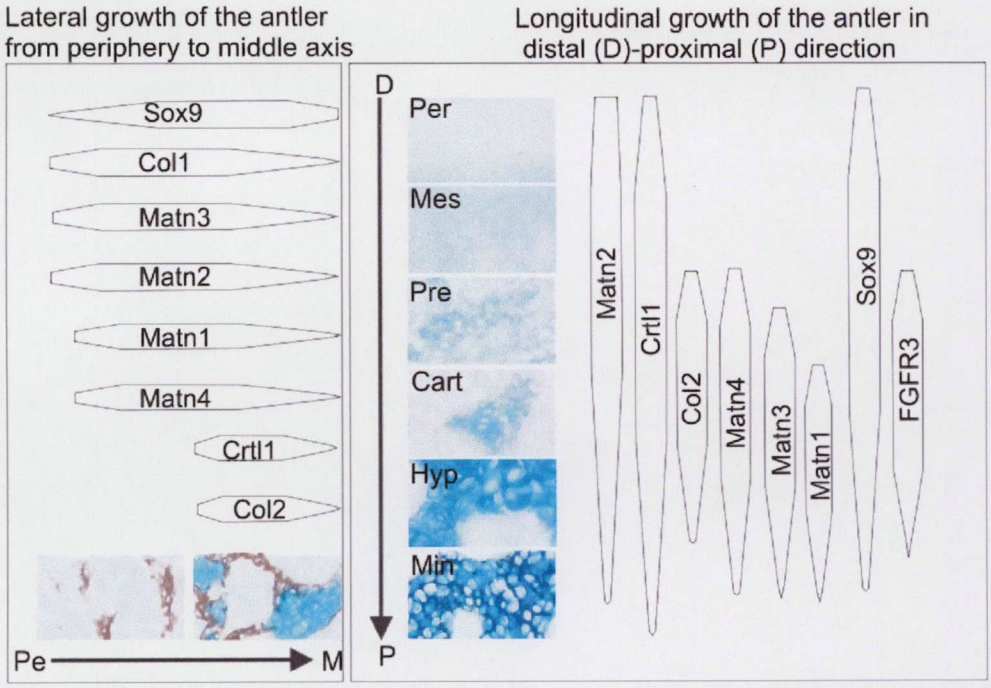

Figure 23. Schematic diagram summarizing the pattern of localization of ECM and regulatory molecules in the growing antler tip. Cart, cartilage; Hyp, hypertophic chondrocytes; Mes, mesenchyme; Min, mineralized cartilage; Per, perichondrium; Pre, precartilage.

6.4. Antlerogenesis: new findings

We observed differences between the apical and lateral mesenchyme of the developing antler. The apical mesenchyme is thicker; it is stained moderately with alcian blue and rich in Crtl1. The subperichondrial lateral mesenchyme is thin, alcian blue negative and Crtl1 negative. Cartilage link protein stabilizes large aggregates of hyaluronan and aggrecan via direct binding to both macromolecular partners (Heinegard et al., 2003). In the study of Banks and Newbrey (1983), the alcianophilia in the apical mesenchyme was hyaluronidase-labile, but neuraminidase resistant. The difference observed between apical and lateral mesenchyme may support a functional divergence. The apical mesenchyme is a morphogenetic center, as it is evident from the appearance of two forming tines. Hyaluronan secreted by the apical mesenchyme cells prevents aggregation and facilitates locomotion necessary for morphogenesis. In the lateral mesenchyme, farther from the tip in our studies than level i, the internal mesenchymal cells of the perichondrium differentiate into osteoblasts and contribute to cortical bone formation.

The antler osseocartilage showed differences from long bone growth plate samples. The epiphyseal cartilage of humerus stained almost homogenously for Matn1, Matn3 and Matn4. On the contrary, in the precartilage zone and cartilage trabeculae of antler at section level g, immunohistochemistry and *in situ* hybridization signals for all three gene products were heterogeneous. Particularly Matn1 showed a moderate level of expression all over the matrix and strong accumulation around particular cells. *Matn1* gene expression in chicken, human and mouse cartilage was previously described. In the epiphyseal growth plate of chicken tibia postproliferative chondrocytes, which have not reached hypertrophy yet, were found to specifically transcribe the *Matn1* (Chen et al., 1995). Analysis of cultured chicken sternum chondrocytes also showed that expression of the Matn1 gene is dependent on cell differentiation stages (Szűts et al., 1988). The observed heterogeneity of matrilin gene expression in the antler osseocartilage can be understood if we assume that the cells did not differentiate uniformly. Chondrocytes may have stayed in close proximity but differentiated at different rates. One cell may have stopped proliferation and reached maturity, the adjacent cell may have kept on dividing. In the cartilaginous anlagen of long bones, morphogenetic signals lead to segmentation and proximodistal zonality. In the antlers, however, signaling centers must be dispersed. Among others, perivascular tissue cells must be mentioned as sources of signal molecules. Previously, expression of retinaldehyde dehydrogenase type 2, responsible for the synthesis of retinoic acid that regulates chondrocyte differentiation and

maturation, was shown in skin, perichondrium and perivascular cells in antler cartilage (Allen et al., 2002). More recently the same laboratory extended this observation and demonstrated that Ihh, PTHrP and transforming growth factor-beta1 (TGF-b1) are expressed in perivascular cells (Faucheux et al., 2004).

Osteoprogenitor cells. We have identified 1-3 cell layers around the vascular channel, which expressed and deposited all four matrilins and collagen I, but did not produce cartilage markers collagen II and link protein. These perivascular cells were identified as separating vascular channels from hypertrophic chondrocytes, which deposited von Kossa-positive matrix within the trabeculae, but have not yet accumulated mineral at the trabecular surface. They were mostly found in a single layer, adjacent to cartilage trabeculae and not localized between two blood vessels within the vascular channels. They are most probably osteoprogenitor cells, which appear at the surface of cartilage trabeculae, but have neither acquired yet a more osteoblast-like morphology nor have they started accumulating mineral at the trabecular surface. These cells are mononuclear and negative for TRAP-staining, therefore not considered as osteoclast progenitors. These flat cells with elongated nuclei resemble in morphology to the cells of the fibrous perichondrium-periosteum. Further, some periosteum cells were also positive for all matrilins and collagen I. Therefore, we believe that these osteoprogenitors must have originated from the perichondrium-periosteum and migrated into the antler shaft along the vascular channels during angiogenesis.

Presumed identity of osteoprogenitor cells with PTHrP-positive perivascular cells. Faucheux et al. (2004) and Barling et al. (2004) studied the same tissue for the presence of some signalling molecules and receptors. They described perivascular cells in the mineralized cartilage area, which were immunopositive for PTHrP, the common receptor of PTHrP and parathyroid hormone, TGF-b1 and Ihh. The matrilin-positive cells may be identical with the PTHrP and the receptor of PTHrP and parathyroid hormone-positive perivascular cells identified during those studies. If so, matrilin positive cells may be PTHrP responders, and matrilin synthesis may be under control of PTHrP and/or TGF-b1 and/or Ihh signaling. This assumption is further supported by the similarity we observed between the gene expression pattern of periosteum cells and osteoblasts: Both expressed all four matrilins in our hand and were positive for PTHrP in the two independent studies (Faucheux et al., 2004; Barling et al., 2004).

Recapitulation of FGFR3 and Sox9 expression during regeneration of deer antler

A gradient of intact and degraded FGFR3 was observed in the ossification process of the antler most probably due to the proteolysis. Intact FGFR3 was found associated to

prechondrocytes and chondrocytes, although the receptor was found mostly in the matrix of hypertrophic chondrocytes. Similar degradation of FGFR3 was described in the bovine growth plate, whereabout the soluble forms of the receptor were the extracellular N-terminal Ig-like domains. They may sequester the ligand, thus reducing the activation of the receptor and signal transmission across the membrane (Pandit et al., 2002). Due to the same expression pattern in the proliferating chondrocytes FGFR3, most probable plays a role in the developing antler similar to that in the epihyseal growth plate (Minina et al., 2002). If so, FGFR3 may inhibit chondrocyte proliferation by suppressing Ihh expression. In antler, Ihh was found associated to chondrolasts/chondrocytes (Faucheux et al., 2004), but not in hypertrophic chondrocytes as in the growth plate (St-Jacques et al., 1999).

It was shown previously that *Sox9* is required for expression of cartilage-specific extracellular matrix components such as collagen II, collagens IX and XI, and the large proteoglycan aggrecan (Bi et al., 1999, Lefebvre and de Crombrugghe, 1998). Col2α1 and Crtl1 show similar expression pattern to Sox9 in prechondrocytes and chondrocytes during antler development. Recenlty it has been demonstrated that Sox9 is a key regulator also of human Crtl1 (Kou and Ikegawa, 2005). We presume that in prechondrocytes and chondrocytes of the antler Sox9 is the main regulator of Col2α1 and Crtl1. Beside the prechondrocytes and chondrocytes in antler, Crtl1 was found to be expressed also by hypertrophic chondrocytes as in the growth plate of long bones, where Sox9 level was very low. The regulation of Crtl1 in hypertrophic chondrocytes of the growth plate most probable depends on other transcription factor/s in Sox9-independent manner.

There is a significant difference between the localization of Sox9 in osteoblast of bones of the skeleton and antler. We found for the first time Sox9 mRNA in the osteoblast of the bone collars at the periphery of the antler formed by intramembranous ossification. During skeletal development, Sox9 was found at early steps of osteogenesis but was not detected in the regions of active bone formation during endochondral bone formation or during remodeling or modeling of intramembranous bone (Yamashiro et al., 2004). To elucidate the function of Sox9 in osteoblast more investigations are needed. Immunolocalization of the Cbfa1, the master regulatory factor of osteogenesis hopefully will help to understand the function of Sox9 in the osteoblast of the cortical bone of the antler. Further investigations are needed to identify molecular mechanisms that underlie the sterically and temporally regulated gene expression pattern during antler development.

In conclusion, the developing antler of cervids is a good system to investigate bone formation. Endochondral and intramembranous ossification can be monitored simultaneously. In addition it is a special ossification process, primarily due to the high growing rate and the presence of blood vessels and perivascular tissue. Matrilins in this study served as specific markers for characterizing the various cell differentiation events during this complicated process. Further investigations are needed to identify molecular mechanisms that underlie the terically and temporally regulated gene expression pattern during antler development.

6.5. Summary of novel results

- We have proven that *MATN2* is transcribed from two alternative promoters, we mapped the second promoter and revealed that it is used at lower frequency than the upstream one.
- We showed Matn2 expression already in extraembryonic tissues of 7.5 dpc mouse embryos and the amount of the transcript increased steadily by age in embryonic tissue.
- All matrilins were found during endochondral and intramembranous ossification, although showed characteristic differences in distribution. Matn2 differed the most from the other three matrilins. 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. Matn1 was slightly further from the articular surface, than Matn3. Furthermore, Matn3 was found to be present in the territorial matrix of the cartilage and in the interterritorial matrix both of them were observed. All four matrilins were found in bone matrix to different extent, Matn2 and Matn3 were abundant, but Matn1 and Matn4 were only at the level of detectability.
- With histochemistry and immunohistochemistry methods using ECM markers a detailed histological analysis of the velvet antler was performed.
    - We observed differences in the apical and lateral mesenchyme of the antler regarding to the expression of link protein and hyaluronan.
    - We localized Matn2 in the basement membranes of blood vessel endothels.

- We also identified Matn2 as a marker of cells with high differentiation potential: it is expressed predominantly by mesenchyme cells, prechondroblasts and preosteoblasts.
- We found accumulation of Matn4 and Crtl1 in the matrix of precartilage cells, however the deposition of Matn1 and Matn3 was detected around particular chondroblasts of the cartilage columns.
- We detected all four matrilin gene activity by *in situ* hybridization and protein deposition by immunohistochemistry in osteoprogenitor cells and osteoblasts.

➢ 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.

➢ We observed the expression pattern of FGFR3 in the developing antler similar to that in the epiphyseal growth plate: FGFR3 was found in prechondrocytes and chondrocytes, however in hypertrophic cartilage columns it was localized to the matrix.

➢ We detected a decrease in the *in situ* hybridization signal intensity of Sox9 transcript from the mesenchyme zone of the antler to the mineralized zone. For the first time we found Sox9 expression in the osteoblasts at the periphery of the antler formed by intramembranous ossification.

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9. 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 matrilin-1, -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

10. SUMMARY OF THE THESIS

10.1. 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. ECM is produced by the cells that are embedded in and influenced by the surrounding extracellular matrices. 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 (Kronenberg et al., 2003). 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 (Sahni et al., 1999; Minina et al., 2002). 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 (reviewed by Deák et al., 1999). Matrilins are able to form homo-oligomers (Hauser et al, 1994; Piecha et al., 1999; Klatt et al., 2000; 2001) and the natural occurrence of hetero-oligomers was also shown in case of matrilin-1 and -3 (Wu et al., 1998; Kleemann-Fischer et al., 2001). Matrilins have been shown to interact with both collagenous and non-collagenous matrix proteins (Hauser et al., 1996; Piecha et al., 1999; 2002, Nitsche et al., 2004).

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 (Wagener et al., 1997; Klatt et al., 2000). Matrilin-2 and -4 form the other subgroup with a much broader tissue distribution. Expression of the mouse matrilin-2 gene was detected by immunostaining in loose and dense connective tissues, and around muscle cells and certain epithelial cell types (Deák et al., 1997; Piecha et al., 1999). Similarly to matrilin-2, matrilin-4 also shows a broad expression in dense and loose connective tissues, bone, cartilage, nervous system and a variety of organs (Wagener et al., 1998a, 1998b; Klatt et al., 2001).

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.

As an introduction to gene regulation studies, it was our aim to verify the existence of the second promoter in the human matrilin-2 (*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 matrilins were found 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. During those studies we gained a better understanding of the potential function of matrilins as well. The antler study was supplemented with tissue distribution of Sox9 and FGFR3.

10.2. Results and discussion

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.

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.

All matrilins were observed during endochondral and intramembranous ossification, although showed characteristic differences. Matrilin-2 and matrilin-3 were abundant in occipital formed by intramembranous ossification. Matrilin-1 and matrilin-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.

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 foetal 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.

ÖSSZEFOGLALÁS

11.1. Bevezetés

A sejtközötti állomány vagy extracelluláris mátrix (ECM) a sejtek által termelt és a sejtközötti térbe kiválasztott makromolekulák jól meghatározott hálózata. A különböző mátrix fehérjék közötti kölcsönhatás létfontosságú az ECM megfelelő összerendeződéséhez. Az ECM meghatározza a kötőszövetek szerkezeti integritását és fizikai tulajdonságait. Összetétele és összerendezettsége révén az ECM a sejtek fizikai támaszául szolgál, továbbá befolyásolja a sejtek adhézióját, vándorlását, proliferációját, differenciálódását.

A vázrendszert felépítő elemekben (porc, csont, inak, ínszalagok), az ECM és a sejtek aránya az előbbi javára tolódik el. A vázrendszer csontos elemei desmális ill. chondrális csontosodás révén alakulnak ki. Mindkét folyamat a mesenchyma sejtek kondezációjával kezdődik. Desmális csontosodáskor a mesenchyma sejtek közvetlenül alakulnak osteoblastokká, majd csontsejtekké, maguk körül kollagén I-ben gazdag mátrixot termelve. A chondrális csontosodás porcos templáton keresztül valósul meg. A mesenchyma sejtek porcsejtekké differenciálódnak, melyek kollagén II-ben gazdag mátrixot termelnek. A porcra jellemző génexpressziót a Sox9 szabályozza más transzkripciós faktorokkal együttesen (Kronenberg és mti, 2003). A porcsejtek proliferációja biztosítja a porc növekedését. Az Indian hedgehog közvetlenül és a parathyroid hormon rokon peptiden keresztül serkenti a sejtek proliferációját. A fibroblast növekedési faktor receptor-3 (FGFR3) révén ható jelmolekulák viszont gátolják a porcsejtek proliferációját (Sahni és mti., 1999; Minina és mti., 2002). A porcos templát belsejében levő porcsejtek nem osztódnak tovább, megduzzadnak, hipertrófizálódnak és kollagén X-et termelnek. A hipertróf porcsejtek irányítják a porcos mátrix elmeszesedését, váltják ki vérerek behatolását a porcba, ezt követően apoptózis útján elhalnak. A megmaradt porcos mátrix tapadási felületet biztosít a vér útján bekerült osteoblastok számára, melyek csontsejtekké differenciálódnak, és új csontos állományt alakítanak ki.

A matrilin fehérje család moduláris szerkezettel bíró, nem-kollagénszerű ECM fehérjéket foglal magában. A matrilineket egy vagy két von Willebrand A modul, változó számú epidermális növekedési faktor és a COOH-végen az oligomerizációért felelős coiled-coil modul építi fel (Deák és mti., 1999). Mind a négy matrilin képes homo-oligomérekké rendeződni (Hauser és mti., 1994; Piecha és mti., 1999; Klatt és

mti., 2000; 2001), a matrilin-1 és -3 együtt hetero-oligoméreket is képez (Wu és mti., 1998; Kleemann-Fischer és mti., 2001). A matrilinek képesek kollagént hordozó, de kollagéntől független sejtközötti fonalas hálózatba is beépülni.

Expressziójukat tekintve a matrilinek két csoportba oszthatók. A matrilin-1 és -3 a porcos ECM alkotói. A matrilin-3 kimutatható a csontos mátrixban is. A másik csoportba a szélesebb szöveti kifejeződést mutató matrilin-2 és -4 tartozik. Immunfestéssel az egér matrilin-2 láthatóvá tehető tömött- és lazarostos kötőszövetben, izomrostok és bizonyos hámsejtek körül. A matrilin-4, hasonlóan a matrilin-2-höz, kimutatható tömött- és lazarostos kötőszövetben, idegszövetben.

A matrilin fehérje család funkciójának megfejtése folyamatban van. Amikor a disszertációban ismertetett munka elkezdődött, kevés információval rendelkeztünk a matrilineket illetően. Csak a matrilin-1 gént célzottan inaktivált formában hordozó egér létezett, mely nem mutatott semmilyen rendellenességet. Továbbá a matrilin-2 részleges kifejeződését is csak az újszülött egérben ismertük.

Kutatásaink legfőbb célkitűzése az volt, hogy közelebb jussunk a matrilinek funkciójának megértéséhez. Megvizsgáltuk a humán matrilin-2 gén (*MATN2*) promoterét; követtük a matrilin-2 gén kifejeződését az egér egyedfejlődése során összehasonlítottuk a matrilinek kifejeződését a vázfejlődés során és jellemeztük az agancs fejlődését, a chondrális csontosodásban ismert ECM fehérjék és szabályozó molekulák segítségével.

Eredmények és az eredmények megvitatása

Összehasonlítva az általunk előállított matrilin-2 cDNS klónokat az adatbázisban lévő szekvenciákkal, arra következtettünk, hogy a matrilin-2 gén vagy két promoterről íródik át vagy az 5'-végi át nem íródó régióban történő alternatív RNS-vágás magyarázza a cDNS szekvenciák különbözőségét. Szekvencia analízis során nem találtunk alternatív akceptor hasító helyet az adott régióban. Annak érdekében, hogy kimutassuk egy második, potenciális promoter (P_d) aktivitását különböző humán sejtvonalakban, RT-PCR analízist végeztünk a sejtvonalakból származó RNS mintákon. A két mRNS izoformának megfelelő oligonukleotid primer párokat terveztünk, hogy meghatározzuk, hogy 5' irányban mennyire terjed ki az átíródó régió, továbbá megvizsgáljuk, hogy az alternatív promoterről képződő transzkriptum milyen arányát képezi az össz matrilin-2 mRNS-nek.

Bebizonyítottuk, hogy a *MATN2* két promoterről íródik át. Betérképeztük a második promotert és kimutattuk az aktivitását a WI-26 tüdő fibroblast és HEp-2 epitél sejtvonalakban. A primer bőr fibroblast tenyészetben viszont a második promoter aktivitását már nem észleltük.

RT-PCR segítségével követtük a matrilin-2 kifejeződését az egér embrionális fejlődése során. A matrilin-2-t megfigyeltük már a 7.5 napos egér embrió extraembrionális szöveteiben. A matrilin-2 transzkriptum mennyisége nőtt az embrionális kor előrehaladtával.

Korábbról tudtuk, hogy a matrilin-1 csaknem kizárólag porcban fordul elő és a matrilin-3 is csontban és porcszövetben fejeződik ki. Ezért célul tűztük ki a matrilin-1, -2 és -3 gének expressziójának összehasonlítását egér magzatok vázfejlődése során. Az összes matrilint kimutattuk mind a chondrális, mind a desmális csontosodási folyamatokban és kifejeződési mintázatukban eltérést tapasztaltunk. Erős matrilin-2 és -3 immunjelet mutattunk ki a desmális csontosodás révén kialakult nyakszirti csontban. Ezzel ellentétben a matrilin-1 és -4 gyenge expressziót mutatott, a porc link protein pedig teljesen hiányzott.

A matrilin-2 mutatta a legnagyobb különbséget a többi matrilinhoz képest a chondrális csontosodásban. Nagy mennyiségben halmozódott fel az ízületi felszínen, a porchártyában, a porcos templát hipertróf zónájában és az inszalagokban. A matrilin-1 és -3 a növekedési porckorong összes zónájában kimutatható, de hiányzik az ízületi porcból. A matrilin-1-et az ízületi felszíntől távolabb figyeltük meg, mint a matrilin-3-at. Továbbá, a matrilin-3 beépülését észleltük a porc territoriális és interterritoriális mátrixában, a matrilin-1-ét pedig csak az utóbbiban.

A fentebb ismertetett eredmények alapján kiderült, hogy a matrilinek alkalmazhatók a fejlődő porc zónáinak azonosítására. A továbbiakban jellemeztük a gímszarvas (*Cervus elaphus*) agancskezdemény porcos-csontos fejlődését a matrilinek és más ECM markerek segítségével. Az agancsképződés az emlős teljes szerv regeneráció ritka példája. Az agancs hosszanti növekedése módosult chondrális csontosodás révén valósul meg, a vastagodása pedig desmális csontosodás következtében. Hisztokémiai, immunhisztokémiai és *in situ* hibridizációs módszerek segítségével megvizsgáltuk az agancs fejlődését. Az antitestek specifikusságát az agancs különböző régióiból származó fehérje mintákon végzett immunblott segítségével teszteltük. A szarvas magzat epifízis porcból származó metszeteken végzett immunfestés is igazolta az antitestek specifikus kötődését a szövetben levő

antigénhez. Hematoxilin-eozin festést alkalmaztunk a szöveti morfológia bemutatására, a porcos mátrixot alcián kék, a mineralizált mátrixot pedig von Kossa festéssel azonosítottuk.

A porc link protein és hialuronsav kifejeződését illetően az agancs csúcsi mesenchyma rétege különbözött az oldal mesenchymától. *In situ* hibridizációval matrilin génaktivitást mutattunk ki az osteoprogenitor sejtekben és az osteoblastokban. Immunfestéssel mindezt fehérje szinten is alátámasztottuk. Fokozott matrilin-2 kifejeződést tapasztaltunk a magas differeciációs potenciállal rendelkező sejtekben, mint a mesenchyma sejtek, prechondroblastokban és preosteoblastokban. A matrilin-2 alkalmazható ezen sejtek azonosítására.

Az összes matrilint kimutattuk a fejlődő szarvas agancs kollagén I-ben gazdag rétegeiben. A porcoszlopok és a perivaszkuláris szövet határzónájában, a kollagén I-hez hasonlóan, az összes matrilin kimutatható volt, viszont a porc link protein és kollagén II nem fejeződött ki.

Kutatásunk kiterjedt a csont regenerációs modell molekuláris hátterének a vizsgálatára is, a csontosodásban fontos szerepet jásztó molekulák segítségével. A disszertáció a porcosodást szabályozó FGFR3 és Sox9 kifejeződését tárgyalja. FGFR3 immunreakciót mutattunk ki a proliferáló sejtekben, mint a prechondrocytákban és chondrocytákban, viszont a hipertróf sejtek negatívak voltak. FGFR3 fragmentumokat mutattunk ki a hipertróf sejtek mátrixában, amelyek feltehetően proteolitikus hasítás következtében kerültek oda. Az FGFR3 extracelluláris fragmentumai megkötik a hozzájuk kapcsolódó ligandot viszont már nem képesek továbbítani sejten belülre a jelet. A hasonlóság az FGFR3 kifejeződését illetően az agancsban és az epifízis porcban, hasonló funkcióra utal a két különböző rendszerben.

A Sox9 kifejeződése szintén hasonlít az epifízis porcbeli mintázatához. Erős *in situ* hibridizációs jelet figyeltünk meg a mesenchyma sejtekben, prechondrocytákban, chondrocytákban, viszont a hipertróf porcsejtek nem termelték a vizsgált mRNS-t. Elöször sikerült kimutatnunk Sox9 transzkriptumot osteoblastokban.

Végkövetkeztetésként elmondhatjuk, hogy a matrilineket, más ECM markerekkel együtt jól tudtuk felhasználni a végtagváz és az agancsfejlődés követésére. Az alapvető sejtdifferenciációs folyamatok hasonlóak voltak mindkét rendszerben, a megfigyelt különbségek hátterében nagyobbrészt Az agancs gazdag vérellátása és a perivaszkuláris szövet befolyása áll. Jelenleg további vizsgálatokat végzünk annak érdekében, hogy meghatározzuk azokat a molekuláris

mechanizmusokat, melyek befolyásolják a térbeli és időbeli gén expressziót az agancs fejlődés során és megadják a matrilinek pontos szerepét a vázrendszer felépítésében és fejlődésében.