

In Vivo Assessment of Class-specific
Inducible Inhibitors of
Metalloproteinases in Osteoarthritis

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ABSTRACT

The role of articular cartilage is providing a painless and attrition-free surface for joints movements. Osteoarthritis (OA) is a debilitating multifactorial pathology characterized by progressive articular cartilage loss, exposition of the bone surface accompanied by pain, and final loss of joint functions. To date there is not an effective alternative to surgical replacement of damaged joints.

Metalloproteinases (MPs) play an important role in cartilage remodelling both in physiological and pathological conditions. Among the metalloproteinases, the aggrecanase family was shown to be the major family responsible for aggrecan breakdown, while matrix metalloproteinases (MMPs) target preferentially the collagen molecules. Tissue inhibitor of metalloproteinases (TIMPs) are the endogenous regulators of those enzymes and in particular TIMP-3 was found to possess the broadest activity. So far all synthetic MP inhibitors failed in trials for OA treatment, and TIMP-3 may prove to be a possible alternative. Notably several synthetic mutants were generated to restrict its activity towards metalloproteinases.

In order to test the potential therapeutic effects of these proteins, I decided to employ transgenic overexpressing mice. However I appreciated that it is of paramount importance to restrict the exogenous expression just to cartilage. Moreover because it was previously observed that overexpression of TIMP-3 in cartilage severely affects bone development and caused embryonic lethality, I decided to generate tissue specific inducible transgenic mice. I focused on the Aggrecan promoter in order to achieve specific cartilage expression and the Cre/loxP system to obtain inducible expression.

Here I show the design and generation of a new aggrecan-driven Cre deleter mouse line. These mice can effectively target floxed genes in cartilage. I also show the generation of inducible TIMP-expressing mouse lines for both a cartilage-specific and ubiquitous expression.

I aim to induce OA in those transgenic mice, after induction of TIMPs expression, to evaluate the inhibitors role in OA treatment.

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STATEMENT OF ORIGINALITY

I declare that the work in this thesis was carried out in accordance with the regulations of Imperial College London. The experimental work presented herein is my own, except where it has otherwise been indicated by reference in the text. Any collaboration and/or assistance of methods or relevant prior experimental findings by others have been acknowledged in the relevant sections. The work has been carried out at the Kennedy Institute of Rheumatology, Imperial College London. No part of this thesis has been submitted for any other degree or examination in the United Kingdom or overseas.

Leandro Lo Cascio

December 2013

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LIST OF ABBREVIATIONS

4-OHT	4-Hydroxy Tamoxifen
Abs	Absorbance
Acan	Aggrecan gene
ADAM	A Disintegrin and Metalloproteinase domain
ADAMTS	A Disintegrin And Metalloproteinase domain with Thrombospondin Motif
AC	Articular chondrocytes
ACL	Anterior cruciate ligament
ACLT	Anterior cruciate ligament transection
Agc1	Aggrecan gene
BMI	Body mass index
BSA	Bovine serum albumin
CAG	CMV early enhancer/chicken β -actin
cDNA	Complementary DNA
cHS4	chicken β -globin hypersensitive site 4
CIP	Calf intestine phosphatase
CMV	Cytomegalovirus
Col2a1	Collagen type II α 1 gene
COMP	Cartilage oligomeric matrix protein
COX-2	cyclooxygenase 2
CRP	Complement regulatory protein
CS	Chondroitin sulphate
CTX-II	C-terminal telopeptide of collagen type II
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMM	Destabilization of medial meniscus

DNA	Deoxyribonucleic acid
Dpc	Days post coitum
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ER	Estrogen receptor
EtBr	Ethidium bromide
Fabp1	Fatty-acid binding protein-1
Fas	First apoptosis signal
FBS	Fetal bovine serum
FTO	fat mass and obesity associated
GAG	Glycosaminoglycan
GDF5	Growth differentiation factor 5
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
HA	Hyaluronan
HABR	Hyaluronan-binding region
Hh	Hedgehog
HMG	High mobility group
Hprt	Hypoxanthine guanine phosphoribosyl transferase
HSP-90	Heat shock protein 90
IGD	Interglobular domain
IGF-2	Insulin-like growth factor 2
Ihh	Indian hedgehog
IL-1 β	Interleukin-1 β
IP	Intraperitoneal
IRES	Internal ribosome entry site

IU	International units
kDa	kilo Dalton
KS	Keratan sulphate
LB	Luria-Bertoni
LBD	Ligand-binding domain
MMP	Matrix metalloproteinase
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MAPK-ERK	Mitogen-activated protein kinases – Extracellular signal-regulated kinases
MEF2C	Myocyte enhancer factor 2C
MRI	Magnetic resonance imaging
MT-MMP	Membrane type MMP
Nls	Nuclear localization sequence
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
OD	Optical density
OSM	Oncostatin M
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Proteoglycan
P/S	Penicillin/Streptomycin
PTHrP	Parathyroid hormone receptor protein
qPCR	Quantitative PCR
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
ROS	Reactive oxygen species

rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
Runx2	Runt-related transcription factor - 2
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SED	Spondyloepiphyseal dysplasia
(SEM(MO))	Spondyloepimetaphyseal dysplasia Missouri type
SOX9	SRY (sex determining region Y chromosome) -box 9
TACE	TNF α converting enzyme
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
TGF β	Transforming growth factor- β
TIMP	Tissue inhibitor of metalloproteinase
TNF α	Tumor necrosis factor- α
TSR	Thrombospondin repeat
TSS	Transcription starting site

Chapter 1

Introduction

1.1 Synovial Joint

1.1.1 Overview

The term “synovial joint” refers to a structure constituted of two bone ends coated with a thin and smooth layer of hyaline cartilage, enclosed by the synovial membrane in a capsule.

The synovial membrane or synovium lines the inside of the joint capsule and is responsible for producing the synovial fluid and offering physical protection. The synovium acts like a sieve allowing water, small molecules and nutrients to pass from the vascular system into the joint cavity but blocking the movement of larger molecules and potentially dangerous microorganisms. The resident cells of the synovium are the synoviocytes; these cells actively secrete proteins such as collagen, fibronectin and hyaluronan (HA) (which confers viscosity and elasticity) into the joint cavity.

Hyaline cartilage functions as a low-friction, wear-resistant tissue designed to bear and distribute loads. It is a highly specialized tissue with unique mechanical behaviour and poor regenerative capacities. Articular cartilage has low metabolic activity; it consists of chondrocytes and a dense extracellular matrix composed primarily of water, collagen, and proteoglycan. A peculiar feature of articular cartilage is the lack of vascularization and innervation (Poole 1997) .

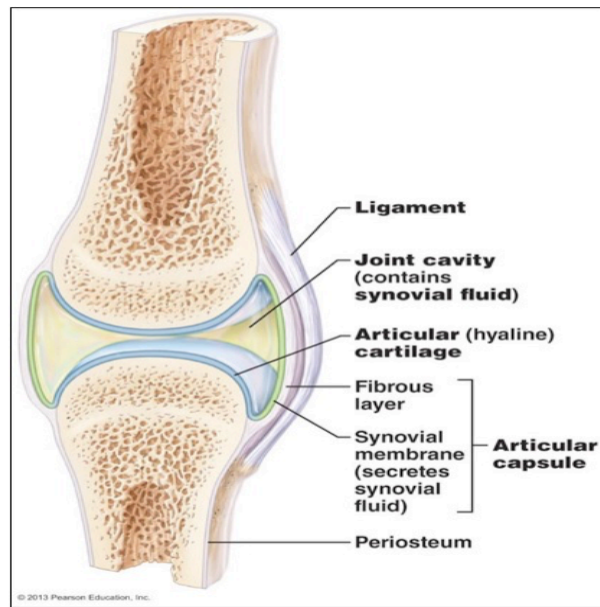


Figure 1.1. Schematic representation of the synovial joint. The diarthrodial joint connects two bones and allows their reciprocal movement. A layer of articular cartilage covers the two bony epiphyses. The joint cavity, containing the synovial fluid, separates the two edges (http://classes.midlandstech.edu/carterp/Courses/bio110/chap06/figure_08_03_labeled.jpg).

1.1.2 Formation and structure of the synovial joint

In vertebrates the synovial joint is a rather remarkable structure that can be heavily specialized depending on location and function. Examples are the joints of the femur head that is encapsulated in the hips or the knee joint formed between femur and tibia bones. Although the macroscopic mechanism of joint formation is a quite linear process, however how mesenchymal cells are directed to form an interzone and how the interzone differentiates into distinct joint components are largely unknown. Research focused to uncover the molecular mechanisms is of importance to understand, and possibly cure, both congenital diseases (e.g. several types of chondrodysplasia), and pathological conditions related to aging that leads to progressive degeneration and loss of function of the joint, as in osteoarthritis (OA).

The first event in vertebrate skeleton generation is the mesenchymal cells condensation in a continuous structure (**Figure 1.2**). Individual skeletal elements then arise through a segmentation process, introducing joints in specific locations. The first sign of the joint development is observed with the appearance

of the interzone where the joints will form. The interzone is recognized as an area where the mesenchymal cells are closely connected by gap junctions suggesting a strong coordination among them. Interzone can be structurally divided into three sub-structures: a central intermediate zone that separates two outer layers. While mesenchymal cells from outer layers seem to differentiate into chondrocytes and become part of the epiphyses, the intermediates zone will form the proper articular cartilage. The formation of the interzone is a process independent from the skeletal formation, as demonstrated by Pacifici and colleagues (Pacifici, Koyama et al. 2006). If the mesenchymal cells from the presumptive interzone of the elbow are removed from during embryogenesis, it prevents the joint formation, but the bones will still be formed (Holder 1977) . The first step in joint formation is cavitation, an event caused mostly by apoptosis of pre-chondrocytes in the presumptive joint region (Archer, Dowthwaite et al. 2003) . The pre-chondrocytes, aligned underneath, differentiate into articular chondroblasts that express high level of lubricin and matrix protein. Morphological observations indicate that once the joint anlagen have separated, those cells flattened and form the articular chondrocytes of the articular cartilage layer (**Figure 1.2**). The mechanism coordinating this event is not fully defined, but is likely to involve changes in ECM composition (Pitsillides, Archer et al. 1995, Capehart 2010). Studies suggest that enzymatic degradation of the ECM is unlikely (Edwards, Wilkinson et al. 1996), but support a selective mechano-dependent activation of the MEK–ERK pathway, leading to local changes in hyaluronan synthesis and binding in cells immediately bordering the presumptive joint, which directly contribute to the loss of tissue cohesion essential for cavitation (Bastow, Lamb et al. 2005). The articular chondrocytes are resting cells and maintain high expression of cartilage genes such as Aggrecan (*Acan*), Lubricin (*Prg4*) and basal level of type II collagen (*Col2a1*).

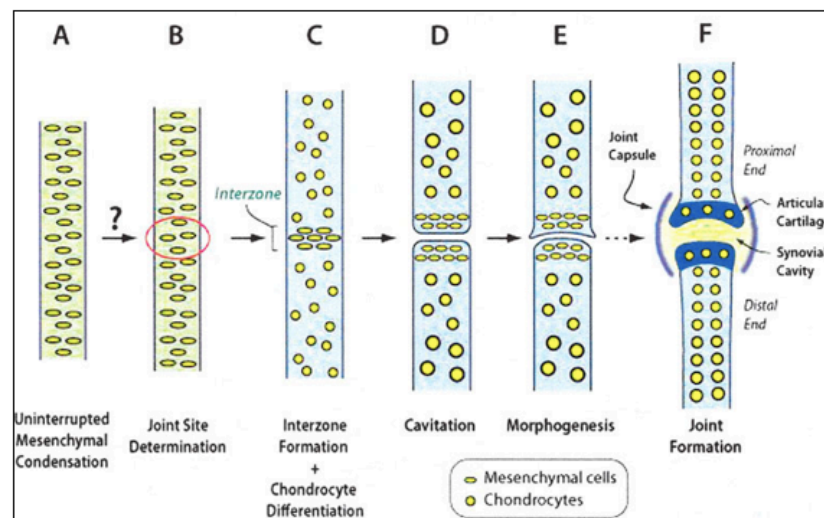


Figure 1.2. Schematic diagram illustrating the major steps in synovial joint formation. A: Mesenchymal condensations produce an uninterrupted layer in the early limb bud. B: Unknown upstream patterning mechanisms determine the exact location for joint initiation. C: Further condensation marks the forming interzone; mesenchymal cells constituting the bulk of the condensations differentiate into chondrocytes. D: A cavitation process begins and eventually leads to the physical separation of the adjacent skeletal anlagen and formation of a synovial cavity. E: The opposing sides of the joint begin to **be molded into reciprocally shaped and interlocking structures**. F: **All the distinct component of a mature joint form and include articular cartilage, capsule and the synovial cavity.** (Adapted from Pacifici et al., 2005).

On the molecular level SOX9 plays a central role in chondrocyte differentiation and commitment. Sox9 is a member of the SRY (sex-determining region of the Y-chromosome) family that contains the HMG (high mobility group) box DNA-binding domain. Targeted loss of SOX9 in mouse early development results in complete absence of both cartilage and bone, whereas SOX9 inactivation at later stages leads to diminished chondrocyte proliferation, chondrodysplasia and joint formation defects (Bi, Deng et al. 1999). SOX9 was observed to activate the expression of type II collagen in mouse embryos from 10 dpc (day post coitum) (Lefebvre, Huang et al. 1997), explaining the parallel expression pattern of the two genes (Zhao, Eberspaecher et al. 1997). More recently it was shown that SOX9, in concert with L- SOX5 and L- SOX6, recognizes a target sequence upstream of the *Acan* enhancer and drives its expression in cartilage (Han and Lefebvre 2008). Sox9 expression persists postnatally and in adult mice to control the homeostasis of connective tissues such as the growth plate, articular cartilage, and the intervertebral disk (Henry, Liang et al. 2012). SOX9 coordinates not only the expression of extracellular matrix genes, but is required to maintain

chondrocyte columnar proliferation and generate cell hypertrophy, two key features of functional growth plates. Sox9 may be critical to shift the activities of Runx2 and β -catenin from osteoblast differentiation to chondrocyte maturation, inhibiting not only progression from proliferation to pre-hypertrophy, but also subsequent acquisition of an osteoblastic phenotype. SOX9 protein outlives its mRNA in upper hypertrophic chondrocytes, where it contributes with MEF2C to directly activate the major marker of these cells, *Col10a1* (Dy, Wang et al. 2012). Moreover SOX9 was observed during attempted repair of articular cartilage damage in a transgenic mouse model of osteoarthritis (Salminen, Vuorio et al. 2001).

Targeting of Sox9 in mouse chondrocytes leads to down-regulation of the levels of expression of Ihh (Indian Hedgehog) and its two downstream targets parathyroid hormone receptor protein genes (PTHrP) (Akiyama, Chaboissier et al. 2002). Both Ihh and PTHrP are expressed postnatally by chondrocytes and are essential for maintaining the growth plate physiology (Maeda, Nakamura et al. 2007) and are shown to play a role in cartilage repair and pathological conditions (Kudo, Mizuta et al. 2011).

Wnt/ β -catenin signalling is also essential in chondrocyte proliferation, differentiation and apoptosis during development (Hartmann and Tabin 2000). The critical role of Wnt/ β -catenin signalling has been elegantly demonstrated in the cartilage of adult mice in which the gene for β -catenin was selectively activated in articular chondrocytes and shown to produce premature chondrocyte differentiation and an OA-like phenotype (Zhu, Tang et al. 2009).

Two genes encoding secreted proteins, Wnt9a and Gdf5, have been shown to play important roles in early joint formation, both position and cavitation. It has been shown that isolated interzone cells expressed typical phenotypic markers, such as GDF-5, WNT-9A and CD-44, and differentiated into chondrocytes over time (Pacifici, Koyama et al. 2006). Although evidence from Wnt9a null mice suggests that it does not initiate joint cavity formation, it is important in specifying joint position. Misexpressing Wnt9a locally within the developing autopods of chick embryos leads to alterations of the skeletal pattern (Hartmann

and Tabin 2001) . Wnt9 can activate expression of CD44 mRNA, the major hyaluronan-binding protein, in knee joint, thus demonstrating a role in joint cavitation (Dowthwaite, Edwards et al. 1998). WNT-9A was shown to induce expression of Gdf5, a member on the TGF β super-family. Gdf5 is required for the normal formation of a number of joints is not sufficient to initiate the joint-forming process. A loss-of-function mutation of the Gdf5 gene causes the loss of several joints in mice (Storm, Huynh et al. 1994). Similarly, a truncated GDF5 protein in humans is known to cause a congenital joint malformation called Grebe-type chondrodysplasia (Basit, Naqvi et al. 2008). However, gain of function of Gdf5 does not result in joint formation in the developing limb (Tsumaki, Nakase et al. 2002).

1.1.3 Hyaline cartilage composition

Hyaline cartilage lacks neural, vascular and lymphatic structures (Buckwalter and Mankin 1997). Articular chondrocytes contribute only 1–5% to the total volume of the articular cartilage. These receive their nutrition by diffusion through the abundant extracellular matrix (ECM). ECM has a slightly basic pH, changes in which can easily disrupt the highly specialized matrix infrastructure. Chondrocytes are very specialized cells responsible for synthesizing and maintaining the surrounding ECM (Chen, Frenkel et al. 1999) .

The articular cartilage can be divided into three components: water, collagens, and proteoglycans.

Water provides about 65-80 % of the wet weight of the cartilage. Such amount of water is contained in the intrafibrillar space produced by the ECM components, and held in place by the negative charges on the proteoglycans. Such amount of water helps to maintain the compression-resistance ability to the tissue. Moreover it helps to generate a low-friction surface and provides nutrition the articular chondrocytes.

Collagens form 10–20% of wet weight of the articular cartilage. Type II collagen is the principal component (90–95%) of the macrofibrillar meshwork, provides the tensile strength of the articular cartilage, and is produced by proliferating chondrocytes. Type VI collagen is found in the pericellular space and helps

chondrocytes to attach to the matrix. Type X collagen provides structural support, aids in cartilage mineralization and is associated to the hypertrophic chondrocytes.

Proteoglycans account for 10–20% of the wet weight and provide compressive strength to the articular cartilage, and maintain the fluid electrolyte balance. They are heavily glycosylated proteins and consist of a "core protein" with one or more covalently attached glycosaminoglycan (GAGs) chains. GAGs are long unbranched polysaccharides, consisting of a repeating disaccharide unit. Being overall highly polar they attract local water molecules, generating an increment in osmolarity that lead to an increase of the concentration of inorganic ions (e.g. sodium) inside the matrix. In articular cartilage, the chief structural proteoglycan is aggrecan, which consists of a long protein core with up to 100 chondroitin sulphate chains and up to 50 keratan sulphate glycosaminoglycan chains. Aggrecan molecules bind via a link protein on the protein core to a hyaluronate molecule. In cartilage, the hyaluronate molecules form a backbone with palisading aggrecan molecules; this macromolecular complex is known as the proteoglycan aggregate. The interaction between the proteoglycan molecules and collagen fibrils creates a fibre-reinforced composite solid matrix. The proteoglycans are entangled and compacted within the collagen interfibrillar space, which helps to maintain a porous-permeable solid matrix and determines the movement of the fluid phase of the matrix.

Finally chondrocytes are the cartilage resident cells. They are high specialized cell type and responsible of deposition of both collagens and proteoglycans. Because the relatively low cell number compared to the total volume of the tissue, chondrocytes are characterized by the lack of cell-to-cell interactions, and receive their nutrition by diffusion from blood vessels at the periphery of the tissue. In mammals such human chondrocytes survive on low oxygen conditions depending on anaerobic metabolism, however in smaller animals such mouse the hypoxic environment is absent. Finally articular chondrocytes produce the enzymes responsible for the matrix degradation.

1.1.4 Hyaline cartilage ultra-structure

Cartilaginous tissues are present throughout the body and are classified into three groups: hyaline, elastic and fibrocartilage cartilage according to the composition of the extracellular matrix (ECM) and on tissue morphology (Bhosale and Richardson 2008). Articular cartilage is a hyaline cartilage and has an organized layered structure that can be functionally and structurally divided into four zones: the superficial zone, the middle (or transitional) zone, the radial (or deep) zone, and the calcified zone. These zones are each characterised by different amounts and organisation of matrix macromolecules (**Figure 1.3**).

The superficial zone is the articulating surface that provides a smooth gliding surface and resists shear. It has the highest collagen content of the zones, and the lowest in proteoglycan; the collagen fibrils in this zone are densely packed and have a highly ordered alignment parallel to the articular surface (Mow, Proctor et al. 1989). Chondrocytes in the superficial zone are elongated and express proteins that have lubricating and protective functions and secrete relatively little proteoglycan. The middle zone covers the majority of the articular cartilage volume. Collagen fibres in this layer are less organized, loosely packed, and aligned obliquely to the surface (Hwang, Li et al. 1992). Chondrocytes are more rounded than in the superficial layer and arranged vertically into columnar clusters. The deep zone makes up to a third of the cartilage volume and consists of large diameter collagen fibrils oriented perpendicular to the articular surface. This layer contains the highest proteoglycan and lowest water concentration. The chondrocytes are arranged, as in the layer above, in columnar clusters parallel to the collagen fibres and perpendicular to the joint line. The calcified cartilage zone separates hyaline cartilage from subchondral bone. A discrete band marks the physical boundary with the deep layer. This line represents the mineralization front of the calcified cartilage and provides a gradual transition between the two dissimilar regions of cartilage (Lyons, Stoddart et al. 2005). It has an affinity for numerous histological stains and appears as the “tidemark” in haematoxylin sections. The calcified cartilage contains large amount of type X collagen and small cells in a chondroid matrix speckled with apatite salts.

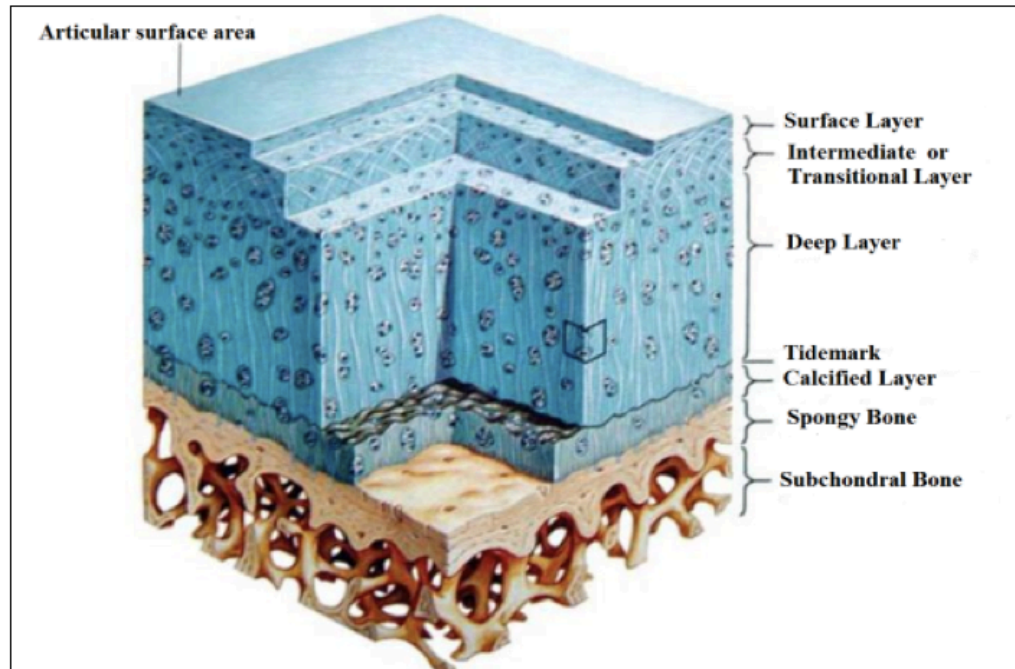


Figure 1.3 Architectural layout of the articular cartilage. Schematic representation of articular cartilage ultrastructure. (Adapted from Sopena-Juncosa et al., 2000).

1.2 Homeostasis of the Cartilage ECM

1.2.1 Overview

In cartilage, water molecules are attracted and maintained by the negatively charged proteoglycans. Both proteoglycans and collagens are synthesized and deposited by chondrocytes. Among collagens the type II is the most abundant and interestingly is deposited quite early during tissue development, remaining virtually stable during the life of the organism. Proteoglycans on the other hand are subjected to a continuous remodelling, with new molecules actively synthesized. The turnover of those structural proteins is maintained by a balance between their synthesis and the production of matrix proteinases that can be divided into: matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs), a disintegrin and metalloproteinase (ADAMs). These enzymes share sequence and structure similarity but differ for specificity of substrate. Chondrocytes maintain such balance between catabolic and anabolic activity using the tissue inhibitors of metalloproteinase (TIMPs). These secreted proteins are the endogenous regulators

of the matrix enzymes and their misregulation can lead to development defect as well as pathological conditions.

1.2.2 Proteoglycans and glycosaminoglycans

The study of cartilage proteins composition proved to be a very difficult task, because the strong interactions between the charged molecules of this tissue. Extraction and purification of the single components required harsh conditions that were matched only with the use of the strong chaotropic agent guanidinium hydrochloride and CsCl density gradient centrifugation.

Proteoglycans are heavily glycosylated proteins consisting of a unit called “core protein” decorated with one or more chains of glycosaminoglycans (GAGs). GAGs are covalently linked to serine residues of the core protein; they are polysaccharides consisting of a repeating disaccharide that contains an amino sugar. GAGs are negatively charged and this is essential for their function. Proteoglycans are found in all connective tissues, extracellular matrix and on the surfaces of many cell types. They are remarkable for their diversity such as different cores, different numbers of GAGs with various lengths and compositions). The protein component of proteoglycans is synthesized by ribosomes on the rough endoplasmic reticulum. Glycosylation occurs in the Golgi apparatus in multiple enzymatic steps. First a special link tetrasaccharide is attached to a serine side chain on the core protein to serve as a primer for polysaccharide growth. Then sugars are added one at a time by glycosyl transferase. The completed proteoglycan is then exported in secretory vesicles to the extracellular matrix of the tissue.

Although cartilage ECM is made by of several proteoglycans, aggrecan is by far the main one and contributes 90% of the total proteoglycan content. In the cartilage ECM aggrecan is bound to a backbone of hyaluronan (hyaluronic acid) and forms huge aggregates (up to 20 aggrecan molecules) filling the interfibrillar space of the cartilage matrix (Hardingham 1979, Roughley 2006). The hyaluronan backbone is itself anchored to chondrocytes via CD44. Aggrecan is

connected to the hyaluronan by a globular protein, highly homologous to the aggrecan globular domain and named link protein.

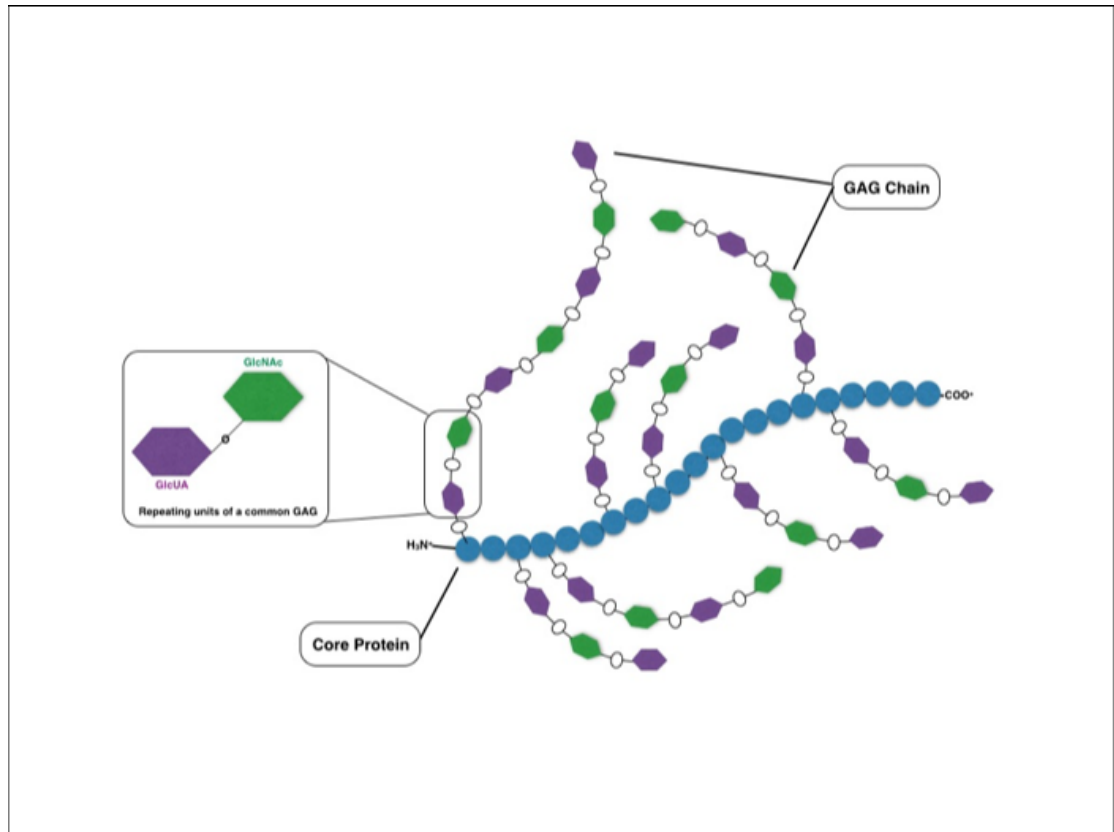


Figure 1.4. Schematic diagram illustrating general proteoglycans structure and GAGs interaction. Proteoglycans are a class of extracellular proteins composed of a core protein (shown in blue) and distinguished by the addition of glycosaminoglycans (GAGs). GAGs are long-chain polysaccharides composed of disaccharide repeats. Here is schematised hyaluronate composed of the repeating disaccharide pair gluronic acid (GlcUA, purple) and N-acetylglucosamine (GlcNAc, green).

1.2.3 Aggrecan

1.2.3.1 *Aggrecan gene and expression*

The aggrecan gene (*ACAN*) has been characterised in several species including *R. norvegicus* (Doege, Sasaki et al. 1990), *H. sapiens* (Valhmu, Palmer et al. 1995) (Valhmu, Palmer et al. 1995), *M. musculus* (Watanabe, Gao et al. 1995), and *G. domesticus* (Li and Schwartz 1995). In human aggrecan gene has been localised to chromosome 15q26.2 by in situ hybridization (Korenberg, Chen et al. 1993) (Korenberg, Chen et al. 1993) and sequencing has revealed that the gene consists of 19 exons and 18 introns.

Aggrecan gene is mainly expressed in cartilaginous tissue; expression can be found in differentiated chondrocytes such as the chondrocytes of the growth plate and the articular cartilage, since embryonic stages and throughout adulthood. However is not expressed in skeletogenic mesenchymal cells, the perichondrium or the presumptive joint cells (Shepard, Krug et al. 2007). Aggrecan gene is also expressed in the fibrocartilage of the menisci. Notably although the expression of aggrecan is mainly found in differentiated chondrocytes, it can be detected before the appearance of cartilage matrix. Aggrecan mRNA was detected in E14.5 mice in aggregation of mesenchymal cell before chondrogenesis (Fukada, Shibata et al. 1999).

Aggrecan mRNA has also been observed in the in non-cartilage tissues such as central nervous system (CNS), liver, adipose tissue and heart. In CNS aggrecan is the major constituents of perineuronal nets in adult rat brain. Matthews et al showed that neurons are the predominant cellular source of aggrecan in the adult CNS. Using *in situ* hybridization they also demonstrated that aggrecan mRNA is not expressed in the CNS until late in embryonic development, with peaks in expression at 1 month of age, followed by stable expression during adulthood (Matthews, Kelly et al. 2002). More recently was found that aggrecan regulates astrocyte development and is expressed by embryonic glial precursors (Domowicz, Sanders et al. 2008). In liver aggrecan mRNA has been shown to be expressed during liver fibrogenesis (Krull and Gressner 1992). In adipose tissue Voros et al showed that aggrecan mRNA is present in murine intra-abdominal and inguinal subcutaneous fat pads. They suggested that aggrecan might contribute to the regulation of lipid uptake and obesity in mice (Voros, Sandy et al. 2006). Studies of chicken embryo hearts at stages 12-21 of development, shown that aggrecan is specifically co-localized with several groups of migrating cells that such as endocardial cushion tissue cells, epicardial cells and a mesenchymal cell population in the inflow tract (Zanin, Bundy et al. 1999). The aggrecan-like molecules that are expressed in such different tissues are all products of the same gene. However, in each tissue the gene product exhibits significantly different chemical and structural properties, indicating that although they share an identical core protein, they are processed differently, yielding to distinct proteoglycans (Schwartz and Domowicz 2004).

The promoter of the aggrecan gene has been studied in different species including *H. sapiens* (Valhmu, Palmer et al. 1995) and *R. norvegicus* (Doege, Garrison et al. 1994). In humans, the basal promoter was shown to lack canonical TATA and CAAT boxes and contain several putative binding sites for ubiquitous transcription factors such as SP-1, AP-2, and AP-4 that are located in both the promoter and untranslated regions. Those alone or in combination may mediate the basal activities of this promoter. Cis and trans-regulatory elements have been also identified by *in vitro* studies and have been shown to regulate the expression of aggrecan by directly driving/repressing promoter activity. The first *in vivo* study to search for these regulatory elements involved the rat aggrecan gene. Initially, this search had focused primarily on a 922 bp DNA fragment across the proximal promoter. This sequence had showed higher promoter activity in transfected chondrocytes than in fibroblasts (Doege, Garrison et al. 1994). However it was predicted that there might be additional regulatory sequences other than the proximal promoter that are responsible for expression of the rat aggrecan gene in chondrocytes. Attention was focused on a 30 kb DNA fragment that span upstream of the TSS (transcription start site) and throughout the first intron, employing both cell transfection assays as well as transgenic mice. Experimental results in cell transfection experiments identified an upstream 5kb tissue specific enhancer/repressor at about 10 kb upstream of the TSS. This upstream enhancer was active also *in vivo* when fused to a proximal promoter sequence (Doege, Hall et al. 2002). More recently an important study on aggrecan cis-regulatory elements was performed by Lefebvre group. A highly conserved and regulatory 359-bp sequence located approximately 10Kb upstream of the mouse *Acan* TSS (partially corresponding to the sequence previously identified in the rat aggrecan gene) was identified. Four tandem repeats of this sequence were able to direct expression of a *Col2a1* minimal promoter in both embryonic and adult cartilage in transgenic mice, in a manner that matches aggrecan expression. Expression levels in transient transfection experiments shown that the identified sequence was almost as active as proper the *Col2a1* enhancer fused to the *Col2a1* minimal promoter (Han and Lefebvre 2008). These findings lay the path to my present work. Harnessing aggrecan gene enhancer sequence I could achieve strong and stable expression of target genes in cartilage.

1.2.3.2 Aggrecan protein

The core protein of aggrecan has a molecular weight of ~230 kDa and consists of three globular domains, G1, G2 and G3, and three interglobular domains, the keratan sulphate (KS) and chondroitin sulphate (CS) glycosaminoglycan attachment domains located between G2 and G3 and a short interglobular domain between G1 and G2. The N-terminus comprises the G1 globular domain, which interacts with hyaluronan and link protein. Because G1 domain is important for interaction with hyaluronan, aggrecan which lacks in the G1 domain, is free to exit the matrix and does not contribute to cartilage function (Sandy, Neame et al. 1991). Mild trypsin treatment generates a protein fragment containing the G1 and G2 domains, which retains hyaluronan binding capacity, and thus is termed the hyaluronan-binding region (HABR). The C-terminus comprises the G3 domain that includes an EGF module, a C-type lectin module and a complement regulatory protein (CRP) module. Deposition of aggrecan is a hallmark of chondrogenesis. Aggrecan has a relatively long half-life of about 3-4 years, and thus its expression is maintained in adult articular chondrocytes. It has been shown that this protein is cleaved by endogenous proteinases in specific sites between G1 and G2 domains. In particular cleavage occurs in the IGD domain at Glu³⁷³-Ala³⁷⁴ (by ADAMTS) and at Asn³⁴¹-Phe³⁴² (by MMPs) (Sandy, Flannery et al. 1992, Fosang, Last et al. 1993). Other cleavage sites were found using *in vitro* studies but are unlikely to be responsible for aggrecan degradation in physiological conditions. Other less abundant small proteoglycans found in the matrix include decorin, biglycan, lumican and fibromodulin which are thought to be involved in fibril assembly, organisation and stabilisation of collagen matrix structure within the cartilage extracellular matrix (Roughley 2006).

1.2.4 Collagens

Collagen is the main component of connective tissues such as the cartilage and bone, and is the most abundant protein type in the vertebrate organism. The formation of collagen-rich extracellular matrices is a complex process that

involves intracellular and extracellular steps. Mutations in genes that encode individual chains of triple-helical collagens present in bone and cartilage are associated with heritable diseases of skeletal tissues. In addition, mutations in genes encoding proteins involved in the intracellular and extracellular modifications of collagens are also responsible for developing skeletal abnormalities (Arnold and Fertala 2013). Mutations in the COL2A1, the gene that encodes collagen II, are associated with spondyloepiphyseal dysplasia (SED), characterized by short stature, myopia, and hearing loss (Royce and Steinmann 2003, Kannu, Bateman et al. 2012). Collagens fibres are deposited early during development and provide tensile strength to the cartilage.

The collagen molecule is composed of a triple helix that may consist of heterotrimer (two identical chains and an additional chain that differs slightly in its chemical composition, eg $[\alpha 1]_2(\alpha 2)$) or homotrimer (eg. $[\alpha 1]_3$). Each chain consists of approximately 300 uninterrupted tandem repeats of -Glycine-X-Y- triplets (where X and Y positions are frequently occupied by proline or hydroxyproline residues). Collagen type I and II are produced as pro-collagens, in which the extended triple-helical domains are flanked by globular N-terminal and C-terminal pro-peptides, this feature prevents that helices interact too early and engulf the cell machinery. As a simultaneous process with the biosynthesis and post-translational modifications in the ER, the nascent pro-collagen α -chains fold into the triple-helical structure in a zipper-like fashion (Engel and Prockop 1991). This occurs via site-specific interactions of domains present within the C pro-peptide, which align individual pro-collagen chains and initiate their folding. Type II collagen is the most abundant collagen in articular cartilage, reaching the 90% of the total amount. It is made by a homotrimer of $\alpha 1$ chains, coded in human by the COL2A1 gene. It has been demonstrated that the gene undergoes alternative splicing, and two forms are expressed in progenitor cells during early embryo development, and in fully differentiated chondrocytes (Mcalinden, Johnstone et al. 2008). Type II collagen is a very stable molecule and therefore it is believed that the synthesis of type II collagen in articular cartilage is down-regulated after development and maintained to basal levels during adulthood. However there are evidences that transcription levels may be upregulated in osteoarthritis (Hermansson, Sawaji et al. 2004).

In addition to type II collagen in cartilage other types are present and necessary to maintain the tissue homeostasis. Type VI collagen has been identified in the pericellular matrix of articular cartilage, but its role is unknown but it may interact with matrix proteoglycans (Soder, Hambach et al. 2002). Type IX collagen decorates type II fibrils allowing cross-linking the adjacent ones. It is thought that type IX role is to increase network mechanical integrity and providing a restraint for entrapped proteoglycan osmotic swelling pressure. Type X collagen is a homotrimer that is highly expressed by hypertrophic chondrocytes within the calcified cartilage (Gannon, Walker et al. 1991, Nerlich, Kirsch et al. 1992). Type XI collagen is thought to reside in the central portion of the collagen fibril, acting as a filamentous core template which regulates fibril diameter through its retained N-propeptide domains (Sandberg, Hirvonen et al. 1993).

1.3 Cartilage metalloproteinases

1.3.1 Overview

Physiological remodelling processes such as development, morphogenesis and tissue repair involve precisely regulated degradation of the ECM. Remodelling also occurs during the course of diseases such as arthritis, cancer and cardiovascular disease, in part in an attempt at repair, but is frequently deregulated and destructive (Sternlicht and Werb 2001, Milner and Cawston 2005, Aiken and Khokha 2010). Although members of four classes of proteinases: serine/threonine proteinases, cysteine proteinases, aspartic proteinases, and metalloproteinases, have been implicated in the degradation of cartilage, current data indicate that the initial steps in matrix breakdown are extracellular processes involving metalloproteinases.

1.3.2 Matrix Metalloproteinases

In human 23 different MMPs are synthesized; these enzymes are defined as multidomain zinc metalloproteinases, because they possess a catalytic domain that contains the Zn^{2+} binding motif HEXXHXXGXXH, that is essential for their activity (Bode, Gomis-Ruth et al. 1993). Most of MMPs require Ca^{2+} ions to

exert their activity; in fact chelating agents that remove such co-factors can inhibit the enzyme activity. MMPs generally consist of a pro-domain, a catalytic domain, a hinge region, and a hemopexin domain. The matrixins are synthesized as pre-proenzymes and the signal peptide is removed during translation to generate proMMPs. The pro-peptides have the 'cysteine switch' motif PRCGXPD in which the cysteine residue coordinates with the catalytic Zn^{2+} in the catalytic domain, keeping the proMMPs inactive (Van Wart and Birkedal-Hansen 1990). However it was observed *in vitro* that some chemical agents may lead to activation (Nagase 1997). Notably among them thiol-modifying agents, oxidized glutathione, SDS (sodium dodecyl sulphate), chaotropic agents and reactive oxygens. These agents most likely work through the disturbance of the cysteine-zinc interaction of the cysteine switch (Van Wart and Birkedal-Hansen 1990). MMPs are either secreted from the cell or anchored to the plasma membrane. On the basis of substrate specificity, sequence similarity, and domain organization, vertebrate MMPs can be divided into six groups: collagenases, gelatinases, stromelysins, matrilysins, membrane-type (MT-MMPs) and others (Visse and Nagase 2003, Parks, Wilson et al. 2004, Murphy and Nagase 2008). Belong to collagenases MMP-1, -8 and -13; these enzymes are able to cleave interstitial collagens types I, II, and III at specific site, but they can also digest other ECM molecules. MMP-13 in particular preferentially cleaves the type II collagen, that is the major collagen constituent of cartilage. MMP-2 and MMP-9, also referred as gelatinase A and B, can digest already denatured collagens, so they may act in concert with collagenases during cartilage breakdown. Substrates of MMP-2 are collagens type I, II and III (Aimes, French et al. 1994). Belong to the stromelysins MMP-3 and MMP-10; these enzymes are characterized by a broad substrate specificity being able to degrade many of the extracellular matrix proteins such as: gelatins, proteoglycans, fibronectin, and type IX collagen. Moreover MMP-3 is involved in the protolytic activation of other MMPs (Suzuki, Enghild et al. 1990). The matrilysins (MMP-7 and -26) are characterized by lack of the hemopexin domain (Uria and Lopez-Otin 2000). Both enzymes digest ECM components but also cell surface molecules such as: Fas-ligand, pro-TNF- α (tumor necrosis factor- α) and E-cadherin. Membrane-type MMPs (MT-MMPs) are subdivided into four trans-membrane proteins (MMP-14, -15, -16 and

-24) and two GPI-anchored (glycosylphosphatidylinositol) (MMP-17 and -25) proteins. These enzymes are able to digest several ECM components but also activate other MMPs via pro-domain cleavage. In particular MMP-14 (also known as MT1-MMP) can digest type I, II, and –III collagen (Ohuchi, Imai et al. 1997). Importance of this enzyme is demonstrated by the presence of skeletal abnormalities during postnatal development in mice *mmp14* deficient (Holmbeck, Bianco et al. 1999).

Designation	Common name	ECM substrates	Non-ECM
MMP-1	Collagenase-I	yes	no
MMP-2	Gelatinase A	no	yes
MMP-3	Stromelysin-1	yes	yes
MMP-7	Matrilysin	yes	yes
MMP-8	Collagenase-II	no	yes
MMP-9	Gelatinase B	yes	yes
MMP-10	Stromelysin-2	ND	ND
MMP-11	Stromelysin-3	ND	ND
MMP-12	Metalloelastase	no	yes
MMP-13	Collagenase-3	yes	no
MMP-14	MT1-MMP	yes	yes
MMP-15	MT2-MMP	no	yes
MMP-16	MT3-MMP	yes	yes
MMP-17	MT4-MMP	ND	ND
MMP-19		ND	ND
MMP-20	Enamelysin	yes	no
MMP-21		ND	ND
MMP-22		ND	ND
MMP-23	CA-MMP	ND	ND
MMP-24	MT5-MMP	ND	ND
MMP-25	Leukolysin, MT6-	ND	ND
MMP-26	Endometase,	ND	ND
MMP-27		ND	ND
MMP-28	Epilysin	ND	ND

Table 1.1. Mammalian Matrix Metalloproteinases (MMPs). Adapted from (Parks, Wilson et al. 2004).

The expression of matrix metalloproteinases in synovial membranes, cartilage, tendon and bone of synovial joints in both rheumatoid arthritis (RA) and OA is well documented and often correlates with tissue destruction (Murphy and

Nagase 2008). The collagenases MMP-1 and MMP-13 are produced by synovial fibroblasts, macrophages and chondrocytes and have important roles in RA and OA because they are able to initiate the degradation of fibrillar collagens, making them accessible to further degradation by gelatinases. Antibodies that recognize terminal amino acid sequences generated by proteolysis (known as neo-epitopes) at specific sites in type II collagen have been since-long used for identifying the proteinases responsible for cartilage breakdown both *in vitro* and *in vivo* (Hollander, Heathfield et al. 1994, Wu, Billingham et al. 2002). Preliminary results with quantitative assays of type II collagen neo-epitopes suggest that they may be useful markers of joint disease in humans (Fosang, Stanton et al. 2003). Interestingly MMP-13 also degrades the proteoglycan molecule, such as aggrecan, giving it a dual role in matrix destruction. Non-collagen matrix components of the joints are also degraded by MMP-2, MMP-3, MMP-9, MMP-10 and MMP-19 (Cawston and Wilson 2006). Generally speaking alterations in MMPs activity were correlated to one or more human diseases; however animal models of human disease studied so far, demonstrated that relevance of MMP expression profiles varies with the nature of the cells, the stage of the disease and the genetic make up of the animal (Lemaitre and D'Armiento 2006, Martin and Matrisian 2007). Targeted gene deletion in mice has shown, with few exceptions, that embryogenesis and subsequent growth happens normally, suggesting a compensatory role of MMPs function during development. On the other hand tilting the balance by inducing external challenges to the systems have proved fruitful in understanding of the roles of MMPs. In light of those consideration is not surprising that only few human genetic disorders of the MMPs and TIMPs have been described and directly correlated to pathological conditions. For example it has been reported that mutations in the MMP2 gene, resulting in the absence of active enzyme, are linked with an autosomal recessive form of multicentric osteolysis (Martignetti, Aqeel et al. 2001). At the same time loss of MMP-2 in mice leads to defects in skeletal and craniofacial development (Mosig, Dowling et al. 2007). In human mutation in the MMP13 gene is correlated to a form of spondyloepimetaphyseal dysplasia, Missouri type (SEMD(MO)) characterized by defective growth and remodelling of long bones (Kennedy, Inada et al. 2005). Similarly mice lacking the Mmp13 gene have defects in

growth plate cartilage with increased hypertrophic domains and delayed endochondral ossification during development (Inada, Wang et al. 2004, Stickens, Behonick et al. 2004).

Due to the role of MMP in pathological conditions, inhibitors of MMPs were investigated for therapeutic potential. MMP inhibitors can broadly be subdivided into non-synthetic (e.g. endogenous) or synthetic. Several potent MMP inhibitors have been identified, including hydroxamates, thiols, carbamoylphosphonates, hydroxyureas, hydrazines, β -lactams, squaric acids and nitrogenous ligands. Although several of these compounds shown appreciable inhibitory effects *in vitro*, most of MMP inhibitors have exhibited serious side effects during preclinical trials. These side effects are caused by insufficient selectivity. Several MMPs, for example, mediate the release of signalling molecules that where shown to inhibit angiogenesis and vasculogenesis in mice, such as angiostatin and endostatin. It is now clear that broad-spectrum inhibition of metzincins is inefficient for effective pharmaceutical intervention in pathological conditions since proteolytic products of MMPs or other metzincins are responsible for physiological processes. It is generally acknowledge that the bulk of inhibitor design efforts need to focus on the improvement of inhibitor selectivity index in order to obtain specific tools for probing metzincin function and develop drug-candidates with better chances in clinical applications. The design of such inhibitors is a difficult task given the conserved active-site topology and among MMPs. However efforts in this direction have already shown results, as a Johnson and colleagues have recently developed a highly selective inhibitor for MMP-13 that effectively reduces cartilage damage *in vivo*. Interestingly rats do not develop musculoskeletal syndrome side effects. This highly selective inhibitor MMP-13 in may overcome the major safety and efficacy challenges that have limited previously tested non-selective MMP inhibitors (Johnson, Pavlovsky et al. 2007).

Characteristic and therapeutic possibilities of endogenous inhibitors, TIMPs, are discussed in a dedicated paragraph.

1.3.3 A Disintegrin And Metalloproteinases with Thrombospondin Motifs

ADAMTSs family of zinc proteins counts 19 members but only 5 were shown to degrade tissues proteoglycans, such as brevican, versican, decorin but also COMP and fibromodulin (Matthews, Gary et al. 2000, Liu, Kong et al. 2006, Liu, Kong et al. 2006, Apte 2009). It has been shown that some ADAMTSs are responsible of the aggrecan cleavage in arthritis, although with great difference in activity, thus some of them are also referred to as aggrecanases: ADAMTS-1, ADAMTS-4, ADAMTS-5, ADAMTS-8, and ADAMTS-9 (Tortorella, Burn et al. 1999, Kuno, Okada et al. 2000). ADAMTSs structure is modular, they consist of: a proteinases domain containing the signal peptide, the pro-peptide and the catalytic site; an ancillary domain consisting of disintegrin-like module, a central TSR (thrombospondin repeat), a cysteine-rich module, a cysteine-free spacer, and a variable number of additional TSRs (Nagase and Kashiwagi 2003, Apte 2004). ADAMTSs share a conserved catalytic domain containing the consensus HEBxHxBGBxH motif, but differ in the ancillary domain, thus the latter plays a critical role in directing these enzymes to their substrates. Moreover C-terminal domain, that may contain thrombospondin motifs, interacts with the cell surface and the ECM (Porter, Clark et al. 2005), but it was also shown to promote association with N-TIMP-3, and thus contribute to the regulation process via endogenous inhibitors (Troeberg, Fushimi et al. 2009).

Aggrecanases are the main proteinases responsible for aggrecan cleavage in the early events of cartilage remodelling, and their activity is considered a hallmark of cartilage degradation during inflammatory joint diseases like OA. While Little and colleagues have shown that mice deficient for ADAMTS-1 are not protected from experimental (Little, Mittaz et al. 2005), it was demonstrated that mice deficient in ADAMTS-5 (but not ADAMTS-4) are protected from early aggrecan loss and cartilage erosion in inflammatory and non-inflammatory models of arthritis (Glasson, Askew et al. 2005, Stanton, Rogerson et al. 2005). However was also shown that Adamts5 null mice cartilage explants if stimulated still release aggrecan neo-epitopes, suggesting that ADAMTS-4 is potentially able to digest aggrecan (East, Stanton et al. 2007). Although some studies suggested that human ADAMTS-5 is substantially more active than ADAMTS-4 (Gendron, Kashiwagi et al. 2007, Song, Tortorella et al. 2007), it is still not clear which

aggrecanase plays the main role in pathological cartilage degradation. As all the matrix-degrading enzymes, also aggrecanases expression in tissues must be a regulated process. Experimental evidence pointed out that while ADAMTS-4 can be up-regulated by IL-1b, TNF-a and TGF-b (Arai, Anderson et al. 2004, Song, Tortorella et al. 2007, Rogerson, Chung et al. 2010), ADAMTS5 mRNA levels seem quite stable and independent from cytokines. Another interesting point is that ADAMTS4 expression seems to be quite ubiquitous among all tissues, while ADAMTS5 is restricted to few tissues. Vankemmelbeke and colleagues have shown that active ADAMTS-5 is expressed in osteoarthritic human synovium, suggesting that this tissue could contribute to articular cartilage destruction (Vankemmelbeke, Holen et al. 2001). *In vitro* studies also evidenced that ADAMTS-5 is almost a thousand time more active than ADAMTS-4 (Gendron, Kashiwagi et al. 2007). Based on these experimental evidences it is still debating if cytokine blocking agents may be a useful tool to alt aggrecan loss derived from aggrecanases-dependent activity. Current studies however suggest that inhibition of both ADAMTS-4 and ADAMTS-5 may be required to achieve maximum drug efficacy in treating OA. Moreover some researchers speculated that ADAMTS inhibitors that block the progression of OA might be more successful than inhibitors that block the initial stages (Cooper, Snow et al. 2000). This is because many patients with early stage OA remain stable for long periods of time and some do not progress at all (Hunter and Felson 2006, Smelter and Hochberg 2013). Recently, AGG-523, an ADAMTS-4/-5 inhibitor developed by Wyeth, was shown to reduce the release of aggrecanase-generated aggrecan fragments into rat joints and has advanced to Phase I clinical trials for the treatment of OA (Chockalingam, Sun et al. 2011).

1.3.4 Tissue inhibitors of metalloproteinases

Four TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) molecules are present in vertebrates and their expression is regulated during development and tissue remodelling (Brew and Nagase 2010). All mammalian TIMPs have two distinct domains, a N-terminal domain of about 125 amino acid residues and a C-terminal domain with about 65 residues; the conformation of each domain is stabilized by three disulphide bonds (Williamson, Marston et al. 1990). The four human

TIMPs share about 40% of identity in sequence to each other. The mechanism by which TIMPs inhibit metalloproteinases was suggested by Gomis-Rüth and colleagues (Gomis-Ruth, Maskos et al. 1997). In this mechanism two pair of four residues in the N-terminal domain of the inhibitor, linked by a disulphide bond, slots into the active site of the enzyme, chelating the zinc atom, and expelling the water in the pocket. Recombinant forms of the N-terminal domains of TIMPs, designated N-TIMPs, have stable native structures and are fully active as inhibitors of MMPs and some ADAMTSs (Murphy, Houbrechts et al. 1991, Kashiwagi, Tortorella et al. 2001).

The four human TIMPs are broad-spectrum inhibitors of the 23 MMPs found in humans, but there are some differences in specificity among them. TIMP-1 for example is more restricted in its inhibitory range than the other three TIMPs, having a relatively low affinity for the membrane-type MMPs. Besides TIMP-3 by far has the broadest activity among all TIMPs; in addition to all the MMPs it inhibits also several ADAMs (ADAM-17 and ADAM-10) as well as aggrecanases (ADAMTS-4 and ADAMTS-5) (Amour, Slocombe et al. 1998, Amour, Knight et al. 2000, Kashiwagi, Tortorella et al. 2001). Kinetic studies indicates that TIMP-3 is a better inhibitor for ADAM-17 and aggrecanases than for MMPs. ADAM-17, also known as TACE (tumor necrosis factor- α converting enzyme) catalyses the release of soluble TNF- α from its membrane-bound precursor, which was shown to be able to increase ADAMTS-4 expression. Being able to inhibit both ADAM-17 and aggrecanases, TIMP-3 plays an important role in regulating inflammatory processes, and targeting this molecule may lead to affect the progression of pathological processes such as cancer, rheumatoid arthritis and osteoarthritis (Murphy and Nagase 2008, Rocks, Paulissen et al. 2008). While TIMP-2 and TIMP-4 do not contain carbohydrates, TIMP-1 and TIMP-3 are glycoproteins; as result TIMP-3 binds tightly to the sulphated glycosaminoglycans of the ECM (Yu, Yu et al. 2000). This feature both affects the protein behaviour but also makes it very difficult to handle *in vitro*, and particularly to purify or extract it from cartilaginous tissue. Although human and mouse TIMPs share an elevated identity in structure, they present a different glycosylation pattern.

TIMPs are natural inhibitors of MMPs, ADAMs and ADAMTSs, that have been linked to multifarious diseases in humans including cancer, rheumatoid arthritis, osteoarthritis, heart and kidney diseases, thus they are attractive target for potential clinical applications. An approach to the therapy of metalloproteinase-related diseases is the use of engineered TIMPs with restricted inhibitory specificities.

1.3.5 TIMP-3

Because of its broad inhibitory spectrum has been extensively studied and it has been indicates as the major regulator of metalloproteinase *in vivo*. Timp3 null mice, generated by Khokha and colleagues, have been shown to suffer of air space enlargement in lungs (Gill, Pape et al. 2003) and enhanced apoptosis of mammary epithelial cells after weaning (Fata, Leco et al. 2001), compared to Timp1 null mice and Timp2 null mice, which do not exhibit obvious abnormalities unless those animals are challenged. Timp3 null mice also suffer of chronic hepatic inflammation and are affected by impaired liver regeneration (Mohammed, Smookler et al. 2004), cardiomyopathy and abnormal vascularization with dilated capillaries (Fedak, Smookler et al. 2004, Janssen, Hoellenriegel et al. 2008). Sahebjam and colleagues have shown that mice lacking Timp3 gene suffer of abnormal collagen and aggrecan degradation that increase with aging (Sahebjam, Khokha et al. 2007). Mutations in the TIMP3 gene in human are associated with Sorsby's fundus dystrophy, an autosomal-dominant disease that causes blindness due to macular degeneration. Mutated TIMP-3 seems to acquire enhanced stability in the extracellular space, affecting the turnover of the surrounding matrix (Majid, Smith et al. 2007). Interestingly all the mutations are found in the C-terminal domain producing either a single amino-acid substitution or a nonsense mutation (Qi, Ebrahim et al. 2003).

1.3.5.1 TIMP-3 as potential target in human diseases

Application of TIMPs as a therapeutic tool for gene therapy or direct protein application is a promising but still not fully developed approach (Baker, Edwards et al. 2002). However, there is a clear potential for the application of TIMPs as endogenous inhibitors, especially because the results of clinical trials with small

molecule inhibitors failed to provide appreciable results. However, expressing wild-type TIMPs could have drawbacks because multiple MMPs may be inhibited, and in the case of TIMP-3, ADAMs and ADAMTSs may be inhibited as well. A promising route to success is the development of engineered TIMPs with altered specificity, to allow targeting of specific proteinases. To date one of the best results was achieved by Wei and colleagues adding an extra alanine residue to the N-terminus of TIMP-3 (Wei, Kashiwagi et al. 2005). The synthetic mutant so generated ([⁻¹Ala]N-TIMP-3) was shown to retain ability to inhibit ADAM-17, but it impaired the inhibition of MMPs. Further studies by Lim and colleagues demonstrated that this synthetic mutant was still able to block ADAMTS-4 and -5 *in vitro* (Lim, Kashiwagi et al. 2010).

1.4 Osteoarthritis

1.4.1 Overview

OA is the most common joint disease (arthropathy) and is characterized by joint pain and loss of joint function (Hunter and Felson 2006). Although it was previously described as a non-inflammatory arthritis, the use of new diagnostic technique as MRI, led to understand that inflammation is very common within the OA joint. In particular inflammation of the synovial lining of the joint (synovitis) is almost ubiquitous in knees affected by OA (Conaghan, Felson et al. 2006)

Evolution of OA leads to loss of articular cartilage, subchondral bone thickening and new bone formation in a synovial joint. Osteoarthritis affects approximately 15% of the total population and greater than 50% of the population over 60 years of age (Poole, Kobayashi et al. 2002). OA represents an enormous socio-economic challenge, with millions of people affected worldwide. The aging of the population, coupled with epidemic increases in other risk factors such as obesity, suggests that the impact on society of OA-related problems will continue to rise (Lawrence, Felson et al. 2008). Severities of pain and loss of cartilage are highly variable among patients, affecting the therapeutical approach. To date drug therapy options are currently limited to palliative treatment with analgesics and NSAIDs (Non-steroidal anti-inflammatory drugs) (Berenbaum 2008). Such

treatment usually lasts until loss of function of the joint, when the only option is partial or total prosthetic replacement.

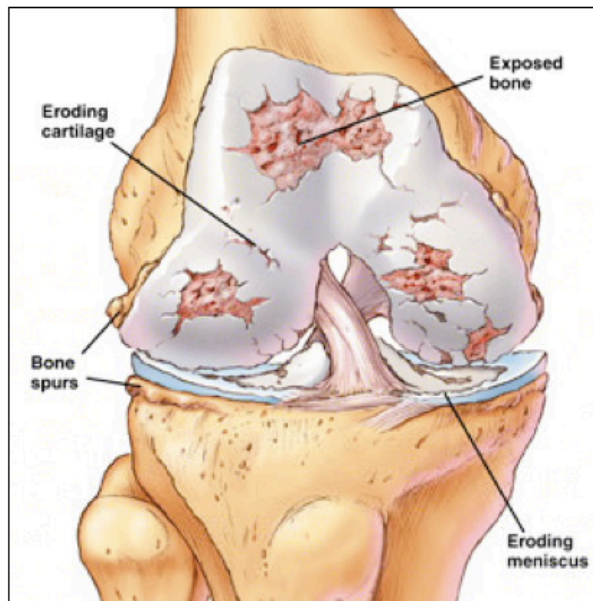


Figure 1.5. Osteoarthritic changes in the anterior of the right knee. OA progression results in erosion of cartilage and exposure of the underlying bone (<http://www.christchurchosteopathy.co.uk/018.html>).

1.4.2 Clinical aspects of osteoarthritis

Because cartilage lacks neural connections, symptoms of OA often do not present until late stage disease. Osteoarthritis can be subdivided as either idiopathic (of an unknown cause) or secondary (caused by another disease or condition). Physical symptoms upon examination of osteoarthritic joints include: pain, decreased range of motion, effusions and tenderness on palpation. Patients may describe localised pain on exercise (which is relieved by rest) and stiffness during periods of inactivity.

The diagnosis of OA revealed to be a quite difficult task and many efforts are made towards the prediction of OA. To date diagnosis is currently based on radiographic criteria (e.g., joint space width) and clinical symptoms (e.g. pain and loss of function). However, the limitations of radiography (e.g. technical issues, precision and sensitivity) (Hunter and Guermazi 2012) have led to research into other biomarkers for monitoring osteoarthritis. Imaging markers such magnetic resonance may be useful tools in the evaluation of OA and in drug development in the field (Eckstein, Le Graverand et al. 2011, Eckstein and Wirth 2011).

A promising outcome is the use of quantitative magnetic resonance imaging (MRI) of the joint structure to detect minute changes in cartilage structure, and thus predict the joint pathogenesis (Hunter, Li et al. 2007, Dam, Loog et al. 2009, Binks, Hodgson et al. 2013). Molecular changes are obviously difficult to detect, because cartilage undergoes continuous remodelling, a process that can hide pathological changes. However, widespread use of MRI is limited by cost, availability and the absence of a validated international score. Another attractive alternative is the measurement of biochemical markers in blood, urine or synovial fluid samples, which could reflect the metabolic changes of the joint and therefore hint to disease progression. To date the biomarkers with the most consistent evidence appear to be those at the end of the pathways of tissue destruction. Belong to this group the C-terminal telopeptide of collagen type II (CTX-II) and Cartilage oligomeric matrix proteins (COMPs) (Posey and Hecht 2008, Sowers, Karvonen-Gutierrez et al. 2009). Pharmacological therapy for osteoarthritis relies on pain relief-drugs such as paracetamol, non-steroidal anti-inflammatory drugs (NSAIDs), cyclooxygenase 2 (COX-2) inhibitors and opioids. Surgical intervention for joint replacement is used in advanced stages of OA (Conaghan, Dickson et al. 2008). There are no currently available disease-modifying osteoarthritis drugs. Trials to regulate endogenous catabolic factors by administering proteinases inhibitors against MMP-13 and ADAMTS-5 targets have been unsuccessful because of insufficient efficiency and adverse events (Little, Meeker et al. 2007, Fosang and Little 2008, Troeberg, Fushimi et al. 2008, Flannery 2010).

1.4.3 Aetiology of osteoarthritis

Aetiology of Osteoarthritis is still a controversial topic. The variability of patients and progression of the condition cannot be explained with a single model, and thus should be addressed as a multifactorial pathological condition.

OA undoubtedly has a genetic element as has been demonstrated in sibling studies, familial aggregation and twin pair studies; however it is clear that occurs a complex interaction between genetic and environmental factors in OA aetiology, with susceptibility differing according to gender, age and quality of life. Susceptibility to OA appears to be influenced by a wide number of genetic

variations (as proved by several mouse transgenic or knockout models). Mutations in genes that encode for cytokines such as IL-1 and IL-6, and polymorphisms in cytokine and metalloproteinase genes are correlated to onset of OA. Mechanical stress, obesity, aging and gender are now generally accepted as risk factors associated to the development and progression of osteoarthritis (Buckwalter and Mankin 1998, Felson 2005).

Some studies pointed toward a connection between high levels of physical activity and incident of knee OA. Anterior cruciate ligament (ACL) or meniscal tissue damage also results in increased risk of developing OA (Englund 2004, Sharma, Eckstein et al. 2008). However, a link between low-impact recreational exercise and OA is controversial. Obesity is a well-established risk factor for OA (Sowers and Karvonen-Gutierrez 2010). In line with the upward trend in mean population Body mass index (BMI), the prevalence of OA is also increasing. Longitudinal data indicate that two in three obese adults will develop symptomatic OA in their lifetime (Murphy, Schwartz et al. 2008). The mechanisms for how obesity influences OA are unclear and a possibility is that increased body mass, increases mechanical stress placed on joints (Wluka, Lombard et al. 2013). However it is known that adipose tissue produces adipokines (such as leptin) as well as inflammatory cytokines. Leptin is found in synovial fluid, chondrocytes and osteophytes in osteoarthritic knees, with levels related to those of insulin-like growth factor-1 (IGF-1) and transforming growth factor- β (TGF- β) (Dumond, Presle et al. 2003). In the leptin-impaired mouse model of OA, obesity did not increase the prevalence of OA (Griffin, Huebner et al. 2009). It has been reported that knee-injection of leptin up-regulates expression of some collagenases and aggrecanases, and is associated with depletion of proteoglycan in rat (Bao, Chen et al. 2010). In summary, excessive wear and tear through joint overuse, obesity, joint instability and finally gross damage to the articular cartilage surface are all strongly associated with the development of OA.

It is now well accepted that aging is correlated to OA onset (Loeser 2013). OA is rare in adults under 40 and prevalence rates of radiographic OA rise steeply after the age of 50 in men and age 40 in women (Felson, Zhang et al. 1995). Gender is also a well-recognised risk factor in OA. Evidences suggest an association

between endogenous oestrogen and cartilage turnover and radiographic OA, and between testosterone and cartilage volume. There is also evidence for an association between exogenous oestrogen and cartilage and bone turnover (Tanamas, Wijethilake et al. 2011). These data suggest oestrogen have a chondro-protective role against OA. A study of cartilage volume in postmenopausal women reported use of long-term oestrogen replacement therapy (ERT) had significantly greater cartilage volume than controls and ERT may prevent knee articular cartilage degradation (Wluka, Davis et al. 2001).

1.4.4 GWAS studies on OA

As previously mentioned osteoarthritis is referred as a complex disease and as such has to be investigated. OA is a set of disorders of the musculoskeletal system characterized by degradation and loss of articular cartilage in synovial joints. OA development appears to be a result of a complex set of interactions between mechanical, biological, biochemical, and molecular factors that destabilize the normal coupling of degradation and synthesis of articular cartilage chondrocytes and extracellular matrix, and subchondral bone. A substantial genetic component for OA has been demonstrated by studies on twin pair, sibling and families. Those studies highlighted that OA is transmitted in a non-Mendelian manner, which is typical of multifactorial diseases.

In the last decade we assisted to a revolution on the investigation of large genomes enabled by the advent of Next generation sequencing (NGS) techniques (Behjati and Tarpey 2013).

This has enabled identification and tagging of single nucleotide polymorphisms (SNPs), non-redundant markers responsible of the majority of common variations across the genome, which led to the development of high throughput genotyping platforms in which hundreds of thousands of SNPs can be concurrently examined for association with pathologies. This new approach was successfully applied to several common complex diseases and traits,¹⁴ leading to the identification of numerous novel associations between genomic variation and disease. OA was relatively late to enter the genome-wide association scans (GWAS) era but recently a very broad study performed by the arcOGEN consortium successful led to the discovery of five loci with genome-wide significant association and of

three near this level (Apte, Hayashi et al. 1994). They identified five genome-wide significant loci for association with osteoarthritis and three loci just below this threshold. The most significant signal was on chromosome 3 where two SNPs were found in perfect linkage disequilibrium (LD) with each other: rs11177, a missense polymorphism within exon three of *GNL3* and rs6976 situated in the 3' untranslated region (UTR) of the *GLT8D1* gene. These SNPs were found consistent in both OA types investigated (knee and hip). The potential role of those two genes in OA is however still unknown. Interestingly another SNP identified was the rs8044769. This SNP was found associated with OA in females. It is located within intron 1 of FTO (fat mass and obesity associated) gene. Variation in the FTO gene is known to play an important role in susceptibility to obesity (Fawcett and Barroso 2010) and overweight/obesity is a well established risk factor for OA susceptibility and it is also a predictor for OA progression, especially of the knee joint (Lohmander, Gerhardsson de Verdier et al. 2009). An earlier study conducted by Miyamoto and colleagues on Japan population identified the allelic variation rs143383 affecting the GDF5 gene in hip OA patients (Miyamoto, Mabuchi et al. 2007). Gdf5 was shown to have multiple functions in mouse skeletogenesis, including roles in joint and cartilage development (Storm and Kingsley 1999).

1.4.5 Cartilage modifications in the OA pathogenesis

Macroscopically, OA cartilage is often yellowish, compared to the whitish of the normal cartilage, typically soft and swollen. Histological changes of osteoarthritis are well characterised. Initially there is an progressive loss of proteoglycan, the articular cartilage surface loses its smooth appearance, and fibrillation starts to appear (Meachim, Ghadially et al. 1965). As disease progress fissures opens in the cartilage starting from the middle zone and advancing inward towards the calcified cartilage (McElligott and Collins 1960). Safranin-O staining of cartilage proteoglycans would show a decrease of red colour until complete white staining, matching the complete loss of proteoglycans. Chondrocytes behaviour is peculiar because cells are seen to form clusters and proliferate, as an attempt to regenerate the tissue (Meachim, Ghadially et al. 1965, Meachim and Sheffield 1969). However a progressive cell death is observed, via apoptosis, which leads

to a hypocellularity of the tissue. Cartilage is now lost, subchondral bone is exposed and the whole joint is no functional anymore. The grading system most commonly used (partly with minor modifications) is the histochemical-histologic grading system designed by Mankin and co-workers in 1971 (Mankin, Dorfman et al. 1971).

1.4.6 ECM modifications in the OA pathogenesis

The destruction of articular cartilage and the loss of its biomechanical function are largely due to the destruction and loss of the territorial cartilage matrix. Our knowledge focuses on degradation processes of the two major components of the interterritorial ECM, the collagen network and the interwoven proteoglycan aggregates. Loss of aggrecan and its negative charges is characteristic of the early stages of cartilage degeneration, whereas the overall content of collagen remains rather constant nearly throughout the disease process. Still, loosening of the collagen network is a major feature also in early cartilage degeneration. So far, it is unknown what happens first if the loss of proteoglycans or the loosening of the collagen network, because each one eventually influences the other as well. Loosening of the collagen network leads to a loss of proteoglycans, and a loss of proteoglycans leads to a mechanical overload and, thus, damage and loosening of the collagen network. In particular, the latter appears to be responsible for the hyperhydration of articular cartilage in the early phases of the disease process, macroscopically visible as softening and swelling of the OA articular cartilage. Degradation processes appear to be specifically prominent in the surface zone and around the chondrocytes in OA cartilage. It has been suggested that the earliest events in OA is the loss of aggrecan core protein from the cartilage ECM, and only after this happens the proteolysis of collagen fibres. However chondrocytes continuously synthesise aggrecan, turning the first event more difficult or rather reversible. Loss of collagens however is considered permanent because, despite repair attempts, the original fibrous collagen network cannot be restored (Karsdal, Madsen et al. 2008). Some study suggested that although broken, proteoglycan fragment could still be maintained in the place by the collagen structure, and thus maintaining the physiological property of the

cartilage. Only when collagens are lost these molecules can be washed away and impair the cartilage physiology.

In OA, it is believed that there is an increase in matrix degradation through increased production of proteinases (ADAMTSs, MMPs), increased proteinases activity (increased cleavage of pro-enzymes) or decreased activity of catabolic regulators, TIMPs. According to this perspective, researches focused on mRNA levels of catabolic enzymes in human cartilage samples from late-stage OA donors has shown an increased expression of MMP1, MMP2, MMP9, MMP13, ADAMTS4 and ADAMTS5 in late stage OA cartilage (Bau, Gebhard et al. 2002, Aigner, Zien et al. 2003).

It has been shown that aggrecan neoepitope, produced by both MMPs and ADAMTSs activity, are found in culture media of cartilage explants *in vitro* as well as human synovial fluid *in vivo* suggesting elevated aggrecanase/MMP levels of activity (Fosang, Last et al. 1996, Lark, Bayne et al. 1997, Sandy and Verscharen 2001, Little, Hughes et al. 2002). Little and colleagues, in a quite elegant study, generated an aggrecan knock-in mouse with a mutation between the G1 and G2 domains obtaining aggrecan molecule resistant to aggrecanase activity. This knock-in mutation significantly inhibited proteoglycan loss, diminished cartilage degradation and promoted cartilage repair (Little, Meeker et al. 2007). Adamts5 knockout mice are significantly resistant to IL-1 α and retinoic acid induced aggrecan cleavage as well as surgically induced cartilage destruction, compared with wild-type mice (Glasson, Askew et al. 2005). However no protection towards OA was observed in Adamts4 knockout mice after surgical-induced OA. Because this evidences Adamts5 is believed to be the most important aggrecanase in mouse cartilage (Glasson, Askew et al. 2004). Interestingly Song and colleagues demonstrated that the depletion of either ADAMTS5 or ADAMTS4 using siRNA in human cartilage explants inhibits proteoglycan release when explants were stimulated with tumor necrosis factor- α (TNF α) and oncostatin M (OSM). This suggests that despite the dominant role of both ADAMTS-4 and ADAMTS-5 may contribute towards cartilage destruction in human osteoarthritis (Song, Tortorella et al. 2007). It has been recently shown that MicroRNA-125b regulates the expression of ADAMTS4 in human

osteoarthritic chondrocytes, making this MicroRNA a possible therapeutic target for OA (Matsukawa, Sakai et al. 2013).

It has been shown in mice and in human cartilage explants that pharmacological or genetic inhibition of HH signalling reduces the severity of OA and that runt-related transcription factor-2 (RUNX-2) potentially mediates this process by regulating ADAMTS5 expression. These findings raise the possibility that HH blockade can be used as a therapeutic approach to inhibit articular cartilage degeneration (Lin, Seeto et al. 2009). Moreover increased expression of RUNX2 in OA cartilage may contribute to increased expression of MMP-13 (Wang, Manner et al. 2004).

MMP-13 is thought to play a critical role in cartilage destruction being more active against type II collagen than other collagenases and chondrocyte expression of MMP13 mRNA is increased in late-stage human OA cartilage in association with cartilage erosion (Aigner, Zien et al. 2001). Constitutively overexpression of active human MMP13 in hyaline cartilage of transgenic mice resulted in OA-like cartilage modification at load bearing sites, similar to human OA including proteoglycan and type II collagen degradation (Neuhold, Killar et al. 2001). Little and colleagues demonstrated that the cartilage of *Mmp13^{-/-}* mice is protected from structural cartilage damage in a surgically induced model of OA, however there was no significant reduction in aggrecanolysis, osteophyte development or chondrocyte hypertrophy (Little, Barai et al. 2009). *Mmp13^{-/-}* mice were able to inhibit cartilage degradation in the presence of aggrecan loss; this may suggest that aggrecan depletion on its own does not drive cartilage degradation.

1.4.7 Mouse models of osteoarthritis

One of the reasons of the lack of knowledge in the osteoarthritis pathology is that sample tissues from patients are usually collected late in the disease process, when most of the cartilage tissue is already destroyed. A second reason is that OA is a slow progressive disease, and it may take years before a positive diagnosis. These evidences explain the major difficult to decide which animal model better represent the OA pathology. As a matter of fact, experimental animal will develop spontaneously OA-like cartilage erosion if left aging until

elderly. However this may take years, making it incompatible with research timeframe. For this reason were developed system that could induce osteoarthritic phenotype in experimental animals, in shorter period of time. Notably researchers identified in the STR/ort mice a model of spontaneous OA, where characteristic cartilage lesions are evident by 6 months of age (Mason, Chambers et al. 2001). This model although resembling the observed variability of the human disease is poor characterized at the molecular level, resulting in a too complex system. Some insight however suggested an important role in cartilage destructions played by reactive oxygen species (ROS) (Regan, Flannelly et al. 2005). An alternative arose with the development of surgical-induced OA models. These models can be applied in a variety of different animals: rats, dogs and mice (Kamekura, Hoshi et al. 2005, Bove, Laemont et al. 2006). However is the elective species for this kind of studies because relative lower cost of colonies maintaining, and because mouse is the primary species for the generation of transgenic animals, both knock-out and over-expressing. Glasson and colleagues demonstrated that severity of surgical-induced OA lesions and rate of the destruction progression can be affected by the type of surgery. For example anterior cruciate ligament transection (ACLT) produced a much more severe OA phenotype compared to the destabilization of the medial meniscus (DMM) surgery. In both system the destabilization of the joint structure, led to friction of the cartilage surface, and progressive erosion of the tissue. The DMM model was shown to provide extremely good reproducibility and a slower progression of disease, resembling more the human OA, and thus was elected as method of choice for many studies. Most of the damage in DMM model is concentrated in the medial tibia plateau and femoral condyles, while the laterals are relatively unaffected. Cartilage damage is evaluated according to the OARSI (Osteoarthritis Research Society International) Histopathology Initiative system that has been recently published (Glasson, Chambers et al. 2010). Most importantly Glasson and colleagues demonstrated that after DMM surgery male mice develop a more severe OA than females, but also ovariectomized female are more protected against OA compared to intact females. These findings suggest that hormones play a critical role in the progression of OA in the murine DMM surgical model (Ma, Blanchet et al. 2007). It has been observed that if joints are immobilized by

sciatic neurectomy, after DMM surgery, the regulation of selected genes, including Adamts4 and Adamts5, was abrogated and OA was prevented up to 12 weeks post surgery (Burleigh, Chanalaris et al. 2012). However a disadvantage of the surgical models is that only knee damage can be studied. Osteoarthritis can be induced in laboratory animals also by intra-articular injection of modifying substances such as collagenase, that some authors describe as more resembling the human disease compared to surgically induced instability. Knee injection of mono-iodoacetate, to alter chondrocyte metabolism, is another method that produces OA-like morphological changes in mice knees (Guingamp, Gegout-Pottie et al. 1997, Bove, Calcaterra et al. 2003). Finally Poulet and colleagues have recently characterised a non-surgical model for controlled mechanical loading of murine knees that may help to identify the contribution made by precise mechanical factors to OA (Poulet, Hamilton et al. 2011). In this model peaks loads of 9N are applied through natural points of articulation, with the contralateral joint experiencing normal gait between loading episodes, providing an internal control. An evident advantage of this model is the non-invasiveness that reduces the risk of infections, and thus of an immune response affecting the articular cartilage itself.

1.5 Transgenic mice

1.5.1 Overview

Transgenic animals for research are extensively used to study *in vivo* gene function as well as to model human diseases. The technology for producing transgenic animals exists for a variety of vertebrate and invertebrate species such as: pig, rabbit, rat mouse, zebra fish, drosophila and nematode. Choosing the best animal model usually rely on previously published literature as well as the level of complexity that can be afforded. Research on epigenetic and molecular biology mechanism is often carried out in drosophila, which require minimum colony space, relatively easy handling and has short time of reproduction. Mice are often employed for drug delivery studies, as well as model of human genetic diseases, but require relatively higher cost in colony maintaining and handling.

The most commonly used techniques for producing transgenic mice involves either the pronuclear injection of transgenes into fertilized oocytes or embryonic stem cell-mediated gene targeting. Embryonic stem cell technology has been most often used to produce null mutants (knock-outs) but may also be used to introduce exotic genes (knock-ins) or modifications down to the level of making single nucleotide changes in endogenous mouse genes. Methods are also available for inducing conditional gene knockouts as well as inducible control of transgene expression. Working with transgenic and knockout mice can be challenging and is often hampered by limitations and technical problems. The most common limitations involved construction of the transgene, generation and characterization of founder lines, and maintenance of a transgenic colony.

1.5.2 Cre/loxP system

The most common method to alter gene expression in a conditional manner is the use of the Cre/loxP system. Cre is an enzyme found in the P1 bacteriophage that acts as a site-specific recombinase; it recognizing a short sequence of DNA called a loxP site. A loxP site is a consensus 34-bp DNA sequence that is not present in the mouse genome and has directionality. Cre-mediated recombination of genes flanked by loxP sites (also called a floxed sequence) can result in the excision, inversion, or translocation of DNA depending upon the location and orientation of the loxP sites. The most common use of Cre recombinase is to excise or delete a floxed DNA sequence (Nagy 2000). Cre-mediated recombination has been shown to occur in a variety of cell types (Sauer 1998, Feltri, D'Antonio et al. 1999) and that only a few Cre molecules per cell are needed to excise the floxed DNA. Cre recombinase mediates a permanent deletion of the floxed DNA, and if cell division occurs after Cre-mediated recombination, all daughter cells will inherit this gene deletion.

To allow for cell type-specific control of gene deletions, mouse models have been created where Cre expression is controlled by a cell type-specific promoter. Crossing them with mouse lines containing a relevant part of a target gene enclosed in loxP sites allows obtaining cell-type specific deletion. Cre-loxP system can be employed to induce the overexpression or ectopic expression of a target gene. In this case, a construct containing a promoter and a floxed “stop”

sequence upstream of target gene or coding sequence, is inserted into the genome; thus, only Cre-positive cells are able to remove the “stop” sequence and overexpress or ectopically express the gene (Gao, Wu et al. 2004).

To gain temporal control of gene expression, the ligand-binding domain of an engineered oestrogen receptor is fused to the Cre enzyme and the Cre molecules are sequestered in the cytoplasm by heat shock protein 90 (Hsp90), keeping Cre inactive. The translocation of the Cre/oestrogen receptor fusion protein to the nucleus, which allows Cre to become active, only occurs in a ligand-dependent manner, thus inducible (Feil, Brocard et al. 1996, Hayashi and McMahon 2002). Improvement to prevent Cre activation by endogenous steroid hormones, leads to introducing specific point mutations that make it insensitive to endogenous oestrogen while retaining binding affinity for synthetic analogues such tamoxifen.

1.5.3 Cartilage specific expression

Many attempts had been made to obtain chondrocytes specific expression both *in vitro* and most importantly *in vivo*.

The study of chondrocytes gene expression in mouse led to identifying several promoters to target this cell type. Such targeted promoters span from transcription factors implicated in early commitment or maintenance of chondrocyte phenotype, as well as genes responsible for structural maintenance of articular cartilage. The former ones include Prx1-Cre (Logan, Martin et al. 2002), Dermo-Cre (Yu, Xu et al. 2003), Gdf5-Cre (Rountree, Schoor et al. 2004), Sox9-Cre (Akiyama, Kim et al. 2005); to the second group belong the Col10a1-Cre (Gebhard, Hattori et al. 2008), and Col11a2-Cre (Fujimaki, Hayashi et al. 2005).

One important notion is that chondrocytes in adult mice could be found at different differentiation stages, so the choice of promoter can determine whether the expression be restricted to a specific subset of cells. Collagen type II and type X promoters were mostly used in studies aimed to proliferative and hypertrophic chondrocytes respectively. More recently the use of Aggrecan promoter/enhancer

has been exploited and revealed itself as the best system to target non-hypertrophic, mature chondrocytes.

Collagen type II is an early marker for chondrogenesis and is expressed from embryonic day E9.5 in mouse in mesenchyme destined to develop in sclerotome of the somites. The collagen type II gene (*Col2a1*) is expressed in all chondrogenic tissues of the axial and appendicular skeleton until onset of endochondral ossification. However, it is also expressed transiently at low levels in certain non-chondrogenic tissues, including notochord, eye, heart, epidermis and discrete areas of the brain (Cheah, Lau et al. 1991). First Zhou and colleagues in 1995 investigated chondrocyte specificity of the *Col2a1* promoter in murine embryos (Zhou, Garofalo et al. 1995). They initially identified a large 6 kb sequence, spanning from putative promoter region to exon 1, which was able to drive β -galactosidase expression in chondrocytes. Following deletion studies revealed that a 182 bp fragment within intron 1 was sufficient to target expression in chondrocytes even using a different promoter, although it showed additional expression in the brain (Leung, Ng et al. 1998). Lefebvre and colleagues in 2008 shown that four copies of a 48 bp intron 1 fragment were enough to drive strong expression in chondrocytes, when linked to a 309 bp *Col2a1* promoter sequence. Their system also drove cartilage specific expression *in vivo*. To date the most used cartilage specific Cre-expressing line is the one generated by Ovchinnikov and colleagues (Ovchinnikov, Deng et al. 2000). They used a 3 kb sequence of the *Col2a1* promoter region and 3.02 kb fragment of intron 1, obtain Cre-recombinase expression early during cartilage development and later in the adult cartilage, however some expression was observed in notochord and cranial mesenchyme as well. A similar mouse line generated by Belteki and colleagues showed high cre recombinase levels in epidermis eye and heart (Belteki, Haigh et al. 2005). In contrast to the vast amount of knowledge on the regulation of *Col2a1* promoter, Aggrecan regulation has been far less researched. Doege and colleagues studied the Aggrecan promoter regulation *in vivo*. They identified cell-specific enhancer elements in a 4.7 kb DNA fragment 12 kb upstream of the transcription start site that strongly stimulated Aggrecan expression in chondrocytes (Doege, Hall et al. 2002). Han and Lefebvre

identified in the same 4.7 kb region a highly conserved region directing specificity of Aggrecan expression in embryonic and adult cartilage (Han and Lefebvre 2008). This region drove expression differently as *Col2a1* reporters, in fact it was not expressed in skeletogenic mesenchymal cells, perichondrium and presumptive joint cells, but was strictly specific for differentiated chondrocytes. Moreover, it remained active through adulthood whereas *Col2a1* was less active upon ageing. This would make this Aggrecan enhancer region an interesting tool for expressing transgenes in differentiated chondrocytes in adulthood. Finally in 2009 Henry and colleagues generated a tamoxifen inducible cre recombinase-expressing mouse, cloning in the enzyme coding sequence downstream the 3' untranslated region of the endogenous mouse Aggrecan gene (Henry, Jang et al. 2009). Aggrecan gene has been shown to be expressed also in other mouse tissues such brain (Domowicz, Sanders et al. 2008).

AIMS

1.a. To design and generate an expression vector driving cartilage specific expression. I will use the sequence identified by Han and Lefebvre (Han and Lefebvre 2008) to generate a vector that drives robust expression of both CreERT² recombinase and Luciferase in chondrocytes.

1.b. To generate transgenic mice as a tool for cartilage specific Cre-mediate recombination *in vivo*. I will use an expression vector driven from the Aggrecan enhancer elements to generate a Cre-deleter.

Hypothesis: The aggrecan enhancer elements are sufficient to drive the expression in chondrocytes. Being the chondrocytes the sole resident cell into cartilage, transgenic expression will be restricted to this tissue only. The transgenic mice line generated will serve as a unique tool to study cartilage development and/or pathologies.

2.a. To generate transgenic mice lines to overexpress TIMPs protein in mouse cartilage. I will use the same Aggrecan enhancer sequences identified before.

Hypothesis: Osteoarthritis is a multifactorial disease affecting a increasing number of people worldwide. Articular cartilage destruction is the key event of this pathology and a predominant role of enzymes that affect the homeostasis of the cartilage protein component is generally accepted. Because most of those enzymes are regulated by TIMPs, I want to overexpress these inhibitors in cartilage to evaluate potential beneficial activities.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 General Reagents

Unless otherwise stated, all chemicals were supplied by Sigma-Aldrich (Dorset, UK) or BDH Chemicals (Dorset, UK) and are of the highest quality. Bovine serum albumin, electrophoresis grade (BSA) was from PAA Laboratories (Pasching, Austria). Phosphate buffered saline (PBS) was obtained as a 10x sterile solution (VWR, Lutterworth, UK). A 1xPBS solution was generated by diluting the 10x stock with ultrapure ddH₂O.

2.1.1.1 *Cloning*

- DH10b Competent cells (Stratagene)
- Luria-Bertani (LB) medium: (Invitrogen)
- Agar (Invitrogen)
- Agarose (Invitrogen)
- 0.5kb DNA Ladder (New England BioLabs)
- 1kb DNA Ladder (New England BioLabs)
- QIAquick PCR purification kit (Qiagen)
- QIAquick gel extraction kit (Qiagen)
- QIAfilter plasmid maxi kit (Qiagen)
- T4 DNA ligase (New England BioLabs)
- T4 DNA Polymerase (New England BioLabs)
- Polymerase I Large (Klenow) Fragment (New England BioLabs)
- Alkaline phosphatase (CIP, calf intestinal, New England BioLabs)
- Restriction enzymes (New England BioLabs)
- Platinum Taq DNA Polymerase (Invitrogen)

2.1.1.2 *Cell culture*

- DMEM (4.5g/l) liquid with Sodium Pyruvate with L-Glutamine (PAA Laboratories)
- FBS (PAA Laboratories)
- Penicillin/streptomycin (PAA)

- Trypsin-EDTA (1x) 0.05% / 0.02% in DPBS (PAA Laboratories)

2.1.1.3 *RT-qPCR*

- High Capacity cDNA Reverse Transcription kit (Applied Biosystems)
- TaqMan® Universal PCR Master Mix (Applied Biosystems)
- ABI PRISM™ 384-Well Clear Optical Reaction Plate with Barcode (Applied Biosystems)
- RNeasy Micro Kit (Qiagen)
- DNase I (Qiagen)
- QIAprep Spin Miniprep Kit (Qiagen)

2.1.1.4 *Immunofluorescence*

- Goat Serum (X0907, Dako)
- antiTIMP-3 Rabbit polyclonal (AB6000, Millipore)
- antiRabbit Goat polyclonal Alexa Fluor® 488 (A-11034, Life Technology)
- DAPI 1:1000 (Sigma)
- ProLong® Gold Antifade Reagent (Life Technology)

2.1.1.5 *Mouse transgenesis*

- Hypnoval (Roche)
- Hypnorm (Janssen)
- Chorulon (Intervet)
- Folligon (Intervet)

2.1.2 Restriction Enzymes

All DNA constructs were digested using Neo England Biolab (NEB) enzymes or Fast Digest® (Fermentas) enzymes. NEB enzymes were allowed to digest the substrate for 4 hours with specific buffers according to the manufacturer specifications working temperature. Digestions employing the Fast Digest® enzymes were performed for 2 hours. All enzymatic digestions were performed in water bath, and using a total volume of 20 µl.

2.1.3 Principal instruments and software

Samples were visualized on an Olympus SZX12 dissecting stereomicroscope with illumination either from below or from a fibre optic light source. Microscope was equipped with 10x/0.40 and 20x/0.70; ∞ /0.17 lenses (Olympus). Images were captured with a digital CCD camera (model QIClick-F-CLR-12) using accompanying software. The 7900HT Fast Real-Time PCR System real-time thermal cycler and software were from Applied Biosystem. Gel Doc XR+ system and Quantity One® software were from Bio-Rad Laboratories. Graphs and statistical analysis were performed using GraphPad Prism 6.0. Neon Transfection system and all buffers related were from Life Technology. Confocal images were acquired using the Ultraview confocal microscope (PerkinElmer Life Sciences) with a 60x objective lens and equipped with a Nikon TE-2000 digital CCD camera.

2.2 **Methods**

2.2.1 Standard manipulation of DNA

2.2.1.1 *Agarose gel electrophoresis*

Digestion products were separated by agarose gel electrophoresis. UltraPure Agarose from Invitrogen was used and percentage was always 1% in Tris-Acetate-EDTA (TAE) buffer, if not otherwise specified. TAE buffer was made as stock solution 50x and diluted to 1x as working solution. To visualize DNA fragment, Ethidium Bromide (EtBr) was added to the agarose solution to a final concentration of 0.3 μ g/ml. Gel electrophoresis was performed using Bio-Rad Wide Mini-Sub Cell GT Systems setting the instrument to 55 V. As molecular weight marker 1kb DNA ladder was used. An UV light trans-illuminator set to 365 nm wavelengths was used to visualize DNA fragment. Exposure was shortest as possible to avoid DNA damage.

2.2.1.2 *DNA recovery*

To recover fragments of interest, the corresponding portion of the agarose gel was cut using a sterile scalpel and transferred into a sterile 1.5 ml tube. DNA was

eluted using QIAquick Gel Extraction Kit according to manufacturer specification. Briefly, sample is dissolved into QG buffer at 50°C, then solution was spun down throughout a column that traps DNA. Column was washed using specific buffer and then DNA eluted in proper volume using Elution Buffer provided or Tris-EDTA buffer.

2.2.1.3 Blunt-ending

In case of blunt-end ligation as final step, digested constructs were treated with DNA Polymerase I Large (Klenow) Fragment, 15 min 37°C, then 20 min 65°C to inactivate the enzyme.

2.2.1.4 De-phosphorylation

Vectors were always de-phosphorylated using Calf Intestine Phosphatase (CIP) 60 min at 37°C.

2.2.1.5 Ligation

Ligations were performed using 20 ng of vector fragment and the amount of insert fragment calculated adjusting for the molar weight; molar ratio insert/vector was usually 3:1. Ligations were performed into a total volume of 20 µl using T4 DNA Ligase. Reactions were performed for 16 hours at 16°C.

2.2.1.6 Transformation into Escherichia coli (E.coli)

Entire ligation product was used to transform competent *E.coli* strain DH10β. The whole ligation product was inoculated into 80 µl of cells suspension and heat shock was performed in water bath at 43°C for 3 min. Then cells were transferred into ice for more than 5 min. Total volume of 100 µl was spread into petri dish coated with agar supplemented with 100 µg/µl ampicillin. Petri dishes were incubated over-night at 37°C. A control ligation without insert was always performed in order to check the vector self-ligation.

2.2.1.7 Plasmid mini- and maxi-preparation and extraction

5 to 10 colonies were usually picked up, and inoculated each into 3 ml Luria Broth (LB) supplemented with 100 µg/µl ampicillin, and incubated 6 hours at

37°C in shaking incubator. Plasmid DNA was extracted using QIAprep Spin Miniprep Kit. Plasmid DNA was re-suspended into 50 µl Tris-EDTA. Enzyme digestion was always performed to check the ligation product as well as sequencing.

2.2.1.8 *Competent cells*

DH10b competent cells for heat shock were prepared growing them in LB agar plate without antibiotic selection overnight at 37°C. Day after one single colony is picked up and inoculate into 2 ml of LB without antibiotic selection, overnight at 37°C. Next day, 500 µl of overnight culture growth are grown into 100 ml LB without antibiotic selection at 37°C, until the OD 600 reaches values between 0.6 and 0.8. Cells are transferred into 50 ml tubes on ice. From this point on, cells must be kept cold at all time. After 1 hour cells are spun down at 3500 rpm, 15 min, 4°C. Pellet is re-suspended in 30 ml ice-cold 100mM CaCl₂ / 15 % glycerol solution and left on ice 1 hour. Than the solution was spun one more time as above and re-suspended in 8 ml ice-cold 100 mM CaCl₂ / 15 % glycerol solution and left on ice 1 hour. Cell suspension is thus transferred into 1.5 ml tube and flash-frozen for 5 min in a mixture of dry ice and 70% Ethanol and stored at -80°C.

2.2.2 Standard tissue cultures procedures

2.2.2.1 *Cells cultures*

HTB-94 cells were used to test the ability of the DNA constructs to express the transgenes *in vitro*. Cells were cultured into DMEM with 10% FCS and 1x Penicillin/Streptomycin (P/S). Cells were kept into incubator providing constant 37°C and 5% CO₂ pressure. Culture medium was usually changed with fresh one every other day. Cells were growth on culture dishes or flasks (Corning Incorporated).

To detach adherent cells from the surface of culture dishes or flasks, trypsin-EDTA was used. Cells were washed two times with sterile PBS. Cells were incubated with trypsin 5 min at 37°C. Complete DMEM was added to neutralize enzymatic activity. Cell suspension was transferred into 15 or 50 ml tube and

centrifuged for 5 min at 300x g. Supernatant was discarded and cell pellet re-suspended into fresh medium and seeded according to experimental plan.

2.2.2.2 *Transfection with plasmid DNA*

HTB-94 cells were transfected by electroporation using the Neon Transfection System (Invitrogen).

Cells were washed twice in sterile PBS and detached using trypsin. Harvested cells were re-suspended in PBS (without Ca^{2+} and Mg^{2+}) after centrifugation and counted. Aliquots of 2.5×10^6 cells each were transferred in sterile 15 ml tube. Cells were centrifuged once more and re-suspended in 225 μl of Buffer R. 50 μg of plasmid DNA were added in a total volume of 25 μl to obtain a final cell density of 1.0×10^7 cells/ml. 100 μl of the cell suspension were loaded in the pipet provided in the kit and electroporated. The best electroporation setting was empirically determined as: 2 pulses of 20 ms at 1000 v. Electroporated cells were transferred in 6 cm dish containing 2 ml of growth medium without P/S. The same tip was used to electroporate another 100 μl aliquot of the cells suspension. Again the transfected cells were transferred in a new 6 cm dish containing medium without P/S. Cells were incubated 24 hours at 37°C.

2.2.2.3 *Transduction with adenovirus*

Adenovirus handling was performed in the specific restricted access “virus room” of the Kennedy Institute of Rheumatology (KIR) and according to the department safety rules.

The adenovirus CMV-Cre was present in the KIR virus archive (#205). It was initially obtained as a gift and expanded by Dr Fred Tam (Imperial College of London). The virus backbone corresponds to the human Adenovirus Type5 (dE1/E3), and is a well-established tool to deliver high efficient recombination in a variety of cells and tissues (Kamei, Ishikawa et al. 2006).

The virus was designed in order to express the Cre recombinase enzyme from the P1 Bacteriophage, modified with a nuclear localization sequence (NLS). A cytomegalovirus (CMV) promoter sequence mediates the gene expression. The virus construct lacks a reporter gene such as Green fluorescence protein (GFP).

After transfection cells were incubated 24 hours and then transferred in the virus room. Cells were washed twice in PBS. Adenovirus was diluted in 1 ml of FCS-free DMEM without P/S in order to have a multiplicity of infection (MOI) of 50 calculated on the number of cells used for the transfection. Infected cells were incubated at 37°C for 2 hours. Cells were washed 3 times in PBS than incubated for 48 hours in serum-free DMEM.

2.2.3 Standard β -galactosidase staining

Fixative solution	0.1 M Na_3PO_4 (sodium phosphate) pH 7.4, 2% formaldehyde, 5 mM EGTA (pH 8.0), 2 mM MgCl_2 , 0.2% glutaraldehyde.
Washing solution	0.1% (w/v) sodium deoxycholate, 0.2% (v/v) NP-40, 0.1 M Na_3PO_4 (sodium phosphate) pH 7.4, 2 mM MgCl_2
Staining solution (in 100 ml water)	184 g potassium ferrocyanide ($\text{K}_4\text{Fe}(\text{CN})_6$), 168 g potassium ferrocyanate ($\text{K}_3\text{Fe}(\text{CN})_6$), 4 ml X-gal substrate solution
X-gal substrate solution	250 mg 5-bromo-4-chloro-indolyl- β -D-galactopyranoside were dissolved in 10 ml dimethylformamide to a final concentration of 25 mg/ml

Table 2.1. Reagents for β -galactosidase staining

All solution were stored at 4°C up to a maximum of one week. X-gal substrate solution was stored at -20°C.

2.2.3.1 *In vitro* β -galactosidase staining (cells)

Cells were washed twice in cold PBS, than incubated in ice-cold fixative solution for 4 min, on ice. Fixative was discarded and cells carefully washed 3 times for 5 min with ice-cold washing solution. Finally cells were incubated at room-temperature over-night staining solution.

2.2.3.2 *In vivo* β -galactosidase staining (embryos)

Pregnant mothers were intra-peritoneal (IP) injected once with 0.5 ml of 10 mg/ml tamoxifen at embryonic day E13.5 or E14.5 and sacrificed 24 hours later. Embryos were harvested and incubated for 30 min in cold fixative solution at room temperature on a rotating shaker. Fixative solution was discarded and embryos were washed for 30 min in washing solution at room temperature on a rotating shaker. This step was repeated 3 times. Embryos were than incubated

over-night in staining solution at room temperature on a rotating shaker. The day after embryos were washed in washing solution and transferred to 10% formalin solution to preserve the tissues.

2.2.3.3 In vivo β -galactosidase staining tissues (adult tissues)

Mice were injected via IP 3 times with 150 μ l of 10 mg/ml tamoxifen and sacrificed 2 weeks afterwards. Several organs and tissues were collected. More in details to hind limbs, they were clean from most of muscular tissues and both cruciate ligaments and tendons were cut in order to expose tibia plateau and femur condyles. Samples were incubated for 90 min in fixative solution at room temperature on a rotating shaker. Fixative solution was discarded and samples were washed three times for 30 min each in washing solution at room temperature on a rotating shaker. Samples were incubated over-night in staining solution at room temperature on a rotating platform. The Day after, tissues were washed in washing solution and transferred to 10% formalin solution to preserve the tissues.

2.2.3.4 Transgenic Mouse analysis

2.2.3.4.1 Collection of embryos from pregnant mothers.

Unless otherwise stated, mice were collected at the E14.5 stage of gestation (14.5 dpc). All the procedures occurred in ice cold PBS. Pregnant mother was sacrificed by vertebra dislocation according to local rules. Once the cadaver was set up supine, the belly was open using sterile forceps, and the uterus isolated. Embryos were removed from the uterus and cleaned from the yolk sack. Embryos were briefly washed in ice-cold PBS and transferred in ice-cold Fixative solution for X-gal staining.

2.2.4 Standard Western Blot analysis protocols

2.2.4.1 Samples collection

Adult 8 weeks-old mice were injected with tamoxifen and sacrificed 2 weeks afterwards. Muscle tissues were removed using a sterile scalpel. Cruciate

ligaments and tendons were cut in order to expose tibia and femur articular cartilage ends. Epiphyses were detached from the long bones inserting the scalpel in the growth plate and twisting. The total of four epiphyses were collected from the hind limbs of each mouse and pulled together. Samples were quickly frozen in liquid nitrogen and stored in -80°C freezer when not used immediately.

2.2.4.2 Extraction of proteins from mouse epiphysis

Samples were pulverized using the freeze-fracturing method and a bio-pulveriser pistol was used. Samples were re-suspended in Tissue Protein Extraction Reagent supplemented with proteinases inhibitors (Pierce). T-PER was used at 10 µl/mg of tissues weight. Tissues suspensions were vortexed for 30 sec and rested in ice. In order to facilitate the proteins extraction, tissues suspensions were sonicated 3 times on a 30sec/30sec on/off cycle in a 4°C water bath. Samples were vortexed for 30 sec and centrifuged at 10000 g for 5 min in a refrigerated centrifuge. Supernatant was collected and transferred in a clean tube. In order to evaluate the protein concentration of the samples the BCA Protein Assay was used according to the manufacturer's instructions.

2.2.4.3 Standard histological analysis protocol

Dissected knee tissues either collected fresh or after X-gal staining, were kept in 10% formalin solution for 48 h. Tissues were decalcified in 10% EDTA solution pH 7.4 for 4 weeks, changing solution every week.

Tissues were then embedded in paraffin wax to be sectioned for histological analysis. The automated, under vacuum tissue processor was set up as follows: tissues were dehydrated sequentially in 80% ethanol for 30 min, 90% ethanol for 30 min, and 100% ethanol 3 times for 30 min. Then they were put in 50% ethanol/ 50% xylene for 15 min, then 100% xylene for other 15 min. Finally, they were placed in molten paraffin wax at 65°C for 4-8 hours. They were then transferred to a plastic mold containing paraffin wax and left to set. Unless otherwise stated, all steps were performed at room temperature. The wax blocks were then cut along the coronal plan at 10 µm. All the embedding and cutting procedures were carried out in the Histology Laboratory of the Kennedy Institute of Rheumatology.

2.2.4.4 *Immunofluorescence techniques*

Selected histological sections were heated at 60°C for at least 40 min (or until paraffin was melted) to bond the tissue to the glass. Three washes in xylene for 5 min each assured complete removal of paraffin wax from the sections. Sections were rehydrated in solution of decreasing concentration of ethanol: 100%, 70%, 30% and then rested in tap water. An antigen retrieval procedure was performed to expose the epitopes on the samples. Slides were treated with bovine testicular hyaluronidase 0.1 mg/ml in PBS for 45 min at 37°C, in a wet chamber to prevent excessive evaporation. Sections were washed 3 x 3 min in TBS-T at room temperature. Non-specific binding of primary or secondary antibodies was prevented by incubation in TBS-T/ 5% BSA, 1% Goat Serum for 1 h at room temperature. Sections were incubated in primary antibody and DAPI (for nuclei staining) diluted in blocking buffer ON at 4°C in a wet chamber. After 3 x 3 min washes in TBS-T sections were incubated in secondary antibody diluted in blocking solution for 1 h at room temperature. Incubations omitting the primary antibody were used as negative control. After three washes of 5 min each in TBS-T, a final wash of 5 min was performed in TBS. Slides were air dried and mounted with ProLong Gold Antifade Reagent. Sections were observed using Ultraview confocal microscope (PerkinElmer Life Sciences) with a 60x objective lens.

2.2.5 Quantitative reverse-transcription PCR (Q-RT-PCR)

2.2.5.1 *Isolation of articular cartilage*

Adult 8 weeks-old mice were injected with tamoxifen and sacrificed 2 weeks afterwards. Muscle tissues were removed using a sterile scalpel. Cruciate ligaments and tendons were cut in order to expose tibia and femur facing ends. Articular cartilage was accurately shaved off the bone surface using a micro-rongeur surgical tool (Friedman-Pearson, cat# 16120-14). Articular cartilage was transferred in RNAlater, frozen in liquid nitrogen and stored at -80°C. Only the lower limbs of each mouse were processed, so each sample contained cartilage from two tibia plateaus and two femoral condyles.

2.2.5.2 *Extraction of RNA from knee cartilage*

Total RNA was extracted from mouse articular cartilage samples using the RNeasy Micro Kit. The manufacturer protocol was partially modified by Dr Anastasios Chanalaris (KIR) increase the RNA quality from small amount of articular cartilage.

RNA later was removed from the tubes and samples were resuspended in 150 µl of 1% β-Mercaptoethanol/RTL lysis buffer. In order to destroy the cartilage structure samples were first sonicated 2 x 15 s (20 s rest), on ice, and then, after addition of 5 µl of Proteinase K and 295 µl of RNase-free H₂O, incubate at 55°C 20 min. Samples tube were centrifuged in a bench centrifuge at 1000 g for 3 min. A total of 400 µl of supernatant was transferred into a clean tube and mixed with 225 µl Ethanol 100%. The whole solution containing nucleic acids was then transferred in the RNeasy Micro column and centrifuged at 7000 g for 15 s. Column was washed with 350 µl of RW1 buffer and DNase I treatment performed in column, to degrade genomic DNA. The column was washed again with 350 µl RW1. Two more washes with 500 µl RPE buffer and with 500 µl ethanol 80% were performed to prepare the RNA for elution. Finally RNA was eluted in 14 µl RNase-free water. A NanoDrop Spectrophotometer was used to assess concentration and purity of RNA samples (**Table 2.2**).

2.2.5.3 *Reverse-Transcription of mRNA (RT-PCR)*

The TaqMan system was the method chosen for the real-time PCR assays. This method principle relies on the 5′–3′ exonuclease activity of Taq polymerase to cleave a probe during hybridization to the complementary target sequence. The probe is designed to anneal a region within the primers set and is dual-labeled with a fluorophore and a quencher, the latter quenching the fluorescence emitted by the former. When the Taq polymerase hydrolyses the bond between the probe and the fluorophore, this is released and is free to emit fluorescence. TaqMan method allows obtaining a direct correlation between number of copies of the gene of interest and the fluorescence signal.

cDNA was generated using the ‘High Capacity cDNA Reverse Transcription Kit’. Briefly, for each RNA sample, 500 ng of RNA was reverse transcribed to cDNA in a 20 µl reaction according to manufacturer instructions. The RT

reaction was made of four steps: 10 min 25°C, 120 min 37°C, 5 min 85°C and cool down to 4°C. cDNA was diluted 1:4 and stored at -20°C.

Sample ID	ng/ul	Abs 260/280	Volume for 500 ng
LLC1.53xRosaCre 22 Knees	168.40	2.10	2.97
LLC1.53xRosaCre 23 Knees	190.82	2.08	2.62
LLC1.53xRosaCre 24 Knees	173.10	2.02	2.89
UbCT3.6xRosaCre 14 Knees	96.24	2.13	5.20
UbCT3.6xRosaCre 15 Knees	181.84	2.08	2.75

Table 2.2. Example of RNA quantification isolated from adult mice knees, obtained using NanoDrop. Abs 260/280: ratio of absorbance at 260 nm and 280 nm; values >2 denote high purity for RNA.

2.2.5.4 Real-Time PCR (qPCR)

cDNA was examined by Real-Time PCR using TaqMan technology. TaqMan primers/probe sets for TIMP3 (Hs), *Acan* (Mm00545794_m1), Hprt (Mm00446968_m1) and lacZ were from Applied Biosystems (USA). The ABI HT9000 thermal cycler and the $\Delta\Delta C_t$ method of relative quantitation were used for mRNA quantitation using Hprt as internal control. For the $\Delta\Delta C_t$ calculation to be valid, the amplification efficiencies of the target and reference must be approximately equal. This was assessed by analysis of the ΔC_t of dilution of the cDNA, which showed a similar slope for the targets and the Hprt reference.

2.2.6 Generation of transgenic mice

2.2.6.1 General notes

Transgenic mice were generated in collaboration with Dr Ke Liu from the Kennedy Institute of Rheumatology (KIR). Dr Liu is the transgenic expert at Kennedy and performed all pronuclei harvesting, DNA microinjections and transfer in foster mothers. My contribution in transgenic generation was restricted to provide high quality DNA, help in hormone stimulation, and post surgical caring. For all procedures, C57BL10 x CBA/J F1 mice were used. All techniques

performed on animals were licensed under the Animals (Scientific Procedures) Act 1986, personal license number (PIL) 70/20608 and project license number (PPL) 70/7288.

2.2.6.2 *Transgenic media*

M2 and M16 media were purchased from Sigma and made up according to manufacturers' instructions. M16 was covered in mineral oil and incubated at 37°C in 5% CO₂, 95% air to simulate *in vivo* conditions during use. M2 was kept at room temperature.

2.2.6.3 *Preparation of DNA for microinjection*

Fragments of linear DNA for microinjection were isolated from their plasmid vectors with the appropriate restriction enzymes and gel-purified in EtBr free gels without UV exposure. This was done by running two identically digested samples alongside each other on an EtBr free gel, cutting the gel vertically between the two samples, staining one of the halves in an EtBr solution and exposing this half to UV to mark by gel excision the location of the desired band. Finally, the desired band was cut from the other non-stained half using the stained gel for guidance. The DNA was then purified from the gel using spin columns. The concentration of the DNA was established, diluted to 2 ng/μl in embryo water from Sigma, and stored at -20°C.

2.2.6.4 *Preparation of egg donors by superovulation*

Superovulation was performed on F1 females between 4-6 weeks of age. On day one, 5 International Unites (IU) of follicle stimulating hormone (folligon) was administered to each mouse via IP injection. On day 3, approximately 46 hours later, 5 IU luteinising hormone (chorulon) was administered to each mouse via IP injection. Following this injection, the mice were mated with F1 fertile males overnight. On the morning of day 4, copulation plugs were checked to identify mated females. From these, oviducts were removed and dissected in M2 media giving an average of 10-15 eggs per oviduct. These were removed by forceps and transferred into clean media. Cumulus cells were removed by treatment with a few drops of hyaluronidase (300 μg/ml) in M2. The eggs were gently shaken

until the cumulus cells had visibly detached from the egg surfaces (>4 min). They were thoroughly rinsed, and transferred to M16 media to be incubated for 1-2 hours.

2.2.6.5 Injection of fertilised eggs

Injection of fertilised eggs was done approximately 72 hours after superovulation. This is described in Hogan (Hogan 1994). Undamaged eggs were transferred to clean M16 and incubated until their transfer into pseudo pregnant females.

2.2.6.6 Transfer to pseudo pregnant recipients

Mating with vasectomized CD1 males overnight on day 3 generated pseudo pregnant CD1 female mice. Females with copulation plugs were selected and used 0.5 day post-coitum. Anaesthetic solution was prepared mixing 50 µl hypnorm, 50 µl hypnoval and 100 µl sterile H₂O. Mice were anaesthetized via IP injection of 200 µl of anaesthetic solution. Microinjected eggs, at 1 cell stage, were transferred through the infundibulum to the oviducts. Each pseudo pregnant female received 20-30 eggs.

2.2.7 Management of mouse colonies

2.2.7.1 Common husbandry procedures

Mice underwent regular health monitoring, a microbiological surveillance programme by competent persons checking cages twice per day. These checks ensured that all sick or injured animals were identified and appropriate action took.

Breeding pairs were set up choosing animals between 8 weeks old and 6 months old. In order to maintain the transgenic lines, mice screened positive by PCR genotyping were mated initially to C57BL/6 wild type mice, and further between each others in order to obtain transgenic homozygous.

Transgenic mice responsible to tamoxifen were generated breeding mice screened positive for the transgene to ROSA-Cre (B6.129-

Gt(ROSA)26Sor^{tm1}(cre/ER^{T2})Tyj/J mice, The Jackson Laboratory). Progenies were screened for both the genetic background.

2.2.7.2 DNA extraction from mouse tissues for genotyping

For genotyping purpose ears biopsy from adult mice and tail fragment from embryos were collected. Tissues were processed using the REDExtract-N-Amp Tissue kit from Sigma.

2.2.7.3 Genotyping by PCR

Multiplex PCR was performed for screening of transgenic mice (adapted from Wayne Barnes, Washington University in St. Louis). Primers were designed to target the lacZ gene (in the expression vector), the Cre recombinase gene and the mouse gene fabp1 (fatty-acid-binding protein) (used as internal control). All primers stock solutions were diluted to a working dilution of 10 pmol. Each PCR reaction contained the following amount of primers:

2 µl each target primer (lacZ or Cre)

1 µl each internal control primer (fabp1)

A 2-step PCR program was used and is described in the following table:

Step	Temperature	Time	
Step 1	93°C	60 s	Denaturation
Step 2	93°C	20 s	Denaturation
Step 3	68°C	180 s	Annealing and Elongation
Step 4		Repeat Step 2 to 3 for 29 times	
Step 5	4°C	hold	

Table 2.3. Thermo cycler settings for genotyping

Nucleotide sequences of all primers' set are shown in the following table:

Gene	Orientaion	Sequence 5'-3'	Product size
LacZ	Forward	GTT GCA GTG CAC GGC AGA TAC ACT TGC TGA	389 bp
	Reverse	GCC ACT GGT GTG GGC CAT AAT TCA ATT CGC	
Cre	Forward	GCA TTA CCG GTC GAT GCA ACG AGT GAT GAG	408 bp
	Reverse	GAG TGA ACG AAC CTG GTC GAA ATC AGT GCG	
Fabp1	Forward	TGG ACA GGA CTG GAC CTC TGC TTT CCT AGA	194 bp
	Reverse	TAG AGC TTT GCC ACA TCA CAG GTC ATT CAG	

Table 2.4. Nucleotide sequences of primers used for genotyping

The total PCR reaction volume of 20 µl was electrophoresed in a 2% Agarose gel. Figure 2.1 is a representative genotyping results. All samples containing genomic DNA show a 200 bp band. Only samples from transgenic mice show an extra band at 400 bp. No template control (NTC) does not show any band (**Figure 2.1**).

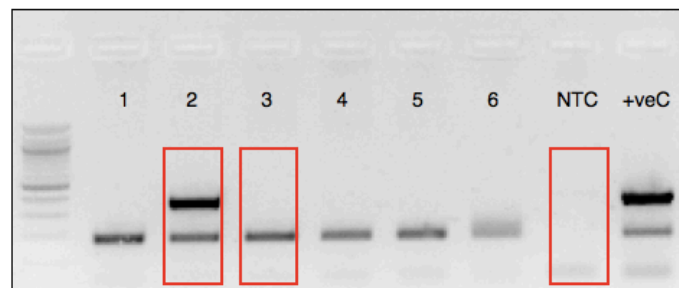


Figure 2.1. Genotyping by Multiplex PCR. Representative genotyping screening by PCR analysis of wildtype and transgenic mice. Primers sets to lacZ gene and fabp1 were used. All samples but NTC show the 200 bp band related to the fabp1 used as internal control. Only sample 2 and the positive control show a 400 bp band related to the lacZ gene. NTC: no template control.

2.2.7.4 Surgical induction of OA through the destabilisation of the medial meniscus (DMM) model

Mice were anaesthetized via IP injection of Hypnorm and Hypnovel. After shaving of the ventral portion of the right knee, a midline incision was made over the ventral aspect and the medial compartment of the knee. The medial meniscus was identified, and using an ophthalmic scalpel, its anterior horn was released by sharp dissection of the attachments to the tibia plateau. Mobility of the anterior

half of the medial meniscus was confirmed by displacement with forceps. The para-patellar incision was closed with an absorbable suture (Ethicon, USA), and the skin closed with fine non-absorbable sutures (Ethicon, USA). A specialized technician at the Kennedy Institute of Rheumatology performed all the DMM surgeries.

Chapter 3

Design and generation of aggrecan-driven CreER^{T2} mice

I have divided the 'Results' section into four chapters. Each chapter contains an overview of the experimental work (background), a report of the data itself (results) and finally a discussion of the data evaluating the findings (discussion). The main aim of the Chapters 3 and 4 is to illustrate the design and production of expression vectors using sequences from the mouse aggrecan enhancer. The designed vectors will drive expression in mouse tissues of different target genes: the Cre-recombinase or the human TIMP-3 and its mutant [-1Ala]TIMP-3. In Chapter 5 I will describe the design of a vector to express TIMPs not only in the articular cartilage but also in other tissues, including the synovial membrane of knee joint and the subchondral bone. To achieve this result I used the promoter of the mouse ubiquitin C gene. Finally in Chapter 6 I will illustrate preliminary results of surgical-induced osteoarthritis in some transgenic lines.

3.1 Background

The first aim of my thesis was to develop an effective inducible Cre/Lox system to target articular cartilage and monitor its expression in real-time. I was well aware that there were at least two CreERT2-expressing cartilage-specific mice already existing, however I decided to generate a new one for four reasons. First, some data from literature suggested that the promoter of the Collagen type II gene could not be so effective in old mice (more than 6 months of age); second, that the Aggrecan knock-in from Henry would not allow to observe the expression *in vivo*; third, my supervisor Dr Bou-Gharios and I were interested to investigate if the Aggrecan enhancer sequences identified by Lefebvre's group (named A1, A2 and A3) were able to regulate the expression of Aggrecan both spatially and temporally; fourth, because I wanted to investigate the expression of TIMP-3 in cartilage I could have used the same genetic elements for both the transgenic mouse systems.

Conditional overexpression or inactivation of genes in the adult mouse has become the method of choice when investigating the role of a genes of interest *in vivo* (Branda and Dymecki 2004). This recombination system has been made possible by the advent of the Cre/loxP technology and more recently the similar

Flp/FRT technology (Dang and Perrimon 1992, Lee, Moding et al. 2012). The Cre recombinase system has become the tool of choice to conditionally activate (“gain-of-function”) or inactivate (“loss-of-function”) gene expression in a temporal, spatial, or tissue-specific manner. Cre recombinase efficiently excises DNA flanked by repeated loxP recognition sites both in cultured cells and in mice (Sauer and Henderson 1988, Orban, Chui et al. 1992). A major enhancement was achieved by conditional gene targeting based on inducible activation of the Cre recombinase obtained by fusing the Cre to the ligand-binding domains (LBDs) of steroid receptors (Metzger, Clifford et al. 1995). Subsequent mutations were introduced into the LBDs of chimeric Cre recombinase to abolish the activation by natural ligands and to increase affinity to the synthetic tamoxifen. Feil and colleagues generated a chimeric protein where the Cre recombinase has been fused to a mutated ligand-binding domain of the human oestrogen receptor (ER) resulting in a tamoxifen-dependent Cre recombinase, Cre-ERT, which is activated by 4-hydroxytamoxifen (4-OHT), but not by estradiol. The chimeric protein is thus trapped in the cytoplasmatic environment until it binds to the receptor’s ligand (Feil, Brocard et al. 1996) (Feil, Brocard et al. 1996). Subsequent mutation studies on the ligand-binding site in the ER portion, allowed to generate chimeric protein highly specific for the 4-OHT (Feil, Wagner et al. 1997, Indra, Warot et al. 1999).

As I will show in the current and next chapters, a tissue-specific Cre deleter was generated to be able to study the effects of several genes in articular cartilage. At the same time a cartilage-specific transgenic mouse was generated to work together with the well-described ROSA-Cre recombinase mouse line (Grover and Roughley 2006).

My aim was to achieve articular chondrocyte-specific expression. Remarkably two target genes were identified to be able to allow restricted expression in the articular cartilage chondrocytes: *Col2a1* and *Acan*. As discussed in the Introduction chapter those are the most abundant structural proteins in the adult articular cartilage and are very likely to be responsible for maintaining the whole

structure. Ever since type II collagen was discovered as a major component of chick cartilage by Miller and Matukas (Miller and Matukas 1969), it has been considered an hallmark for chondrocyte differentiation and a chondrocyte-specific marker. However, later studies demonstrated that type II collagen was expressed much earlier than the onset of chondrogenesis, which occurs at the time of condensation of the mesenchyme. Moreover type II collagen has been detected in numerous tissues that never undergo chondrogenic differentiation. Expression of *Col2a1* mRNA was also observed in notochord that may be responsible for inducing chondrogenesis in somitic mesoderm, neural retina, the corneal and conjunctival epithelia and sclera of the developing eye. Transient expression was also found in the heart at 9.5-12.5 days, and the epidermis at 10.5-14.5 days, the inner ear at 14.5 days and the fetal brain from 9.5-14.5 days. (Cheah, Lau et al. 1991). In 2000 Behringer's group generated a Cre-deleter transgenic mouse using the promoter of the collagen type II (Ovchinnikov, Deng et al. 2000). Both in my experience and in literature, the expression of collagen type II is sustained during the pre-natal development until early adult age (4 to 6 weeks of age). Following that, expression drops to lower levels as suggested by Chambers, although in unpublished data (Chambers, Kuffner et al. 2002). Although the decrease of expression is a physiological event and does not affect the structure of the adult joint, I foresaw a caveat in use the promoter for adult expression of the transgenes of interest. Moreover some authors detected Cre recombinase expression in kidney epithelium in Col2-Cre mice during development, which reflects the transient expression of the endogenous *Col2a1* gene in the embryonic renal tubules (Kolpakova-Hart, Nicolae et al. 2008). As an alternative approach several authors investigated the capacity of aggrecan response elements to drive cartilage specific expression in mouse cartilage since aggrecan is a major extracellular matrix proteoglycan of both the growth plate and articular chondrocytes. Henry et al in 2009 cloned the Cre recombinase coding sequence downstream of the mouse aggrecan gene achieving cartilage specific deletion (Henry, Jang et al. 2009). In 2008 Han and Lefebvre identified a 400-bp long cis-acting element upstream of the aggrecan mouse gene, recognized by Sox9 and sufficient to drive cartilage specific expression (Han and Lefebvre

2008). I decided to take advantage of these findings to generate an aggrecan-driven cartilage specific transgenic mouse.

As mentioned above an inducible system allows one to achieve temporal regulation to delete or overexpress target genes. Engineering the Cre recombinase enzyme was the key to obtaining this feature.

In the first inducible Col2a1-CreER^T generated (Grover and Roughley 2006) transgene was not highly expressed in adult (Nakamura, Nguyen et al. 2006). However, more recent work by Chen and colleagues presented a Col2a1-driven CreER^{T2} transgenic line in which they achieve efficient tamoxifen induced recombination in articular cartilage in older animals (Chen, Lichtler et al. 2007) .

In 2008, Han and Lefebvre identified an enhancer upstream of the mouse aggrecan gene that was recognized by Sox9, L-Sox5 and Sox6 (Han and Lefebvre 2008). When this enhancer was used to generate lacZ reporter mouse, the transgenic mouse stained for β -galactosidase in all cartilaginous tissues. Henry et al (2009) generated a deleter mouse line by knocking-in the CreERT² downstream of the stop codon of the endogenous mouse aggrecan gene creating Agc1 tm(IRES-creERT2) (Henry, Jang et al. 2009).

3.2 Results

3.2.1 Design and construction of expression vectors

To design and generate our own cartilage-specific Cre deleter mouse strain, I took advantage of Han and Lefebvre paper where they describe the design of a construct containing four tandem repeats of the sequence identified in the mouse ‘aggrecan enhancer’ (which they named A1), fused downstream of the *Col2a1* minimal promoter (-309 / +308 base pairs relative to the mouse *Col2a1* transcriptional start site) (**Figure 3.1 A**).

The original vector containing Col2-4xA1 sequence was obtained from Veronique Lefebvre's group (Han and Lefebvre 2008). As described in their paper, the A1 sequence was amplified by PCR performed on 129 mouse genomic DNA. Four tandem copies were cloned into a LacZ reporter plasmid containing a *Col2a1* promoter sequence that was previously identified to drive chondrocyte expression in mouse embryos (Zhou, Garofalo et al. 1995).

I designed a three-steps plan to construct the final vector (**Figure 3.1 B**).

- The firefly Luciferase reporter gene was cloned in a pIRES plasmid, downstream of the IRES sequence and upstream of a polyA DNA sequence. The Luciferase gene sequence and pIRES plasmid belong to the Matrix Biology plasmids archive at KIR.
- The CreERT² fusion gene was lifted from its original plasmids (Feil, Wagner et al. 1997) and cloned upstream of the IRES sequence of the modified pIRES plasmid.
- The Col2a1-4xA1 DNA sequence was lifted from the original vector and cloned upstream of the CreERT² sequence in the modified pIRES plasmid.

The final construct was named Col2a1-4xA1-CreER^{T2} (**Figure 3.1 B**).

In the CreER^{T2} the Cre enzyme has been fused to a mutated ligand-binding domain of the human oestrogen receptor (ER) resulting in a tamoxifen-dependent Cre recombinase, Cre-ER^{T2}, which is activated by tamoxifen, but not by estradiol or endogenous hormones (Feil, Brocard et al. 1996, Feil, Wagner et al. 1997). The new vector featured an IRES sequence and the firefly (*Photinus pyralis*) luciferase reporter gene. The advantages of using the firefly luciferase as a reporter gene are the high sensitivity in detection of expression of the gene, the absence of luciferase activity inside most cell types, the wide dynamic range, rapidity and low costs. Also, the protein does not require any posttranslational modification to be enzymatically active and is not toxic in high concentrations *in*

vivo (de Wet, Wood et al. 1987). The IRES sequence ensured the transcription of bi-cistronic mRNA in which two proteins are translated from one mRNA sequence. The expression and recombinase activity of this construct was tested and used to generate transgenic mice for cartilage specific Cre-mediated deletion of floxed sequences (Lo Cascio, Liu et al. 2013). The DNA construct was transfected into embryonic cells isolated during development from embryos at 11.5 days post coitum of ROSA26-lacZ reporter mouse (Rosa26R). This latter mouse expresses β -galactosidase after Cre-mediated excision of a transcriptional STOP cassette, knocked in the ROSA26 locus (Grover and Roughley 2006). In this system the enzyme Cre recombinase deletes the transcriptional STOP, then β -galactosidase is expressed and converts its substrate (X-gal), in the presence of iron, into blue staining. The staining occurs only in cells expressing the Cre recombinase enzyme. In order to activate the Cre recombinase enzyme, 4-Hydroxytamoxifen (4-OHT) was added to one of the transfected cell cultures (PBS was added as control) for 48h. 4-OHT is the metabolic product of the tamoxifen that in mammals is metabolised by cytochrome P450 in the liver to the active form. Because this activation process is missing in cell cultures, the active 4-OHT has to be used. Transfected cells were fixed and X-gal staining showed that in the presence of tamoxifen, the majority of cells were expressing β -galactosidase (**Figure 3.1 C**), indicating that the inducible construct can remove the floxed transcriptional STOP *in vitro*.

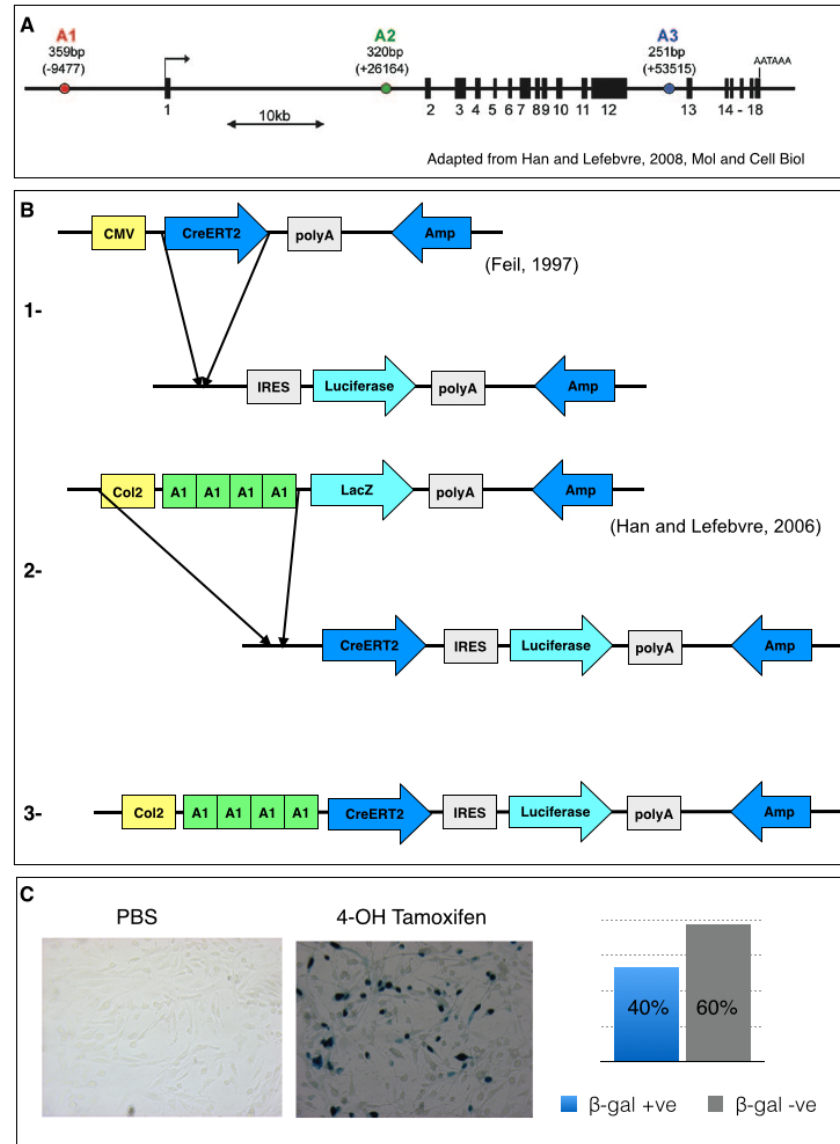


Figure 3. 1. Schematic diagram of the Col2a1-4xA1-CreERT² expression vector and its testing *in vitro*. A: Diagram of the *Acan* gene showing the locations (in parentheses) and sizes of A1, A2, and A3 elements. The horizontal line represents nonexonic sequences. Black boxes with numbers underneath represent exons. The arrow symbolizes the start of transcription, and AATAAA indicates the polyadenylation site. B: Vector consists of a *Col2a1* minimal promoter (Col2) plus four tandem repeats of mouse aggrecan enhancer A1; CreERT2 fusion protein; IRES; Firefly Luciferase coding sequence; polyA sequence. Ampicillin resistance gene is also indicated (Amp). Arrows denote the direction of transcription. *E.coli* origin of replication is not shown. IRES: internal ribosome entry site; polyA: polyadenylation sequence. C: Representative X-gal staining. Mesenchymal cells previously isolated from E10 ROSA Reporter embryos were transfected with the Col2a1-4xA1-CreERT2 expression vector. Cells were incubated either in PBS (left) or in 4-OH tamoxifen (right) for 48h. Cells were fixed and X-gal substrate added. Only cells treated with 4-OH tamoxifen showed blue staining. Graph on the right is an estimate of the β-gal positive cells.

3.2.2 Generation of transgenic mice

In order to generate a transgenic mouse expressing this construct, the linearized plasmid was injected in the pro-nucleus of a one day fertilised egg. Founder animals were identified by genotyping and mated with Rosa26R mouse to be tested for Cre activation. Pregnant mothers were given 3 mg tamoxifen and 1.5mg of progesterone (to counteract the mixed oestrogen agonist effects of tamoxifen injections, which can result in late foetal abortions in pregnant mice) at different times during development (E13.5 to E16.5) and sacrificed 24 hours later. At each time point, embryos were harvested and stained for β -galactosidase. The results showed that three lines out of twenty positive were able to delete the transcriptional STOP and express the β -galactosidase albeit with varying level of luciferase activity and number of chondrocytes that can efficiently delete in adult chondrocytes. In **Figure 3.2** are shown representative images of the line that showed highest expression. Blue staining was observed in fore and hind limbs at E13.5 (**Figure 3.2 A**, left panel, arrows). At E14.5, the transgene expression is more visible in the limbs and the developing ribs (**Figure 3.2 A**, middle panel). By E15.5 almost all the cartilaginous centres were stained, including the nasal (n) and otic (o) region, as well as, ribs (r) and all developing vertebra down to the tail (t) (**Figure 3.2 A**, right panel). E16.5 embryos were de-skinned to show expression in the head region where the base of the trachea and the mandible were stained with X-gal (**Figure 3.2 B**). The skeletal elements were well defined and stained bone formation of the torso including the developing clavicles (**Figure 3.2 C**, c), the forelimbs developing bones, such as the humerus (h) radius (r) and ulna (u) and the forming digits (*). The ribs, sternum and the developing vertebra of the spine were also intensely stained (**Figure 3.2 D**, arrows).

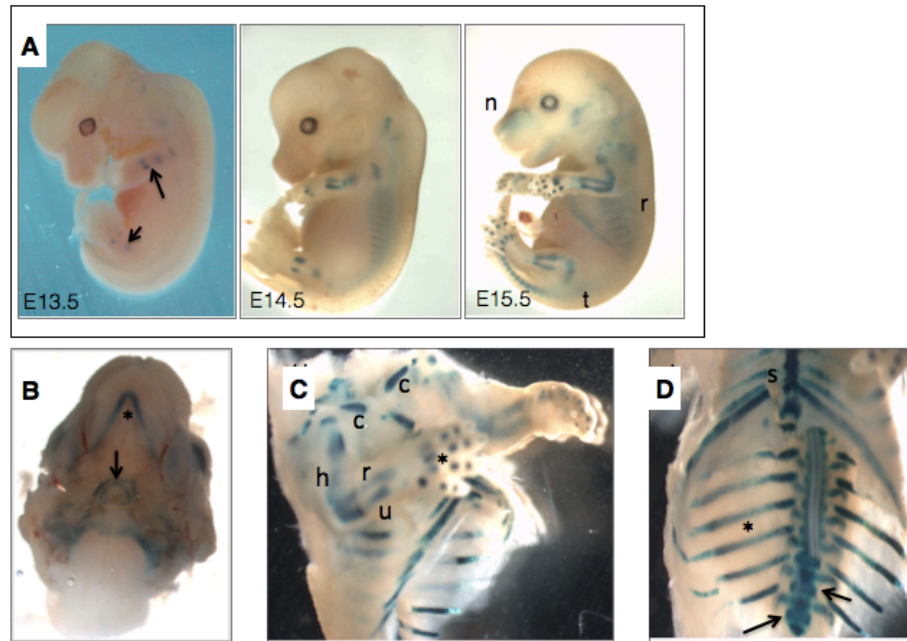


Figure 3.2. Inducible expression of Cre recombinase in adult double transgenic mice confirmed by X-gal staining. A-D: Representative whole-mount Col2a1-4xA1-CreERT² x ROSA26R embryos expressing β -galactosidase (blue staining). A: X-gal staining can be seen in the developing embryo at E13.5 in limbs (arrows), E14.5 and E15.5 the nasal region (n), developing ear (e) and ribs (r) as well as vertebra of the tail (t). B-D: At E16.5 the mouse was de skinned and dissected to reveal the staining at base of trachea, (B, arrow) and mandibles (B, *). The forelimbs and shoulder cartilaginous staining is seen in developing clavicles (C,c), humerus (C,h) radius and ulna (C, r and u) and in digits (C, *). Staining is also seen in sternum (D, s) and developing dorsal ribs (D, *) and vertebra of spine (D, arrows).

Expression of Cre recombinase was tested in adult mice at eight weeks of age. The mature double transgenic mice were injected intra-peritoneally three times, every other day, with 2 mg of tamoxifen and sacrificed one week after last injection. The skeleton was isolated, fixed and stained for β -galactosidase overnight. Whole mount isolated tissues and histological cross-sections showed high levels of β -galactosidase expression only in the tamoxifen injected double transgenic animals. These data prove that the inducible, conditional Cre recombinase under the control of *Acan* enhancer is activated, as expected, in the mouse adult tissues (**Figure 3.3**). The expression was evident in the knee joint tibia plateau (**Figure 3.3 A**). The condyles, as well as the fibrocartilage of the patella and menisci were stained (**Figure 3.3 B-C**). Histological sections show the expression of the transgene in the majority of the chondrocytes above the tidemark (**Figure 3.3 C**). There was no expression of the transgene in calcified cartilage. The majority of the chondrocytes in ribs were also stained with X-gal

(Figure 3.3 D-E). Isolated whole mount tails and longitudinal sections stained with X-gal, shown staining in the intervertebral disc nucleus pulposus (np), annulus fibrosis (af) showing as punctate staining of cells expressing transgene and in the growth plate **(Figure 3.3 F-G)**. Tendons did not stain uniformly.

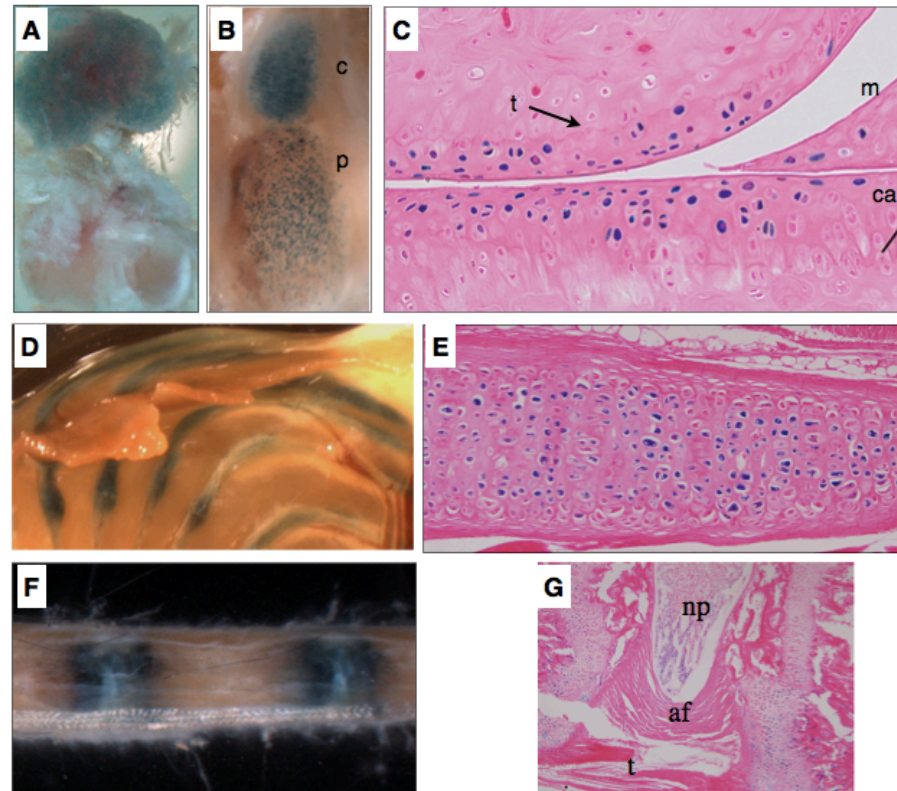


Figure 3.3: Inducible expression of Cre recombinase in adult double transgenic mice showed by X-gal staining. Col2a1-4xA1-CreERT² mice mated with Rosa26R mice were given tamoxifen at eight weeks and stained for β -galactosidase a week later. **A:** Isolated tibia plateau shows staining only in the presence of tamoxifen (right) compared to just vehicle (left). **B:** Blue staining was observed in condyle (c) and patellar cartilage (p). **C:** Coronal section of the knee shows chondrocytes in the uncalcified cartilage (ca, above the tidemark, t) express the transgene and as well as cells in meniscus (m). **D-E:** Rib cage shows blue staining in whole mount (D) and in cross section (E). **F-G:** Blue staining was observed in the vertebral rings of the tail. **G:** Histological coronal sections of tails highlight the cells stained in the nucleus pulposus (np) and annulus fibrosus (af). By comparison, only occasional cell stained blue in tendon (t).

The added advantage in this mouse model is the inclusion of the firefly (*Photinus pyralis*) luciferase coding sequence downstream to the Cre recombinase, allowing the visualisation of expression in real time, in live animals. The chemical reaction catalysed by firefly luciferase takes place in two steps and the final product is the oxydation of the substrate luciferin to oxyluciferin. Light is emitted because oxyluciferin jumps in an electronically excited state and when it returns to the ground state, it releases a photon of light. Compared to other detection systems such as fluorescence, luciferase offers inferior resolution in tissues, but produces a higher level of energy, allowing better penetration of tissues, and thus detection.

Upon IP injection, the D-luciferin is slowly absorbed into circulation, perfuses into tissues and is excreted by the kidneys, because it is not metabolised. The emission plateau phase usually occurs at 15 min post luciferin injection and lasts for 15-20 min. Signal detection was performed between 20 and 30 min post injection in a Kodak In Vivo FX Pro machine. Bioluminescence was detected in the fore and hind limbs, as well as, the tail. The most discernable luciferase signal was in the knees, heels and metatarsals. Bioluminescence was also detected in every vertebra of the tail as well as the cartilage of the sternum (**Figure 3.4 A**). I studied the levels of luciferase expression of five mice, from two weeks until eight weeks of age. Every two weeks mice were anaesthetized and injected with D-luciferin. Because in the DNA construct the Luciferase coding sequence was under the control of the Aggrecan enhancer, it is possible to speculate that in both knees and in the tail vertebra the aggrecan expression was reduced between two and four weeks of age but remained constant thereafter (**Figure 3.4 B-C**).

At 10 weeks of age the same mice were subjected to a surgical procedure (DMM, Destabilization of the Medial Meniscus) that induces the development of osteoarthritis-like changes in the medial compartment of the knee (Indra, Warot et al. 1999). The rationale was to evaluate whether the onset of osteoarthritis would induce changes in expression of Aggrecan gene, and thus luciferase, in the operated knee (right knee) compared with the left (un-operated) knee. Luminescence was measured from the right and the left knee over eight week period post-operatively (**Figure 3.4 D**) and the amount of luminescence emitted from the right knee was compared to the left knee as well as the third vertebrae from the base of the tail. The results showed that there is no significant change in luminescence *in vivo* between the operated and un-operated knees for the duration of the experiment (**Figure 3.4 D**). Using quantitative PCR, we found that there was no significant change in the *Acan* mRNA level between the two knees at 2, 4, and 8 weeks post-surgery, confirming the bioluminescence observations (**Figure 3.4 E**).

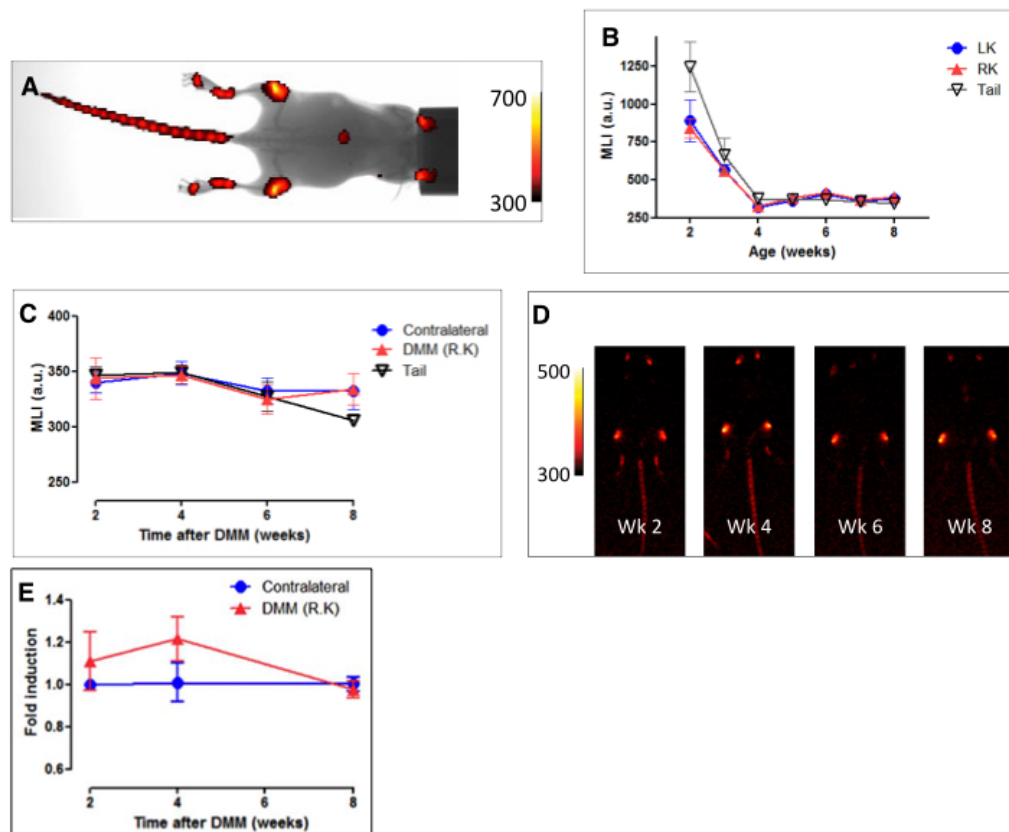


Figure 3.4. Bioluminescence as a tool to track expression *in vivo*. Double transgenic mice were anaesthetised and injected with D-luciferin. **A:** luminescence can be observed in joints of the limbs, tail and cartilage of the sternum. **B:** The expression was tracked *in vivo* showing that transgene level decreased at weaning age (4wks) and remained steady thereafter. **C-D:** Transgenic mice that have undergone DMM at ten weeks of age ($n=5$ in each group) were tracked every other week for the following eight weeks. Bioluminescence was plotted in injured, right knee and left untreated knee and compared with tail expression. **E:** qPCR analysis of endogenous *Acan* gene in articular cartilage at 2, 4, and 8 weeks after DMM show no significant difference in Aggrecan mRNA level between right (operated) and left (un-operated) knees ($n=3$ in each group). Data shown that transgene expression is stable over eight weeks after DMM in both knees.

The osteoarthritis-like injury induced by the surgical model was validated. Five mice were sacrificed at eight weeks after DMM. The knee joints were removed, fixed and embedded in paraffin wax. Coronal sections were cut and stained with Safranin-O and fast-green, and scored using the Osteoarthritis Research Society International (OARSI) scoring system (Glasson, Chambers et al. 2010) (**Table 3.1**). I and Dr Darren Plumb at KIR blind-scored the same histological sections.

Grade	Osteoarthritic damage
0	Normal
5	Loss of Safranin-O without structural changes
1	Small fibrillations without loss of cartilage
2	Vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina
3	Vertical clefts/erosion to the calcified cartilage extending to <25% of the articular surface
4	Vertical clefts/erosion to the calcified cartilage extending to 25-50% of the articular surface
5	Vertical clefts/erosion to the calcified cartilage extending to 50-75% of the articular surface
6	Vertical clefts/erosion to the calcified cartilage extending to >75% of the articular surface

Table 3.1. The recommended semi-quantitative scoring system. (Adapted from Glasson et al., 2010)

Eight consecutive sections per sample were scored and summed. High magnification of the medial compartment shows areas of fibrillation with loss of Safranin-O, indicating loss of proteoglycan in the right operated knee (**Figure 3.5 B,D**) compared with the left (un-operated) (**Figure 3.5 A,C**). This is also reflected in the histologic scores of all five mice where it shows significant increase $P < 0.05$ in degradation of articular cartilage in the medial tibia plateau of right operated knees of transgenic mice at 8 weeks after DMM surgery (**Figure 3.5 E**).

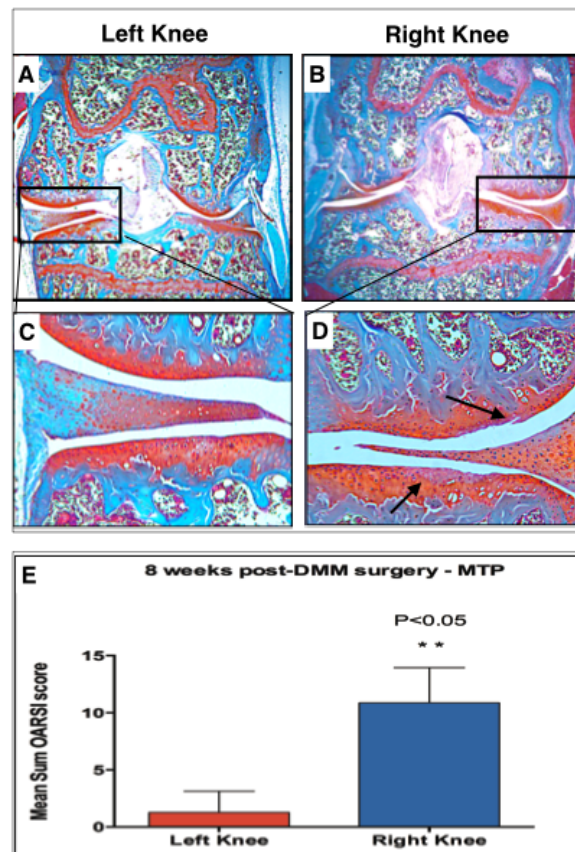


Figure 3.5. Photomicrographs of representative histologic sections of knee joints, eight weeks after DMM. A-B: Low magnification micrographs of the left (un-operated) and right (operated) knees. **C-D:** Higher magnification micrographs of the medial compartment (of A, B respectively) shows areas of fibrillation (arrows) with loss of Safranin O-fast green staining, indicating loss of proteoglycan in the operated knee only. **E:** Histologic scores of transgenic mice at 8 weeks after DMM surgery, using the Osteoarthritis Research Society International (OARSI) scoring system. Values are the mean of standard deviation (SD) of 5 mice per group. P value determined by student T-test shows significant degradation in operated knee joints.

3.3 Discussion

The rationale to generate this mouse line was to develop an effective inducible Cre/Lox system to target articular cartilage, and monitor its expression in real-time. One of the advantages of the inducible system is to circumvent potential lethality and skeletal defects during development of cartilage.

At the time I started my PhD there were already a number of cartilage-specific Cre mice. Most of them relied on Col2a1 promoter to deliver the Cre recombinase in chondrocytes. Both Ovchinnikov (2000) and Sakai (2001) employed DNA sequences of the Col2a1 promoter/first intron to deliver Cre

recombinase in cartilage. Both manuscripts demonstrate cartilage-specific recombination in embryos, as shown by β -galactosidase activity. In both models Cre recombinase expression was detected as early as 9.0 dpc, matching previous finding by Zhao in 1997, which investigated Col2a1 expression by in situ hybridization. Sakai however observed β -galactosidase activity in osteoblasts, speculating that this may be explained by the common progenitor that osteoblast and chondrocytes share. Both Ovchinnikov' and Sakai's groups did not shown data on Cre-mediated recombination in adult mice. In 2007 and 2008 Di Chen's group described a mouse model where the inducible CreERT2 protein was expressed in cartilage, driven by a 1kb DNA sequence of the Col2a1 promoter (Chen 2007, Zhou 2008). This Cre deleter mouse line was efficient to achieve recombination in both embryos and young adult mice (up to 8 weeks of age). The authors observed that the percentage of X-Gal-positive articular chondrocytes was similar at 1 and 6 months after tamoxifen induction, suggesting that it may be due by the minimal turnover in articular cartilage, consistent with the limited reparative potential of this tissue. At about the same time the first Aggrecan-driven CreERT2 mouse line was described by Henry, here he generated a mouse by targeted homologous recombination knocking-in the inducible Cre recombinase CreERT2, in the 3' untranslated region of the endogenous mouse Aggrecan gene. From the data shown in the manuscript, recombination is achieved both in embryo and in adult. In adult mice X-gal staining was clearly visible in articular cartilage, as well as menisci and growth plate, intervertebral discs and annulus fibrosis.

In this Chapter I demonstrate the generation of inducible Cre deleter where high and cartilage-specific expression is driven by Aggrecan enhancer. This was illustrated in the double transgenic Aggrecan Cre ER^{T2} /Rosa26-lacZ reporter. When tamoxifen is injected IP to pregnant mother or adult mice, the tamoxifen induces the translocation of the Cre recombinase to the nucleus allowing it to recombine floxed sequences of DNA, in this case the transcriptional STOP that precedes the β -galactosidase gene in the ROSA26 locus (Grover and Roughley 2006). This β -galactosidase activity was visualised by X-gal staining of tissues during development and postnatally. As with previous aggrecan-driven constructs

reported in the literature, including the knock in mouse *Agc1*(tm(IRES-creERT2)), where the expression of the Cre recombinase is driven by the endogenous gene (Henry, Jang et al. 2009). The expression pattern of our transgenic was similar to the endogenous Cre deleter in developing embryos, and in adult tissues where expression was evident in cartilaginous tissues, including growth plate chondrocytes, articular cartilage, and fibrocartilage cells of the meniscus and of the intervertebral disks. Like the knock-in mouse *Agc1*(tm(IRES-creERT2)), I did not observed any staining in brain or the brain envelop, nor did I find consistent staining in tendons.

The unique feature of this mouse is that it is the first *Acan*-driven transgenic line that allows *in vivo* visualization of *Acan* gene transcriptional activity in real-time.

Aggrecan expression has been studied previously. Chambers and colleagues (Chambers, Kuffner et al. 2002) investigated by in situ hybridization, the expression of both *Col2a1* and *Acan* in young (below 9 weeks of age) and adult (above 36 weeks of age) CBA mice. They could not detect differences in the distribution of transcripts for *Acan* and *Co2a1* in articular cartilages from young mice. However while *Acan* expression was maintained in adults mice unchanged, the *Col2a1* mRNA signal was undetectable. These previously findings support my observation of stable luciferase expression in mice adult mice up to 16 weeks of age.

Although I do not know whether the level of this transgene is equivalent to the endogenous gene, it appear to be expressed under the same conditions as the endogenous aggrecan gene, and thus could be used to provide insights on changes in endogenous *Acan* gene as a read-out of druggable target gene expression that increase *Acan* transcriptional activity in osteoarthritis.

I tested the bioluminescence of this mouse using the surgical model of osteoarthritis, on the assumption that as the medial plateau of the operated knee joint is degraded during the progression of the disease, I may be able to detect

changes in the luciferase transgene, as a reflection of endogenous *Acan* gene expression.

Our bioluminescence results indicated that the transgene level between the operated and non-operated knee remained the same, suggesting that transcription of *Acan* gene did not change in operated knee at any time up to eight weeks following surgery. The histological data, however, showed discernable anatomical differences on the surfaces of the articular cartilage of the operated knee in both the tibial plateau and femoral condyle. This change was significant when eight consecutive slides were scored between the operated and un-operated knee. This discrepancy between the bioluminescence data and the histology may be explained by the inability to distinguish between the luciferase expression in articular cartilage versus the growth plate because the resolution of the Kodak machine used could not separate one from the other due to close proximity and the scatter of the light signal from the tissue. It can be argued that if the luciferase signal is far stronger in the growth plate cartilage than that from the articular cartilage, it may be impossible to detect change in luciferase signals in the articular cartilage of the operated knee. Quantitative PCR analysis on mRNA extracted from articular cartilage confirmed stable expression of Aggrecan in operated articular cartilage. Being qPCR by far more sensitive than bioluminescence, although is an *in vitro* technique, provide additional strength on the *in vivo* tracking of the gene expression. However it is important to stress that stability in Aggrecan expression is contextual to loss of aggrecan protein from the articular cartilage. A possible explanation is that although the cartilage ECM is affected by surgery, chondrocytes are still alive and maintain the expression levels of matrix proteins such Aggrecan.

It has been already demonstrated that Aggrecan is expressed at detectable levels in tissues other than cartilage such as brain. My main interest was to generate a cartilage-specific Cre deleter, and I did not investigate expression in other tissues. However research focused in brain and neurodegenerative disease is an expanding field, and this mouse model may provide useful insight on the

expression of Aggrecan in brain cells. Detecting the bioluminescence signal from brain or other tissues *in vivo* may prove difficult for technical reasons such distance from the detecting surface of the machine or external tissues shielding the signal. However organs could be rapidly dissected and observed. Also cells could be isolated and cultured before detection of luminescence. Western blot analysis on aggrecan is usually difficult due to the strong interactions between matrix proteins, lucifrasease however would be easier to detect and indirectly shown if a tissue or cell type express Aggrecan.

Here I investigated if Aggrecan enhancer elements previously studied by Veronique Lefebvre's group could be suitable to deliver target proteins in articular cartilage. Compared to previous reported mouse models, the one I generated has expression localization and recombinase activity very similar to the one generated by Henry, very likely because both models use aggrecan-driven expression. In both β -galactosidase expression is strongly visible in adults mice in both articular cartilage and intervertebral discs, as well as menisci and other structures such annulus fibrosus. As a direct comparison of expression levels of the Cre recombinase cannot be performed, the two models could be comparable as cartilage specific deleter. Although my model may seams redundant it offers a clear novelty: it allows tracking *in vivo* of Aggrecan expression, and could be employed in other research fields to investigate the involvement of this gene in other tissues.

According to my aim, I generated a Cre deleter inducible mouse that could be useful in studies aimed to better understanding cartilage pathologies such as osteoarthritis. I have demonstrated that the vector designed is able to express Cre recombinase even in its episomal form, when transfected in mouse embryonic cells isolated from ROSA26 Reporter mice. I have also demonstrated that transgenic mice generated from this DNA construct, upon tamoxifen induction, express both the transgenes either in embryos than in adult. Finally I have shown that such expression is stable during the adult life of animals. Taken together

those data demonstrate that the transgenic mouse generated is suitable for studies focused on the articular cartilage.

Chapter 4

Design and Generation of an inducible aggrecan-driven TIMP-3 and [-1Ala]TIMP-3 transgenic mice

4.1 Background

The major threat to the cartilage matrix and thus to the integrity of articular cartilage are matrix-degrading enzymes destroying the collagen and proteoglycan meshwork. To date, our knowledge focuses mostly on degradation processes of collagen type II and aggrecan. Loss of aggrecan is characteristic of the early stages of cartilage degeneration, whereas the overall content of collagen remains rather constant nearly throughout the disease process. Still, weakening of the collagen network is a major feature also in early cartilage degeneration. So far, it is unknown what happens first, the loss of proteoglycans or the loosening of the collagen network, because both do eventually influence the other. Enhanced levels of many MMPs (matrix metalloproteinases), as well as ADAMs (a disintegrin and metalloproteinase domain) and ADAMTSs (a disintegrin and metalloproteinase domain with thrombospondin type-1 motifs) are the most likely candidate enzymes responsible for the increased matrix degradation in OA cartilage (Burrage and Brinckerhoff 2007). To date is still unknown which proteinases are really crucial for the degradation of the various cartilage matrix components, although MMP-13 is certainly a top candidate for primary collagen type II fibril degradation, MMP-2 (gelatinase A) is a good candidate for subsequent cleavage of denatured collagen fibrils (gelatins), whereas ADAMTS-4 and ADAMTS-5 are favored to be the major aggrecanases responsible for the aggrecan breakdown. This lack of knowledge is particularly striking considering that the incidence and prevalence of osteoarthritis will continue to rise because the general aging of the population, and unless measures are taken to improve disease prevention and/or cure, Based on current trends, osteoarthritis is forecast to become the fourth leading cause of disability worldwide by the year 2020 (Woolf and Pfleger 2003).

So far several broad range inhibitory molecules were tested for osteoarthritis treatment. A whole class of them was designed to target MMPs, but the clinical development of broad-spectrum synthetic inhibitors has been hampered by adverse events, in particular the pain associated with joint fibroplasia in the musculoskeletal syndrome (MSS) (Renkiewicz, Qiu et al. 2003).

All the aforementioned enzymes are regulated, in physiological conditions, by the Tissue Inhibitors of MetalloProteinases (TIMPs). Interest in the research group led by my supervisor Dr George Bou-Gharios in collaboration with Prof Hideaki Nagase focused on the use of these endogenous inhibitors and specifically the TIMP-3 protein and its synthetic mutant [-1Ala]TIMP-3 as potential treatments of osteoarthritis. TIMP-3 was shown to be a major regulator of MMPs and ADAMTSs and mice lacking their homologous protein seem to face age-related increase in collagen and aggrecan degradation (Sahebjam, Khokha et al. 2007). Previous studies have shown that *in vitro* [-1Ala]TIMP-3 could block only aggrecanases (ADAMTS-4 and -5) and ADAMs (TACE/ADAM-10) (Lim, Kashiwagi et al. 2010). As mentioned in the Introduction chapter, ADAMTS-5 has been shown to be one of the enzymes mostly responsible for cartilage degradation in mice because mice deficient in ADAMTS-5 (but not ADAMTS-4) are protected from early aggrecan loss and cartilage erosion in a surgically-induced model of osteoarthritis (Glasson, Askew et al. 2005).

I decided to test *in vivo* the potential role of TIMPs in treatment of osteoarthritis and to generate transgenic mice that overexpress those proteins.

My aim was to generate a conditional/inducible cartilage-specific transgenic mouse in which TIMPs can be over-expressed at any given time in adult mice, such as before DMM surgery (Glasson, Blanchet et al. 2007). It was of paramount importance to use a promoter that would drive cartilage-specific expression throughout adulthood, as osteoarthritis is a late-onset disease. I demonstrated my own Cre deleter mouse strain (Chapter 3) was sufficient to drive the expression in cartilaginous tissues, I decided to use the same elements to generate the TIMP-expressing conditional mice.

4.2 Results

4.2.1 Design and construction of expression vectors

The DNA construct was designed to harbour the aggrecan regulatory elements, a floxed transcriptional STOP, the human TIMP-3 (or its mutant) coding sequence, an IRES, the *E.coli* lacZ gene and the SV40 (Simian Virus-40) polyadenylation (polyA) sequence. Both TIMP-3 and its synthetic mutant were FLAG tagged at the C-terminal, while the lacZ gene was modified to have a nuclear localization sequence (nls) that allows the protein to translocate and be retained into the nucleus. Protein tagging has been used widely in many research filed to help detect proteins of interest because the lack of strong antibody against the protein target as well as because of specific techniques such as protein purification or immunoprecipitation. FLAG system utilizes a short, hydrophilic 8-amino acid peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) that is fused to the recombinant protein of interest when expressed from a pFLAG vector (Terpe 2003, Brizzard 2008). FLAG tag's structure has been optimized to be more hydrophilic than other common epitope tags, therefore is more likely to be located on the surface of a fusion protein and less likely to denature or inactivate proteins to which it is appended. Finally because of the small size of the FLAG peptide tag, it is not likely to obscure other epitopes, domains, or alter function, secretion, or transport of the fusion protein. In addition, N-terminal FLAG tags can be removed readily from proteins once they have been isolated, by treatment with the specific protease, enterokinase. Many papers had shown that FLAG tag does not influence the target protein and specifically published and unpublished works in our lab shown that it does not affect the TIMPs activity *in vitro* (Troeberg, Fushimi *et al.* 2009, Scilabra, Troeberg *et al.* 2013). The structure of the vector is schematized in **Figure 4.1** (up). TIMP-3-Flag sequence was present in the Matrix Laboratory archive at KIR. The sequence was lifted from an original plasmid construct and cloned in a pIRES plasmid (originally from Clontech Laboratories) harbouring the LacZ gene downstream to the IRES sequence (also present in the same archive). The TIMP-3-Flag/IRES/LacZ was then lifted and cloned in downstream to Aggrecan enhancer sequences. Two constructs were generated to express either the TIMP-3 and its synthetic mutant [-1Ala]TIMP-3. These

constructs cannot constitutively express the transgenes because the presence of the transcriptional STOP, made by several repeated sequences of polyA. The loxP sites that flank the transcriptional STOP are recognized by the Cre recombinase from the P1 Bacteriophage. The loxP sites are 34 bp-long and consist of two 13 bp palindromic sequences that flank an 8bp spacer region. The Cre recombinase, a 38 kDa enzyme and member of the integrase family, catalyzes a site-specific recombination via a topoisomerase I-like mechanism (**Figure 4.1**, down). The outcome of the Cre-mediated recombination is dependent upon the location and relative orientation of the loxP sites. Recombination could occur between two different DNA species if located close enough. Interesting DNA found between two loxP sites oriented in the same direction will be excised as a circular loop of DNA whilst DNA between two loxP sites that are opposing-orientated will be inverted.

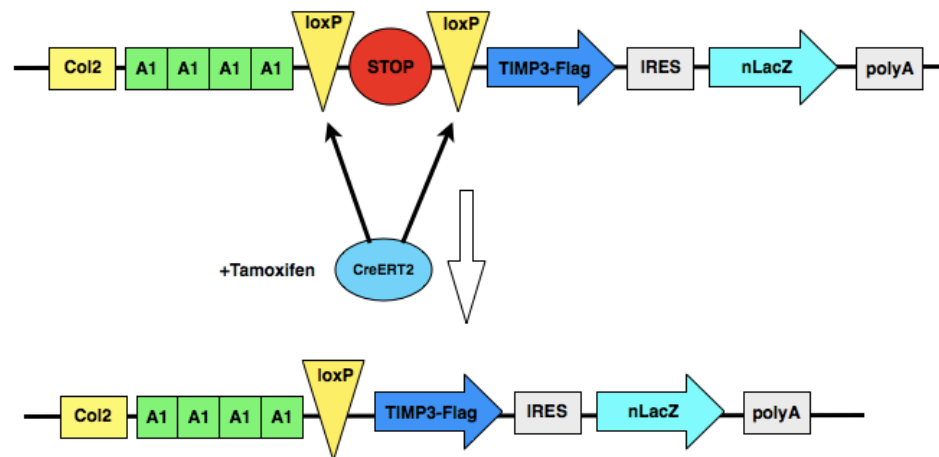


Figure 4.1: Schematic diagram of the Col2-4xA1-TIMP-3 IRES nlacZ plasmid and its mechanism. It consists of a *Col2a1* minimal promoter (Col2) plus four tandem repeats of mouse Aggrecan enhancer A1; transcriptional STOP sequences flanked by loxP sites; C-term flagged hTIMP-3 coding sequence; IRES; *E.coli* lacZ gene containing a nuclear localization sequence; polyA sequence. After Cre-mediated intrachromosomal recombination, the STOP sequence is excised allowing the expression of both of the downstream genes as a single mRNA. Arrows denote the direction of transcription. *E.coli* origin of replication and ampicillin resistance gene are not shown. IRES: internal ribosome entry site; polyA: polyadenylation sequence; *E.coli* origin of replication and Ampicillin resistance gene are not shown.

The original Col2a1-4xA1-CreER^{T2} plasmid was digested with BglII/SapI restriction enzymes, in order to isolate the aggrecan regulatory elements. This DNA fragment of 5kb also contained the origin of replication site, and it was handled as the vector fragment (**Figure 4.2 A**, left). The TIMP-3 coding sequence, the IRES and the lacZ gene together were isolated from a plasmid previously generated in our group using the EcoNI/AscI restriction enzymes. This DNA fragment was handled as the insert fragment (**Figure 4.2 A**, right). Both vector and insert were subjected to treatment with the Klenow enzyme, that has a 5'-3' polymerase activity, in order to make 5' overhang extremities blunt. Vector fragment was also treated with Calf Intestine Phosphatase (CIP), which catalyzes the removal phosphate groups at the 5' termini, reducing the ability of the vector fragment to self-ligate. The vector and insert fragments were ligated together using the T4 ligase enzyme. Digestion with XmnI restriction enzyme produced the expected digestion fragments (**Figure 4.2 B**). The plasmids generated as described were named LLC1 Col2-4xA1 hTIMP-3 IRES nlacZ

(LLC1 in short) and LLC2 Col2-4xA1 [-1Ala]hTIMP-3 IRES nlacZ (LLC2 in short). Both vectors were sequenced in order to confirm the sequences of the backbone and discriminate between the coding sequences of the two genes (**Figure 4.2 C**).

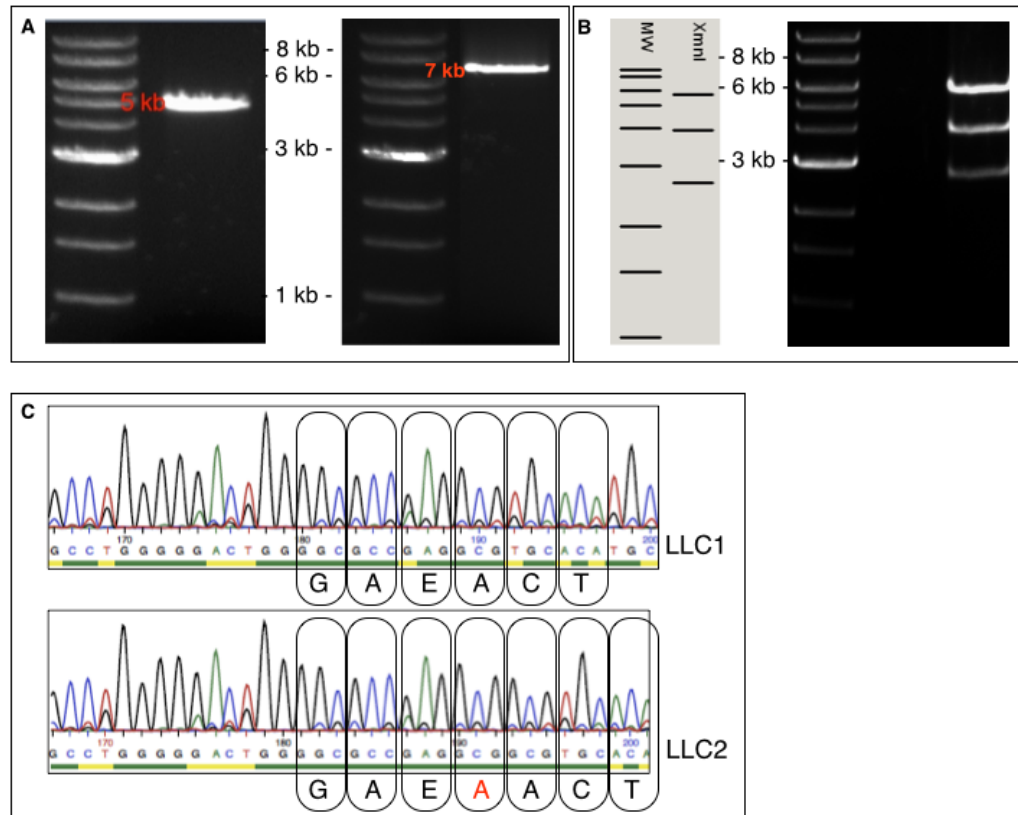


Figure 4.2: Cloning strategy used to generate the Col2-4xA1-TIMP-3 IRES nlacZ. A: Col2a1-4xA1-CreERT2 plasmid was digested with SapI/BglII restriction enzymes, to isolate the aggrecan elements in the 5 kb fragment (left); A plasmid containing hTIMP-3-Flag coding sequence was digested with EcoNI/AscI restriction enzymes, to lift the hTIMP-3-F IRES lacZ sequence in the 7,2 kb fragment (right). **B:** LLC1 and LLC2 plasmid were digested with XmnI restriction enzyme in order to confirm the correct direction of the ligation: three fragments were expected: 5.7 kb, 3.9 kb and 2.6 kb. **C:** LLC1 (above) and LLC2 (below) plasmids were sequenced in order to confirm the presence of the extra Alanine in position -1.

The first question that I wanted to answer was whether the plasmids were able to express the reporter gene in mammalian HTB94 chondrosarcoma cells. These cells were transfected using the Invitrogen Neon System (electroporation). Cultured cells were counted and re-suspended in a proprietary buffer. 1.0×10^6 cells were transiently transfected with 30 μ g of the chosen vector and seeded in two 6 cm of diameter culture dishes, in whole growing media and allowed to rest

24 h. In order to activate the transgene expression, the transcriptional STOP has to be removed by the Cre recombinase enzyme. Transfected cells were then transduced with an adenovirus expressing a Cre recombinase featuring a NLS, therefore not dependent on tamoxifen administration. Cells were incubated for 48 h and were stained for X-gal in order to evaluate the result of the experiment. I have optimized the infection procedure using MEF (mouse epithelial fibroblast) isolated from Rosa26 Reporter mice (Grover and Roughley 2006). In optimizing the adenovirus transduction my main concern was on cell viability. I arbitrarily selected 4 different MOIs (10, 25, 50, 100) and infected MEF cells isolated from Rosa26 Reporter embryos. The number of positive cells was increasing up to 50 MOI, but not much between 50 and 100 MOI. However I could observe several floating cells when the highest MOI was used, thus I decided to use 50 MOI that provide by observation the highest result with least side effect. I used as negative controls MEF from WT embryos treated with virus and MEF from Rosa26R not treated with virus.

Interestingly, although cells looked healthy, and the same experiment was repeated several times, the observed amount of cells which stained in blue after transfection and transduction, was quite low (**Figure 4.3 A,B**). Transfection of different DNA constructs of similar size but driven by different promoter, however shown a higher number of stained cells (**Figure 4.3 C**). A possible explanation could be the episomal structure of the plasmid, that might make it more difficult for the enhancer to induce the genes, or a combination of this event plus the recombination event driven by the Cre recombinase. Because TIMP-3 is an extracellular protein, I tried to detect it in the conditioned media by western blot analysis. Troeberg and Scilabra demonstrated that TIMP-3 is actively internalized, however that process could be mostly inhibited by adding heparin to the growth media (Scilabra, Troeberg et al. 2013). Experimental evidences demonstrated no appreciable difference in the TIMP-3 amount between LLC1- or LLC2-transfected and mock-transfected cells.

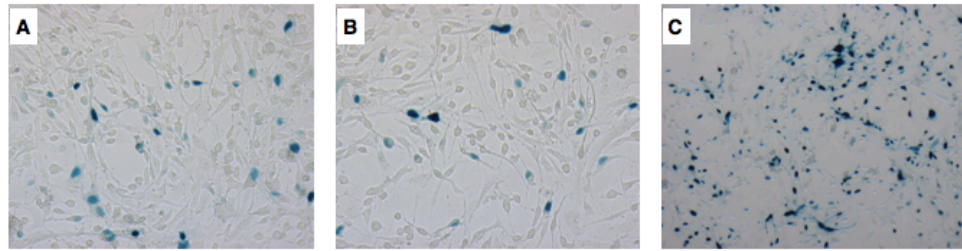


Figure 4.3: Representative bright field images of HTB94 cells transfected with the aggrecan-driven plasmid. HTB94 cells were transfected with an target plasmid using the Neon electroporation system (Invitrogen) e 24 hours later were transduced with a CMV-driven adenovirus expressing nCre recombinase. After 48 hours cells were fixed and stained for X-gal. A-B: Random fields from two independent experiments of LLC1 transfected cells. C: Random field from a control experiment using a general promoter.

4.2.2 Generation of transgenic mice

I decided to use both LLC1 and LLC2 plasmid to generate transgenic mice. Dr Ke Liu performed the whole procedure in the KIR animal facility. Both LLC1 and LLC2 plasmid were linearized by digestion with NaeI/SphI restriction enzymes, obtaining a 10 kb fragment. Linearization allowed removing the region of the vector backbone containing both the *E.coli* origin of replication and the ampicillin resistance gene. As described in Chapter 2 linearized vectors were injected in the male pronucleus of fertilized oocyte to generate the transgenic mice. When microinjected into pronuclei, transgenes are integrated at random into the genome. Thus, each founder will have a different site(s) of integration. The number of copies of the transgene possessed by each founder may also be different and there may be more than one site of integration within the genome. For all these reasons, each founder should be treated as a separate line and bred independently of other founders carrying the same transgene. Offspring obtained from each founder is required to be tested for transmission and expression of the transgene. Due to position effects and different copy numbers, each founder line can have a different level of expression. A percentage of positive founders of 11,68 % (7 of 60) and 8,16 % (4 of 49) for LLC1 and LLC2 constructs, was obtained respectively.

Once the mouse lines were established I wanted to evaluate the expression of the reporter gene, thus founder mice were mated to Cre recombinase expressing mice (ROSA-Cre, B6.129-Gt(ROSA)^{26Sortm1(cre/ERT2)Tyj/J} mice, The Jackson Laboratory,

USA). Pregnant mothers were injected with 5 mg of tamoxifen 14 days after mating (E14.5). These mothers were sacrificed at E15.5 and the embryos were fixed and stained with X-gal. If blue staining was evident and uniform in cartilaginous structures, the transgenic line was maintained. I identified two transgenic lines for the LLC1 construct (lines 53 and 58) and two lines for the LLC2 (lines 18 and 47). Transgenic line LLC2.47 was discarded afterwards because expression levels were not judged sufficient to deliver the transgene (**Figure 4.6 J**). All the founders were bred for homozygous lines. At the same time transgenic mice were crossed to homozygous ROSA-Cre mice to obtain double transgenic adult mice. The transgenic mouse lines used for breeding were heterozygous and only after few generations I obtained homozygous mice. Using heterozygous in the ROSA-Cre breeding led to a progeny positive of only on 50% for the Cre recombinase.

I compared the different lines in order to evaluate if the staining was uniform among the transgenic lines and eventually comparable. Particular attention was paid to the patterning of the staining. Because transgenic mice are generated by pronuclear microinjection, as already discussed in the Introduction chapter, position variegation effects (PVEs) may occur if vector copies land in compact chromatin regions.

All lines generated from the aggrecan-driven vectors expressed β -galactosidase in all the cartilaginous structures of the embryos after tamoxifen administration to the pregnant mother. However not all of them were stained similarly. Here I will describe the study of the LLC1 #53 mouse line that overall performed best. The remaining transgenic lines shown variable results and will require further investigation. A strong blue color was observed in the developing cartilage of the long bones of the fore and hind limbs, the ribs, the scapulae, the digits and some craniofacial cartilages such as olfactory, otic and jaws cartilages. Spine and tail were also stained. It was clearly visible the formation of secondary centers of ossification in the long bones. Both tibia plateau and femur condyles showed blue staining (**Figure 4.4**).

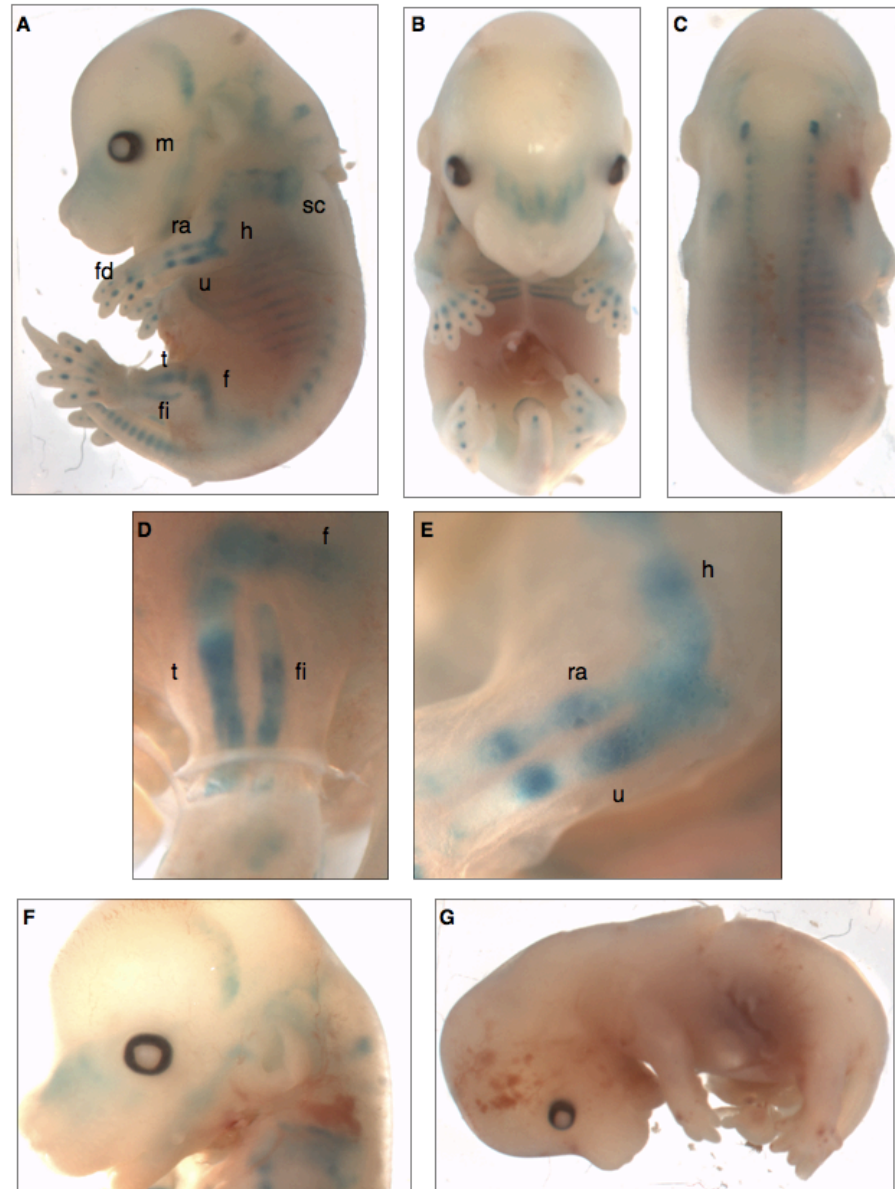


Figure 4.4: Representative E15.5 wholemount embryos expressing β -galactosidase (blue staining) driven by the Aggrecan enhancer. A-F: Double transgenic embryos were showing identical and symmetrical staining. G: Double transgenic embryos without tamoxifen administration did not expressed β -galactosidase. A,D,E,F,G are lateral images while B and C are frontal and dorsal images. f (femur), fd (forelimb digits), fi (fibula), h (humerus), hd (hindlimb digits), m (Meckel's cartilage), r (rib cartilage), ra (radius), sc (scapular cartilage), t (tibia), u (ulna).

Furthermore I wanted to know how the β -galactosidase expression was distributed among the chondrocytes. Whole embryos were embedded in wax and histological sections cut along the sagittal plan. Observation at higher magnification showed that the nuclei of all chondrocytes were stained in blue (**Figure 4.5**), and thus were expressing the transgene.

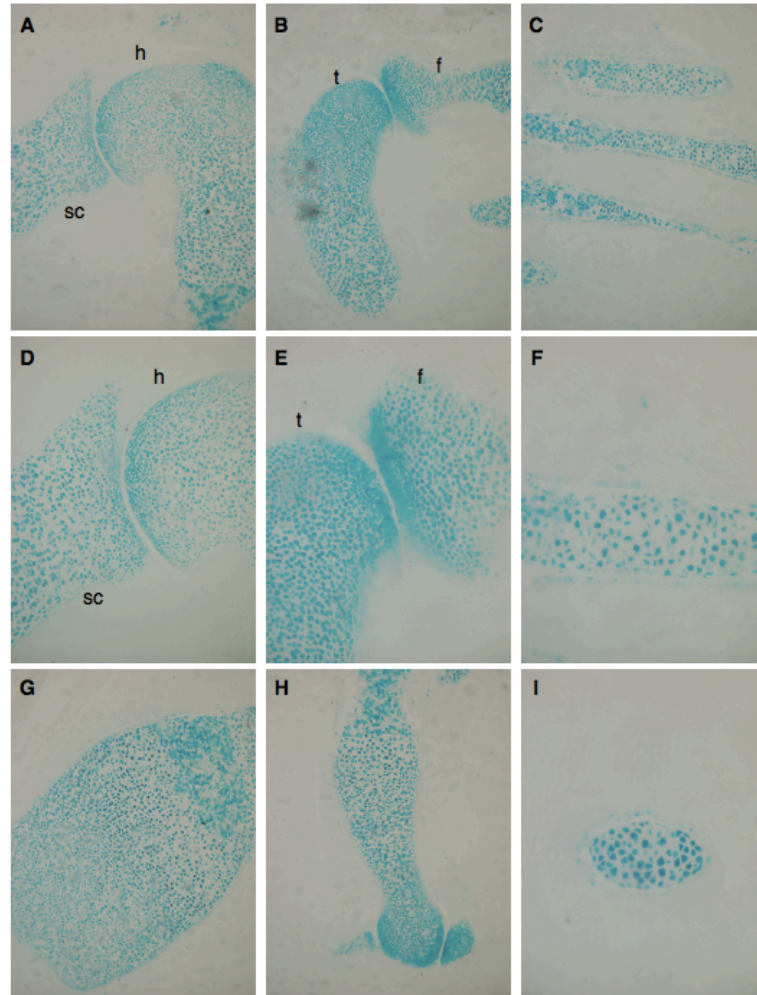


Figure 4.5: Representative histological sections of E15.5 double transgenic embryo expressing β -galactosidase (blue staining) driven by the Aggrecan enhancer. A-C: representative images of shoulder articulation, knee joint and ribs cage, respectively. D-F: higher magnification images of A-C. G-I are representative images of scapula, humerus and rib, respectively. f (femur), h (humerus), sc (scapular cartilage), t (tibia).

Because the aim of this particular section was to obtain cartilage-specific expression in adult mice, 6 week old double transgenic mice were injected with 1.5 mg Tamoxifen three times every 48 h. Mice were sacrificed two weeks later (8 weeks old) and several tissues harvested. Knee joints were exposed to increase penetration of X-gal staining solutions, which was carried out according to the protocol (Chapter 2.2, Methods). Whole mount X-gal staining demonstrated that expression of the lacZ gene was in hyaline cartilage. As the major aim was to express in the knee joint articular cartilage, staining demonstrated both tibia plateau and femur condyles from double-transgenic mice were stained blue but not the non-double-transgenic littermates (**Figure 4.6 A-C**). Cartilage surrounding the hip was also strongly stained. In double transgenic mice without

tamoxifen administration, no x-gal staining was observed in cartilage (**Figure 4.6 D-F**). Finally I looked for expression in non-cartilaginous tissues and confirmed that the transgene is not expressed outside cartilage (**Figure 4.6 G-I**).

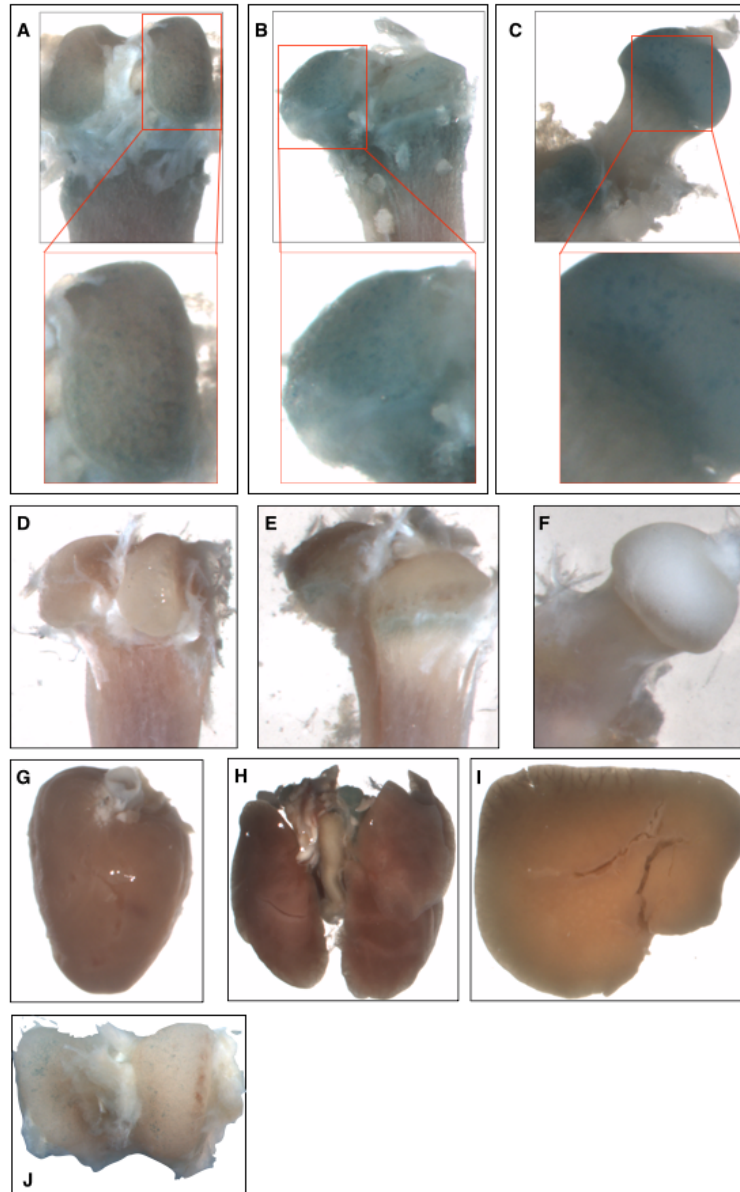


Figure 4.6: Representative pictures of adult double transgenic mice expressing β - galactosidase (blue staining) driven by the Aggrecan enhancer. 8-weeks old double transgenic mice and wildtype littermates were injected with Tamoxifen and sacrificed 2 weeks later. Tissues were isolated and stained for X-gal. A-I: Line LLC1.53. J: LINE LLC2.47. A-C representative images of femur condyles, tibia plateau and hip head from double transgenic transgenic mice; red boxes are zoomed particulars. D-F: representative images of femur condyles, tibia plateau and hip head from double transgenic mice without tamoxifen administration. G-I: representative images of heart, lungs and liver from double transgenic transgenic mice treated with tamoxifen. J: Representative image of femur condyles of a double transgenic mice LLC2.47, showing much lower staining compared to line LLC1.53.

4.2.3 Quantification of the expression levels and detection of TIMP-3 protein in adult tissues

A key question to address was about the TIMP expression levels in those mice. Six weeks old double transgenic mice were injected with 1.5 mg tamoxifen IP, three times every 48 h. Mice were sacrificed two weeks later (8 weeks old). Knees were isolated and cartilage was micro-dissected and stored in RNAlater solution. RNA was extracted, purified and reverse transcribed into cDNA, as described in the Methods. Micro-dissection of the articular cartilage proved to be a challenging method. Articular cartilage is just few tens of micron thick (Stockwell 1971) and thus avoiding damage to the underneath bone is very difficult. Using a micro-rongeur, allows gently scraping the surface to the joint, collecting the overlying articular cartilage. Moreover extracting RNA from the dissected cartilage is a very difficult task because the huge amount of charged protein of the tissue. The protocol for RNA purification is described in the Chapter 2.2. The amount of RNA extracted from the articular cartilage of two knee joints was sometime insufficient; nevertheless I decided not to mix individual mice, in order to highlight the expected individual variability among the same group of transgenic mice. All primers/probe sets for human TIMP3 and mouse Hprt were bought from Applied Biosystems. Although the primer set was designed for human TIMP3 gene, it was able to detect the endogenous mouse gene. The explanation relies on the fact that mouse and human TIMP-3 proteins share 95% identity in the aminoacid sequences. Real-time PCR analysis showed an increase of the Timp3 expression of ~4 fold. The data were analysed using the paired T-test, and proved to be statistically significant (**Figure 4.7**).

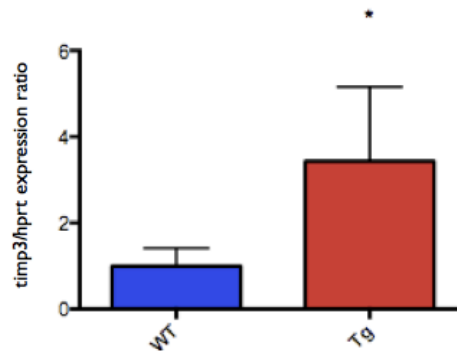


Figure 4.7: Timp3 upregulation in double transgenic mice after Tamoxifen administration. Real-time PCR analysis of Timp3 in mouse articular cartilage of 10-weeks old double transgenic mice and control mice (WT). Tamoxifen was administrated at 8-weeks of age. Y values represent fold difference of timp3 expression compared to endogenous Hprt expression. n=3. P < 0.05. Error bars represent (SD). Hprt: hypoxanthine guanine phosphoribosyl transferase.

The last evidence for the analysis of the transgenic line was to detect the TIMP protein in the articular cartilage. Six-weeks old double transgenic mice were injected with 1.5 mg Tamoxifen via IP, three times every 48 h. Mice were sacrificed two weeks later (8 weeks old). Knees were isolated fixed in neutral buffered formalin 10% for 24 h and decalcified in 10% EDTA pH 7.4 for 4-5 weeks. Subsequently tissues were embedded in paraffin and sectioned. As previously stated RNA extraction, the main difficulty in cartilage work is given by the high amount of charged proteins in the ECM. To detect the TIMP-3 protein by immunofluorescence, the ECM was partially digested by hyaluronidase treatment. The concentration of anti-TIMP-3 antibody was optimized to reduce the tissue background signal, so that it was very low, although not completely absent, in sections samples from TIMP3-KO mice. The fluorescence signal was observed in the chondrocytes pericellular space transgenic mice, but not in wild type littermate (**Figure 4.8**).

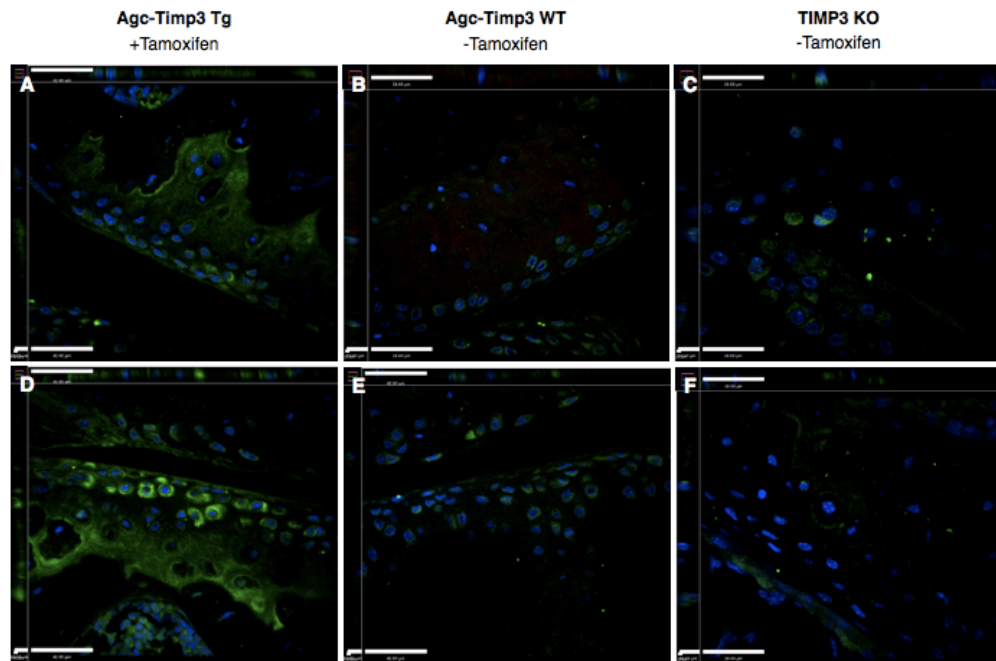


Figure 4.8: Representative confocal images of coronal histological sections from knee joint from 10-weeks old mice. 8-weeks old double transgenic mice and wildtype littermates were injected with Tamoxifen and sacrificed 2 weeks later. Knee tissues were isolated and after decalcification embedded in wax. Increase in TIMP-3 (green) was observed in double-transgenic mice tissues (A,D) but not in wildtype littermates (B,E). Very low background signal was detected in tissues from TIMP-3 KO mice (C,F). A-C are representative images for femur condyles, D-E for tibia plateau. Tissues were counter stained with DAPI to highlight cells nuclei.

4.3 Discussion

In this chapter I described all the steps undertaken to generate an inducible transgenic mouse that express TIMP-3 specifically into the articular cartilage. I generated several transgenic lines that express either human TIMP-3 or [-1Ala]TIMP-3, and selected the most promising ones in terms of expression levels and localization.

Because of their role in cartilage remodelling, both metalloproteinases and aggrecanases were targeted in clinical trials for treatment of osteoarthritis. Several synthetic inhibitors were designed and tested, but data have shown that such inhibitors may produce musculoskeletal side effects characterized by joint stiffness, joint fibroplasias and accumulation of type I collagen in the affected joints (Renkiewicz, Qiu et al. 2003). Possible reasons could be the low selectivity of the inhibitors, this data are pushing research groups to develop selective inhibitors of proteinases rather than broad proteinases inhibition.

I selected TIMP-3 as possible target for osteoarthritis treatment because it regulates the pro-MMP-2 activation and demonstrates high affinity inhibition of both ADAMTS-4 and ADAMTS-5. Moreover knowing that its broad activity towards most of MMPs and aggrecanases could results in a less clear phenotype, I decided to take advantage of synthetic mutant of TIMP-3 that were generated and studied in Gill Murphy's research groups (Lee, Verma et al. 2002). Particularly the [-1Ala]TIMP-3 was shown to be able to inhibit *in vitro* aggrecanases and TACE enzymes, but not MMPs (Lim, Kashiwagi et al. 2010).

I decided to generate a transgenic mouse that could express TIMP-3 specifically in the cartilage tissue, in order to minimize the effects of expression from other tissues such as subchondral bone, or synovial tissue. I take advantage of the Cre deleter described in the previous Chapter, and generated two plasmids named LLC1 and LLC2, that could express TIMP-3 and [-1Ala]TIMP-3 respectively. I tested if the plasmids could express the transgene or the reporter gene (*lacZ*) *in vitro*, transfecting HTB94 (chondrosarcoma) cells. Because a floxed transcriptional STOP region prevented the expression, transfected cells were also transduced with an adenovirus expressing the Cre recombinase, to mediate the recombination. X-gal staining on those cells showed that *E.coli lacZ* gene was expressed as nuclei were stained of blue. Troeberg and Scilabra in their experiments on TIMP-3, succeeded in detecting the protein in the stable transfected cell media treated with heparin. They also have shown that TIMP-3 is actively removed from the pericellular space by direct endocytosis. I was not able to detect TIMP-3 in transfected cell media, probably because of the low amount of protein produced. The discrepancy with the strong blue staining of the nuclei could be explained by the fact that the β -galactosidase enzyme is accumulated in the nuclei, instead TIMP-3 is degraded once internalized. Both the plasmids were used to generate transgenic mice that were mated to a ROSA-Cre deleter. Mouse lines showing blue staining in the progenies were kept, all the rest were cull. Among all the mice obtained I decided to maintain two lines for the LLC1 plasmid (#53 and #58) and one for the LLC2 plasmid (#47). Among all LLC1 #53 shown best expression patterning, the other lines will require further investigation. From whole-mount X-gal staining, expression was evident in all

cartilaginous structures. Spine and ribs, jaw and otic cartilage as well as knee and elbow were clearly stained. Because these results were promising I decided to analyse the adult mice. Whole-mount X-gal staining as well as sagittal sections of the knees demonstrated that those mice expressed the lacZ gene also in adult tissue. Blue staining was observed in the cartilage surrounding hips, but not in other tissues such as liver or kidney. I expected different levels of expression of the transgene among the different mouse lines. Such difference could not be appreciated in X-gal staining because it is an end-point assay, and measures did not correlate with the real values. Transgenic mice expressed about 5 times more Timp3 mRNA, after two weeks activation via tamoxifen, compared to the wild-type ones. TIMP-3 protein localization was investigated by immunofluorescence, and I was able to detect an increase in signal in the transgenic mice compared to the wild-type ones.

There is small amount of literature on TIMP-3 mRNA expression in mouse embryos. The mayor study on TIMP-3 expression and localization in mouse has been conducted by Suneel Apte and colleagues in 1994 (Apte, Hayashi et al. 1994). Their paper was the first to describe genomic localization and expression pattern of TIMP3 in mouse. Northern Blot analysis of mRNA in several mouse adult tissues shown that kidney is by far the organ with the highest expression of Timp3 transcript, followed by lung, and brain. They also shown that Timp3 message is highly expressed both in developing and new-born mouse tissues. Localization of the Timp3 transcript was observed by in situ hybridization (ISH) in both embryo and newborn mice. Expression of Timp3 mRNA was observed in E14.5 embryos in Meckel's cartilage, otic capsule, intervertebral disc and hypertrophic chondrocytes of the vertebral cartilage. Timp3 mRNA was also observed in intervertebral disc and hypertrophic chondrocytes of the ribs and sternum in new-born mice. In 1997 Chin and Werb performed in situ hybridization (ISH) on mouse dissected mandibles from mouse embryos, obtaining evidences that TIMP-3 mRNA is expressed intensely in the mesenchyme in the pre-osteoid region and along the medial axis of the tongue of E12-E14 embryos, but not in Meckel's cartilage (Chin and Werb 1997). Blavier and DeClerk in 1997 investigated the expression of TIMP-3 in adult mouse

tissues by Northern Blot: kidney was by far the organ with the highest expression, followed by lung (Blavier and DeClerck 1997). Weaker signal was detected also in brain, eye, heart and ovary. Although I did not performed any ISH studies, from the β -galactosidase staining pattern seems evident that in my model TIMP-3 could be expressed in different tissues in respect of wild-type, and this support furthermore my choice to generate and inducible model where TIMP-3 expression does not affect the physiological expression pattern during early and late embryogenesis and early development.

Taken together all those data prove that the approach I pursued was right and I can deliver exogenous TIMP-3 in mouse knee articular chondrocytes. Because there is not literature on this specific field I did not knew if such increase will be sufficient to produce a phenotype in OA-induced animal. I do know that TIMP-3 is physiologically regulated not just at the transcription level, but it is actively endocytosed via more than one pathway (Scilabra, Troeberg et al. 2013). Among the transgenic mouse lines generated so far, I foresee that line LLC1 #53, is a good candidate to undergo further studies and investigate the potential role of TIMPs overexpression in osteoarthritis.

Chapter 5

Design and Generation of an
inducible Ubiquitin C-driven TIMP-3
and
[-1Ala]TIMP-3 transgenic mice

5.1 Background

As previously stated the major aim I tried to fulfil was to generate an inducible system specific for mouse articular cartilage transgenic expression. However I was well aware that in the content of the knee joint, articular cartilage is not the only tissue responsible of the maintenance of the joint physiology. Although articular cartilage is the main tissue affected during early stage OA, the subchondral bone region underneath influences and is influenced in turn, not only by mechanical effects, but also by exchange of catabolic factors. *In vivo* observations in human OA patients demonstrates interdependence between the loss of cartilage and the deterioration of the subchondral bone structure (Sharif, George et al. 1995, Wildi, Raynauld et al. 2010). Emerging evidence suggests that very early in the OA process, biological and morphological disturbances occur at the subchondral bone level that play a role in modulating articular cartilage metabolism.

In OA, the role of synovial inflammation in the pathophysiology of OA is now widely accepted. Synovitis has been considered secondary to the cartilage changes yet findings indicate that synovial inflammation could be a component of the early events leading to the clinical stage of OA. Synovial inflammation leads to the production and release of pro-inflammatory cytokines and several other inflammatory mediators. Some of these factors, including the pro-inflammatory cytokines, diffuse through the synovial fluid into the cartilage, where they activate chondrocyte production of the catabolic factors through auto- and paracrine mechanisms.

This evidence led me to design a second strategy of transgene delivering using a ubiquitous promoter in order to deliver the transgene in other tissues. A peculiarity of ubiquitous promoters is that they are expressed in virtually all cells of the organism because they usually belong to those genes responsible for important cell processes that are little altered by external stimuli. One of the most common system to express a transgene is the cytomegalovirus (CMV) enhancer/chicken β -actin promoter (CAG) (Niwa, Yamamura et al. 1991). Another example is the human promoter of the ubiquitin C, as was shown by Schorpp, it

is able to drive expression of target genes both *in vitro* and *in vivo* (Schorpp, Jager et al. 1996).

5.2 Results

5.2.1 Design and construction of expression vectors

The expression vector containing the human Ubiquitin promoter was kindly donated by Richard Behringer's group, and is described in Stewart et al. (Stewart, Jang et al. 2009) (**Figure 5.1 A**). Here the human Ubiquitin C (UBC) promoter fragment was used to direct expression of the HS-GR cassette (UBC-HS-GR) in a wide variety of embryonic cell types (Schorpp, Jager et al. 1996). To reduce position effects at the site of transgene integration, the transgene was flanked by two copies of cHS4 (chicken hypersensitive site 4) transcriptional insulators (Potts, Tucker et al. 2000).

I designed a three-steps plan to construct the final vector (**Figure 5.1 B**).

- I first lifted the whole “STOP-TIMP3-IRES-LacZ-polyA” sequence from the Col2-4xA1 hTIMP-3 IRES nLacZ described in Chapter 4.
- I then digested the UBC-HS-GR expression vector from Stewart, removing all sequence in between the UbC promoter and the cHS4 site.
- Finally I ligated the “STOP-TIMP3-IRES-LacZ” sequence into the empty UbC-driven vector.

The final construct was named UbiquitinC hTIMP-3 IRES nLacZ.

The expression vector designed for expression of TIMP-3 is schematized in **Figure 5.1**. Similarly to the plasmid described in Chapter 4, here the human TIMP-3 coding sequence is linked to the lacZ gene by an IRES sequence. The lacZ gene, modified with a nuclear localizing sequence is followed by a polyadenylation sequence (polyA) to allow expression in eukaryotic cells. The expression vector harbours two tandem repeats of the chicken β -globin

hypersensitive site 4 (cHS4) that have been shown to function as transcriptional insulators, thus increase shielding of transgenes from position effects in cell-lines and transgenic mice (Potts, Tucker et al. 2000). Expression is driven by the Ubiquitin C promoter/first intron. The expression vectors were generated similarly to the aggrecan-driven ones, and named Ubiquitin C hTIMP-3 IRES nlacZ (UbCT3 in short) and Ubiquitin C [-1Ala]hTIMP-3 IRES nlacZ (UbCAla in short). As for the Aggrecan-driven expression vectors, in order for these constructs to express the transgenes, a recombination event mediated by the Cre recombinase is required because of a floxed transcriptional STOP.

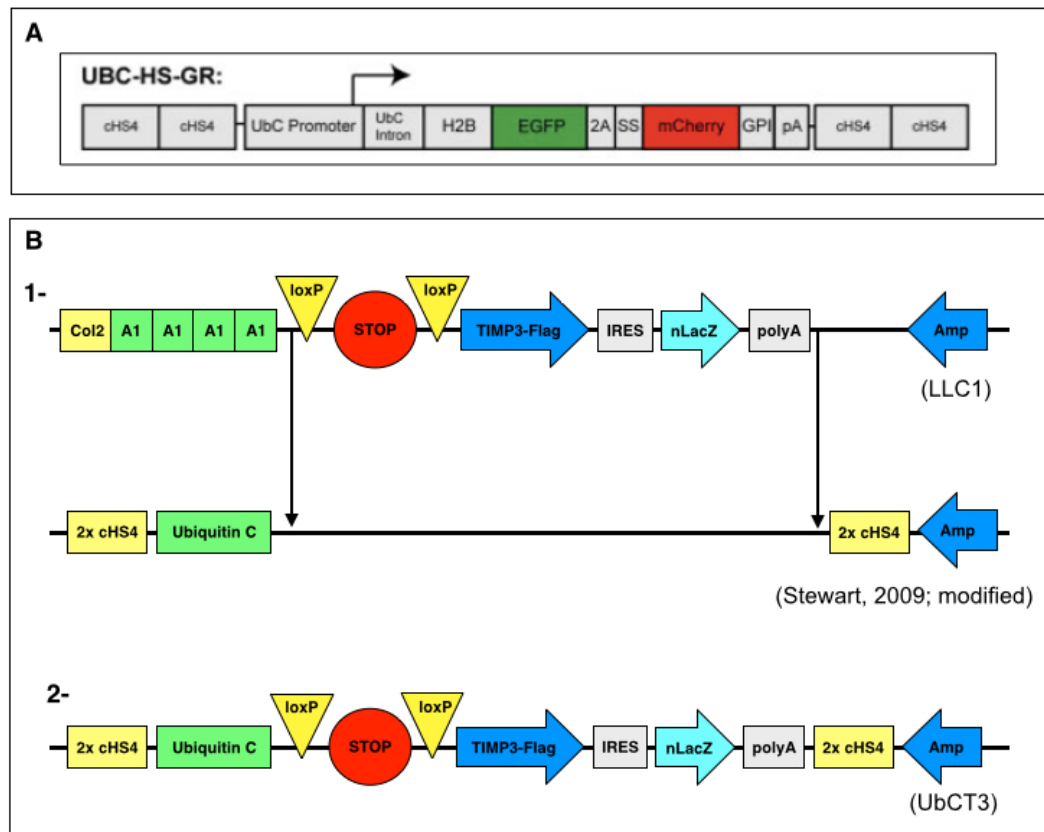


Figure 5.1: Schematic diagram of the UbC-TIMP-3 IRES nlacZ expression vector. It consists of a Ubiquitin C minimal promoter/first intron to drive expression; transcriptional STOP sequences flanked by loxP sites; C-term flagged hTIMP-3 CDS; IRES; E.coli lacZ gene plus NLS; polyA sequence; cHS4 chicken hypersensitive site 4. After Cre-mediated intrachromosomal recombination, STOP sequence is excised allowing the expression of both of the downstream genes as a single mRNA. Arrows denote the direction of transcription. E.coli origin of replication and ampicillin resistance gene are not shown. IRES: internal ribosome entry site; polyA: polyadenylation sequence.

The first question that I wanted to answer was if the plasmids were able to express the reporter gene in mammalian HTB94 chondrosarcoma cells. Those cells were transfected using the Neon System (Invitrogen) as described in the Methods chapter. In brief cultured cells were counted and resuspended in a proprietary buffer and 1.0×10^6 cells were transiently transfected with 30mg of the chosen vector and seeded in two 6 cm of diameter culture dishes, in complete medium and incubated for 24 h. In order to activate the transgene expression, the transcriptional STOP has to be removed by the Cre recombinase enzyme. Transfected cells were then transduced with a CMV-driven adenovirus expressing the Cre recombinase enzyme modified with a nuclear localizing sequence, therefore not dependent on tamoxifen administration for relocating in

the nucleus. Cells were incubated 48 h and tested for lacZ expression. As expected the UbC-driven vectors generated were able to express β -galactosidase protein in very high levels (**Figure 5.2**). Particularly striking was the number of stained cells compared to the Aggrecan-driven vector. Based on these evidences I decided to generate transgenic mice from these expression vectors.

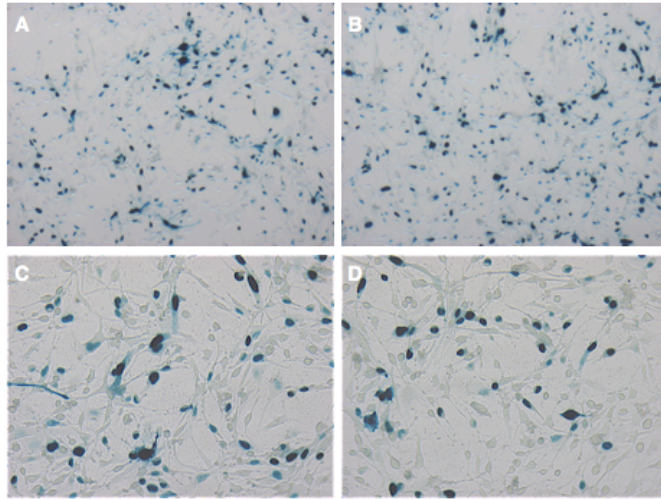


Figure 5.2: Representative bright field images of HTB94 cells transfected with the Ubiquitin C-driven plasmid. HTB94 cells were transfected with an target plasmid using the Neon electroporation system (Invitrogen). 24 hours later cells were transduced with a CMV-driven adenovirus expressing nCre recombinase. After 48 hours cells were fixed and stained for X-gal. **A-D:** Random fields from four independent experiments.

5.2.2 Generation of transgenic mice

Both UbC-TIMP-3 and UbC-1AlaTIMP-3 vectors were linearized after digestion to remove part of the plasmid backbone containing both the origin of replication and the ampicillin-resistance gene. As previously described Dr Ke injected the linearized DNA in the male pronuclei of fertilized oocytes, which were than transferred in surrogate mothers (C57BL/6 background). Progeny was screened by PCR for the lacZ gene to identify the positive transgenics.

Transgenic mice, identified by PCR, were mated to ROSA-CreR^{T2} mice in order to obtain double transgenic animals. Pregnant female were injected once with 5 mg of tamoxifen at embryonic day E14.5 and sacrificed the day after. Embryos were collected and stained for β -galactosidase. As expected I observed variability in β -galactosidase staining and the least promising transgenic mouse lines were

culled. Transgenic line UbCAla #38 proved to be the most promising line, so I will describe the characterization of this particular line.

The use of a non-tissue specific promoter allowed the transgene to be expressed in most of the mouse tissues. Embryos were found positive for X-gal staining in skin, cartilage, and some internal organs (**Figure 5.3**).

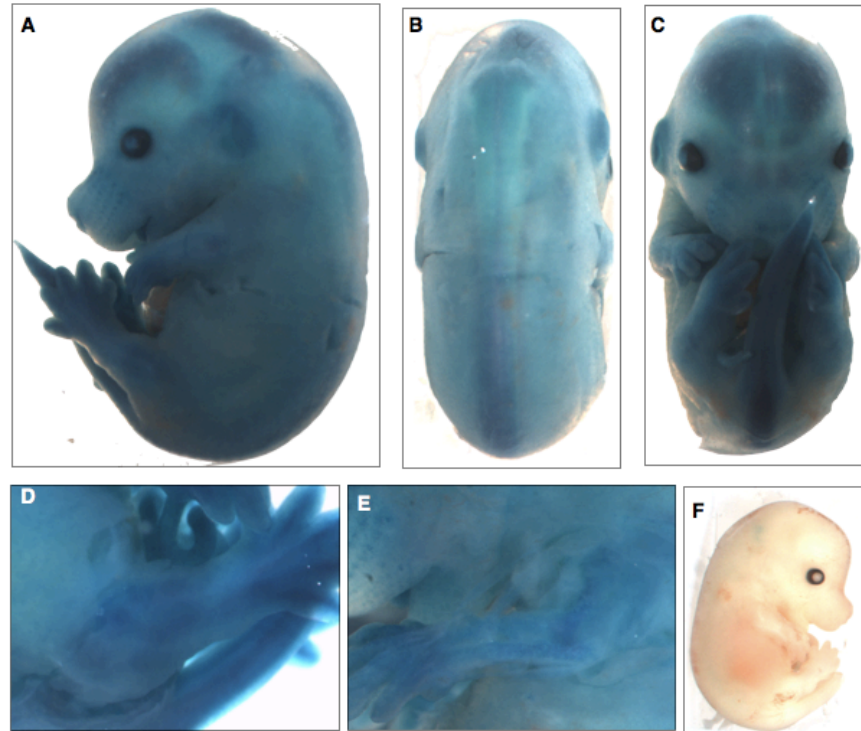


Figure 5.3: Representative E15.5 wholemount embryos expressing β -galactosidase (blue staining) driven by the UbiquitinC promoter. A-E: Images from a double transgenic embryo after tamoxifen administration. **F:** Representative double transgenic embryo without tamoxifen administration. Transgenic mice shown identical and symmetrical staining. Compared to the Aggrecan-driven construct where only cartilage was stained, here the staining appears diffused.

To evaluate localization and levels of lacZ expression in adult tissues 8-week-old double transgenic mice were injected 3 times every other day 1.5 mg of tamoxifen and sacrificed 2 weeks later. Several tissues were collected and stained for X-gal. Strong blue staining was observed in the articular cartilage of tibias, femurs and hips. Some X-gal positive staining was observed also in ribs, and tendons. However no staining was observed in heart or lung (**Figure 5.4**). Staining in the heart was expected as Stewart and colleagues in their 2009 paper have shown that a similar construct is able to deliver eGFP in the heart of E11.5 mouse embryos (Stewart, Jang et al. 2009). Further investigation has to be done to address this discrepancy, although it may diverge from the target of the present work.

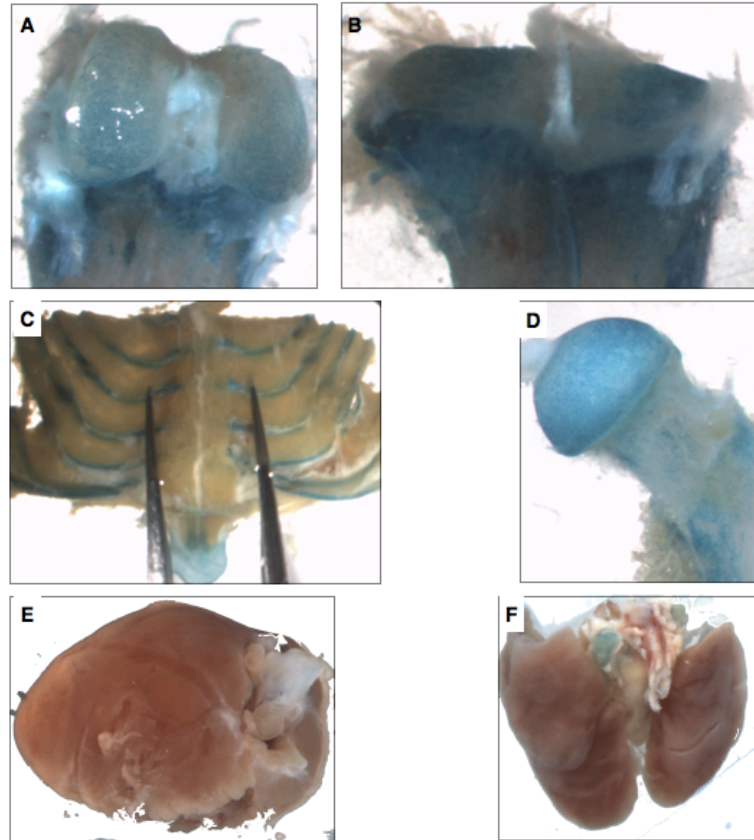


Figure 5.4: Representative pictures of adult double transgenic mice expressing β - galactosidase (blue staining) driven by the *Ubiquitin C* promoter. 8-weeks old double transgenic mice were injected with Tamoxifen and sacrificed 2 weeks later. Tissues were isolated and stained for X-gal. A-D strong staining was observed in both femur condyles and tibia plateau as well as rib cage and hip head of double transgenic transgenic mice. E-F: Non-cartilagenous tissues such as heart and lungs were not showing any blue staining.

As for the Aggrecan-driven vectors I wanted to evaluate the expression level of Timp3 in cartilage. As described in the previous chapter I micro-dissected the articular cartilage both from tibia plateau and femur condyles of 10-week old double transgenic mice injected with tamoxifen. RNA was extracted, purified and reverse-transcribed to produce cDNA library, as described in Methods (Chapter 2.2). Relative levels of expression of Timp3 were evaluated and compared to the endogenous Hprt levels. Timp3 levels of expression in the UbCA1a #38 mouse line were calculated to be 15-20 times upregulated compared to the control. These values are higher compared to the LLC1 #53 mouse line (**Figure 5.5**).

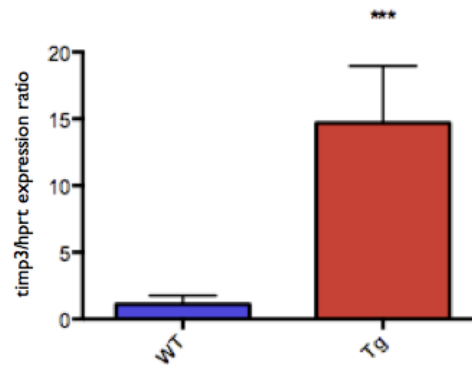


Figure 5.5: Timp3 upregulation in UbCA1a #38 transgenic mice after tamoxifen administration. Real-time PCR analysis of Timp3 in mouse articular cartilage of 10-weeks old double transgenic mice and control mice (WT). Tamoxifen was administrated at 8-weeks of age. Y values represent fold difference of Timp3 expression compared to endogenous Hprt expression. n=4. $P < 0.05$. Error bars represent standard deviation. Hprt: hypoxanthine guanine phosphoribosyl transferase

Finally TIMP-3 protein levels were investigated in mouse knees samples. Knees from 10-weeks old mice, injected with tamoxifen two weeks prior to collection. Epiphysis were dislodged from the underneath bone and collected. All four epiphysis from the lower limbs of each animal were pulled together and processed as described in Methods (Chapter 2.2). Western blot analysis on these samples showed an increase of TIMP-3 protein of 4-5 times (**Figure 5.6**).

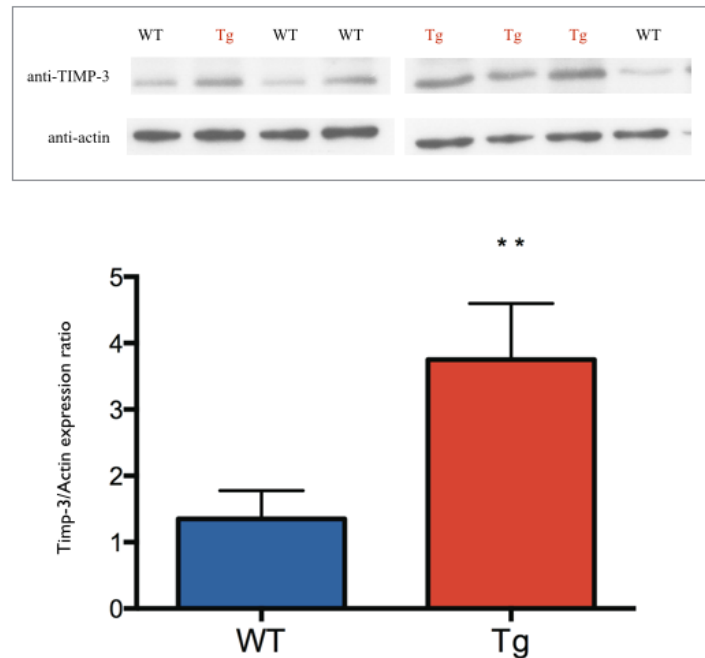


Figure 5.6: Timp-3 protein is upregulation in UbCAla #38 transgenic mice after tamoxifen administration. Western blot analysis of TIMP-3 in mouse epiphysis of 10-weeks old double transgenic mice and control mice (WT). Tamoxifen was administrated at 8-weeks of age. Y values represent fold difference of TIMP-3 signal compared to endogenous Actin signal. n=4. P < 0.05. Error bars represent standard deviation (SD). Upper: Representative Western Blot. Lower: representative statistical analysis.

5.3 Discussion

In this chapter I described how I generated a mouse model for general TIMP overexpression. I decided to use the ubiquitin C promoter that would allow a virtually ubiquitous expression. Similarly to the vector described in Chapter 4, two vectors were designed to express TIMP-3 or [-1Ala]TIMP-3. Data on the localization of the β -galactosidase staining in HTB94 transfected cells, suggested that this promoter might provide a stronger expression of the transgene compared to the Aggrecan one. Transgenic mice were generated from both the plasmids, and only the ones expressing β -galactosidase at embryonic stage were kept. Moreover I selected only the ones that presented stronger blue staining. Among them the UbC [-1Ala]TIMP-3 #38 (UbCAla #38 in short) proved to be the most promising line, and I focused further studies on that one line.

The rationale to employ a general promoter is to achieve expression in other tissue of the joint other than the articular cartilage itself. Expression of the transgene in the synovial membrane may allow the TIMP protein to be present in

the synovial fluid and increase the binding to the cartilage ECM. However a bias would be if the protein would be able to reach deeper layer of the tissue. On the other side if TIMP is expressed in the sub-chondral bone, it may help to protect the ECM in the deepest layers of the cartilage.

In eukaryotic cells, not all the genetic information is readily available for transcription. It is known that in the nucleus DNA is organized in two structurally and transcriptionally different states: heterochromatic and euchromatic. The first is usually associated with an “open” structure that allows the transcription machinery (including all the co-factors) to bind the target regions and promote gene expression; the latter is associated with “closed” structure and silencing of genes. It has been proposed that genes and gene clusters may achieve regulatory autonomy by organization into chromatin domains that are maintained independently of their surroundings, through the establishment of chromatin boundaries. Chromatin boundaries are observed as a physical transition from transcriptionally repressive condensed chromatin to more permissive open chromatin structures. Such boundaries vary in position and would appear as a broad transition in chromatin states (fuzzy boundaries) when studying a population of cells. The lack of fixed boundaries is the basis of position-effect variegation (PEV), where the stochastic spread of heterochromatin formation results in the heritable silencing of a neighbouring gene. Boundaries can be the result of a balance between countervailing chromatin opening and condensation processes that are nucleated at neighbouring gene loci. Such boundaries would not be fixed so large intergenic regions would be required to buffer a gene from its neighbours. The boundaries of heterochromatin domains can be fixed by the action of cis-regulatory elements. In some cases, these could be promoters or enhancers with specific characteristics that allow them to resist heterochromatin formation. Heterochromatin boundaries would be formed by autonomous elements known as insulators. Insulators can be defined as DNA elements that have a common ability to protect a gene from undesirable regulatory influences present in their chromosomal environment. There are at least five types of insulators in *Drosophila* that have been studied in details: the sequences flanking the heat shock hsp70 locus, the gypsy insulator (identified in the gypsy

retrotransposon), the Fab 8 insulator in the bithorax complex and the SF1 insulator described in the Antennapedia complex. In vertebrates the most studied and characterized thus far is the CTCF-mediated insulator (as in the chicken β -globin locus) that groups components and mechanisms of action of several of the invertebrates ones (Barkess and West 2012). Randomly integrated transgenes that lack insulation tend to silence over time in culture, but transgenes shielded by barrier insulators may retain their expression, regardless of chromosomal position. Barrier insulators must be placed on both flanks of the transgene as chromosomal silencing can encroach from either direction. Studies on the chicken HS4 (cHS4) element from the β -globin locus control region displayed classic insulator activity: it insulates reporter genes from the activating effects of proximal enhancers, promotes uniform transgene expression in cell lines, and protects genes from chromosomal PEV in transgenic *Drosophila* and mice (Chung, Whiteley et al. 1993). The native cHS4 element is associated with a peak of histone hyperacetylation and other hallmarks of an “open” histone code, which in turn is thought to prevent the invasion of transcriptionally repressive heterochromatin from an adjoining region into the chicken β -globin locus.

The PEV effect thus could affect the generation of transgenic mice. However being the site of integration of the transgene essentially a stochastic event, and being multiple integration events, a transgenic may escape to PEV, as illustrated by the mouse lines described in chapters 3 and 4. Nevertheless I decided to include in a transgene meant for ubiquitous expression two tandem repeats of the cHS4 insulator, with the aim to potentially decrease PEV effect in this transgenic line.

First I observed the expression of β -galactosidase in embryos. Blue staining was observed in the cartilaginous skeleton of embryos similarly to the Aggrecan-driven mouse lines. However here staining was also present in skin tissues. Other organs were difficult to assess because the diffuse blue staining, however I would investigate them in older animals. When I investigated the adults, two weeks after tamoxifen administration, I could observe a strong staining in the articular cartilage of femur and tibia facing surface as well as in the hip head. Diffused

staining was observed in the sub-chondral bone, down to the growth plate that it is known to be positive to x-gal staining due to osteoclast expressing β -galactosidase, as it is found positive also in wild-type mice (Kopp, Hooper et al. 2007). When spine and rib cage was stained blue staining was observed only in the ribs and intervertebral disks, but not in intercostal muscles. Internal organs such as heart, lungs or kidney were not stained. I did not investigate further the reason why those organs were not X-gal positive however, as diverged from the aim of the project. To assess the levels of Timp3 expression I extracted total mRNA from tibia and femur articular cartilage. Every sample contained cartilage from the lower hind of a single mouse, in order to highlight the variability among the single individuals. I observed in transgenic line UbCAla #38 an increase of Timp3 messenger of 15 times, a value clearly higher compared to the Aggrecan-driven LLC1 #53. Interestingly being the mRNA extracted only from articular cartilage this result was not affected by other tissues that could express the transgene. Finally I investigated by Western Blotting analysis the TIMP-3 protein levels in femur and tibia epiphyses and found it was increased four times in the transgenic mice compared to the wild-type ones.

Taken together those data shown that the generally speaking the Ubiquitin C promoter can be employed to target mouse articular cartilage, although expression may not be restricted to just that tissue. Role of other tissues of the knee joint need to be further investigated. However the expression levels of the transgene could be higher compared to the Aggrecan-driven one. Finally TIMP-3 protein levels were found also increased. Discrepancy between mRNA and protein levels were expected as TIMP-3 is known to be regulated at both transcriptional and translational levels, but is also actively endocytosed, limiting its presence in the extracellular space.

Chapter 6

Concluding remarks and future directions

6.1 Introduction

Articular cartilage is fundamental for the functionality of the joint articulation. Although cartilage was shown to possess a certain degree of regeneration, major and continued insults can tilt the balance between catabolic and anabolic processes, leading to erosion of the tissue. Among the pathologies that affect the articular cartilage, osteoarthritis (OA) is the most common, and the number of patients is increasing dramatically in the industrialized countries. Although OA in human is thought to be a multifactorial disease, deeply affected by style and quality of life, it has been shown experimentally in animal models that OA-like phenotype can be obtained by alteration of single specific genes. Particularly was observed that targeting genes coding for cartilage metalloproteinases such MMPs and ADAMTSs can prevent or delay experimental induced OA in animal models. Even more importantly was showed that targeting gene coding for endogenous inhibitors of those enzymes can predispose or accelerate the onset of the pathology.

Metalloproteinases are implicated in the natural turnover of the extracellular macromolecules and collectively they can degrade all the major macromolecules of the extracellular matrix. Some of these enzymes are synthesized as pro-enzymes, and generally they are soluble proteins.

In cartilage ECM collagens are mostly represented by collagen type II and type X in the most inner region, close to the bone interface. Among the proteoglycans the most abundant is aggrecan. Available data suggest that while proteoglycan molecules are actively synthesized and degraded, the collagen components is more static, being deposited mostly during embryonic development. This evidence lead to the consequence that collagen structure cannot be deposited anew and once is degraded is lost.

To date no therapeutic treatments that directly target OA are available, but not for lacking of candidates. Many research groups focused their efforts in generating synthetic approaches to controlling the level of MMP activity. Developing

proteinases inhibitors that are therapeutically active is very challenging as, in addition to ensuring that the molecule has the required potency, it must also be orally active, specific to the targeted enzyme family, and has no significant toxicity. These molecules are designed to target the catalytic pocket of the enzyme, limiting their action. The first generations of these inhibitors were broad-spectrum inhibitors and failed clinical trials. They were shown to produce musculoskeletal side effects characterized by joint stiffness, joint fibroplasias and accumulation of type I collagen in the affected joints, as well as mild anaemia and elevated levels of liver enzymes (Renkiewicz, Qiu et al. 2003, Georgiadis and Yiotakis 2008). Drug development programs are now exploring the use of selective inhibitors of proteinases rather than broad proteinases inhibition, aiming to reduce the side effects. However this may be a quite difficult task due to the high homology in the enzymes catalytic sites. An orally active MMP-13 inhibitor was shown *in vivo* in a rat meniscectomy model to exert chondro-protective effects and, interestingly, without observable musculoskeletal toxicity (Baragi, Becher et al. 2009).

While MMPs are mostly targeting cartilage collagens, ADAMTSs (mostly -4 and -5) were shown to be responsible for most of aggrecan loss, according to the neo-epitope resulting from aggrecan degradation, found in synovial joints of OA patients (Fosang, Stanton et al. 2003). In mice *Adamts5* deficiency causes protection from OA, making the coded enzyme a key target for OA treatment.

A different approach from design new inhibitory molecules is focusing on the endogenous inhibitors of cartilage catabolic enzymes. Increasing the local synthesis of TIMPs has been suggested to be an effective way of preventing articular tissue turnover and OA progression. Among the four mammalian TIMPs, TIMP-3 was shown to have high affinity inhibition toward both ADAMTS-4 and ADAMTS-5 (Kashiwagi, Tortorella et al. 2001) and MMPs. It has been shown that TIMP-3 deficiency in mice results in cartilage degradation similar to OA (Sahebjam, Khokha et al. 2007). Because its broad spectrum of activity TIMP-3 may not be the best candidate for therapeutic purpose, however

researchers have looked at engineering the TIMP-3 molecule to be selective to a specific MMP (Nagase and Brew 2002, Murphy and Nagase 2008). It has been recently demonstrated *in vitro* that mutating TIMP-3 adding an extra Alanine residue at the N-terminal, restricts the activity toward MMPs, maintaining inhibitory ability towards aggrecanases.

6.2 Summary of current work and future perspectives

The main aim of this study was to generate a tool to assess the potential of TIMP-3 in altering the progression of experimentally induced osteoarthritis in mice. I chose TIMP-3 because its broad inhibitory activity and selectivity against cartilage-degrading enzymes, and thus a potential treatment for some type of OA.

In this study I demonstrate that I could achieve cartilage-specific expression using Aggrecan regulatory elements, and that they are sufficient to deliver a stable and detectable amount of target protein in mouse knee articular cartilage. The first construct that I designed was used to generate a mouse cartilage-specific Cre-deleter. Although it was not the first of such systems present in literature (Henry, Jang et al. 2009), it was the first to use specific enhancer sequences found upstream to the Aggrecan gene. Unpublished work from our group suggested that different enhancer sequences could differently modulate the time-space expression of the downstream gene. In my study I used the sequence that Han and Lefebvre designed as “A1” (Han and Lefebvre 2008). Henry and colleagues generated a different cartilage specific mouse line, opting to knock-in the Cre recombinase coding sequence downstream of the whole Aggrecan gene. Doing so they obtained at best only two copies per genome of the recombinase coding sequence. The recombination event requires very small amount of enzyme per cell, if not a single molecule, to be performed. It could be speculated that my system may have more copy per genome and thus possibly a higher affectivity, but comparing X-gal staining pattern from both embryos and adult knees does not highlight such differences. I also demonstrated that double transgenic mice (Col2a1-4xA1-CreERT² x ROSA26R) are still prone to develop OA-like lesions in cartilage after DMM. What may be an interesting question is what happens to

aggrecan protein and gene expression after DMM surgery and during early OA. The Luciferase gene present in the construct is subjected to the same Aggrecan regulation thus I could detect bioluminescence emission in the mouse knee before and after DMM. The data recorded do not show large variations in the emission levels in a period of several weeks. Using fluorescence proteins may provide a more sensitive reading and accurate localization, but also require a more expensive hardware to be detected. In my experience the Kodak In Vivo FX Pro machine we currently employ, failed to detect mCherry protein fluorescence emission in adult mouse knee, although it could detect localized fluorescence from E16 embryos. Although some authors had shown Aggrecan expression in mouse brain, I never observed any staining in mouse embryos.

Taking together all the data shown that this mouse line could be trustily used in study that depend on Cre recombination in cartilage, both in embryos that in adults. The DMM technique in adult, and very likely other surgical or mechanical techniques, could be used to investigate the osteoarthritis pathogenesis.

In order to investigate the effect of TIMP-3 overexpression in an osteoarthritis model, I designed and generate a conditional TIMP-3-expressing mouse line. TIMP-3 is a potent endogenous inhibitor of matrix-degrading enzymes such as MMPs and ADAMTSs, with inhibitory activity concentration on the nanomolar. Those classes of enzymes have been associated with osteoarthritis both in mouse and human, and their regulation could be a potential treatment for OA. TIMP-3 has been investigated because its broad activity range against metalloproteinases, however taking in account the failure of broad range drugs in OA treatment, some research group aimed to modify the protein sequence to restrict the activity to just one class of metalloproteinases or even single enzymes. It has been reported previously that a N-terminal reactive-site mutants of N-TIMP-3, [-N-te-TIMP-3 (N-TIMP-3 with an extra alanine residue appended to Cys1) is a poor MMP inhibitors but still retain inhibitory activity against ADAMs and ADAMTSs (Wei, Kashiwagi et al. 2005, Lim, Kashiwagi et al. 2010).

The conditional model I generated allows cartilage specific expression driven by the same Aggrecan enhancer elements previously used. I was thus quite confident on the specificity of the target delivery. The most important question was about the amount of TIMP-3 that would be delivered in cartilage, dependent on expression levels and protein turnover. As I mentioned earlier to obtain recombination in a Cre system, it is needed as little as a molecule per cells. The amount of TIMP-3 necessary to obtain inhibition of matrix molecules is unknown and can be assessed only empirically. A transgenic system has characteristic to harbour a higher number of copy compared to a knock-in, and thus lead to higher expression levels. Cartilage tissue proved to be difficult to investigate due to its composition of strongly charged proteoglycans and collagen fibers organized in a meshwork. I was able to optimize a protocol to extract mRNA from the thin layer of mouse articular cartilage, and observe about 4-fold increase compared to control. I failed to detect TIMP-3 protein in articular cartilage by Western Blot. Reason maybe due to the small amount of sample that did not allow stronger processing to separate the highly charged proteins. However I detected an increase in protein signal by Immunofluorescence of mouse knee sections. As I already mentioned the amount of protein in the cartilage is of paramount importance, and expression is not the only factor that may limit the inhibitory activity. It has been shown by Scilabra and co-workers that TIMP-3 is actively internalized by two different pathways (Scilabra, Troeberg et al. 2013). It is not clear however if the internalization can boost a self-limiting loop to reduce the amount of TIMP-3-scavenger proteins on the cell surface. Data so obtained strongly suggested that the model generated is able to deliver TIMP-3 in adult mouse articular cartilage.

The obvious future plan would be to perform DMM surgery on this mouse model to challenge the hypothesis that TIMP-3 delivered in mouse cartilage could ameliorate the OA outcome. However several points need to be taken in account:

1) Tamoxifen administration.

- a. To obtain the maximum amount of TIMP-3 it could be speculated that tamoxifen has to be given as earlier as possible but after full mouse development to avoid structural defects.
- b. Overexpressing TIMP-3 before the OA onset could lead to positive results in terms of cartilage protection, but would not resemble any real clinical fact. Thus will be required to evaluate the effect of tamoxifen administration at the same time and even after the DMM.

2) TIMP-3 delivery

- a. In my model chondrocytes are induced to express an exogenous protein, providing a proof of concept. However an alternative delivery method have to be devised. In my group we are working in order to produce both Adenoviral and Lentiviral vectors to overexpress TIMP-3.
- b. Viral vector-based delivery in articular cartilage is affected by several technical problems. First, mouse articular capsule is extremely small and only minute amount of volume can be injected. Using rats or rabbits as animal model may help on this point. Second, both adeno- or lenti- viruses have to travel across the surface of the joint to approach articular chondrocytes (and probably synoviocytes). Deeper chondrocytes could be infected at very low rates or not at all. Increasing the amount of virus injected however can increase also the toxicity on cells closer to the surface. Finally, adenovirus vectors produce a temporary increase in the expression of the target protein, and we could speculate that more injections could be necessary; on the other hand lentivirus vectors integrate in the DNA of the target cell, maintaining a more stable expression. Articular chondrocytes posses a relatively low proliferating ratio and movement ability, both are quality that could enhance the safety of viral vector-based gene therapy.

Many studies are pointing out that direct therapeutic gene transfer could be a promising tool to treat articular cartilage defects. Very recently it has been shown the successful ability of a recombinant adeno-associated virus (rAAV) insulin-like growth factor I (IGF-I) vector to improve the early repair of cartilage lesions in rabbit *in vivo* (Cucchiaroni and Madry 2014).

TIMP-3 and its mutant such the [-1A]TIMP-3 should be strongly investigated as promising target to balance the enzymatic degradation of cartilage macromolecules.

Chapter 7

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