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Expressão das proteínas da matriz na discondroplasia da tíbia

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palavras-chave

Ossificação endocondral, discondroplasia da tíbia, metaloproteinases da matriz, factores de crescimento, matriz extracelular, placa de crescimento.

resumo

A discondroplasia da tíbia (TD) em aves consiste numa anomalia do esqueleto onde existe uma falha nos processos normais da ossificação endocondral. Esta patologia é caracterizada pela formação de uma cartilagem não vascularizada e não mineralizada que se estende até à metáfise. Uma vez que existem várias anomalias do esqueleto em mamíferos com lesões semelhantes às apresentadas pela TD, este trabalho teve como objectivo a caracterização desta patologia em termos das moléculas que podem estar envolvidas no seu desenvolvimento. Assim, foi estudada a expressão das macromoléculas da matriz extracelular, das enzimas degradadoras da matriz (metaloproteinases da matriz: MMPs), bem como das moléculas envolvidas na proliferação e diferenciação celular, na angiogénese e apoptose. A expressão génica foi realizada, por PCR quantitativo em tempo real, em placas de crescimento normais e discondroplásicas obtidas a partir de frangos de carne (broilers) da estirpe Cobb. Os níveis proteicos de algumas MMPs foram analisados por immunoblotting e zimografia de gelatina. No presente estudo não se verificou alteração na expressão dos genes dos colagénios do tipo II, IX, X e XI, bem como do agrecano, nas lesões discondroplásicas. Observou-se uma redução acentuada nos níveis de mRNA da gelatinase-B (MMP-9), da colagenase-3 (MMP-13) e das estromalisinas -2 (MMP-10) e -3 (MMP-11), bem como nos níveis proteicos da gelatinase-A (MMP-2) e da MMP-13. Por outro lado, a MMP-7 aumentou drasticamente a expressão do seu gene. As moléculas envolvidas na proliferação e diferenciação dos condrócitos, tais como a PTHrP, o Ihh, o Cbfa-1 e o Sox-9, mantiveram a sua expressão génica nas lesões. Por outro lado, o TGF-β reduziu a sua expressão. A caspase-3 também dimimuiu a sua expressão na patologia. Em relação aos factores angiogénicos, o FGF manteve a sua expressão e o VEGF aumentou significativamente nas lesões. Este aumento do VEGF juntamente com o aumento da MMP-7 sugere um aumento da hipoxia nas lesões. Os nossos resultados sugerem que a acumulação da cartilagem observada na discondroplasia é devida a uma diminuição da proteólise da matriz, resultado de uma sub-expressão das MMPs, e não de um aumento da produção das macromoléculas da matriz. Desta forma, os nossos resultados sugerem que a falha na expressão e/ou activação das MMPs poderá estar associada ao desenvolvimento da discondroplasia da tíbia em aves. Finalmente, os nossos resultados vêm suportar os resultados anteriores que sugerem uma ligação entre a expressão das MMPs e anomalias no processo de ossificação endocondral.

keywords

Endochondral ossification, tibial dyschondroplasia, matrix metalloproteinases, growth factors, extracellular matrix, growth plate.

abstract

Avian tibial dyschondroplasia (TD) is a skeletal disease where the normal events of endochondral bone formation are disrupted. It is characterized by the formation of a lesion composed of nonvascularized and nonmineralized cartilage that can extend into the metaphysis. Because there are several mammalian skeletal diseases with lesions similar to TD, the present work aimed to characterize the disease in terms of the molecules that may be involved in its development. Thus, the expression of extracellular matrix (ECM) macromolecules, ECM-degrading enzymes (matrix metalloproteinases: MMPs), and the regulatory molecules involved in cell proliferation and differentiation, angiogenesis, and apoptosis, were studied. Gene expression was performed by real-time quantitative PCR in normal and TD-affected growth plates from 3-week-old broiler chicks (Cobb strain). The protein levels of some of the MMPs studied were analysed by immunoblotting and gelatin zymography. The collagen types II, IX, X, and XI as well as aggrecan did not change their gene expression in dyschondroplastic lesions. There was a pronounced reduction in the mRNA levels of gelatinase-B (MMP-9), collagenase-3 (MMP-13), and of stromelysins-2 (MMP-10) and -3 (MMP-11), as well as in the protein levels of gelatinase-A (MMP-2) and MMP-13. On the other hand, MMP-7 mRNA has increased significantly. The molecules involved in chondrocyte proliferation and differentiation, such as PTHrP, Ihh, Cbfa-1, and Sox-9 have maintained their mRNA levels in the pathology. On the other hand, TGF-β has decreased its gene expression. Caspase-3 also showed diminished mRNA levels in the pathology. Regarding the angiogenic factors, FGF has maintained its expression and VEGF has increased significantly in the lesions. The increment in VEGF in conjunction with the increased expression of MMP-7 suggests the formation of a hypoxic environment in the lesions. Our results suggest that the accumulated cartilage observed in dyschondroplasia seems to be the result of decreased matrix proteolysis due to the downregulation of MMPs and not to an increased production of the matrix macromolecules. Thus, our results suggest that the failure in the expression or lack in the activation of MMPs might be associated with the development of avian tibial dyschondroplasia. Furthermore, our results strengthen the link between the lack in MMP expression and abnormal endochondral bone formation.

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ABBREVIATIONS

ALP	alkaline phosphatase
APMA	4-aminophenylmercuric acetate
BLAST	Basic Local Alignment Search Tool
BMP	bone morphogenetic protein
Cbfa1	core binding factor-1
cDNA	complementary deoxyribonucleic acid
COMP	cartilage oligomeric matrix protein
Ct	threshold cycle
DNA	deoxyribonucleic acid
ECM	extracellular matrix
FACIT	fibril associated collagen with interrupted triple helix
FGF	fibroblast growth factor
GAG	glycosaminoglycan
Ihh	Indian hedgehog
IL	interleukin
LTBP	latent TGF-β binding protein
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MT-MMP	membrane type-matrix metalloproteinase
PCR	polymerase chain reaction

Ptc	patched
PTH	parathyroid hormone
PTHrP	parathyroid hormone-related peptide
RT-PCR	reverse transcription-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Smo	smoothened
TD	tibial dyschondroplasia
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of matrix metalloproteinase
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor

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1 INTRODUCTION

1.1 BONE FORMATION

Bone develops by replacement of a preexisting connective tissue. There are two processes of bone formation (osteogenesis) in the embryo. In intramembranous bone formation the bone tissue is laid down directly in primitive connective tissue or mesenchyme without passing through a cartilage stage. Examples of bones developed by intramembranous ossification are the membrane bones such as the flat bones of the skull. On the other hand, in endochondral bone formation the mesenchymal cells can differentiate into hyaline cartilage which then provides the template or anlage for the future bone (Kierszenbaum, 2002). This process is responsible for the formation of most of the vertebrate appendicular and axial skeleton (Ortega et al., 2004).

Long bones are separated in epiphysis, metaphysis, and diaphysis (Fig. 1.1). The epiphyses are the ends of the long bones and consist of spongy bone covered by a thin layer of compact bone. The epiphysis is covered by the articular cartilage constituted by hyaline cartilage. Between the epiphysis and diaphysis exists the metaphysis. During growth, the epiphysis and metaphysis are separated by the epiphyseal growth plate which is responsible for the growth in length of the long bone. The epiphyseal growth plate becomes reduced to an epiphyseal line from puberty to maturity, and the long bone can no longer grows in length. The diaphysis consists of compact bone forming a hollow cylinder with a central marrow space, the marrow cavity (Kierszenbaum, 2002).

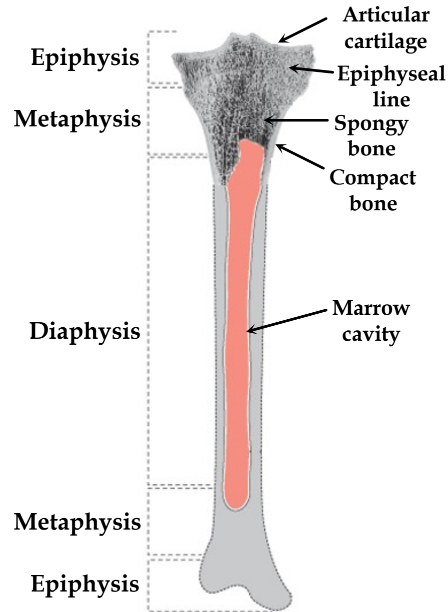


Figure 1.1 – General aspect of a long bone. Adapted from Schenk RK, Felix R, Hofstetter W: Connective Tissue and its Heritable Disorders. New York, Wiley-Liss, 1993 *in* Abraham L. Kierszenbaum: Histology and Cell Biology - An Introduction to Pathology. Missouri, Mosby, 2002.

1.2 ENDOCHONDRAL BONE FORMATION

1.2.1 Primary Center of Ossification

The process of endochondral ossification begins with the proliferation of chondrocytes in the middle of the diaphysis leading to the formation of the primary center of ossification (Fig. 1.2). The proliferated chondrocytes deposit an extracellular matrix containing type II collagen in this region. Afterwards, these chondrocytes undergo maturation to hypertrophy and starts to synthesize a matrix containing type X collagen, a marker of hypertrophy. The hypertrophic chondrocytes produce angiogenic factors such as the vascular endothelial cell growth factor (VEGF) to induce the formation of blood vessels from the perichondrium, a layer of undifferentiated cells that can differentiate into chondroblasts which surrounds the cartilage tissue. The newly formed blood vessels bring with them the osteoprogenitor and hematopoietic cells. Then, hypertrophic chondrocytes undergo apoptosis as calcification of the matrix in the middle of the diaphysis of the cartilage template occurs. At the same time, the osteoprogenitor cells of the perichondrium form a thin periosteal collar of bone around the midpoint of the diaphysis. After that, blood vessels invade the space formerly occupied by the hypertrophic chondrocytes, and then they branch and project toward either end of the center of ossification. Blind capillary ends extend into the cavities formed within the calcified cartilage. Osteoprogenitor cells and hematopoietic stem cells reach the core of the calcified cartilage through the perivascular connective tissue surrounding the invading blood vessels. Then, osteoprogenitor cells differentiate into osteoblasts that aggregate on the surfaces of the calcified cartilage and begin to deposit bone matrix (osteoid). The osteoid contains abundant type I collagen fibers embedded in the extracellular matrix. The deposit of osteoid denotes the beginning of osteogenesis and results in the formation of bone spicules and later, in trabeculae. As a consequence, cancellous bone appears in the midsection of the template. As the ossification process advances toward the adjacent proliferative zones, the bone marrow cavity increases in size owing to loss of cartilage and erosion of newly formed bone spicules by osteoclasts. The periosteal collar grows in length and thickness at the midsection of the shaft and compensates for the loss of endochondral bone while also strengthening the gradually eroding cartilage template. In this phase, the primary center of ossification is formed at the diaphysis which corresponds to the center of ossification in the interior of the cartilage template (Kierszenbaum, 2002).

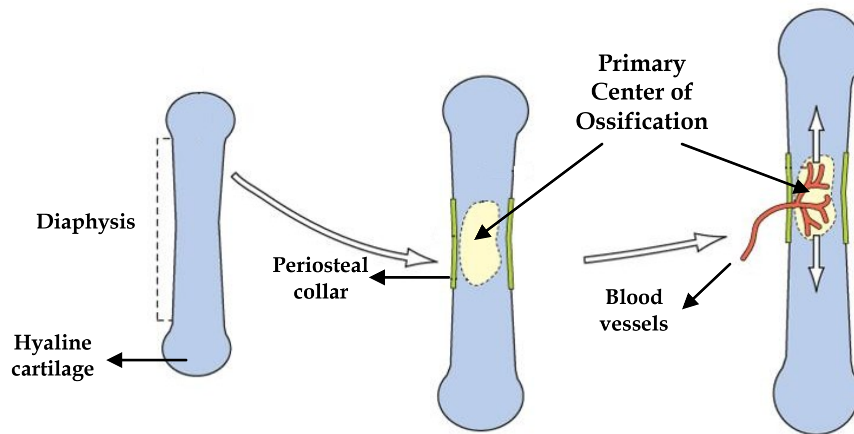


Figure 1.2 – Primary center of ossification in a long bone. Adapted from Abraham L. Kierszenbaum: Histology and Cell Biology - An Introduction to Pathology. Missouri, Mosby, 2002.

Primary centers of ossification occur in the diaphysis of long bones by the third month of fetal life. After birth, secondary centers of ossification develop in the epiphyses.

1.2.2 Secondary Centers of Ossification

During the process of formation of secondary centers of ossification in the epiphyses, blood vessels and osteoprogenitor cells, coming from the perichondrium, invade the space occupied by hypertrophic chondrocytes and one of the secondary centers of ossification is formed in one of the epiphyses (Fig. 1.3-A). Later, a similar secondary center of ossification appears in the opposite epiphysis (Fig. 1.3-B). Most of the hyaline cartilage of the epiphyses is replaced by the spongy bone with the exception of the articular cartilage and the epiphyseal growth plate (Fig. 1.3-B). The epiphyseal growth plate is located between the epiphysis and the metaphysis and is responsible for subsequent growth in length of the bone. This structure becomes reduced to an epiphyseal line from puberty to maturity, and the long bone no longer grows in length (Fig. 1.3-C). At the end, blood vessels from the diaphysis and epiphysis intercommunicate and all the epiphyseal cartilage is replaced by bone with the exception of articular cartilage (Fig. 1.3-D) (Kierszenbaum, 2002)

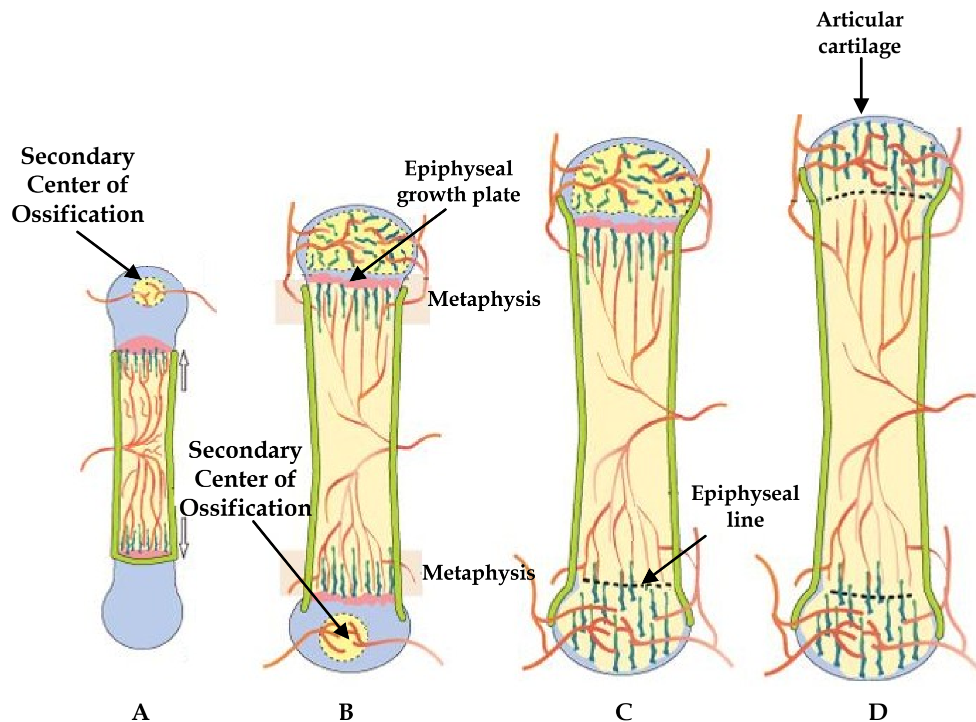


Figure 1.3 – Secondary centers of ossification in a long bone. Adapted from Abraham L. Kierszenbaum: Histology and Cell Biology - An Introduction to Pathology. Missouri, Mosby, 2002.

1.2.3 Zones of Endochondral Ossification

During the process of cartilage erosion and bone deposition, four major zones can be distinguished (Fig. 1.4).

The reserve zone, the region more distant from the erosion center, is composed of primitive hyaline cartilage and is responsible for the growth in length of the bone as the erosion and bone deposition process advances.

The proliferative zone is characterized by active proliferation of chondrocytes. Here, chondrocytes are aligned in cellular columns parallel to the long axis of the cartilage template.

The hypertrophic zone is the region where calcification of matrix surrounding the columns of previously proliferated chondrocytes takes place and where chondrocytes undergo apoptosis. Here, the chondrocytes increase significantly in size, that is, they become hypertrophic, and start to produce type X collagen which is considered a marker of hypertrophic chondrocytes. As a result of chondrocyte enlargement in this region, the septa separating adjacent columns appear thinner. Calcification begins in the longitudinal septa. The deepest layer of the hypertrophic zone, proximal

to the vascular invasion zone, displays the blind end of capillary sprouts derived from the developing bone marrow cavity occupied by hematopoietic cells.

In the zone of vascular invasion, blood vessels penetrate the transverse septa and carry migrating osteoprogenitor cells with them. These zones will be discussed in more detail in section 1.3.

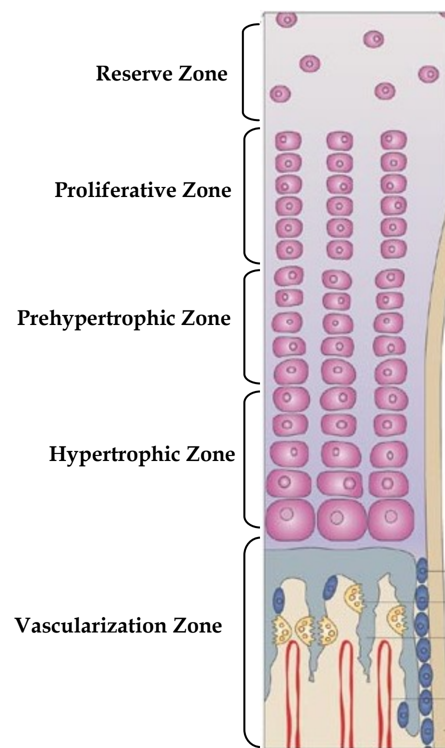


Figure 1.4 – Zones of endochondral ossification. Adapted from Abraham L. Kierszenbaum: Histology and Cell Biology - An Introduction to Pathology. Missouri, Mosby, 2002.

1.3 The Growth Plate

The growth plate is a specialized tissue responsible for the growth in length of long bones until the time of growth plate closure, that is, at the time that long bones can no longer grows in length. This structure functions through a highly complex and synchronous mechanism of continuous cell proliferation, cell enlargement, or hypertrophy, and cell removal (Howell and Dean, 1992). In this way, the growth plate can be divided into different zones that distinguish unique morphological and biochemical stages during the process of endochondral ossification (Ballock and O'Keefe, 2003). These zones are the reserve, proliferative, hypertrophic, and vascularisation zones. Each chondrocyte of the growth plate moves through the entire spectrum of maturational stages while remaining in a spatially fixed location throughout its existence (Farquharson and Jefferies, 2000). The description of the structure of the epiphyseal growth plate in mammals making the comparison with the analogous structure in birds will be presented in section 1.3.2. Basically, avian and mammalian growth plates are quite similar, however some differences are noted (Leach and Gay, 1987).

1.3.1 Matrix Composition

The extracellular matrix of cartilage is a combination of collagens, noncollagenous glycoproteins, and proteoglycans surrounding cells and fibers of the connective tissue (Ballock and O'Keefe, 2003) (Fig. 1.5). Essentially, the matrix consists of fibrillar networks of collagen type II and the main proteoglycan of cartilage, aggrecan (Heinegard et al., 1999).

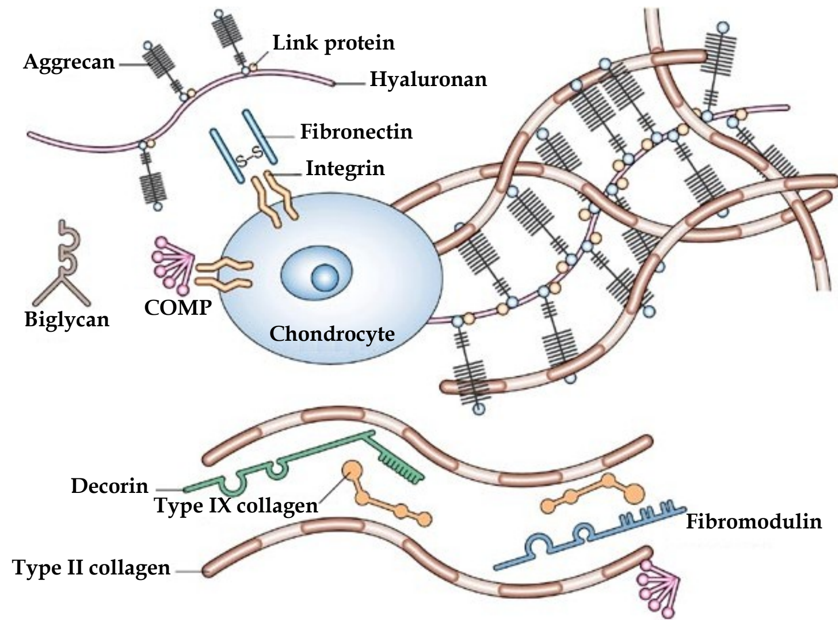


Figure 1.5 – Extracellular matrix of cartilage. Adapted from Chen FH *et al.* (2006) Technology Insight: adult stem cells in cartilage regeneration and tissue engineering, *Nat Clin Pract Rheumatol* **2:** 373–382 doi:10.1038/ncprheum0216.

1.3.1.1 Collagen type II

The major macromolecule of cartilage is collagen type II. It is composed of three identical polypeptide chains – α1(II) – that intertwine along most of their length to form a triple helix. The α-chains are synthesized as pro-α-chains, which possess large propeptides at both their *N* and *C* termini. The propeptides are separated from the central triple-helix-forming region by short telopeptides. The propeptides are required for normal trimer assembly during synthesis within the chondrocyte, but are removed from the resulting procollagen by proteolysis following secretion into the extracellular space, leaving only the telopeptides attached to the triple helix (Martel-Pelletier et al., 2008). The triple helical region of the type II collagen molecule is resistant to degradation by most proteases but can be cleaved by the action of collagenases. Collagenases act consecutively at a single site in each of the three α-chains to yield fragments that are about three-quarters and one-quarter the length of the intact molecule. Three mammalian collagenases exist and are named matrix metalloproteinase (MMP)-1, MMP-8, and MMP-13. MMP-13 has preferential action on type II collagen over the other fibrillar collagens (Martel-Pelletier et al., 2008). Among these collagenases, only MMP-13 has yet been identified in the chicken (Lei et al., 1999).

The importance of type II collagen in cartilage formation and function is best illustrated by the consequence of type II collagen gene mutations which give growth plate cartilage

abnormalities (Byers, 2001). For example, when type II collagen is mutated, spondyloepiphyseal dysplasia, Kniest dysplasia, and Stickler syndrome may occur (Holden et al., 1999).

1.3.1.2 Collagen type XI

Type XI collagen is, like type II collagen, a fibrillar collagen that is cartilage specific. It is less abundant than type II collagen and it is composed of three different α-chains that intertwine to form a heterotrimeric triple helix. This molecule possesses the composition α1(XI), α2(XI), α3(XI). Type XI collagen is synthesized and secreted as a procollagen in a similar manner to type II collagen. However, it undergoes proteolytic removal of only its *C*-propeptide. Type XI collagen molecules do not form unique fibrils but occur in heterotypic fibrils in association with type II collagen molecules. Type XI collagen molecules reside at the center of the heterotypic fibril with their *N*-propeptides protruding from its surface and limiting its lateral growth. This collagen is most concentrated in the thin collagen fibrils that form the pericellular network (Martel-Pelletier et al., 2008).

Stickler syndrome has been associated with mutations in type XI collagen (Sirko-Osadsa et al., 1998).

1.3.1.3 Collagen type IX

Type IX collagen is a member of the fibril-associated collagen with interrupted triple helix (FACIT) family. This collagen is also cartilage specific. It does not form a collagen fibril itself but is instead present at the surface of the type II/type XI fibril. It is composed of three different α-chains that form a heterotrimeric molecule possessing the composition α1(IX), α2(IX), α3(IX). Type IX collagen molecule consists of three triple helical domains bordered by nonhelical domains (Martel-Pelletier et al., 2008).

Some mutations have been described in type IX collagen genes giving rise to multiple epiphyseal dysplasias (Byers, 2001).

1.3.1.4 Collagen type X

Type X collagen is a cartilage-specific collagen that, under normal circumstances, is confined to the hypertrophic zone of the growth plate, where it participates in endochondral ossification. It is a homotrimeric molecule with one central triple helical domain and nonhelical termini. Type X collagen can be cleaved within its triple helical region by mammalian MMP-1. In the chicken, MMP-1 has not yet been identified. In mammals, mutations in type X collagen gene lead to various forms of metaphyseal dysplasia (Martel-Pelletier et al., 2008).

1.3.1.5 Proteoglycans (Aggrecan)

Cartilage is resistant to compression which is achieved by filling the interfibrillar matrix with a very high content of proteoglycans. Thus, proteoglycans contribute to the biomechanical properties of cartilage (Hardingham, 1999). These molecules are major components of the extracellular matrix in cartilage and they form a special class of glycoproteins with attached long unbranched and highly charged glycosaminoglycan (GAGs) chains (Hardingham, 1999) (Fig. 1.6). GAGs are linear polymers of disaccharides with sulfate residues. GAGs control the biological functions of proteoglycans by establishing links with cell surface components, growth factors, and other ECM constituents. Different types of GAGs are attached to a core protein to form a proteoglycan. Proteoglycans are named according to the prevalent GAG (for example, proteoglycan chondroitin sulfate, proteoglycan dermatan sulfate, proteoglycan heparin sulfate) (Kierszenbaum, 2002). The core protein, in turn, is linked to a hyaluronan molecule by a linker protein. The hyaluronan molecule is, in this manner, the axis of a proteoglycan aggregate (Kierszenbaum, 2002). This molecule is synthesized at the plasma membrane of the cell via a hyaluronan synthase and extruded directly into the extracellular space where proteoglycan aggregate formation occurs in the pericellular matrix (Martel-Pelletier et al., 2008). The linker protein is, as mentioned above, responsible for interaction with the core protein of the proteoglycan and the hyaluronan molecule. The linker protein stabilizes the proteoglycan aggregate and prevents its dissociation under physiological conditions. It also forms a protein coat covering the surface of hyaluronan which helps protect the hyaluronan from hyaluronidases and free radical mediated degradation (Martel-Pelletier et al., 2008).

The main proteoglycan in cartilage is aggrecan. Aggrecan is a proteoglycan with a core protein of high molecular weight (~250 000) and is expressed predominantly in cartilaginous tissues (Hardingham and Fosang, 1992). Because aggrecan is a highly negatively charged molecule it provides fixed charged density and therefore an osmotic environment that creates a swelling pressure (Doege et al., 1997). In this way, aggrecan at high concentration draws water into the tissue as it creates the large osmotic swelling pressure. Thus, it helps to maintain structural integrity during mechanical loading and also regulate cation concentration in the matrix (Hardingham, 1999). In the matrix of growth plate, aggrecan is enmeshed in the collagen fibril network composed of types II, IX, and XI collagen (Orth and Cook, 1994).

The essential role of aggrecan in cartilage function is demonstrated by mutations in the aggrecan gene that lead to chondrodysplasias (Martel-Pelletier et al., 2008).

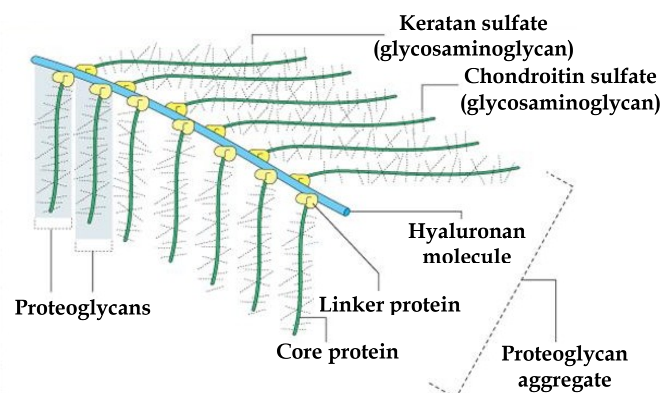


Figure 1.6 – Proteoglycan aggregate. Adapted from Abraham L. Kierszenbaum: Histology and Cell Biology - An Introduction to Pathology. Missouri, Mosby, 2002.

1.3.1.6 Small leucine-rich repeat proteoglycans

Cartilage contains a variety of small leucine-rich repeat proteoglycans such as decorin, biglycan, fibromodulin, and lumican. These molecules may contribute to the stabilization of collagen network because they interact with adjacent collagen fibers. Indeed, decorin, fibromodulin, and lumican interact with the fibrillar collagen via their core proteins. The core proteins bind to one collagen fiber and the negatively charged side chains may participate in

interactions with the neighboring fibril. Consequently, these interactions will provide a tight, multiple-site linkage between adjacent collagen fibers contributing to regulate fibril diameter and fibril-fibril interaction (Heinegard et al., 1999). Thus, these interactions contribute to stabilize the network and to maintain the integrity of the tissue. These interactions might also limit access of the collagenases to their unique cleavage site on each collagen molecule, and therefore help protect the fibrils from proteolytic damage. Fibromodulin and lumican interact with the same region of the collagen molecule, whereas decorin interacts at a distinct site. Biglycan is able to interact with type VI collagen and so participate in the formation of an organized network. The glycosaminoglycan chains have been associated with the interaction with several growth factors, and they enable the small leucine-rich repeat proteoglycans to provide a sink for growth factor accumulation within the extracellular matrix, thus regulating growth factor access to the cells (Martel-Pelletier et al., 2008).

1.3.1.7 Cartilage oligomeric matrix protein (COMP)

Cartilage oligomeric matrix protein (COMP) is one of the major proteins in cartilage. This protein is a homopentamer of five subunits. It has been shown that COMP interacts with triple helical collagen. Therefore, COMP may have a role in stabilizing the collagen network and/or in promoting the collagen fibril assembly. In addition, it has been also proposed that COMP may bind chondrocytes. The protein has been found close to the cells in the proliferative region of the growth plate, where it thus may have a role in cell interactions (Heinegard et al., 1999).

1.3.2 Structural Organization

1.3.2.1 Reserve Zone

The reserve zone, or resting zone, lies underneath the articular cartilage. The chondrocytes in this zone are spherical and are approximately the same size as the proliferating chondrocytes (Orth and Cook, 1994) (Fig. 1.7). They are in a relatively quiescent state (Ballock and O'Keefe, 2003) and are scattered irregularly throughout the matrix (Howell and Dean, 1992) which is in a high volume when compared with cell volume (Ballock and O'Keefe, 2003). The collagen matrix in this region consists of randomly oriented fibers (Howell and Dean, 1992). The vascularisation of this zone is poor (Orth and Cook, 1994).

In terms of composition, this zone contains the lowest amount of proteoglycan, however it has the highest concentration of collagen fibrils, composed of type II collagen. As mentioned before the fibrils are randomly distributed and oriented.

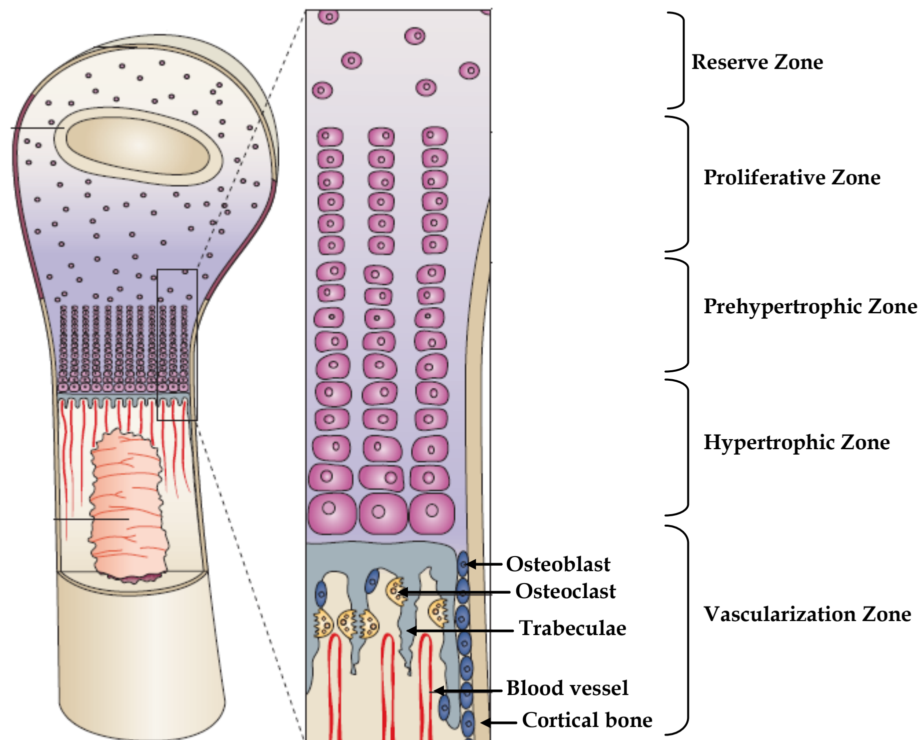


Figure 1.7 – The morphology of the growth plate. Adapted from Page-McCaw et al. (2007) Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol* **8**(3):221-233.

1.3.2.2 Proliferative Zone

In the proliferative zone chondrocytes are flattened in shape (Ballock and O'Keefe, 2003) and divide rapidly forming well-oriented columns lying in the longitudinal axis of the bone (Fig. 1.7). Matrix is produced at a rapid rate and the random collagenous matrix found in the reserve zone becomes more ordered, forming longitudinal septa, and by the end of the proliferating zone the predominant fiber direction is longitudinal (Howell and Dean, 1992).

The proliferative zone has the highest percentage of extracellular matrix volume (Orth and Cook, 1994). Proliferating chondrocytes synthesize types II, IX, and XI collagen and aggrecan (Orth, 1999).

Proliferative chondrocytes of avian growth plates are arranged into columns, but unlike mammals, they are not easily distinguishable due to a much higher density and less extracellular matrix. Like in mammals, the cells have a flattened, oblate spheroid shape (Farquharson and Jefferies, 2000).

1.3.2.3 Hypertrophic Zone

In the hypertrophic zone, flattened cells from the proliferative zone become spherical in shape increasing significantly their size and maintaining their columnar orientation (Fig. 1.7). They may become super ellipses having their long axis parallel with the longitudinal septa. During differentiation, chondrocytes have a five to tenfold increase in intracellular volume (Ballock and O'Keefe, 2003). The cytoplasm, endoplasmic reticulum, cisternae, Golgi, and mitochondria also become greatly expanded (Howell and Dean, 1992). It has been suggested that the factors that contribute to cell enlargement may involve alterations in ion channels that lead to an ingress of water (Hunziker, 1994). The environment surrounding the cells becomes increasingly anaerobic and void of a nutrient supply. In this manner, the cells undergo anaerobic glycolysis, using glycogen stores as their fuel source (Orth and Cook, 1994). Transverse expansion of the lacunae occurs at the expense of narrowing the longitudinal septa adjacent to the chondrocytes. In the zone of mineralization, cell expansion reaches the maximum and calcification of the matrix takes place (Howell and Dean, 1992).

In the hypertrophic and calcifying zones of the avian growth plate there are longer columns of cells that become randomly oriented and therefore cell columns are no longer apparent (Leach and Gay, 1987).

Chondrocyte differentiation and hypertrophy are crucial steps in the longitudinal growth of long bones (Ballock and O'Keefe, 2003). The growth of a long bone occurs mainly due to the increase in chondrocyte height although matrix synthesis and chondrocyte proliferation also contribute to the growth. It has been shown that bones with different growth rates have also chondrocytes with different sizes. In other words, chondrocytes in bones with rapid growth, such as the femur, increase more in size that do chondrocytes in growth plates in bones with lower growth (Ballock and O'Keefe, 2003).

During differentiation, chondrocytes begin to synthesize type X collagen and decrease the production of collagen types II and IX (Orth and Cook, 1994). Thus, terminally differentiated hypertrophic chondrocytes exclusively express type X collagen (Ballock and O'Keefe, 2003).

Mineralization of the extracellular matrix occurs in the lower hypertrophic zone, approximately 100 µm from the tip of the most advanced metaphyseal blood vessels (Howlett, 1979). The function of extracellular matrix is to promote calcification of cartilage that serves as a template for bone formation by osteoblasts. The mineralization of cartilage occurs primarily in the

matrix located between distinct hypertrophic chondrocyte columns and not in the interzone between hypertrophic chondrocytes in the same column (Johnstone et al., 2000).

Hypertrophic chondrocytes have a role in preparing the extracellular matrix for calcification. As mentioned above, they express exclusively type X collagen as well as the enzyme alkaline phosphatase (ALP). Both type X collagen and ALP are involved in mineralization (Orth and Cook, 1994) being ALP present at high concentrations in matrix vesicles. This enzyme has been suggested to be important for the release of phosphorus into the matrix. Extracellular matrix vesicles are small membranes vesicles secreted by hypertrophic chondrocytes into the surrounding matrix (Anderson, 1995) which are thought to be the initial sites of mineralization in the hypertrophic zone of the growth plate (Anderson, 1976). These particles also contain calcium binding proteins (annexins) (Ballock and O'Keefe, 2003). Annexins, in particular annexin V, mediate the binding between type II and type X collagen and matrix vesicles. Type X collagen stimulates the activity of the annexin-V calcium channel (Kirsch et al., 2000). In this way, and as mentioned before, one of the roles suggested for type X collagen (present only in the hypertrophic zone) is to contribute for deposition of calcium in the matrix having therefore an important role in mineralization (Ballock and O'Keefe, 2003).

Apoptosis is the mechanism through which cells undergo programmed death, a process necessary for the homeostasis of most organs, including the growth plate (Ballock and O'Keefe, 2003). Terminally differentiated chondrocytes undergo apoptosis at the lower region of the hypertrophic zone (Hatori et al., 1995, Gibson et al., 1997). As already referred, these chondrocytes prepare the matrix for calcification which is then used as a template for osteoblastic bone formation. Once the cartilage matrix calcifies, terminally differentiated hypertrophic chondrocytes die by apoptosis and its removal provides space for the invasion of blood vessels and bone-marrow stromal cells. Thus, apoptosis of terminally differentiated chondrocytes in the hypertrophic zone is necessary for vascularisation and bone formation to occur (Ballock and O'Keefe, 2003). On the other hand, the penetrating blood vessels from subchondral bone could initiate apoptosis (Orth, 1999). Terminally differentiated chondrocytes in the hypertrophic zone undergoing apoptosis show morphological features such as condensation of the nuclear chromatin, cell shrinkage, and plasma membrane blebbing (Hatori et al., 1995, Gibson et al., 1997).

The enzymes that initiate apoptosis are named caspases. Caspases are a family of cysteine proteases that cleave target proteins with high specificity. These enzymes are present in an inactive (zymogenic) form in the cytoplasm of all cells and thus the cells are primed to undergo apoptosis (Ballock and O'Keefe, 2003).

1.3.2.4 Vascularization Zone

Beneath the last row of hypertrophic chondrocytes the metaphyseal vascular channels are found in compartments bounded by calcified cartilage (Fig. 1.7). The vascular channels are aligned along the longitudinal axis of the bone and contain an ascending and descending capillary system (Ballock and O'Keefe, 2003).

The avian growth plate is more vascular than the mammalian one, being the metaphyseal blood vessels larger and penetrating deeper into the growth plate (Pines and Hurwitz, 1991).

The growth plate is an avascular structure where cells receive nutrients and oxygen by diffusion through the extracellular matrix from the blood vessels coming from the metaphyseal side of the growth plate. Vascular invasion plays an important role in the regulation of the endochondral ossification process.

In summary, and regarding the differences between the mammalian and avian growth plates, the last one contains longer columns of cells than the mammalian one. In contrast to mammals, cell columns of the avian growth plate become randomly oriented and in the hypertrophic and calcifying zones they are no longer apparent. More cells are found in each zone of the avian growth plate and the metaphyseal blood vessels penetrate more deeply. The growth plate of a 4- to 7-week-old chicken contains approximately 200 cells per column compared with 25 cells in that of a 6-week-old rat. Increasing disorder occurs phylogenetically, avian epiphyseal cartilages show less order than mammalian, reptilian less than avian and so on (Leach and Gay, 1987). The time taken for a chondrocyte to move from a proliferative to a terminally differentiated phenotype is species dependent. It is approximately 21 hours in the broiler chick (Thorp, 1988) and 2 days and 20 days for the rat and the man, respectively (Kember and Sissons, 1976).

It has been suggested that although differences in structure between the avian and mammalian growth plate were noted, the physiological control mechanisms might be the same since electron microscopy has revealed many similarities. In this way, the process of endochondral bone growth in the mammalian and avian growth plates has been reported to be functionally similar (Leach and Lilburn, 1992).

1.3.3 Regulatory Molecules

1.3.3.1 Indian hedgehog and Parathyroid hormone-related peptide

Indian hedgehog (Ihh) belongs to the family of hedgehog proteins which are morphogens that play crucial roles in embryonic development. Ihh is expressed by prehypertrophic chondrocytes. It stimulates chondrocyte proliferation and inhibits chondrocyte hypertrophy. The inhibition of chondrocyte hypertrophy is dependent on induction by Ihh of expression of parathyroid hormone-related peptide (PTHrP). Hedgehogs bind to a receptor called patched (Ptc), thereby releasing smoothened (Smo), a membrane protein with an intrinsic intracellular activity that is abrogated by Ptc in the absence of hedgehogs. Releasing Smo results in its conformational change and a downstream signal to activate its intracellular targets (van der Eerden et al., 2003).

PTHrP is expressed by perichondrial cells and early proliferating chondrocytes. As mentioned above, Ihh is expressed by chondrocytes making the transition from a proliferating into a hypertrophic phenotype (prehypertrophic chondrocytes). Ihh activates adjacent chondrocytes and diffuses toward the lateral perichondrium where it can bind to its receptor Ptc. PTHrP production is then stimulated in the periarticular perichondrium. After that, PTHrP diffuses toward the prehypertrophic zone, which expresses high levels of PTH/PTHrP receptors and inhibits differentiation of proliferating chondrocytes. By limiting the transition from proliferation to hypertrophy, PTHrP limits the number of cells capable of expressing Ihh, and thus participates in a negative feedback loop regulating its own expression and the rate of chondrocyte differentiation (Mackie et al., 2008).

1.3.3.2 Transforming growth factor-β

Transforming growth factor-β (TGF-β) is a superfamily of growth factors which includes the family of bone morphogenetic proteins (BMPs). All these proteins regulate diverse cellular processes, such as proliferation, differentiation, apoptosis, and extracellular matrix formation during embryogenesis. TGF-β signaling has been implicated in cartilage and bone formation (Matsunobu et al., 2009). TGF-β is produced by chondrocytes as a latent, high molecular weight molecule in association with latent TGF-β binding protein (LTBP). In growth plate chondrocytes, the storage of TGF-β by LTBP is cell maturation dependent. In the human epiphyseal growth plate, TGF-β2 exists in the resting, proliferating, and hypertrophic zones with the highest expression seen in hypertrophic and mineralizing zones. TGF-β3 is expressed in the chondrocytes of proliferative

and hypertrophic zones. TGF-β1 is only found in the proliferative and upper hypertrophic zones (Li et al., 2005).

1.3.3.3 Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a chemoattractant for endothelial cells and is one of the most important growth factors for endothelial cells. It is considered a major factor initiating angiogenesis in the epiphyseal growth plate. Hypertrophic chondrocytes secrete VEGF to attract metaphyseal blood vessels to the chondro-osseous junction. The invading vasculature in turn promotes further hypertrophic differentiation to terminal hypertrophic cells and eventual apoptosis, thereby creating space for invading osteoclasts and osteoblasts to remodel and build bone on the remaining mineralized hypertrophic extracellular matrix (Evans and Oberbauer, 2008).

1.3.3.4 Sox9 and Core binding factor

Sox9 is a member of the Sox family of transcription factors that are characterized by a high-mobility-group (HMG)-box DNA binding domain whose sequence is at least 50% identical with that of the sex determining factor SRY. The Sox family is composed of several subgroups based on sequence homologies both within and outside the HMG box domain. Sox9 is a typical transcription factor. Indeed, Sox9 harbors a potent transcription activation domain and, like other Sox proteins, binds to a specific sequence in the minor groove of DNA (Crombrugghe et al., 2001). Sox9 is expressed in cells of mesenchymal condensations and in proliferating chondrocytes, buy not in hypertrophic chondrocytes. In cultured cells, Sox9 stimulates transcription of a number of cartilage matrix genes, including *Col2a1*, *Col11a2* and aggrecan (Kronenberg, 2003). Sox9 is required for normal rates of chondrocyte proliferation, and for delaying the onset of hypertrophy (Mackie et al., 2008).

Core binding factor (CBfa1), also known as Runx2, is a transcriptional regulatory factor that belongs to the Runt-domain gene family. It has a DNA-binding domain, *runt*, that is homologous with the *Drosophila* pair-rule gene *runt* (Enomoto et al., 2000). 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 osteoblasts (Kronenberg, 2003). Cbfa1 is a transcriptional activator of hypertrophic chondrocyte markers such as the collagen type X gene *Col10a1* (Mackie et al., 2008). Furthermore, Cbfa1 has been shown to stimulate hypertrophic

chondrocyte differentiation. By stimulating hypertrophic chondrocyte differentiation, Cbfa1 primes the cartilage skeleton for its subsequent invasion by osteoblasts and its replacement by a bone specific matrix. Indeed, once chondrocytes become hypertrophic they are destined for rapid cell death and replacement by bone (Crombrugghe et al., 2001).

1.3.3.5 Fibroblast growth factors

The family of fibroblast growth factors (FGF) comprises at least 22 members that interact with at least four receptors (FGFR) and are major regulators of embryonic bone development. Many of these 22 distinct FGF genes and four FGF receptors genes are expressed at every stage of endochondral bone formation (Kronenberg, 2003). Both FGF1 and FGF2, as well as FGF receptor-1 (FGFR1), FGFR2, and FGFR3 are expressed in chondrocytes. FGF signaling crucially regulates chondrocyte proliferation and differentiation. FGF2 signaling, independently, inhibits chondrocyte proliferation and accelerates hypertrophic chondrocyte differentiation (Kronenberg, 2003). However, part of the effects of FGF signaling is mediated by suppression of Ihh expression (Kronenberg, 2003; van der Eerden et al., 2003).

1.3.3.6 Fibronectin

Fibronectin is an adhesive dimeric glycoprotein found in the extracellular matrix of cartilage. Although fibronectin has no catabolic effect on cartilage, it is readily degraded into fragments by multiple proteases and, once fragmented, acquires catalytic activities. Fibronectin can bind several integrins and other cell-surface protein ligands. Integrins are adhesion molecules which mediate cell-matrix and cell-cell interactions. The integrin family consists of receptors that share as basic structure two noncovalently linked transmembrane glycoproteins (α and β subunits). Both subunits have a single transmembrane domain and, with the exception of β4, have short cytoplasmic regions (Helfrich and Horton, 1999). The role of these molecules in cartilage include roles in cartilage differentiation during fetal development, maintenance of tissue architecture and integrity including matrix synthesis and assembly and cell adhesion, and regulation of chondrocyte gene expression. Previous studies demonstrated that production of matrix metalloproteinases is mediated by interaction of fibronectin-fragments with α5β1 integrin (Martel-Pelletier et al., 2008).

1.3.3.7 Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases

Matrix metalloproteinases have been demonstrated to be involved in extracellular matrix degradation. They constitute a family of zinc-dependent proteases secreted as latent precursors (zymogens) which are proteolytically activated in the extracellular matrix. The activity of matrix metalloproteinases in the extracellular space can be specifically inhibited by tissue inhibitors of metalloproteinases. The expression of matrix metalloproteinases genes is regulated by cytokines, growth factors and cell contact with the extracellular matrix. The degradation of the extracellular matrix occurs normally during the development, growth, and repair of tissues. MMPs are divided into subgroups according to their structure and function. For example, collagenases 1, 2 and 3 can degrade types I, II, III, and V collagens. Collagenase-1 is synthesized by fibroblasts, chondrocytes, keratinocytes, monocytes and macrophages, hepatocytes, and tumor cells. Collagenase-2 is stored in cytoplasmic granules of polymorphonuclear leukocytes and released in response to a stimulus. Collagenase-3 can degrade several collagens (types I, II, III, IV, IX, X, and XI), laminin and fibronectin, and other extracellular matrix components. Stromelysins (1, 2, and metalloelastase) degrade basement membrane components (type IV collagen and fibronectin) and elastin. Gelatinases A and B can cleave degraded collagen (gelatin) (Kierszenbaum, 2002). MMPs and TIMPs will be discussed in more detail in section 1.4.

1.4 MATRIX METALLOPROTEINASES

The matrix metalloproteinases (MMPs) are a family of extracellular matrix (ECM)-degrading enzymes that share common structural and functional domains and are products of different genes (Massova et al., 1998). Collectively, MMPs are capable *in vitro* and *in vivo* of degrading all kinds of extracellular matrix protein components such as interstitial and basement membrane collagens, proteoglycans, fibronectin and laminin, as well as nonmatrix proteins (Bode et al., 1999). These are Ca^{2+}- and Zn^{2+}- dependent endopeptidases that are active at neutral pH (Vu and Werb, 2000). They are believed to play a crucial role in many physiological processes, including angiogenesis, embryogenesis, wound healing, and bone growth (Woessner, 1991, Matrisian, 1992, Birkedal-Hansen et al., 1993). The human MMPs have counterparts in other vertebrates including chicken and they have even been identified in invertebrates (Lepage and Gache, 1990; Nomura et al., 1997) and three have been sequenced from plant sources (Massova et al., 1998).

1.4.1 Structure of MMPs

The MMPs have a common domain structure which is organized into five distinctive, basic domains: a signal peptide, an amino-terminal propeptide, a catalytic domain, a hemopexin-like domain at the carboxy-terminal, and a hinge region (Visse and Nagase, 2003).

The signal peptide of approximately 20 amino acid residues is involved in protein secretion (Lemaître and D’Armiento, 2006).

The N-terminal propeptide of approximately 80 to 100 amino acids (Massova et al., 1998) is responsible for the latency of the zymogen and contains a highly conserved sequence called cysteine switch motif (… PRCGXPD…) (Lemaître and D’Armiento, 2006).

The catalytic domain of about 170 to 200 amino acids (Nagase et al., 2006) contains a conserved motif (HEXGHXXGXXH) binding a zinc ion involved in proteolysis (Lemaître and D’Armiento, 2006).

The C-terminal hemopexin-like domain of about 300 amino acids is highly conserved and shows sequence similarity to the plasma protein, hemopexin. The hemopexin-like domain has been shown to play a functional role in substrate binding and/or interactions with the tissue inhibitors of metalloproteinases (TIMPs), a family of specific MMP protein inhibitors (Borden and Heller, 1997).

The hinge region varies in length between the different MMPs and it separates the catalytic and the hemopexin-like domains. However, variations on this general structure may occur and therefore they can be divided into colagenases, gelatinases, stromelysins, matrilysin, and membrane-type MMPs, according to their domain structure but also according to their substrate specificity and sequence homology. In this way, at least 24 distinct MMPs have been identified in humans (Lemaître and D'Armiento, 2006).

1.4.2 Groups of MMPs

1.4.2.1 Collagenases

Collagenases possess the five basic domains of MMPs, that is, the signal peptide, the propeptide, the catalytic domain, the hinge region, and the hemopexin-like domain (Fig. 1.8). This group includes interstitial collagenase or collagenase-1 (MMP-1), neutrophil collagenase or collagenase-2 (MMP-8), and collagenase-3 (MMP-13). A fourth type of collagenase (MMP-18) has been identified in *Xenopus*, but has no known human homolog (Stolow et al., 1996). These proteases are called collagenases due to their capability to cleave the major fibrillar collagens (types I, II, and III) at a specific site in their very resistant triple-helical domain at neutral pH, generating fragments that are three-fourths N-terminal and one-fourth C-terminal of the intact molecule (Lemaître and D'Armiento, 2006). Collagenases can also digest a number of other ECM and non-ECM molecules (Visse and Nagase, 2003). MMP-1 also digests noncollagenous substrates including aggrecan and perlecan, and activates cytokines, such as interleukin (IL)1-β and tumor necrosis factor (TNF)-α (Gearing et al., 1994). MMP-13 cleaves a broad range of substrates, including proteoglycans, fibronectin, laminin, elastin, and type IV collagen (Knäuper et al., 1996).

The chicken MMP-13 protein shares 71% identity with human MMP-13 (Zijlstra et al., 2004). The highest homology exists between the catalytic domains (82%), whereas the propeptide and hemopexin domain homology is only 58 and 67%, respectively. MMP-13 has been cloned in the chicken (D'Angelo et al., 2000; Simsa et al., 2007a) but its involvement in the growth plate has not been studied extensively.

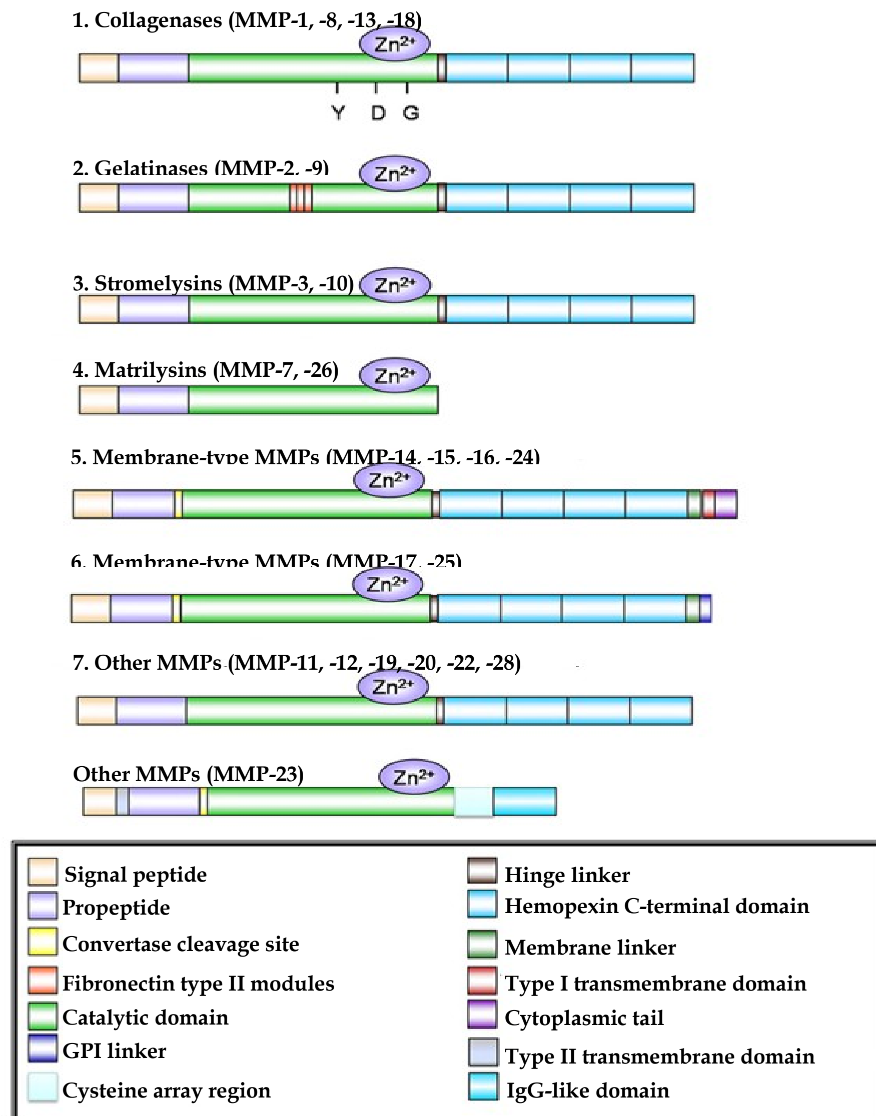


Figure 1.8 – Domain structure of the matrix metalloproteinases. Adapted from Huang et al. (2009) Extracellular matrix proteases - cytokine regulation role in cancer and pregnancy. *Front Biosci* **14:**1571-88.

1.4.2.2 Gelatinases

Two gelatinases are part of this group, gelatinase-A (MMP-2) and gelatinase-B (MMP-9). Besides the five general domains of MMPs, MMP-2 and MMP-9 have an additional domain: three repeats of a type II fibronectin domain inserted in the catalytic domain, which bind to gelatin (denatured collagen), collagens, and laminin (Allan et al., 1995) (Fig. 1.8). In addition to the fibronectin domain, MMP-9 contains a type V collagen-like sequence located between the catalytic

and hemopexin domains. They are denominated gelatinases because they digest gelatin very efficiently. They also digest type IV and V collagens and, additionally, MMP-2 even digests type VII and type X collagens and MMP-9 degrades type XI collagen and the N-telopeptides of type I collagen (Lemaître and D'Armiento, 2006). MMP-2, but not MMP-9, digests collagens I, II, and III in a similar manner to the collagenases (Aimes and Quigley, 1995; Patterson et al., 2001). MMP-2 degrades noncollagenous molecules, such as elastin, fibronectin, and laminin, and non-ECM molecules, such as several chemokines. MMP-9 also degrades noncollagenous proteins like aggrecan, the cartilage link protein, and elastin (Visse and Nagase, 2003; Lemaître and D'Armiento, 2006). MMP-9 also activates cytokines such as IL1-β (Ito et al., 1996) and TGF-β (Yu and Stamenkovic, 2000), and converts plasminogen into angiostatin (Patterson and Sang, 1997).

The degree of identity of the chicken MMP-2 to human MMP-2 is 84% at the protein and 75% at the nucleotide level. The avian and mammalian proteinases diverge significantly in the C-terminal, hemopexin-like domain. The last 100 residues of the chicken gelatinase are only 66% identical with mammalian gelatinases. The level of identity between chicken and human MMP-2 proteins, however, is not distributed homogeneously over the entire amino acid sequence. These two enzymes are more similar in the N-terminal 430 residues of the molecule (approximately 90% identity) than in the C-terminal 200 amino acids (less than 70% identity). Chicken gelatinase does cleave the denatured forms of most collagens but, unlike human MMP-2, it does not appear to cleave native type-IV collagen (Chen et al., 1991; Aimes et al., 1994).

Chicken MMP-9 has a molecular weight of 75 kDa whereas human MMP-9 has 92 kDa (Tong et al., 2003). The chicken MMP-9/75-kDa gelatinase B-like enzyme shares comparatively low homology (59%) with the human gelatinase-B (MMP-9). It lacks the type V collagen domain that is found in all mammalian gelatinases B, and the activated enzyme can cleave fibronectin, which is not a substrate for mammalian gelatinase-B. Thus, although this enzyme resembles MMP-9/gelatinase-B in its overall domain structure, it seems to be biochemically divergent from the mammalian analogue (Hahn-Dantona et al., 2000, Tong et al., 2003).

1.4.2.3 Stromelysins

The group of stromelysins consists of stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), and stromelysin-3 (MMP-11). These proteases have a domain arrangement similar to that of collagenases, that is, with the five general domains of MMPs, but contrarily to collagenases, they do not cleave interstitial collagens (Fig. 1.8). MMP-3 and MMP-10 are similar in structure and substrate specificity, on the other hand, and based on these two features, MMP-11 diverges from

these two proteases, and therefore, it is usually inserted into the "other MMPs" subgroup. In addition to the five general domains, MMP-11 has a furin recognition motif RX[R/K]R at the C-terminal end of the propeptide and therefore it is activated intracellularly (Nagase et al., 2006). MMP-3 and MMP-10 digest proteoglycans, fibronectin, nidogen, laminin, casein, gelatin, collagens IV, IX, and X, tenascin, vitronectin, and decorin (Lemaître and D'Armiento, 2006). MMP-11, on the other hand, has very weak activity toward ECM molecules but cleaves serpins more readily (Nagase et al., 2006).

The only stromelysin so far cloned and studied in the avian growth plate was MMP-10 (Simsa et al., 2007a) which is 52% identical to human MMP-10. MMP-3 and MMP-11 have 55 and 82% of identity to human MMP-3 and MM-11, respectively. These have not so far been identified in the chicken growth plate, and both sequences deposited in the *GenBank* were predicted by automated computational analysis.

1.4.2.4 Matrilysins

Matrilysin-1 (MMP-7) and matrilysin-2 or endometase (MMP-26) form the group of matrilysins. Of the five general domains of MMPs the matrilysins lack the hinge region and the hemopexin-like domain (Fig. 1.8). Both MMP-7 and MMP-26 digest type IV collagen. Additionally, MMP-7 degrades laminin and entactin, and MMP-26 cleaves fibronectin, vitronectin, and fibrinogen (Lemaître and D'Armiento, 2006). Besides ECM components, MMP-7 can also process cell surface molecules such as pro-α-defensin, Fas-ligand, pro-tumor necrosis factor (TNF)-α, and E-cadherin (Nagase et al., 2006).

The chicken MMP-7 protein is 53% identical to human MMP-7. It was identified in the chicken and its nucleotidic sequence deposited in the *GenBank*. However it has not so far been studied in the avian growth plate.

1.4.2.5 Membrane-Type (MT)-MMPs

This group includes MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT4-MMP (MMP-17), MT5-MMP (MMP-24), and MT6-MMP (MMP-25). MMP-14, -15, -16, and -24 are type I transmembrane proteins having, besides the five general domains of MMPs, two more domains: the type I transmembrane domain and the cytoplasmic domain (Fig. 1.8). MMP-17 and -

25 are glycosylphosphatidylinositol (GPI) anchored proteins possessing, in addition to the five domains, the GPI anchor domain (Lemaître and D'Armiento, 2006). Additionally, they all have a furin recognition sequence RX[R/K]R at the C-terminus of the propeptide, and they are therefore activated intracellularly and active enzymes are likely to be expressed on the cell surface. These enzymes can digest a number of ECM molecules, and MMP-14 has collagenolytic activity on type I, II, and III collagens (Visse and Nagase 2003).

MMP-14 has not yet been identified in the chicken (Simsa et al., 2007).

1.4.2.6 Other MMPs

Seven MMPs are not classified in the above categories because they diverge in sequence and substrate specificity compared to them (Fig. 1.8).

Metalloelastase (MMP-12), MMP-19, enamelysin (MMP-20), and MMP-22 (CMMP - chicken MMP) (Yang and Kurkinen, 1998; Lemaître and D'Armiento, 2006) have the five domains of MMPs, as collagenases. MMP-12 digests several ECM molecules including elastin, aggrecan, fibronectin, laminin, and type IV collagen. MMP-19 can hydrolyze type IV collagen, gelatin, tenascin, laminin, nidogen, aggrecan (Lemaître and D'Armiento, 2006) and components of basement membranes (Nagase et al., 2006). Enamelysin (MMP-20) performs the cleavage of enamel matrix proteins (Lemaître and D'Armiento, 2006). MMP-22 (CMMP) was first found in chicken embryo fibroblasts (Yang and Kurkinen, 1998). MMP-21 and epilysin (MMP-28) have a furin recognition sequence before the catalytic domain and therefore they are likely to be activated intracellularly and secreted as active enzymes. MMP-21 digests gelatin (Nagase et al., 2006).

MMP-23 is a unique member within the MMP family having a very unique molecular structure. Instead of a hemopexin-like domain, MMP-23 has a cysteine-rich domain followed by an immunoglobulin (IgG)-like domain. The propeptide of this enzyme lacks the cysteine switch motif but it has a type II transmembrane domain in its N-terminal and a furin recognition sequence in its C-terminal (Lemaître and D'Armiento, 2006).

1.4.3 Activation of MMPs

Like other proteolytic enzymes, MMPs are first synthesized as inactive proenzymes or zymogens. Their latency is maintained because their propeptide domain blocks the active site in

the catalytic domain. The catalytic domain of MMPs contains one catalytic zinc ion, one structural zinc ion, and up to three calcium ions which stabilize the structure (Visse and Nagase, 2003). Three histidines coordinate with the catalytic zinc ion in the active site. In the latent enzyme, a cysteine residue in the N-terminal propeptide coordinates with the catalytic zinc ion, forming the fourth ligand for the active site zinc ion, and activation requires that this cysteine-to-zinc switch be opened by normal proteolytic removal of the propeptide domain or by ectopic perturbation of the cysteine-zinc interaction (Van Wart and Birkedal-Hansen, 1990). This Cys-Zn^{2+} coordination keeps proMMPs inactive by preventing a water molecule, essential for catalysis, from binding to the zinc ion (Nagase et al., 2006). In the active enzyme, water coordinated with this zinc ion is important in the mechanism of peptide bond hydrolysis (Clark and Murphy, 1999). The water molecule is displaced after substrate binding into the catalytic site cleft (Visse and Nagase, 2003).

Most of the MMPs are secreted in their latent forms and activation takes place in the extracellular space, except for MMP-11 which is activated intracellularly by furin prior to secretion (Clark and Murphy, 1999, 98). The MT-MMPs have a furin-cleavage site similar to that of MMP-11 and may also be activated before leaving the cell (Clark and Murphy, 1999).

ProMMPs without the furin-cleavage site are secreted from the cell into the extracellular space as latent MMPs. MMPs activation probably takes place upon the enzymic cleavage of the propeptide (Clark and Murphy, 1999). Once a part of the propeptide is removed, this probably destabilizes the rest of the propeptide, including the cysteine switch-zinc interaction, which allows the intermolecular processing by partially activated MMP intermediates or other active MMPs (Visse and Nagase, 2003). Indeed, many of the MMPs share common activators and many are activated by other MMPs. In this way, it has been suggested that MMPs are involved in activation cascades. An important cascade is initiated by the action of tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA) on plasminogen, which results in the active enzyme plasmin (Fig. 1.9). This enzyme can activate several MMPs and this activation is a relevant pathway *in vivo* (Clark and Murphy, 1999). Both plasminogen and urokinase plasminogen activator are membrane-associated, thereby creating localized proMMP activation and subsequent ECM turnover. Plasmin has been reported to activate proMMP-1, proMMP-3, proMMP-7, proMMP-9, proMMP-10, and proMMP-13 (Lijnen, 2001).

Activation *in vitro* of proMMP-9 by metalloproteinases other than MMP-3 and MMP-13 has been reported for several cell and organ types which include MMP-1, MMP-2, MMP-7, MMP-10, and MMP-26 (Dreier et al., 2004). Activated forms of MMP-2 can activate proMMP-9 (Wucherpfennig et al., 1994). A candidate proteinase for activating procollagenases is MMP-3 which is also a proven activator of proMMP-9 (Dreier et al., 2001).

MMP-2 is not involved in the plasmin cascade and appears to be regulated differently than the plasmin-activated MMPs (Clark and Murphy, 1999). ProMMP-2 activation is mainly performed on the cell surface and is mediated by MT-MMPs, including MT1-MMP, MT2-MMP, MT3-MMP, MT5-MMP, and MT6-MMP. MT4-MMP does not activate proMMP-2. MT1-MMP-mediated activation of proMMP-2 has been widely studied because it requires the presence of an MMP protein inhibitor, the TIMP-2. ProMMP-2 forms a tight complex with TIMP-2 through their C-terminal domains, therefore permitting the N-terminal inhibitory domain of TIMP-2 in the complex to bind to MT1-MMP on the cell surface. The cell surface-bound proMMP-2 is then activated by an MT1-MMP that is free of TIMP-2. Alternatively, MT1-MMP inhibited by TIMP-2 can act as a "receptor" of proMMP-2. This MT1-MMP/TIMP-2/proMMP-2 complex is then presented to an adjacent free MT1-MMP for activation (Visse and Nagase, 2003).

MT1-MMP also activates proMMP-13 on the cell surface, and this activation is more efficient in the presence of active MMP-2 (Knauper et al., 1996b). MMP-2 can also activate MMP-9. MMP-10 can activate MMP-7, MMP-9, and MM-13 (Chakraborti et al., 2003).

In vitro, the MMPs can be activated by chemical agents that modify cysteine residues, such as the organomercurial 4-aminophenylmercuric acetate (APMA). Activation may also occur when the conformation of the proenzyme is perturbed, as is evident from the SDS-activation of proMMP-2 and proMMP-9 during zymography (Clark and Murphy, 1999).

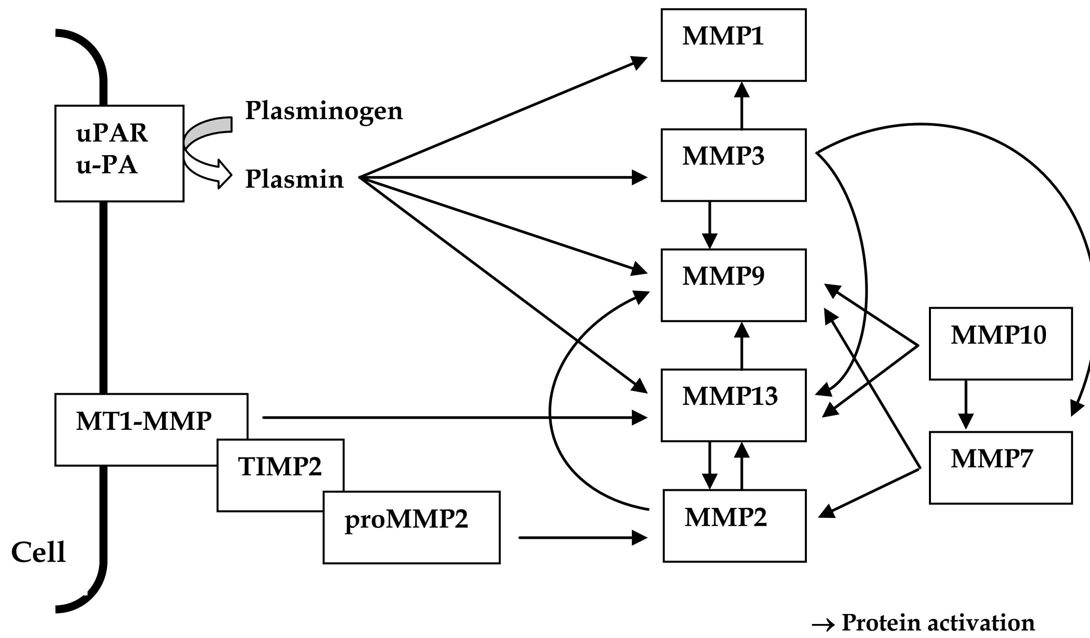


Figure 1.9 – Activation cascade of matrix metalloproteinases. Adapted from Chakraborti et al. (2003) Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem* **253**(1-2):269-85.

1.4.4 Regulation of MMPs

The release of proteolytic enzymes into the extracellular matrix originates some risks of uncontrolled breakdown and therefore a number of mechanisms have evolved in order to avoid uncontrolled proteolysis.

MMPs are produced by most cell types and are regulated at the transcriptional level by hormones, growth factors and cytokines (Chakraborti et al., 2003, Lemaître and D'Armiento, 2006) and at the translational, and posttranslational level by zymogen activation (Schubayev et al., 2004). Unlike other MMPs, MMP-2 is constitutively expressed in several tissues and is not usually induced by inflammatory stimuli (Fabumni et al., 1996; Matsumoto et al., 1996). In almost all evaluated cells, many growth factors and cytokines do not enhance MMP-2 synthesis even when they induce other MMPs *in vitro*. Thus, MMP-2 may be involved in normal turnover of the extracellular matrix in several tissues. However, in a few cell types, TGF-β enhances MMP-2 synthesis. TGF-β stimulates MMP-2 synthesis in gingival fibroblasts and some tumor cells (Overall et al., 1989).

In addition to the regulation of MMPs activity at the level of gene expression and activation of its zymogen, MMPs activity is also regulated by endogenous inhibitors. These include general inhibitors such as α2-macroglobulin family members and the more specific tissue inhibitors of metalloproteinases (TIMPs) (Woessner, 2002).

TIMPs are specific inhibitors that bind to MMPs in a 1:1 stoichiometry. Four TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) have been identified in vertebrates (Brew et al., 2000). TIMPs inhibit all MMPs tested so far, except that TIMP-1 fails to inhibit MT1-MMP. TIMP-1 and TIMP-2 are unique in that they also bind to the pro-forms of MMP-9 and MMP-2, respectively (Will et al., 1996). TIMPs consist of approximately 184 to 194 amino acids (Nagase et al., 2006) having a weight of approximately 21 to 29 kDa (Visse and Nagase, 2003). They are subdivided into an N-terminal (~ 125 amino acids) and C-terminal (~ 65 amino acids) subdomain, with each containing three conserved disulfide bonds (Williamson et al., 1990). The N-terminal domain folds as an independent unit with MMP inhibitory activity (Nagase et al., 2006). TIMPs form complexes with matrix metalloproteinases and interact with the active site plus, a site in the carboxyl terminal hemopexin-like domain (Willenbrock et al., 1993). In addition to the metalloproteinase-inhibiting activities of TIMPs, they may have other biological functions such as growth factor activity (Woessner et al., 2002), anti- (Guedez et al., 1998; Visse and Nagase, 2003) or pro-apoptotic activity (Ahonen et al., 2003; Visse and Nagase, 2003), and antiangiogenic activity (Bode et al., 1999).

The balanced activity of the metalloproteinases and their inhibitors is thought to be critical for the maintenance of the integrity and composition of the ECM and normal cellular functions. In fact, any change in this equilibrium may result in a variety of pathological states including

rheumatoid and ostoarthritis, atherosclerosis, liver fibrosis, tumor growth (Visse and Nagase, 2003).

1.4.5 Biological Activities of MMPs

Because MMPs can degrade ECM molecules, their main function has been presumed to be the remodeling of the ECM (Vu and Werb, 2000). However, the ECM is not simply an extracellular scaffold; it also acts as a reservoir of biologically active molecules, such as growth factors (Visse and Nagase, 2003) and cytokines. MMPs may control the function of these molecules by their action on ECM substrates and by proteolytically activating them. It is now clear that MMPs not only remodel the ECM but also influence many cellular functions (Ortega et al., 2004).

MMPs may affect cell migration by degrading the ECM and thus breaking down physical barriers (Vu and Werb, 2000), and by removal of sites of adhesion or by exposing a binding site (Fig. 10). MMP activity may also modulate attachment and migration by cleaving cell-cell or cell-matrix receptors directly (McCawley and Matrisian, 2001).

MMPs may regulate the function of biologically active molecules such as growth factors or growth factors receptors. They may control the bioavailability of these molecules by releasing them from bound proteins or ECM stores. For example, MMP-1 and MMP-3 can degrade perlecan and release bound FGF (Whitelock et al., 1996; Vu and Werb, 2000) and cleavage of decorin by MMP-2, MMP-3, and MMP-7 releases TGF-β bound to decorin (Imai et al., 1997; Vu and Werb, 2000). Recently, it has been shown in ovarian carcinoma cells that MMP-2 and MMP-9 induce the release of VEGF *in vitro* (Belotti et al., 2003; Haeusler et al., 2005). MMP-9 is required for the release of VEGF during long bone development (Engsig et al., 2000).

MMPs may also regulate the activity of biologically active molecules by proteolytically activating them. For example, *in vitro* studies show that MMP-2 and MMP-9 can proteolytically cleave latent TGF-β (Yu and Stamenkovic, 2000). Similarly, IL1-β precursor can be processed to its active form by MMP-2, MMP-3, and MMP-9 (Schönbeck et al., 1998; Vu and Werb, 2000). Karsdal et al. (2002) observed that latent TGF-β is activated by MT1-MMP. Moreover, MT1-MMP has been shown to be able to activate MMP-2, MMP-9, and MMP-13 (Knauper et al., 1996b). When activated, these MMPs have also the opportunity to activate latent TGF-β (D'Angelo et al., 2001a,b; Maeda et al., 2001; Maeda et al., 2002). Therefore, this proteolytic potential of MT1-MMP, with respect to other MMPs and latent TGF-β, qualifies MT1-MMP as an obvious candidate for the control of TGF-β (Karsdal et al., 2002).

MMPs may also affect cell proliferation, survival, apoptosis, and differentiation through their action on the ECM (Vu and Werb, 2000).

Because MMPs are involved in ECM degradation and consequently in cell migration, release of proteins from ECM stores or in regulating the activity of biologically active molecules, they have been shown to play a crucial role during the process of endochondral bone development. This process involves steps such as chondrocyte proliferation and differentiation, vascularisation and apoptosis in which MMPs have been demonstrated to be implicated. The involvement of MMPs in endochondral bone development will be discussed in the next section.

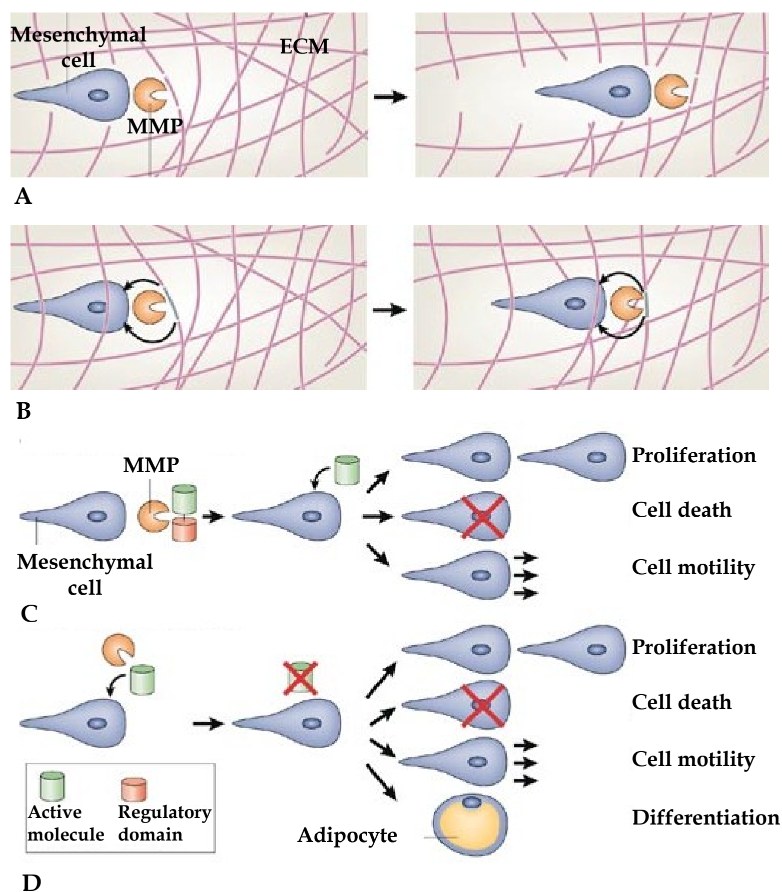


Figure 1.10 – Modes of action of matrix metalloproteinases. **A)** MMPs can cleave components of the extracellular matrix (ECM), resulting in increased space for cell or tissue movement; **B)** MMP proteolysis can generate specific cleavage products that then signal in an autocrine or paracrine manner; **C)** MMPs can activate or modify the action of latent signalling molecules, resulting in diverse cellular consequences; **D)** MMPs can deactivate or modify the action of active signalling molecules, resulting in changes in proliferation, cell death, differentiation or cell motility. Adapted from Page-McCaw et al. (2007) Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol* **8**(3):221-233.

1.5 MMPs in Endochondral Bone Formation

MMPs have been shown to play a crucial role in endochondral ossification since they are involved in the remodeling of the extracellular matrix. In fact, MMPs play a role in the normal maintenance and remodeling of the cartilage matrix and therefore its proteolytic activity has been considered to be essential for normal bone development.

Previous biochemical and immunohistochemical studies have shown that MMPs play a number of important roles in the growth plate. Collagenase and a collagenase inhibitor were identified in the early 1980s in growth plate cartilage from embryonic chick bone (Yasui et al., 1981). Later, collagenase was shown to be highly expressed in the hypertrophic zone of the rat growth plate and suggested to be involved in removing pericellular collagen so the cells could enlarge (Blair et al., 1989; Dean et al., 1990). In 1985, Ehrlich et al. found that proteinases that were capable of degrading aggecan at neutral pH were demonstrated in calf growth plate cartilage (Maeda et al., 2001). Later, the MMPs stromelysin, gelatinase, and collagenase, were immunolocalized in the distal femoral growth plate of rabbits (Brown et al., 1989).

Collectively, MMPs are capable *in vitro* and *in vivo* of degrading all kinds of extracellular matrix protein components such as interstitial and basement membrane collagens, proteoglycans, fibronectin and laminin (Bode et al., 1999). ECM degradation is required for proliferation and enlargement of chondrocytes (Mwale et al., 2000; Wu et al., 2002; Tchetina et al., 2003), migration of chondrocytes in the growth plate (Álvarez et al., 2000), matrix calcification (Hunter, 1991; D'Angelo et al., 2000), matrix invasion by blood vessels (Bittner et al., 1998; Tong et al., 2003), and for the release of stored signalling molecules (Vu et al., 1998). Degradation of cartilage matrix to permit all these functions should require MMPs capable of attacking the ECM components. In addition to the extracellular matrix molecules, MMPs have other substrates such as, other proteinases (for example MMPs), proteinase inhibitors, chemotactic molecules, latent and active growth factors, growth factor-binding proteins, cell surface receptors, and cell-cell adhesion molecules (Sternlicht and Werb, 2001). All these targets of MMPs have contributed to implicate MMPs in a variety of biological processes such as, in cell proliferation (Malemud, 2006), differentiation (D'Angelo et al., 2000), attachment (Haeusler et al., 2005), and apoptosis (Kerkela et al., 2001; Malemud, 2006) of growth plate chondrocytes, as well as in angiogenesis (Vu et al., 1998; Pufe et al., 2004), processes that are present during endochondral ossification. MMPs may directly facilitate angiogenesis by stimulating matrix degradation and, indirectly, by releasing matrix-associated endothelial growth factors and inhibitors (Pluijm et al., 2003). In this way, the proteolytic activity of MMPs is essential for normal bone development and many studies on the expression patterns of MMPs during endochondral ossification have been reported. Several MMPs are expressed during the

endochondral ossification process, including collagenases (MMP-1 and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3 and MMP-10) and MT1-MMP (MMP-14) (Ortega et al., 2004). Among these MMPs, MMP-9, MMP-13, and MMP-14 have been shown to be highly expressed during endochondral ossification suggesting that they play an important role in this process.

In human development, MMP-13 is expressed in mineralizing skeletal tissue, in hypertrophic chondrocytes, and in osteoblasts involved in ossification. In osteoblasts, MMP-14 and MMP-2 were colocalized with MMP-13. This protein was therefore proposed to function in the degradation of type II collagen in primary ossification, skeletal remodeling, and joint disease. Expression of MMPs, particularly MMP-9 but also MMP-1, -2, -13 and -14, has also been noted in osteoclasts (Clark and Murphy, 1999)

MMP-9 plays an important role in connective tissue remodeling and basement membrane turnover. It degrades collagens of type IV, V, and XI, and elastin, but not native type I collagen, proteoglycans, or laminins (Vu et al., 1998). The enzyme shows, however, high specific activity for denatured collagen fragments (gelatin) being considered the gelatinase required for the removal of gelatin generated by the action of interstitial collagenases (Reponen et al., 1994). MMP-9 is highly expressed in osteoclasts and chondroclasts during mouse development (Vu et al., 1998; Ortega et al., 2004), and endothelial cells (Engsig et al., 2000), and the active enzyme is concentrated at sites of cartilage resorption, proximal to the chondro-osseous junction, where vascular invasion occurs (Fig. 1.11). This expression pattern suggests a role for MMP-9 not only in ECM degradation but also in invasion and migration of endothelial cells and osteoclasts (Ortega et al., 2004). Previous studies have shown that gelatinase B recruits osteoclasts to the hypertrophic zone of the cartilage anlage to initiate the process of endochondral bone formation (Engsig et al., 2000). This is confirmed by studies using null mice for MMP-9 that shows a delay in the recruitment of osteoclasts and endothelial cells at the early stages of endochondral ossification. These mice also display substantially more hypertrophic cartilage, impaired endochondral ossification and a delay in the formation of the bone marrow cavity (Ortega et al., 2004).

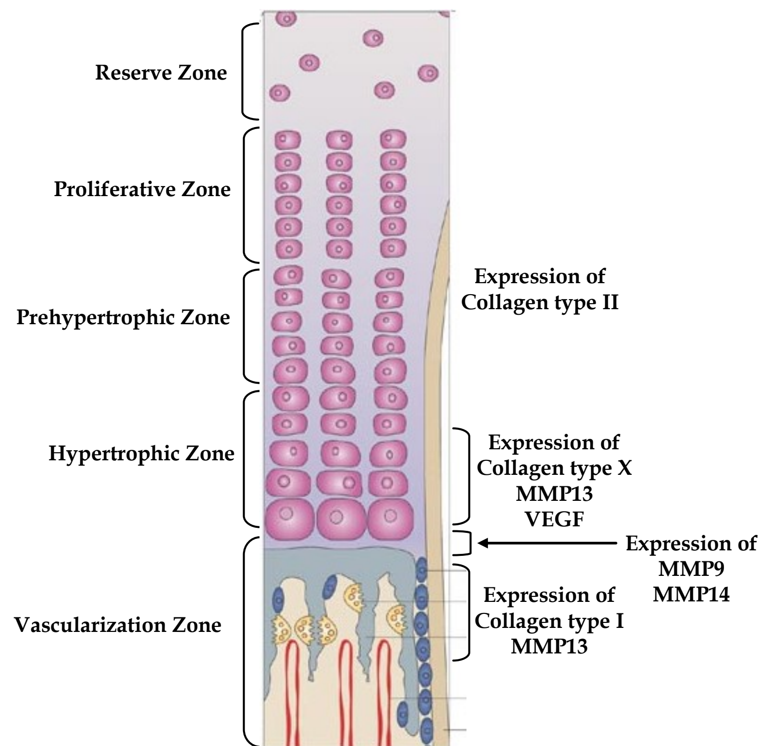


Figure 1.11 – The distribution of molecules in the growth plate. Adapted from Page-McCaw et al. (2007) Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol* **8**(3):221-233 and Ortega N, Behonick DJ, and Werb Z (2004) Matrix remodeling during endochondral ossification. *Trends Cell Biol* **14**(2):86-93.

It has been reported that MMP-9 is a key regulator of hypertrophic chondrocyte apoptosis and growth plate angiogenesis because in MMP-9-null mice, hypertrophic chondrocytes differentiate normally, but apoptosis, vascularization, and ossification are delayed, resulting in progressive lengthening of the hypertrophic chondrocyte region in the bones (Vu et al., 1998). It has been suggested that the molecular mode of action of this enzyme includes releasing VEGF or other growth factors from the matrix (Engsig et al., 2000; Sternlicht and Werb, 2001).

Other MMP also highly expressed during endochondral ossification is MMP-13. The expression of MMP-13 during development has been reported to be confined to skeletal tissues (Stahle-Bäckdahl et al., 1997), and it is the only collagenase that has been implicated in degradation of the collagenous matrices during development of bone and cartilage (Mattot et al., 1995). MMP-13 is capable of cleaving in the native triple helical region of the fibrillar collagens type I, II, and III, generating fragments approximately ¾ and ¼ the size of the original molecule (Knäuper et al., 1996a; Pendas et al., 1997). This enzyme has preferential activity on type II collagen which is the

major protein in cartilage. MMP-13 can also degrade type X collagen and cartilage aggrecan, and may therefore contribute to both collagen and proteoglycan degradation in the hypertrophying and calcifying chondrocyte matrix (Knäuper et al., 1996a, D'Angelo et al., 2000). These substrate specificities make collagenase-3 a likely candidate to participate in the degradation of cartilage extracellular matrix during the endochondral ossification process.

The enzyme is expressed in the hypertrophic chondrocytes and osteoblasts (Johansson et al., 1997) and its localization has been found in hypertrophic and calcifying cartilage of mammalian growth plate (Wu et al., 2002) (Fig. 1.11).

Mutation in human MMP-13 causes the Missouri variant of spondyloepimetaphyseal dysplasia with abnormalities in the development and growth of endochondral skeletal elements (Simsa et al., 2007). MMP-13-deficient mice show altered endochondral bone development and profound defects in the growth plate cartilage with markedly increased hypertrophic domains (Inada et al., 2004; Stickens et al., 2004).

There is evidence supporting the view that MMP-9 can synergize with MMP-13 in regulating growth plate development (Stickens et al., 2004). It has been proposed that MMP-9 and MMP-13 cooperate during the degradation of the unmineralized septa of hypertrophic chondrocytes (Lee et al., 1999). Stickens et al. (2004) suggest that these enzymes may first act to degrade aggrecan in the hypertrophic zone of the growth plate, making collagen type II accessible to cleavage by MMP-13. MMP-9 may then act downstream to clear denatured collagen cleavage products generated by MMP-13. Indeed, mice deficient in both MMP-9 and MMP-13 have severely impaired endochondral bone formation, characterized by prolonged survival of "prehypertrophic" chondrocytes, delayed vascular formation and impaired trabecular bone formation (Malemud, 2006).

MMP-13 is activated by MMP-2 during chondrocyte maturation and the combination of both proteinases is required to prepare cartilage matrix for subsequent calcification, before endochondral ossification. Therefore, MMP-2 has been suggested to act as a regulator of MMP-13 activity in hypertrophic cartilage (D'Angelo et al., 2000).

MMP-2 is a gelatinase with widespread expression that is thought to serve a housekeeping function in removing abnormal or degraded collagen (Matrisian, 1994, D'Angelo et al., 2000). MMP-2 activity is high in normal growth plates (Wardale and Duance, 1996), however, its importance in skeletal development remains unclear (Malemud, 2006). The high activity of MMP-2 against gelatin has led to the hypothesis that it plays a role in the removal of denatured collagen fragments (gelatin) after cleavage of native collagen by the other metalloproteinases (Reponen et al., 1994). Nevertheless, although gelatinase A has been considered to be important to complete

collagen degradation after the specific cleavage of the triple helical region of the fibrillar collagen molecules by collagenase, mice deficient in this enzyme by targeted mutagenesis have not shown significant developmental impairment (Álvarez et al., 2000).

MMP-14 is the unique activator of proMMP-2 and is found on the cell surface (Strongin et al., 1995; Kinoh et al., 1996; Sato et al.; 1996). MMP-14 is activated intracellularly by furin or furin-like enzymes (Apte et al., 1997) and once activated is transported to the cell surface (Cao et al., 1996). Here, MMP-14 can either directly activate MMP-13 or cleave proMMP-13 via activation of proMMP-2 (Knäuper et al., 1996b; D'Angelo et al., 2000). Thus, expression of MMP-14 is a first step in a pathway leading to activation of metalloproteinases which is a key event in the regulation of their enzymatic activity (Álvarez et al., 2000).

MMP-14 displays broad collagenolytic, glycoproteolytic, gelatinolytic and fibrinolytic activities (Maquoi et al., 2003). This protein has been shown to be expressed in the hypertrophic chondrocytes (Kinoh et al., 1996) (Fig. 1.11). The importance of MMP-14 in the process of angiogenesis and in bone growth has been shown through the study of MMP-14-null mice. These, lack activation of proMMP-2 and have severe defects in skeletal development and angiogenesis (Holmbeck et al., 1999; Zhou et al., 2000).

To date, neither MMP-15 (MT2-MMP) nor MMP-16 (MT3-MMP)-deficiencies have been linked to either mouse or human growth plate disturbances (Malemud, 2006).

In regard to TIMPs, the major TIMP expressed during bone development is TIMP-2 (Apte et al., 1997; Blavier and DeClerck, 1997) although TIMP-1, TIMP-3, and TIMP-4 are also expressed in skeletal elements during mouse and human development (Huang et al., 2002). Nevertheless, to date no analysis of mice deficient for one or several TIMPs has reported abnormal bone phenotype (Ortega et al., 2004).

In regard to stromelysins, these enzymes have been localized in the rabbit growth plate (Brown et al., 1989) and investigated further in human fetal rib (Bord et al., 1998) and bovine growth plate (Armstrong et al., 2002; Haeusler et al., 2005). They have broad substrate specificity including proteoglycans, laminin, and fibronectin, and additionally MMP-3 can also cleave type II collagen (Fukui et al., 2002). MMP-3 has been considered to be a major proteoglycan degrading enzyme in cartilage and may also be involved in tissue remodeling in the growth plate. *In vitro* studies have shown that MMP-3 is produced by stimulated human and mouse osteoblasts and isolated rabbit osteoclasts. MMP-11 was detected in proliferating chondrocytes (Bord et al., 1998; Haeusler et al., 2005).

The cooperative effects of collagenase (MMP-13), gelatinases (MMP-2 and MMP-9), and stromelysins (MMP-3 and MMP-11) are considered to be essential for matrix degradation during endochondral ossification (Ortega et al., 2004).

Matrilysin-1 (MMP-7) has a wide range of substrates, including gelatins of types I, III, IV, and V, collagen type IV, laminin (Wilson and Matrisian, 1996), proteoglycans, fibronectin, elastin, and casein (Gaire et al., 1994). It has been reported that among the MMP gene family members, MMP-7 has the highest specific activity against many ECM components (Fujita et al., 2006). These results suggested that MMP-7 might play a role in ECM protein turnover under normal conditions and in accelerated ECM protein degradation in inflammation (Malemud, 2006). MMP-7 has little, if any, activity against native fibrillar collagens. In addition to its ability to degrade matrix components, MMP-7 may have an indirect role in matrix remodeling by activating the latent forms of other MMPs. Indeed, previous studies have demonstrated that MMP-7 can activate proMMP-1 and proMMP-9 (Fujita et al., 2006).

1.6 AVIAN TIBIAL DYSCHONDROPLASIA

1.6.1 Characterization

Avian species which have to grow rapidly may develop a disease associated with the development of long bones resulting in deformed bones and lameness. Avian tibial dyschondroplasia was first described by Leach and Nesheim (1965) as a cartilage abnormality that occurred spontaneously in rapidly growing broiler chickens. This disease is a skeletal abnormality where the normal events of endochondral bone formation are disrupted and is characterized by the formation of a lesion composed of noncalcified, nonvascularized cartilage that can extend from the epiphyseal growth plate into the metaphysis (Fig. 1.12). Dyschondroplastic lesions affect only the growth plate with no incidence on the articular cartilage (Wardale and Duance, 1996). The lesion takes the appearance of a plug of avascular cartilage, white and opaque, underlying the bone growth plate. It can vary considerably in size from a small focal lesion in one part of the growth plate to a large mass occupying the full width of the growth plate (Farquharson et al., 1993) which may result in an epiphysis comprising solely by cartilage and periosteum (Wardale and Duance, 1996). The incidence of tibial dyschondroplasia within the proximal tibiotarsi is similar in both legs (Farquharson and Jefferies, 2000).

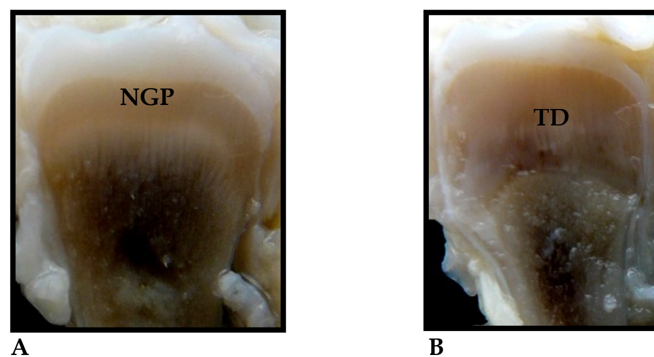


Figure 1.12 – Avian tibial dyschondroplasia. **A)** Normal growth plate; **B)** growth plate affected with tibial dyschondroplasia. NGP – Normal growth plate; TD – tibial dyschondroplasia.

Although TD has been reported initially to occur spontaneously, it is now known that it can be induced by selective breeding and by manipulation of nutritional factors. For example, TD can be induced by altered calcium-to-phosphorus ratio (Rennie et al., 1993; Pines et al., 2005). The cysteine, homocysteine, salicylic acid, or copper content can also contribute to the incidence of TD, as well as the inclusion of mycotoxins (Fusarium species) (Farquharson and Jefferies, 2000). It was reported that a certain dithiocarbamate fungicide, the tetramethylthiuram disulfide (thiram or thiuram), when fed to chickens, increase the incidence of TD. Thus, this dithiocarbamate has been used to induce very high incidence of this pathology in chickens and therefore used as a research model for experimental studies (Vargas et al., 1983).

The way how thiram acts to induce TD is still uncertain. Initially, it has been thought that the chemical and amino acid induction of TD was via Cu chelation and thus Cu deficiency because thiram is a known Cu chelator (Brown and Strain, 1990; Cook et al., 1994). Indeed, some studies have demonstrated that when feeding high dietary levels of Cu this would prevent thiram-induced dyschondroplasia (Cook et al., 1994). However, later it was suggested that TD is not caused by Cu deficiency but instead by a decreased bioavailability of thiram at the gastrointestinal level. Because thiram is a copper chelator it can form a complex with copper in the gut which would prevent its absorption and consequently TD induction (Orth and Cook, 1994).

1.6.2 Morphology

The growth plate of chicken, like the mammalian one, is composed of chondrocytes at different stages of development. Chondrocytes from the resting or reserve zone start to proliferate forming a columnar zone of flattened cells. Then, these cells start to enlarge, or differentiate, into prehypertrophic chondrocytes which then mature to become fully hypertrophic (Whitehead, 1997).

Dyschondroplastic lesions are characterized by the accumulation of chondrocytes that differ in morphological and histochemical properties comparing with normal chondrocytes (Leach and Nesheim, 1965, Hargest et al., 1985; Praul et al., 2000). It seems that the cells comprising dyschondroplastic lesions are prehypertrophic chondrocytes that have not matured into hypertrophic chondrocytes. Because chondrocytes continue to proliferate normally, there is an increase in the number of prehypertrophic chondrocytes forming a layer of accumulated cartilage that is resistant to vascularisation and that does not calcify (Praul et al., 2000).

At the ultrastructural level, chondrocytes from dyschondroplastic lesions undergo specific morphological changes. In the proximal region of the lesion, cells began to undergo necrotic changes probably due to energy depletion. The changes include dilation and vesiculation of the

endoplasmic reticulum, enlargement of the paranuclear space, mitochondrial swelling with dilation of the intracristal spaces and the appearance of electron-dense, flocculent material in the mitochondrial matrix, chromatin margination, and dilation of the Golgi saccules (Leach and Lilburn, 1992). Chondrocytes can show shrinkage and the chondrocyte lacunae may become empty. In addition, the nuclei may become pyknotic and shrunken in size (Hargest et al., 1985, Thorp et al., 1991, Leach and Lilburn, 1992; Rath et al., 2004).

1.6.3 Biochemistry

The matrix of chicken growth plate is composed mainly by the structural proteins of collagen type II, type X, and aggrecan, as observed in the mammalian growth plate. Collagen type II is the principal structural protein and predominant collagen of the growth plate matrix. It is present throughout the normal growth plate (Farquharson and Jefferies, 2000). This collagen interacts with minor collagens such as collagen types IX and XI forming heterotypic fibrils that are distributed throughout the cartilage matrix. This collagen is synthesized mainly by proliferating chondrocytes and an increase in this protein is indicative of chondrocyte differentiation from proliferative to prehypertrophic form (Thorp et al., 1992; Thorp et al., 1993). As chondrocyte maturation occurs, collagen type II expression decreases and the hypertrophic chondrocytes initiate the synthesis of collagen type X (Farquharson and Jefferies, 2000).

In the TD growth plate, the expression pattern of collagen type II is similar to the normal growth plate, being present throughout the affected growth plate, as well (Chen et al., 1993, Farquharson et al., 1995, Tselepis et al., 1996). In accordance with these results, collagen type II mRNA has been reported to maintain its expression in dyschondroplastic cartilage comparing with normal cartilage (Rath et al., 2005). Because it has been observed little difference in the expression of type II collagen between normal and dyschondroplastic growth plates, it has been suggested that the disorder occurs after differentiation to the prehypertrophic form (Thorp et al., 1993). In contradiction, some investigators found no collagen type II within the lesion (Chen et al., 1993).

Collagen type X has been the center of several investigations because of two main reasons. First, because tibial dyschondroplasia resembles metaphyseal dysplasia, a disease in humans caused by a mutation in collagen type X gene. Secondly, because type X collagen is a marker of hypertrophy and TD is a disease where the transition of chondrocytes from prehypertrophy to hypertrophy is inhibited.

Immunohystochemistry studies showed collagen type X expression within chondrocytes of dyschondroplastic lesions and in regions proximal to these lesions. However, no positive staining was observed in extracellular matrix which suggests that a defect in its secretion or incorporation into the matrix may occur (Chen et al., 1993; Farquharson et al., 1995; Tselepis et al., 1996; Reginato et al., 1998). The distribution of the expression obtained for collagen type X mRNA was similar to the one achieved for collagen type X protein by immunohystochemical analysis. The mRNA of collagen type X was found within the proximal lesion bordering the junction with the normal growth plate (Farquharson et al., 1995). In regard to this protein there are some contradictory results. For example, Bashey et al. (1989) have reported a reduction in the amount of collagen type X within the TD lesion comparing with normal growth plate. On the other hand, some authors have reported an elevated transcription of the collagen type X gene in dyschondroplasia (Thorp et al., 1993) whereas others showed no change in the expression of the gene in this disorder (Rath et al., 2005).

In addition to collagens, the extracellular matrix of the growth plate is constituted also by proteoglycans, mainly by aggrecan, the large aggregating proteoglycan. This protein has been detected by using antibodies to its potential glycosaminoglycan epitopes, such as chondroitin sulfate and keratan sulfate. It was observed decreased staining for these epitopes in TD lesions with some staining localized intracellularly. Similarly, in situ hybridization studies showed that aggrecan gene expression was reduced in TD lesions (Tselepis et al., 1996). Contradictory results have demonstrated no alteration in aggrecan gene expression in dyschondroplasia (Rath et al., 2005).

Growth factors are involved in the regulation of the differentiation of growth plate chondrocytes (Gelb et al., 1990; Thorp et al., 1993) and therefore it has been suggested that the development of dyschondroplasia may be related to altered expression of these proteins.

In growing chickens, TGF-β3 has been localized to the prehypertrophic and hypertrophic chondrocytes of the growth plate (Thorp et al., 1995). Several studies have reported a marked reduction in the expression of both TGF-β3 mRNA and protein in dyschondroplastic lesions (Loveridge et al., 1993, Thorp et al., 1993, Thorp et al., 1995, Law et al., 1996).

The expression of PTHrP was found in the pre-hypertrophic zone of the normal avian growth plate (Vortkamp et al., 1996; Ben-Bassat et al., 1999; Webster et al., 2003). Several studies have demonstrated that PTHrP acts a negative regulator of chondrocyte terminal differentiation in longitudinal bone growth (Vortkamp et al., 1996). Thus, PTHrP functions to slow the rate of differentiation of pre-hypertrophic to hypertrophic chondrocytes within the growth plate. In dyschondroplasia, the expression of PTHrP was found to be normal in regions proximal to the

lesion and reduced within the lesion itself (Farquharson et al., 2001). Based on these results, the authors concluded that the initiation of TD lesions is not due to alterations in the distribution and gene expression of PTHrP.

There are a number of other proteins that are reported to be greatly diminished in dyschondroplastic lesions compared with the normal cartilage. Among these are alkaline phosphatase, fibroblast growth factor, biglycan, bone sialoprotein, carbonic anhydrase, osteonectin, osteopontin, and matrix metalloproteinases (Gay et al., 1985; Thorp and Jakowlew, 1994; Knopov et al., 1995; Tselepis et al., 1996; Twal et al., 1996; Wu et al., 1996; Rath et al., 1997; Pines et al., 1998; Praul et al., 2000).

1.6.4 Tibial Dyschondroplasia-like Mammalian Skeletal Dysplasias

There are several human skeletal diseases with cartilage lesions similar to those observed in tibial dyschondroplasia. Schmid metaphyseal chondrodysplasia is caused by a mutation in the collagen type X gene. This collagen is specific of hypertrophic chondrocytes being considered a marker of hypertrophy. The pathology is characterized by delayed endochondral maturation (Chan and Jacenko, 1998; Leach and Monsonego-Ornan, 2007). Jansen's metaphyseal chondrodysplasia is a disease where the mutated gene is the PTH/PTHrP receptor. The mutation in a single nucleotide results in a constitutively active receptor (Schipani et al., 1995; Leach and Monsonego-Ornan, 2007). When PTHrP is overexpressed, a delay in chondrocyte maturation and subsequent vascularisation occur. On the other hand, deletion of this gene causes accelerated maturation of growth plate chondrocytes (Praul et al., 2000).

There are also transgenic models with ablation of genes involved in the normal endochondral bone growth and that produce lesions similar to dyschondroplasia. For example, it has been demonstrated that mice lacking MMP-9, MMP-13, or both enzymes, show a phenotype which resembles dyschondroplastic lesions. MMP-9-null mice exhibit abnormal pattern of growth plate vascularisation and ossification resulting in progressive lengthening of the growth plate. Furthermore, apoptotic cells appear in the center of the extended hypertrophic zone, as also observed in dyschondroplastic lesions (Vu et al., 1998). In MMP-13 knockout mice, the chondrocytes differentiate normally but their exit from the growth plate is delayed. Mice lacking both MMP-9 and MMP-13 revealed a more pronounced phenotype, being the lesions characterized by diminished extracellular matrix remodeling and delayed vascular recruitment (Inada et al., 2004; Stickens et al., 2004).

1.6.5 MMPs in Tibial Dyschondroplasia

As described in section 1.4, MMPs are a multigene family of secreted or cell-surface enzymes which the main function is to degrade numerous pericellular substrates that are required during bone development. The targets of MMPs include other proteinases, proteinase inhibitors, clotting factors, chemotactic molecules, latent growth factors, growth factor-binding proteins, cell surface receptors, cell-cell adhesion molecules, and virtually all structural extracellular matrix proteins (Sternlicht and Werb, 2001; Pines et al., 2005). Having all these targets MMPs play, in this manner, a crucial role during bone development, a process that requires active remodelling of the growth plate.

The earliest report about the involvement of MMPs in dyschondroplasia showed a severe reduction in MMP-2 activity in TD-affected growth plate cartilage (Rath et al., 1997). Accordingly, and more recently, it was also demonstrated a reduction in MMP-2 activity (Pines et al., 2005, Simsa et al., 2007b) in dyschondroplasia. However, and in regard to the mRNA expression, Pines et al. (2005) demonstrated only a slight reduction in the MMP-2 gene expression whereas Rath et al. (2005) found no changes in the mRNA expression of this enzyme in the disorder. MMP-2 gene expression was observed in the proliferative zone of the chicken growth plate and in regions surrounding the blood vessels (Pines et al., 2005).

In regard to other MMPs, only MMP-9 and MMP-13 were also studied in dyschondroplasia. MMP-9 is of particular importance since it was reported to be a key regulator in the final step of endochondral bone development (Vu et al., 1998). This enzyme has been found in cells surrounding the blood vessels that penetrate the chicken growth plate and by chondrocytes located in the front of vascularisation, between the bone and the cartilage. This distribution pattern is similar to the one found in the mouse. On the other hand, MMP-13 is localized in the hypertrophic zone of the chicken growth plate (Pines et al., 2005). For both MMP-9 and MMP-13, a reduction in their activities (Simsa et al., 2007b) and gene expressions (Pines et al., 2005) in TD were observed. In contrast, a study has revealed an increased gene expression for MMP-13 in dyschondroplastic growth plates (Rath et al., 2005).

2 OBJECTIVES

Avian tibial dyschondroplasia (TD) is a skeletal disease where the normal events of endochondral bone formation are disrupted. There are several mammalian skeletal diseases with lesions similar to TD (Leach and Monsonego-Ornan, 2007). Thus, the main goal of this work is to characterize the disease in terms of the molecules that may be involved in its development.

More specifically, in order to know if the accumulated cartilage (a feature of TD) is due to: 1) an increased synthesis of the cartilage matrix or; 2) a lack in the extracellular matrix degradation, the expression of the matrix macromolecules (collagen types II, IX, X, XI, and aggrecan) and the extracellular matrix-degrading enzymes (matrix metalloproteinases) will be investigated in normal and TD-affected growth plates.

Additionally, because TD is also characterized by a failure in terminal differentiation from prehypertrophic to hypertrophic chondrocytes (Poulos et al., 1978; Hargest et al., 1985; Thorp et al., 1993), the expression of hormones, growth and transcription factors, known to be expressed in the growth plate and to be involved in the regulation of chondrocyte proliferation and differentiation will be also investigated. These molecules include the PTHrP, Ihh, FGF2, TGF-β3, Cbfa1, and Sox9.

Finally, the expression of the VEGF and caspase-3 will be also analysed in order to explain the avascular lesions, as well as the accumulated pre-hypertrophic chondrocytes observed in affected growth plates.

3 MATERIALS AND METHODS

3.1 ANIMAL RAISING AND PATHOLOGY INDUCTION

All animal procedures were performed according to the approved guidelines of national ethical requirements for animal research and to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

Day-old *broiler* chickens (Cobb strain) were obtained from commercial hatcheries and raised in two separate groups for 21 days in constant-temperature rooms at 24ºC. Chickens from one group were fed *ad libitum* on a normal diet (control group) and chickens from the other group were fed, also *ad libitum*, on a diet containing 25mg/kg of the fungicide tetramethylthiuram disulfide (thiram) (Sigma), which is known to induce a high incidence of tibial dyschondroplasia (TD) (Vargas et al., 1983).

3.2 EXTRACTION AND PRESERVATION OF TISSUE

At 3 weeks of age chickens were sacrificed by cervical dislocation under ether anaesthesia. After their sacrifice birds were weighed. Tibia growth plates were taken according to Wardale and co-workers (1996) (Wardale and Duance, 1996) and stored at -80ºC for protein analysis studies and placed in *RNAlater* for gene expression analysis studies.

RNA*later* is an aqueous, nontoxic, tissue storage reagent that rapidly permeates most tissues to stabilize and protect RNA in fresh specimens. This reagent protects cellular RNA from degradation, and stabilizes RNA avoiding new induction of genes, which prevents unwanted changes in the gene-expression pattern.

Under sterile conditions the right and left proximal tibiotarsi were dissected free of muscle, tendons and ligament, and a single cut was made centrally through the tibial plateau into the diaphysis using a bistoury (Wardale and Duance, 1996), and examined for the presence of the dyschondroplastic lesions. The presence of dyschondroplastic lesions was assessed visually, being the lesions easily identified because of its opaque nonmineralized, avascular nature, as described by Berry and collaborators (Berry et al., 1996). Normal growth plates from control birds and TD-affected growth plates were carefully removed free from the upper articular cartilage and free from the lower bone with a bistoury. Both tissues were cut into 1mm cubes and placed immediately in liquid nitrogen and then transferred to -80ºC until use for protein analysis studies. For gene expression analysis studies the tissue was dissected, as quickly as possible, into 1mm cubes and immediately submerged in 10 volumes of RNA*later®* (Ambion, Austin, USA) solution, which was pipetted into a collection vessel, and then incubated for 16 hours at 4ºC to allow thorough penetration of the tissue. Samples were then transferred, in the reagent, to -20ºC for storage.

3.3. GENE EXPRESSION ANALYSIS

Real-time PCR technology is based on the detection of a fluorescent signal produced proportionally during the amplification of a DNA target (Bustin et al., 2005). To accomplish this, it is necessary to have a method of detecting the accumulation of PCR product, an instrument to monitor the amplification in real time and the appropriate software to analyze the data.

In the present work, it was used the SYBR green (*QuantiTect SYBR Green PCR* (Qiagen)) chemistry for detecting the PCR products. This is an intercalating dye that binds to the double-stranded DNA and emits a fluorescence signal that increases in intensity in direct proportion to the amount of specific amplified product. In solution, the unbound dye exhibits little fluorescence. However, during the PCR assay, increasing amounts of dye bind to the nascent double-stranded DNA. When monitored in real-time, this results in an increase in the fluorescence signal as the polymerization proceeds (Bustin and Mueller, 2005).

An instrument measures the cycle-to-cycle changes in fluorescence in each sample and generates a sigmoidal-shaped amplification plot (when using a linear scale), in which the fluorescence signal is plotted against the number of PCR cycles. In the present study, it was used the *ABI Prism 7900HT Sequence Detection System* (Applied Biosystems, Foster City, USA).

The fractional PCR cycle number at which the fluorescence signal passes a fixed threshold above baseline is defined as the threshold cycle (Ct) for a sample. The Ct values are determined automatically by the used software. In the present study, the *SDS 2.1 software* (Applied Biosystems, Foster, USA) was used.

3.3.1 Extraction of Total RNA

The successful isolation of total RNA from tissues involves the complete disruption of tissues; the effective denaturation of the nuclear protein complex; the inactivation of endogenous ribonuclease (RNase) activity; and purification of the RNA from the contaminating DNA and proteins.

In the current study, total RNA was extracted from growth plate cartilage stored at -20ºC in *RNAlater®* (Ambion, Austin, USA) using the *RNeasy® Plant Mini Kit* (Qiagen, Hilden, Germany) in agreement with the supplier's instructions.

The *RNeasy* technology is based on the selective binding properties of a silica-gel-based membrane and on the speed of a microspin technology. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine isothiocyanate (GITC)-containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an *RNeasy* mini column where the total RNA binds to the membrane and contaminants are efficiently washed away. The RNeasy Plant Mini kit has an additional column, the QIAshredder homogenizer, which simultaneously removes insoluble material and reduces the viscosity of the lysates by disrupting gelatinous material prior the lysate to be applied to the RNeasy mini column.

The RNeasy Plant Mini assay was specifically chosen for this work because Huang and his collaborators (Huang et al., 2002) have proved the efficiency of this method for extraction of total RNA from cartilage samples. According to these authors, the spin columns of this system allow the elimination of the interfering glycosaminoglycans in cartilage whose analogues are also found in plants. However, some modifications were made to this protocol in order to adjust it to the different type of tissue used in this assay. These modifications were based on the protocol indicated for RNA extraction from animal tissues from the same supplier.

Total cellular RNA was extracted from 30mg of both normal and TD-affected growth plate cartilages stored at -20ºC in RNA*later®*, excised from animals with approximately the same weight. Tissue was disrupted and simultaneously homogenized with a rotor-stator homogenizer *Ystral X10/25* (Ystral, Ballrechten-Dottingen, Germany) in a glass *cuvette* (Abbott Laboratories, Abbott Park, USA) containing 600µl of a guanidine isothiocyanate-containing buffer (component of the *RNeasy® Plant Mini Kit*) to which was added 6µl of β-mercaptoethanol, until the sample is uniformly homogeneous. The probes used for tissue disruption were previously soaked in 0.1% DEPC (diethylpyrocarbonate) (Sigma-Aldrich, Steinheim, Germany) for 2hours at 37ºC, and then autoclaved for 15minutes at 120ºC, in order to destroy ribonucleases (RNases). The tissue lysate

was then centrifuged for 1 minute at 16 200g in a microcentrifuge *Eppendorf 5415D* (Eppendorf, Hamburg, Germany) and the resultant supernatant was further used following the instructions of the RNeasy Plant Mini Kit protocol. At the end, RNA was eluted in a 50µl volume of RNase-free water (Ambion) and stored at -80ºC.

3.3.2 Determination of RNA Quality and Concentration

RNA intended for use in quantitative RT-PCR assays must be of high quality in terms of purity and sequence integrity. It is well know that RNA is extremely susceptible to degradation once removed from its cellular environment. Therefore, every RNA sample was rigorously evaluated for its degree of degradation before using it in the subsequent steps of quantitative RT-PCR assays.

To assess both the quality and concentration of total RNA, a microcapillary electrophoresis was carried out by using the *Agilent 2100 bioanalyzer* (Agilent Technologies, Waldbronn, Germany) in conjunction with the *RNA 6000 Nano LabChip® Kit* (Agilent Technologies, Waldbronn, Germany) following the manufacturer's instructions.

The Agilent 2100 bioanalyzer associated with the RNA 6000 labchips allow the electrophoretic separation of RNA samples according to their molecular weight in gel-filled microchannels of the microfabricated chips. RNA samples are subsequently detected via laser-induced fluorescence detection due to the presence of a dye which intercalates with RNA molecules. The result is visualized as an electropherogram where the amount of measured fluorescence correlates with the amount of RNA of a given size (Schroeder et al., 2006). These results are given by the software *Agilent 2100 expert software* (Agilent Technologies, Waldbronn, Germany).

Total RNA concentration for each sample is given automatically by the software, as well as the RNA Integrity Number (RIN). This number for evaluation of the integrity of RNA is determined by the RIN software algorithm, a tool of the *Agilent 2100 expert software.* This algorithm is based on a selection of features obtained from the electropherogram. The output RIN is a decimal or integer number in the range of 1-10, where a RIN of 1 corresponds to a completely degraded RNA samples and a RIN of 10 corresponds to an intact RNA samples (Imbeaud et al., 2005; Schroeder et al., 2006).

RNA samples were denatured at 70ºC for 2 minutes and then subjected to a microcapillary electrophoresis by using the *Agilent 2100 bioanalyzer* and the *RNA 6000 Nano LabChip® Kit*, following the manufacturer's instructions, in order to determine the quality and concentration of RNA.

3.3.3 DNase Treatment of RNA

A prerequisite for RNA template to be suitable for inclusion in an RT-PCR assay is that it should be free of DNA, especially if the target is an intronless gene. Thus, to eliminate any presence of contaminating genomic DNA in RNA samples, the digestion of RNA samples with deoxyribonuclease was carried out by using the *Deoxyribonuclease I, Amplification Grade kit* (Invitrogen) in accordance with the manufacturer's instructions.

A mixture was prepared by using 1µg of total RNA to which was added 1µl of DNase I (1U/µl) and 1µl of 10 fold concentrated DNase I reaction buffer. A final volume of 8µl was adjusted with RNase-free water. After an incubation at room temperature for 15minutes, DNase I was inactivated by the addition of 1µl of 25mM EDTA solution, followed by an incubation at 65ºC for 10minutes carried out in a *Thermomixer confort* (Eppendorf, Hamburg, Germany). The DNase-treated RNA was ready to use in reverse transcription reaction (cDNA synthesis).

3.3.4 Complementary DNA Synthesis

In reverse transcription, an oligodeoxynucleotide primer is hybridized to the mRNA and is then extended by an RNA-dependent DNA polymerase (reverse transcriptase) to create a complementary DNA (cDNA) copy that can be amplified by PCR.

In the present study, RNA was reverse transcribed to cDNA by using the *SuperScript™ III First-Strand Synthesis System for RT-PCR kit* (Invitrogen, California, USA) according to the procedure supplied by the manufacturer.

The SuperScript commercial kit (Invitrogen) for cDNA synthesis uses the SuperScript III reverse transcriptase which is a version of M-MLV reverse transcriptase that has been engineered to reduce RNase activity and provide increased thermal stability. These variants of M-MLV reverse

transcriptases that lack RNase H activity are modified enzymes that transcribe a greater proportion of the template molecules and synthesize longer cDNA molecules than the wild-type enzyme. In addition, they are capable of cDNA synthesis at high temperatures (up to 50ºC in some cases), which is an advantage when the template RNA is rucked into secondary structures (Sambrook and Russel, 2001).

RT reaction was carried out using the 8µl (1µg of RNA) volume obtained from DNase treatment, to which was added 1µl of a oligo(dT) (50µM) and random hexamers (50ng/µl) primers mixture (1:1) and 1µl of dNTP mix (10mM), obtaining a final volume of 10µl. Following an incubation at 65ºC for 5minutes, the RT reaction was completed by adding 10µl of RT reaction mix, containing 2µl of 10 fold concentrated RT buffer, 4µl of $MgCl_2$ (25mM), 2µl of dithiothreitol (DTT; 0.1M), 1µl of *RNaseOUT™* (40U/µl), and 1µl of *SuperScript™ III RT* (200U/µl, *M-MLV Reverse Transcriptase*). The reaction was incubated at 25ºC for 10minutes in order to primers anneal to the RNA template. The RNA was subsequently reverse transcribed into cDNA during the incubation at 50ºC for 50minutes. Following the 50ºC incubation the *M-MLV Reverse Transcriptase* was denatured by incubating the reaction at 85ºC for 5minutes. All the incubations were carried out in the *Gene Amp PCR System 9600* thermocycler (Perkin Elmer, Norwalk, USA). Finally, 1µl of RNase H (2U/µl) was added to the reaction in order to remove the RNA template from the cDNA:RNA hybrid molecule and thus increase the sensitivity of the PCR step, followed by an incubation at 37ºC for 20minutes. The resultant cDNA was kept at -20ºC until used in downstream PCR.

3.3.5 Primer Design

Optimal design of the PCR primers is essential for accurate and specific quantification using real-time PCR. In the assay described here it was used the SYBR green chemistry for detection of the PCR products. The SYBR green dye binds to any double-stranded DNA increasing its fluorescence strongly. Thus, is of particular importance to minimize the formation of primer-dimers as well as non-specific PCR products, by careful primer design.

For this reason, the *Primer Express® version 2.0* software (Applied Biosystems) was chosen in order to design the primers used in the present study. This software allows the design of PCR primers using a combination of criteria based on the general guidelines for primers design, such as, the primer length, the guanine and cytosine content, the melting temperature, among others. It also includes a primer test document that allows evaluating primers for their melting temperature as well as for their secondary structure and primer-dimer formation.

The primers were synthesized by MWG-Biotech AG. Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) was used to confirm the specificity of the primers.

Each pair of primers was designed following the manufacturer's instructions using the default supplied criteria of the program, except for the length of the PCR product which was set between 100 and 150 base pairs. Among the set of primers given by the software it was selected those ones that have a melting temperature of about 60ºC (5ºC above the annealing temperature) and that had the smallest probability to form secondary structures and primer-dimers, according to the primer test document of the software.

TABLE 1 - Oligonucleotide sequences for real-time quantitative RT-PCR analysis.

Gene	Accession Number	Oligonucleotide sequences[1]	Concentration (nM)	Product size (bp)
MMP-2	NM_204420	for: 5′-GCTCTGCAAGCACGACATTG-3′ rev: 5′-CTCGAGGGTTTACAGTCCTCCAC-3′	300 300	101
MMP-7	NM_001006278	for: 5′-CGCTGCGCTTCAAAAGAGTT-3′ rev: 5′-GCCACCTCTTCCATCAAAAGG-3′	900 100	101
MMP-9	NM_204667	for: 5′-GCCATCACTGAGATCAATGGAG-3′ rev: 5′-GATAGAGAAGGCGCCCTGAGT-3′	300 300	102
MMP-10	XM_417175	for: 5′-TGCTTCTGGATTTCACGGTG-3′ rev: 5′-AGTGGGCATCCCCTCCTATC-3′	300 300	101
MMP-11	XM_001232776	for: 5′-CAAGGTACTGGCATGGTGACA-3′ rev: 5′-AAAATGGACATCTCCTTCCCG-3′	300 300	101
MMP-13	AF070478	for: 5′-GGAACACTCCAGAGACCCTGG-3′ rev: 5′-CTTGGATCCCTTGCACATCAT-3′	100 100	101
TIMP-2	NM_204298	for: 5′-GACATTTATGGGAACCCCATCA-3′ rev: 5′-GGCGCCGTGTAGATGAATTC-3′	300 300	101
Collagen II	NM_204426	for: 5′-GATGCCACCCTCAAATCCCT-3′ rev: 5′-CAGAGTTTGATGTCGCGGC-3′	100 100	101
Collagen IX	NM_205305	for: 5′-TCAATCACCTCACCTTCCCTG-3′ rev: 5′-AAAAAGCTGCGCTAGTACACCC-3′	100 300	103
Collagen X	M13496	for: 5′-CTGGCCAATCCACAATCCC-3′ rev: 5′-TCCTGTGAGAGCTTGATTTGCTC-3′	100 100	101
Collagen XI	XM_422303	for: 5′-GGTCCCCCAGGTACCATGTT-3′ rev: 5′-GAAGAATAGCTTGTGCTTGAGCC-3′	100 100	101
Aggrecan	NM_204955	for: 5′-CCAAGGGAAGAGAACGTGACC-3′ rev: 5′-CATTCCGGTGGTGAAAGCA-3′	100 300	102
Ihh	NM_204957	for: 5′-GACCGCAACAAGTACGGCAT-3′ rev: 5′-TGACTTGACGGAGCAGTGGA-3′	300 300	102
PTHrP	AB175678	for: 5′-GGAACAGCCCCTGAAGGTATC-3′ rev: 5′-TTAGCCAAGCTGAGCGAGCT-3′	300 300	101
FGF2	NM_205433	for: 5′-AAAGGCGTAAGTGCAAACCG-3′ rev: 5′-TCCAAGCGCTCGAAAAAGAA-3′	100 300	101
VEGF	NM_205042	for: 5′-CGATGAGGGCCTAGAATGTGTC-3′ rev: 5′-AGCTCATGTGCGCTATGTGC-3′	300 300	101
TGFβ-3	NM_205454	for: 5′-CTGCGGCCAGATGAGCATAT-3′ rev: 5′-GTGTCGGTGACATCGAAGGA-3′	300 300	101
Caspase-3	NM_204725	for: 5′-GGAACACGCCAGGAAACTTG-3′ rev: 5′-TTGCATTGAAATCCTGTCGAGT-3′	300 300	101
Cbfa1	AF445419	for: 5′-CTAGTTTGTTCCCTGAACGCCT-3′ rev: 5′-AGAGTTCAGAGAGGGCCGTG-3′	300 300	101
Sox9	NM_204281	for: 5′-CCTCCCCACATCGATTTCC-3′ rev: 5′-AGGTATTGGTCGAACTCGTTGAC-3′	300 300	101
ACTB	NM_205518	for: 5′-AGAAGCTGTGCTACGTCGCA-3′ rev: 5′-GACCTGACCATCAGGGAGTTCA-3′	300 300	101
GAPDH	NM_204305	for: 5′-TCAGCAATGCATCGTGCAC-3′ rev: 5′-GGCATGGACAGTGGTCATAAGAC-3′	300 300	101
HPRT1	NM_204848	for: 5′-TGTAACCACCCAGTGCAATTCT-3′ rev: 5′-CGGAGCTCACAAACAGCACA-3′	900 300	101
TBP	NM_205103	for: 5′-TGAAAAGGCATTGCATATGGC-3′ rev: 5′-CAGGGAAATAGGCACTAACTGGG-3′	300 300	101
18S rRNA	AF173612	for: 5′-GGCTACCACATCCAAGGAAGG-3′ rev: 5′-AGGGCCTCGAAAGAGTCCTG-3′	100 300	101
28S rRNA	DQ018756	for: 5′-CTTAACGGTTTCACGCCCTCT-3′ rev: 5′-AAGGCTAAATACCGGCACGAG-3′	100 100	101

[1]Primers correspond to cDNA sequences deposited in GeneBank; for=forward primer; rev=reverse primer

3.3.6 Optimization of Primer Concentration

The optimisation of primer concentration is essential since each set of primers work best under different concentration conditions.

3.3.6.1 Preparation of Reaction Mixture

For every primer set it was prepared nine PCR mastermixes, each one with a different combination of concentrations for the forward and reverse primers. For example, 100nM of the forward primer/100nM of the reverse primer, 100nM of the forward primer/300nM of the reverse primer, 100nM of the forward primer/900nM of the reverse primer, and so on.

The PCR mastermix was prepared by mixing 12.5µl of 2 fold concentrated *QuantiTect SYBR Green PCR Master Mix* (which contains the enzyme *HotStarTaq Polymerase*, the PCR buffer, the dNTP mix and the fluorescent dye SYBR Green I), the appropriate volume of the forward and reverse primers to achieve the combination of concentrations to be tested, and RNase-free water to a final volume of 22.5µl. Each PCR mastermix was then dispensed in *96-Well Optical Reaction Plates* (Applied Biosystems, Foster City, USA) containing 2.5µl (20ng) of cDNA, obtaining a 25µl-final volume of reaction. All samples were run in duplicate along with no template controls where the sample was replaced by RNase-free water.

3.3.6.2 Thermocycler Conditions

The real-time PCR reactions were carried out with the *ABI Prism 7900HT Sequence Detection System* (Applied Biosystems, Foster City, USA). The thermocycler conditions used were the ones recommended by the manufacturer.

In order to achieve the best combination of concentrations for the forward and reverse primers, a melting curve analysis was carried out.

The ABI Prism 7900HT instrument generates a melting curve for each sample in which the fluorescence is plotted as a function of temperature. These melting curves or dissociation curves are useful for determining the presence of multiple species in the sample since every PCR product melts at a characteristic temperature, its Tm (melting temperature). Curves with peaks at a Tm

lower than that of the specific PCR product indicate the formation of primer-dimers, while diverse peaks with different Tms or plateaus indicate production of non-specific products or a smear. To create a melting curve the temperature is increased very slowly from a low temperature of 65ºC to a high temperature of 95ºC.

The amplification reactions were carry out under the following real-time cycler conditions: an initial heating step of 95ºC for 15minutes to activate the *HotStarTaq DNA Polymerase*, followed by 40 cycles of denaturation at 94ºC for 15seconds, annealing at 55ºC for 30seconds, and extension at 72ºC for 30seconds. Following amplification, melting curve analysis was performed by increasing temperature very slowly from a low temperature of 65ºC to a high temperature of 95ºC.

3.3.6.3 Data Analysis

Analysing the dissociation curves, primer concentration was determined to be optimal when specific amplification was maximal for a given primers concentration and when primer-dimers were absent in the corresponding no template control. The combination of concentrations for the forward and reverse primers optimized for each set of primers is indicated in Table 1.

3.3.7 Selection of Optimal Internal Control Genes

In order to control for differences in the amount of amplifiable cDNA in individual samples generated by: i) different amounts of starting material; ii) the quality of the starting material; and iii) differences in RNA preparation and cDNA synthesis (Radonic et al., 2004), the expression levels of six housekeeping genes in a given set of samples were studied. The aim was to identify three housekeeping genes with minimal variability under the current experimental treatment and thus to validate their presumed stability of expression. It was selected six commonly used reference genes which fall roughly into four different groups: 1) structure-related genes: beta actin (ACTB); 2) metabolism-related genes: hypoxanthine phosphoribosyl-transferase (HPRT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH); 3) transcription-related genes: TATA box binding protein (TBP); and 4) ribosomal subunits: 18S rRNA and 28S rRNA.

3.3.7.1 Preparation of Reaction Mixture

For each primer set a PCR mastermix was prepared by mixing 12.5µl of 2 fold concentrated *QuantiTect SYBR Green PCR Master Mix* (which contains the enzyme *HotStarTaq Polymerase*, the PCR buffer, the dNTP mix and the fluorescent dye SYBR Green I), the appropriate volume of the forward and reverse primers to achieve the optimized final concentration (Table 1), and RNase-free water to a final volume of 22.5µl. The mastermix was then dispensed in *96-Well Optical Reaction Plates* (Applied Biosystems, Foster City, USA) containing 2.5µl (20ng) of cDNA from each sample, obtaining a 25µl-final volume of reaction. For each gene, all samples were run in duplicate along with no template controls where the sample was replaced by RNase-free water.

3.3.7.2 Thermocycler Conditions

The amplification reactions were carried out as described before in section 3.3.6.2.

3.3.7.3 Data Analysis

To select the best-performing housekeeping genes, it was used the program *geNorm®* version 3.4, a Visual Basic Application (VBA) for Microsoft Excel that automatically calculates a gene-stability measure for all control genes in a given set of samples.

The gene-stability measure *M* calculated by the *geNorm®* program to determine the expression stability of all control genes relies on the principle that the expression ratio of two ideal internal control genes is identical in all samples, regardless of the experimental condition or cell type. In this way, variation of the expression ratios of two real-life housekeeping genes reflects the fact that one (or both) of the genes is (are) not constantly expressed, with increasing variation in ratio corresponding to decreasing expression stability. For every control gene the program determined the pairwise variation with all other control genes as the standard deviation of the logarithmically transformed expression ratios, and defined the internal control gene-stability measure *M* as the average pairwise variation of a particular gene with all other control genes. Genes with the lowest *M* values have the most stable expression. Assuming that the control genes are not co-regulated, stepwise exclusion of the gene with the highest *M* value results in a combination of two constitutively expressed housekeeping genes that have the most stable expression in the tested samples (Vandesompele et al., 2002).

The threshold cycle (Ct) values given by the *SDS 2.1 software* (Applied Biosystems, Foster, USA) were exported as tab-delimited text files and imported into Microsoft Excel. These values were transformed to relative quantities by using the comparative Ct method and setting to 1, for each internal control gene, the sample with highest expression level (lowest Ct value). The formula used was: $Q = E^{\Delta Ct}$, $Q = E^{(minCt - sampleCt)}$, where Q = sample quantity relative to sample with highest expression; E = amplification efficiency (2=100%); and minCt = lowest Ct value = Ct value of sample with highest expression. These relative quantities values were then introduced in *geNorm®* and the program automatically ranked the control genes according to their expression stability, starting from the least stable gene and ending with the two most stable genes (GeNorm – User's Manual, 2004). The three most stable internal control genes given by the software (GAPDH, HPRT1, and TBP) were used for the normalization of gene expression levels, as recommended by the authors (Vandesompele et al., 2002).

3.3.8 Relative Gene Expression Analysis

To calculate relative changes in gene expression for the proteins in study, both in normal and dyschondroplastic growth plates, real-time quantitative PCR was carried out with the *ABI Prism 7900HT Sequence Detection System* (Applied Biosystems, Foster City, USA) and by using the *QuantiTect SYBR Green PCR kit* (Qiagen), as described in earlier sections.

3.3.8.1 Preparation of Reaction Mixture

For each primer set a PCR mastermix was prepared by mixing 12.5µl of 2 fold concentrated *QuantiTect SYBR Green PCR Master Mix* (which contains the enzyme *HotStarTaq Polymerase*, the PCR buffer, the dNTP mix and the fluorescent dye SYBR Green I), the appropriate volume of the forward and reverse primers to achieve the optimized final concentration (Table 1), and RNase-free water, to a final volume of 22.5µl. The mastermix was then dispensed in *96-Well Optical Reaction Plates* (Applied Biosystems, Foster City, USA) containing 2.5µl (20ng) of cDNA from each sample, obtaining a 25µl-final volume of reaction. For each gene, all samples were run in duplicate along with no template controls where the sample was replaced by RNase-free water.

3.3.8.2 Thermocycler Conditions

The amplification reactions were carried out as described before in section 3.3.6.2.

3.3.8.3 Data Analysis

The calculation of relative changes in gene expression, determined from real-time quantitative PCR, was made by the comparative threshold cycle (Ct) method through the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{GOI} - Ct_{HKG})_{sample} - (Ct_{GOI} - Ct_{HKG})_{control\ sample}$. The result obtained by this formula represents the fold change in expression of a gene of interest (GOI), normalized to an endogenous reference (HKG, housekeeping gene), in a given sample (sample) relative to the untreated control (control sample).

The threshold cycle (Ct) values given by the *SDS 2.1 software* (Applied Biosystems, Foster, USA) were exported as tab-delimited text files and imported into Microsoft Excel where all the calculations were done. The $2^{-\Delta\Delta Ct}$ formula was done three times between the gene of interest and the three most stable housekeeping genes (GAPDH, HPRT1, and TBP) determined above in section 3.3.7. The geometric mean of the three relative quantifications was taken to obtain the normalized gene of interest expression levels.

3.3.8.4 Statistical Analysis

Differences between the means of control group (n = 6) (normal growth plates) and experimental group (n = 6) (TD-affected growth plates) for the normalized expression levels of the genes of interest were evaluated with the Student's *t*-test. Differences were considered statistically significant at $P \leq 0.05$ and when there was a two-fold or greater difference in expression between the control and experimental groups. Each experiment of real-time quantitative PCR was performed twice. The graphical data show a representative experiment.

3.4 Protein Analysis

3.4.1 Extraction of Proteins

In order to purify and characterize the proteins in study, a simple method of protein extraction was applied in growth plate cartilages. This was based on a simple maceration of the tissue in a mortar with liquid nitrogen and then a homogenization in a buffer, according to the protocol used by others (Ohkubo et al., 2003).

Frozen tibial growth plates were macerated in a mortar in liquid nitrogen and then homogenized in ice-cold buffer containing 20mM Tris, pH 8.0, 0.5mM $CaCl_2$, and 0.5% NP-40 (Ohkubo et al., 2003). The homogenate was incubated at 4ºC for 2 hours with gentle shaking. After centrifugation at 10 000 x g for 10 min the supernatant was collected and stored at -80C.

3.4.2 Protein Quantification

BCA™ protein assay (Pierce) was used to determine protein concentration according to the manufacturer's instructions. BSA was used as standard protein.

3.4.3 Analysis of MMPs Activity– Gelatin Zymography

Gelatin zymography was used to detect the activity of MMPs. Indeed, this assay is commonly used to detect the gelatinase activity of members of the matrix metalloproteinases family. This technique is based on an electrophoresis for the separation of the proteases coupled with the detection of the activity within the polyacrylamide gel. Gelatin was used as the protease substrate which was copolymerized within the polyacrylamide gel. Following electrophoresis, the gel is washed to remove detergent which may interfere with the protease activity and then it is incubated in buffer which favors proteolytic activity for the enzymes of interest. Protease activity is revealed by areas of clearing following staining of the gel (Snoek-van Beurden and von den Hoff, 2005).

Ten-μg aliquots of protein extracts of normal and TD-affected growth plates were mixed with equal volumes of sample loading buffer (100 mM Tris, pH 6.8, 5% SDS, 20% glycerol, 0.1% bromophenol blue) and subjected to sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) (Hoefer, San Francisco, USA) on a 4% stacking, 10% separating (containing 0.1% gelatin) gels, under nonreducing conditions. The gels were washed for 30 min at room temperature in 2.5% Triton X-100, were rinsed in distilled water and were developed for 16 hours at 37ºC in 50 mM Tris pH 7.4, 5 mM NaCl, 10 mM $CaCl_2$, 1μM $ZnCl_2$, 0.02% (v/v) Triton X-100. Finally, gels were stained with Coomassie brilliant blue R250 (Pierce, Steinheim, Germany) to visualize protease activity and were scanned on a Gel Image Analyzer (FX-710 scanner, Quantity ONE software - Bio-Rad) in order to estimate protein bands molecular weights and optical densities.

3.4.3.1 Statistical Analysis

Differences between the means of control group (n = 6) (normal growth plates) and experimental group (n = 6) (TD-affected growth plates) for the optical density (OD) values were evaluated with the Student's *t*-test. Differences were considered statistically significant at $P \leq 0.05$. Each experiment of zymography was performed at least 3 times. The graphical data and the images show a representative experiment.

3.4.4 SDS-PAGE and Immunoblotting

Immunoblotting was performed in order to identify a specific protein in extracts from normal and dyschondroplastic cartilages as well as to obtain semiquantitative data about this protein.

Twenty four-μg aliquots of protein extracts of normal and TD-affected growth plates were separated by SDS-PAGE on a 4% stacking, 10% separating gels, under reducing conditions. Proteins were electrotransferred at 90 mA to poly(vinylidene difluoride) (PVDF) membranes (Bio-Rad, Hercules, USA) overnight at 4ºC. The membranes were blocked for 1 hour at room temperature with 5% dried milk (w/v) in TBS-T. Blocked membranes were incubated for 1 hour at room temperature with an anti-chicken MMP-2 antibody (kindly provided by Dr. J. P. Quigley and Dr. E. I. Deryugina, The Scripps Research Institute, La Jolla, CA, USA), diluted 1:1000 in TBS-T. The membranes were washed three times with TBS-T, and then incubated for 1 hour at room temperature with the horseradish peroxidase (HRP)-labelled secondary antibody, diluted 1:1000 in

TBS-T. After washing, as described above, membranes were developed with ECL reagents (Amersham Pharmacia Biotech, Freiburg, Germany) following the manufacturer's instructions. Protein bands detection was performed by short exposures to blue-light sensitive autoradiography film. The films were scanned on a Gel Image Analyzer (FX-710 scanner, Quantity ONE software - Bio-Rad) in order to estimate protein bands molecular weights and optical densities, and the results were expressed relative to the β-actin signals.

3.4.4.1 Statistical Analysis

Differences between the means of control group (n = 6) (normal growth plates) and experimental group (n = 6) (TD-affected growth plates) for the optical density (OD) values were evaluated with the Student's *t*-test. Differences were considered statistically significant at $P \leq 0.05$. Each experiment of immunoblotting was performed at least 3 times. The graphical data and the images show a representative experiment.

4 RESULTS AND DISCUSSION

4.1 ANALYSIS OF GROWTH PLATES FROM NORMAL AND THIRAM-FED CHICKENS

4.1.1 Morphological Analysis

Investigations of gross pathology indicate that tibial dyschondroplasia is characterized by a thickened growth plate comprising a mass of opaque cartilage extending from the distal edge of the growth plate into the underlying metaphysis of the long bone. This mass of cartilage can either be focal and limited to a small area within the metaphysis or can occupy the entire metaphyseal region. In all cases, the cartilage is unmineralized and devoid of blood vessels, giving it a soft and pliable texture (Leach and Nesheim, 1965; Leach and Nesheim, 1972; Poulos et al., 1978).

In the present study, when the proximal tibiotarsi were dissected by sectioning the bone lengthwise, a mass of opaque cartilage was observed below the epiphyseal plate extending into the metaphysis (Fig. 4.1). We could see a uniform band across the width of the bone that affected whole the growth plate resulting in an epiphysis comprising solely by cartilage and periosteum. Our observations are in agreement with those reported by other authors (Leach and Nesheim, 1965; Wardale and Duance, 1996) regarding to tibial dyschondroplasia. Articular cartilage was not affected by tibial dyschondroplasia, which is in accord with the observations of Wardale and Duance (1996).

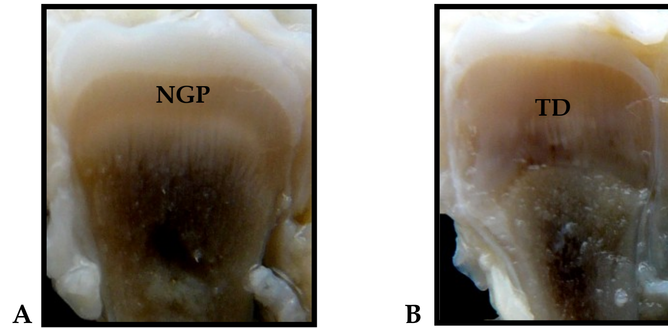


Figure 4.1 – Comparison of normal (A) and dyschondroplastic (B) growth plates. The tibial dyschondroplasia lesion is an opaque and avascular cartilaginous mass, which occupies a large area of the metaphysis under the growth plate. Tibial dyschondroplasia (TD) was induced in broiler chickens by adding thiram to the diet at 25mg/kg. NGP – Normal growth plate; TD – tibial dyschondroplasia.

4.1.2 Histological Analysis

The histological analysis of growth plates from thiram-fed chickens revealed an accumulation of prehypertrophic chondrocytes and the lesion seems to contain more matrix than normal cartilage (Fig. 4.2). Our observations are in agreement with previous studies describing the histology of growth plates affected with tibial dyschondroplasia (Poulos et al., 1978; Thorp et al., 1992; Tselepis et al., 1996).

Additionally, chondrocytes from lesions were different from those of normal growth plates. They were necrotic and had pyknotic and shrunken nuclei and sometimes showed emptied chondrocyte lacunae. According to Riddell (1981), Hargest et al. (1985), Thorp et al. (1991), and Leach and Lilburn (1992) these are some of the hallmark features of tibial dyschondroplastic lesions.

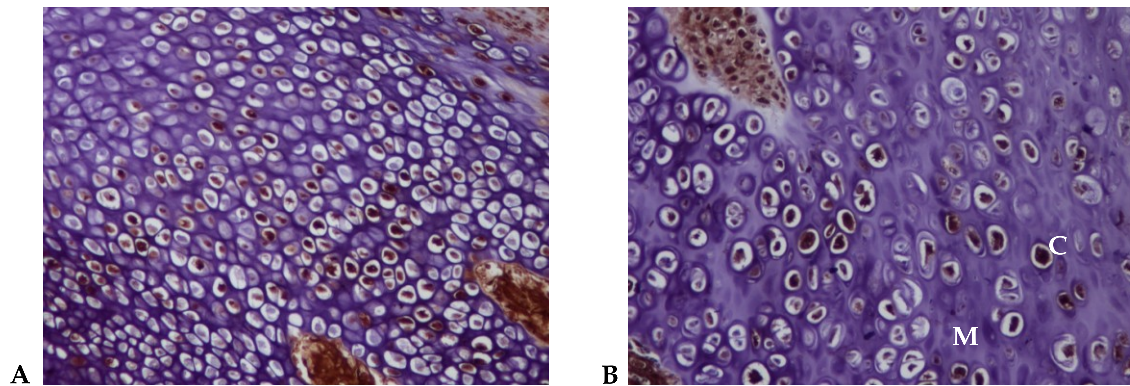


Figure 4.2 – Histology of the growth plates of (A) normal and (B) thiram-fed chickens. Tibial dyschondroplasia (TD) was induced in broiler chickens by adding thiram to the diet at 25mg/kg. The histological sections were stained with hematoxilyn-eosin. M – matrix; C – chondrocyte. Adapted from Capela-Silva, F. (2003) Discondroplasia da tíbia em aves - Avaliação de um modelo de patologia experimental. PhD Thesis, Dep of Biology, University of Évora, Évora, pp 264.

The results from morphological and histological analysis confirm that the growth plates obtained from thiram-fed chickens were indeed affected with tibial dyschondroplasia.

4.2 EXPRESSION OF MATRIX MACROMOLECULES

Each stage of the process of endochondral ossification is accompanied by changes in chondrocyte morphology and by variations in the biosynthetic activity of the cells which lead to alterations in the composition of the extracellular matrix. The complex organization of the extracellular matrix in the growth plate and the structure-function relationship is still not fully understood. Changes in molecular composition within the cartilage matrix may contribute to the normal skeletal development as well as to pathological situations (Tselepis et al., 1996).

Since TD is characterized by an accumulation of cartilage extending from the growth plate into the mataphysis (Leach and Nesheim, 1965; Tselepis et al., 1996) we aimed to investigate whether the disease was related to altered expression of the macromolecules in the growth plate matrix. On that regard, we analyzed the mRNA expression of collagens type II, type IX, type X, and type XI, and aggrecan in normal and TD-affected growth plates. For that purpose, we designed specific primers for chicken collagens and aggrecan using the corresponding mRNA published sequence (*GenBank* accession number NM_204426 for collagen type II, *GenBank* accession number M13496 for collagen type X, *GenBank* accession number NM_205305 for collagen type IX, *GenBank* accession number XM_422303 for collagen type XI, and *GenBank* accession number NM_204955 for aggrecan). A BLAST analysis was performed in order to confirm the specificity of the primers. Then, a real-time PCR analysis was performed using these specific primers, SYBR green as the detection chemistry, and cDNA reverse transcribed from total RNA isolated from the normal and TD-affected growth plates, as described in Materials and Methods chapter.

4.2.1 Collagen type X

With regard to collagen type X, it is exclusively expressed by hypertrophic chondrocytes in the growth plate (Velleman, 2000) and therefore it has been widely studied in TD, a disease where pre-hypertrophic chondrocytes fail to undergo full hypertrophy.

In the present study, we analyzed the expression of collagen type X at the mRNA level and we found a statistically significant ($P < 0.05$) increment in TD cartilage (Fig. 4.3). However, this increment was very low (1.7-fold) and therefore it was considered not relevant.

Thorp et al. (1993) and Reginato et al. (1998) observed, by northern hybridization, increased mRNA levels of collagen type X in TD cartilage. However, these authors have found that

despite the higher levels of expression of the collagen type X mRNA, the production of its protein has decreased in TD. Based on these results, the authors suggested that probably a defect in collagen type X secretion or incorporation into the extracellular matrix may occur. In this way, collagen type X would not be able to interact with the existing extracellular type II collagen network. This change in extracellular matrix architecture may result in a matrix that does not have a permissive structure to support vascularization that is necessary for bone formation (Velleman, 2000). Accordingly, Thorp et al. (1993) and Tselepis et al. (1996), through immunocytochemistry and immunohystochemistry, respectively, also showed a reduction in the protein levels of collagen type X in TD cartilage. A great controversy exists among the authors regarding the expression and distribution of collagen type X in TD lesions. For example, and in contrast, Wardale and Duance (1996) and Reginato et al. (1998) have reported, by determination of the relative amount of collagen, an increase in the protein level of collagen type X in TD cartilage. These authors even suggest that the raised levels of type X collagen in a cartilage which has failed to calcify indicate that collagen type X does not have a primary role in calcification.

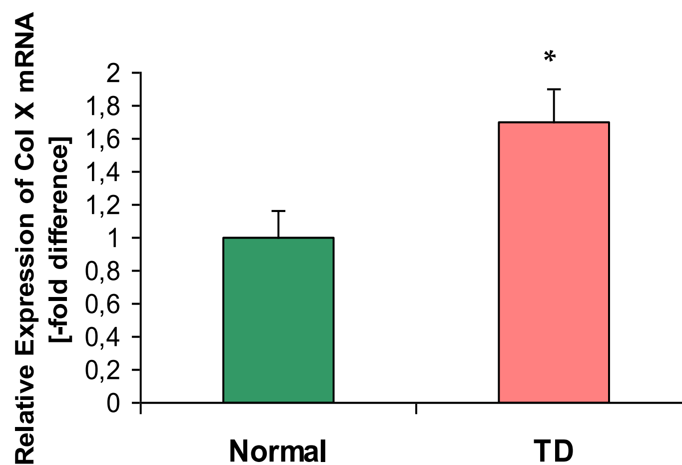


Figure 4.3 – Relative expression of collagen type X mRNA. Total RNA was extracted from normal and TD-affected growth plates of 3-week-old chickens. One microgram (1µg) of total RNA was reverse transcribed into cDNA and twenty nanograms (20ng) of this were used to perform real-time quantitative RT-PCR analysis. Expression levels were normalised to the levels of GAPDH, HPRT1, and TBP gene expression. Values are shown as mean ± SD (n = 6 for each group). $^{*}P < 0.05$. Results are shown relative to mRNA expression levels from control group (normal growth plates), set to 1, thus corresponding to the N-fold difference in relation to the control group. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates.

As mentioned above, it has been reported that collagen type X is exclusively expressed by hypertrophic chondrocytes in the growth plate (Velleman, 2000). However, in the chicken normal growth plate the collagen type X mRNA has been localized in both prehypertrophic and hypertrophic chondrocytes (Webster et al., 2003). Thus, based on our results we suggest that the low raise of collagen type X mRNA levels in TD is the result of an accumulation of prehypertrophic chondrocytes in the growth plate and not an overexpression of the gene in the cell.

4.2.2 Collagen type II

In regard to collagen type II, we have shown no significant differences in the levels of collagen type II mRNA between normal and TD-affected growth plates which is in agreement with previous reports (Fig. 4.4). Indeed, previous studies have shown that collagen type II has an expression pattern in TD cartilages similar to the normal cartilages being present throughout the affected growth plate as well (Chen et al., 1993; Farquharson et al., 1995; Tselepis et al., 1996). Accordingly, Rath et al. (2005) demonstrated similar mRNA levels for collagen type II in both normal and dyschondroplastic growth plates.

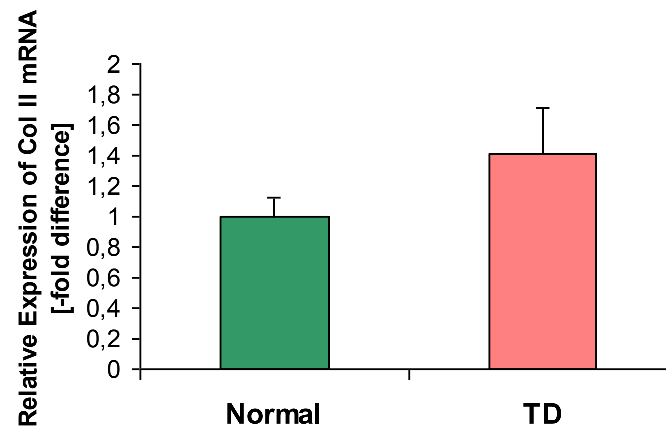


Figure 4.4 – Relative expression of collagen type II mRNA. Total RNA was extracted from normal and TD-affected growth plates of 3-week-old chickens. One microgram (1µg) of total RNA was reverse transcribed into cDNA and twenty nanograms (20ng) of this were used to perform real-time quantitative RT-PCR analysis. Expression levels were normalised to the levels of GAPDH, HPRT1, and TBP gene expression. Values are shown as mean ± SD (n = 6 for each group). Results are shown relative to mRNA expression levels from control group (normal growth plates), set to 1, thus corresponding to the N-fold difference in relation to the control group. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates.

It has been reported that a marked increase in type II collagen is an indicator of normal differentiation of chondrocytes from a proliferative to prehypertrophied form (Thorp et al., 1992; Thorp et al., 1993). Hence, because it has been observed, by other authors and also by us, little difference in the expression of type II collagen between normal and dyschondroplastic cartilages, it has been suggested that differentiation from proliferative to prehypertrophic chondrocytes occurs normally in the disorder. In other words, dyschondroplasia occurs after differentiation to the prehypertrophic form (Thorp et al., 1993).

4.2.3 Collagen types IX and XI

It has been reported the importance of collagens type IX and XI in the growth plate. In the prehypertrophic cartilage, collagen type II interacts with collagens type IX and XI to form heterotypic fibrils that are distributed throughout the cartilage matrix (van der Rest and Mayne, 1988; Vaughan et al., 1988; Farquharson and Jefferies, 2000). A recent study has shown a markedly disturbance of the growth plate cartilage morphology in mice lacking collagen IX (Dreier et al.,

2008). In regard to collagen type XI, identification of the gene causing autosomal recessive chondrodysplasia in mice demonstrates that the quantitatively minor fibrillar collagen XI is essential for the cohesive properties of cartilage and the normal differentiation and spatial organization of chondrocytes in growth plates (Olsen, 1995). Similarly, Li et al. (1995) suggested that the normal differentiation and spatial organization of growth plate chondrocytes is critically dependent on the presence of type XI collagen in cartilage extracellular matrix.

From our study, it appears that both type IX and type XI collagens do not change their mRNA expression levels in TD cartilage (Fig. 4.5). However, Wardale and Duance (1996) found a reduction in the protein levels for these collagens in dyschondroplasia. The fact that the protein levels for collagens type IX and XI are reduced despite their unchanged mRNA levels may imply that, as suggested for collagen type X, a defect in the secretion and incorporation of these collagens into the extracellular matrix may occur.

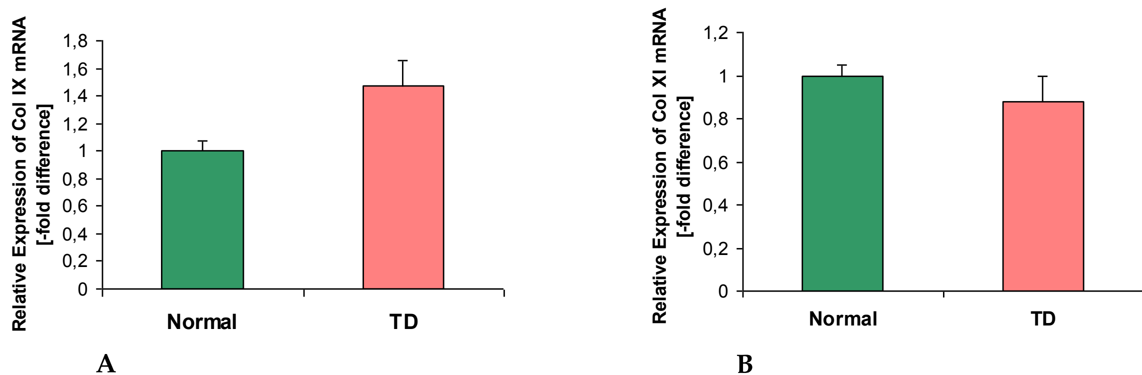


Figure 4.5 – Relative expression of collagen type IX mRNA (A) and collagen type XI mRNA (B). Total RNA was extracted from normal and TD-affected growth plates of 3-week-old chickens. One microgram (1μg) of total RNA was reverse transcribed into cDNA and twenty nanograms (20ng) of this were used to perform real-time quantitative RT-PCR analysis. Expression levels were normalised to the levels of GAPDH, HPRT1, and TBP gene expression. Values are shown as mean ± SD (n = 6 for each group). Results are shown relative to mRNA expression levels from control group (normal growth plates), set to 1, thus corresponding to the N-fold difference in relation to the control group. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates.

4.2.4 Aggrecan

There are very few reports dealing with the expression of aggrecan in dyschondroplasia. The present study has shown a very low increment (1.6-fold), although statistically significant ($P < 0.05$), in the mRNA levels of aggrecan in TD cartilage (Fig. 4.6). Because this increment was very low, it was considered not relevant. These data are in contradiction with the results obtained by Tselepis et al. (1996), which have shown a marked decrease in the mRNA and protein levels of aggrecan within the dyschondroplastic lesion when compared to that of normal growth plate. Additionally, it has been suggested that, since proteoglycans have been implicated in the process of mineral deposition (Matsui et al., 1991; Hunter and Szigety, 1992), a decrease in aggrecan concentration may have an inhibitory effect on mineralization in the TD cartilage and also may lead to altered mechanical properties of the growth plate. This may lead to the breakdown in tissue integrity and therefore to the progression of the disease (Tselepis et al., 2000). On the other hand, Farquharson et al. (1994) have demonstrated that in TD cartilage the concentration of sulphated glycosaminoglycans, determined by alcian blue staining, is higher than that of normal growth plate cartilage.

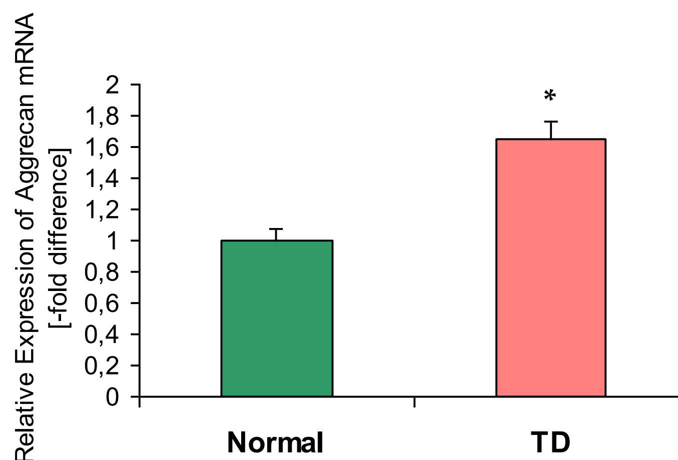


Figure 4.6 – Relative expression of aggrecan mRNA. Total RNA was extracted from normal and TD-affected growth plates of 3-week-old chickens. One microgram (1µg) of total RNA was reverse transcribed into cDNA and twenty nanograms (20ng) of this were used to perform real-time quantitative RT-PCR analysis. Expression levels were normalised to the levels of GAPDH, HPRT1, and TBP gene expression. Values are shown as mean ± SD (n = 6 for each group). *$P < 0.05$. Results are shown relative to mRNA expression levels from control group (normal growth plates), set to 1, thus corresponding to the N-fold difference in relation to the control group. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates.

In summary, several studies have suggested an altered extracellular matrix composition within the cartilage lesion in TD which may have implications in the initiation and the progression of the disease (Lowther et al., 1974; Thorp et al., 1993). Indeed, changes in the molecular composition of the matrix will alter the patterns of intermolecular interactions or cell-matrix interactions, leading to the breakdown in tissue integrity, which may conduct, at the end, to the disruption of matrix mineralization (Tselepis et al., 2000), and, eventually, to the development of TD. It has been generally reported a reduction in the protein levels of the matrix macromolecules in TD growth plate, especially for collagens type IX, X, and XI, and aggrecan. This reduction has been reported to be the result of decreased gene expression or, on the other hand, a defect in the secretion and incorporation of the proteins into the matrix. Our results demonstrated that collagens type II, type X, type IX and type XI, and aggrecan did not have relevant changes in their mRNA levels in dyschondroplasia. Thus, we may suggest that the reduced protein levels of matrix macromolecules in TD cartilage reported by other authors are not the result of decreased gene expression levels. Furthermore, the possibility of the occurrence of a defect in the secretion or incorporation of these matrix proteins into the extracellular matrix can be raised.

4.3 EXPRESSION OF REGULATORY MOLECULES

Chondrocyte proliferation and differentiation are regulated by local factors that include Ihh, PTHrP, FGF, TGF-β, and VEGF. All of these signaling systems have been shown to be present in the avian growth plate (Thorp et al., 1995; Law et al., 1996; Praul et al., 2002). Transcription factors that play crucial roles in regulation of chondrocyte gene expression under the control of these extracellular factors include Cbfa1 and Sox9. The invasion of cartilage matrix by the ossification front is dependent on its resorption by members of the MMP family. These proteases will be discussed in section 4.4.

In order to study the expression of these regulatory molecules in dyschondroplasia we designed specific primers for the growth and transcription factors in study using the corresponding mRNA published sequence (chicken TGF-β3: *GenBank* accession number NM_205454, chicken Cbfa1: *GenBank* accession number AF445419, chicken VEGF: *GenBank* accession number NM_205042; chicken PTHrP: *GenBank* accession number AB175678; chicken Ihh: *GenBank* accession number NM_204957; chicken Sox9: *GenBank* accession number NM_204281; chicken FGF2: *GenBank* accession number CK610935). A BLAST analysis was performed in order to confirm the specificity of the primers. Then, a real-time PCR analysis was performed using these specific primers, SYBR green as the detection chemistry, and cDNA reverse transcribed from total RNA isolated from the normal and TD-affected growth plates, as described in Materials and Methods chapter.

4.3.1 Ihh and PTHrP

The results of Ihh and PTHrP mRNA expression in TD-affected growth plates (TD) are shown in Figure 4.7 relative to the Ihh and PTHrP mRNA expression from control group (normal growth plates) which was set to 1.

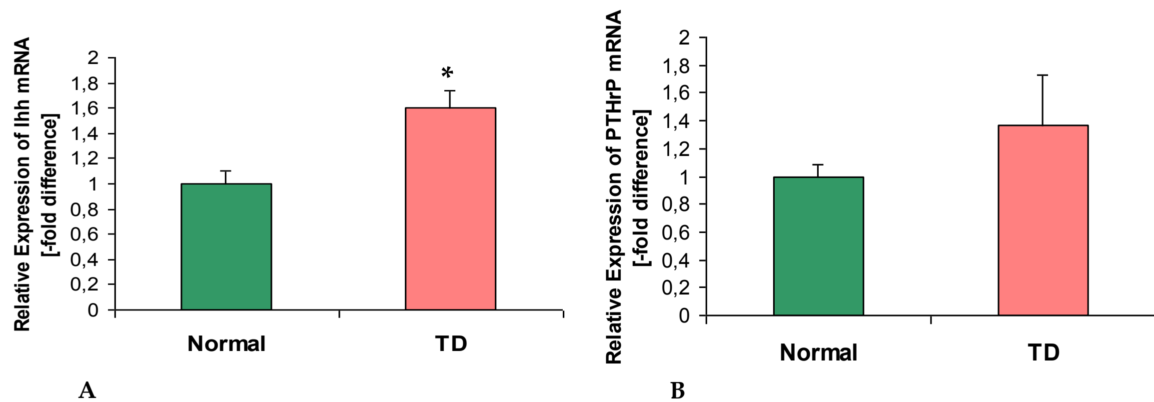


Figure 4.7 – Relative expression of Ihh mRNA (**A**) and PTHrP mRNA (**B**). Total RNA was extracted from normal and TD-affected growth plates of 3-week-old chickens. One microgram (1µg) of total RNA was reverse transcribed into cDNA and twenty nanograms (20ng) of this were used to perform real-time quantitative RT-PCR analysis. Expression levels were normalised to the levels of GAPDH, HPRT1, and TBP gene expression. Values are shown as mean ± SD (n = 6 for each group). * $P < 0.05$. Results are shown relative to mRNA expression levels from control group (normal growth plates), set to 1, thus corresponding to the N-fold difference in relation to the control group. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates.

To our knowledge, there are no previous studies about changes in the gene expression of Ihh molecule in dyschondroplasia. One study was performed by in situ hybridization in order to examine the spatial expression of mRNA for Ihh in normal and TD growth plate (Webster et al., 2003). The authors found that Ihh mRNA was expressed by pre-hypertrophic chondrocytes in normal cartilage. Indeed, Ihh is widely reported to be synthesized by prehypertrophic chondrocytes in the growth plate (Mackie et al., 2008). In dyschondroplastic cartilage the mRNA for Ihh was also seen in pre-hypertrophic chondrocytes and throughout the lesion (Webster et al., 2003). Based on these data, the authors suggested that chondrocyte differentiation is arrested at the transitional stage (pre-hypertrophy) just prior to hypertrophy. In the present study we found an increase in the expression of Ihh mRNA in TD. Although this increment has been statistically significant ($P < 0.05$) it was very slight (1.6-fold) and therefore it was considered few relevant. Taking into account the previous study, we suggest that this slight increment is the result of increased number of prehypertrophic cells that accumulate in the dyschondroplastic growth plate and not an increased expression of the Ihh gene in the cell.

Regarding the PTHrP, we found that the expression of its mRNA did not change in the pathology. This result is in agreement with the previous results of Ben-Bassat et al. (1999). Indeed, the authors showed normal expression of the PTHrP gene in the lower proliferating chondrocytes situated proximal to the lesion.

Ihh and PTHrP interact o each other in order to control chondrocyte proliferation and differentiation in the chicken growth plate. This interaction is made through a negative feedback loop. Based on our and previous results, we suggest that there are no relevant changes in the expression of Ihh and PTHrP in dyschondroplasia. Therefore, the interaction between these two molecules is not altered and the negative feedback loop is maintained. Furthermore, we may conclude that these two molecules are not involved in the development of the pathology.

4.3.2 FGF2

The results of FGF2 mRNA expression in TD-affected growth plates (TD) are shown in Figure 4.8 relative to the FGF2 mRNA expression from control group (normal growth plates) which was set to 1.

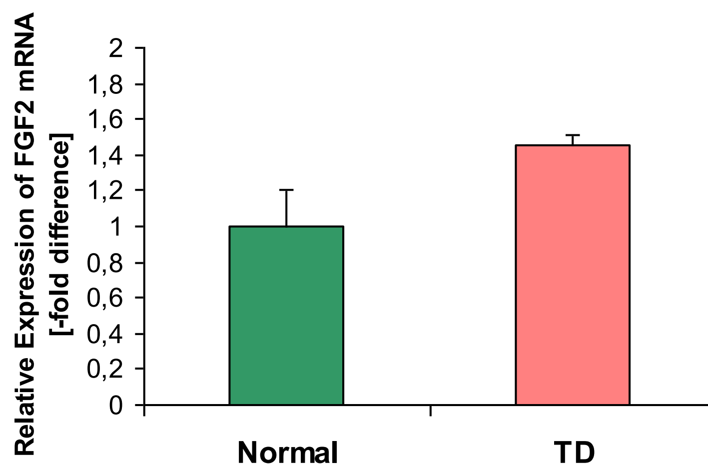


Figure 4.8 – Relative expression of FGF2 mRNA. Total RNA was extracted from normal and TD-affected growth plates of 3-week-old chickens. One microgram (1μg) of total RNA was reverse transcribed into cDNA and twenty nanograms (20ng) of this were used to perform real-time quantitative RT-PCR analysis. Expression levels were normalised to the levels of GAPDH, HPRT1, and TBP gene expression. Values are shown as mean ± SD (n = 6 for each group). Results are shown relative to mRNA expression levels from control group (normal growth plates), set to 1, thus corresponding to the N-fold difference in relation to the control group. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates.

FGF signaling, like Ihh and PTHrP signaling, also regulates chondrocyte proliferation and differentiation. Our results have demonstrated no significant changes in the mRNA expression for FGF2 in dyschondroplasia comparing with normal cartilage. Our results seem to be in contradiction with previous data. Indeed, several authors noticed by immunocytochemistry that in TD the intensity of staining for FGF2 was greatly reduced (Thorp et al., 1995; Twal et al., 1996; Ren et al., 1997) as was the number of positively FGF2 stained chondrocytes (Thorp et al., 1995; Twal et al., 1996). However, increased staining of FGF2 was noted in areas of repair at the periphery of the lesion (Loveridge et al., 1993).

It has been observed, using cell culture systems, that FGF signaling acts upstream of Ihh expression. FGF inhibits chondrocyte proliferation by downregulating Ihh expression. Furthermore, FGF signaling also suppresses chondrocyte proliferation independently of Ihh (van der Eerden et al., 2003; Mackie et al., 2008). These conclusions were obtained based on studies where FGF2 treatment of limb explant cultures leaded to a reduction in the number of cells expressing Ihh (Mackie et al., 2008). Furthermore, studies on bone explants *in vitro* demonstrated that FGF signaling, independent of effects on Ihh and PTHrP, accelerates terminal differentiation of hypertrophic chondrocytes (Kronenberg, 2003). Because in the present study we have not seen any significant changes in the gene expression of FGF and Ihh in TD, we may not speculate about the regulation of Ihh by FGF in the avian growth plate, as observed in other cell systems. Additionally, we may suggest that the failure in chondrocyte differentiation, characteristic of TD, is not a consequence of abnormal expression of FGF2. Thus, this growth factor is not responsible for the development of the disease.

4.3.3 TGF-β

The results of TGF-β3 mRNA expression in TD-affected growth plates (TD) are shown in Figure 4.9 relative to the TGF-β3 mRNA expression from control group (normal growth plates) which was set to 1.

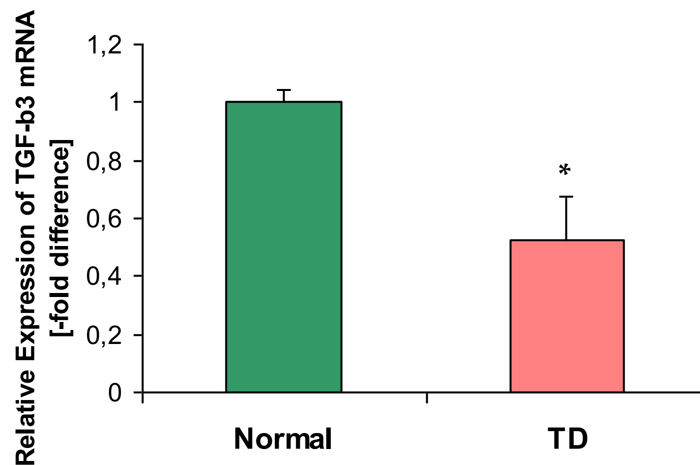


Figure 4.9 – Relative expression of TGF-β3 mRNA. Total RNA was extracted from normal and TD-affected growth plates of 3-week-old chickens. One microgram (1μg) of total RNA was reverse transcribed into cDNA and twenty nanograms (20ng) of this were used to perform real-time quantitative RT-PCR analysis. Expression levels were normalised to the levels of GAPDH, HPRT1, and TBP gene expression. Values are shown as mean ± SD (n = 6 for each group). * $P < 0.05$. Results are shown relative to mRNA expression levels from control group (normal growth plates), set to 1, thus corresponding to the N-fold difference in relation to the control group. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates.

TGF-β enhances chondrocyte proliferation and inhibits terminal differentiation of chondrocytes and therefore it helps chondrocytes to remain in the pre-hypertrophic stage. In the mouse, it has been observed that inhibition of chondrocyte differentiation by TGF-β is, at least in part, mediated by induction of PTHrP expression. TGF-β1, -2, and -3, all stimulate the expression of PTHrP (Li et al., 2005). However, TGF-β also inhibits chondrocyte maturation directly and independently of PTHrP. Thus, it has been reported that TGF-β acts downstream of Ihh and upstream of PTHrP (Alvarez et al., 2002) and therefore TGF-β may have an effect on the Ihh/PTHrP feedback loop. In tibial dyschondroplasia it has been widely demonstrated a reduction in TGF-β, both at the mRNA and protein levels (Loveridge et al., 1993; Thorp et al., 1993; Thorp et al., 1995; Law et al., 1996). The results obtained from our study were similar to these previous results showing decreased levels of about 2-fold ($P < 0.05$) of TGF-β3 mRNA in dyschondroplasia. Although the levels of mRNA for TGF-β have decreased in TD, no significant alterations in the mRNA levels for Ihh and PTHrP were observed, as mentioned before. However, it has been demonstrated that TGF-β also regulates the expression of PTHrP in avian endochondral ossification (Pateder et al., 2001). Thus, we speculate that, besides TGF-β, other molecules may be also regulating the expression of PTHrP in the avian growth plate. Furthermore, since TGF-β inhibits terminal differentiation of chondrocytes we suggest that the reduced gene expression of

this factor in dyschondroplasia is the response of the tissue in order to control the failure of hypertrophic chondrocyte differentiation.

4.3.4 Cbfa1 and Sox9

Cbfa1 has the ability to stimulate hypertrophic chondrocyte differentiation (Mackie et al., 2008). Given that in dyschondroplasia there is a failure in chondrocyte differentiation we hypothesized that the expression of Cbfa1 could be downregulated in the pathology. However, the present study found that the gene expression of Cbfa1 was not altered (Fig. 4.10 A). Thus, we may imply that Cbfa1 is not responsible for the development of the disease.

It has been suggested that Cbfa1 contributes to the Ihh/PTHrP negative feedback loop because it activates the Ihh promoter and therefore stimulating Ihh expression. Additionally, the expression of Cbfa1 is inhibited by PTHrP which contribute to the ability of PTHrP to delay chondrocyte hypertrophy. In this way, Cbfa1 is suggested to play an important role in maintaining an appropriate balance between continued proliferation and progression to hypertrophy in chondrocytes in mice growth cartilage (Mackie et al., 2008). As mentioned above, we found that any of these three factors has changed its mRNA expression in TD. Probably in the avian growth plate they may interact among them as in the mice growth plate. Furthermore, they are not involved in the development of the pathology.

TGF-β has an effect on Cbfa1 expression and this effect varies in different cells (Li et al., 2005). For example, in rat mesenchymal cells TGF-β induces Cbfa1 expression. On the other hand, in osteoblasts TGF-β inhibits the expression of the transcription factor. In the present study, the reduced gene expression of TGF-β does not allow to any change in the Cbfa1 gene expression. Thus, it seems that in chondrocytes of the avian growth plate TGF-β does not act upstream of Cbfa1.

The activity of Cbfa1 is repressed by Sox9 through a direct interaction between the two transcription factors (Zhou et al., 2006). As described above, the initiation of dyschondroplasia was not the result of decreased expression of the transcription factor Cbfa1. Another hypothesis raised was if the Cbfa1 activity could be inhibited by increased expression of Sox9 leading consequently to the failure in chondrocyte differentiation. In the present study, the mRNA expression for Sox9 was not altered in TD (Fig. 4.10 B). Thus, we may imply that this transcription factor is not involved in the initiation of the disease both through inhibition of Cbfa1 activity, as directly and

independently of this factor. Indeed, it has been suggested that Sox9 is required for normal rates of chondrocyte proliferation and for delaying the onset of hypertrophy (Mackie et al., 2008).

These two transcription factors, Cbfa1 and Sox9, stimulate the transcription of a number of cartilage matrix genes such as *Col10a1* (regulated by Cbfa1) (Mackie et al., 2008) and *Col2a1*, *Col11a2*, and *aggrecan* (regulated by Sox9) (Kronenberg, 2003). As discussed before, like their transcription factors, these matrix macromolecules have not altered their gene expression in dyschondroplasia. These results suggest that, in the avian growth plate, these matrix macromolecules may be regulated by the same transcription molecules as in the mice growth plate.

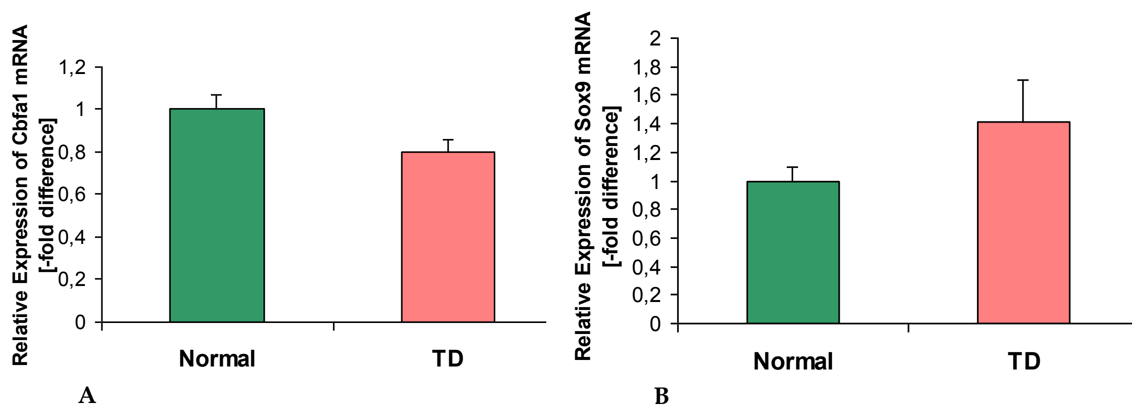


Figure 4.10 – Relative expression of Cbfa1 mRNA (**A**) and Sox9 mRNA (**B**). Total RNA was extracted from normal and TD-affected growth plates of 3-week-old chickens. One microgram (1µg) of total RNA was reverse transcribed into cDNA and twenty nanograms (20ng) of this were used to perform real-time quantitative RT-PCR analysis. Expression levels were normalised to the levels of GAPDH, HPRT1, and TBP gene expression. Values are shown as mean ± SD (n = 6 for each group). Results are shown relative to mRNA expression levels from control group (normal growth plates), set to 1, thus corresponding to the N-fold difference in relation to the control group. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates.

4.3.5 VEGF

The present study has demonstrated a statistically significant ($P < 0.01$) upregulation of about 2.8-fold for VEGF mRNA expression in dyschondroplastic growth plates comparing with normal growth plates (Fig. 4.11).

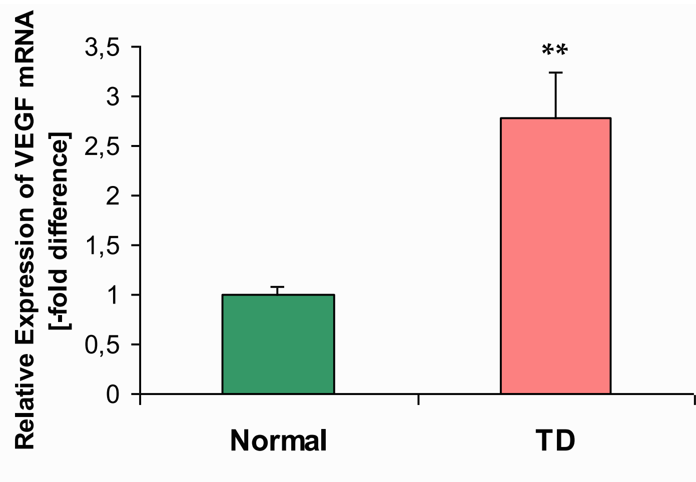


Figure 4.11 – Relative expression of VEGF mRNA. Total RNA was extracted from normal and TD-affected growth plates of 3-week-old chickens. One microgram (1µg) of total RNA was reverse transcribed into cDNA and twenty nanograms (20ng) of this were used to perform real-time quantitative RT-PCR analysis. Expression levels were normalised to the levels of GAPDH, HPRT1, and TBP gene expression. Values are shown as mean ± SD (n = 6 for each group). $^{**}P < 0.01$. Results are shown relative to mRNA expression levels from control group (normal growth plates), set to 1, thus corresponding to the N-fold difference in relation to the control group. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates.

The expression of VEGF was shown to be regulated by Cbfa1 in growth plate chondrocytes in mice (Zelzer et al., 2001). As already discussed before, we observed no alteration in the expression of Cbfa1 mRNA in dyschondroplasia. Thus, we may imply that in dyschondroplastic growth plates the mRNA levels for VEGF were not induced by the transcription factor Cbfa1. Thus, other transcription factors may be responsible for the induction of VEGF expression in the pathology.

It is well established that hypoxia is one of the principal stimuli for the expression of VEGF (Gerber et al., 1999). It has been reported the existence of a hypoxic (low oxygen tension (Burke et al., 2003)) environment (Stewart et al., 2006; Provot and Schipani, 2007) around the chondrocytes in the growth plate because the blood supply to these cells is limited (Shapiro et al., 1997). Because hypertrophic chondrocytes are avascular, these cells are a potential source of VEGF within the growth plate (Gerber et al., 1999). However, Shapiro et al. (1997) have demonstrated that chondrocytes in chicken growth plate are not hypoxic. This is related to structural differences in mammalian and avian growth plates, as already mentioned before. We are tempted to speculate that, despite normal avian growth plate is not hypoxic, dyschondroplastic growth plates may became a hypoxic tissue owing to the increased accumulation of cartilage matrix and therefore it is induced to produce VEGF. Indeed, several studies have demonstrated an upregulation of several

genes in response to hypoxia, being VEGF the most common. It was demonstrated through a study using cDNA array hybridization, to investigate the effects of hypoxia on the mRNAs of several genes, that VEGF was increased in primary human monocyte-derived macrophages during exposure to hypoxia (Burke et al., 2003). Indeed, macrophages accumulate in large number in hypoxic tissues (Lewis et al., 1999; Burke et al., 2003) and respond to hypoxia by up-regulating a number of transcription factors (Burke et al., 2003) which then stimulate the transcription of genes, such as the VEGF. In accordance, Cramer et al. (2004) found that hypoxia is able to induce the synthesis of soluble VEGF isoforms by epiphyseal chondrocytes.

In summary, figure 4.12 shows a scheme with the interactions of growth and transcription factors in the modulation of chondrocyte proliferation and differentiation during endochondral bone formation. The interactions mentioned above are presented here. The results are obtained from mouse and human studies. The mRNA expression of most of the growth factors studied in the present study has not changed in dyschondroplasia. These include Ihh, PTHrP, and FGF. Regarding the transcription factors, both the molecules studied, Cbfa1 and Sox9, have also maintained their mRNA levels in dyschondroplasia. In the same manner, the macromolecules of the matrix which are regulated by these transcription factors did not change their gene expression in the pathology. Among the growth factors analysed in the present study, only VEGF and TGF-β have changed their mRNA levels in dyschondroplasia. VEGF has increased (green box) significantly in the pathology which was related to higher levels of hypoxia in dyschondroplastic cartilage. TGF-β showed decreased (red box) mRNA levels in the pathology. Although TGF-β mRNA has decreased in TD, the molecules which are known to interact with this factor in other cell systems have not changed their mRNA levels in the disease. It has been observed that in the avian endochondral ossification TGF-β also regulates the expression of PTHrP (Pateder et al., 2001). We speculate that, besides TGF-β, other molecules may be regulating the expression of PTHrP in the avian growth plate.

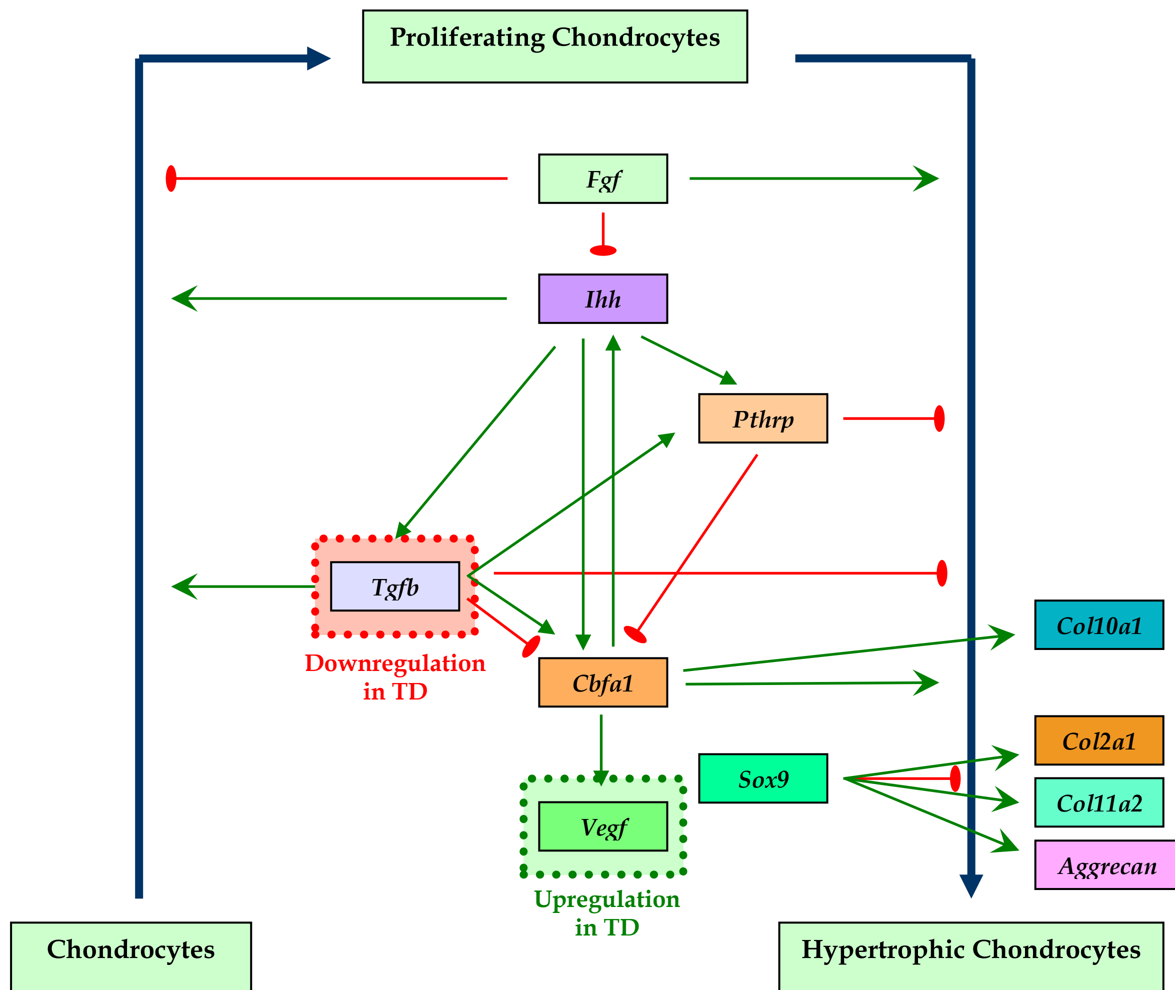


Figure 4.12 – Interaction of growth and transcription factors in the modulation of chondrocyte proliferation and differentiation during endochondral bone formation. Data obtained from mouse and human studies. Induction of gene expression (↓); induction of chondrocyte differentiation (↓); inhibition of gene expression (⊥); inhibition of chondrocyte differentiation (⊥). Red box - molecules which mRNA levels are decreased in TD. Green box – molecules which mRNA levels are increased in TD. Adapted from Crombrugghe et al. (2001) *Curr Opin Cell Biol* **13**:721-727; Mackie et al. (2008) *Int J Biochem Cell Biol* **40**(1):46-62; Kronemberg (2003) *Nature* **423**(6937):332-6; Karsdal et al. (2002) *J Biol Chem* **277**(46):44061-7.

4.4 EXPRESSION OF MATRIX METALLOPROTEINASES

The expression of MMPs were analysed in dyschondroplastic cartilages in order to investigate whether the accumulated cartilage, vascularisation inhibition, and failure in hypertrophy could be related to changes in the expression of these ECM-degrading enzymes. For gene expression studies, we designed specific primers for chicken MMPs using the corresponding mRNA published sequence (*GenBank* accession number AF070478 for chicken MMP-13; *GenBank* accession number NM_204667 for chicken MMP-9; *GenBank* accession number NM_204420 for chicken MMP-2; *GenBank* accession number BX950347/XM_417175 for chicken MMP-10; *GenBank* accession number XM_001232776 for chicken MMP-11; *GenBank* accession number NM_001006278 for chicken MMP-7). Additionally, we also analysed chicken caspase-3 (*GenBank* accession number NM_204725) and chicken TIMP-2 (*GenBank* accession number NM_204298). A BLAST analysis was performed in order to confirm the specificity of the primers. Then, a real-time PCR analysis was performed using these specific primers, SYBR green as the detection chemistry, and cDNA reverse transcribed from total RNA isolated from the normal and TD-affected growth plates, as described in Materials and Methods chapter.

4.4.1 Expression of Collagenase-3 (MMP-13)

MMP-13 (collagenase-3) in combination with MMP-1 (collagenase-1/interstitial collagenase/fibroblast collagenase) and MMP-8 (collagenase-2/ neutrophil collagenase) form the group of collagenases of the MMP family. Among the collagenases, only the MMP-13 was already identified in the chicken (Lei et al., 1999). The MMP-8 was not yet identified and the gene sequence for MMP-1 was recently predicted by automated computational analysis. For this reason, only MMP-13 was studied in the present work. The chicken MMP-13 is 71% identical to human MMP-13 at the protein level (Zijlstra et al., 2004). The highest homology exists between the catalytic domains (82%), whereas the propeptide and hemopexin domain homology is only 58 and 67%, respectively.

MMP-13 has been reported to be confined to skeletal tissues during development, implying that it has a specific function in bone formation (D'Angelo et al., 2000). In the chicken, the MMP-13 was firstly identified in embryonic membranes and extraembryonic fluid and was implicated in the matrix remodeling of embryonic membranes through its collagenolytic action (Lei et al., 1999).

Previous studies have investigated the expression of MMP-13 in dyschondroplasia at the protein level by gelatin-impregnated gels (gelatin zymography) (Simsa et al., 2007b), and at the mRNA level by RT-PCR (Rath et al., 2005). The results obtained by these two studies were contradictory. Simsa et al. (2007b) have verified a reduction in MMP-13 activity in TD lesions whereas Rath et al. (2005) have observed an increased expression of MMP-13 gene. Thus, in the present study, we intended to investigate the expression of MMP-13 both at the protein and mRNA levels in parallel in order to elucidate these previous contradictory results.

In this way, we first analysed the protease activity of MMP-13 in chicken growth plates (normal and affected with TD). For that purpose, aliquots of protein extracts of normal and TD-affected growth plates from 3-week-old chickens were mixed with equal volumes of sample loading and subjected to SDS-PAGE under nonreducing conditions. The gels were then treated and stained in order to visualise protease activity, as described in Materials and Methods chapter. The analysis of molecular masses and optical density of protein bands was made by densitometry. The results are shown in Figure 4.13.

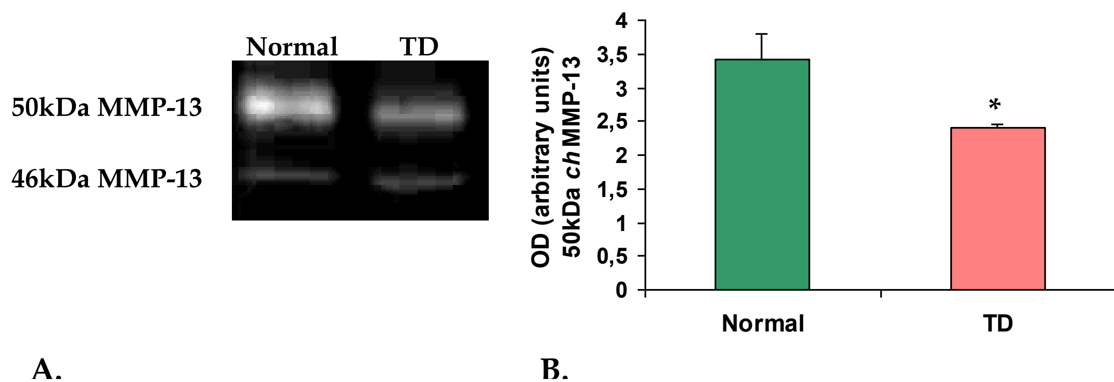


Figure 4.13 – Downregulation of MMP-13 activity. Gelatin zymographic analysis of normal and TD-affected growth plate extracts. Protein extracts were subjected to SDS-PAGE on a 4% stacking, 10% separating (containing 0.1% gelatin) gels, under nonreducing conditions. **(A)** The zymogram was developed and stained as described in Materials and Methods and shows the protein bands corresponding to the 46- and 50kDa MMP-13. **(B)** The optical density (OD) of the band corresponding to the 50kDa MMP-13 was calculated, for normal and TD-affected growth plate extracts, by scanning densitometry and is given as arbitrary units. Values are shown as mean ± SD (n = 6 for each group). * $P < 0.05$. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates; MMP: matrix metalloproteinase.

As we can see in Figure 4.13, there was a statistically significant decrease ($P < 0.05$) in the activity of the 50kDa MMP in TD-affected growth plate extracts. Based on its molecular weight, the 50kDa protein has been suggested to be MMP-13 (Zijlstra et al., 2004). Unfortunately, it was not possible to confirm the identity of the protein by using antibodies because anti-chicken antibodies are not yet commercially available and the anti-human antibodies used did not recognise the chicken protein. The 46kDa gelatinolytic band is probably an active degradation product of MMP-13, which suggests rapid autodegradation of this protein.

Afterwards, in order to verify whether the decreased MMP-13 activity was related to a downregulation of the expression of its gene, we analysed the mRNA expression of MMP-13 in tibial growth plates (normal and affected with TD) from chickens.

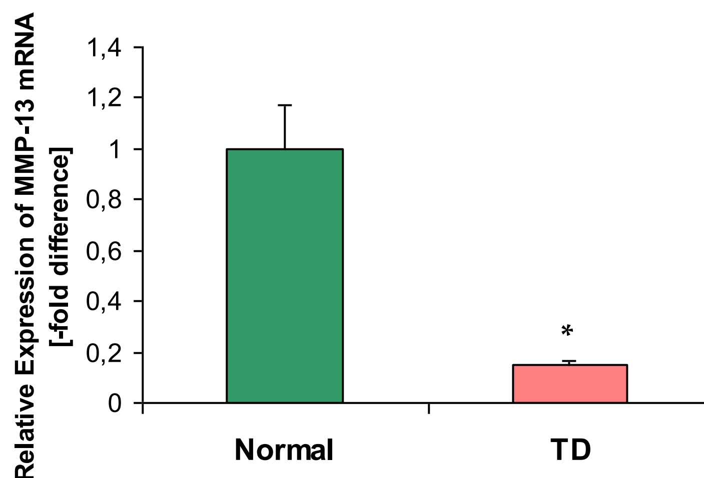


Figure 4.14 – Downregulation of MMP-13 mRNA expression. Total RNA was extracted from normal and TD-affected growth plates of 3-week-old chickens. One microgram (1μg) of total RNA was reverse transcribed into cDNA and twenty nanograms (20ng) of this were used to perform real-time quantitative RT-PCR analysis. Expression levels were normalised to the levels of GAPDH, HPRT1, and TBP gene expression. Values are shown as mean ± SD (n = 6 for each group). $^*P < 0.05$. Results are shown relative to mRNA expression levels from control group (normal growth plates), set to 1, thus corresponding to the N-fold difference in relation to the control group. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates.

Our results have demonstrated a pronounced downregulation (of about 6.7-fold), statistically significant ($P < 0.05$), in the mRNA levels of MMP-13 in dyschondroplasia (Fig. 4.14).

The present study has shown that in tibial dyschondroplasia, where there is a failure in terminal differentiation from prehypertrophic to hypertrophic chondrocytes (Poulos et al., 1978; Hargest et al., 1985; Thorp et al., 1993), the mRNA and protein levels for MMP-13 were downregulated. Our results support the previous results about the involvement of MMP-13 with chondrocyte hypertrophy. In fact, D'Angelo et al. (2000) observed, using chondrocytes from prehypertrophic cartilage of chick embryo sternae that, when hypertrophy was induced by serum-free culture with inducers of hypertrophy, MMP-13 mRNA levels were increased. In agreement, Wu et al. (2002) have shown, using bovine fetal chondrocytes isolated from physeal cartilages, that the expression and synthesis of MMP-13 mRNA was upregulated on chondrocyte differentiation into hypertrophic cells. Additionally, these authors have shown that a nontoxic carboxylate inhibitor of MMP-13 inhibited type II collagen degradation and consequently suppressed chondrocyte differentiation. In fact, it has been reported that matrix degradation is required for chondrocyte differentiation. The reason why matrix degradation is required for chondrocyte differentiation may rely on the fact that it permits the release of growth factors sequestered in the matrix (Whitelock et al., 1996; Álvarez et al., 2000).

Based on our results we could speculate that the decreased expression of MMP-13 could be involved in the development of the pathology. However, we cannot affirm that the reduced expression of MMP-13 in dyschondroplasia is responsible for the initiation of the failure in chondrocyte maturation. To clarify this question, it would be necessary to study the expression of this protein at earlier stages of the disorder, for instance, at 1 and 2 weeks of age. Here, we have studied the expression of MMP-13 in a later stage (3 weeks of age). For this reason, we cannot rule out the hypothesis that the decreased expression of MMP-13 in TD is simply a consequence of the delay of hypertrophy in TD and that other regulatory molecules are involved in the initiation of the failure. In other words, because in TD there is a failure in the maturation of hypertrophic chondrocytes and being MMP-13 expressed only by hypertrophic chondrocytes, the reduction of its expression in TD may be simply the result of decreased MMP-13-producing cells. However, and taking into account the functions of MMP-13 in matrix degradation and its implication in chondrocyte differentiation we may speculate that the reduced expression of MMP-13 in TD, although not being responsible for the initiation of the disorder, it may contribute to its aggravation.

Indeed, and regarding the functions of MMP-13 in matrix degradation, MMP-13 has been seen as the only collagenase implicated in degradation of the collagenous matrices during development of bone and cartilage. In the human, MMP-13 has the ability to hydrolyze the native helix of types I, II, and III fibrillar collagens, generating fragments approximately ¾ and ¼ of the

original molecule. However, MMP-13 preferentially cleaves type II collagen over type I and III. In addition to its degrading activity on fibrillar collagens this MMP has been reported to have also a gelatinase activity. This gelatinolytic activity could contribute to degrade further the initial cleavage products of the enzyme on native collagens to small fragments suitable for subsequent metabolism (Knäuper et al., 1996a; Pendas et al., 1997). It has been observed that MMP-13 can also degrade type X collagen and cartilage aggrecan (Mitchell et al., 1996; Knäuper et al., 1996a; Fosang et al., 1996; D'Angelo et al., 2000). Taken together, these substrate specificities make MMP-13 a likely candidate to participate in collagen and proteoglycan degradation during endochondral ossification. One of the processes that could account for increase in the hypertrophic zone is decreased proteolysis of the ECM. Thus, we may suggest that the accumulated cartilage in dyschondroplastic growth plates is the result of increased deposition of collagen and proteoglycans (mainly aggrecan) due to the lack of matrix proteolysis involving MMP-13. In fact, studies performed using transgenic mice where MMP-13 is ablated revealed that these mice showed abnormal skeletal growth plate development with a delay in endochondral ossification (Inada et al., 2004; Stickens et al., 2004). Inada et al. (2004) have demonstrated that MMP-13-/- mice had growth plates in long bones almost double in length, accounted for by increases in the zone of hypertrophy. In the same way, in the endochondral bones of MMP-13-/- mice, chondrocytes proliferate and differentiate normally, but accumulate in the most terminally differentiated hypertrophic state as their exit from the growth plate is delayed (Stickens et al., 2004).

MMP-13 has been reported to mediate the process of angiogenesis (Zijlstra et al., 2004). These authors, through studies on the chorioallantoic membrane of the chick embryos, found that in addition to its role in cartilage/bone resorption MMP-13 can also mediate the process of angiogenesis through its action on collagen remodeling. In fact, Zijlstra et al. (2004) have demonstrated that when purified MMP-13 was added directly to the CAM onplants, at low quantities, it induced substantial levels of new blood vessel formation. They also found that MMP-13 stimulated blood vessel formation in the CAM onplants in the absence of supplemental FGF/VEGF. The precise mechanisms underlying angiogenic stimulation by active MMP-13 have yet to be identified. It is unlikely that the MMP-13 activity simply substitutes for the effects of FGF and/or VEGF. The more likely event is that exogenous MMP-13, upon activation in the CAM tissue, cleaves the fibrillar collagen and further initiates a cascade of events that result in the accessibility of endogenous FGF and VEGF or other angiogenic stimulatory factors (Ziljstra et al., 2004). Thus, in the present study, and based on this previous report, we may imply that the failure of vascularization observed in dyschondroplasia may be the result of decreased expression of MMP-13 in the growth plates. That is, since MMP-13 is a potent ECM-degrading enzyme, its downregulation in the tissue leads to a decrease in matrix proteolysis which may prevent the

release of angiogenic factors sequestered in the matrix. Among these factors are VEGF and FGF proteins. Indeed, the failure of vascularization could be due to a reduction in the expression of these proteins. However, in the present study it was demonstrated that VEGF expression was increased (section 4.3.5) and FGF expression was not altered (section 4.3.2) in dyschondroplasia. Thus, these results support our suggestion that MMP-13 downregulation may be responsible for the impairment of vascularization in the disease. In fact, Ziljstra et al. (2004) have demonstrated that the addition of MMP-13 to CAM onplants containing collagenase-resistant r/r collagen failed to induce angiogenesis, implying that the cleavage of fibrillar collagen by MMP-13 had to be the initiating event.

Our results are in agreement with those reported by Simsa et al. (2007b) who also showed a decrease in the MMP-13 activity in TD. However, they are in contradiction with a previous study which has demonstrated an increased expression of MMP-13 mRNA in dyschondroplasia (Rath et al., 2005). In fact, the authors have considered this result very intriguing because MMP-13 is a pro-angiogenic protein and in dyschondroplasia the process of vascularization is interrupted. In order to explain the unexpected result, Rath et al. (2005) suggested that the increased expression of MMP-13 mRNA may not reflect an increased production of the corresponding protein that is eventually responsible for angiogenesis. However, the authors have not investigated the expression of MMP-13 at the protein level. We have performed this study on MMP-13 expression both at the protein and mRNA levels in parallel, and we found a direct correlation between the protein and mRNA expressions, being both downregulated. This may imply that the decreased activity of MMP-13 is the result of decreased expression of the corresponding gene.

The expression of MMP-13 is regulated by the transcription factor Cbfa1 (Selvamurugan et al., 1998; Porte et al., 1999; Wu et al., 2002) and it has been demonstrated that Cbfa1 is involved in the expression of MMP-13 during the process of bone formation (Jiménez et al., 1999). Here, the authors showed that in mutant mice deficient in Cbfa1, hypertrophic chondrocytes do not express MMP-13 during fetal ossification. Considering the potent proteolytic activity of MMP-13 on diverse collagenous and noncollagenous bone and cartilage components this may explain why Cbfa1-deficient embryos exhibit lack of endochondral ossification.

As already discussed, the present study has demonstrated no significant differences in the Cbfa1 mRNA expression levels between normal and TD-affected growth plates (section 4.3.4). Thus, the reduced MMP-13 mRNA in dyschondroplasia is not related to a decreased gene expression of the Cbfa1. To our knowledge, it was not yet described in the literature the involvement of Cbfa1 in the regulation of MMP-13 expression in the chicken. Thus, we may speculate that there may be differences in the regulatory mechanisms in the chicken comparing

with human and rat, and probably other regulatory molecules, besides Cbfa1, may be involved in the regulation of chicken MMP-13. In fact, it has been reported that, in case of the human and rat species, the minor structural differences between human and rat MMP-13 promoters led to different properties in terms of regulatory mechanisms. In agreement, it has been reported that the human and murine MMP-13 genes are subjected to different regulatory controls, the human gene being more restricted in its expression in normal tissues (Johansson et al., 1997; Ståhle-Bäckdahl et al., 1997; Jiménez et al., 1999).

4.4.2 Expression of Gelatinase B (MMP-9)

MMP-9 (gelatinase B/92kDa type IV collagenase) in combination with MMP-2 (gelatinase A/72kDa type IV collagenase) form the group of gelatinases of the MMP family.

One function of MMP-9 is to degrade cartilage matrix. It has been reported that MMP-9 acts in conjunction with MMP-13, first degrading aggrecan in the hypertrophic zone of the growth plate, making collagen II accessible to cleavage by MMP-13. Then, MMP-9 may act downstream to clear denatured collagen cleavage products generated by MMP-13. Thus, MMP-9 can synergize with MMP-13 in matrix degradation during endochondral ossification (Engsig et al., 2000; Stickens et al., 2004). Furthermore, these authors demonstrated that mice lacking both MMP-13 and MMP-9 had severely impaired endochondral bone, characterized by diminished ECM remodeling, prolonged chondrocyte survival, delayed vascular recruitment and defective trabecular bone formation (Stickens et al., 2004). In the present study we demonstrated a pronounced downregulation (of about 3.0-fold), statistically significant ($P < 0.01$), of MMP-9 mRNA levels in TD-affected growth plates (Fig. 4.15). In this way, we may suggest that the downregulation of this enzyme in TD, together with the diminished expression of MMP-13 (as demonstrated before in this study in section 4.4.1), would contribute to the lack of ECM remodeling and consequently to the increased accumulation of cartilage characteristic of TD. In fact, this accumulated cartilage is unlikely to be due solely to MMP-9 deficiency since it has been described that type II collagen (one of the major collagens of cartilage matrix) appears to have limited susceptibility to degradation by MMP-9 (Hahn-Dantona et al., 2000). Indeed, Hahn-Dantona et al. (2000) have compared the substrate specificity of human MMP-9 and chicken MMP-9 and concluded that both enzymes were unable to digest collagens type I and IV. However, collagen type II appeared to have limited susceptibility to degradation by both enzymes. The authors found a difference between the two enzymes in regard to fibronectin, being this substrate digested by chicken MMP-9, but not by

human MMP-9. They also demonstrated that chicken MMP-9 prefers to cleave denatured collagens (gelatin) rather than native collagens, as happen for mammalian MMP-9.

MMP-9 was identified in the chicken by Hahn-Dantona et al. (2000). The chicken gelatinase B was firstly denominated as 75kDa gelatinase B because it has a different size (75kDa) compared with the 92kDa for the human one (Tong et al., 2003). The chicken 75kDa gelatinase B-like enzyme shares comparatively low homology (59%) with the human gelatinase B, in contrast to other members of the MMP family. These exhibit much higher levels of homology: 84% for MMP-2 (Chen et al., 1991; Aimes et al., 1994), 89% for MMP-16 (Yang et al., 1996), and 71% for MMP-13 (Zijlstra et al., 2004). Cloning and characterization of the 75kDa gelatinase has demonstrated that it appeared to be gelatinase B-like in its overall structure, however, it possessed a number of biochemical and amino acid sequence features that differed from all known mammalian gelatinase B enzymes. The chicken gelatinase B lacks the type V collagen domain that is found in all mammalian gelatinases B (Hahn-Dantona et al., 2000).

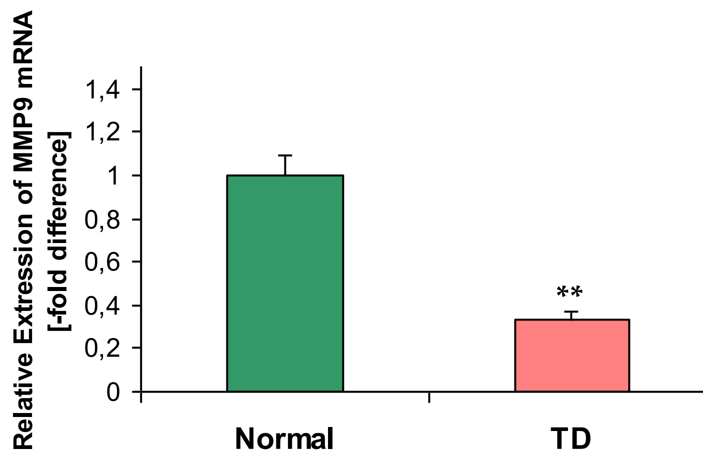


Figure 4.15 – Downregulation of MMP-9 mRNA expression. Total RNA was extracted from normal and TD-affected growth plates of 3-week-old chickens. One microgram (1µg) of total RNA was reverse transcribed into cDNA and twenty nanograms (20ng) of this were used to perform real-time quantitative RT-PCR analysis. Expression levels were normalised to the levels of GAPDH, HPRT1, and TBP gene expression. Values are shown as mean ± SD (n = 6 for each group). ***P* < 0.01. Results are shown relative to mRNA expression levels from control group (normal growth plates), set to 1, thus corresponding to the N-fold difference in relation to the control group. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates.

MMP-9 has been reported to be a key regulator of growth plate angiogenesis in mice (Vu et al., 1998). These authors found that explants of hypertrophic cartilage from MMP-9-null mice showed a delay in the release of angiogenic activity in *in vitro* angiogenesis assays, demonstrating that mice lacking MMP-9 show delayed vascularization and ossification, resulting in an abnormal accumulation of hypertrophic cartilage (Vu et al., 1998). In regard to its expression, MMP-9 has been reported to be expressed by osteoclasts during mouse embryonic development and after birth (Reponen et al., 1994) and in mouse chondroclasts (Vu et al., 1998). Chondroclasts refer to the cells against the nonmineralized transversal septae, whereas osteoclasts refer to the cells along the mineralized longitudinal trabeculae (Vu et al., 1998; Engsig et al., 2000). In mice, its expression has been localized at the junction between hypertrophic cartilage and the vascular invasion front (Vu et al., 1998; Takahara et al., 2004).

In the chicken, it was also demonstrated an involvement of MMP-9 in angiogenesis, demonstrating a positive correlation between substances that promote vascularization of the growth plate and the expression of the chicken MMP-9 (Tong et al., 2003). Exactly, Tong et al. (2003) showed that angiogenesis promoting factors (such as retinoic acid) increased the expression and activity of MMP-9 in cultured chicken chondrocytes. Regarding the localization of MMP-9 in the chicken, this protein was expressed by cells surrounding the blood vessels penetrating the growth plate, by the endothelial cells in the vessels, and by chondrocytes located adjacent to these vascular invasions (Tong et al., 2003). Based on these results, the authors suggested that MMP-9, despite the divergences in structure and substrate specificity seems to perform the same role in the process of angiogenesis during avian endochondral bone formation as its homolog in mammals.

In the present study, the decreased levels of MMP-9 mRNA in dyschondroplasia, a disease where vascularisation is interrupted, support these previous suggestions about the involvement of MMP-9 in growth plate vascularization. In fact, the phenotype of MMP-9-null mice observed by Vu et al. (1998) resembles the one observed in dyschondroplasia. Therefore, it is likely that the decreased mRNA expression of MMP-9 may be involved in the failure of vascularisation in the growth plate and consequently in the development of dyschondroplasia.

To our knowledge there are only two studies dealing with the expression of MMP-9 in dyschondroplasia and only at the protein level. Through immunohystochemistry studies, Capela-Silva (2003) showed a reduction in the amount of MMP-9 in dyschondroplasia. Accordingly, Simsa et al. (2007b) by gelatin zymography found decreased MMP-9 activity in dyschondroplastic lesions. Our results are in agreement with these previous findings.

It has been suggested that MMP-9 exerts its role in vascular invasion of the growth plate through the release of angiogenic molecules sequestered in the extracellular matrix. The release of these molecules is done through the proteolytic action of MMP-9 on extracellular matrix (Vu et al., 1998). VEGF is one such molecule. As discussed before, the present study has shown increased

levels of VEGF mRNA in dyschondroplasia (section 4.3.5) resultant from the creation of a hypoxic environment in dyschondroplastic lesions. Our results are in agreement with previous reports (Rath et al., 2005; Rath et al., 2007) that also observed an increased VEGF mRNA expression in dyschondroplasia. The question that may arise is why the increment of VEGF mRNA in dyschondroplastic growth plates does not stimulate their vascularisation. To answer that question, Rath et al. (2005) suggested that the increased expression of the gene could not reflect an increased production of the corresponding protein. Based on our findings, we suggest that even VEGF mRNA is upregulated in dyschondroplastic lesions, the corresponding protein may be arrested in ECM storage sites due to the reduced expression of MMP-9. Therefore, the growth factor cannot exert its activity. Indeed, it has been reported that MMP-9 may release VEGF that is bound to the hypertrophic cartilage matrix, thereby regulating vascular invasion (Colnot et al., 2003). Once released, VEGF could bind to its receptors on endothelial cells, osteoclasts, and osteoblasts (Nakagawa et al., 2000), stimulating their migration and activity (Colnot et al., 2003). In fact, VEGF, which is expressed by hypertrophic chondrocytes, has been shown to be essential for osteoclast recruitment into the hypertrophic cartilage, in addition to its widely known function in recruitment of endothelial cells (Engsig et al., 2000). Chondroclasts/osteoclasts are involved in remodeling hypertrophic cartilage and endothelial cells in forming vascular channels (Colnot et al., 2003). The chondroclasts/osteoclasts express MMP-9 which in turn releases ECM-bound VEGF. Therefore, it has been suggested an interaction between VEGF and MMP-9 in regulating vascular invasion and ossification during normal longitudinal growth (Haeusler et al., 2005).

Because in the chicken VEGF is also secreted by hypertrophic chondrocytes (Carlevaro et al., 2000), we may speculate that the reduced expression of MMP-9 in dyschondroplastic cartilages may be involved in the retention of VEGF in the hypertrophic cartilage matrix. In other words, the impaired vascularization observed in dyschondroplasia is neither related to a failure in the VEGF gene expression nor to a failure in its protein production. Instead, it is related with a lack in the VEGF protein bioavailability owing to the reduced expression of MMP-9.

4.4.2.1 Caspase-3

During endochondral ossification, terminal hypertrophic chondrocytes, the cells situated in the last row of hypertrophic cartilage in proximity to the invading capillary loops, undergo apoptosis. Apoptosis of terminal chondrocytes may serve as a signal leading to vascular invasion. Alternatively, vascular invasion may cause apoptosis of the chondrocytes in contact with invading vessels, perhaps from dissolution of the surrounding ECM. Thus, it is suggested that apoptosis of

terminal hypertrophic chondrocytes is functionally coupled to vascularization and ossification so that only the cells in contact with the vascular invasion front undergo cell death (Vu et al., 1998).

In order to investigate if chondrocyte apoptosis is disrupted in dyschondroplasia, the extent of this process in TD-affected growth plates was studied using a molecular marker of apoptosis. We analysed the expression of caspase-3, an enzyme that mediates the final stage of cell death by apoptosis (Sharif et al., 2004). Since caspase-3 expression is a crucial mediator of programmed cell death (Porter et al., 1999), it is plausible that altered expression of this gene may indicate a defect in the apoptotic mechanism. The results are shown in Figure 4.16.

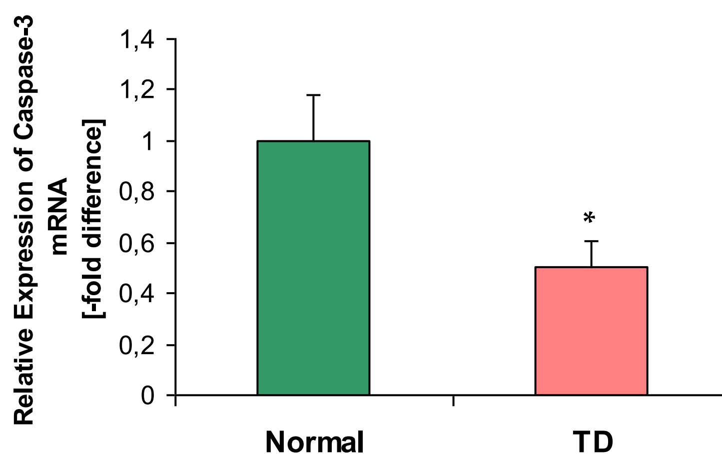


Figure 4.16 – Downregulation of caspase-3 mRNA expression. Total RNA was extracted from normal and TD-affected growth plates of 3-week-old chickens. One microgram (1μg) of total RNA was reverse transcribed into cDNA and twenty nanograms (20ng) of this were used to perform real-time quantitative RT-PCR analysis. Expression levels were normalised to the levels of GAPDH, HPRT1, and TBP gene expression. Values are shown as mean ± SD (n = 6 for each group). *$P < 0.05$. Results are shown relative to mRNA expression levels from control group (normal growth plates), set to 1, thus corresponding to the N-fold difference in relation to the control group. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates.

In the present study, it was shown a statistically significant ($P < 0.05$) reduction of about 2.0-fold in caspase-3 mRNA expression in dyschondroplasia. Thus, the reduced expression of caspase-3 in TD-affected growth plates may be an indicator of impaired apoptosis in the pathology. The disruption of this process can explain the accumulation of cartilage in the metaphysis. In other words, the accumulated cartilage characteristic of TD may be due to an accumulation of chondrocytes that do not mature and do not undergo apoptosis. Our results are in contradiction

with the studies of Rath et al. (2005) that have demonstrated, through procedures like DNA fragmentation and nuclei labeling, that thiram induced endothelial cell apoptosis in the capillary vessels of the growth plates which was followed by a massive death of chondrocytes in the transition zone of the growth plate. On the contrary, our results are in agreement with those obtained by Ohyama et al. (1997) that, through the same methods as those used by Rath et al. (2005), have shown that there was a minimal level of apoptosis in the dyschondroplastic cartilage. On the other hand, Praul et al. (1997) and Rath et al. (1998) have found that small TD lesions contain few, if any, apoptotic chondrocytes, whereas in severe lesions numerous apoptotic cells were present. These authors suggested that these results imply that apoptosis was not a primary cause of TD but was secondary to the formation of the cartilaginous plug. Therefore, we can speculate that the decreased expression of MMP-9 in TD lesions may be involved not only in the failure of vascularization, as discussed above, but also in the impairment of apoptosis of chondrocytes, through decreased degradation of the surrounding extracellular matrix. In fact, it has been reported that proteinases, and the associated ECM degradation, may lead to apoptosis and this happen in several systems including development, neuronal death, and mammary gland involution (Vu et al., 1998). Indeed, a previous report has shown that in the growth plates of MMP-9-null mice there is a delay in hypertrophic chondrocyte apoptosis which is coupled with a delay in vascularization and ossification (Vu et al., 1998). The authors suggested that MMP-9 could function to cause chondrocyte apoptosis and indirectly allow vascularization and ossification.

4.4.3 Expression of Gelatinase A (MMP-2)

As mentioned before (section 4.4.2) MMP-2 (72kDa type IV collagenase/gelatinase A) in combination with MMP-9 (92kDa type IV collagenase/gelatinase B) form the group of gelatinases of the MMP family, degrading denatured collagen fragments (gelatin).

It has been reported that MMP-2 is found at high levels in growth plate (Kawashima-Ohya et al., 1998), however, the importance of its activity in skeletal development remains still unclear (Malemud, 2006). For this reason and because MMP-2, like most MMPs, has been implicated in the ECM remodeling during endochondral ossification, we aimed to investigate the expression of its gene and protein in normal and TD-affected tibial growth plates.

We analysed the protease activity of MMP-2 in chicken tibial growth plates (normal and affected with TD) using gelatin-impregnated gels (gelatin zymography). Aliquots of protein extracts of normal and TD-affected growth plates from 3-week-old chickens were mixed with equal volumes of sample loading and subjected to SDS-PAGE, under nonreducing conditions. The gels were then treated and stained in order to visualise protease activity, as described in Materials and Methods chapter. The analysis of molecular masses and optical density of protein bands was made by densitometry. The results are shown in Figure 4.17.

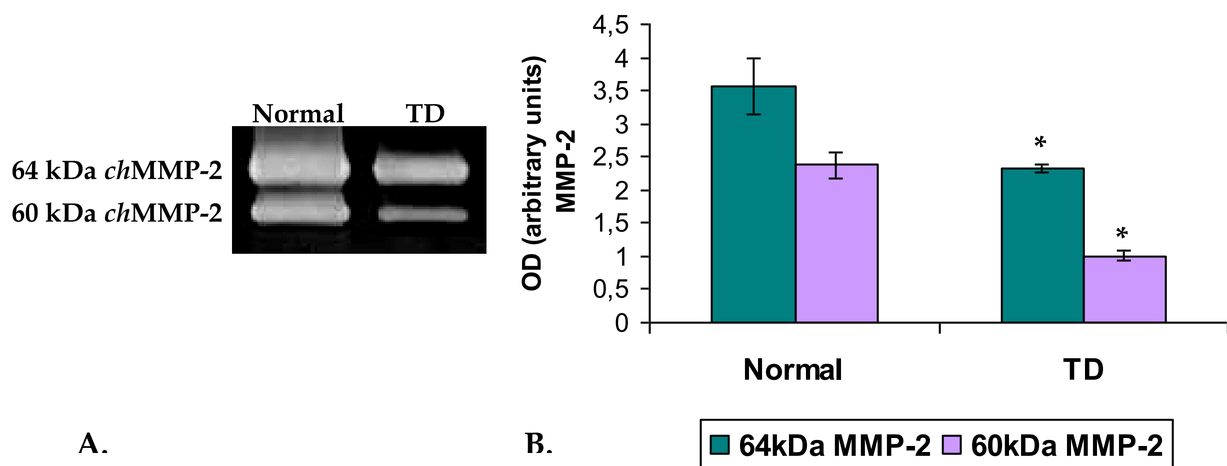


Figure 4.17 – Downregulation of MMP-2 activity. Gelatin zymographic analysis of normal and TD-affected growth plate extracts. Protein extracts were subjected to SDS-PAGE on a 4% stacking, 10% separating (containing 0.1% gelatin) gels, under nonreducing conditions. **(A)** The zymogram was developed and stained as described in Materials and Methods and shows the protein bands corresponding to the 64- and 60kDa MMP-2. **(B)** The optical density (OD) of the bands corresponding to the 64- and 60kDa MMP-2 was calculated, for normal and TD-affected growth plate extracts, by scanning densitometry and is given as arbitrary units. Values are shown as mean ± SD (n = 6 for each group). $^*P < 0.05$. Normal: normal growth plates; tibial dyschondroplasia: tibial dyschondroplasia-affected growth plates; MMP: matrix metalloproteinase.

By gelatin zymography, it was possible to observe high activity of 64- and 60kDa MMPs in normal growth plates (Fig. 4.17, A). Based on their molecular weights, these bands have been suggested to correspond both to the MMP-2 protein (D'Angelo et al., 2000; Simsa et al., 2007b). In TD-affected growth plates it was detected a statistically significant reduction ($P < 0.05$) in the activity of both 64- and 60kDa MMP-2 (Fig. 4.17, A and B). The 60kDa gelatinolytic band is probably an active degradation product of MMP-2, as suggested by Simsa et al. (2007b). Our

results are in accordance with the findings of these authors who also showed a decrease in the MMP-2 activity in TD lesions.

As mentioned above, the protein bands observed by zymography have been suggested to be both MMP-2, based on their molecular weights. In order to confirm this, *immunoblotting* analysis was performed by using an anti-chicken MMP-2 antibody. Thus, aliquots of protein extracts of normal and TD-affected growth plates from 3-week-old chickens were separated by SDS-PAGE, under reducing conditions. After electrotransference of proteins to PVDF membranes, these were treated, incubated with the anti-chicken MMP-2 antibody, and developed (as described in Material and Methods chapter) in order to detect protein bands. The analysis of molecular masses and optical density of protein bands was made by densitometry. The results are shown in Figure 4.18.

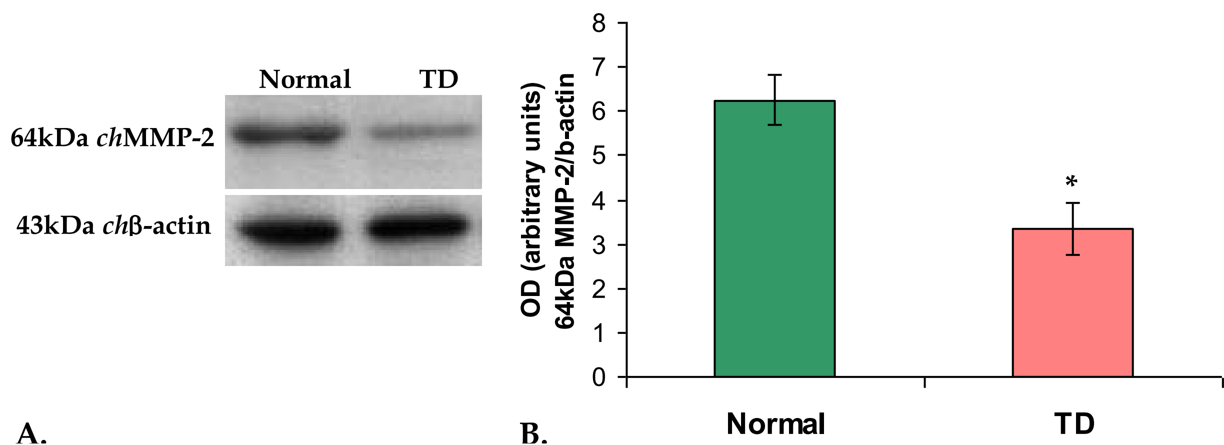


Figure 4.18 – Downregulation of MMP-2 protein. **(A)** Immunoblotting analysis of normal and TD-affected growth plate extracts. Protein extracts were subjected to SDS-PAGE on a 4% stacking, 10% separating gels, under reducing conditions. Proteins were transferred to PVDF membranes and probed with an anti-chicken MMP-2 antibody (kindly provided by Dr. J. P. Quigley and Dr. E. I. Deryugina, The Scripps Research Institute, La Jolla, CA, USA), which recognized the band corresponding to the 64 kDa MMP-2. Membranes were also probed with an antibody against to the 43 kDa β-actin to verify that equal amounts of proteins were loaded. **(B)** The optical density (OD) of the band corresponding to the 64 kDa MMP-2 was calculated, for normal and TD-affected growth plate extracts, by scanning densitometry, and was normalized relative to the amount of β-actin protein, and is given as arbitrary units. Values are shown as mean ± SD (n = 6 for each group). *$P < 0.05$. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates; MMP: matrix metalloproteinase.

The band with an apparent molecular weight of 64kDa was recognized by the antibody, but the 60kDa one was not. As mentioned above, the 60kDa gelatinolytic band is probably an active degradation product of MMP-2 (as suggested by Simsa et al. (2007b)) and therefore it was not identified by the antibody perhaps because the protein degradation process may have involved the binding site to the antibody.

The *immunoblotting* analysis also revealed a reduction, statistically significant ($P < 0.05$), in the protein amount of the 64kDa MMP-2 in TD-affected growth plates (Fig. 4.18, A and B) which confirm the results obtained by zymography.

In order to verify whether decreased MMP-2 protein amount was related to a downregulation of the expression of its gene, we analysed the mRNA levels of MMP-2 in tibial growth plates (normal and affected with TD).

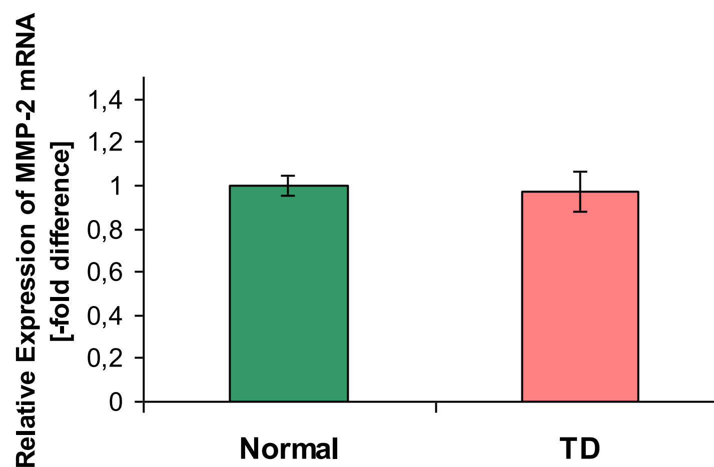


Figure 4.19 – Relative expression of MMP-2 mRNA. Total RNA was extracted from normal and TD-affected growth plates of 3-week-old chickens. One microgram (1µg) of total RNA was reverse transcribed into cDNA and twenty nanograms (20ng) of this were used to perform real-time quantitative RT-PCR analysis. Expression levels were normalised to the levels of GAPDH, HPRT1, and TBP gene expression. Values are shown as mean ± SD (n = 6 for each group). Results are shown relative to mRNA expression levels from control group (normal growth plates), set to 1, thus corresponding to the N-fold difference in relation to the control group. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates.

In the present study, no changes in the mRNA expression levels of MMP-2 were found in TD-affected growth plates (Fig. 4.19). This result is in accordance with the findings of Rath et al.

(2005). The fact that MMP-2 gene expression is not changing in dyschondroplastic lesions is consistent with its reported housekeeping function in the normal turnover of cartilage (Birkedal-Hansen et al., 1993; Gepstein et al., 2002). Indeed, MMP-2 has been described as being a gelatinase with a widespread expression that is thought to serve a housekeeping function in removing degraded collagen (Matrisian, 1994). Unlike other MMPs, MMP-2 is constitutively expressed in several tissues and is not usually induced by inflammatory stimuli (Fabunmi et al., 1996; Matsumoto et al., 1996; Kawashima-Ohya et al., 1998). In almost all evaluated cells, many growth factors and cytokines do not enhance MMP-2 synthesis even when they induce other MMPs *in vitro* (Overall et al., 1989; Marti et al., 1993; Kohn et al., 1994). Therefore, we may imply that MMP-2 plays the same housekeeping function in the chicken tibial growth plate as its homolog in mammals.

From our results, the decreased MMP-2 protein amount in TD-affected growth plates may contribute to the reduced resorption of the ECM and consequently to the persistence of the cartilage characteristic of dyschondroplasia. These results are in agreement with previous reports (Wardale and Duance, 1996; Simsa et al., 2007b). In fact, MMP-2 has been shown to be involved in ECM remodeling in the growth plate. In the human, MMP-2 was shown to cleave gelatin with high specific activity and basement-membrane type IV collagen at a reduced, yet significant rate (Aimes et al., 1994). Additionally, human MMP-2 has been shown to cleave also type X collagen (Welgus et al., 1990; Reponen et al., 1992). In the chicken, MMP-2 was firstly identified in embryo fibroblasts by Chen et al. (1991) and later on cloned and sequenced from the same cells (Aimes et al., 1994). Chen et al. (1991) found that the protein digests the denatured forms of most collagens but, unlike human MMP-2, it does not cleave native type IV collagen (Chen et al., 1991). Both human and chicken MMP-2, free of tissue inhibitors of metalloproteinases (TIMPs), are capable of cleaving soluble triple helical type I collagen (Aimes and Quigley, 1995).

Additionally, MMP-2 may also have an indirect role in the ECM remodeling through activation of proMMP-13. Indeed, it was suggested that MMP-2 can activate proMMP-13 (Knäuper et al., 1996b; D'Angelo et al., 2000) during chondrocyte maturation, and that the combination of both proteinases is required to prepare cartilage matrix for subsequent calcification, before endochondral ossification (D'Angelo et al., 2000). As discussed before, our study has demonstrated a reduction in the MMP-13 activity is dyschondroplasia. We may imply that the reduced activity of MMP13 was not solely due to a decreased expression of its mRNA but also due to a reduction in the amount of its activator, MMP-2.

To our knowledge, our study is the first one where the expression of MMP-2 at the protein and mRNA levels was evaluated in parallel. Therefore, we were able to demonstrate that the

reduced amount of MMP-2 protein in TD was not a consequence of decreased expression of its gene. Furthermore, we may speculate that the reduction of MMP-2 protein levels without a concomitant decrease in the levels of its mRNA may be the result of decreased translation, increased degradation of protein, or decreased protein activation.

4.4.3.1 Expression of TIMP-2

We have just seen that, in dyschondroplasia, the reduced MMP-2 protein levels without a concomitant decrease in the level of its mRNA may be the result of decreased translation, increased degradation of protein, or decreased protein activation. In order to discuss this last possibility it is important to know that, unlike most other MMPs where activation occurs in the extracellular milieu, activation of MMP-2 takes place on the cell surface mediated by membrane-type 1 matrix metalloproteinase (MT1-MMP or MMP-14), that is bound to the tissue inhibitor of metalloproteinases-2 (TIMP-2), thereby forming a ternary complex. A free molecule of MMP-14 closely located to the ternary complex then activates proMMP-2 on the cell surface (Strongin et al., 1995; Nagase et al., 1998). Thus, two molecules contribute to proMMP-2 activation and these are MMP-14 and TIMP-2 (Strongin et al., 1995) and so, one possible explanation for the reduced levels of MMP-2 active protein in TD could be a failure in the expression of these two molecules.

In regard to TIMP-2, this protein was cloned and characterized in the chicken by Aimes et al. (1998). TIMPs are a family of proteins that block the enzymatic activity of the MMPs. The balanced activity of the metalloproteinases and their inhibitors is thought to be critical for the maintenance of the ECM and normal cellular functions (Zeng et al., 1998). TIMP-2 is the most abundantly expressed TIMP and has been suggested to provide the basic and constant antimetalloproteinase activity in tissues (Hammani et al., 1996). Basically, TIMP-2 inhibits the activity of all MMPs (Kähäri and Saarialho-Kere, 1999; Joronen et al., 2000) being, nevertheless, a potent inhibitor of MMP-2 (Birkedal-Hansen et al., 1993). However, besides the inhibitory activity of TIMP-2 against MMP-2 at high concentrations, this inhibitor has been shown to promote also the activation of MMP-2 at low concentrations (Strongin et al., 1995; Kinoshita et al., 1998; Maquoi et al., 2003).

In the present study, we analysed the gene expression of TIMP-2 in tibial growth plates. The results are shown in Figure 4.20.

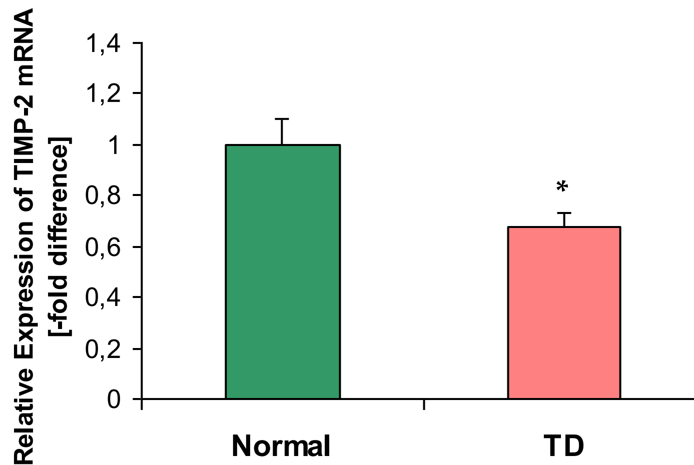


Figure 4.20 – Relative expression of TIMP-2 mRNA. Total RNA was extracted from normal and TD-affected growth plates of 3-week-old chickens. One microgram (1μg) of total RNA was reverse transcribed into cDNA and twenty nanograms (20ng) of this were used to perform real-time quantitative RT-PCR analysis. Expression levels were normalised to the levels of GAPDH, HPRT1, and TBP gene expression. Values are shown as mean ± SD (n = 6 for each group). **P* < 0.05. Results are shown relative to mRNA expression levels from control group (normal growth plates), set to 1, thus corresponding to the N-fold difference in relation to the control group. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates.

From our results, a statistically significant reduction ($P < 0.05$) was observed in the mRNA expression levels of TIMP-2 in TD-affected growth plates (Fig. 4.20). However, this reduction was very low (1.5-fold) and therefore it was considered not relevant. Thus, it seems that the reduced levels of MMP-2 active protein in lesions are not related to changes in TIMP-2 gene expression. Furthermore, we suggest that TIMP-2 is not involved in the development of dyschondroplasia. Accordingly, it has been reported that although TIMP-2 is the major TIMP expressed during bone development (Apte et al., 1997; Blavier and DeClerck, 1997) no analysis of mice deficient for TIMP-2 has reported abnormal bone phenotype (Ortega et al., 2004).

Regarding the MMP-14, unfortunately the software Primer Express was unable to find specific primers for the mRNA published sequence of chicken MMP-14 (*GenBank* accession number BM489822). Thus, it was not possible to perform the gene expression analysis of this protein in tibial growth plates. Furthermore, up to now, MMP-14 has not yet been cloned in the chicken.

4.4.4 Expression of Stromelysin-2 (MMP-10) and Stromelysin-3 (MMP-11)

MMP-3 (stromelysin-1) in combination with MMP-10 (stromelysin-2) and MMP-11 (stromelysin-3) form the group of stromelysins, members of the MMP family. The only stromelysin so far cloned and studied in the avian growth plate was MMP-10 (BX950347/XM_417175) (Simsa et al., 2007a). MMP-3 (XM_425644) and MMP-11 (XM_001232776) have not so far been identified in the chicken growth plate, and both sequences deposited in the *GenBank* were predicted by automated computational analysis. Additionally, their involvement in the avian growth plate has not been studied.

Stromelysins have been localized in the rabbit growth plate (Brown et al., 1989; Haeusler et al., 2005) and investigated further in human fetal rib (Bord et al., 1998) and bovine growth plates (Armstrong et al., 2002; Haeusler et al., 2005).

Cartilage extracellular matrix is composed primarily of collagen type II and proteoglycans (mainly aggrecan). Given that stromelysins are potent proteoglycanases, the expression of stromelysin-2 (MMP-10) and stromelysin-3 (MMP-11) were investigated in TD-affected growth plates. The gene expression of MMP-3 was not analysed because the mRNA published sequence initially deposited in the *GenBank* as corresponding to MMP-3 was further redefined as corresponding to MMP-10. The results are shown in Figures 4.21 and 4.22.

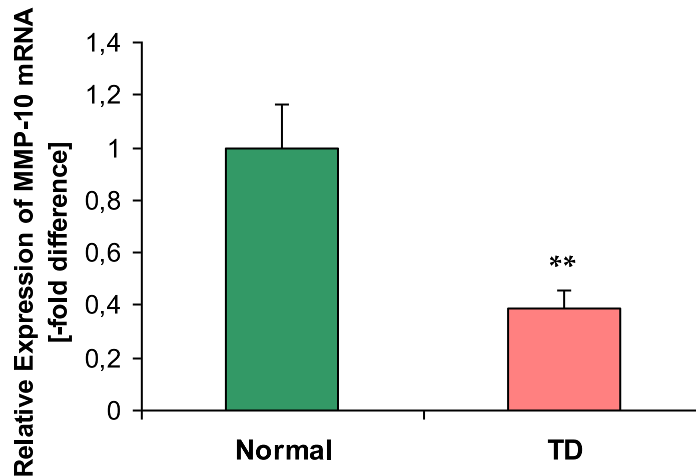


Figure 4.21 – Downregulation of MMP-10 mRNA expression. Total RNA was extracted from normal and TD-affected growth plates of 3-week-old chickens. One microgram (1µg) of total RNA was reverse transcribed into cDNA and twenty nanograms (20ng) of this were used to perform real-time quantitative RT-PCR analysis. Expression levels were normalised to the levels of GAPDH, HPRT1, and TBP gene expression. Values are shown as mean ± SD (n = 6 for each group). **$P < 0.01$. Results are shown relative to mRNA expression levels from control group (normal growth plates), set to 1, thus corresponding to the N-fold difference in relation to the control group. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates.

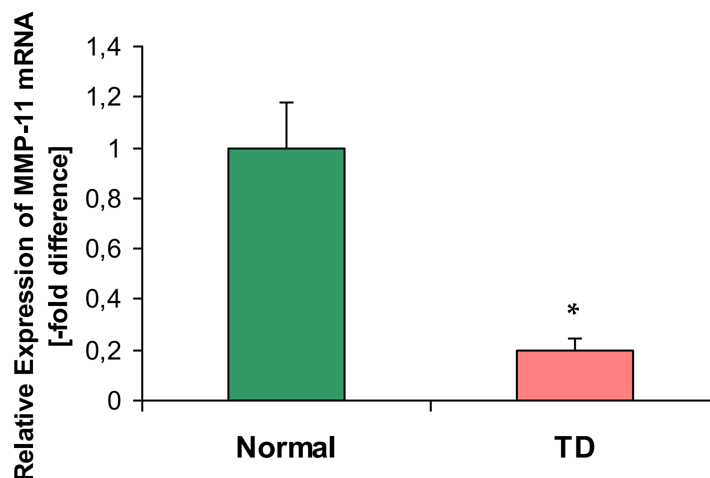


Figure 4.22 – Downregulation of MMP-11 mRNA expression. Total RNA was extracted from normal and TD-affected growth plates of 3-week-old chickens. One microgram (1µg) of total RNA was reverse transcribed into cDNA and twenty nanograms (20ng) of this were used to perform real-time quantitative RT-PCR analysis. Expression levels were normalised to the levels of GAPDH, HPRT1, and TBP gene expression. Values are shown as mean ± SD (n = 6 for each group). *$P < 0.05$. Results are shown relative to mRNA expression levels from control group (normal growth plates), set to 1, thus corresponding to the N-fold difference in relation to the control group. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates.

As it can be seen, there was a statistically significant reduction in the mRNA expression levels of about 2.6-fold ($P < 0.01$) for MMP-10 (Fig. 4.21) and of about 5.0-fold ($P < 0.05$) for MMP-11 (Fig. 4.22) in TD lesions.

Stromelysins demonstrate wide substrate specificity with the ability to degrade proteoglycans, fibronectin, laminin, casein, and the nonhelical region of collagen (Bord et al., 1998). It has been postulated that stromelysins may modulate proteoglycans degradation prior to mineralization during endochondral ossification (Bord et al., 1998). Because stromelysins are considered potent proteoglycanases we may speculate that the accumulated cartilage observed in dyschondroplasia may be the result of a delay in proteoglycans proteolysis due to the decreased expression of these enzymes.

The only stromelysin cloned and studied so far in the avian growth plate was stromelysin-2 (MMP-10) by Simsa et al. (2007a). The authors found its expression in the turkey growth plate in cells surrounding the blood vessels penetrating the growth plate and in hypertrophic chondrocytes, mainly those adjacent to the compact bone. In dyschondroplasia, the vascularisation is inhibited and chondrocytes fail to undergo hypertrophy. Thus, the reduced gene expression of MMP-10 associated with this pathology support these previous results and imply that the enzyme may have the same distribution in the chicken growth plate as its counterpart in the turkey growth plate.

4.4.5 Expression of Matrilysin-1 (MMP-7)

MMP-7 (matrilysin-1) is a member of the stromelysin subclass of enzymes and is the smallest member of the matrix metalloproteinase family. In fact, activated MMP-7 is distinct in that it contains only the catalytic domain with the zinc-binding region required for proteolytic activity, in contrast to other members of the family, which comprise additional carboxyl-terminal domains (Muller et al., 1988; Gaire et al., 1994).

The MMP-7 was identified in the chicken and its nucleotidic sequence deposited in the *GenBank* however, at the time of writing, it has never been studied in the avian growth plate.

Very few data exist concerning the involvement of MMP-7 in bone development. However, MMP-7 was found to be expressed in cultured human chondrocytes (Ohta et al., 1998) and localized in hypertrophic chondrocytes of the rodent femur growth plate (Shubayev et al., 2004).

In the present study, we have demonstrated a pronounced upregulation (7.3-fold), statistically significant ($P < 0.05$), of MMP-7 mRNA expression in dyschondroplastic growth plates (Fig 4.23). This result goes somehow in contradiction with previous reports which associate

increased MMP-7 mRNA levels with diseases where there is destruction of cartilage, such as human osteoarthritic (Ohta et al., 1998). In dyschondroplasia, there is, contrarily, an accumulation of cartilage. However, our data are in accordance with a previous study which, by immunohistochemistry, showed an upregulation of MMP-7 protein levels in dyschondroplasia (Capela-Silva, 2003).

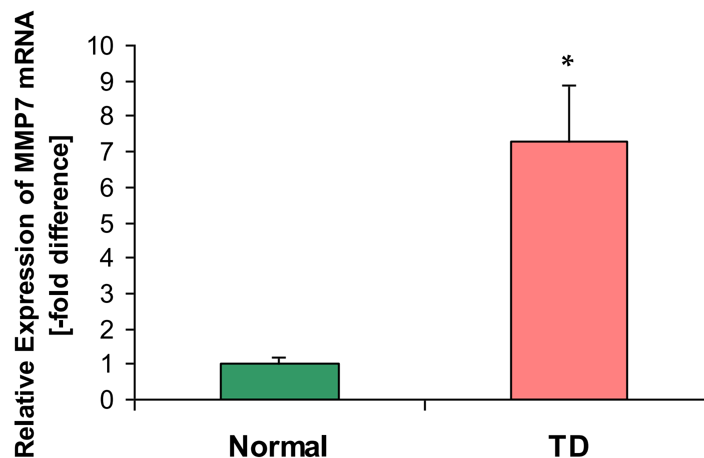
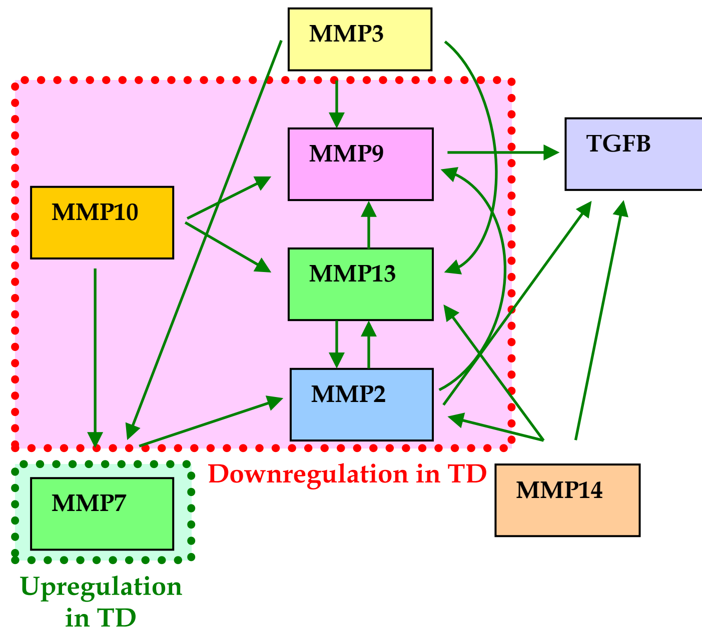


Figure 4.23 – Upregulation of MMP-7 mRNA expression. Total RNA was extracted from normal and TD-affected growth plates of 3-week-old chickens. One microgram (1µg) of total RNA was reverse transcribed into cDNA and twenty nanograms (20ng) of this were used to perform real-time quantitative RT-PCR analysis. Expression levels were normalised to the levels of GAPDH, HPRT1, and TBP gene expression. Values are shown as mean ± SD (n = 6 for each group). *P < 0.05. Results are shown relative to mRNA expression levels from control group (normal growth plates), set to 1, thus corresponding to the N-fold difference in relation to the control group. Normal: normal growth plates; TD: tibial dyschondroplasia-affected growth plates.

Regarding its substrate specificity, MMP-7 has the highest specific activity against many ECM components (Imai et al., 1995a; Imai et al., 1995b; Imai et al., 1997; Fujita et al., 2006), including the main proteoglycan of cartilage ECM, aggrecan (Wilson and Matrisian, 1996). Indeed, MMP-7 has a wide range of substrates such as fibronectin, elastin, casein (Gaire et al., 1994), gelatins of types I, III, IV and V, laminin, and aggrecan (Wilson and Matrisian, 1996). This enzyme has little if any activity against native fibrillar collagens (Wilson and Matrisian, 1996). This substrate specificity suggests that MMP-7 may play a role in ECM turnover under normal

conditions (Malemud, 2006). Thus, given that MMP-7 is an ECM-degrading enzyme its upregulation associated with a disease (tibial dyschondroplasia) where cartilage ECM is accumulated is an intriguing result. One possible explanation is that MMP-7 mRNA may be induced by the hypoxic environment existent in dyschondroplastic cartilages. As already discussed before (section 4.3.5), we speculate that dyschondroplastic growth plates may became a hypoxic tissue, which was demonstrated by the induced expression of VEGF. Indeed, Burke et al. (2003) revealed that, in addition to the VEGF mRNA upregulation, the mRNA of MMP-7 was also upregulated by hypoxia in primary human monocyte-derived macrophages. These authors have shown for the first time a highly upregulation of MMP-7 by hypoxia along with that of such known hypoxia-regulated gene, the VEGF.

As described in the Introduction chapter, matrix metalloproteinases are involved in activation cascades where many MMPs share common activators. The general activation cascade of MMPs is shown in Figure 4.24 A. The scheme is based on data obtained from human and mouse studies. Inside the red box are the MMPs analysed in this study which protein and/or mRNA levels were downregulated in dyschondroplasia. Alterations in the MMP gene expression lead to changes in the activation cascade. For example, the reduced gene expression of MMP-13 in TD conducts to decreased levels of the corresponding active protein. Thus, the activation of MMPs, known to be activated by MMP-13, such as MMP-2 and MMP-9, will also decrease. Indeed, the present study has shown decreased levels of the MMP-2 active protein in the lesions besides the unchanged mRNA levels. The reduction in the MMP-10 gene expression can also contribute to the reduced activation of MMP-9 and MMP-13. It is shown that MMPs are also able to activate latent TGF-β, such as the MMP-14 (Karsdal et al., 2002), and the MMP-2 and MMP-9 (Yu and Stamenkovic, 2000). The activation of latent TGF-β by MMP-2 and MMP-9 was also observed in the chick embryo chondrocytes in culture (D'Angelo et al., 2001). We speculate that the reduced gene expression of TGF-β in dyschondroplasia conducts to diminished levels of the corresponding protein. Additionally, TGF-β active protein is reduced in TD, not only due to the downregulation of the gene but also to the reduced levels of their activators, MMP2 and MMP-9. Like VEGF, MMP-7 had a pronounced increase in dyschondroplasia (green box) which was related to the creation of a hypoxic environment in dyschondroplastic cartilage. Besides the reduced mRNA levels for MMP-13 in TD, the gene expression of Cbfa1 was not altered (Fig. 4.24 B). Thus, in the avian growth plate it seems that MMP-13 may be regulated by other regulatory molecules besides Cbfa1.



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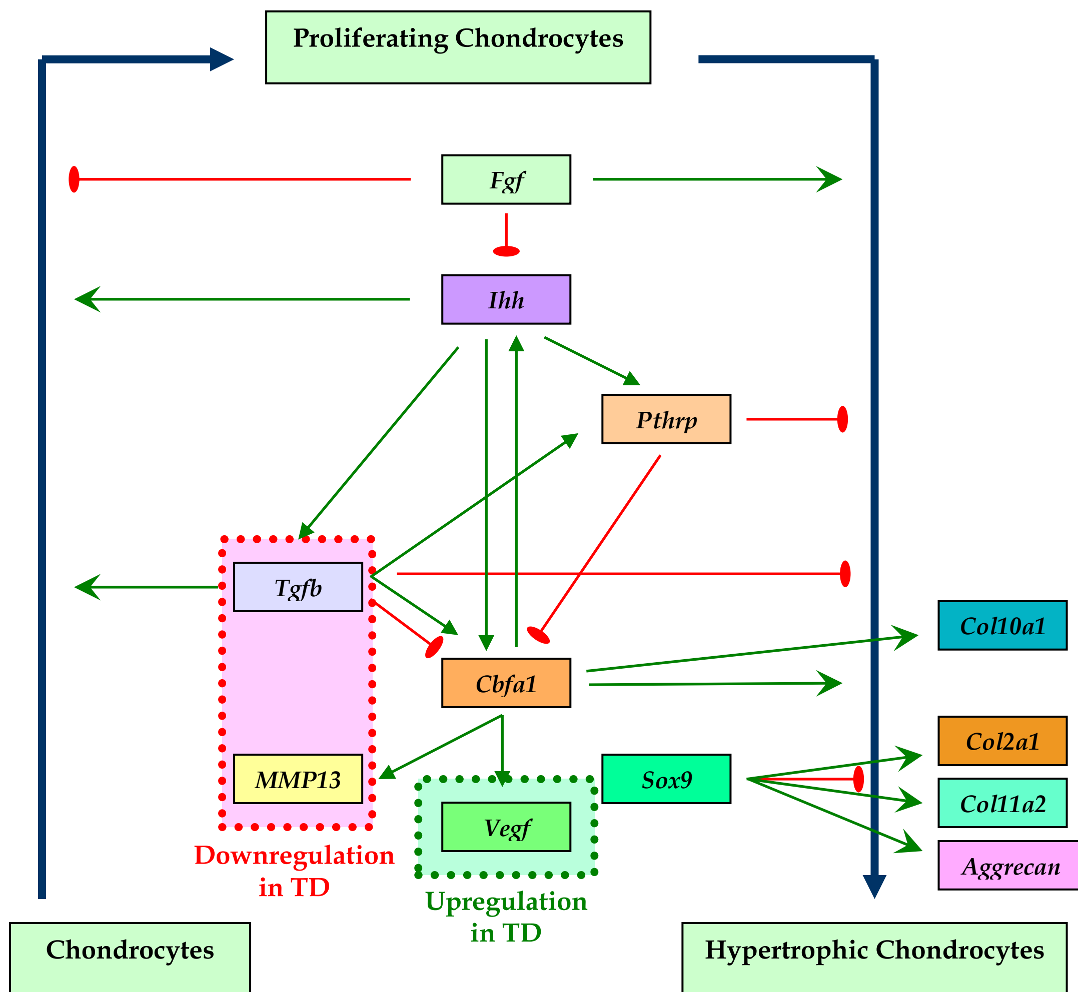

B

Figure 4.24 – Activation cascade of matrix metalloproteinases and the interaction of growth and transcription factors. **(A)** Activation cascade of matrix metalloproteinases and the proteolytic activation of latent TGFB by MMP-2 and MMP-9. Adapted from Chakraborti et al. (2003) Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem* **253**(1-2)**:**269-85. **(B)** Interaction of growth and transcription factors in the modulation of chondrocyte proliferation and differentiation during endochondral bone formation. Data obtained from mouse and human studies. Italic type letter – gene; capital letter – protein. (↓) – protein activation, induction of gene expression, and induction of chondrocyte differentiation. (⊥) - inhibition of gene expression, and inhibition of chondrocyte differentiation. Red box - molecules which protein or mRNA levels are decreased in TD. Green box – molecules which protein or mRNA levels are increased in TD. Adapted from Crombrugghe et al. (2001) *Curr Opin Cell Biol* **13**:721-727; Mackie et al. (2008) *Int J Biochem Cell Biol* **40**(1)**:**46-62; Kronemberg (2003) *Nature* **423**(6937)**:**332-6; Karsdal et al. (2002) *J Biol Chem* **277**(46)**:**44061-7.

5 CONCLUSIONS

Avian tibial dyschondroplasia has been widely characterized by a failure in terminal differentiation from prehypertrophic to hypertrophic chondrocytes (Poulos et al., 1978; Hargest et al., 1985; Thorp et al., 1993). Additionally, Rath et al. (2005) also suggest that a metabolic dysfunction may lead to the destruction of blood capillaries in the transition zone and Orth and Cook (1994) propose that chondrocytes secrete an immature cartilage that becomes highly cross-linked and is resistant to resorption and vascularisation by the metaphyseal vessels. Dyschondroplastic growth plates used in this study were obtained accordingly with the procedure of Capela-Silva (2003). This author has found, histologically, an accumulation of prehypertrophic chondrocytes in the lesions as well as an accumulated extracellular matrix. Our study has shown no increase in the gene expression of the macromolecules of the matrix such as collagens type II, IX, X, and XI as well of the main proteoglycan of the cartilage matrix, aggrecan. In the literature, it has been reported a decrease in the protein levels of these macromolecules in dyschondroplasia with the exception of collagen type II. Thus, our study showed that the lower protein levels of macromolecules in the lesions, reported by other authors, are not the result of decreased expression of their genes. It has been reported that a failure in the secretion or incorporation of these proteins into the matrix may occur which may lead to the development of the disease. In mammals, it has been described the regulation of collagens type II, X, and XI and aggrecan by the transcription factors Cbfa1 and Sox9. In the chicken, there is no information in the literature about this regulation. Nevertheless, the present study has also shown no changes in the gene expression of these factors in dyschondroplastic growth plates.

From our data, the failure in the differentiation of hypertrophic chondrocytes that characterize tibial dyschondroplasia, as mentioned above, seems not to be due to an alteration in the gene expression of the hormones and growth factors expressed in the growth plate and that regulate the proliferation and differentiation of chondrocytes. These include PTHrP, Ihh, and FGF. However, TGF-β and VEGF have changed their mRNA levels in dyschondroplasia. TGF-β has decreased and VEGF has increased significantly in the pathology. Since TGF-β inhibits terminal differentiation of chondrocytes we suggest that the reduced gene expression of this factor in dyschondroplasia is the

response of the tissue in order to control the failure of hypertrophic chondrocyte differentiation. The drastic increase of VEGF and MMP-7 (matrilysin-1) mRNA levels in TD leads us to suggest that dyschondroplastic lesions are in a hypoxic environment due to the accumulated cartilage. Indeed, these two proteins have been reported as being induced by hypoxia in other cell systems (Burke et al., 2003).

Regarding the MMPs analysed in the present study, we found a general downregulation in their gene expression in dyschondroplasia, with the exception of MMP-7, as discussed above, and MMP-2 (gelatinase A) which has maintained its gene expression in the pathology. Because MMP-2 mRNA levels did not change in TD we suggest that this protein may play the same housekeeping function in the avian growth plate during endochondral bone formation as its homolog in mammals. MMP-9 (gelatinase B), MMP-13 (collagenase-3), and the MMP-10 and MMP-11 (stromelysin-2 and -3, respectively) have demonstrated a pronounced reduction in their mRNA levels in dyschondroplasia. Only MMP-2 and MMP-13 were analysed in terms of their protein levels in dyschondroplasia and both showed reduced levels in the lesions. However, although we have not analysed the other MMPs in terms of protein levels, we believe that the decreased gene expression will correspond to reduced levels of the corresponding proteins. It is known that MMPs are involved in activation cascades in which many MMPs have common activators. Thus, any alteration in the protein levels of one MMP will implicate changes in the active protein levels of the MMPs activated by that MMP. We have discussed above that the accumulated cartilage characteristic of TD seems not to be due to increased production of the matrix macromolecules. Since the main function of MMPs is the degradation of extracellular matrix we may suggest that the accumulated cartilage of TD is the result of decreased proteolysis of the matrix due to diminished expression of these ECM-degrading enzymes. The lack in ECM proteolysis may lead to the failure in growth plate vascularisation as well as to impaired chondrocyte apoptosis due to the failure in the release of regulatory molecules sequestered in the matrix. These include VEGF and FGF which are consequently prevented to exert their function in the vascularisation of the growth plate. Indeed, our study has shown that the failure in the vascularisation of the affected growth plate was not due to a downregulation of these factors. Indeed, and as mentioned before, FGF has maintained unchanged and VEGF has increased drastically in TD. As already discussed, the result of VEGF was related to the formation of a hypoxic environment in affected cartilages. Additionally, the accumulated cartilage characteristic of TD may be also due to an accumulation of chondrocytes that do not mature and do not undergo apoptosis. The impaired apoptosis in dyschondroplastic cartilages was demonstrated by the reduced gene expression of caspase-3.

We speculate a possible scenario in dyschondroplasia which is the following. Since MMP-13 is exclusively synthesized by hypertrophic chondrocytes we suggest that the reduced mRNA and

protein levels of the enzyme in the lesions is a consequence of the delay in chondrocyte hypertrophy characteristic of dyschondroplasia. Indeed, we cannot assert that MMPs are responsible for the initiation of dyschondroplasia. For this, it would be necessary to perform the analysis at earlier stages of the pathology. The reduced expression of MMP-13 leads to reduced activation of MMP-2 and MMP-9 which are activated by MMP-13. Indeed, we found decreased levels of the MMP-2 active protein in the lesions besides the expression of the corresponding gene has maintained unchanged. Additionally, the reduced expression of MMP-10 can also contribute to the reduced activation of MMP-9 and MMP-13. The general downregulation of MMPs will contribute to the lack in ECM proteolysis and consequently to the accumulation of cartilage and to the failure of vascularisation and chondrocyte apoptosis in dyschondroplastic growth plates.

In conclusion, the present study has identified, for the first time, MMP-7 and MMP-11 in the avian tibial growth plate. It seems that the MMPs expressed in the mammalian growth plate are also expressed in the avian growth plate and that the MMPs expressed in the avian growth plate may play the same role as their counterparts in mammals. This occurred for MMP-2, which plays the same housekeeping function, for MMP-13, which plays the same role in chondrocyte hypertrophy, and for MMP-9, which plays the same role in the vascularization of the growth plate. Our results suggest that dyschondroplastic cartilages are in a hypoxic environment and that the accumulated cartilage observed in dyschondroplasia seems to be the result of decreased matrix proteolysis, due to the downregulation of the ECM-remodeling enzymes (MMPs), and not of an increased production of the ECM macromolecules. Thus, our results imply that the failure in the expression or lack in the activation of MMPs might be associated with the development of avian tibial dyschondroplasia. Furthermore, the data of this work strengthen the link between the lack in MMP expression and impaired endochondral bone formation, which might be pivotal findings for further research on pathologies related to abnormal bone growth.

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