AGRIN CONTRIBUTES TO ARTICULAR CARTILAGE HOMEOSTASIS

By Suzanne Eldridge

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Abstract

Osteoarthritis is a leading cause of disability for which there is no cure. We have discovered that the multidomain signalling protein Agrin, most commonly known for its requirement at the neuromuscular junction, strongly promotes chondrocyte differentiation and cartilage formation *in vivo*. Agrin is expressed in normal cartilage but absent in osteoarthritis. *In vitro*, Agrin knockdown resulted in the downregulation of the cartilage transcription factor SOX9 and other cartilage-specific extracellular matrix molecules. Conversely, the addition of exogenous Agrin supported cartilage differentiation *in vitro* and ectopic cartilage formation *in vivo.* In contrast to other biological contexts where Agrin signalling requires the interaction with either LRP4 or α-dystroglycan, chondrocytes require the presence of both receptors. Our results identify Agrin as a novel potent anabolic growth factor with strong therapeutic potential in cartilage regeneration.

Acknowledgements

I owe a huge thanks to my supervisor Prof Francesco Dell'Accio for giving me the opportunity to be part of such a fantastic project and providing me with continuous guidance, support and enthusiasm. I also wish to thank Prof Costantino Pitzalis for his feedback and encouragement during my time in his lab.

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Finally, I would like to thank my funders, the MRC and the Rosetrees trust who continue to support my research.

Declaration

The work presented in this thesis is less than 100,000 words and was performed and analysed by the candidate except for the murine DMM surgery, injury of human cartilage explants and the microarray analysis which were performed by Prof Francesco Dell'Accio. LRP4 silencing experiments were performed in collaboration by Habib Ismail (Figure 67 and Figure 68). The immunohistochemistry in Figure 82 was carried out in collaboration with Giovanna Nalesso. The experiment carried out in Figure 89 was carried out in collaboration with Elspeth Petrycki.

Prof Francesco Dell'Accio and Dr Giovanna Nalesso closely supervised the project providing scientific guidance and advice regarding experimental design and planning as well as interpretation of the results and critical review of the manuscript.

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Contents

Abbreviations and Aliases

ACAN	Aggrecan
AChR	Acetylcholine Receptor
ACI	Autologous chondrocyte implantation
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motif
AGRN	Agrin
AHAC	Adult human articular chondrocytes
APLP2	Amyloid precursor-like protein 2
APP	Amyloid precursor protein
BMP	Bone morphogenic protein
BSA	Bovine albumin serum
COL	Collagen
COMP	Cartilage oligomeric matrix protein
CS	Chondroitin sulphate
DAG	Dystroglycan
DKK	Dickkopf
DMEM	Dulbecco's modified Eagle's medium
DMM	Destabilisation of the medial meniscus
DNA	deoxyribonucleic acid
DS	Dermatan sulphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra-acetic acid
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FZD	Frizzled receptor
GAG	Gylcosaminoglycan
GDF	Growth differentiation factor
GFP	Green fluorescent protein
HA	Hyaluronic acid
HS	Heparan sulphate
HSPG	Heparan sulphate proteoglycan
IGF	Insulin-like growth factor
Ihh	Indian hedgehog
IL	Interleukin
kDa	Kilodalton
KO	Knockout
KS	Keratan sulphate
LAM	Laminin
LB	Luria broth
LRP	Low-density lipoprotein receptor

MMP	Matrix metalloprotinases
MSC	Mesenchymal stem cell
MuSK	Muscle-specific tyrosine kinase receptor
OA	Osteoarthritis
PCR	Polymerase chain reaction
pERK	Phosphorylated ERK
PFA	Paraformaldehyde
PG	Proteoglycan
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SEA	Sea urchin sperm protein, enterokinase and Agrin
Shh	Sonic hedgehog
siRNA	Small-interfering RNA
SOST	Sclerostin
SOX9	SRY (sex determining region Y)-box
TGF	Transforming growth factor
TIMP	Tissue inhibitors of metalloproteinases
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
WISE	Sclerostin domain containing 1 (SOSTDC1)
WNT	Wingless type MMTV integration site family member
WT	Wildtype

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Background

Osteoarthritis is a chronic disabling disease, characterized by cartilage breakdown, for which there is no cure. Disruption of the gene encoding for the heparan sulphate proteoglycan Agrin results in embryonic skeletal dysplasia suggesting a role for Agrin in cartilage biology and prompting us to study its role in articular cartilage biology and osteoarthritis. Agrin and its known receptors were expressed in healthy adult human articular cartilage and downregulated in human and experimental murine osteoarthritis. Silencing of Agrin by siRNA resulted in reduced GAG production in C28/I2 cells and in chondrocyte de-differentiation characterised by decreased expression of SOX9, Col2A1 and Aggrecan mRNA. Overexpression of Agrin in the human chondrocyte cell line C28/I2 cells and primary bovine articular chondrocytes resulted in enhanced GAG production and SOX9, COL2A1 and ACAN upregulation. Importantly, the hypertrophic marker COL10A1 was not upregulated by Agrin overexpression. Delivering Agrin in an *in vivo* model of cartilage formation resulted in enhanced formation of ectopic cartilage, which did not display signs of hypertrophy, vascular invasion, or endochondral bone formation. These findings demonstrate that Agrin is essential for the maintenance of the chondrocytic phenotype and extracellular matrix production. Furthermore exogenous Agrin enhances chondrocyte differentiation and cartilage formation *in vitro* and *in vivo*; and therefore may be a valuable chondrogenic molecule in tissue engineering technologies.

CHAPTER 1: Introduction

Limb development

To understand the biology of articular cartilage it is necessary to understand the processes which occur during development of the skeleton and formation of synovial joints. During embryonic limb formation, the skeleton is formed through a step-wise process consisting of cell migration, adhesion, proliferation and growth. These processes are regulated by signals provided by WNTs, hedgehogs, fibroblast growth factors (FGF), SOX9 and members of the transforming growth factor-β (TGF-β) superfamily (de Crombrugghe, et al., 2001).

Skeletal tissues in the limbs are derived from the lateral plate mesoderm, whereas other tissues such as blood vessels, nerves and muscle are formed by cells derived from somites which then migrate into the to developing limb bud (Cohn, & Tickle, 1996; Ng, et al., 1998).

Interactions between the mesenchyme and overlying epithelium dictate the patterning of the mesenchyme in the limb and the shaping of the skeletal elements in the limbs. The proximodistal outgrowth is supported by FGF signalling originating by a distal structure called apical ectoderm ridge while cells in the posterior area of the limb ectoderm constitute the zone of polarizing activity, which directs the anteroposterior patterning through Sonic hedgehog signalling (Shh). Dorsoventral patterning is largely dependent on Wnt7a signalling and radical fringe from the dorsal ectoderm combined with expression of the Homeobox-containing (HOX) transcription factor Engrailed from the ventral ectoderm (Loomis, et al., 1998; Parr, & Mcmahon, 1995; Riddle, et al., 1993; Rodriguez-Esteban, et al., 1997).

Both the limbs and the cartilage anlagen develop in a proximodistal manner, with the humerus/femur developing first and the phalangeal anlagen segmentation occurring last. Mesenchymal cells within the limb bud condense to form the anlagen before undergoing chondrogenesis. Chondrogenesis is preceded by the expression of the transcription factor SOX9, which directly drives the expression of the cartilage-specific extracellular matrix

molecules collagen type II and Aggrecan (Bi, et al., 1999; Hall, & Miyake, 2000; Kronenberg, 2003; Onyekwelu, et al., 2009).

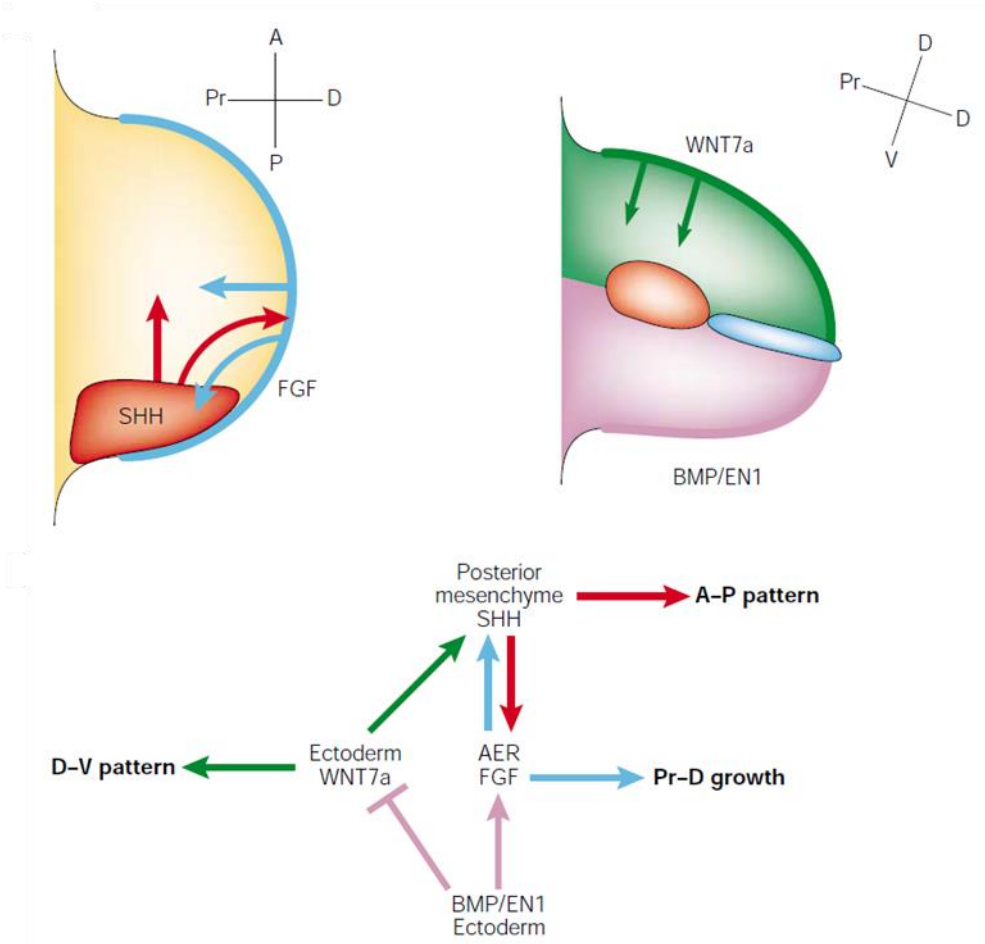


Figure 1. Signalling pathways in vertebrate limb development.

Limb growth and patterning along the three limb axes: proximal–distal (Pr–D) axis is under the control of fibroblast growth factors (FGFs; blue) from the apical ectodermal ridge (AER), the anterior–posterior (A–P) axis is under the control of Sonic hedgehog (SHH; red) from the posterior mesenchyme, and the dorsal–ventral (D–V) axis is under the control of bone morphogenetic proteins (BMPs) and Engrailed1 (EN1; both in pink) from the ventral ectoderm and WNT7a (green) from the dorsal ectoderm. (Niswander, 2003); (3691270148629).

Synovial joint formation

Synovial joints are composed of a number of different tissues and structures including synovial membrane, ligaments, cartilage and bone (Hyde, et al., 2007). The first step towards the formation of the synovial joint is the determination of the anatomical location where the uninterrupted anlagen will be subsequently segmented. In this area, a transient structure composed of densely packed mesenchymal cells, called the joint interzone forms, and subsequently becomes cavitated, thereby separating the two skeletal elements (see Figure 2).

How the location is determined is not yet fully understood, however, it is believed that signalling events involving several molecular pathways including Wnts, TGF-β, BMP and FGF are involved (Dell' Accio, et al., 1999). However, one of the earliest known genes to be characteristic of the joint interzone location is WNT14 (Hartmann, & Tabin, 2001). Additional genes including, GDF5, Wnt16, Wnt4, Noggin, matrilin, autotoxin and collagen type 2α1 are also expressed by cells residing in the interzone (Brunet, 1998; Francis-West, et al., 1999; Guo, et al., 2004; Hyde, et al., 2007; Pacifici, et al., 2006).

The interzone can be further separated into three distinct layers of cells. The central layer expresses increased levels of hyaluronan (HA), which cause the saturation of the cell surface receptor CD44. This high concentration of HA combined with mechanical stimulation to the developing limb results in cell separation and apoptosis and the resultant joint cavitation (Dowthwaite, GP, et al., 1998; Dowthwaite, G., et al., 2003; Pitsillides, et al., 1995; Underhill, & Toole, 1981)

The cells in the two layers either side or the cavitation further differentiate, becoming articular chondrocytes, synovial membrane and ligaments (Hyde, et al., 2007; Koyama, et al., 2008; Mundy, et al., 2011).

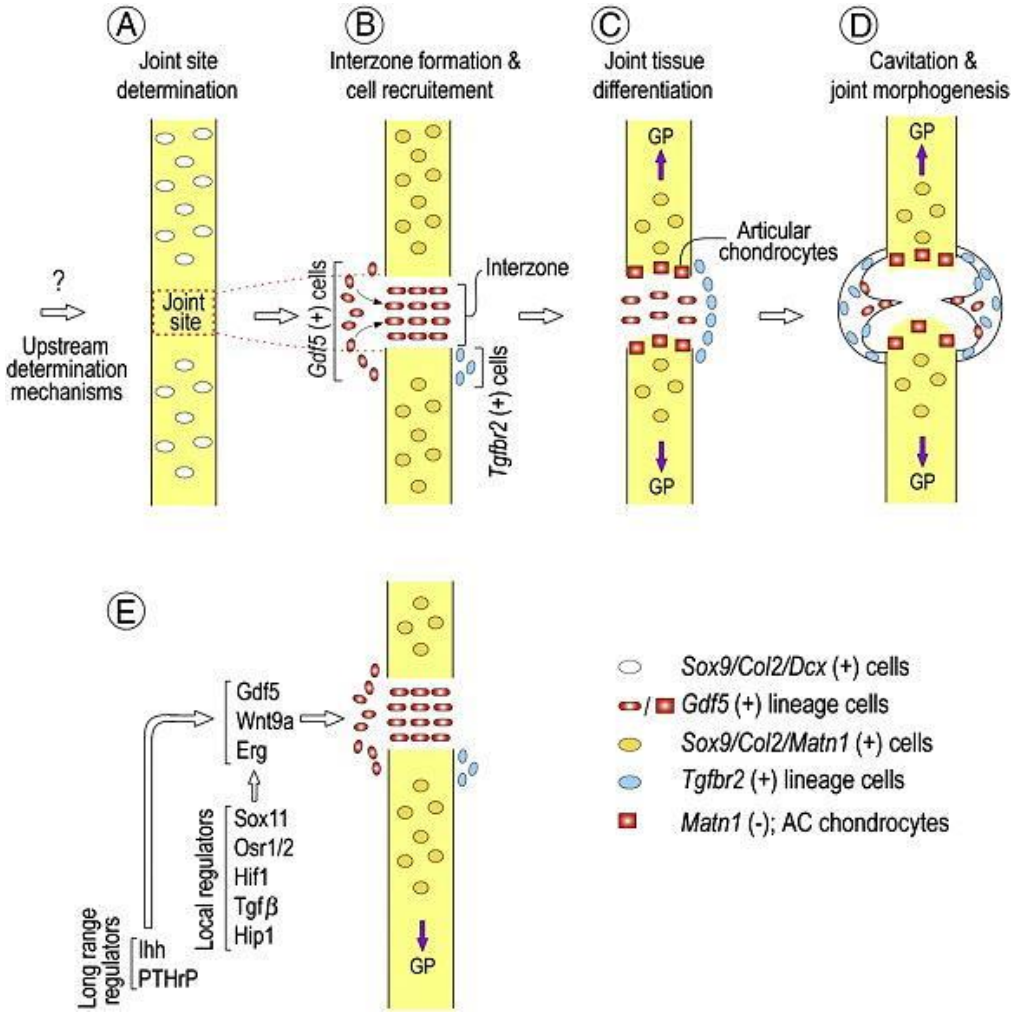


Figure 2. Synovial joint formation.

Within the uninterrupted anlagen (A) During joint determination, yet partially understood signalling mechanisms induce the condensation of mesenchymal cells oriented perpendicularly to the skeletal element marks the location of the future joint (B). The interzone further develops in a three layer structure (C), the central of which will eventually cavitate, while the lateral ones will give rise to the articular cartilage. The cavitation process begins and leads to physical separation of the adjacent skeletal anlagen and formation of the synovial cavity (D). Morphogenetic processes begin to reorganise the structure of the opposing sides of the joint into reciprocally-shaped and interlocking structures (E). (Decker, Koyama, & Pacifici, 2014); (3584720825496).

Endochondral ossification

With the exception of the articular cartilage, which remains stable and avascular throughout life, the rest of the epiphyseal cartilage is replaced by bone through endochondral ossification. This process begins in the centre of the mesenchymal condensations and progresses towards the epiphyses of the anlagen thereby creating distinct chondrocytic layers throughout the length of the skeletal element.

Under the control of the SOX9 transcription factor, cells within the centre of the mesenchymal condensations differentiate into proliferating chondrocytes which can be identified by their unique expression of collagen type II and proteoglycans (Archer, & Francis-West, 2003; Bi, et al., 1999; Eyre, David R, 2004; Lefebvre, V, et al., 1997).

Following proliferation, the central chondrocytes further differentiate into prehypertrophic chondrocytes, expressing Indian hedgehog (Ihh), and ultimately into hypertrophic chondrocytes, expressing collagen type X, matrix metalloprotinases (MMPs), CBFA1 and Runx2 (Ikegami, et al., 2011; Kronenberg, 2003; Lefebvre, V, et al., 1997; Onyekwelu, et al., 2009). These terminal hypertrophic chondrocytes then undergo apoptosis allowing the surrounding ECM to become vascularised. This vascularisation results in several cell types invading the cartilage anlagen including chondroclasts/osteoclasts which degrade the matrix and osteoblasts which initiate the calcification of the remaining tissue to form bone (Karsenty, 2003; Olsen, et al., 2000).

During this process, prehypertrophic chondrocytes begin expressing Indian hedgehog (Ihh), a secreted factor, which stimulates the proliferating chondrocytes at the anlagen epiphyses to express parathyroid hormone-related peptide (PTHrP). The induction of PTHrP expression, through use of this negative feedback loop, is able to keep these chondrocytes in their proliferative state and ensure continued production of an ECM which is resistant to vascular

invasion and mineralisation and forms the permanent articular cartilage (Vortkamp, et al., 1996).

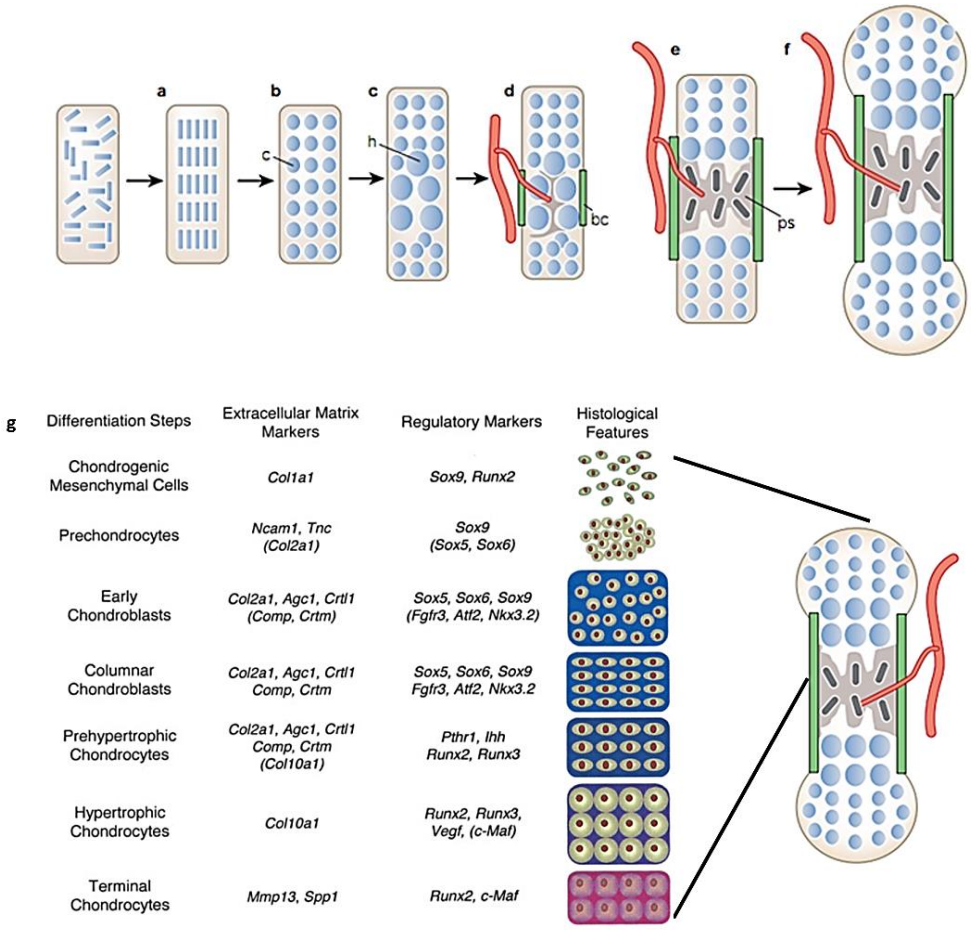


Figure 3. Endochondral bone formation.

Diagrammatic representation of the main steps involved in appendicular skeletal development; (a) condensation of mesenchymal cells; (b) chondrocyte differentiation; (c) chondrocyte hypertrophy; (d) hypertrophic chondrocytes are replaced by osteoblasts and vascular invasion; (e) formation of the primary spongiosa; (f) continued chondrocyte proliferation. (g) selection of genes expressed by chondrocytes in the different zones of the developing cartilage (adapted from (Kronenberg, 2003; Lefebvre, Véronique, & Smits, 2005); (3547111197582; 3547111339078).

Cartilage biology: structure & function

Articular cartilage covers the ends of diarthroidal joints (Figure 4). Its main function is to provide lubrication while dispersing and transferring loads through the joint (Han, et al., 2011). Articular cartilage is avascular and has no lymphatic vessels or nerves. Human articular cartilage is on average 2 to 4mm in thickness and is predominantly composed of a complex extracellular matrix (ECM), surrounding sparsely distributed chondrocytes (Kubakaddi, et al., 2013; Lefebvre, Véronique, & Smits, 2005).

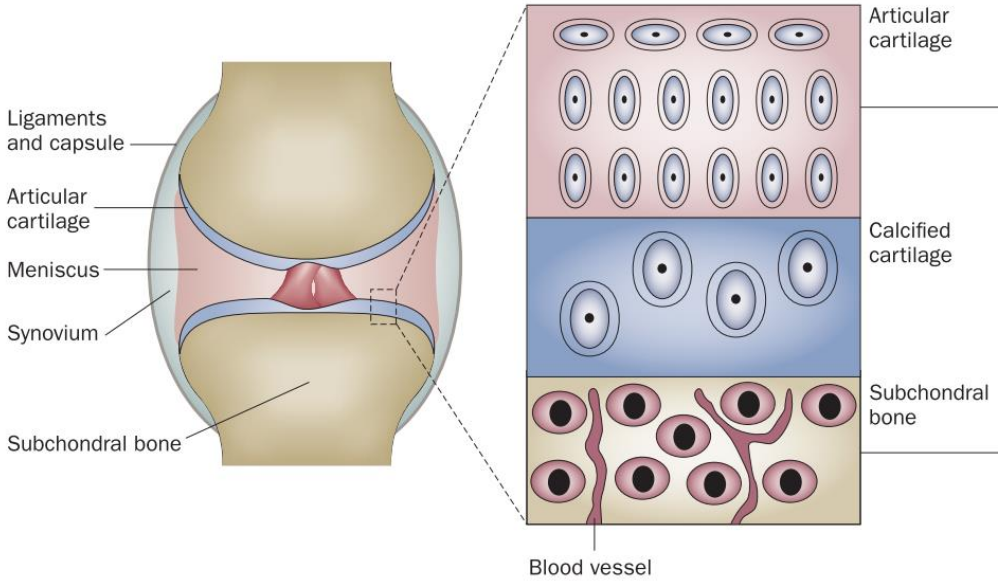


Figure 4. The bone-cartilage unit of the articular joint.

The subchondral bone is covered in a calcified cartilage layer which in turn is covered by hyaline articular cartilage (Lories, & Luyten, 2011); (3547120452923).

The ECM is constituted primarily of water (up to 90%), collagens, proteoglycans (PGs) and, in smaller quantities, numerous non-collagenous proteins and glycoproteins. The ability of the ECM to retain water is of paramount importance in enabling cartilage to function effectively. The structure of cartilage varies throughout the depth of the tissue and is commonly divided

into three distinct zones. The zone directly beneath the articular surface is known as the superficial (tangential) zone and it is here where the nutrients initially diffuse across from the synovial fluid.

The tangential zone is the thinnest of the three zones (see Figure 5) and can further be divided into two regions. A thin (200μm), cell-free region (lamina splendens) consisting of disorganised bundles of fine collagen which allows a low coefficient of friction to be produced; and a deeper region which is composed of bundles of flat collagen fibrils that are oriented parallel to the surface. The chondrocytes in this zone are small, comparable to fibroblasts, and also run parallel to the cartilage surface. They are flat, very densely packed and in the absence of injury, relatively metabolically inactive. This zone has the highest water content and the lowest PG aggregate content (Eyre, 2002; Goldring, & Goldring, 2010; Goldring, 2006). The main known function of this layer is to produce the joint lubricant known as lubricin. Lubricin is essential for joint lubrication and loss of function mutations in humans results in camptodactyly-arthropathy-coxa vara-pericarditis syndrome (Rhee, et al., 2005). In mice deficient of lubricin, although the cartilage appears relatively normal at birth, at 12 weeks of age there is enhanced chondrocyte apoptosis (Coles, et al., 2010; Waller, et al., 2013)

Furthermore, studies have shown that intra-articular injections of recombinant lubricin and cartilage specific overexpression of lubricin are able to protect the cartilage from breakdown in rat and murine models of OA respectively (Flannery, et al., 2009; Ruan, et al., 2013). In addition, the cells of this layer are considered the "cartilage stem cells" (Koyama, et al., 2008) and therefore, the preservation of this layer of cells is essential for cartilage homeostasis.

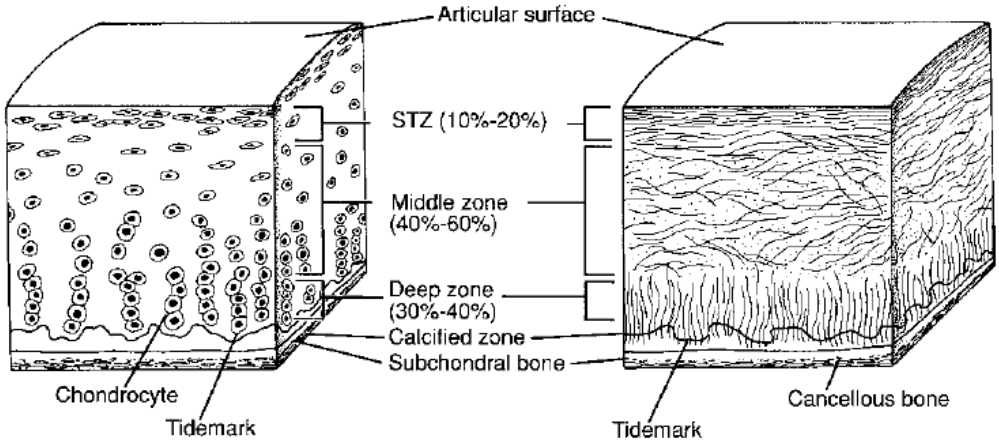


Figure 5. Cross-sectional of healthy articular cartilage.

Schematic showing the cellular organisation and the architectural variations of the collagen bundles through the different zones (Newman, 1998); (CC-BY).

Below the tangential zone is the intermediate (transitional) zone, here the chondrocytes become more spherical and synthesise much more matrix. The collagen fibres change their orientation throughout the depth of this section, running parallel to the surface in the most superficial sections and becoming perpendicular to the surface deeper towards the bone (Eyre, DR, et al., 2006; Sharma, et al., 2007).

The deepest zone (basal) is highly structured, with large collagen fibrils perpendicular to the subchondral bone. The chondrocytes are spherical and organised in columns, they are the most metabolically active in this state. This zone has the highest PG content and the lowest water content (Eyre, DR, et al., 2006). This zone is separated from the subchondral bone by a tidemark. The tidemark is a visible structural difference created due to a change in tissue composition, namely from decalcified to calcified tissue (see Figure 5) (Onyekwelu, et al., 2009).

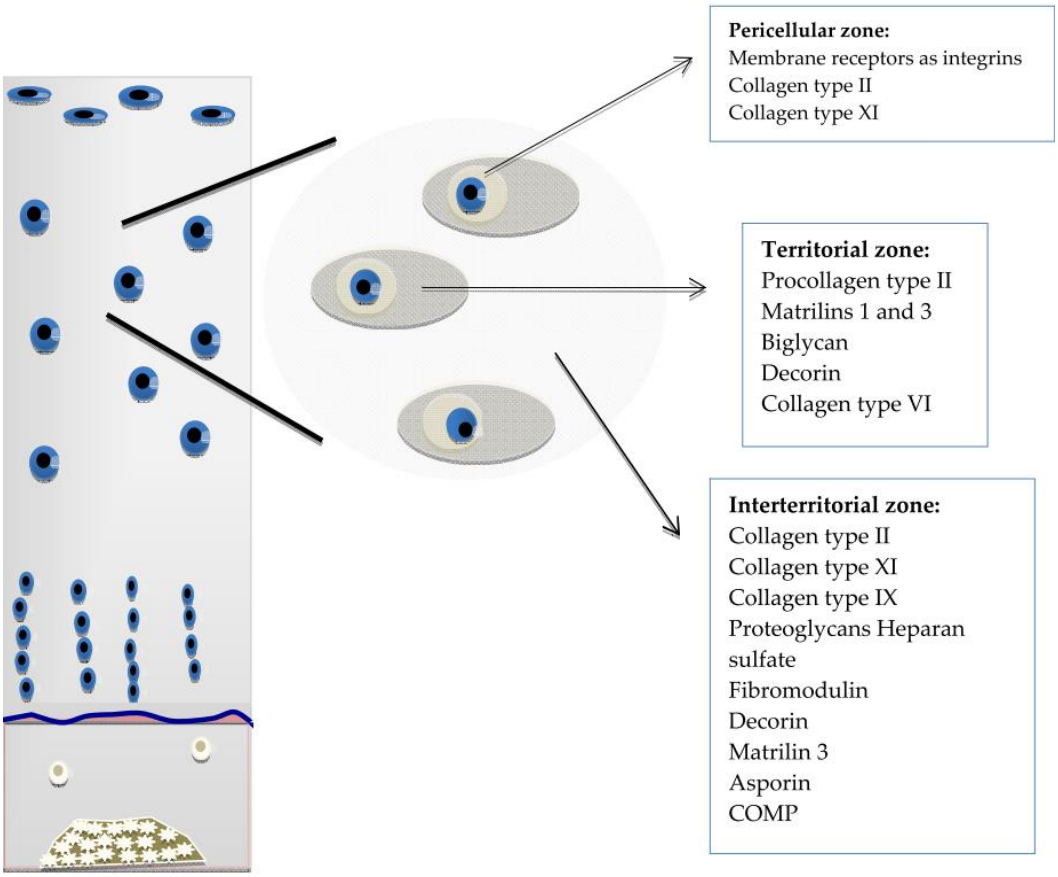


Figure 6. Zone-dependent chondrocyte morphology

Cellular zones defined as the distance from the chondrocyte and expression of different molecules within those zones (García-carvajal, et al., 2013); (CC-BY).

The ECM surrounding chondrocytes in healthy articular cartilage can be further divided into zones defined by their distance from the cell (Figure 6). The pericellular matrix is closest to the chondrocytes and is where molecules that interact with cell surface receptors are located, including FGF-2 (Vincent, et al., 2007). Further away from the chondrocyte is the territorial matrix and further still lies the interterritorial matrix (Heinegård, & Saxne, 2011).

The Chondrocyte

Chondrocytes are the only currently defined cell type present in cartilage; they reside in hypoxic conditions, have a rounded morphology, are approximately 13μm in diameter and account for just 5-10% of the total volume of the tissue (Chen, et al., 2006; Lin, et al., 2006). There is increasing evidence that additional populations of progenitor cells also reside within the articular cartilage, however whether these are cells are not in fact chondrocytes has not been fully established (Dowthwaite, Gary P, et al., 2004; Seol, et al., 2012; Williams, Rebecca, et al., 2010; Yasuhara, et al., 2011). The chondrocyte is unique in that it is isolated from its neighbouring cells, has no access to a vascular system and lacks innervation. Cartilage is often known as an 'embryonic' tissue as it is extensively distributed in the foetus. It provides the template for the skeletal elements during development via endochondral ossification with the chondrocytes being responsible for longitudinal growth in the epiphyseal growth plates. In adulthood, post-endochondral ossification, the cartilage is distributed much more sparsely however its presence remains and its function is mainly to provide mechanical support (Archer, & Francis-West, 2003). Chondrocytes are metabolically active cells, synthesising and controlling degradation of glycoproteins, collagens, proteoglycans and hyaluronan in the large surrounding matrix (Stockwell, 1979).

Due to the lack of vascularisation in articular cartilage, the chondrocyte must rely on nutrient and metabolite exchange through the articular surface. Low oxygen tension is therefore the ideal environment for chondrocyte metabolism with the main source of the chondrocyte energy being obtained through glycolysis resulting in low levels of mitochondria (Archer, & Francis-West, 2003). It has been shown that changes to the oxygen tension in chondrocyte culture can result in the regulation of genes such as TGF-β and connective growth factor, with lower oxygen tension being more anabolic (Grimshaw, & Mason, 2001).

The Extracellular Matrix

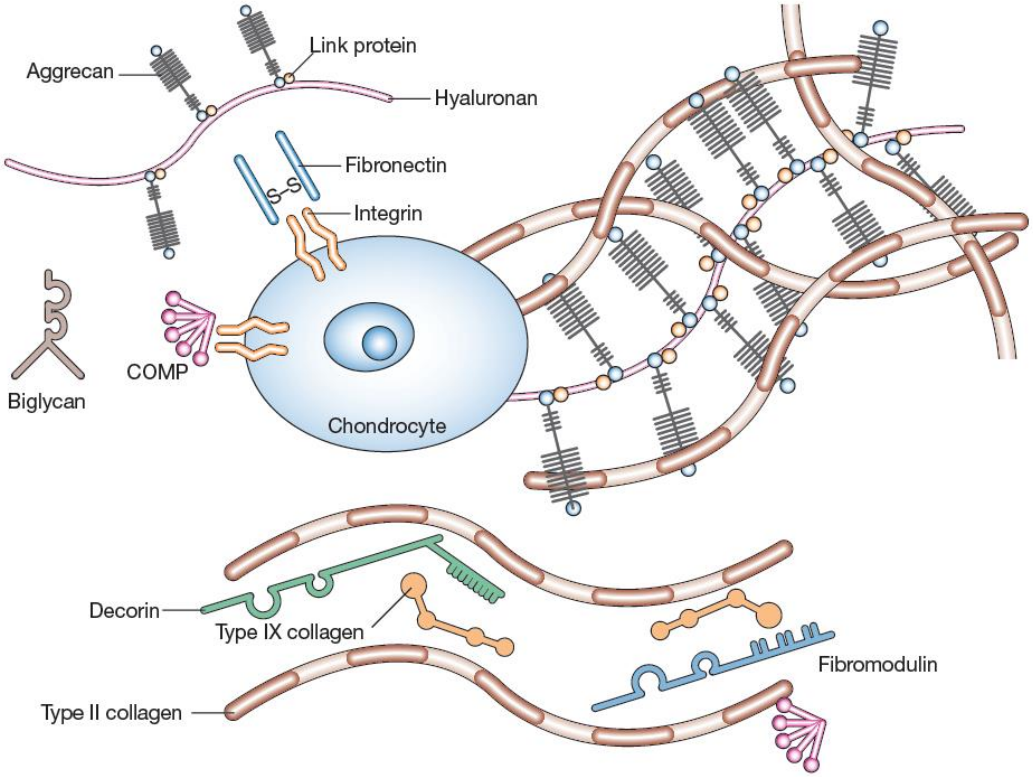


Figure 7. Proteins in the extracellular matrix

Representation of cartilage ECM and the three classes of proteins: collagens (mostly type II collagen); PGs (primarily Aggrecan); and other non-collagenous proteins (including link protein, fibronectin, cartilage oligomeric matrix protein) and the smaller proteoglycans (biglycan, decorin and fibromodulin). Abbreviation: COMP, cartilage oligomeric matrix protein (Chen, et al., 2006); (3547131096146).

Articular cartilage ECM comprises many proteins, however the most abundant are collagens (60–86% of dry weight), PGs (15–40% of dry weight), non-collagenous proteins and smaller PGs (Figure 7). Due to the negative charge of the sulphated glycosaminoglycan (GAG) chains of PGs in cartilage, water is drawn into the ECM. This gives rise to a high osmotic pressure which is retained by the collagen fibre network resulting in a complex PG network able to withstand compressive loading. Articular cartilage also has tensile properties which are primarily due to the network of type II collagen fibres. The biomechanical properties of articular cartilage thus depend on the maintenance of high PG and collagen contents within the matrix (Chen, et al., 2006; Han, et al., 2011).

Collagens

Collagen is the most abundant structural macromolecule in the ECM which accounts for two-third of the dry weight (Figure 8) (Eyre, 2002). Collagen type II is the most abundant and accounts for 90-95% of all collagen in articular cartilage, however types I, IV, V, VI, IX and XI are also present.

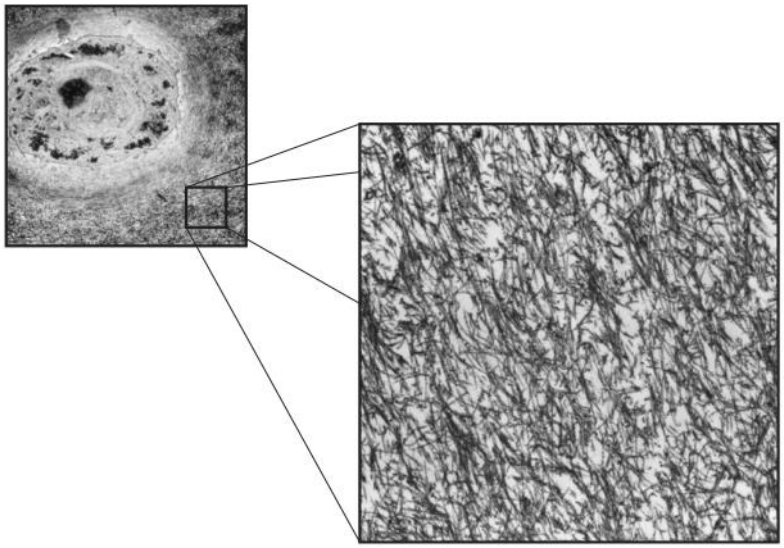


Figure 8. The collagen fibril meshwork in the extracellular matrix

Transmission electron microscopy of collagen fibrils in the ECM of articular cartilage produced by the chondrocytes (Eyre, 2002); (CC-BY).

All collagens contain amino acid triple helix polypeptide chains (α-chains) composed mainly of glycine, proline and hydroxyproline which provides stability through hydrogen bonds (Eyre, 2002; Han, et al., 2011). Within cartilage the majority of collagen is arranged in cross-linked fibrils consisting of collagen types II, IX and XI which enable structural stabilisation of the tissue (Figure 9). Aside from these types, type IV is localised around cells, in particular in the pericellular matrix of chondrocytes and defines the pericellular matrix (Eyre, 2002).

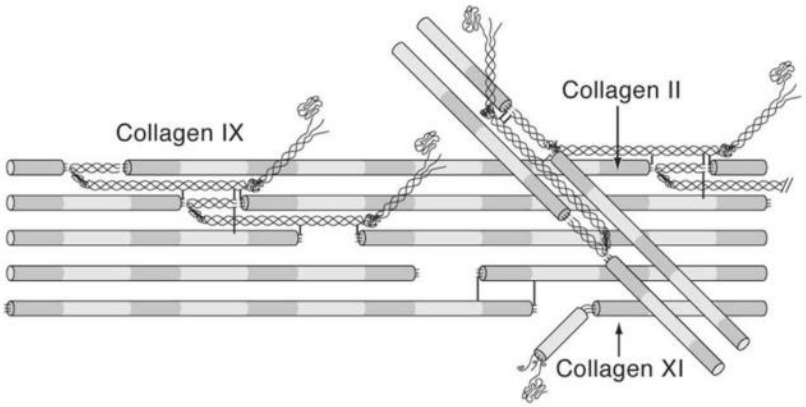


Figure 9. The collagen II/IX/XI heteromer

These types of collagen have individual fibril-to-fibril interactions within articular cartilage (Eyre, David R, 2004); (3547140075256).

Non-collagenous structural proteins

In addition to collagens, there are a number of non-collagenous proteins which provide further structural properties and biological functions within the ECM such as cartilage oligomeric protein (COMP), fibronectin and chondronectin. COMP is a glycoprotein found mainly in the chondrocyte territorial matrix (Rosenberg, 1998). It increases the structural integrity of the articular cartilage by promoting collagen crosslinking and binding type I and II collagen through zinc-dependent interactions (Shen, et al., 1995). Fibronectin is a heterodimeric protein which is present mainly in the pericellular matrix of the chondrocytes in the superficial layer of the articular cartilage. Its main known function is to provide phenotypic support to progenitor cells in this layer and may promote cartilage homeostasis and aid in cartilage repair (Dowthwaite, Gary P, et al., 2004; Williams, Rebecca, et al., 2010). Chondronectin is a high molecular weight glycoprotein involved in the adherence of chondrocytes to the ECM via the interactions of chondroitin sulphate, hyaluronic acid and type II collagen (Chevalier, 1993; Hewitts, et al., 1982).

Proteoglycans

Proteoglycans are synthesised by cells in many tissues and provide structure and function by means of lubrication and mechanical strength. PGs are heavily glycosylated proteins which account for approximately 10% of the wet weight of cartilage ECM and occupy a large area due to their size and the negative charge of the GAGs (Chen, et al., 2006; Han, et al., 2011). This negative charge is caused by extremely high levels of sulphation and enables them not only to remain hydrated but this also allows filtration of molecules in and out of the tissue (Hunziker, 2002; Mankin, & Lippiello, 1971). They are required in articular cartilage to provide both structural and signalling properties via various chemical properties provided by different GAG chains and receptor interactions (Perrimon, & Bernfield, 2001).

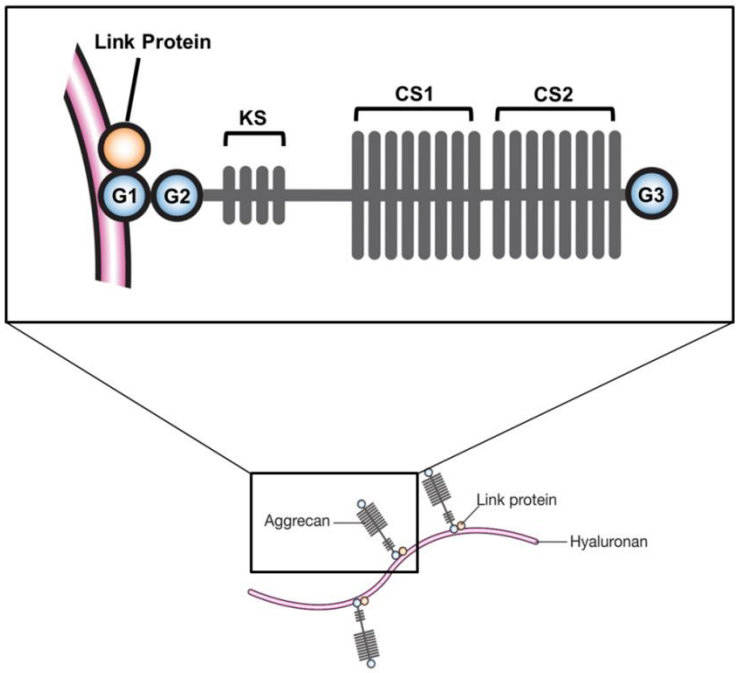


Figure 10. The structure of aggrecan

Aggrecan consists of a large protein core with three globular domains (G) and a link protein to facilitate the binding to hyaluronan. A group of keratin (KS) and two groups of chondroitin sulphate (CS) side chains are attached between G2 and G3 (adapted from Chen et al. 2006); (3547131096146).

Cartilage ECM is home to a number of different PGs, the most abundant being aggrecan (see Figure 10), however many more are also present. Aggrecan consists of a protein core of approximately 220kDa with three globular domains (G1-G3) which can participate in the formation of disulphide bonds (Doege, et al., 1991). Between these domains there is a group of keratan sulphate side chains and two groups of chondroitin sulphate chains which add approximately 2300kda to its molecular mass (Mankin, & Lippiello, 1971; Tselepis, et al., 2000). Vast numbers of aggrecan molecules can bind to a single hyaluronan via link proteins (Figure 10) and occupy the interfibrillar space, giving rise to the osmotic pressure required to provide articular cartilage with its mechanical properties (Han, et al., 2011; Morgelin, et al., 1988).

Table 1. Proteoglycan sub-populations (adapted from Kjeuin et al. 1991; Halfter et al. 1998; Tsen et al. 1995). Abbreviations: PRELP, Proline/arginine-rich end leucine-rich repeat protein.

Proteoglycan	GAG chains	Gene product
Small leucine-rich	Chondroitin sulphate	Decorin
	Chondroitin sulphate/dermatan sulphate	Biglycan Epiphycan
	Keratin sulphate	Fibromodulin PRELP Keratocan Osteoadherin Osteoglycin chondroadherin
Hyalectins or hyalaherins	Chondroitin sulphate/keratin sulphate	Aggrecan
	Chondroitin sulphate	Versican Neurocan Brevican
Basement membrane	Heparan sulphate	Agrin Collagen XVIII
	Heparan sulphate/ chondroitin sulphate	Perlecan
	Chondroitin sulphate	Bamacan
Cell surface	Heparan sulphate	Syndecans Glypicans

Proteoglycans are a superfamily of proteins that are commonly found on the cells surface, basement membrane or within the ECM. They are complex biological macromolecules composed of glycosaminoglycan (GAG) chains which are covalently bound to a protein core (Oh, et al., 2011; Shortkroff, & Yates, 2007). GAGs are linear, unbranched, negatively charged polysaccharides composed of repeating disaccharides. The repeating disaccharides normally consist of acetylated amino sugar moieties and uronic acid. GAGs can be non-sulphated, such as hyaluronan; or sulphated, comprising of chondroitin sulphate, dermatan sulphate, keratan sulphate, heparin, and heparan sulphate (Belcher, et al., 1997; Schaefer, & Schaefer, 2010). PGs are commonly categorised into sub-families depending on the structural properties of their protein core (See Table 1).

Heparan Sulphate Proteoglycans

Heparan sulphate proteoglycans (HSPGs) have a wide range of roles in various tissues and diseases within the human body including, but not limited to, the skeletal, circulatory, muscular, digestive, immune, reproductive and respiratory systems (Bishop, et al., 2007; Iozzo, Renato V, 2001). They are present in abundance on the cell surface and in the ECM, in both vertebrate and invertebrate species (Dreyfuss, et al., 2009).

HSPGs, by definition, consist of a core protein and one or more unique heparan sulphate (HS) glycosaminoglycan (GAG) chains. GAGs are linear polysaccharides composed of alternate units of α-D-glucosamine (GlcN), which can be N-acetylated or N-sulphated; and uronic acid, either β-D-glucuronic acid (GlcA) or α-L-iduronic acid (IdoA). These uronic acid and disaccharide units are quarter staggered and joined by glycosidic linkages. GAG chains have the ability to move laterally. This allows these linear polymers to reach targets hundreds of nanometres away; this could include crossing a full-thickness membrane and even touching adjacent cells (Bishop, et al., 2007; Dreyfuss, et al., 2009; Iozzo, R V, 1998; Iozzo, Renato V, 2001). This ability gives rise to a number of biological activities that are modulated by the interaction of proteins with heparan sulphates; including endocytosis, matrix assembly and cell survival, other cellular activities are depicted in Figure 11 (Bernfield, et al., 1999; Dreyfuss, et al., 2009; Häcker, et al., 2005). HSPGs can be further divided into three groups: transmembrane PGs (e.g. syndecans), glycophosphatidyl-inositol (GPI)-linked PGs (e.g. glypicans) and secreted ECM PGs (e.g. Agrin) (Bishop, et al., 2007; Dreyfuss, et al., 2009; Perrimon, & Bernfield, 2001; Schaefer, & Schaefer, 2010).

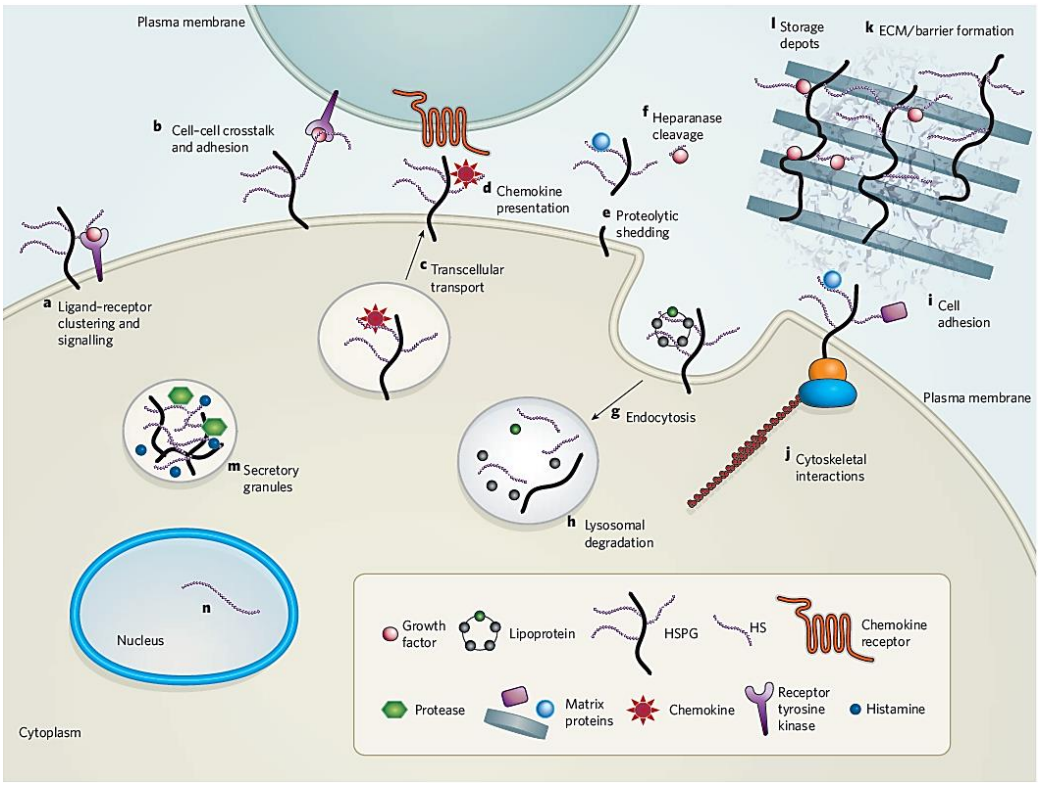


Figure 11. Heparan sulphate proteoglycan cellular activities.

HSPGs can act as co-receptors for growth factors and their receptor tyrosine kinases present either on the same cell (a) or on adjacent cells (b); chemokine transportation across cells (c), chemokine presentation on the cell surface (d); proteolytic cleavage (e), heparanase cleavage of HS chains freeing growth factors (f); endocytosis (g), lysosomal degradation (h); cell adhesion to the extracellular matrix (i), cytoskeletal interactions (j); ECM barrier formation (k) storage of growth factors and morphogens (l); highly sulphated heparin chains are packaged into secretory granules of haematopoietic cells (m); HS chains can exist in the nucleus (n) (Bishop, et al., 2007); (3547140504398).

Basement membrane HSPGs

Basement membrane PGs are a group of high molecular weight proteins, namely Agrin, collagen XVIII, perlecan and bamacan. Other than bamacan, which has additional chondroitin sulphate chains, they exclusively display heparan side chains (Figure 12) (Iozzo, Renato V, et al., 2009).

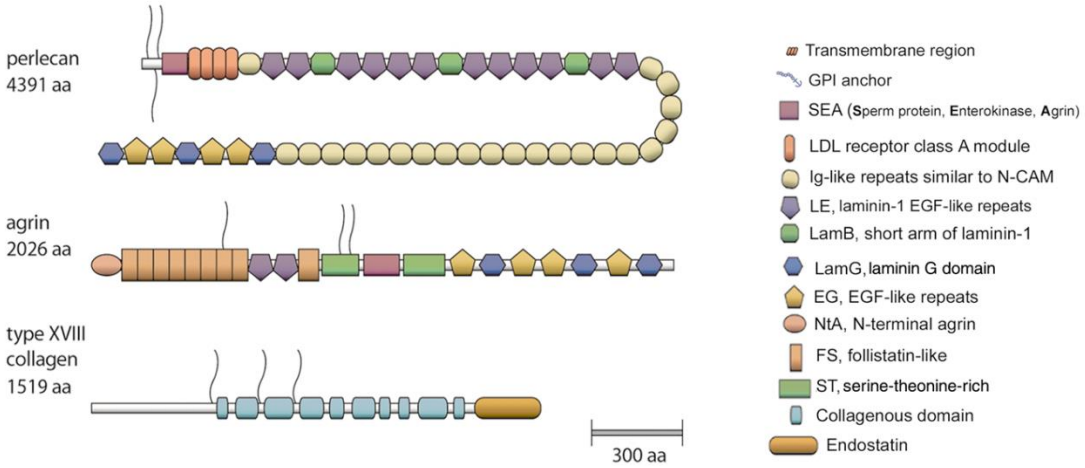


Figure 12. Key structural domains of human perlecan, Agrin and collagen XVIII

(Iozzo, Renato V, 2001); (3547141155698).

These HSPGs are largely associated with the plasma membrane but most HSPG lack a transmembrane domain and therefore their link to the cell membrane may be indirect binding mediated by integrins or other cell surface receptors such as laminin and the dystrophin complex (Bezakova, & Ruegg, 2003). Basement membranes are thin, continuous sheets of multimodular molecules that separate epithelium from stroma and surround nerves, muscle fibres, smooth muscle cells, and fat cells (Kleinman, et al., 1986). These cell-associated sheets have the ability to form a barrier to charged molecules thus creating a biological filter; while

providing structural support to surrounding tissues (Iozzo, Renato V, 2001; Kleinman, et al., 1986; Wiradjaja, et al., 2010). It is made up of three layers known as the lamina lucida, the lamina densa, and the lamina reticularis (See Figure 13). HSPGs reside in the lamina densa and the laminal reticularis, with the exception of Agrin which is only found in the lamina densa due to the negative charge of its protein core and associated laminin binding interactions (Evans, et al., 2010; Loeb, et al., 1999).

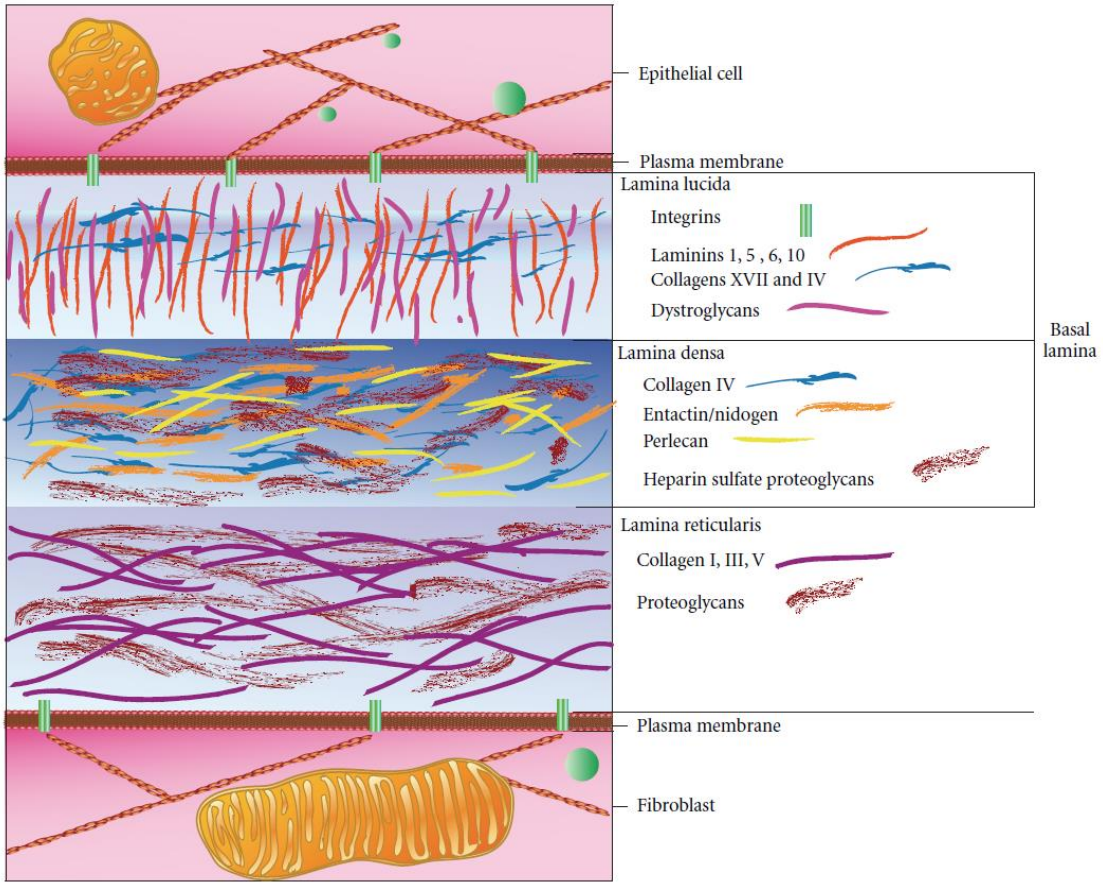


Figure 13. The basement membrane

(Menter, & Dubois, 2012); (CC-BY).

Basement membrane HSPGs and their associated proteins have been studied extensively using genetic models in mouse. Laminin is known to be a key component of the basement membrane, structurally and functionally interacting with the plasma membrane, collagens and dystroglycans, which in turn bind proteins in the lamina densa (Menter, & Dubois, 2012). The absence or even deficiency of any laminin isoforms other than laminin α4 results in pre or postnatal lethality as early as E5.5 for laminin γ1 whose subunit is common to most laminins (Smyth, et al., 1999). Dystroglycan is another key protein which binds laminin using a complex comprised of both α and β-dystroglycan and dystrophin to anchor the complex to the membrane (Bezakova, & Ruegg, 2003; Hohenester, et al., 1999). Dystroglycan mutations in both humans and mice result in marked skeletomuscular phenotypes, in humans a dystroglycan deficiency presents as muscular dystrophy and muscle wasting diseases; while dystroglycan mutant mice have severe disruption to embryonic development resulting in prenatal death (Williamson, et al., 1997; Winder, 2001).

Perlecan knockout mice exhibit defects in the brain, heart and cartilage and up to 40% do not survive past E10.5 (Arikawa-Hirasawa, et al., 1999; Costell, et al., 1999). Although the organs appear normal in Agrin knockout mice, the failure of neuromuscular synapse formation results in perinatal lethality (Gautam, et al., 1996). Transgenic rescue of Agrin in the neurons increase the lifespan of these mice to 3months, however their skeletal growth in significantly reduced postnatally (Hausser, et al., 2007). These data show the significant impact of the absence of these proteins, often resulting in pre or postnatal lethality. Other proteins in the basement membrane such as nidogen and some types of collagen have much milder phenotypes not resulting in lethality, indicating their role in the basement membrane is not necessary for survival (Bishop, et al., 2007; Yurchenco, et al., 2004).

ECM turnover

Under normal, resting conditions cartilage metabolism is a very slow and delicately balanced process of ECM synthesis and degradation which is controlled by a number of anabolic and catabolic factors (Westacott, et al., 1997). In fact, the estimated half-life COL2A1 is approximately 117 years (Verzijl, et al., 2000). However, following injury this turnover is greatly accelerated. The main catabolic mediators responsible for cleaving components of the ECM are proteolytic enzymes (proteases) (Ali, 1964). Proteases are synthesised by chondrocytes, synovial cells and by inflammatory cells and can be found either intracellularly, extracellularly or in lysosomes (Dijkgraaf, et al., 1995a).

There are four main classes of proteases; aspartic, cysteine, serine and metalloproteases (Mort, & Billington, 2001). The most widely studied in cartilage are the matrix metalloproteases (MMPs) which include matrix metallopreotease-1 (MMP-1), also known as collagenase, gelatinase (MMP2) and proteoglycanase/stromelysin-1 (MMP3). The expression of MMPs is activated by a variety of inflammatory cytokines, growth factors and hormones. MMPs are synthesised in their inactive form, requiring activation. Once activated, the MMPs can be inhibited by either tissue inhibitor of metalloproteases (TIMPs) or by plasminogen activator inhibitor (PAI), with the former being the best studied (Dean, et al., 1989; Dijkgraaf, et al., 1995a)

The balance of MMPs and their inhibitors is of significant importance to the integrity of the cartilage and their mediators are of equal importance. Inflammatory cytokines such as Interleukin 1 (IL-1) and tumour necrosis factor (TNF-α) have the capacity to induce the synthesis of proteases, in turn causing ECM degradation (Fernandes, et al., 2002). Inflammatory cytokines can also reduce the synthesis of PGs and other ECM products, causing further cartilage breakdown. Cytokines, like proteases, are also synthesised by chondrocytes, synovial cells and inflammatory cells. The main cytokines present in articular cartilage are

interleukins (IL) I-XII, tumour necrosis factor alpha (TNFα) and interferon gamma (IFγ). The effects caused by cytokines can be antagonised by receptor antagonists, such as IL-1 ra; or also by soluble binding proteins, like TNF-BP (Dijkgraaf, et al., 1995a).

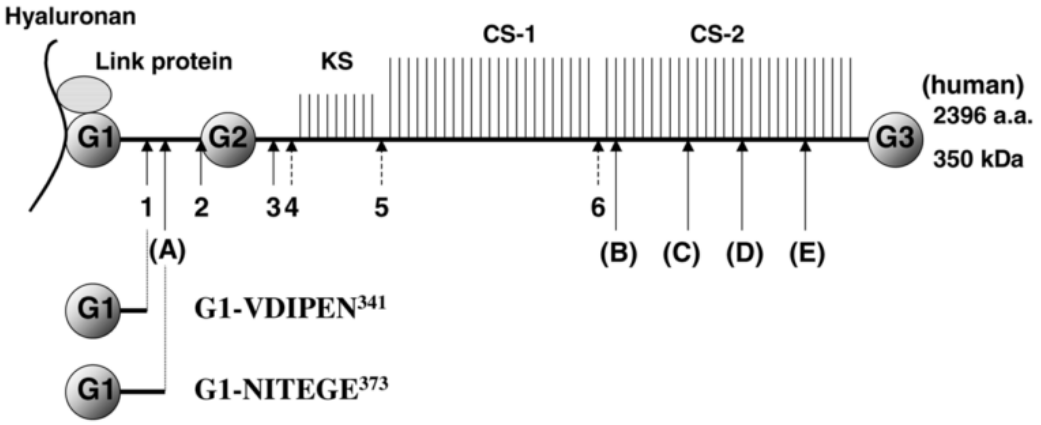


Figure 14. Aggrecan cleavage sites

Schematic of Aggrecan cleavage by ADAMs (A-E) and MMP(1-6) (Nagase, & Kashiwagi, 2003); (CC-BY).

More importantly and much more commonly, growth factors acts as antagonists by increasing the rate of ECM synthesis and TIMPs. Insulin-like growth factor (IGF), transforming growth factor (TGF), bone morphogenetic proteins (BMP), platelet-derived growth factor (PDGF) and connective-tissue-activating peptides (CTAPs) all have the ability to antagonise the catabolic cytokines in cartilage (Chen, et al., 2006). The TGF-β superfamily plays an important role in chondrocyte growth and differentiation. This family of growth factors includes TGF-β1-5 and the subfamily of BMPs. Within this family,BMP-2, BMP-7 and TGF-β are the best studied growth factors that are able to induce proteoglycan synthesis *in vitro* and *in vivo* (van Beuningen, et al., 1998). *In vivo* it has been shown that TGF-β is able to overrule the catabolic effects of IL-1 and enhance cartilage repair whereas BMP-2 can only stimulate PG synthesis in the absence of IL-1 (van der Kraan, et al., 2000). Healthy cartilage has relatively small amounts of TGF-β but in pathological conditions, such as fractures, this level can be increased dramatically (Grimaud, et al., 2002).

A family of peptidases which has been implicated in the degradation of cartilage are disintegrin-like and metalloproteinase with thrombospondin type I motifs (ADAMTS). This family have a role in cell-cell interactions as well as the shedding of various proteoglycans. ADAMTSs have the ability cleave aggrecan, but ADAMTS-4 and 5 have been shown to play a much more dominant role in the breakdown of cartilage. ADAMTS-4 and 5 are particularly involved in the cleavage of aggrecan I and aggrecan II, respectively. ADAMTS-4 is upregulated in osteoarthritis in humans and has a higher aggrecanase activity in humans than ADAMTS-5; however it is the absence of ADAMTS-5 that prevents the progression of osteoarthritis in a mouse model (Bertrand, et al., 2010; Caterson, et al., 2000; Echtermeyer, et al., 2009; Glasson, et al., 2005; Jones, & Riley, 2005; Kuno, et al., 2000; Song, et al., 2007; Stanton, et al., 2005).

Cartilage homeostasis

There are a number of factors that influence cartilage homeostasis and can stem from injury, disease, ageing and other unknown causes. The main problems arise from biomechanical, biochemical, inflammatory or immunological insults that disrupt the chondrocyte balance of ECM synthesis and degradation (Dijkgraaf, et al., 1995b). Following such catabolic events, the cartilage attempts to restore the balance through anabolic mechanisms (Buckwalter, & Mankin, 1998; Dell'Accio, & Vincent, 2010). However, this anabolic response is often not great enough to counteract the catabolism in order to return the joint to a stable state; and following persistent futile attempts, osteoarthritis (OA) develops (Bay-Jensen, et al., 2010; Dijkgraaf, et al., 1995b).

Osteoarthritis

Osteoarthritis is the most common form of arthritis and affects millions of people worldwide (Lawrence, et al., 2008). It is the leading cause of disability in people aged over 65 (60% of men and 70% of women) and is a great health and economic burden (Goldring, et al., 2006; Kinds, et al., 2011).

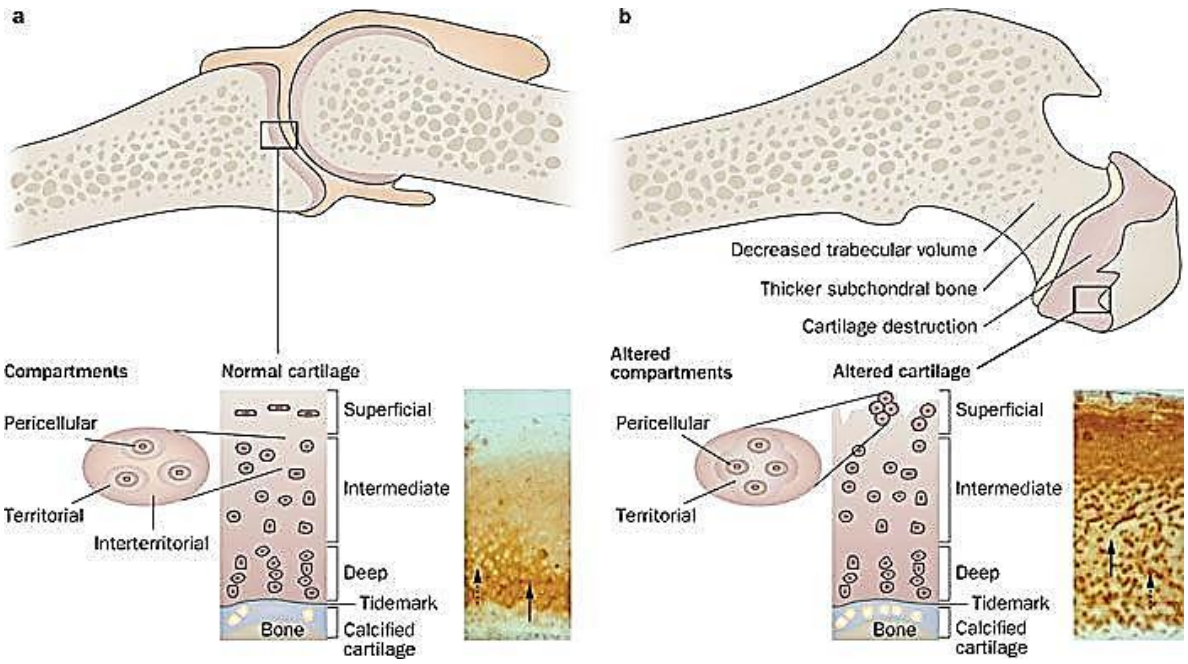


Figure 15. Articular cartilage

A healthy (a) and an osteoarthritic joint (b). The cartilage is divided into distinct zones, the superficial, intermediate and deep. Immunohistochemical staining for cartilage oligomeric matrix protein show a lack of binding in the territorial and pericelluar matrices (dashed arrow), and positive staining in the interterritotrial matrix (solid arrow). An osteoarthritic joint showing the loss or cartilage and changes in the subchondral bone (b). The cartilage zones change their structure. Immunohistochemistry shows that staining is not present in the interterritorial matrix (solid arrow) however, there is positive staining in the pericellular matrix (dashed arrow) (Heinegård, & Saxne, 2011); (3550230924472).

Articular cartilage breakdown is the pathological hallmark of OA. It is often associated with other features such as changes to the subchondral bone, including sclerosis, “bone marrow oedema”, osteophytosis, a modest degree of synovitis, changes to the joint capsule and ligaments. The symptoms of OA include pain, stiffness, swelling, joint instability and impaired range of motion. The most common localizations include the knee, the hand and the hip (Bennell, & Hinman, 2011; Blagojevic, et al., 2010; Goldring, et al., 2006). OA can develop in the absence of other apparent causes (primary OA), or can it be secondary to a variety of other conditions including injury, metabolic or developmental abnormalities (Goldring, et al., 2006).

Dysregulation of ECM turnover in OA

During the progression of OA, the cells, matrix, structural and mechanical properties of the cartilage are all detrimentally affected. Of particular importance is the overall balance of catabolic and anabolic factors which is heavily disrupted and ultimately leads to the loss of cartilage. Although it must be acknowledged that the whole joint is affected during OA. Changes can often be seen in the synovial membrane, joint capsule, ligaments, peri-articular muscles and tendons and the subchondral bone (Goldring, & Goldring, 2010; Goldring, et al., 2006; Martin, & Buckwalter, 2001).

During normal cartilage turnover, genes including Collagen type II, Aggrecan and SOX9 are expressed by chondrocytes but the expression of these, and other, genes is altered dramatically during the progression of OA (Brew, et al., 2010).

In OA both matrix degradation and synthesis is usually enhanced, in an uncontrolled fashion. Cytokines such at TNF-α, IL-1, IL-17 and IL-18 increase MMP production, in particular MMP-13, while decreasing their inhibitors. Anabolic factors such as IGF-1, TGF-β1, 2 and 3 and FGF2, 4 and 8, and BMPs increase matrix synthesis (Sandell, & Aigner, 2001).

Changes in phenotypic markers in OA

During OA articular chondrocytes lose their stable phenotype and increase a number of markers associated with terminal differentiation (Saito, et al., 2010; Yang, et al., 2010). Overall proteoglycan production is reduced, particularly aggrecan; however, both collagen type I and II expression is increased, as well as MMP-13. However the greatest difference in gene expression seen during OA is the production of SOX9 which can be down-regulated 38-fold (Brew, et al., 2010; Saito, et al., 2010). It has been shown that the transcription factor hypoxia-inducible factor-α2 (HIF-2α) is also a catabolic factor which is responsible for an increase in expression of MMPs (namely 1, 3, 9, 12 and13), ADAMTS-4 and nitric oxide synthase-2 (NOS2). The upregulation of these genes by HIF-2α is detrimental to healthy cartilage homeostasis (Saito, et al., 2010; Yang, et al., 2010).

The phenotypic changes are not only related to the articular cartilage, changes in gene expression and ECM homeostasis and seen throughout the calcified layer and down into the subchondral bone as a result of OA. Osteoarthritic subchondral bone and osteoblasts are also known to have an altered gene expression in comparison to the healthy joint. Angiogenic factors such as VEGF as well as IL-6, TGF-β1 and MMPs are upregulated in bone-cartilage unit during OA while at the molecular level, there is some evidence cross talk between osteoblasts and chondrocytes via the MAPK pathways. While the articular cartilage is downregulating COL2A1 and upregulating COLX, the calcified layer increases expression of pERK and downregulates p38 (Lories, & Luyten, 2011).

Table 2. Articular cartilage degeneration responsible for OA (adapted from Martin & Buckwalter 2001).

Structural • Fibrillation and fragmentation extending to subchondral bone • Loss of tissue (decreased cartilage thickness and complete loss of cartilage on some regions • Formation of fibro-cartilaginous repair in tissue
Matrix • Initial increase in water content • Disruption of collagenous macromolecular organisation • Progressive degradation and loss of proteoglycans and hyaluronan • Progressive degradation and loss of collagens • Increase fibronectin concentration
Mechanical • Increased permeability and loss of tensile and compressive stiffness and strength
Cells • Initial increase in synthetic and proliferative activity • Loss of chondrocytes • Eventual decreased synthetic activity • Appearance of fibroblast like cells in regions of fibro-cartilaginous repair tissue

Microarray data

Many of the genes which are affected during OA are the same ones which are stimulated during response to injury. As previously mentioned, the cartilage-specific transcription factor SOX9 and its direct targets COL2A1 and ACAN are known to be upregulated immediately following injury in attempt the enhance ECM production and to stem the effects of catabolism. Previously published data (Dell'Accio, et al., 2008) produced in this lab discovered by microarray analysis that Agrin, amongst others, is one of the genes to be regulated by injury, suggesting that Agrin expression maybe play a role in cartilage homeostasis and in disease.

CHAPTER 2: Introduction to Agrin biology

Agrin: Structure and splicing

Agrin is one of three highly glycosylated basement membrane heparan sulphate proteoglycan (HSPG), together with perlecan and collagen XVIII (Iozzo, Renato V, et al., 2009). It is expressed in many tissues including the kidney, lungs, muscle, brain, nervous system and immune system (Groffen, Buskens, et al., 1998; Hausser, et al., 2007). Its structure, as shown in Figure 16, consists of a large core protein, with a molecular weight of 225kDa, which has four distinct structural domains (Ruegg, et al., 1992; Rupp, et al., 1992). However, in its fully glycosylated form this can increase to 600kDa (Bezakova, & Ruegg, 2003).

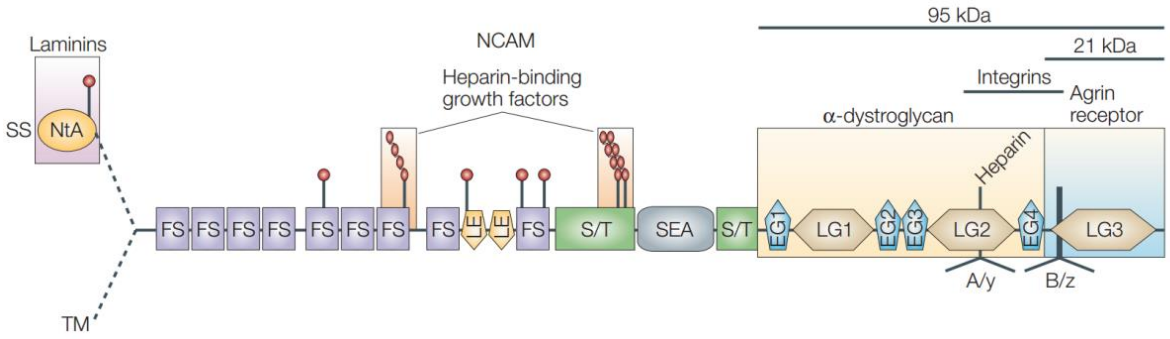


Figure 16. The structure of Agrin

FS, Follistatin-like domain; LE, Laminin EGF-like domain; S/T, serine/threonine; LG, laminin globular domain; SEA, sperm protein, enterokinase and Agrin domain (adapted from Bezakova & Ruegg 2003); (3550221322048).

Agrin exists both as a secreted protein and also as a transmembrane protein dependant on the alternative mRNA splicing, producing a N-terminal consisting of 49 or 150aa respectively (Burgess, et al., 2000). The secreted form of Agrin has a signal sequence (SS-Agrin), responsible for the release of the protein, which is followed by the N-terminal Agrin domain (NtA) which

allows binding to laminin in the basement laminae. The NtA domain is of significant importance and has been shown to be up to 90% conserved in chick, mice and humans (Denzer, et al., 1997).

However in tissues devoid of a basement membrane, such as the brain, Agrin is alternatively spliced to produce a type II transmembrane protein (TM-Agrin) which is anchored to the cell membrane in an N_{cyto}/C_{exo} manner (Bezakova, & Ruegg, 2003; Neumann, et al., 2001). However, this splice variant has only been identified in rat brain tissues despite screening of other tissues and species (Neumann, et al., 2001; Rupp, et al., 1991). The SS and NtA domain are encoded by two separate exons, whereas the TM region is encoded by a single exon which lies more than 8kb downstream allowing alternate splicing (Burgess, et al., 2000; Neumann, et al., 2001). It is suggested that due to the presence of this large intron separating the exons, the splice variants would require individual promoters to regulate their expression (Bezakova, & Ruegg, 2003).

The next domain has five potential glycosylation sites and two glycosaminoglycan attachment sites. It comprises nine follistatin-like repeats, two laminin-EGF-like domains and a central SEA which is between two serine/threonine rich domains. This region is involved in binding to neural-cell adhesion molecule (NCAM) and heparin-binding growth factors. In its most glycosylated and sulphated form, the molecular weight of Agrin can become upwards of 600kDa (Gesemann, et al., 1998). The final domain comprises of three laminin G-like modules which are separated by EGF-like domains (Iozzo, R V, 1998; Jury, & Kabouridis, 2010; Kröger, & Schröder, 2002). These domains are homologous to those found in laminin and perlecan (see Figure 17) (Timpl, et al., 2000).

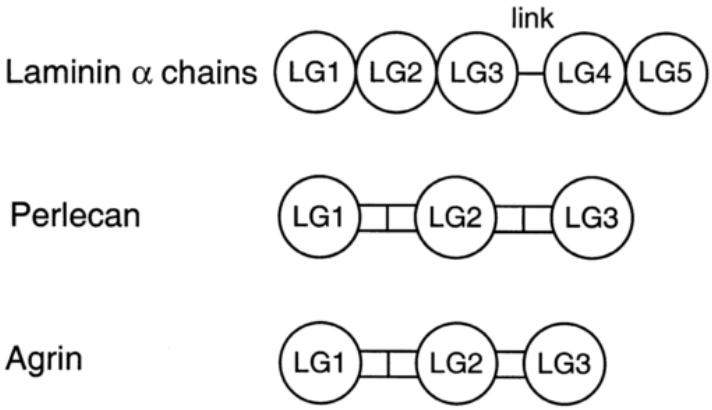


Figure 17. Structural similarities of laminin, perlecan and Agrin

Schematic showing the arrangement of LG modules in laminin, perlecan and Agrin. EGF-like modules are represented by squares. Link represents 35-60 residue insertion (Timpl, et al., 2000); (3550231237150).

The C-terminal portion of Agrin (Figure 17) has three potential amino acid (aa) insert sites known as x, y and z in mammals (A=y and B=z in chick) and are again encoded by separate exons (Ruegg, et al., 1992; Rupp, et al., 1992). Inserts of 3 or 12 amino acids have been discovered at the x site, however their function is currently unknown (Hoch, et al., 1993). Conversely, inserts at the y (4aa) site and z (8, 11 or 19aa) site are known to be responsible for the functionality of Agrin at the neuromuscular junction (see Figure 18) (Ferns, et al., 1993).

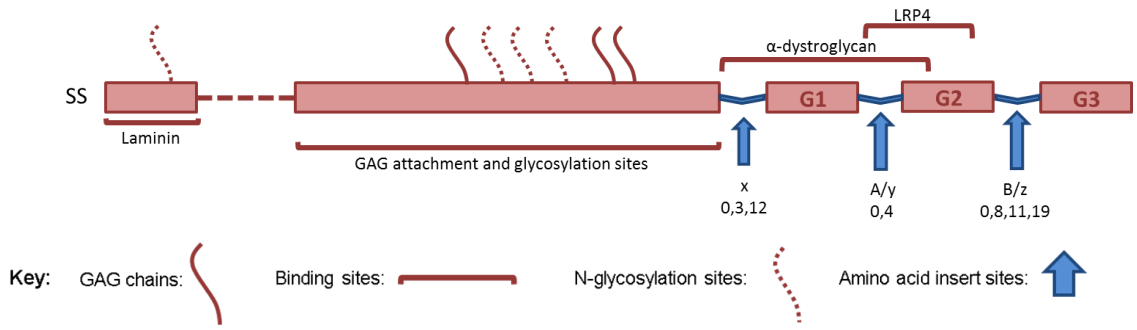


Figure 18. Agrin splice sites

Agrin at the neuromuscular junction

Agrin was first discovered and subsequently isolated from the basal lamina of the Torpedo californica (electric ray) where it was studied for its ability to induce differentiation of the postsynaptic membrane of muscle cells; however it is now known to have an essential function in neuromuscular synapse formation and signalling (Godfrey, et al., 1984).

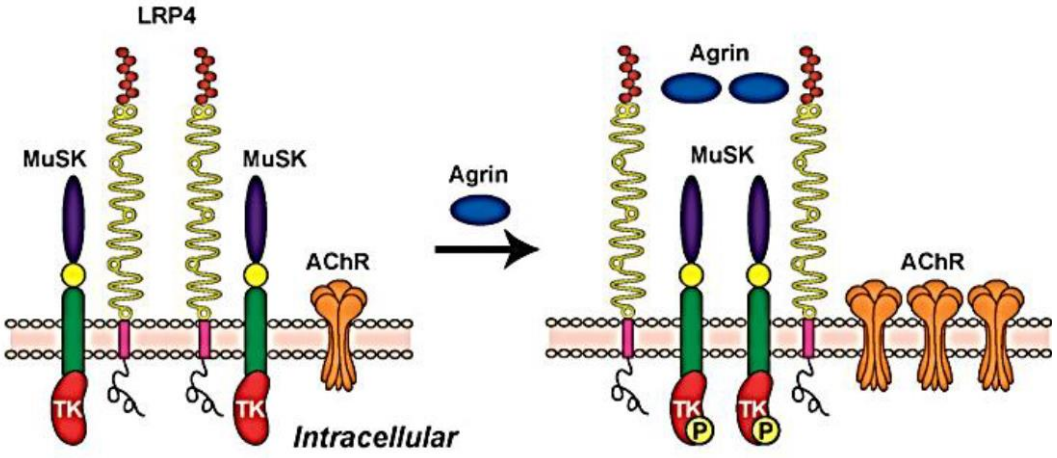


Figure 19. Agrin signalling at the neuromuscular junction

LRP4 is a receptor for Agrin and a co-receptor for MuSK. Activation of MuSK is required for the induction of AChR clustering during the formation of NMJs. MUSK forms a complex with LRP4, but does not bind Agrin directly. Agrin binding to LRP4 enhances complex formation of MuSK with LRP4 and induces transphosphorylation of MuSK (Herz, Joachim, et al., 2009).

At the neuromuscular junction (NMJ) Agrin binds a receptor complex comprised of the muscle-specific kinase (MuSK) and the low-density lipoprotein receptor-related protein 4 (LRP4), inducing the aggregation of acetylcholine receptors (AChRs) on the membrane of myotubes (Jury & Kabouridis, 2010). As mentioned earlier, for Agrin to be functionally active at

the neuromuscular junctions, it must contain the amino acid inserts at the y and z regions. However, it must be noted that Agrin (y0, z0) is still able to cluster AChRs but at an affinity reduced by about 1000 fold (Ferns, et al., 1993).

In nature, amino acid inserts at the z site are only observed when the 4aa at the y site are present. However a number of studies have shown that by mutagenizing the y region to lack the 4aa inserts while retaining the amino acids of 9 or more at the z region are able to retain the same effect on AChR clustering, proving these y inserts are not necessary for the function of Agrin but may be required for the correct translation of the z region (Ferns, et al., 1992, 1993; Hoch, et al., 1993).

It has recently been discovered that the ApoE LRP4 receptor and APP are also required for the normal formation and maintenance of the neuromuscular junction. This is achieved through the use of Agrin as a ligand for both LRP4 and APP which allows the receptors to synergistically induce AChR clustering in myotubes (Choi, et al., 2013).

Agrin at the basement membrane

The LG1 and LG2 domains of C-Agrin comprise the α-dystroglycan binding site required at the basement membrane (Jury and Kabouridis, 2010; Iozzo, 1998; Kroger and Schroder, 2002); while the N-terminal region of Agrin is required for the binding of laminin (Groffen, Buskens, et al., 1998). Agrin has been found to be highly expressed in the basement membranes of a number of tissues such as lung, kidneys, blood vessels and embryonic muscle (Eusebio, et al., 2003). In particular, the presence of Agrin in the glomerular basement membrane has been shown to be 6 times higher than that of perlecan (Groffen, Ruegg, et al., 1998). The isoform of Agrin at the basement membrane is devoid of amino acid inserts at the y and z region and has been shown to have a 10,000 fold higher affinity for α-dystroglycan than Agrin (y4, z8) isoform (Gesemann, et al., 1998).

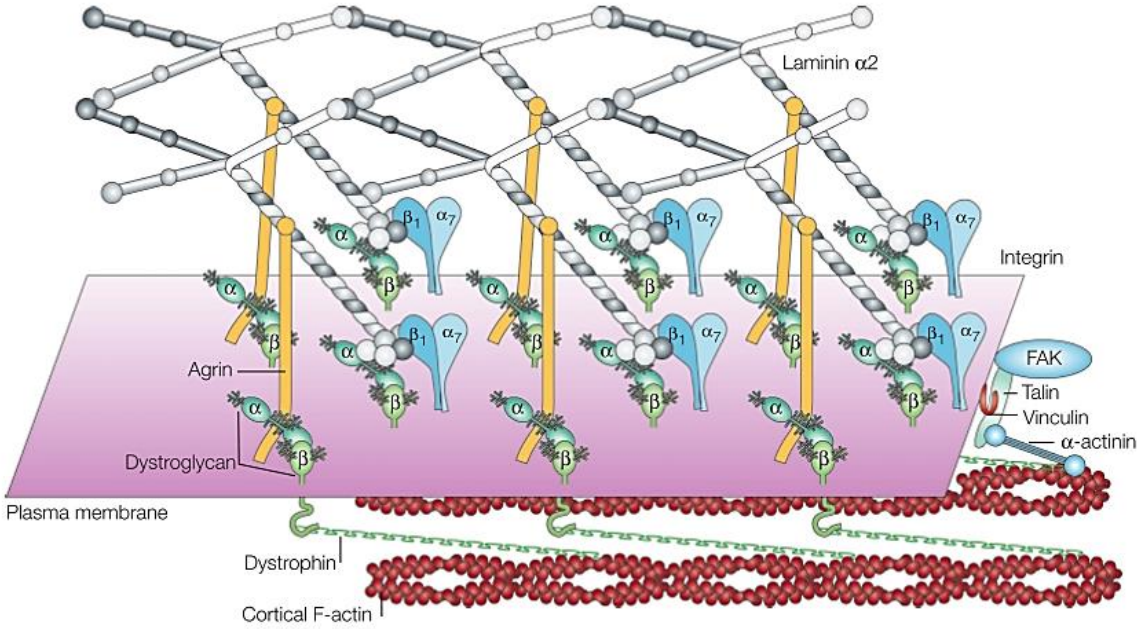


Figure 20. Agrin at the basement membrane

The C-terminal portion of Agrin is bound to the basement membrane through an α- and β-dystroglycan complex. The N-terminal portion of Agrin binds laminin which, in turn, is bound to the basement membrane through binding to α and β-dystroglycan as well as α and β integrin (Bezakova, & Ruegg, 2003) (3550221322048).

MMPs & TIMPs

In 2000 it was discovered that MMP3 is able to remove Agrin from the synaptic basal lamina and *in vitro* studies confirmed that MMP3 was able to directly cleave Agrin (Vansaun, & Werle, 2000). It was found that the final 60kDa portion of Agrin, consisting of the final two LG domains – and the y and z regions - was cleaved in frog anterior tibialis muscle following MMP3 treatment. Further studies in MMP3 knockout mice revealed that these mice displayed increased levels of clustered AChRs and excess levels of C-Agrin (y4, z8) (VanSaun, et al., 2003; Vansaun, & Werle, 2000); also, the denervated motor endplates of the MMP3 knockout mice were protected following traumatic nerve injury revealing a potential target for therapeutic intervention in patients suffering following nerve injury (Chao, et al., 2013).

MMP3 and its inhibitor, TIMP1, are expressed in bone during normal skeletal development and are believed to regulate the bone remodelling (Bord, et al., 1999, 1998). Their expression is retained in the articular cartilage into adulthood with MMP3 becoming the most predominant MMP synthesised in adult human articular cartilage. Both MMP3 and TIMP1 are upregulated during the inflammatory period of an injury in response to IL-1 expression, however, in conditions such as mechanically-induced OA, these inflammatory effects become dysregulated creating a catabolic imbalance of MMPs and TIMPs (Martel-Pelletier, et al., 1994). In addition to the increased levels of MMP3 in the cartilage, synovial fluid and plasma of patients following injury, during OA and in patients with pseudogout the levels of MMP3 are significantly increased (Naito, et al., 1999; Tchetverikov, et al., 2005).

LRP4 and Agrin biology

LRP4 is a receptor for Agrin which forms a complex with MuSK to activate MuSK phosphorylation (Kim, et al., 2008). Agrin is also known to bind APP and enhance the interaction between APP and its co-receptor LRP4 in cultured myotubes (Choi, et al., 2013). A reduction of LRP4 in muscle cells results in reduced Agrin binding and Agrin-induced MuSK tyrosine phosphorylation and AChR clusters (Zhang, Bin, et al., 2008). Agrin binds the N-terminal portion of LRP4, an interaction which promotes the association of LRP4 with MuSK, even in the absence of transmembrane and intracellular domains of LRP4. This shows that an Agrin-Lrp4-MuSK signalling complex can form extracellularly to the cell surface while not affecting its ability to active MuSK. The dispensability of the intracellular portion of LRP4 makes it distinctly different from other LRPs such as LRP5/6 which require their intracellular domains to function in signal transduction (Herz, & Strickland, 2001; Howell, & Herz, 2001; Zhang, Wei, et al., 2011).

Studies on LRP4 knockout mice showed that LRP4 is required for normal skeletal growth and mice deficient of LRP4 have severe skeletal deformities. They are growth-retarded, with polysyndactyly in their fore and hind limbs, and have abnormal tooth development. Furthermore, the lack of LRP4 results in ectopic chondrocyte condensation and subsequent fusion and duplication of digits. This study also showed that LRP4 is able to potently supress canonical Wnt signalling in cells cultured *in vitro* and suggest that LRP4 may function as a negative regulator of Wnt signalling during limb development (Johnson, et al., 2005).

A recent study by Asai et al., showed the requirement for LRP4 in chondrocyte differentiation. They showed that LRP4 overexpression in the murine chondrocyte cell line ATDC5s resulted in upregulation of COL2A1 and ACAN and COLX while LRP4 silencing resulted in the opposite effect. They showed that LRP4 was able to block the Wnt/β -catenin signalling activity in ATDC5 cells suggesting that LRP4 is an important regulator for extracellular matrix

productions and chondrocyte differentiation by suppressing Wnt/b-catenin signalling. (Asai, et al., 2014)

Wnts are a family of 19 morphogens ubiquitously expressed during embryonic development and are required in adulthood for normal tissue homeostasis. Wnt signalling is essential to joint development and, in postnatal life, to joint homeostasis by supporting populations of chondro- and osteo-progenitor cells and regulating their fate (Bowman, et al., 2013; Koyama, et al., 2008). Functional polymorphism in molecules regulating Wnt signalling such as the Wnt inhibitor FRZB are genetically associated with a predisposition to develop OA (Luyten, et al., 2009). At the same time, Wnt signalling, together with Agrin, is involved in the formation and maintenance of the NMJ. Wnt3, Wnt9a, Wnt9b, Wnt10b, Wnt11, and Wnt16 have been shown to be able to stimulate the AChR clustering in a dose dependent manner which is non-additive to that of Agrin (Korkut, & Budnik, 2009). The most potent Wnts in the AChR clustering assays, Wnt9a and Wnt11, bind directly to MuSK to induce MuSK dimerization and subsequent tyrosine phosphorylation of the kinase but interestingly still require the binding of LRP4 to MuSK despite no direct interaction (Zhang, Bin, et al., 2012).

Agrin in skeletal development

Agrin expression has been reported in a wide range of tissues including, but not limited to, the lungs, muscle, brain and the glomular basement membrane of the kidneys (Groffen, 1998). Agrin was first documented as an important player in developmental processes before being directly related to cartilage homeostasis. In 2007 Hausser et al. showed in a mouse model that Agrin is highly expressed by chondrocytes of the growth plate and that lack of Agrin results in a functional impairment of the growth plate chondrocytes and restricted skeletal growth.

Preliminary investigations in our laboratory revealed not only that Agrin, as well as most of its receptors are expressed also in the articular cartilage in adult life, but also that Agrin expression is upregulated after injury. These expression pattern data, together with the already existing data on growth plate development, led to the question of whether Agrin has a role in the articular cartilage.

CHAPTER 3: Hypothesis and Aims

Hypothesis:

Agrin is required for chondrocyte differentiation and supports cartilage formation *in vitro* and *in vivo*.

Aims:

1. Establish the expression pattern of Agrin in normal and osteoarthritic cartilage
    - By sequencing analysis, determine the Agrin splice isoform(s) present in human articular chondrocytes.
    - Investigate the presence and localisation of Agrin in healthy, injured and diseased cartilage in humans and murine models using immunohistochemistry, microarray and qPCR analysis.

2. Determine the effects of silencing and overexpressing Agrin in articular chondrocytes *in vitro* and *in vivo*.
    - Identify a suitable cell type for functional assays.
    - Determine if Agrin can regulate chondrocyte extracellular matrix production by carrying out gain and loss of function experiments using Agrin overexpression plasmids and siRNAs respectively.
    - Develop a method by which to potently and persistently expose Agrin to chondrocytes, without direct transfection, to perform an ectopic cartilage formation assay *in vivo.*

3. Investigate whether α-dystroglycan, LRP4 or both are utilised by Agrin in articular chondrocytes.
    - Using SOX9 expression as a read out, determine the chondrogenic potential of Agrin: in the presence of a α-dystroglycan blocking antibody; and when LRP4 is silenced by siRNA.

4. Investigate Agrin cleavage in osteoarthritis and its effect on the function of Agrin in chondrocytes.
    - Treat *in vitro* cultures of chondrocytes and cartilage explants with MMP3 and stain for Agrin using antibodies specific to the cleaved and non-cleaved portions.
    - Using SOX9 expression as a read out, determine whether the cleavage of Agrin by MMP3 is detrimental to the anabolic function.

CHAPTER 4: Methods & Materials

In vitro culture assays

Cartilage harvest and chondrocyte isolation

Adult human articular cartilage was obtained from patients undergoing joint replacement for knee OA after obtaining informed consent. The cartilage samples were provided by Mr Y. Pengas, Mr P. Achan and Mr M. Ramachandran (Barts and the London National Health Service Trust, London, England, UK). All procedures were approved by the East London and The City Research Ethics Committee 3 (REC 07/Q0605/29). Surgical samples containing bone, cartilage menisci and synovial membrane were immediately placed in a closed sterile jar, to maintain moisture, sterility and overall tissue integrity during transportation. Samples were processed within 4hrs post-surgery. Cartilage tissue was dissected from preserved areas of the femoral condyles and the patellar groove. The cartilage was sliced full thickness, excluding the mineralized cartilage and the subchondral bone. From each sample, full thickness specimens were snap frozen for RNA isolation or fixed in 4% PFA overnight and embedded in paraffin for histological grading.

Bovine chondrocytes were isolated from the metatarsal joints of adult bovine obtained within 6hrs of death from a local abattoir. Full thickness sections were harvested for histological analysis. The articular cartilage was dissected in sterile conditions and processed for cell isolation. For chondrocyte isolation, the cartilage was washed twice in high-glucose DMEM (DMEM/F-12 1:1 plus GlutaMax; Invitrogen) containing 10% FBS, and 2% antibiotic antimycotic solution (Invitrogen). Cartilage was then digested for 30min at 37°C in 1 mg/ml pronase (Roche) and then overnight at 37°C in 1 mg/ml collagenase P (Roche) prepared in complete medium (same composition as above, with 1% antibiotic/antimycotic solution) under agitation. The chondrocytes recovered from the digestion were then resuspended in complete medium and seeded at a density of 10,000 cells/cm^2 or used immediately. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO2. The full thickness explants

taken for histological scoring were fixed in 4% paraformaldehyde in PBS at pH 7.4, paraffin embedded, sectioned, stained, and scored for features of OA as described previously (Mankin et al., 1971, Dell'Accio et al., 2008). All experiments were performed using freshly isolated or P0 cells.

Cells lines

The C28/I2 immortalised human costal chondrocyte cell line was a kind gift from Dr Mary Goldring. The COS7 cell line is a fibroblast-like cell line derived from the kidney of the African green monkey. Cells were split twice weekly at a 1:10 dilution and cultured in normal DMEM in the presence of 10% FBS and 1% antibiotic/antimycotics.

Cell counting and trypan blue exclusion assay

Cells were trypsinised, detached, centrifuged and resuspended in a small volume (1ml) to allow counting. The concentrated cell suspension was mixed at a ratio of 1:1 with 0.5% Trypan blue (approx. 20ul total) and counted using a Neubauer haemocytometer. Cell viability was evaluated by Trypan blue exclusion.

Micromass culture, Alcian blue and Alizarin red staining

C28/I2 cells were transfected 24hrs before being resuspended at a density of 2.0×10^7 cells/ml in complete medium, and micromass cultures were obtained by pipetting 15-µl drops of cell suspension into each well of a 24-well plate. The cells were left to attach for 3hrs and then 1ml of complete medium was added. Micromasses were cultured for 4days (cell lines) or 5days (primary chondrocytes), changing medium every 48hrs. Micromasses were harvested for RT-PCR gene expression analysis or fixed with 4% PFA and whole-mount stained with Alcian blue (0.5%, pH 0.2) or Alizarin red S (2%, pH 4.2) overnight as described previously (De Bari, et al., 2001). After proteoglycan extraction with 6M guanidine HCl, the absorbance was read at 630nm for Alcian blue or 570nm for Alizarin red with a GENios spectrophotometer (Tecan). Protein or DNA was quantified (see below) to normalise the amount of Alcian blue or Alizarin red measured for each sample. Images of the micromasses were acquired at room temperature with a camera (Coolpix 4500; Nikon), with the macro setting and daylight. The contrast of the pictures was then modified with Photoshop 7.0 for best graphic rendering, equally for all treatments.

Protein and DNA quantification

DNA quantification by Sybr green:

Preparation of master mix solution:

	Volume (µl)
TE (10mM Tris HCl, 1mM EDTA, pH8)	94
Sybr Green	1
Sample	5

Preparation of standards:

For each standard, in duplicate, use 5ul of each standard into a black, flat bottomed 96 well plate. Add 95ul of the mastermix to each well. Read fluorescence at: excitation 485nm and emmision 535nm.

Standard	Concentration (ng/µl)	Volume (µl)	Total DNA (µg)
A	10	5	50
B	5	5	25
C	2.5	5	12.5
D	1.25	5	6.25
E	0.625	5	3.13
F	0.313	5	1.56
G	0.156	5	0.78
H	0	5	0

Protein quantification with BCA:

1. For the BCA assay, 25ul of each sample and standard was mixed with 200ul of the BCA reagent in a single well of 96-well clear flat bottomed plate
2. The plate was incubated for 30min at 37°C then measured using the spectrophotometer at 570nm

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20–2,000 µg/ml)

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	0	300 µl of Stock	2,000 µg/ml
B	125 µl	375 µl of Stock	1,500 µg/ml
C	325 µl	325 µl of Stock	1,000 µg/ml
D	175 µl	175 µl of vial B dilution	750 µg/ml
E	325 µl	325 µl of vial C dilution	500 µg/ml
F	325 µl	325 µl of vial E dilution	250 µg/ml
G	325 µl	325 µl of vial F dilution	125 µg/ml
H	400 µl	100 µl of vial G dilution	25 µg/ml
I	400 µl	0	0 µg/ml = Blank

Pellet culture

As an alternative method to micromasses for enhancing chondrogenesis, micropellets were generated by centrifuging cells 200,000 cells at 750RPM or 400,000 cells at 750RPM in sterile Eppendorf's containing 1ml growth medium. The pelleted cells were then cultured in the same conical tube enclosed in a tip box in a humidified 5% CO2 atmosphere. The medium was changed every 2-3days as necessary and the pellets were harvested after 21days. They were placed in individual wells of 24 well plates before weighing, fixing with 4% paraformaldehyde (PFA) and paraffin embedded.

MMP3 treatment

Cells were seeded in monolayer on multi-well FBS coated glass slides and allowed to attach for 24hrs before a further 24hrs treatment with 200ul of 0.5ug/ml MMP3 (Life Technologies)(based on (Sternlicht, et al., 1999)). Distilled water served as vehicle.

Chondrocyte micromasses were plated and cultured for 4days before 24hrs treatment with 500ul of 0.5ug/ml MMP3. Distilled water served as control.

Full thickness bovine articular cartilage explants were harvested and halved. Explants were cultured in normal medium for 4days before 24hrs treatment with 500ul of 0.5ug/ml MMP3. Paired controls were treated with distilled water as vehicle. Explants were then halved and used for paraffin embedding and sectioning or RNA extraction.

Bacterial cultures and plasmid amplification

Preparation of LB-Agar plates:

Ampicillin or kanamycin was added to autoclaved LB-Agar medium to a final concentration of 150µg or 50µg respectively. Individual 300mm bacterial culture plates were filled with approximately 15ml of the antibiotic-containing LB-Agar mixture. After leaving to set for around 2hrs, the plates were inverted and either used immediately, or stored at 4°C.

Plasmid Transformation:

1ul plasmid DNA was mixed with 10ul competent cells on ice for 5minutes. The mix was then placed at 37°C for 1min and added to 600ul LB medium and placed under agitation for a further 45min. The mixture was then poured onto an ampicillin or kanamycin resistant containing LB agar plate for overnight bacterial culture.

Miniprep using Quiagen Kit:

Following overnight bacterial culture growth, a sterile tip was used to touch the colony of choice on the plate and place in a falcon containing 2ml LB medium and the required antibiotic. The colony was grown in the medium at 37°C under agitation overnight. The plasmid was purified from the culture medium using the Quiagen miniprep kit according to manufacturer's instructions.

Agrin plasmids

Both the full length Agrin (FL-Agrin) and the C-terminal Agrin (C-Agrin) plasmids were kind gifts from Dr Michael Ferns (UC Davis Health System, USA).

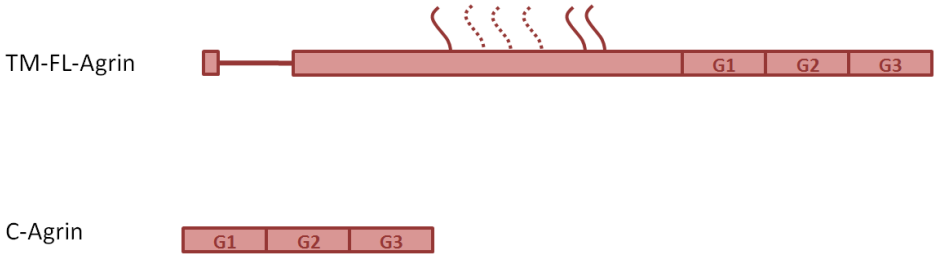


Figure 21. Representation of full length (FL-Agrin) and C-terminal Agrin (C-Agrin) plasmids

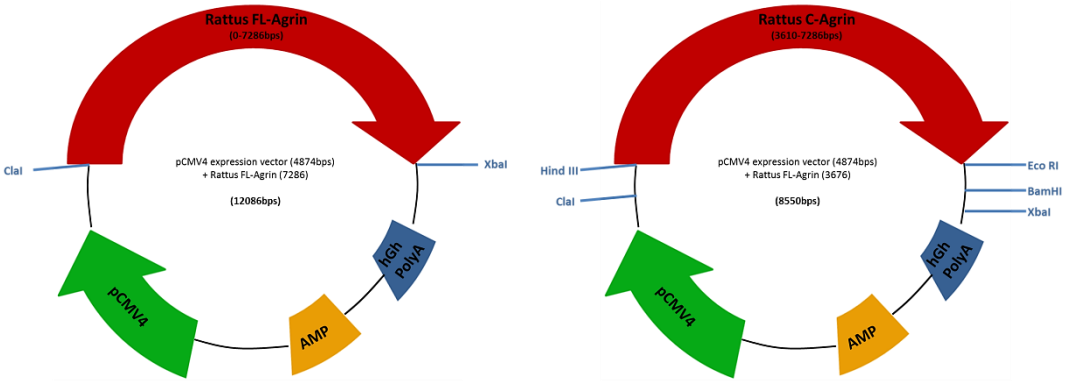


Figure 22. Circular plasmid maps of FL-Agrin and C-Agrin depicting restriction sites

Transfections

Subconfluent (70%) C28/I2 cells, primary bovine articular chondrocytes or COS7 cells were transfected with Full-length Agrin (FL-Agrin), C-terminal Agrin (C-Agrin), BMP2, SOX9 (kind gift from Simon Tew, Liverpool), LRP4, GFP or siRNA's by using JetPrime transfection reagent (PolyPlus transfection, France) according to the manufacturer's instructions. 24hrs after transfection, the medium was replaced and the cells were used in micromass or pellet experiments. Observed transfection efficiency by GFP fluorescence was 25-30% for C28/I2 cells and bovine chondrocytes and around 80-90% for COS7 cells.

Gene expression analysis

Total RNA extraction

Total RNA was extracted after one wash in PBS (for cultured cells) or following homogenisation (for harvested implants), the cells were lysed by direct addition of TRIzol reagent (Invitrogen) (1ml), and each sample was gently sheared with a syringe five times and collected in an Eppendorf tube. After a first centrifugation at 20, 000g for 10min, the supernatant was collected and the debris, if present, discarded. Two hundred µl of chloroform were then added to each sample. The samples were mixed thoroughly by shaking for 30seconds and incubated on ice for 2minutes. The samples were centrifuged for 15minutes at 10, 000g. After centrifugation three different phases are distinguishable in the tubes: an upper aqueous phase, containing the RNA, an interphase containing genomic DNA and a lower phenolic phase containing proteins. The RNA-containing phase was collected, mixed with 500µl of ice-cold isopropanol and left on ice for 20min, to allow the RNA to precipitate. The samples were centrifuged at maximum speed for 40min at 4°C and the RNA pellet was overlayed with ice cold ethanol (70%) and centrifuged at maximum speed for 10min at 4°C. After centrifugation the ethanol was carefully removed and the pellets were allowed to air dry for 15minutes at room temperature, and subsequently resuspended in 12µl of ultrapure water. The concentration of the RNA was evaluated by reading the absorbance at 260nm using a Nanodrop (Nanodrop) spectrophotometer and considering that pure RNA will have a 260/280 ratio between 1.8 and 2.

Reverse transcription cDNA synthesis

Following quantification of the RNA, 500 ng of total RNA from each sample were reverse transcribed by using Thermoscript Reverse Transcriptase Kit (Invitrogen) with oligo dT primers.

Semi-quantitative PCR and qPCR conditions

Cycling was preceded by 2min at 50°C and by a first denaturation step at 96°C, which was also required to activate the hot-start polymerase. The cycling conditions were optimized to achieve the highest efficiency coupled with the highest specificity as evaluated by the generation of a single amplicon of the expected size on agarose gel electrophoresis.

Cycling conditions:

All PCRs were carried out using one of two cycling conditions consisting of 2 or 3 steps with varying annealing and elongation conditions:

2 Step: 96°C for 30seconds; *variable*°C for 1.5min – 40cycles

3 Step: 96°C for 30seconds; *variable*°C for 30seconds; 72°C for 1min – 40cycles

Table 3. Real time qPCR primer cycling conditions

Gene	Species	Step	Variable temperature
ACTIN	Human	3	55
ACTIN	Bovine	3	55/60
AGRN	Human	3	55
AGRN	Bovine	3	55
ACAN	Human	2	68
ACAN	Bovine	3	60
COL2A1	Human	2	68
COL2A1	Bovine	3	55/60
COLX	Bovine	2	60
LRP4	Bovine	3	55
MMP-13	Bovine	2	60
SOX9	Human	3	55
SOX9	Bovine	3	55/60

At the end of the cycling, a melting curve was performed from 90 until 72 degrees. Comparing the melting profile of the positive control (which was also checked for size by agarose gel electrophoresis) with that of the test wells helped identifying individual wells in which nonspecific amplification had occurred. Every sample and standard was run in triplicate and a water control was used to check for reagents contamination.

Gene expression was calculated using a standard curve generated with a positive control or a specific PCR product diluted 1:1, 1:32, 1:1000 and water control.

The expression level of each gene was normalized for the correspondent one of β-actin. The Ct values of β-actin of all the samples were all between 18 and 20. No variation of β-actin values were noticed between treated and not-treated samples.

Table 4. Primers used for semi-quantitative and real-time qPCR.

Gene	Species	Sense	Antisense	Amp (bps)
ACTIN	Human	TGACGGGGTCACCCACACTGTGCCCATCTA	CTAGAAGCATTTGCGGTGGACGATGGAGG	661
ACTIN	Bovine	AGGAGTCGGTTGGATCGAGCA	GGGAAGGCAAAGGACTTCCTGTAAC	136
AGRN	Human	CCTGACCCTCAGCTGGCCCT	AGATACCCAGGCAGGCGGCA	136
AGRN	Bovine	GGCCAAGGAGCAGGTGCAGG	GTTGCCACCCCCACCACGAG	127
ACAN	Human	GTTGTCATCAGCACCAGCATC	ACCACACAGTCCTCTCCAGC	509
ACAN	Bovine	GATGCTTCTATCCCAGCCTCCGC	CGGTCCGGGAAGTGGCGGTAA	125
COL2A1	Human	CTGCTCGTCGCCGCTGTCCTT	AAGGGTCCCAGGTTCTCCATC	432
COL2A1	Bovine	ACGTCCAGATGACCTTCCTG	GGATGAGCAGAGCCTTCTTG	126
COLX	Bovine	AAAGGTCTAAGTGGCCCCTTTTGTC	GAGGTTCATGACAAAAGCACCTTGC	138
LRP4	Bovine	CCTTGGTGGACTCCCGGTCCT	GGGGAGCAGTTAACGGGGTGG	99
MMP-3	Bovine	TGTGTGTCTTGCCCACTAGC	TGCCTGTTGCAGAATGCTAA	118
MMP-13	Bovine	TTGAGGATTCAGGGAAGACG	TCACCAATTCCTGGGAAGAC	124
SOX9	Human	GAACGCACATCAAGACGGAG	TCTCGTTGATTTCGCTGCTC	631
SOX9	Bovine	ACTCTGGGCAAGCTCTGGAGACT	GGCGCGGCTGGTACTTGTAGTCC	121

siRNA design

RNA sequences were chosen based on the following requirements:

- Begin with AA (based on Elbashir et al. 2001)
- Total length of 21 nucleotides (including the AA)
- GC content around 30-50% GC content (high GC content sequences are not as potent)

The chosen sequences were assessed using BLAST against the whole bovine genome to check for any off-target effects. For negative control, the nucleotide sequence was scrambled and checked for off-target effects. Both templates had 5'-CCTGTCTC-3' added to the 3' end.

siRNA sequences

Table 5. siRNA sequences

Gene	Species	Sense	Antisense
AGRN siRNA1	Human	CAUACGGCAACGAGUGUCAGCUGAA	UUCAGCUGACACUCGUUGCCGUAUG
AGRN siRNA2	Human	CCUUUGUCGAGUACCUCAACGCUGU	ACAGCGUUGAGGUACUCGACAAAGG
AGRN Scrambled	Bovine	UUUGGGGUAGUAAGCCGAAAGGGACAGAG	UUCUUUCGGCUUACUACCCCAGGACAGAG
AGRN siRNA1	Bovine	UUUGUAAGCCCAAGGGGUAAGGGACAGAG	UUCUUACCCCUUCGGCUUACAGGACAGAG
AGRN siRNA2	Bovine	UUCAGGAAGAAUUGCAGGCUCGGACAGAG	UUGAGCCUGCAAUUCUUCCUGGGACAGAG
AGRN siRNA3	Bovine	UUCGGUAGACAGAAGACAGGUGGACAGAG	UUACCUGUCUUCUGUCUACCGGGACAGAG
LRP4 Scrambled	Bovine	AAUAUUGUUCAGCACUGACUGCCUGUCUC	AACAGUCAGUGCUGAACAAUACCUGUCUC
LRP4 siRNA1	Bovine	AAGUUCUUGCCAGUAAAUUUGCCUGUCUC	AACAAAUUUACUGGCAAGAACCCUGUCUC
LRP4 siRNA2	Bovine	UUCGAUAACAACUAGUCGUGAGGACAGAG	UUUCACGACUAGUUGUUAUCGGGACAGAG
LRP4 siRNA3	Bovine	UUGAACUACGAAGCUACCAUAGGACAGAG	UUUAUGGUAGCUUCGUAGUUCGGACAGAG

A Stealth™ RNAi negative control duplex of medium GC content was used as a negative control (Invitrogen).

Sequencing

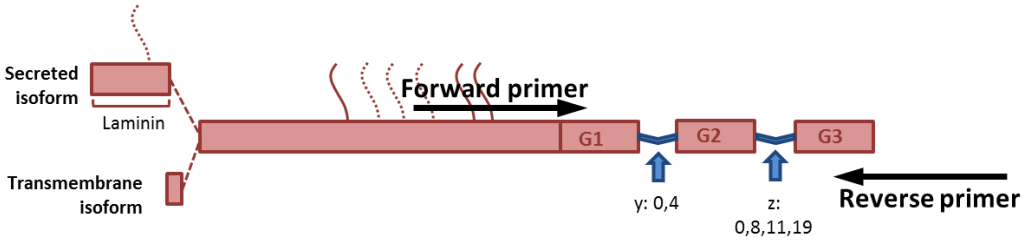


Figure 23. Schematic depicting the region of Agrin to be sequenced following PCR

Table 6. Sequencing primers

Gene	Species	Sense	Antisense	Amp (bps)
AGRN	Human	GGGTGCCCTGCGTGTGGGCG	CGCAGGCTCAGTTCAAAGTGGTT	489

The results were aligned with the known sequence for rat Agrin (y4, z19) (NCBI reference sequence: NP_786930.1) using online software Basic Local Alignment Search Tool (BLAST) (Altschul, 1997). Software can be found at: http://blast.ncbi.nlm.nih.gov/Blast.cgi (correct on Jan 2015).

Processing of histological samples

Fixation and decalcification

Murine knee joints were dissected immediately after the animals were killed and fixed in 4% PFA overnight. The joints were then thoroughly washed in PBS and decalcified in 4% buffered EDTA (ethylenediaminetetra-acetic acid) for 17days. The solution was changed every 2-3days. Bovine and human cartilage explants were fixed in 4% Paraformaldehyde (PFA) at 4°C overnight immediately after dissection from the joints.

Paraffin embedding

Post fixation, explants were processed overnight using the Leica TP 1050 tissue processor as follows:

- 70% Ethanol 1hr x2
- 80% Ethanol 1hr x2
- 95% Ethanol 1hr x2
- 100% Ethanol 1hr x3
- Xylene 1hr x3
- Paraffin (56-58 C) 1hr x2 and 30min x2

Samples were then embedded in paraffin blocks and cooled for 2hrs at 4°C. Bovine and human explants were positioned with the lateral side facing the edge of the blocks so sections produced would be full thickness. Murine joints were orientated in a "kneeling position" with the anterior aspect of the tibia facing down, and the femur angles at 90° with the tibia directed upward. This orientation allowed sections to contain the femur, menisci and tibial plateau. Paraffin embedded samples were stored at room temperature until use.

Paraffin sample processing

Paraffin embedded samples serially sectioned at 5µm intervals using Leica RM 2135 microtome 74 (Knowlhill, UK). The sections were then floated on a warm water bath (45°C), and collected onto Superfrost microscopic slides and dried at 70°C on a hot plate. After 2-3hrs, the slides were removed from the hot plate and stored at room temperature until use.

Histology

Deparaffinisation

Before any histological analysis was performed, the sections on slides were deparaffinised as follows:

- Xylene 5min x2
- Ethanol 5min x2
- Distilled water 5min
- Air dried flat 30min

Toluidine blue

One drop (200ul) of toluidine blue prepared in acetate buffer (pH 4.0) was put on each section for a maximum of 20seconds. The excess of dye was removed by washing the slides in distilled water (5min) and the sections were left to air dry. The sections were then differentiated in ethanol 100% (5min x2), then in xylene (5min x2) and mounted with DPX (Sigma).

Safranin O

Sections were stained with one drop (200ul) Safranin O (Raymond A. Lamb Ltd, UK) for 5min. The excess of dye was removed by washing the slides in distilled water (5min) and the sections were left to air dry. The sections were then differentiated in ethanol 100% (5min x2), then in xylene (5min x2) and mounted with DPX.

Masson's trichrome

Reagents required:

- Ponceau-acid fuchsin
- 0.5% Ponceau 2R (Sigma)
- 0.5% acid fuchsin (Sigma)
- In 1% acetic acid
- 1% aqueous phosphomolybdic acid (Sigma)
- 2% light green (Sigma) in acetic acid >> then use a 1:10 dilution for the staining
- Haematoxylin (Sigma)

Slides were deparaffinised and left to air dry before staining with haematoxylin for 2min before:

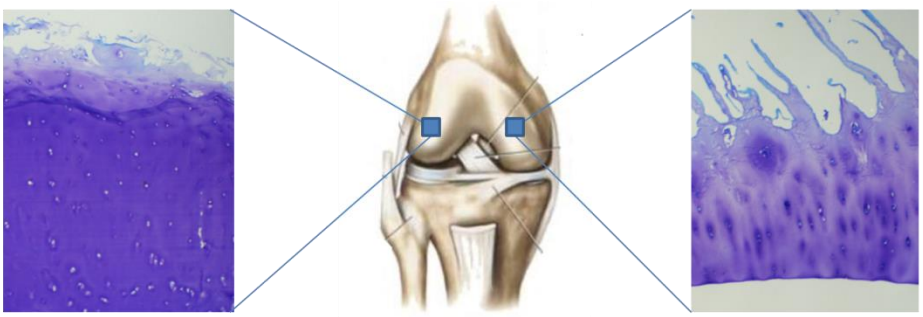
- Washed with distilled water and air dry
- Stained with ponceau-acid fuchsin for 2-3min
- Washed with distilled water
- Stained with phosphomolybdic acid for 5-15min
- Washed with distilled water
- Stained with light green 30seconds
- Washed with distilled water
- Dehydrated ethanol 5min x2 and xylene 5min x2
- Mounted with DPX

Histological scoring

Table 7. Mankin scoring table

Structure	
Normal	0
Surface irregularity	1
Pannus and irregularities	2
Clefts to transitional zone	3
Clefts to calcified zone	5
Complete disorganization	6
Cells	
Normal	0
Diffuse hypercellularity	1
Cloning	2
Hypocellularity	3
Safranin O/ Toluidine blue	
Normal	0
Slightly reduced	1
Moderately reduced	2
Severely reduced	3
No dye	4
Tidemark	
Intact	0
Crossed by blood vessels	1
Total	**/ 14**

Mankin scoring example



Structure	Irregular surface (2)
Cells	Normal (0)
Matrix Staining	Reduced staining in the interterritorial matrix (2)

Mankin score ≤4

Structure	Superficial layers absent (4)
Cells	Clusters (2)
Matrix Staining	Staining only in the pericellular matrix (3)

Mankin score ≥8

Figure 24. Example of Mankin scoring

Paired samples of full thickness adult human articular cartilage sections which, when stained with toluidine blue, show changes to the cartilage surface, cell morphology and reduced matrix staining. Samples are scored using the Mankin scoring system.

Immunohistochemistry

Cells

Primary bovine chondrocytes and C28/I2 cells used for immunofluorescence were seeded on FBS coated chamber slides (LabTek) and cultured for 24hrs in complete growth medium. The cells were then washed in PBS and fixed in 4% buffered PFA, pH 7.4, for 5min at room temperature.

Immunocytochemistry protocol:

- Remove medium
- Wash by dipping gently in PBS
- Air dry thoroughly
- Fix in PFA for 5min
- Wash in PBS for 5min
- Quench ammonium chloride 10min (2.5mg/ml) 5min x2
- Wash in PBS for 5min
- Incubate in blocking solution (PBS+20% FBS) for 1hr
- Incubate with primary antibody (in blocking solution) for 1hr
- Wash in PBS for 5min
- 45min secondary antibody (in blocking solution)
- Wash in PBS for 5min
- Incubate with DAPI (in PBS) for 5min
    - Add triton to PBS in all steps to permeabilise

Tissue

For paraffin embedded tissue samples, sections were deparaffinised:

- Air dry at room temperature for 40min
- Fix in PFA for 5min
- Wash in PBS on rocker 5min x2

Digestion and antigen retrieval:

- Make up 0.02% HCl
- Make up Pepsin (Sigma) solution in 0.02% HCl (15mg/ml)
- Equilibrate both solutions at 37°C for 20min
- Put slides in 0.02% HCl at 37°C for 7min
- Transfer slides to pepsin solution at 37°C for 45min
- Gently dip slides in PBS and then distilled water
- Air dry for 20min
- Fix again with PFA for 5min

Blocking:

- Wash in PBS for 5min x2
- Quench ammonium chloride for 10min (2.5mg/ml) 5min x2
- Block in 3% H2O2 in distilled water for 15min
- Wash in PBS+0.02% Triton-X 5min x2
- Put 1 drop of Avidin-block (Vector Labs, Peterborough, UK) for 10min on each slide
- Flick off and put 1 drop Biotin-block (Vector Labs) for 10min on each slide
- Wash PBS+0.02% Triton-X 5min x2 or 3
- Block in PBS+0.02% Triton-X+20% FBS at room temperature for 1hr+

Antibodies:

- Dilute in blocking buffer (PBS+0.02% Triton-X+20% FBS)
- Incubate at room temp or overnight at 4°C for 1hr
- Wash in PBS+0.2%Triton X for 10min x3
- Dilute biotinylated secondary antibody (Dako) in blocking buffer (PBS+0.02% Triton-X+20% FBS) and incubate at room temp for 30min
- Wash in PBS+0.2%Triton X for 10min x3
- Dilute Streptavidin conjugated with Alexa 555 (Invitrogen) (in the dark) in blocking buffer (PBS+0.02% Triton-X+20% FBS) and incubate at room temp for 30min
- Wash in PBS+0.2%Triton X (in the dark) 10min x3
- Incubate slides with DAPI (Invitrogen) (1/1000 in PBS+Triton X) at room temp for 5min
- Wash in PBS+0.2Tritin X in the dark 10min x3
- Then mount the slides with coverslips using Mowiol
- Leave overnight to dry, in the dark at 4°C

Antibodies

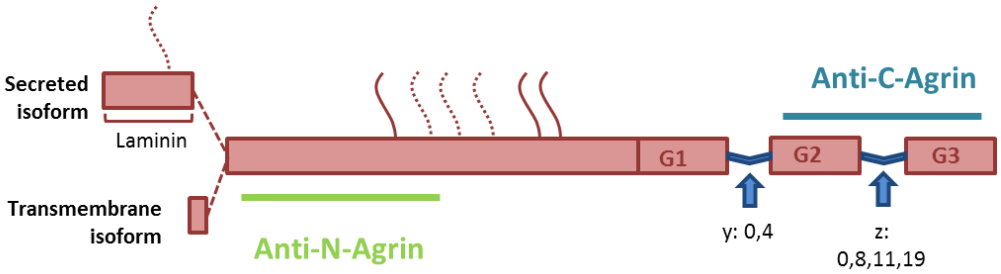


Figure 25. Schematic of where the N-Agrin and C-Agrin antibodies bind

Primary antibodies and concentrations used:

N-Agrin, Rabbit, 500ug/ml, (Abcam ab85174) - 1:250 =2ug/ml

C-Agrin, Rabbit, 200ug/ml, (Santa Cruz H300) - 1:100 = 2ug/ml

Negative control:

IgG, Rabbit, 200ug/ml, (Dako) - 1:100= 2ug/ml

Secondary:

Biotinylated goat ant rabbit (Dako) - (1:300)

Tertiary:

Streptavidin conjugated Alexa 555 (Invitrogen) - (1:300)

Inhibition of dystroglycan binding

The blocking of the binding of Agrin to the basement membrane was carried out using an anti-α-dystroglycan antibody. This was validated using the inhibition of the production of fillamentous actin (F-actin) as a readout, by staining with phalloidin (see Figure 26).

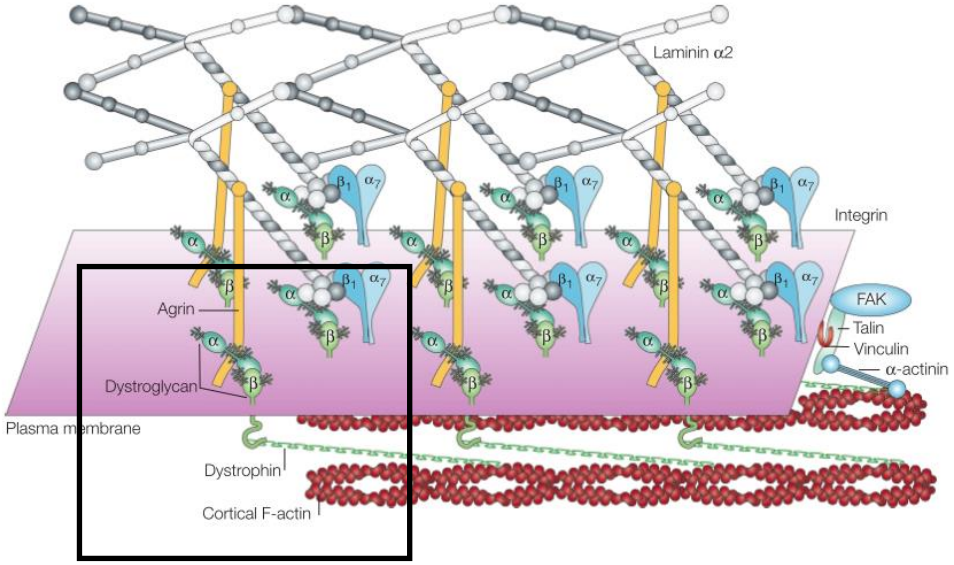


Figure 26. Agrin-dystroglycan binding induces the production of filamentous actin (F-actin)

(Bezakova, & Ruegg, 2003)

To stain the chondrocytes f-actin with phalloidin (Alexa Fluor® 488 Phalloidin, Invitrogen), the following protocol was used:

- Remove medium and wash gently with PBS
- Fix in 4% PFA at room temperature for 15mins
- Wash 1x PBS
- Quench ammonium chloride 10min (2.5mg/ml)
- Wash 1x PBS
- Permeabilise in PBS+ 0.2% Triton-X at room temperature for 10min
- Wash 1x PBS
- Block in PBS+20% FBS for 15min
- Dilute the phalloidin in PBS+20%FBS (1:100)
- Place minimal amount of diluted phalloidin on cells (approx. 500ul per well of a 6 well plate)
- Look under microscope

Antibody treatment:

Preliminary dose-response experiments were carried out using 2.5, 5 or 10ug/ml of either anti-α-dystroglycan or IgM. Following optimisation, subsequent experiments were carried out using a concentration of 2.5ug/ml.

Bovine primary chondrocytes were treated in monolayer or micromass with 2.5ug/ml for 24hrs or 5days respectively in normal culture medium. Chondrocytes treated with 2.5ug/ml IgM served as controls.

Antibodies:

α-dystroglycan, mouse, 200ug/ml, (Santa Cruz, IIH6)

- 1:80= 2.5ug/ml

Negative control:

- IgM, mouse, 200ug/ml, (Santa Cruz)
- 1:800= 2.5ug/ml

Microarray

This experiment was previously carried out by Prof Dell'Accio and published in 2008 (Dell'Accio, et al., 2008). In brief human articular cartilage explants were obtained from 3 donors undergoing total knee replacement surgery for OA. Total RNA was controlled for integrity and purity using an Agilent Bioanalyzer and a NanoDrop spectrophotometer, respectively. RNA amplification was performed by *in vitro* transcription (IVT) using a 2-cycle complementary DNA synthesis kit, according manufacturer instructions (Affymetrix, High Wycombe, UK). Probes were biotin-labelled during the second IVT reaction using the Affymetrix IVT labelling kit. The probes were purified and analysed the resulting antisense RNA was fragmented with alkaline hydrolysis and resuspended with control spikes hybridization buffer (Eukaryotic Hybridization Control Kit; Affymetrix). Human Genome U133 Plus 2.0 array (Affymetrix) is a single array representing around 47,000 transcripts. The gene array chips were hybridized in a rotisserie oven at 45°C and washed and stained in GeneChip Fluidics Stations 400 and 450 (Affymetrix) using the EukGE-WS2v4 protocol. A GeneChip Scanner 3000 (Affymetrix) was used for scanning, and image analysis was performed with GCOS (Affymetrix). Data pre- processing, including normalization, modelling, and "presence/ absence" calls, was determined with MAS5 software (Affymetrix). The target value was set at 100.

In vivo destabilisation of the medial meniscus

Eight-week-old 129/Sv male mice received destabilisation of the medial meniscus (DMM) and sham surgery to the contralateral limb as described previously (Glasson, et al., 2007). After 8wks, mice were killed and the joints were fixed in 4% PFA and decalcified before being embedded in paraffin block. A minimum of five sections per joint were stained using toluidine blue for histological analysis and Osteoarthritis Research Society International (OARSI) scoring for osteoarthritis severity by two independent investigators. This was performed by Prof Dell'Accio and Dr Nalesso.

Generation of transfected growth arrested cell line

COS7 cells were plated in monolayer and allowed to attach for 24hrs. The cells were transfected with either FL-Agrin or GFP and cultured for a further 24hrs. The COS7 cells were then treated with 5, 7.5 or 10ug/ml Mitomycin C for 2hrs at 37°C. The Mitomycin C was removed and cells were washed twice with PBS and cultured in normal culture medium, changing every 2-3days for a further two weeks for viability, proliferation and protein detection.

In vivo ectopic cartilage formation assay

The ectopic cartilage formation assay was performed as described previously (Dell'Accio, et al., 2001). In brief, for each injection 5million freshly isolated bovine chondrocytes were mixed with either 500,000 growth-arrested COS7 cells overexpressing FL-Agrin or with the same amount of Mitomycin C–treated GFP transfected COS7 cells. Each sample was resuspended in 50µl of sterile PBS and injected intramuscularly in the posterior compartment of the thigh of 3wk-old female CD1nu/nu mice (Dell'Accio et al., 2001) (see Figure 27). We performed 12 injections per condition (24 in total). Animals were maintained in isolator cages under an unrestricted diet. The animals were then killed after 2wks and the cartilage implants were retrieved. All procedures were approved by the Local Ethics committee and the UK Home Office. The implants were weighed and cut in half along the longest axis. Half of each implant was embedded in paraffin while the other was used for RNA extraction.

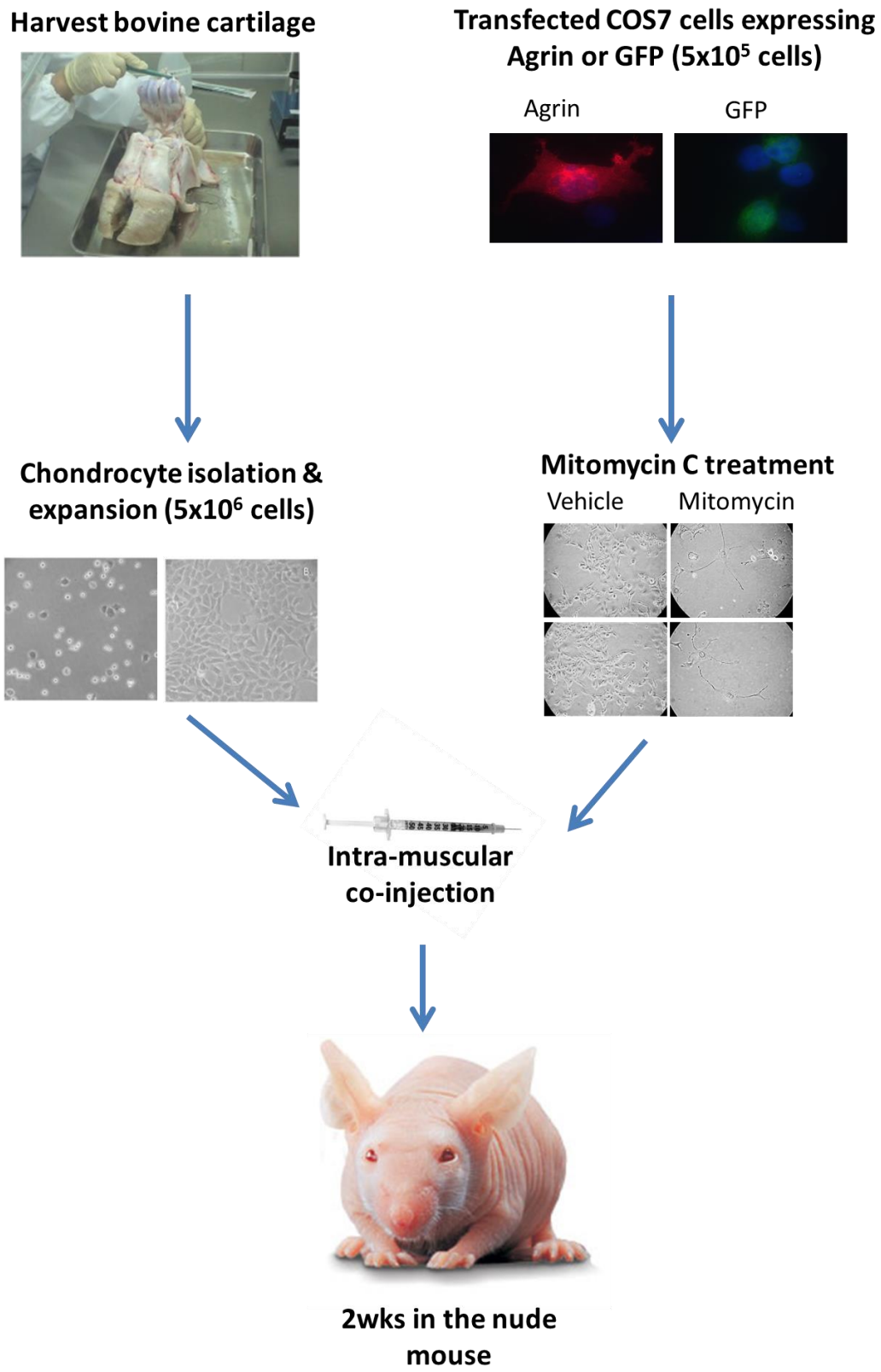


Figure 27. Schematic summarising the *in vivo* ectopic cartilage formation assay

Implant histological analysis

Upon removal from the mice, the implants were weighed, dissected in half and either used for RNA extraction or paraffin embedding. Following sectioning of the paraffin embedded implants, 5 non-consecutive sections were chosen through the centre of the explants. The sections were stained with Safranin O and the cartilage area was isolated by thresholding using Image J software. The differentiated and total area was measured.

Microscopy image acquisition

Images were acquired with a fluorescence microscope (BX61; Olympus) using Uplan-Fluor 4x, 10x, 20x or 40× NA 0.85 objective lenses. Images were acquired at room temperature, by using an F-View II (SIS) camera and Cell P/Sense software. The acquisition parameters were determined using the positive and negative control slides to achieve the maximum sensitivity and specificity. After acquisition, the contrast of the images was enhanced for best graphic rendering using Photoshop 7.0, all with the same parameters, without altering the relationship of the target to control the images. Image analysis was performed using image J software for best rendering, all images were treated the same. Original black and white images were used to quantify fluorescence. Original colour images were used to quantify morphological parameters and Safranin O staining. Nomarski images were produced by using darkfield optics.

Statistical Analysis

Parametric data were compared with a student t-test. For multiple comparisons, analysis of variance (ANOVA) analysis was used, with a Dunnet post-test. P-values <0.05 were considered significant. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$. All the data in the graphs are expressed as mean ± SEM.

Buffers and Media

Luria broth (LB) medium:

- 10g Tryptone
- 5g NaCl
- 5g Yeast extract
- 1000ml distilled water

For LB plates, add 16g Agar to 500ml with the relevant antibiotic

4% Paraformaldehyde (PFA):

- 8g PFA
- 160ml Baxters water
- 40ul 10M NaOH
- 4010ul 1M HCl
- Top up to 200ml with Baxters water

Mowiol mounting medium:

- 6g glyercol
- 2.4g Mowiol (Sigma)
- 6ml distilled water
- 12ml 0.2 M Tris (pH 8.5) (Sigma)

CHAPTER 5: Results section 1

Agrin expression pattern in cartilage

Results: Agrin expression pattern in cartilage

Rationale:

In order to investigate the role of Agrin in the articular cartilage it was first necessary to determine its expression levels and localisation in healthy articular chondrocytes. Previously unpublished microarray data from our laboratory revealed the presence of Agrin in human articular chondrocytes, however, it was important to determine the precise pattern of expression in the different joint tissues and identify which splice variant was expressed in cartilage specifically as Agrin exists in two distinct alternatively spliced isoforms. At the neuromuscular junction it is expressed with the addition of up to 23 amino acids giving rise to the high affinity for LRP4, the binding of which is required for synapse formation and maintenance. However, at the basement membrane, the absence of these splice inserts result an isoform with a high affinity for α-dystroglycan, allowing Agrin to enhance the linkage between the ECM and the cytoskeleton. As the function of Agrin varies greatly depending on the presence or absence of these inserts it was important to sequence across the insert sites and determine the isoform expressed by articular chondrocytes. This would and to allow the overexpression of the correct isoform and enable receptor usage experiments in subsequent chapters.

The following experiments in this chapter investigated the changes in Agrin expression during human and murine models of osteoarthritis and following injury, giving an insight into the function of Agrin in the articular cartilage.

Experimental aims

Determine:

- the expression of Agrin and its receptors in the articular cartilage
- the splice isoforms of Agrin present in human articular chondrocytes
- the expression pattern in human OA and OA in a murine model
- the expression pattern in acute injury

Agrin expression

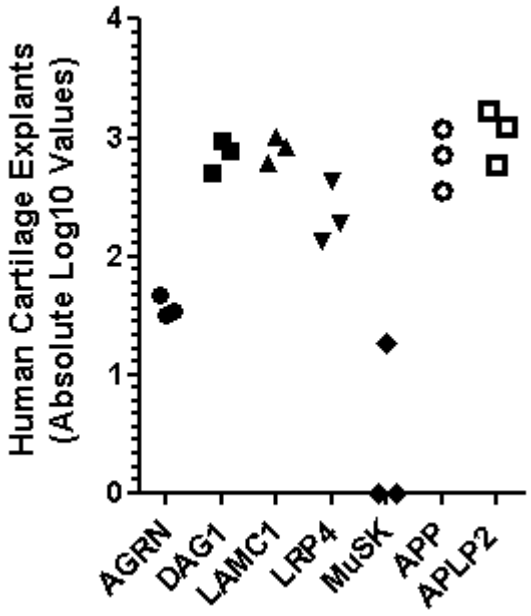


Figure 28. Gene expression of Agrin and its receptors in human cartilage

Gene expression levels of AGRN, DAG1, LAMC, LRP4, MuSK APP and APLP2 in adult human articular cartilage explants measured by microarray (n=3) (Dell'Accio, et al., 2008).

Data from a microarray carried out by Prof. Dell'Accio (unpublished and Dell'Accio et al. 2008) determined that Agrin and its known receptors (LRP4, MuSK, APP, APLP2, LAMC and DAG1) are expressed in freshly isolated human articular cartilage. All genes were determined to be expressed in each patient sample with the exception of MuSK which was only detected in one of the three samples, suggesting its role may not be of significance in the articular cartilage.

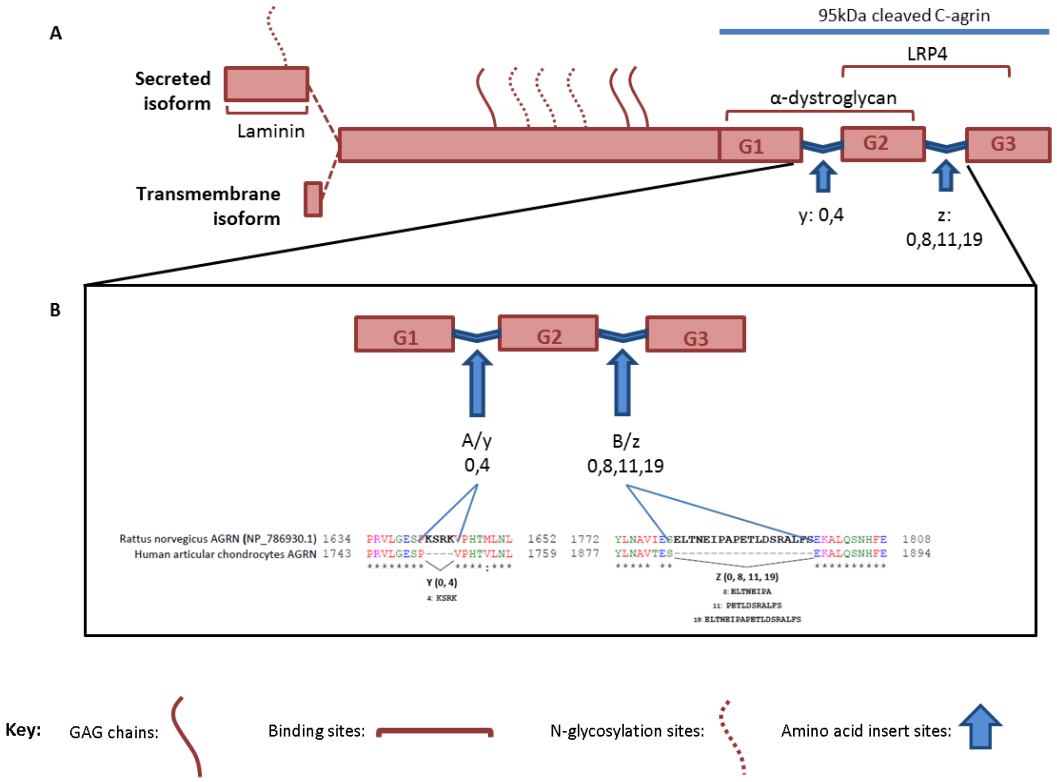


Figure 29. Splice variant of Agrin in human articular chondrocytes

Schematic representation of Agrin (A); cDNA from primary human articular chondrocytes was used to amplify by PCR the portion of Agrin containing the y and z splicing using the following primers (B). The amplicon was then sequenced and the sequence was aligned to that of rat neuronal Agrin. This sequence analysis demonstrated the absence of the y and z inserts in human articular chondrocytes.

Agrin is expressed in a number functionally distinct isoforms in different tissues. In non-neuronal tissues Agrin lacks amino acid inserts at the y and z (y:4, z: 8, 11 or 19) (Figure 29), whereas their presence at the neuromuscular junction is required for the formation and maintenance of the neuromuscular synapses. To identify the isoform expressed in cartilage, primers spanning the potential y and z insert regions were designed and the subsequently obtained cDNA was sequenced (see Appendix for raw data). This established that the Agrin isoform expressed in human articular chondrocytes does not contain the neuronally-required y and z inserts at these sites (y0, z0).

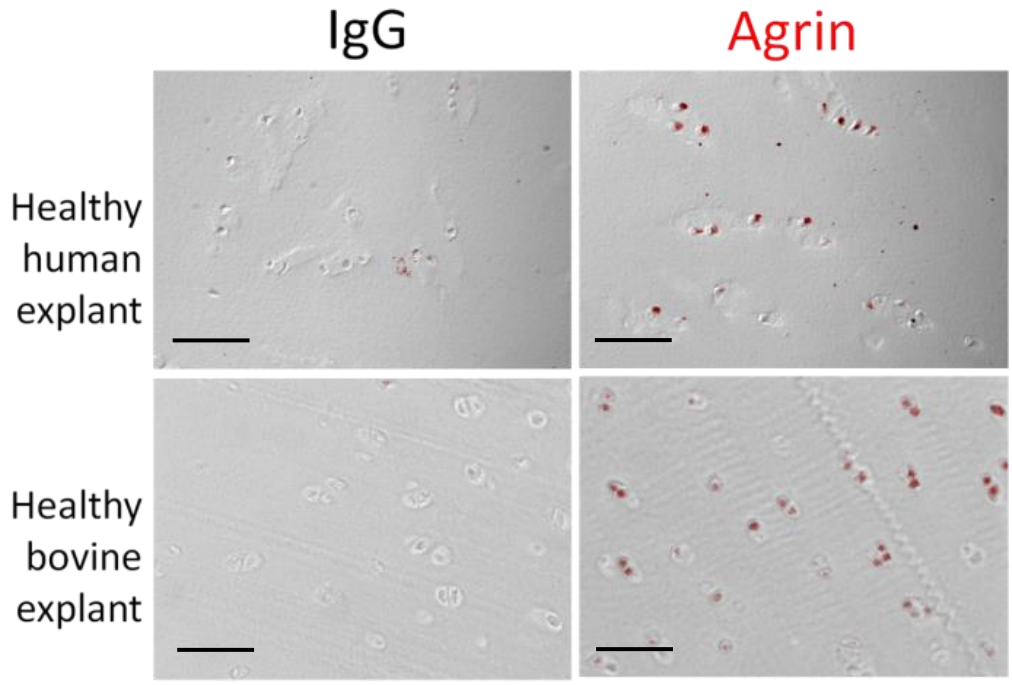


Figure 30. Agrin protein detection in healthy human and bovine cartilage explants

Agrin protein detection in healthy adult human (left) and bovine (right) articular cartilage explants. Nomarski imaging technique was used to obtain high definition of anatomical details, as would be achieved using immunohistochemistry, coupled with the much more rigorous acquisition of the expression data that can be achieved by immunofluorescence (fixed exposure time and parameters for all samples). Bar, 50µm.

Agrin protein was detected in cartilage explants taken from healthy human donors and from young bovine joints (Figure 30). Confirming the conservation of Agrin across the species, it was also detected within the murine articular cartilage, meniscus and growth plate (Figure 31). The staining within the murine joint differs from that within the bovine and human cartilage explants, indicating a degree of difference in the localization of Agrin within different species. Whereas the expression pattern in the healthy murine joint results in a matrix associated staining, in the bovine and human cartilage explants the staining appears to be nuclear.

However, the staining in the murine joint following either sham or DMM surgery (Figure 33), the localisation is altered to be nuclear. This could be an artefact due to the differential access of the antibodies in normal and partially degraded extracellular matrix, but could also implicate intriguing biological mechanism. As is detailed in Chapter 9, Agrin can be cleaved by different proteases *in vivo* and there is evidence of cleaving in OA joints. Therefore the difference in staining when using antibodies directed to the N-terminus and a C-terminus may result in different patterns because whereas the N-terminus will bind to laminin and will remain pericellular, however the C-terminus is soluble and may either diffuse or, as the staining in Figure 30 suggests, may be internalized.

Alpha and beta dystroglycan are generated by autoproteolysis of a monomeric precursor within the SEA motif called sea urchin sperm protein–enterokinase–Agrin (SEA) domain. Beta dystroglycan can be transported to the nucleus with a mechanism that involves autoproteolysis through the SEA domain and two nuclear localization signals (Oppizzi, et al., 2008). Interestingly, Agrin also contains a SEA domain, immediately preceding the biologically active C-terminus, which is flanked by two large S/T rich domains. The function of these domains is currently unknown but it could be involved in nuclear import. Therefore, it is possible that the interaction of Agrin with α-dystroglycan may result in autoproteolysis of the SEA domain and nuclear transportation of the receptor complex comprising C-terminal Agrin.

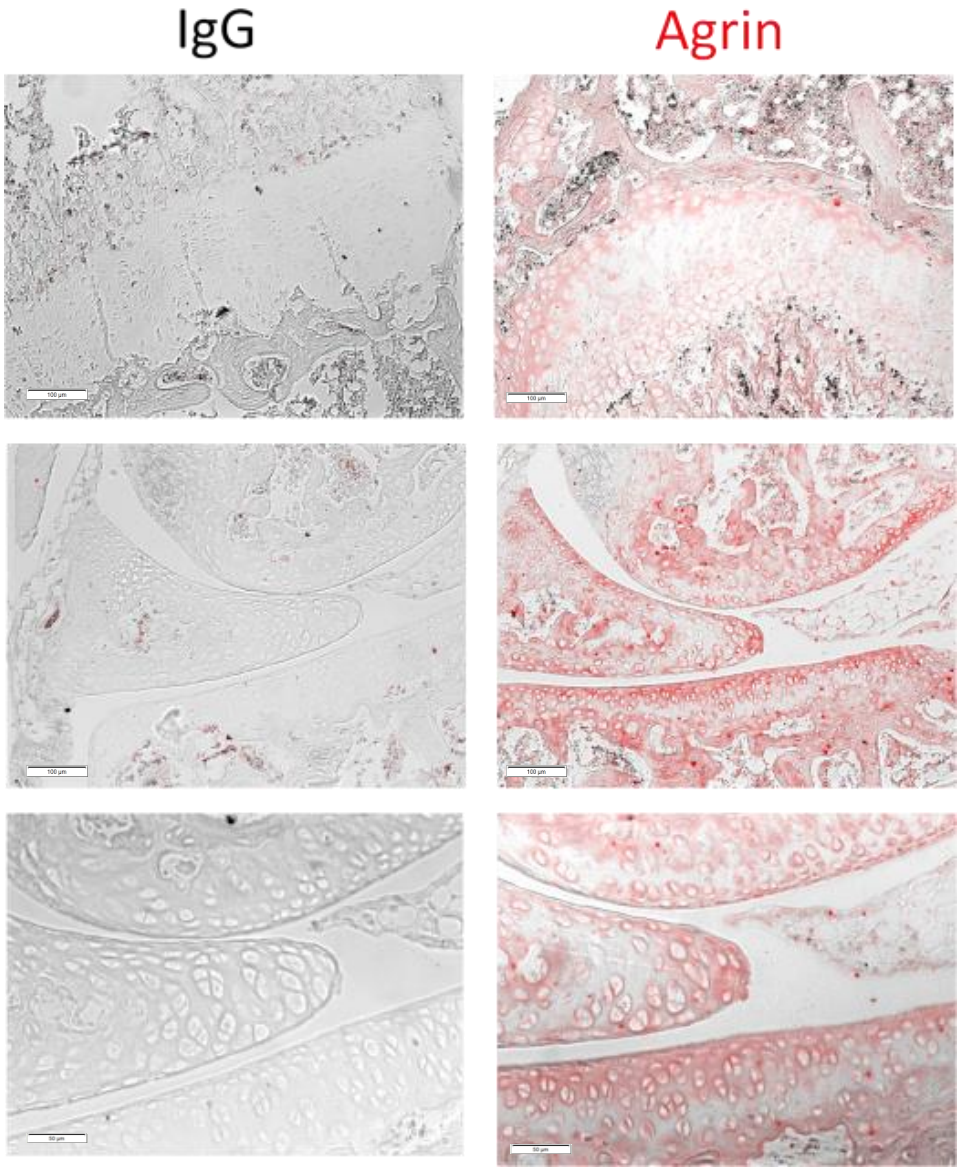


Figure 31. Agrin protein detection in a healthy murine articular joint

Agrin protein detection in healthy 129/Sv murine growth plate and knee joint. Nomarski imaging technique was applied to immunofluorescence photos. Original fluorescence images can be found in Appendix Figure 83.

Agrin in human and murine osteoarthritis

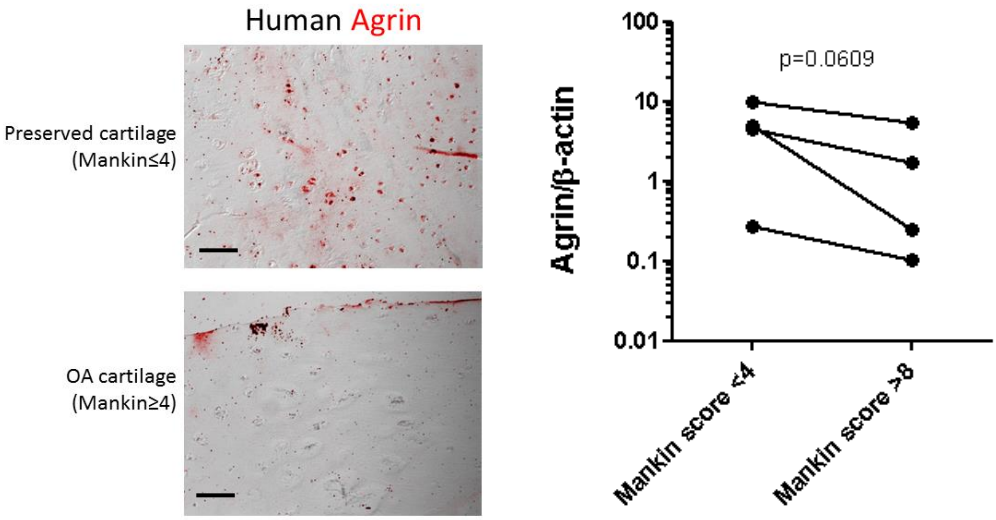


Figure 32. Analysis of Agrin in paired human articular cartilage explants

Comparison of Agrin protein detection in mild (Mankin score <4) and severe (Mankin score >8) human osteoarthritis. Comparison of the gene expression level of Agrin in mild (Mankin score <4) and severe (Mankin score >8) human osteoarthritis (paired two-tailed t-test) (n=4). Bar, 50µm.

To investigate whether Agrin is modulated in diseased cartilage, samples were obtained from patients undergoing total knee replacement for osteoarthritis. Cartilage was explanted from areas of low and high levels of OA damage (determined using the Mankin scoring system) for each donor and were stained for Agrin (Figure 32 – left). In these samples, Agrin was predominantly detectable in explants obtained from the preserved areas of the joints; however it was almost undetectable in areas of cartilage severely affected by osteoarthritis – suggesting Agrin breakdown or reduced production during the progression of OA. Real-time qPCR data did not show a statistically significant downregulation at mRNA level, although this could also be due to insufficient power with only 4 samples per group (Figure 32 - right).

It is often speculated that, as OA is a temporal and often progressive disease, the preserved or less affected areas are representative of earlier disease stages. This is speculative because these cartilage areas are and have been exposed to the same milieu (including inflammatory cytokines and proteinases) as the severely affected areas and of course are derived from different anatomical locations. Therefore, to better study the temporal modulation of Agrin in OA, histological sections obtained at different time points from a murine model of OA induced by the destabilization of the medial meniscus were stained for Agrin (Figure 33).

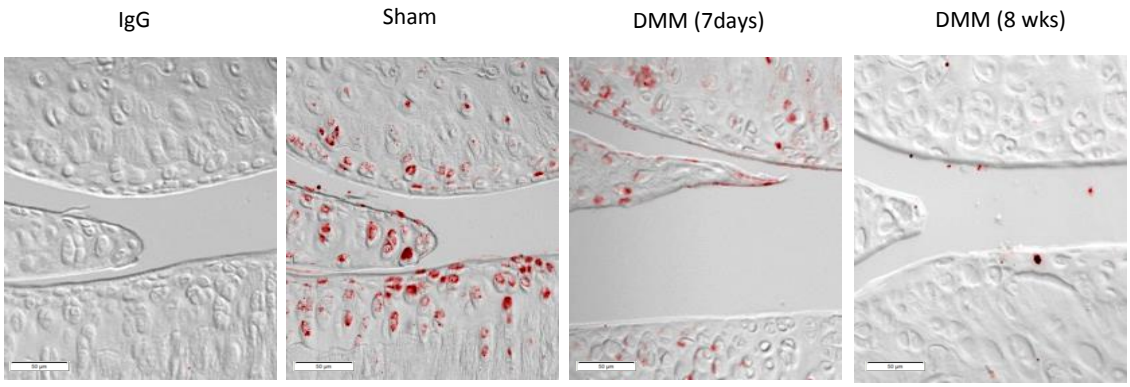


Figure 33. Agrin protein detection in the murine articular joint during osteoarthritis

Agrin protein detection the knee joint of 129/Sv mice 7days and 8 weeks post osteoarthritis induced by DMM surgery. Sham operated served as positive control; non-immune IgGs served as negative control antibody.

Agrin protein detection was reduced in the articular cartilage of mice which had undergone destabilisation of the medial meniscus (DMM) as early as 7 days post-surgery and was completely abolished at 8 weeks (Figure 33). However, the expression of Agrin in the sham operated knees persisted.

Agrin in acute cartilage injury

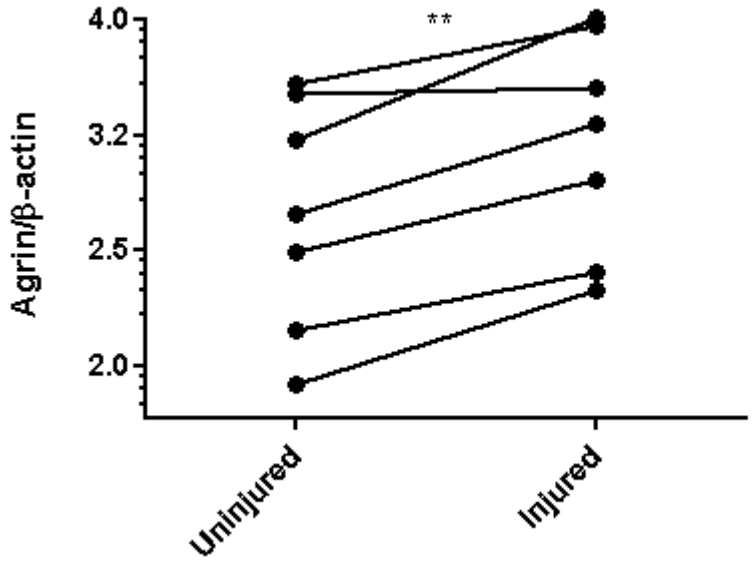


Figure 34. Gene expression analysis of Agrin in human cartilage following injury

Quantitative real-time reverse transcriptase–polymerase chain reaction for Agrin 7 independent pairs of uninjured and injured adult human articular cartilage explants. Results were normalized to the housekeeping gene β-actin. The P value is for the comparison of the rested control versus the injured explants.

At protein level, in both human and murine OA, Agrin was consistently downregulated and undetectable at late time points. It has previously been shown that a number of "repair genes", which are ultimately lost during OA, are initially upregulated at early time points when studied using an *ex-vivo* explant model of injury in human cartilage. These "repair genes" exhibit biphasic behaviour characterized by transient upregulation soon after injury which is ultimately followed by persistent downregulation at longer time points (Dell'Accio, et al., 2008). Therefore it was tested whether this was the case for Agrin using an *ex-vivo* model of cartilage injury.

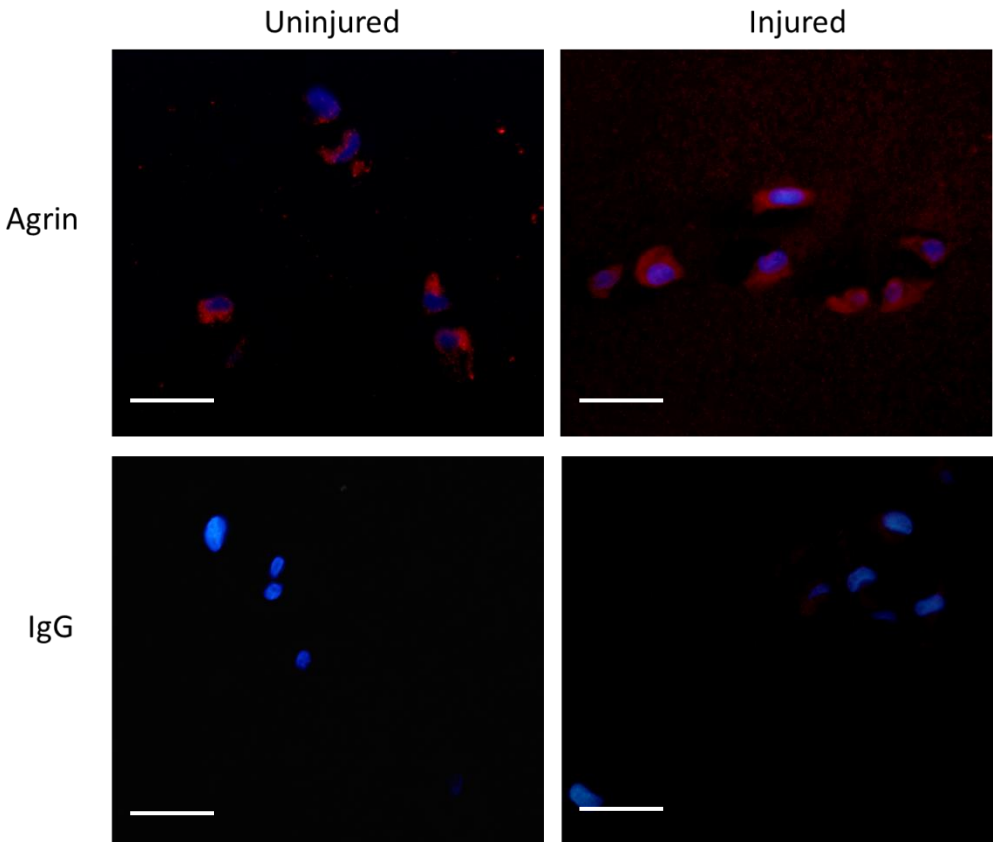


Figure 35. Agrin protein detection in human articular cartilage following injury

Immunofluorescence of Agrin in human articular cartilage 24hrs following *ex-vivo* injury (n=3). Bar, 20µm.

In this model, human cartilage explants were dissected, rested in culture for 7 days to allow the molecular response to injury to subside, and either remained uninjured or were re-injured. The transcriptomic profile obtained the following day reflected those genes which are transiently activated by injury but that reverted to normal or even lower levels within the 7 days of resting (Dell'Accio, et al., 2008). Many of these genes are now known or became known to be involved in cartilage homeostasis and anabolism.

Therefore, to ascertain if also Agrin possesses the same behaviour, these additional unpublished microarray data were utilised. Agrin was modestly but consistently and statistically significantly upregulated in the injured explants at this time point (Figure 34 and Figure 35). To

address the question of whether Agrin is also regulated in early osteoarthritis, samples of murine cartilage was obtained from 2 and 7 days post DMM for qPCR analysis and revealed that Agrin mRNA expression level was unchanged at 2 days post induction of OA by DMM but was upregulated at 7days in the DMM treated group.

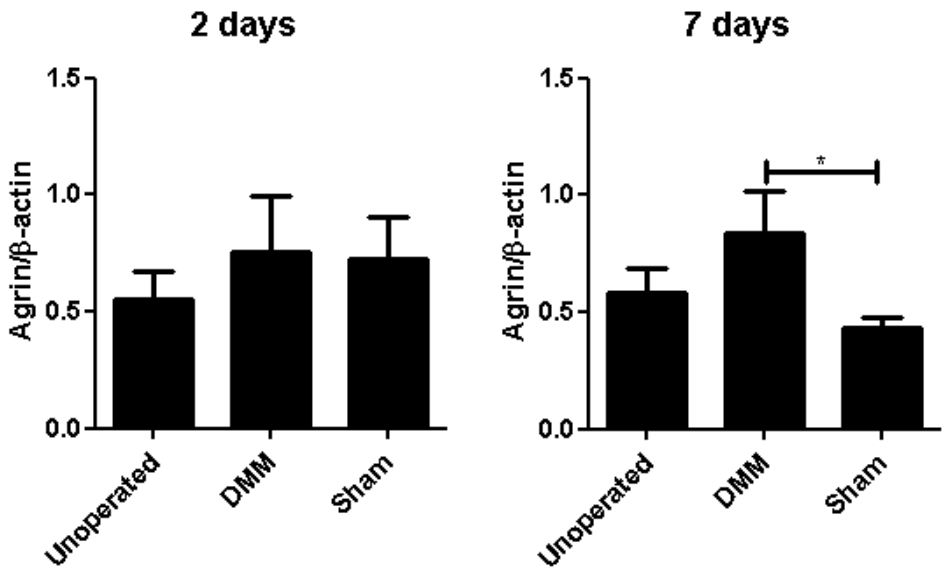


Figure 36. Agrin gene expression 2 and 7 days post DMM surgery

RNA was extracted from the knee joints of 129/Sv mice 2 or 7days post DMM or Sham surgery for gene expression analysis by real time qPCR (n=11).

These data are in contrast with the data at protein level (Figure 33) which suggest reduced Agrin at 7 days. This discrepancy could either be due to the fact that the PCR data are obtained from the entire joint including cartilage and bone, or due to the possibility that in spite of increased transcription (and possibly transduction), at 7 days the proteolysis of Agrin within the diseased joint eventually results in lower detection especially when using an antibody directed to the C-terminus. This hypothesis is tested in Chapter 9.

Discussion

It was discovered that mRNA encoding for Agrin and its receptors LRP4, APP, APLP2, DAG1 and LAMC1 were expressed in human adult articular cartilage raising the question of whether Agrin signalling has a role in cartilage biology. MuSK, which is essential at the neuromuscular junction, was not detected in 2 out of 3 samples.

At the neuromuscular junction, presence of the alternatively spliced inserts y and z results in a 1000 fold higher affinity of Agrin to LRP4 and therefore neuromuscular synapse stability via AChR clustering (Gesemann et al. 1995; Kim et al. 2008). To investigate whether these inserts (y4:KSRK; z8:ELTNEIPA or 11:PETLDRSALFS or 19: ELTNEIPAPETLDRSALFS) were present in chondrocytes, we sequenced across the potential insert sites of Agrin from human articular chondrocyte cDNA. This analysis revealed - as in other non-neural tissues - absence of the alternatively spliced y and z inserts in chondrocytes, where Agrin has a preferred, but not exclusive, affinity for dystroglycan binding compared to the y4, z8 isoform (Gesemann et al. 1998). Due to the absence of these inserts it was expected that cartilage would not express the MuSK or LRP4 as they would be largely redundant, instead we discovered high levels of LRP4 mRNA. The role of the different receptors in Agrin cartilage biology will be investigated further in Chapter 8.

To investigate the protein expression pattern of Agrin in normal joints we used immunofluorescence. Agrin was abundantly expressed in healthy human, bovine and murine articular cartilage. In the mouse, Agrin was detected within the superficial and deep layers of the articular cartilage, within the osteoid in the subchondral bone, and in the menisci. However, in human OA cartilage, Agrin protein was markedly reduced (almost undetectable) in the more severely affected areas (Mankin score >8) compared to the relatively preserved ones (Mankin score <4). In a validated murine model of OA induced by surgical destabilization of the medial meniscus (DMM) (Glasson 2007; Glasson et al. 2005), Agrin protein levels were rapidly

reduced in the articular cartilage as early as 7 days after surgery, reaching undetectable levels after 8 weeks.

Interestingly, at earlier time points of DMM (2 and 7 days) mRNA level of Agrin was increased, these data were complemented by data obtained from an *ex-vivo* injury model of cartilage injury in humans, where mRNA levels were higher at early time points post injury. Therefore it is possible that this initial upregulation is part of the well-known adaptive response that cartilage deploys after injury. The downregulation detected thereafter at protein levels may not be the result is reduced transcription mechanism, but may be due to the proteolytic activities that are abundant in the synovial environment. Indeed it is well documented that MMPs, specifically MMP3, can cleave Agrin (VanSaun, et al., 2003; Vansaun, & Werle, 2000), and this will be discussed further in Chapter 9.

CHAPTER 6: Results section 2

In vitro functional data

Results: In vitro functional data

Rationale:

The regulation of Agrin in the articular cartilage during disease is a novel finding, however, whether the loss of Agrin is a cause or effect of cartilage loss remained to be addressed. Chondrocytes were obtained from patients undergoing knee replacement and therefore, since the endogenous levels of Agrin may play a role in the severity of disease; it was predicted that the use of these samples for gain and loss of function studies could introduce large variability and inconsistencies. To set up a more reliable system for functional studies, chondrocytes obtained from adult cartilage from adult bovine metatarsal joints as primary cells and the human chondrocytic cell line C28/I2 were utilised.

To achieve silencing and overexpression of Agrin, siRNA oligonucleotides and plasmids must be transfected into the recipient cells respectively, and although this has commonly been carried out using the C28/I2 cell line, it is less commonly carried out using bovine primary chondrocytes. As it is known some transfection reagents can be detrimental to cell survival and metabolism, initial experiments investigating the direct effect of JetPrime, which has been previously validated in primary chondrocytes (Sherwood, et al., 2014), were carried out on bovine primary chondrocytes in micromass culture. Agrin was silenced using commercial Stealth siRNA in C28/I2 cells, and to validate the findings in primary cells, in-house created siRNA oligo's were used in bovine primary chondrocytes. The resultant Agrin dose-dependent loss of micromass ECM accumulation and the downregulation of key chondrocyte genes introduced the hypothesis that Agrin may also be able to enhance these parameters. Agrin was overexpressed in C28/I2 cells, bovine primary chondrocytes and human primary chondrocytes and cultured in micromass. This series of experiments revealed that Agrin is potently able to enhance ECM production as well as upregulating key chondrocyte genes.

Experimental aims

Determine:

- a suitable cell type for functional experiments
- the effect of transfection reagents on primary bovine chondrocytes
- the effect of Agrin knockdown by siRNA on chondrocytes
- if exogenous overexpression of Agrin can enhance chondrocyte extracellular matrix production

Chondrocyte and cell line source validation

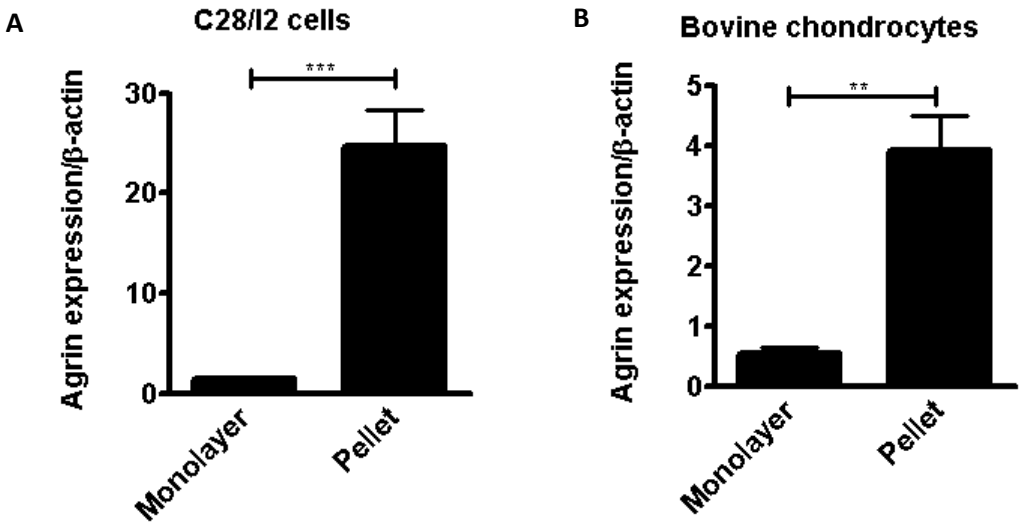


Figure 37. Gene expression of Agrin in monolayer and pellet culture in bovine chondrocytes and C28/I2 cells

Gene expression levels of Agrin in C28/I2 and bovine chondrocytes cultured in monolayer for 5 days or pellet culture for 14 days (n=4)

The expression of Agrin was confirmed in C28/I2 cells and bovine chondrocytes in both monolayer and pellet culture at mRNA level and by protein detection (Figure 37 and Figure 38).

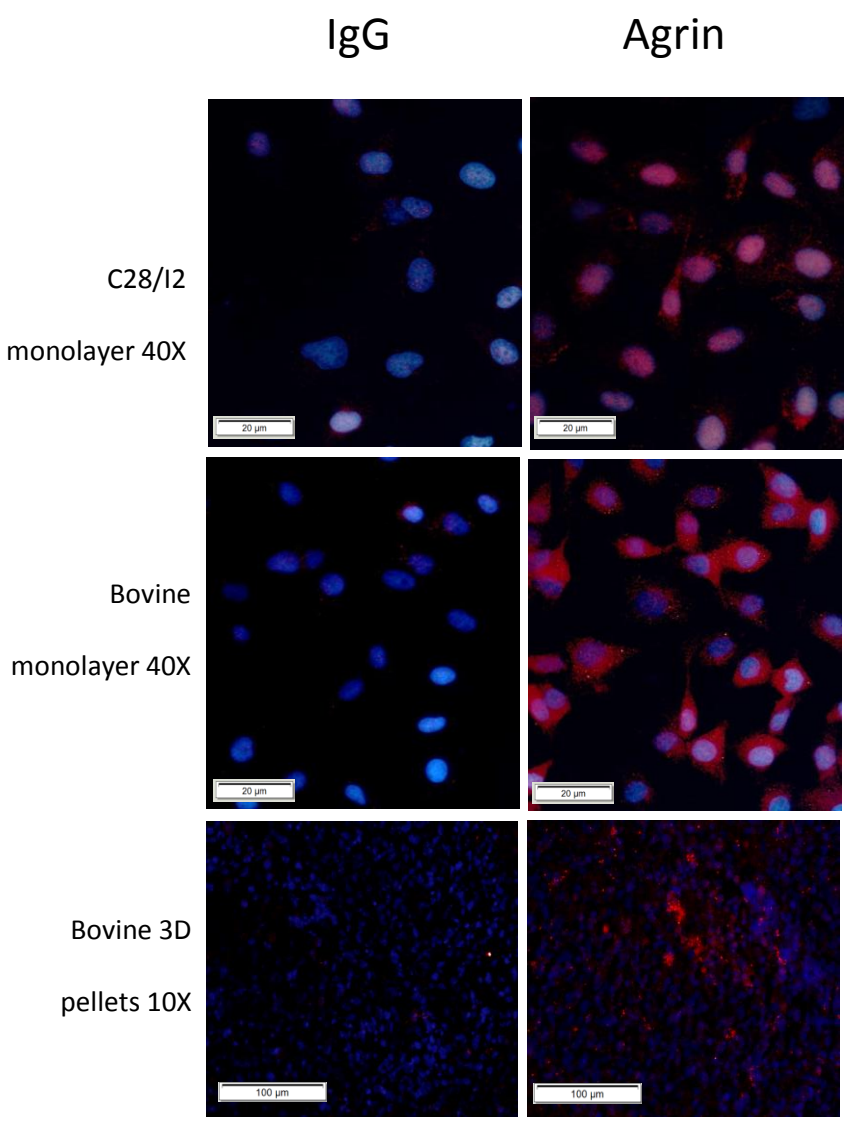


Figure 38. Agrin protein in primary bovine chondrocytes and C28/I2 cells

Immunofluorescence staining of Agrin protein in isolated non-permeabilised primary bovine chondrocytes and C28/I2 cells in monolayer (top) and pellet culture (bottom); Blue DAPI staining; IgG as negative control (n=4).

Fluorescent immunohistochemical analysis C28/I2 cells and freshly isolated primary bovine chondrocytes shows that Agrin expression at protein levels is retained in the pericellular matrix of the articular chondrocytes following isolation from the ECM. These data suggest that these cell types are suitable for experiments to study the role of Agrin by loss of function.

Validation of Agrin transfection in articular chondrocytes

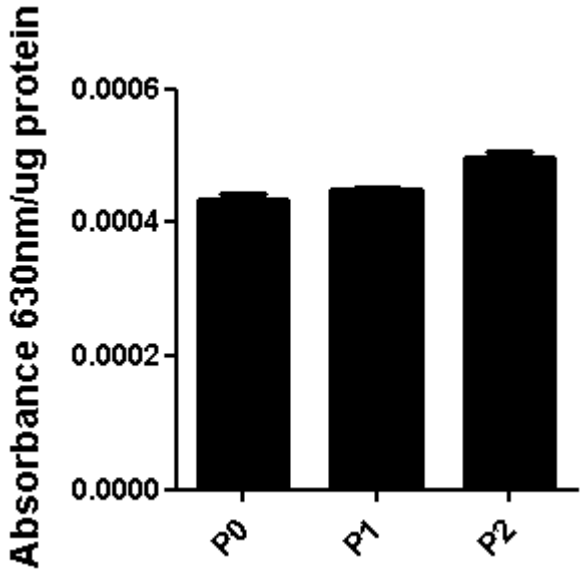


Figure 39. GAG analysis of the effect of passaging primary bovine chondrocytes

Passaged primary bovine chondrocytes (P0-P2) were cultured in micromass for 5days and stained with Alcian blue. Alcian blue was quantified spectrophotometrically following guanidine extraction and normalised for protein content (n=4).

As with many primary cells, the effects of passaging chondrocytes and exposure to transfection reagents can induce dedifferentiation (Lin, et al., 2008). This process is very rapid in human chondrocytes (Benya, PD, & Shaffer, 1982; Dell'Accio, et al., 2001). As *in vitro* culture is required for transfection, the maximum number of passages possible - before any significant changes in chondrocyte behaviour or substantial loss of differentiation are exhibited - was determined by Alcian blue staining of micromass culture of bovine primary chondrocytes. Figure 39 shows the levels of GAG content is unchanged in primary bovine chondrocyte micromasses at P0, P1 and P2, when normalised for protein content. These data demonstrated that bovine chondrocytes can be used up to at least 2 full passages.

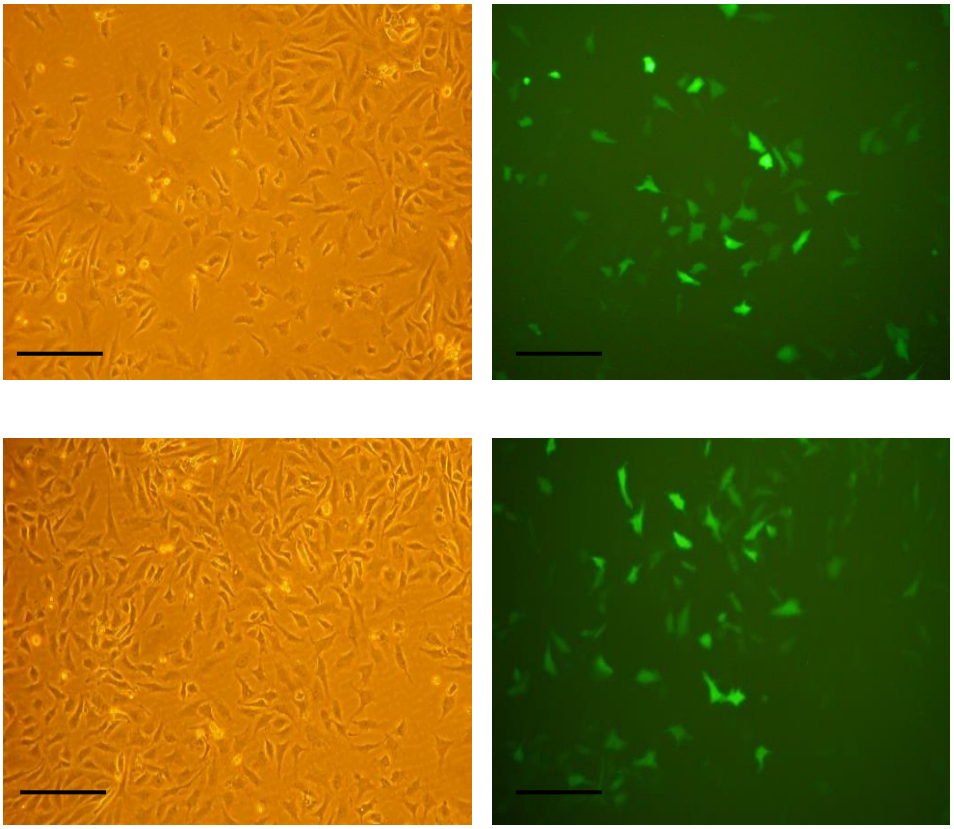


Figure 40. Transfection efficiency of primary bovine chondrocytes

Primary bovine chondrocytes were transfected with GFP using JetPrime and cultured for 24 or 48hrs in monolayer. Representative images in bright field (left) and fluorescence (right) are shown. Bar, 50µm.

Transfection of primary bovine chondrocytes with JetPrime produced levels of transfection efficiency around 15-30% by eye (Figure 40), at both 24hrs (top) and 48hrs (bottom) respectively, following transfection with GFP using an optimised JetPrime protocol. Even transfection levels this low may be sufficient as Agrin is a secreted protein, even non-transfected cells will be exposed to Agrin.

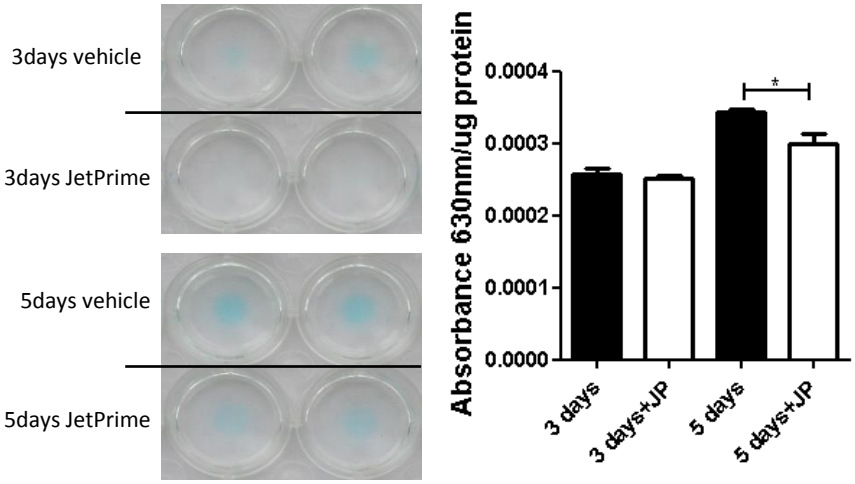


Figure 41. The effect of JetPrime exposure on primary bovine chondrocytes

Primary bovine chondrocytes were cultured in the presence or absence of JetPrime (no plasmid) for 3 or 5 days in micromass culture and stained with Alcian blue. Alcian blue was quantified spectrophotometrically following guanidine extraction and normalised for protein content (n=4).

To assess whether the transfection procedure itself influenced GAG accumulation in primary bovine chondrocytes, preliminary experiments were carried out where micromass cultures were exposed to JetPrime for 3 or 5 days in the presence or absence of this transfection reagent. The results show that the JetPrime reagent has little or no effect on the chondrocytes ability to produce and accumulate GAG at 3 days and 5 days (Figure 41) following exposure to the transfection reagent. The culture medium containing JetPrime was removed 48hrs post transfection and replaced with fresh complete culture medium for the remainder of the experiment.

Agrin knockdown

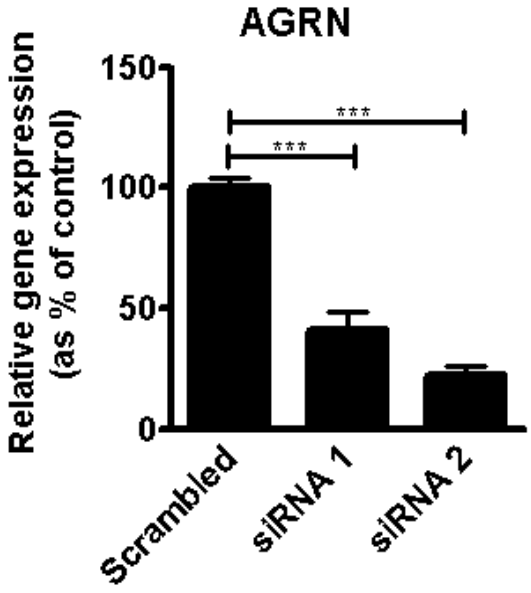


Figure 42. Agrin knockdown using siRNA in C28/I2 cells

C28/I2 cells were transfected with 2 Agrin siRNA's or a scrambled siRNA as control and cultured in micromass for 5days. Real time qPCR was used to measure Agrin gene expression levels (n=3).

To determine if Agrin is required for chondrocyte homeostasis, Agrin was knocked down in the human costal chondrocyte cell line C28/I2 cells using two commercial siRNA's. Transfection with siRNA1 and siRNA2 resulted in the silencing of Agrin at mRNA level of 59% and 73% respectively (Figure 42). The fact that the two siRNA had differing potencies allowed dose-dependent knockdown experiments to be performed.

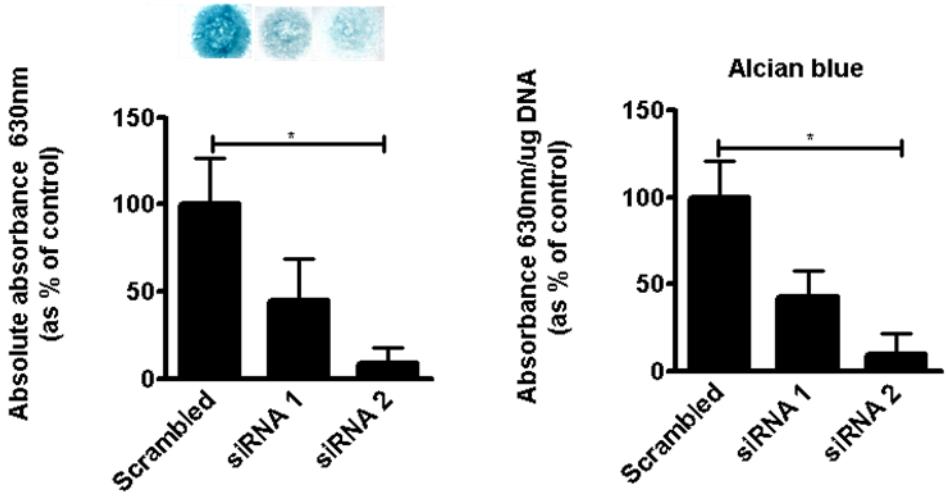


Figure 43. GAG analysis of Agrin knockdown in C28/I2 cells

C28/I2 cells were transfected with 1 of 2 Agrin siRNA's or a scrambled siRNA as control and cultured in micromass for 5days and stained overnight with Alcian blue. Alcian blue was quantified spectrophotometrically following guanidine extraction and normalised for DNA content (n=3).

This dose-dependent knockdown of Agrin in C28/I2 micromass cultures resulted in the corresponding decrease in sulphated proteoglycan content in the culture ECM (Figure 43). This was true at absolute levels but also when normalised for DNA content implying this is not due to decreased cell number. However, it is unclear if this decreased ECM accumulation is due to reduced synthesis or enhanced degradation and it is likely both mechanisms play a role as will become apparent in subsequent chapters.

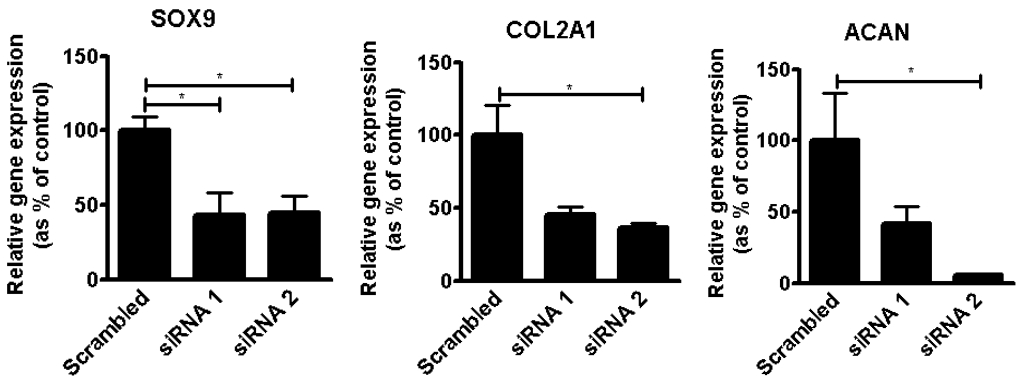


Figure 44. Gene expression analysis of Agrin knockdown in C28/I2 cells

C28/I2 cells were transfected with 2 Agrin siRNA's or a scrambled siRNA as control and cultured in micromass for 5days. RNA was extracted and real time qPCR analysis of SOX9, ACAN and COL2A1 expression mRNA levels was carried out (n=3).

To investigate if the decreased GAG content in the micromasses that had undergone Agrin silencing was associated with decreased expression of chondrocyte differentiation markers, qPCR was carried out on these micromass cultures. Transfection with both siRNA's resulted in the decreased expression of SOX9, COL2A1 and ACAN when compared to transfection with the scrambled oligonucleotide (Figure 44). Interestingly, although the downregulation of SOX9 was not dose-dependent at this time point, the downregulation of its transcriptional targets ACAN and COL2A1 was.

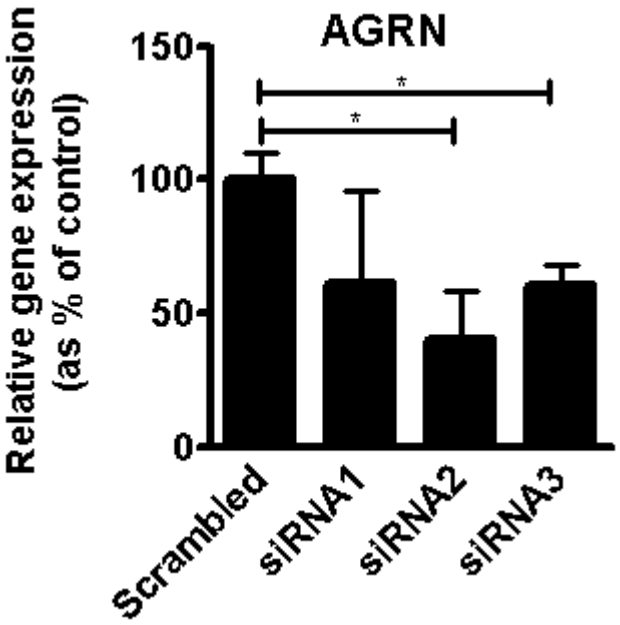


Figure 45. Gene expression analysis of Agrin knockdown in primary bovine chondrocytes

Primary bovine chondrocytes were transfected with 3 in-house designed siRNA or scrambled control (made using Ambion kit (Invitrogen)) and cultured in micromass for 5days. Agrin gene expression levels were measured using real time qPCR following RNA extraction (n=3).

The C28/I2 cell line is derived from costal chondrocytes and therefore may not truthfully represent the biology of primary articular chondrocytes. To validate the results in healthy primary chondrocytes of articular cartilage origin, in-house designed bovine specific siRNA oligonucleotides were created which successfully silenced Agrin by siRNA's 2 and 3 by 50 and 40% respectively (Figure 45).

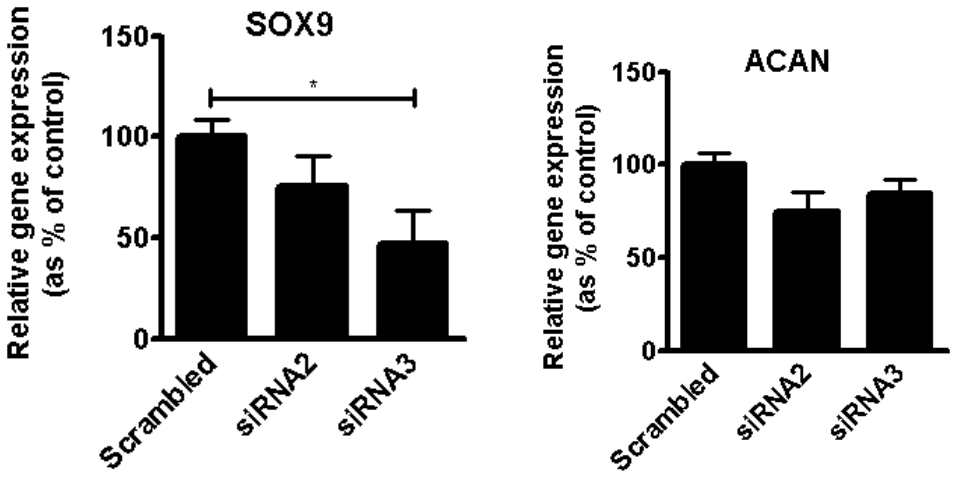


Figure 46. Gene expression analysis of Agrin knockdown in primary bovine chondrocytes

Bovine primary chondrocytes were transfected with 2 Agrin siRNA's or scrambled control and cultured in micromass for 5 days. RNA was extracted and gene expression analysis of SOX9 and ACAN was carried out using real time qPCR (n=3).

As was seen in the C28/I2 cell line, Agrin silencing also resulted in reduced SOX9 expression in primary bovine chondrocytes (Figure 46 – left). However, at this time-point, ACAN expression remained only slightly reduced which is likely due to the time point of 5 days chosen (right). This not only validated that the effect seen in the costal chondrocyte cell line is comparable to that seen in primary articular chondrocytes, but also demonstrated that these biological functions are conserved across the species.

Agrin overexpression

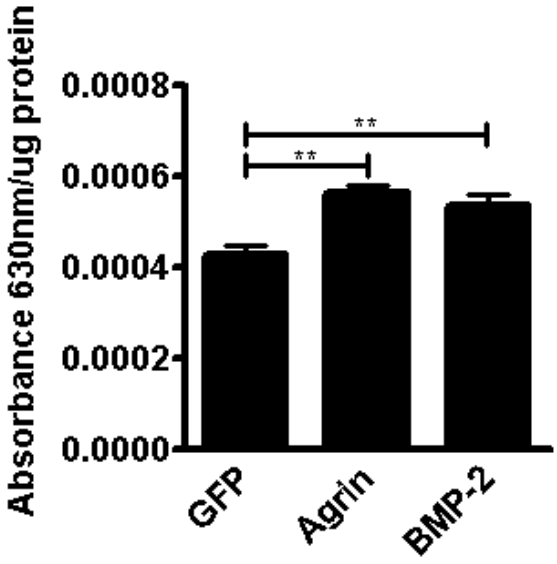


Figure 47. GAG analysis of Agrin overexpression in adult human articular chondrocytes

Adult human articular chondrocytes were transfected with GFP, Agrin or BMP2 and cultured in micromass, in the presence of 10% FBS, for 5days and stained with Alcian blue. Alcian blue was quantified spectrophotometrically following guanidine extraction and normalised for protein content (n=4).

As shown by the Agrin knockdown experiments, Agrin is required for the regulation of key articular chondrocytic genes. To investigate if exogenous Agrin was sufficient to enhance chondrocyte differentiation and GAG production overexpression experiments were carried out.

Two plasmids encoding full-length (FL-Agrin) or C-terminal (C-Agrin) mammalian non-neuronal Agrin (kind gifts from Dr Michael Ferns) were tested for their ability to enhance GAG production (results shown in Figure 88 in the Appendix). Micromass experiments showed FL-Agrin increased GAG production more potently than C-Agrin, and thus FL-Agrin was used in subsequent experiments. Figure 47 shows the results of transfection of primary human

articular chondrocytes transfected with FL-Agrin and cultured in micromass. This resulted in increased ECM production on par with that produced by BMP2 (kind gift from Dr Gerard Gross). In subsequent experiments, for reproducibility, C28/I2 cells and primary bovine chondrocytes were utilised to reduce the variability between patient samples and degree of osteoarthritis.

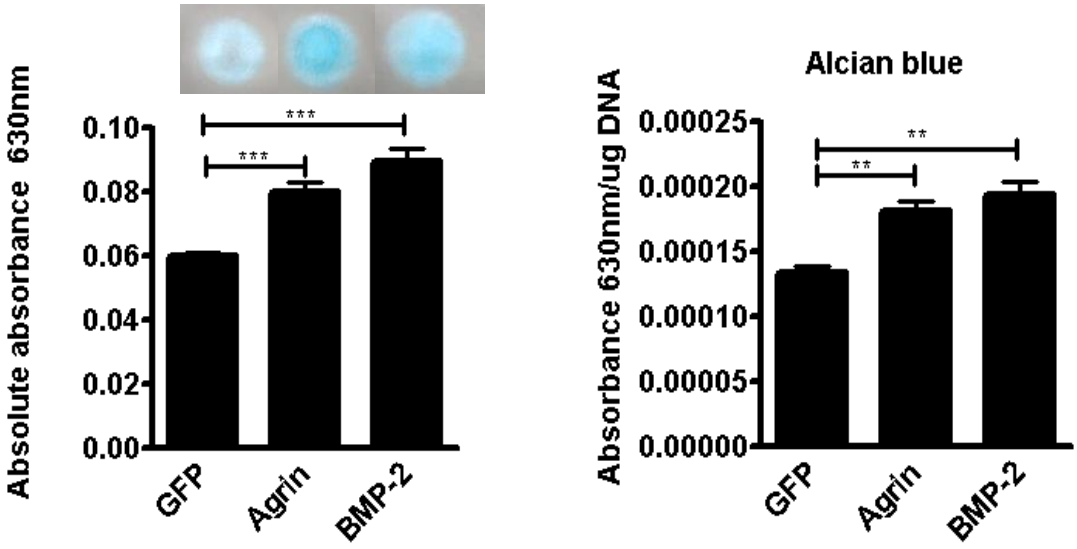


Figure 48. GAG content following Agrin overexpression in C28/I2 cells

C28/I2 cells were transfected with GFP, Agrin or BMP and cultured in micromass for 4days before overnight staining with Alcian blue. Alcian blue was quantified spectrophotometrically following guanidine extraction and normalised for DNA content (n=4).

The results in primary human chondrocytes were replicated in the C28/I2 cell line (Figure 48), showing both a total increase in GAG content (left) and also when normalised for DNA content per micromass (right).

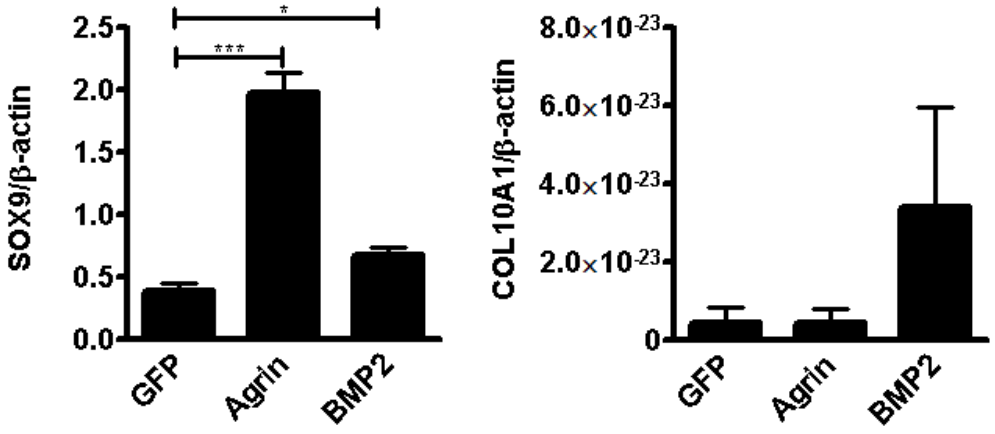


Figure 49. Gene expression analysis following Agrin overexpression in C28/I2 cells

C28/I2 cells were transfected with GFP, Agrin or BMP2 and cultured in micromass for 4days. RNA was extracted and gene expression analysis of SOX9 and COLX was carried out using real time qPCR (n=4).

Real time qPCR analysis of the C28/I2 cell micromasses showed a significant four-fold increase in SOX9 expression while COL10A1 remained stable. Unfortunately, due to the nature of the C28/I2 cell line, they rarely express COL2A1 or ACAN unless differentiated in micromass in the absence of serum for at least 4-5 days. As these conditions were not well suited for the transient expression system that was achieved using plasmid transfection, primary bovine chondrocytes were used for further overexpression experiments.

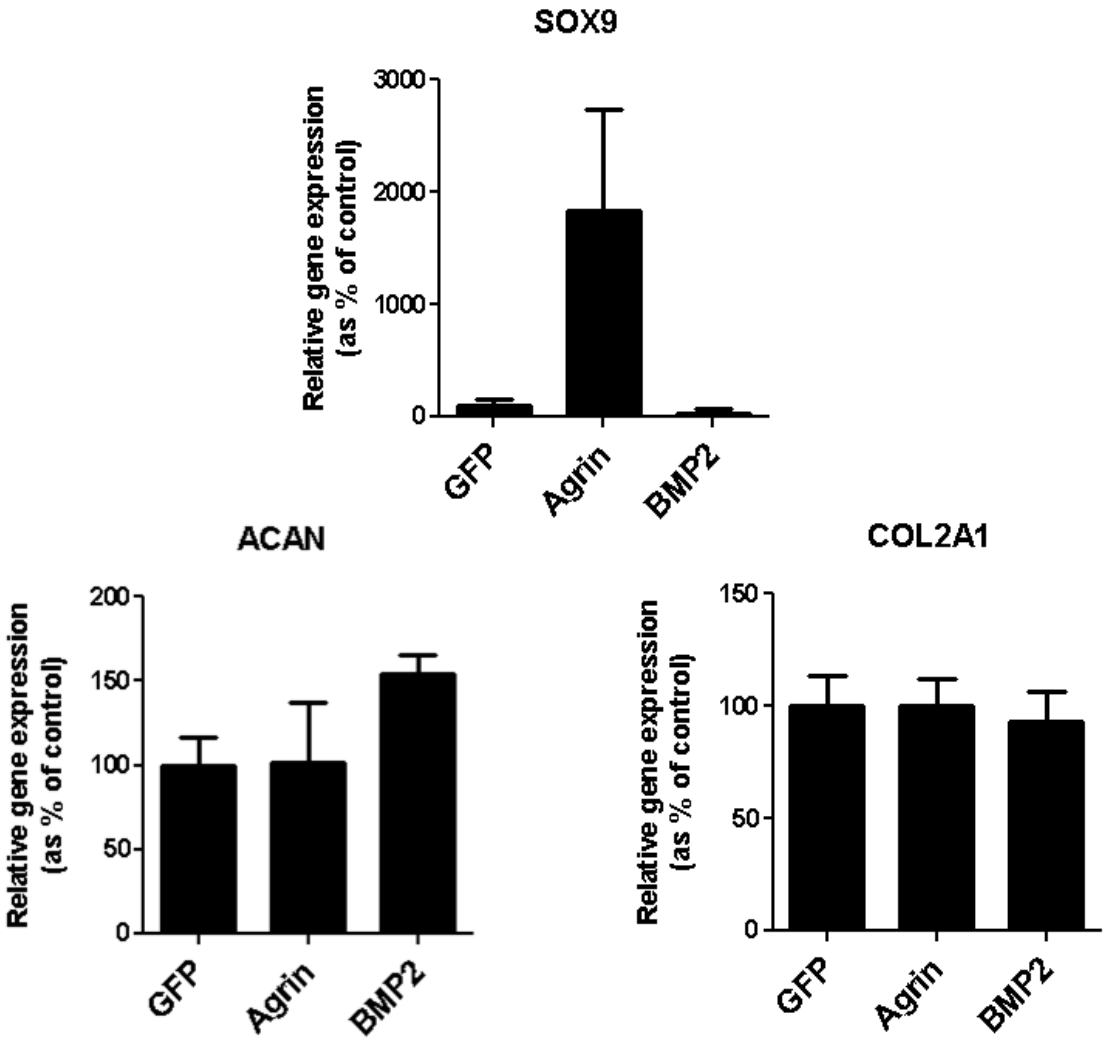


Figure 50. Gene expression analysis of Agrin overexpression in bovine chondrocytes – 3 days

Primary bovine chondrocytes were transfected with GFP, Agrin or BMP2 and cultured in micromass for 3 days. Gene expression analysis of SOX9, COL2A and ACAN was measured by real time qPCR (n=4).

Real time qPCR analysis of primary bovine chondrocytes transfected with full length Agrin and cultured in micromass for 3 days showed an increased expression on SOX9, although this was not significant. At this time point no effect was seen on COL2A1 and ACAN.

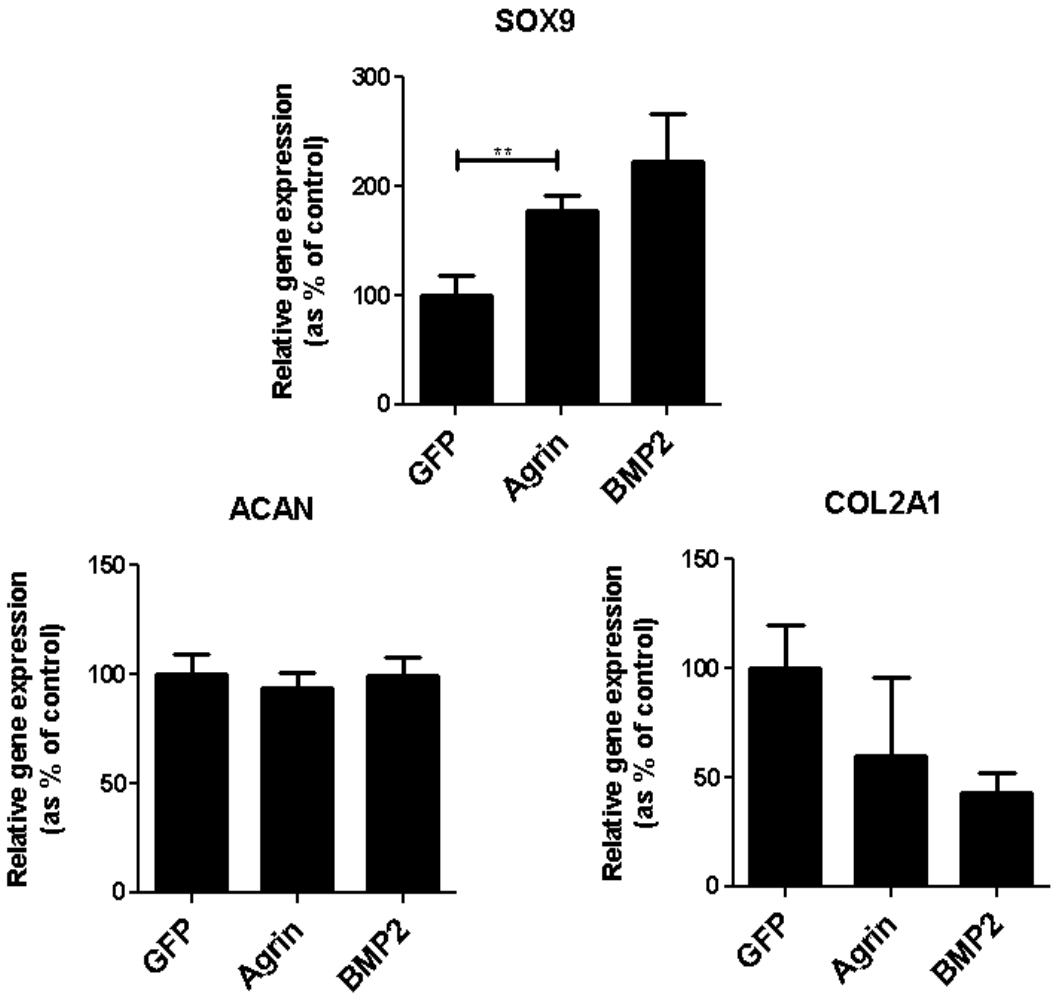


Figure 51. Gene expression analysis of Agrin overexpression in bovine chondrocytes – 5 days

Primary bovine chondrocytes were transfected with GFP, Agrin or BMP2 and cultured in micromass for 5 days. Gene expression analysis of SOX9, COL2A and ACAN was measured by real time qPCR (n=4).

Culturing primary bovine chondrocytes with Agrin for 5 days in micromass did induce a significant upregulation of SOX9, as measured by real-time qPCR. While changes in the mRNA expression levels of COL2A1 and ACAN were not observed.

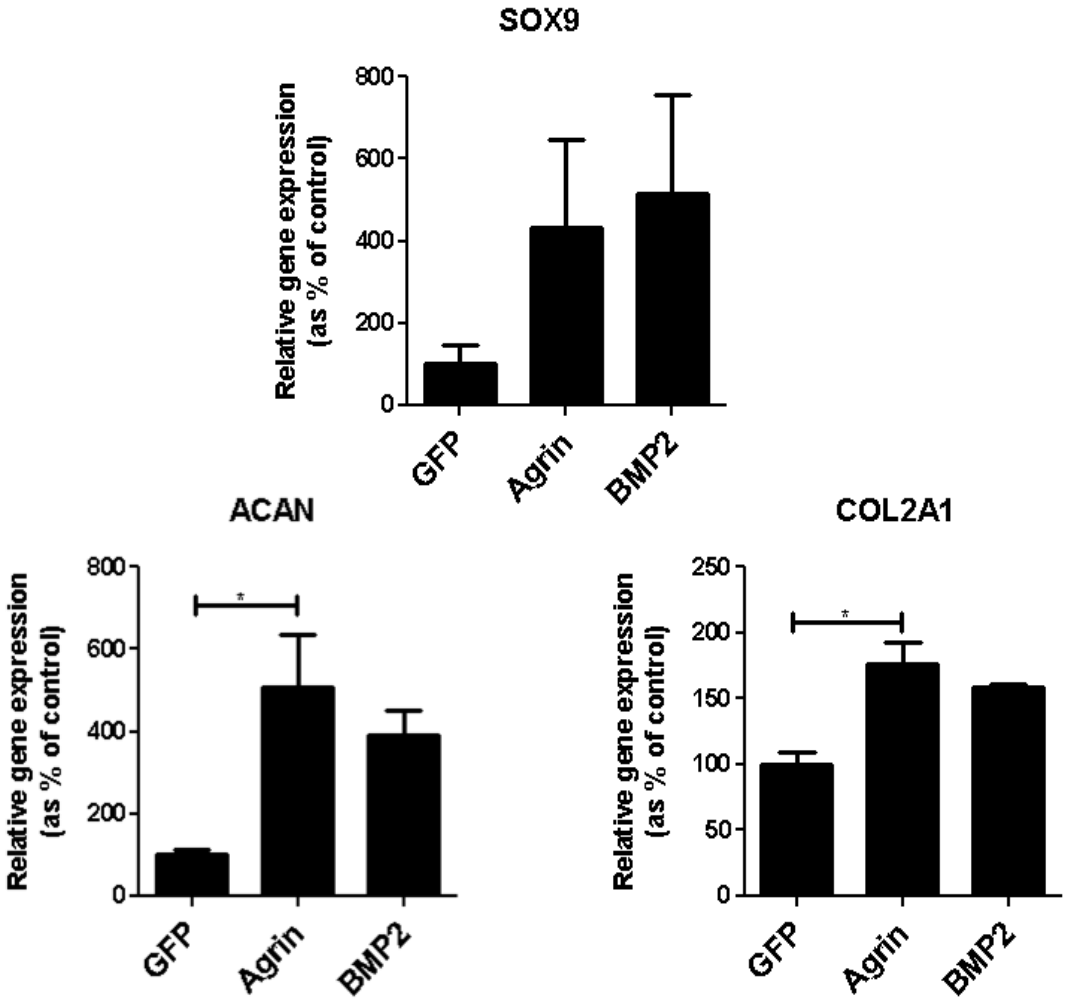


Figure 52. Gene expression analysis of Agrin overexpression in bovine chondrocytes – 7 days

Primary bovine chondrocytes were transfected with GFP, Agrin or BMP2 and cultured in micromass for 7 days. Gene expression analysis of SOX9, COL2A and ACAN was measured by real time qPCR (n=4).

Increasing the culture time further, to 7 days, resulted in a decrease in SOX9 expression levels. This is likely due to the short-lived transcription of the plasmid. However, at this time point a significant upregulation of the mRNA expression levels of COL2A1 and ACAN were observed (Figure 52). In summary, accumulation of Agrin in the pericellular matrix resulted in a slow upregulation of SOX9 which became significant only after 5 days, and which, in turn, resulted in upregulation of COL2A1 and ACAN, direct transcriptional targets of SOX9, after 7 days.

Discussion

The loss of Agrin in OA cartilage prompted us to investigate whether Agrin could have a function in chondrocyte differentiation and ECM matrix formation. To investigate whether Agrin has a cell-autonomous function in chondrocytes a loss of function approach was applied. Silencing Agrin in C28/I2 cells using either of two independent siRNA oligonucleotide pairs (siRNA-1 and siRNA-2), resulted in a reduction of Agrin mRNA by 59% and 78% respectively, allowing dose-response experiments. Agrin knockdown resulted in a dose-dependent decrease of absolute sulphated glycosaminoglycan (GAG) content in C28/I2 cells micromass cultures as evaluated by Alcian blue staining (De Bari, Dell'Accio, and Luyten 2001). Total DNA content was not changed thereby suggesting that such difference did not result from a decreased cell number.

Agrin silencing also resulted in reduced expression of the cartilage transcription factor SOX9 mRNA and dose-dependent downregulation of its transcriptional targets COL2A1 and ACAN mRNA.

Conversely, overexpression of full length Agrin (y0, z0) in primary bovine chondrocytes resulted in significantly increased sulphated glycosaminoglycan (GAG) content compared to GFP transfected controls, both as total amount per micromass and when normalised for DNA content. Moreover, after 5 days culture, SOX9 mRNA was significantly upregulated and 2 days later so were its direct transcriptional targets COL2A1 and ACAN (Bi et al. 1999).

These data also demonstrate that Agrin function is cell autonomous as it takes place *in vitro*, in the absence of potential exogenous sources of Agrin (e.g. nerves). It is also noteworthy that, although in a quite unusually delayed fashion, Agrin could further enhance the differentiation and ECM production of fully mature chondrocytes and even in the presence of FBS. These conditions are very challenging even for BMPs (Wozney, & Seeherman, 2004).

CHAPTER 7: Results section 3

In vivo ectopic cartilage formation assay

Results: In vivo ectopic cartilage formation assay

Rationale:

The primary aim of this chapter was to test whether the capacity of Agrin to support extracellular matrix production was limited to *in vitro* settings or if it was also detectable *in vivo*. To this end we chose a well-established adoptive transfer model in which phenotypically stable chondrocytes implanted in suspension in the muscle of nude mice form hyaline-like cartilage implants that are resistant to vascular invasion and endochondral bone formation for at least 6 months (Dell'Accio, et al., 2001). This system was chosen for the following reasons:

-It is clinically relevant. This assay represented the technology platform for the optimization and efficacy quality control of a cell product for autologous chondrocyte implantation which succeeded in a multicentric randomized clinical trial vs microfracture. Molecular markers associated with this assay correlated with clinical outcomes at 3 years (Saris, et al., 2009).

The main features of this assay include:

- It allows the use of human chondrocytes (when available) and human molecules.

- The outcome can be quantified and it is amenable to biochemical analysis.

- It is a very stringent assay and can distinguish between the formation of hyaline-like cartilage and epiphyseal-like cartilage (Dell'Accio, unpublished).

One problem associated with this assay was the modality of Agrin delivery to the chondrocytes. Agrin is a very large and highly glycosylated molecule that is very difficult to produce as recombinant molecule. In fact early experiments using recombinant Agrin from R&D Systems failed to reproduce the results obtained with the plasmids (data not shown). Other

collaborators have also confirmed that the R&D Systems recombinant molecule failed to work in other assays in their laboratories. Transfection or transduction of chondrocytes would also be problematic. Indeed, chondrocytes lose their capacity to form cartilage in this assay within a few passages (Dell'Accio, et al., 2001), thereby making both these technologies impractical. Adenoviral transduction would probably have represented an alternative, but it has two major limiting factors: first, it induces a great deal of inflammation and cellular stress, both likely to compromise the cartilage-forming ability of chondrocytes, but especially the overexpression would have been way too short to consistently deliver Agrin throughout the minimum duration of the assay (14 days). Therefore a delivery system based on the co-implantation of chondrocytes together with a growth-arrested cell line transfected with the Agrin plasmid was implemented. COS7 cells produce low levels of Agrin endogenously, and therefore GFP transfected cells were an excellent control; treatment with 7.5 mg/ml Mitomycin C achieved not only excellent growth arrest necessary to prevent tumour formation, but also stabilized the expression of Agrin because the plasmid was not lost due to cell division. This system has been used successfully in the past with human and porcine chondrocytes to test the effect of WNT-3A and it is therefore a well-optimized system in our laboratory.

The concept is to transfect a cell line which allows a high efficiency transcription of the plasmid of choice, and growth-arrest them. This enables the non-proliferative, plasmid-overexpressing cell line to be utilised as delivery system to any surrounding cells.

Experimental aims

Determine:

- how to potently and persistently expose Agrin to chondrocytes
- if exogenous Agrin can induce chondrocyte homeostasis long-term *in vitro*
- if exogenous Agrin can enhance stable cartilage formation *in vivo*

Generation of growth-arrested Agrin-overexpressing cells

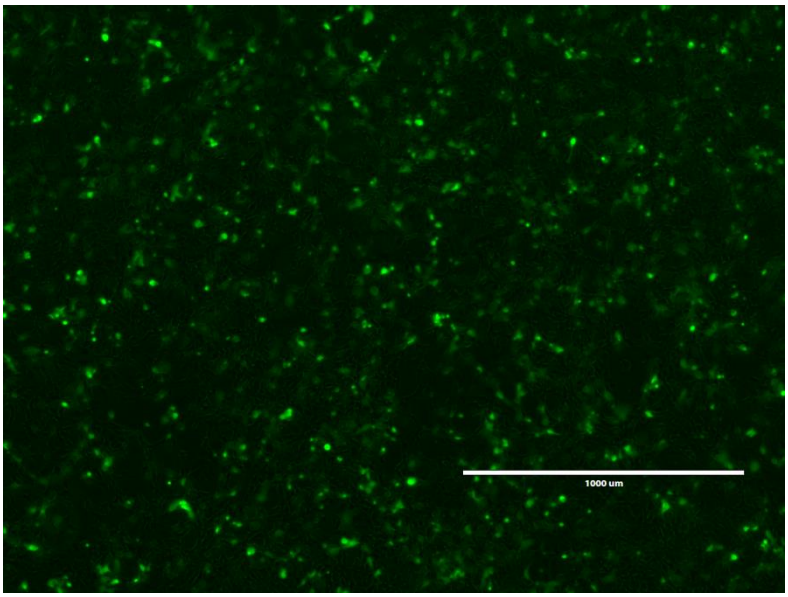


Figure 53. COS7 cells transfected with GFP

COS7 cells plated in monolayer were transfected with GFP using JetPrime and the efficiency checked after 24hrs in culture.

The first step in this series of experiments was the selection of a suitable cell line to deliver Agrin. COS7 cells were selected as these are mammalian cells that yield high amounts of proteins following transfection. In fact, together with CHO cells, COS7 cells are amongst the most used mammalian cells routinely used for recombinant protein production.

To determine the transfection efficiency, COS7 cells were transfected with a GFP plasmid in monolayer using JetPrime. Figure 53 shows the COS7 cell line transfected with GFP after 24hrs. Compared to the level of transfection efficiency in primary chondrocytes (Figure 40), the number of positive cells was greatly increased (to around 99%).

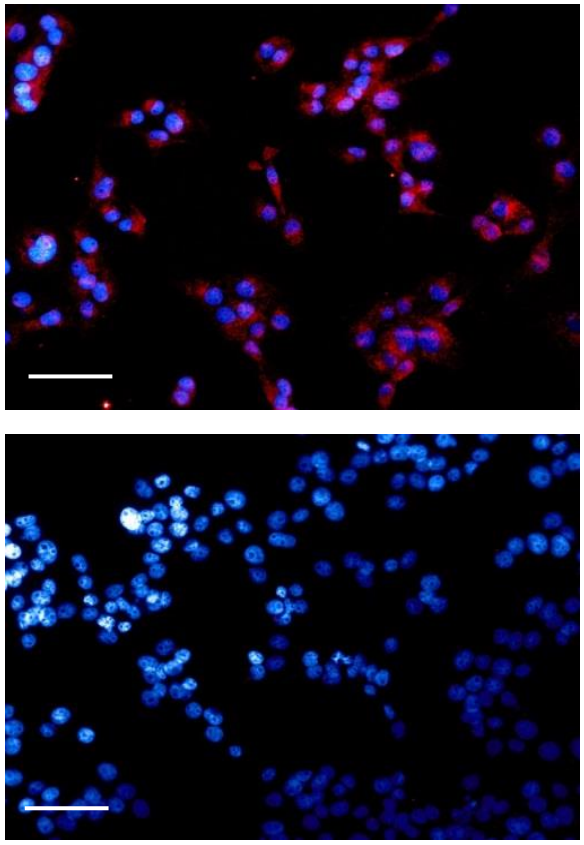


Figure 54. COS7 cells transfected with Agrin

COS7 cells were plated in monolayer on multi-well glass slides and transfected with FL-Agrin. After 48hrs in culture the cells were stained for Agrin using immunofluorescence. Bar, 50µm.

Immunofluorescence staining for Agrin in Agrin-overexpressing COS7 cells 48hrs after transfection with demonstrated that these cells are able to produce the full-length Agrin protein encoded by the plasmid (Figure 54).

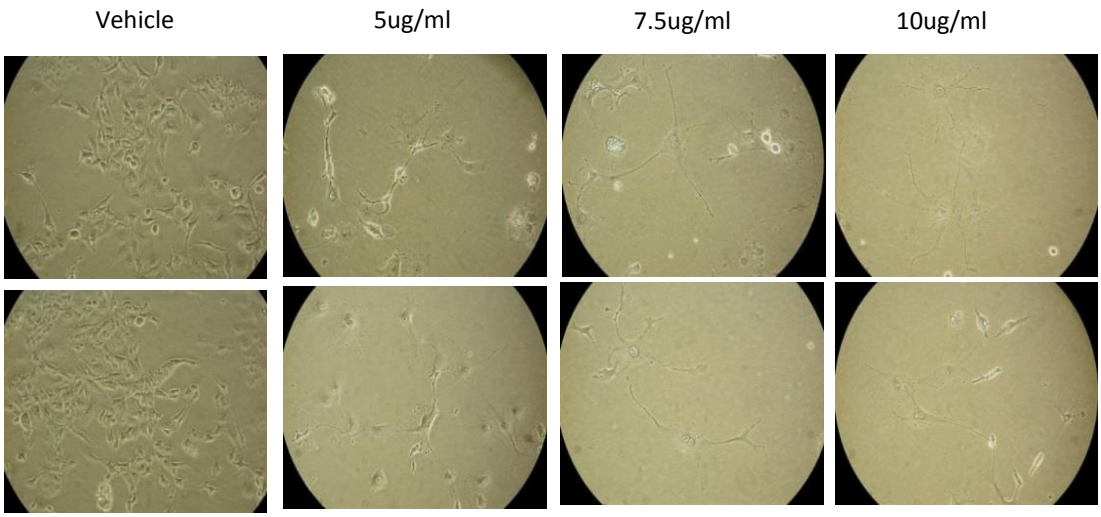


Figure 55. COS7 cells treated with Mitomycin **C**

COS7 cells were plated in monolayer and allowed to attach for 24hrs. Cells were treated with vehicle, 5ug/mg, 7.5ug/ml or 10ug/ml Mitomycin C. Cell proliferation was assessed by eye after 24hrs.

Before COS7 cells could be used as an *in vivo* delivery system, it was chosen to growth arrest them while still preserving their capacity to produce Agrin for at least 2 weeks (the duration of the cartilage formation assay in nude mice). This was necessary for two reasons:

- to prevent that COS7 cells from forming a tumour once vivo and completely taking over chondrocytes

- to prevent proliferation which would ensure the plasmid would not be progressively diluted, with consequent loss of transgene expression.

Therefore, to stop proliferation while preserving optimal Agrin expression, Mitomycin C was titrated to determine the minimum concentration of Mitomycin C needed to growth arrest the COS7 cells. Figure 55 shows COS7 cells treated with vehicle or 5, 7.5 or 10µg/ml Mitomycin for 24hrs. The intermediate dose of 7.5µg/ml was chosen as this did not permit proliferation, which 5µg/ml did, nor did it induce excessive cell death, as was seen using the 10µg/ml dose when monitored for an additional 7days (data not shown).

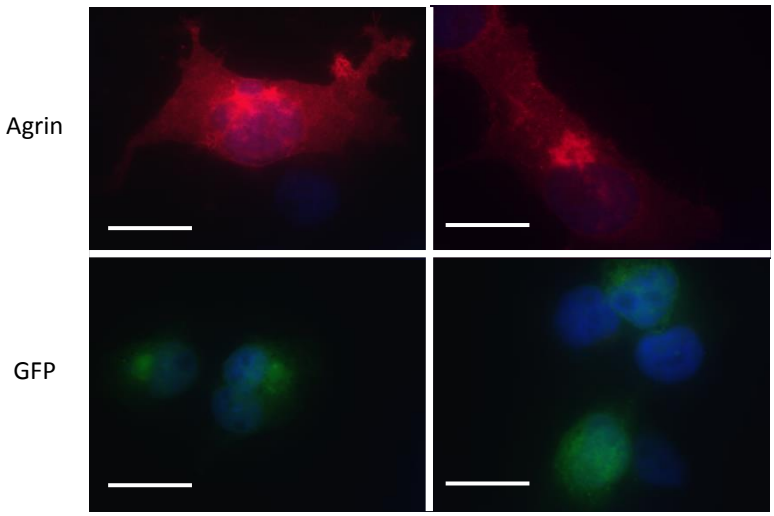


Figure 56. Immunofluorescence of growth arrested COS7 cells transfected with Agrin or GFP

COS7 cells were transfected with Agrin or GFP and growth arrested with Mitomycin C after 24hrs. Agrin protein production was detected by immunofluorescence 3 weeks post transfection, GFP was detected by direct fluorescence microscopy. Bar, 5µm.

COS7 cells transfected with Agrin or GFP and treated with Mitomycin C were able to persistently and potently express the respective protein for up to 3 weeks post treatment (Figure 56). The COS7 cells remained non-proliferative and cell death was limited to around 5% at this time point.

Method validation in vitro

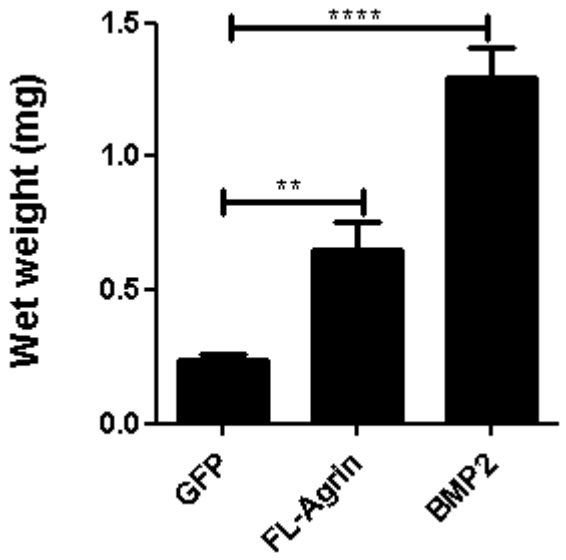


Figure 57. Pellet culture of primary bovine chondrocytes exposed to Agrin, BMP2 or GFP

Primary bovine chondrocytes exposed to Mitomycin C treated COS7 cells overexpressing GFP, Agrin or BMP2 were weighed following 21days in pellet culture (n=4).

Having optimized transfection and Mitomycin C treatment prior to beginning the *in vivo* experiment, confirmation that the Agrin-transfected Mitomycin C treated COS7 cells were capable to enhance ECM formation in co-cultured chondrocytes at least *in vitro* was obtained. To this end, 3D pellet cultures with primary bovine chondrocytes were mixed with COS7 cells (ratio 10:1) overexpressing Agrin, BMP2 or GFP. GFP-transfected COS7 cells were used as control and BMP2 was used as a positive control. The pellets were cultured for 3 weeks, which is one week longer than the length of time required for the *in vivo* assay in nude mice. Comparison of the wet weight of the pellets showed that the chondrocytes exposed to Agrin and BMP2 were able to significantly increase the wet weight of the pellets (Figure 57).

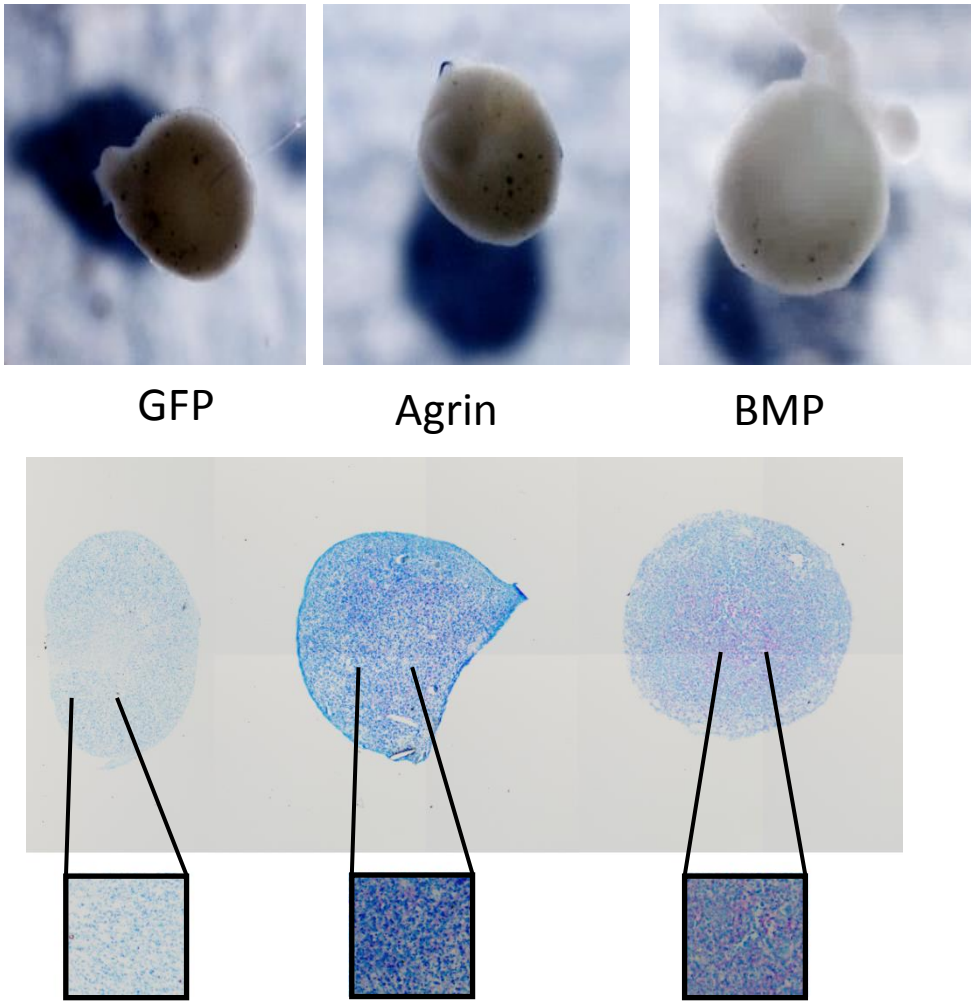


Figure 58. Toluidine blue staining of primary bovine pellet cultures

Primary bovine chondrocytes exposed to Mitomycin C treated COS7 cells overexpressing GFP, Agrin or BMP-2 were sectioned and stained with toluidine blue following 21days in pellet culture.

Histochemical analysis using toluidine blue staining confirmed accumulation of highly sulphated GAGs through the presence of metachromatic staining in the pellets containing Agrin-transfected COS7 cells and those exposed to BMP2 (Figure 58).

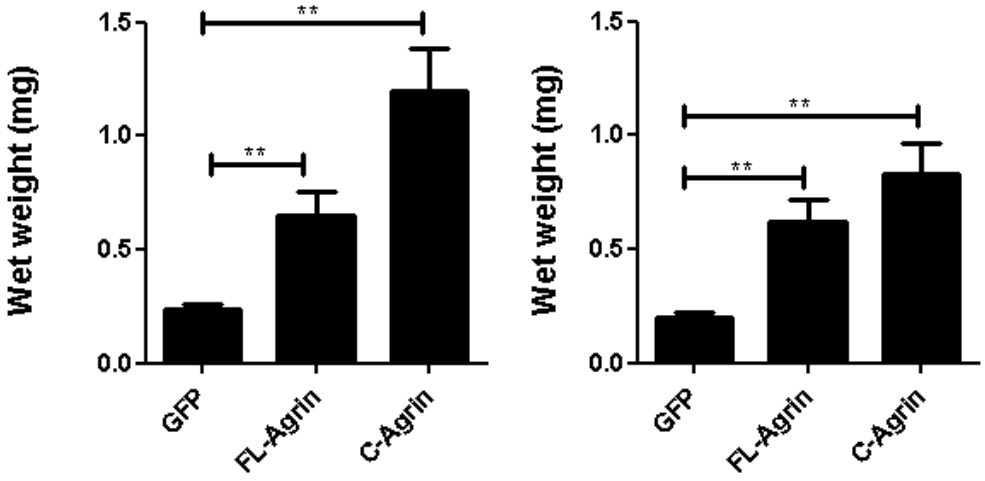


Figure 59. Comparison of direct transfection and COS7 delivery in primary bovine chondrocyte pellets

Primary bovine chondrocytes were transfected with, GFP,FL-Agrin or C-Agrin (left); primary bovine chondrocytes exposed to Mitomycin C treated COS7 cells transfected with GFP, FL-Agrin or C-Agrin (right). The wet weight was measured following 21 days in pellet culture.

For further validation of the COS7 cells as delivery system for Agrin, this system was compared with direct transfection of bovine chondrocytes *in vitro*. Primary bovine chondrocytes were transfected directly with the plasmids and cultured for 21 days (Figure 59 – left) and the transfected, growth-arrested COS7 cells were cultured with non-transfected primary bovine chondrocytes for 21 days (right). Although direct transfection is marginally more potent than COS7 cell delivery, the results are remarkably similar, indicating that this method was sufficient to deliver Agrin to chondrocytes and enhance ECM production.

In vivo ectopic cartilage formation assay

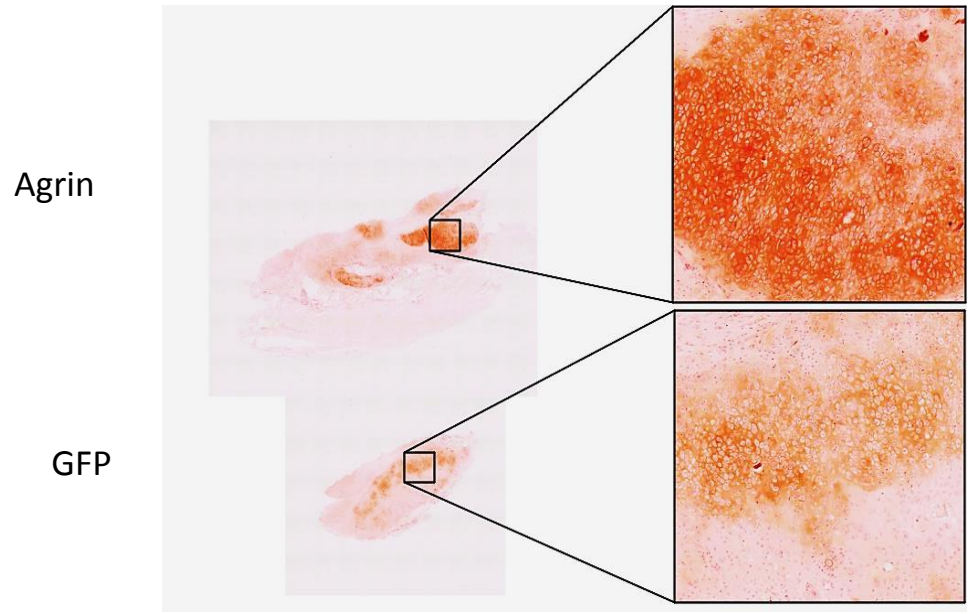


Figure 60. Safranin O staining of *in vivo* ectopic cartilage implants

Implants produced from the ectopic cartilage formation assay were removed from quadriceps two weeks post injection of bovine articular chondrocytes exposed to Agrin or GFP secreting COS7 cells. Differentiation was determined by staining sections with Safranin O (n=12).

With the technology was sufficiently tested and optimized to be applied *in vivo*, aliquots of $5x10^6$ bovine articular chondrocytes were co-implanted with $5X10^5$ growth arrested COS7 cells transfected with either Agrin or GFP. After 14 days, the implants were retrieved, weighed, sectioned through the middle and stained with Safranin O. Safranin O stained sections showed that the chondrocytes exposed to Agrinproduced larger implants with a more intensly stained cartilage matrix (Figure 60).

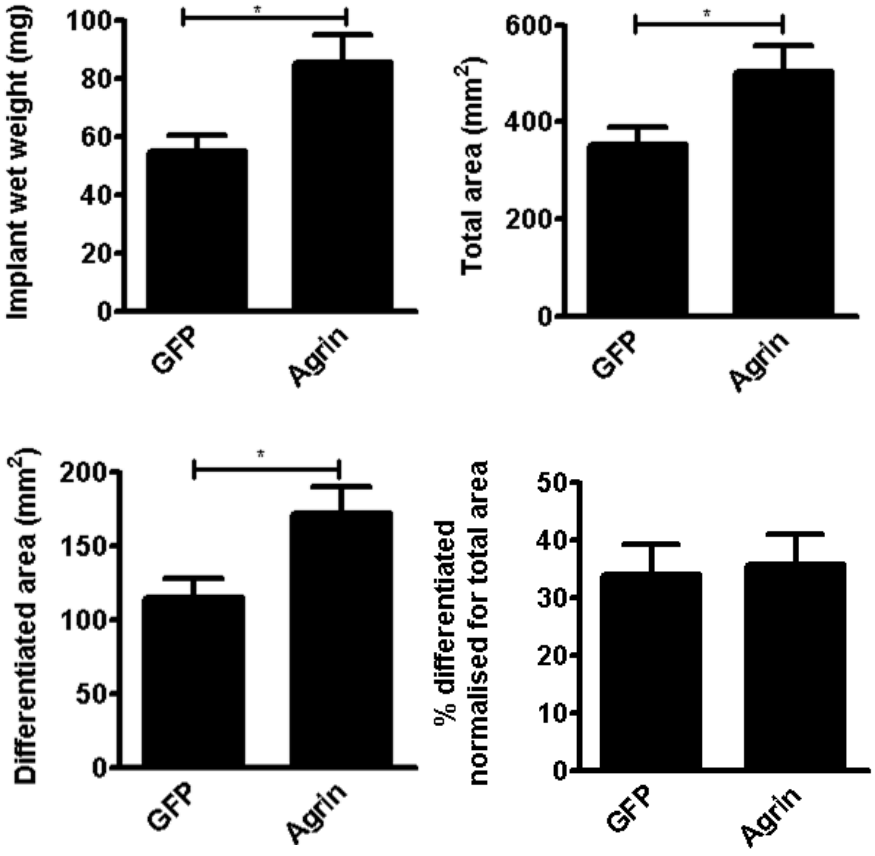


Figure 61. Analysis of *in vivo* ectopic cartilage implants

Implants produced from the ectopic cartilage formation assay were removed from quadriceps two weeks post injection of bovine articular chondrocytes exposed to Agrin or GFP secreting COS7 cells. Implants were weighed, and measured for total area, differentiated area and differentiated are normalised for total area shown as a percentage were measured (n=12).

Analysis of the implants revealed the chondrocytes exposed to Agrin-producing COS7 cells had an increased wet weight. To further the observation that the Agrin-stimulated pellets were heavier, the central (hence largest) section of each implant was used to obtain histomorphometry measurements, revealing Agrin induced both a greater total area and greater differentiated area when compared to GFP exposed chondrocytes (Figure 61). However, the samples were not over-differentiated as the ratio of differentiated tissue remained constant in both treatment groups.

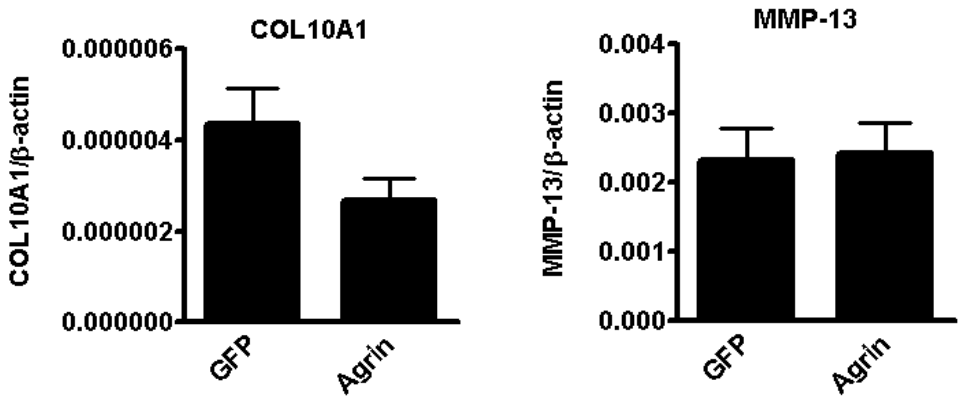


Figure 62. Gene expression analysis of *in vivo* ectopic cartilage implants

RNA was extracted from the retrieved implants and real time qPCR analysis of was used to determine bovine-specific COL10A1 and MMP-13 mRNA expression levels (n=12).

Real time qPCR analysis of gene expression of the implants revealed that Agrin did not increase the expression genes which are known to induce hypertrophy, namely COL10A1 and MMP13 *in vivo* (Figure 62); this an important finding because chondrocyte hypertrophy is a driver of cartilage breakdown in OA (Saito, et al., 2010; Yang, et al., 2010) and during skeletal development is a characteristic of cartilage that is destined to be replaced by bone. Therefore the fact that Agrin does not induce chondrocyte hypertrophy suggests that it supports a more stable hence more durable cartilage phenotype; potentially revealing a new treatment option for cartilage repair.

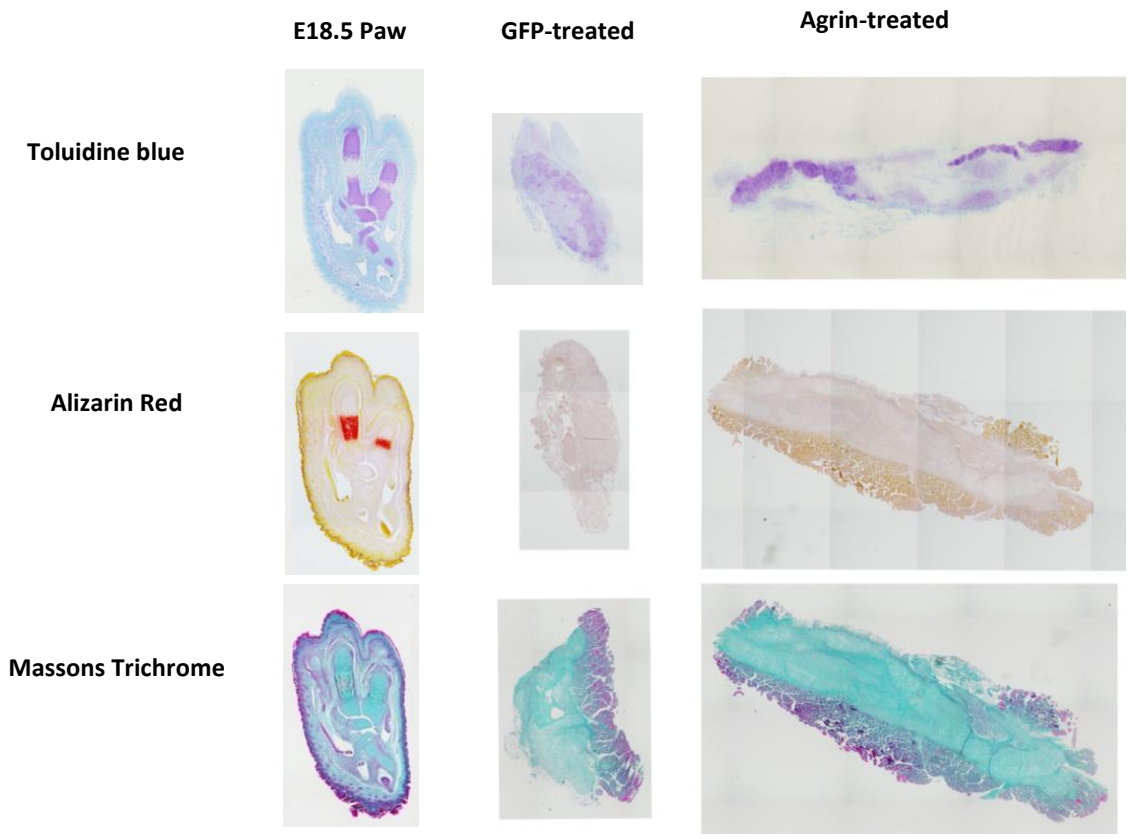


Figure 63. Histological staining of *in vivo* ectopic cartilage implants

Implants produced from the ectopic cartilage formation assay were removed from quadriceps two weeks post injection of bovine articular chondrocytes exposed to Agrin or GFP secreting COS7 cells. Histological staining was performed to confirm the presence of cartilage (toluidine blue) and the absence of bone (alizarin red) and vascular invasion (masons trichrome); E18.5 murine forepaw was used as control (n=12).

To further confirm that Agrin did not support other features of epiphyseal cartilage such as vascular invasion and calcification, specific staining's were performed (Figure 63). Masson's trichrome stains the cartilage in white or green depending on the collagen content and the vessels, including capillaries in bright red. Alizarin red staining stains any calcified tissue in bright red. Therefore implants were compared our implants to the epiphyses of mouse developing paw (E18.5). Alizarin red staining was absent in the implants retrieved but was

clearly visible in the murine paw where the cartilage has undergone hypertrophy before becoming the beginnings of calcified bone. Confirming that Agrin does not support the epiphyseal phenotype, vessels or calcification could not be detected within the implants. One caveat with this experiment is the short time frame.

Discussion

The delivery of secreted molecules using growth arrested cell lines, in our laboratory, has proven to be a very reliable, fast, simple, inexpensive, long term delivery system that circumvents many problems related to delivering bioactive molecules locally, within cartilage implants. This system does not induce harsh treatment to chondrocytes which, at least those coming from human donors, rapidly lose their capacity to form cartilage in this assay with routine procedures such as cryopreservation or after just one or two passages. This method also enables the delivery of the molecule of interest within the implant itself, at a satisfactory concentration and without the uncertainty and hassle of daily local injection of recombinant molecules or installation of osmotic pumps.

This issue is particularly important for large and insoluble molecules such as full length Agrin, which, besides the difficulty of manufacturing a >600kD heavily glycosylated protein and the issue of penetration within the dense and avascular cartilage ECM, would avidly adhere to the pericellular ECM through laminin binding and have very little chances to diffuse. The poor solubility and stability of full length Agrin may, however, be turned to an advantage from the therapeutic point of view: the fact that with simple transfection of bovine chondrocytes with an Agrin plasmid produced larger pellets two weeks later *in vitro* (Figure 58 and Figure 59) is at odds with the transient nature of plasmid transfection. This result could be explained in different ways:

- Chondrocytes in micromass display little if any proliferation. Therefore it is possible that the plasmid persisted in the non-dividing cells.

- It is possible that exogenous Agrin enhanced chondrocyte differentiation for a few days but the advantage gained by Agrin-transfected chondrocytes persisted later on until the end of the culture period.

- Agrin is a large extracellular molecule that tightly adheres to pericellular ECM and particularly to laminin. Therefore it is possible that although the protein accumulated during the first few days, once made, it persisted within the pericellular matrix.

The current data do not support any of these possible explanations over another and they are not mutually exclusive, however the third possibility, that Agrin is an insoluble, long-persisting protein that supports chondrocyte anabolism for a long time, is particularly interesting because, if true, opens the opportunity to "coat" the surface of scaffold material to support chondrocyte anabolism in cartilage repair. This is indeed the main subject of my current research.

This assay does have specific limitations, namely:

- The assay is able to test whether any treatment induces mineralization and bone formation, however, it does not allow the study of the interaction between cartilage and other joint tissues such as the subchondral bone and the synovial membrane.
- The normal joint biomechanics cannot be represented using this system.
- A key restriction is the requirement of a large number of primary chondrocytes. This can be particularly prohibitive when the use of human chondrocytes is necessary. Additionally, the use of human chondrocytes introduces a degree of donor variability

One other important consideration is that the molecular mechanism through which Agrin supports chondrocyte differentiation is still unknown. The next chapter of this thesis represents the first step towards the molecular dissection of Agrin signalling in chondrocytes and deals with the identification of which Agrin receptors mediate the Agrin-dependent chondrocyte differentiation.

CHAPTER 8: Results section 4

Agrin receptor usage in chondrocytes

Results: Agrin receptor usage in chondrocytes

Rationale:

As a first step towards dissecting the molecular mechanism through which Agrin supports chondrocyte differentiation, the Agrin receptors required for this function of Agrin were investigated by taking a loss of function approach. Whereby, Agrin was overexpressed while simultaneously the expression of its receptors was silenced. The ability of Agrin to upregulate SOX9 in the absence of each receptor was assessed.

Experimental aims

Determine whether:

- the basement membrane receptor complex (α-dystroglycan) is required for the function of Agrin in chondrocytes
- or the neuronal receptor complex (LRP4) is required for the function of Agrin in chondrocytes

The role of α-dystroglycan and Agrin in chondrocytes

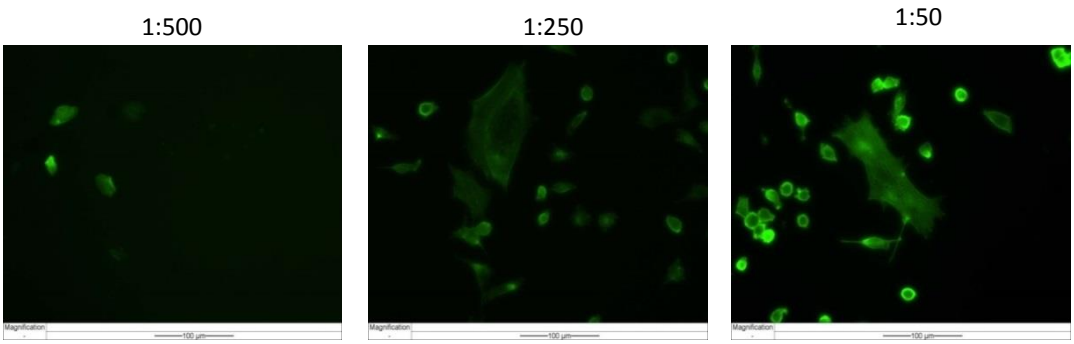


Figure 64. Phalloidin staining of primary bovine chondrocytes

Primary bovine chondrocytes were cultured in monolayer and allowed to attach for 24hrs. Chondrocytes were permeabilised and filamentous actin was stained with phalloidin (dilutions 1:50, 1:250 and 1:500).

Chapter 5 revealed that the Agrin isoform expressed by the articular chondrocytes is lacking the inserts which are responsible for high affinity binding to LRP4, thus making Agrin in chondrocytes more likely to have a higher affinity for α-dystroglycan binding. This isoform of Agrin (y0, z0) is important in connecting the basal membrane of muscle fibres to the actin cytoskeleton and, in cartilage, the functional status of the actin cytoskeleton is an important determinant of chondrocyte differentiation (Benya, PD, & Shaffer, 1982; Benya, PD, 1988) and therefore a similar mechanism in chondrocytes was also a plausible possibility.

Alpha-dystroglycan can be reliably blocked using a well validated blocking antibody (Williams, Stacey, & Jacobson, 2010). In order to optimise this blocking antibody for chondrocytes, phalloidin staining was be used as a readout of α-dystroglycan function. Since α-dystroglycan links the membrane to the cytoskeleton, when its function is disrupted α-actin filaments (which can be stained using phalloidin) are also disrupted. Therefore, the stainability of actin filaments with phalloidin was used as a readout for the α-dystroglycan blockade experiment. To optimise the staining, three initial concentrations were tested on bovine

primary chondrocytes and a dilution of 1:100 was estimated to be sufficient to obtain a reliable readout of F-actin production based on the findings in Figure 64.

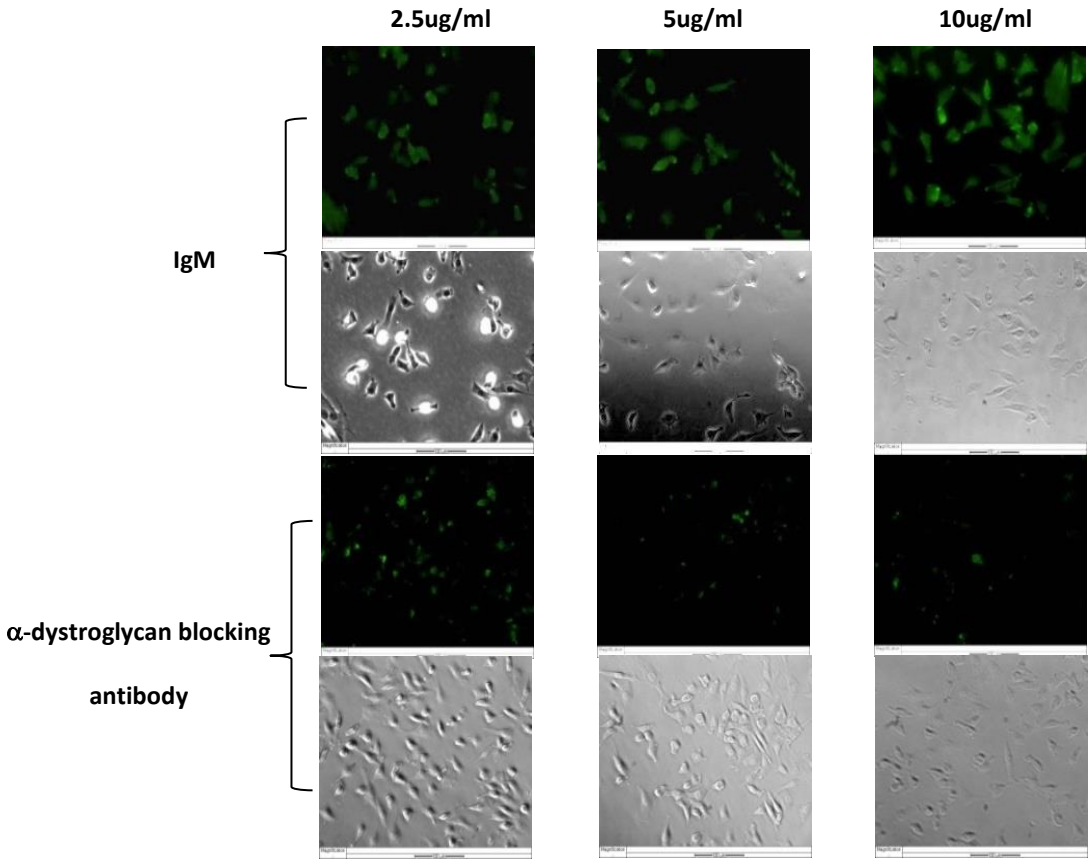


Figure 65. Primary bovine chondrocytes treated with α-dystroglycan blocking antibody

Primary bovine chondrocytes were plated in monolayer and allowed to attach for 24hrs before the addition of 2.5ug/ml, 5ug/ml or 10ug/ml of α-dystroglycan blocking antibody. IgM antibody served as control. Chondrocytes were stained with phalloidin after 24hrs treatment.

Chondrocytes were cultured in monolayer and treated with 2.5ug/ml, which was sufficient to effectively reduce binding of α-dystroglycan to produce the dystrophin complex required for the production of F-actin as shown by phalloidin staining (Figure 65). Higher doses of the α-dystroglycan blocking antibody resulted in total cell detachment.

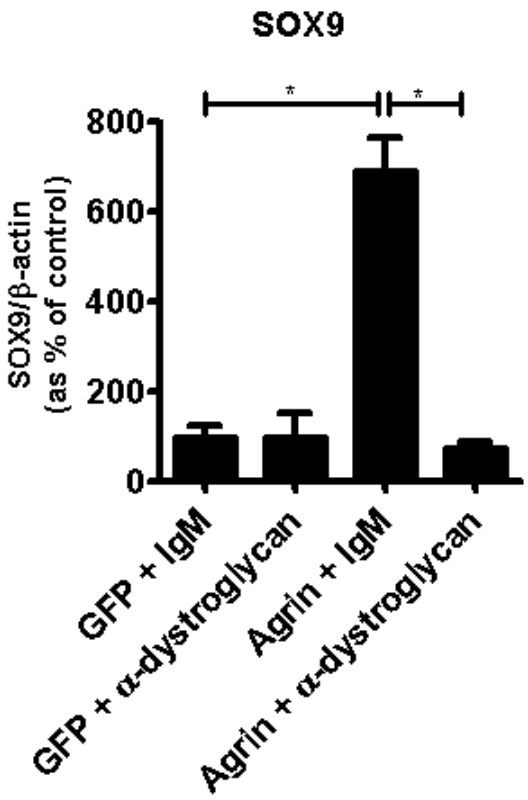


Figure 66. SOX9 gene expression analysis of α-dystroglycan inhibition in primary bovine chondrocytes

Primary bovine chondrocytes were transfected with GFP or Agrin and cultured in micromass for 5days in the presence of 2.5ug/ml α-dystroglycan blocking antibody or IgM control. RNA was extracted and real time qPCR analysis of SOX9 expression mRNA levels was carried out (n=4).

To investigate whether the inability of Agrin to bind α-dystroglycan affected the function of Agrin in the articular chondrocytes, primary bovine chondrocytes were transfected with GFP or Agrin overexpression plasmids whilst in the presence of the α-dystroglycan blocking antibody or IgM control. By blocking this binding interaction the Agrin-induced SOX9 upregulation was completely abolished (Figure 66). These data show that α-dystroglycan is required for the ability of Agrin to upregulate SOX9 mRNA.

The role of LRP4 and Agrin in chondrocytes

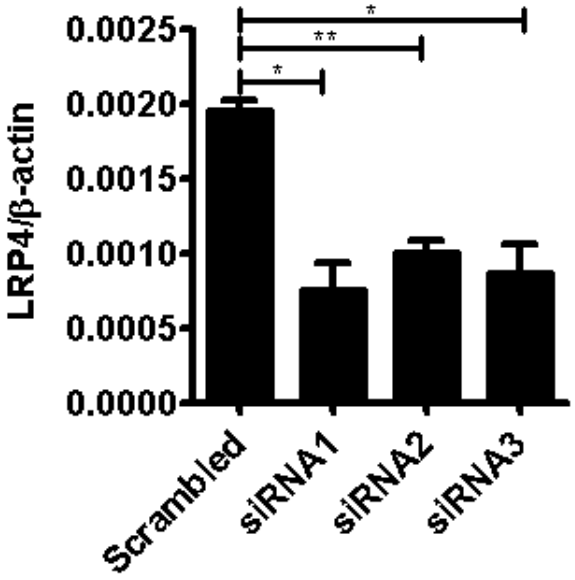


Figure 67. LRP4 knockdown using siRNA in primary bovine chondrocytes

Primary bovine chondrocytes were transfected with 3 Agrin siRNA's or a scrambled siRNA as control and cultured in micromass for 5days. Real time qPCR was used to measure LRP4 gene expression levels (n=4).

Despite discovering that α-dystroglycan is required for the chondrocytic effect of Agrin, it was necessary to verify whether LRP4 also played a role as this receptor was also found to be expressed at high levels in the articular chondrocytes (Figure 28). LRP4 was silenced using three independent in-house designed siRNA oligonucleotide pairs and a corresponding Scrambled RNA to serve as control. All siRNA's reduced the level of LRP4 mRNA by over 50%, with siRNA1 being the most potent (Figure 67).

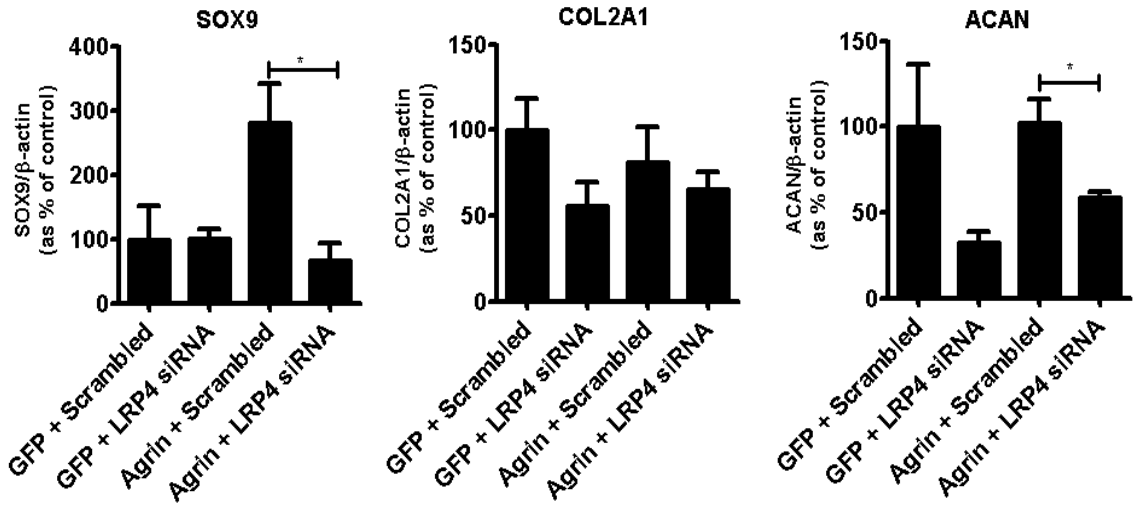


Figure 68. Gene expression analysis of LRP4 knockdown in primary bovine chondrocytes

Primary bovine chondrocytes were transfected with LRP4 siRNA1 or a Scrambled siRNA as control and co-transfected with GFP or Agrin and cultured in micromass for 5days. RNA was extracted and real time qPCR analysis of SOX9, ACAN and COL2A1 mRNA expression levels was carried out (n=4).

To investigate whether the loss of LRP4 mRNA affected the function of Agrin in the articular chondrocytes, primary bovine chondrocytes were co-transfected with GFP or Agrin overexpression plasmids and either Scrambled siRNA or LRP4 siRNA1. While there was no change to the gene expression levels of SOX9 in the GFP transfected whether LRP4 is silenced or not, the upregulation of SOX9 induced by Agrin overexpression was reduced to baseline GFP levels in the absence of normal levels of LRP4 in the chondrocytes (Figure 68 – left). The levels of COL2A1 (middle) and ACAN (right) were affected when LRP4 was silenced in both GFP and Agrin expressing cultures.

These data demonstrate that, although the Agrin isoform expressed in cartilage is predicted to have a relatively low affinity for LRP4, LRP4 function is required for the Agrin-induced upregulation of SOX9.

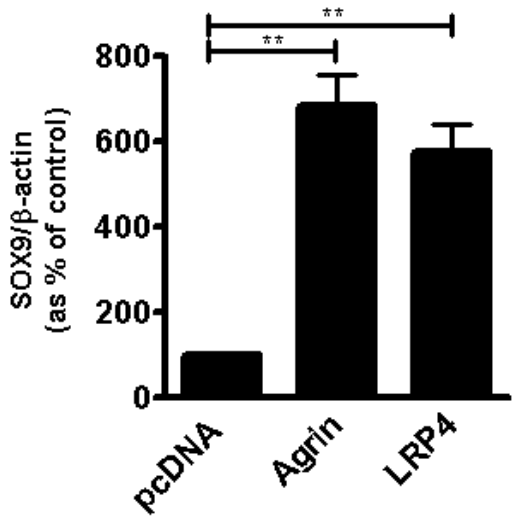


Figure 69. Gene expression analysis of LRP4 overexpression in primary bovine chondrocytes

Primary bovine chondrocytes were transfected with pcDNA, Agrin or LRP4 and cultured in micromass for 5days. SOX9 mRNA expression levels were assessed by real time qPCR (n=4).

A recent study by Asai et al. (Asai, et al., 2014) showed that, in ATDC5 cells, transfection of just LRP4 was sufficient to induce chondrogenic differentiation. Agrin is not the only ligand of LRP4; SOST and DKK1 also function by binding to LRP4 (Choi, et al., 2009; Mason, & Williams, 2010). This data was confirmed in primary bovine chondrocytes, as with ATDC5 cells (Asai, et al., 2014), LRP4 overexpression resulted in a robust upregulation of SOX9 (Figure 69). Assuming that Agrin is indeed the main chondrogenic ligand for LRP4, then it could be expected that SOX9 upregulation should not take place if, simultaneously to LRP4 overexpression I also silenced Agrin.

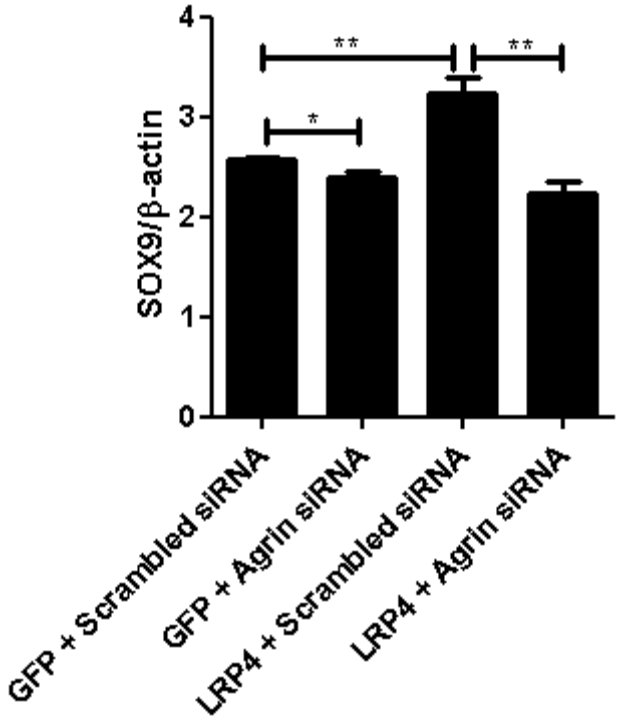


Figure 70. SOX9 expression analysis of LRP4 overexpression and Agrin knockdown in C28/I2 cells

C28/I2 cells were transfected with Agrin siRNA1 or a Scrambled siRNA as control and co-transfected with GFP or LRP4 and cultured in micromass for 5days. RNA was extracted and real time qPCR analysis of SOX9 mRNA expression levels was carried out (n=3).

Replication of the Asai et al., group experiments showed that overexpression of LRP4 alone is sufficient to upregulate SOX9 in bovine chondrocytes as well as the ATDC5 cell line (see Figure 69). However, Figure 68 shows that the loss of LRP4 alone is not sufficient to reduce SOX9 levels lower than basal levels in GFP treated samples suggesting that Agrin, whether it be endogenous or exogenous, is required for the upregulation of SOX9 in chondrocytes. Whereas LRP4 overexpression + Scrambled siRNA resulted in SOX9 upregulation, this upregulation was much reduced after knockdown of Agrin (Figure 70).

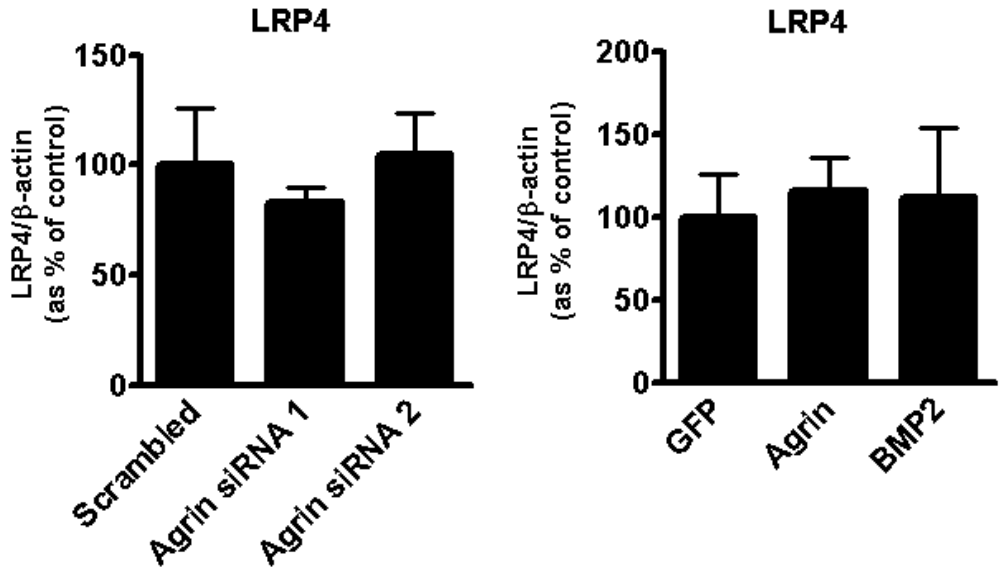


Figure 71. Gene expression regulation of LRP4 by Agrin

Primary bovine chondrocytes were transfected with siRNA's (left) or overexpression plasmids (right) and cultured in micromass for 5days. Gene expression levels of LRP4 were measured using real time qPCR (n=3).

Finally, to exclude the possibility that Agrin was able to induce LRP4 upregulation and this was in turn responsible of SOX9 upregulation through a mechanism independent of Agrin (for instance by binding to SOST), qPCR was carried out to determine whether LRP4 was a transcriptional target of Agrin. Primary bovine chondrocytes transfected with Agrin siRNA or Agrin, however, did not regulate LRP4 mRNA, thereby excluding this possibility (Figure 71).

Discussion

At the neuromuscular junction, neuronal Agrin (y4, z8) binds to LRP4 and signals through MuSK, while, in muscle cells, non-neuronal Agrin (y0, z0) links the cytoskeleton to the basement membrane through binding α-dystroglycan. However, it was unknown which of these two mechanisms was engaged for the anabolic functions of Agrin in chondrocytes. Treatment with an antibody which blocks the interaction of α-dystroglycan with Agrin (Campanelli, Gayer, and Scheller 1996; Gesemann et al. 1996) abrogated Agrin-induced upregulation of SOX9. Surprisingly, also LRP4 silencing by siRNA completely inhibited Agrin-induced SOX9 upregulation suggesting that both LRP4 and α-dystroglycan are required for Agrin-induced chondrocyte differentiation.

The requirement of LRP4 was unexpected because the (y0, z0) variant of Agrin has low affinity for LRP4 and a reduced capacity for clustering the acetylcholine receptor at the neuromuscular junction. To mitigate this concern, however, it has been shown that the co-receptor APP, also expressed by articular chondrocytes (Figure 28) increases the affinity of the (y0, z0) Agrin isoform (Choi, et al., 2013). Therefore it is possible that also in chondrocytes APP may enable the interaction of Agrin with LRP4. However, an alternative and fascinating hypothesis is that this co-receptor role, in cartilage, is taken by α-dystroglycan. This would not only explain the interaction of LRP4 with the (y0, z0) Agrin isoform but also the potential reason for the requirement of α-dystroglycan.

To demonstrate this hypothesis, and also to investigate whether Agrin interacts with LRP4 and α-dystroglycan in a single multimeric complex or separately or whether α-dystroglycan is required for completely different reasons but does not interact with Agrin, a series of co-immunoprecipitation experiments would be required. The hypothesis is that in the presence of Agrin, LRP4 and α-dystroglycan (and perhaps APP) interact in a multimeric complex. Preliminary co-immunoprecipitation experiments with endogenous levels of LRP4 and α-dystroglycan have

failed. This is likely due to the poor affinity of the antibodies, the low copy-number of these receptors, and to the very large size of the complex as a whole (approx. 1000kDa).

CHAPTER 9: Results section 5

Effect of MMP3 on Agrin in chondrocytes

Results: Effect of MMP3 on Agrin in chondrocytes

Rationale:

Despite demonstrating the requirement of Agrin in articular chondrocytes (Chapter 6), the mechanism by which Agrin is lost during OA remains unclear. One possibility is that chondrocytes respond to cartilage damage by reducing the amount of Agrin produced, however this is not necessarily the case as Agrin, both at mRNA and protein levels, in early OA (Figure 36) and also in the acute phases following cartilage injury (Figure 34) suggesting another mechanism is taking place.

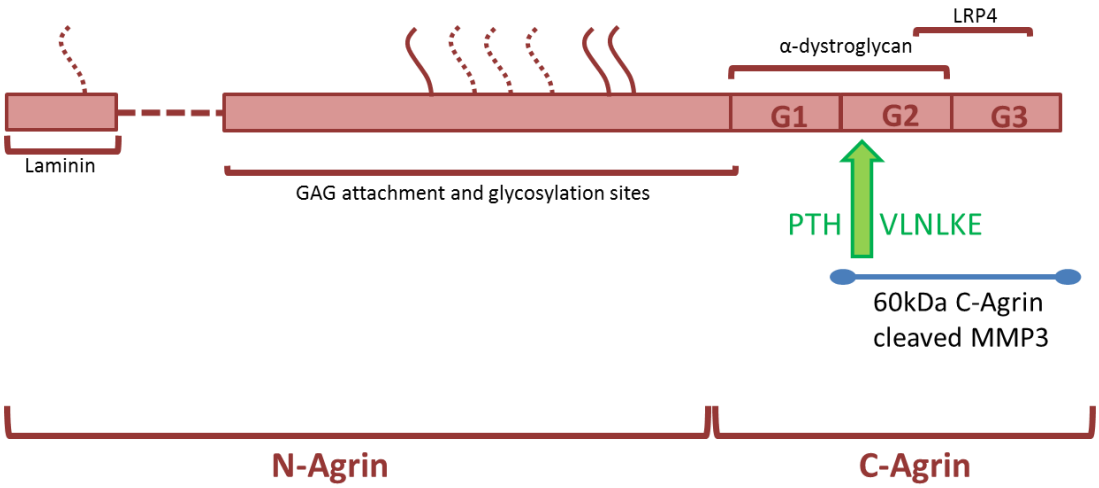


Figure 72. The MMP3 cleavage site of Agrin

In other biological contexts (VanSaun, et al., 2003; Vansaun, & Werle, 2000) Agrin can be cleaved by metalloproteinases, and in particular by MMP3. Since MMP3 is strongly upregulated and enzymatically active in OA (Flannelly, et al., 2002), it is possible that MMP3 mediated cleavage of Agrin may account, at least in part, for its loss in OA (see Figure 72). This is highly

plausible as previously published microarray data (Dell'Accio, et al., 2008) shows that MMP3 is upregulated in a model of cartilage injury (Figure 73) while further publications have reported long-term MMP3 upregulation in the synovial fluid of osteoarthritic joints (Tchetverikov, et al., 2005).

This phenomenon was investigated using two antibodies, one binding the N-terminal portion of Agrin, proximal to the MMP3 cleavage site, and the other one binding the C-terminal portion, distal to the cleavage site. If proteolytic cleavage is taking place, the C-terminal portion would be lost much sooner than the N-terminal portion.

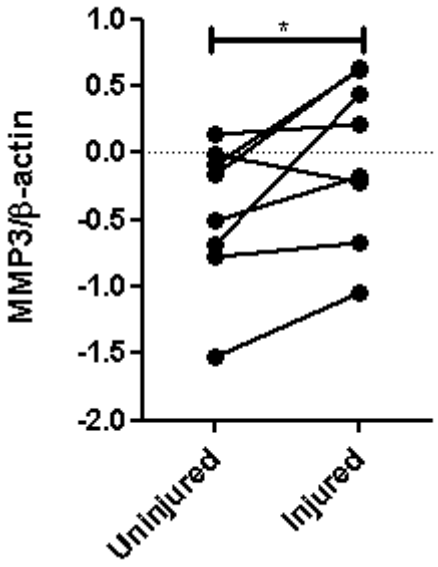


Figure 73. Gene expression analysis of MMP3 in human cartilage following *ex-vivo* injury

Paired gene expression analysis of MMP3 in human articular cartilage 24hrs following *ex-vivo* injury (n=7).

Experimental aims

Determine:

- if Agrin is cleaved by MMP3 in articular chondrocytes
- if Agrin gene expression is dependent on MMP3
- if cleaved Agrin is released into the supernatant or retains cell-association
- if Agrin cleavage by MMP3 is detrimental to the anabolic function

Agrin cleavage in osteoarthritis

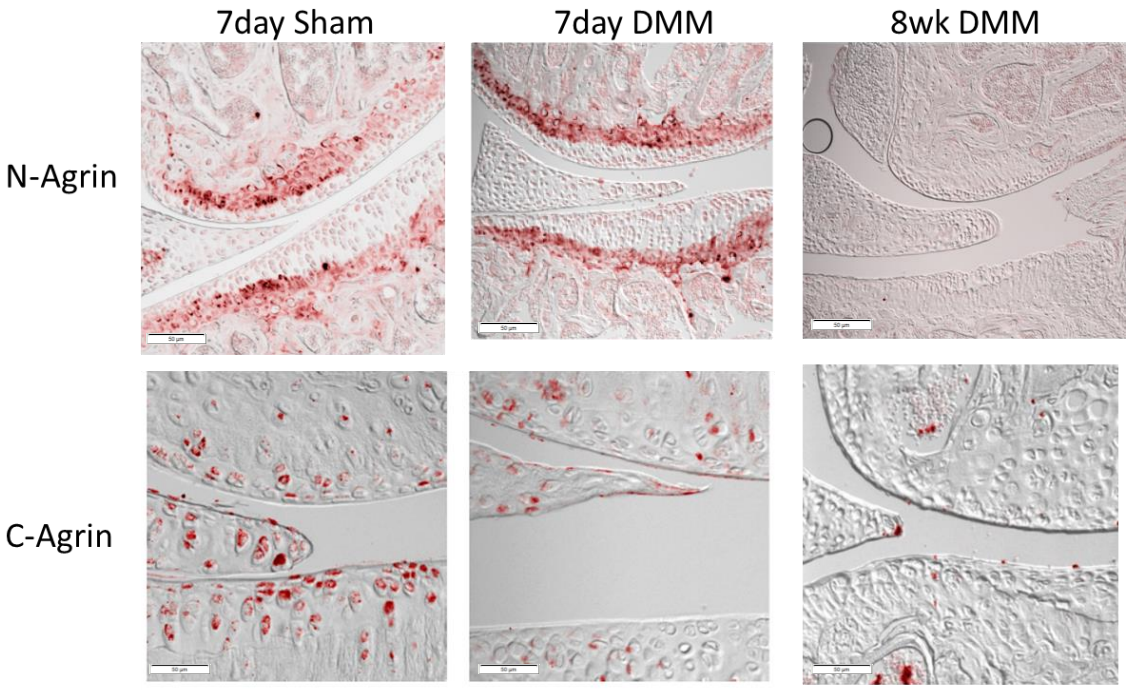


Figure 74. N-Agrin and C-Agrin protein detection in 129/Sv mice articular cartilage following DMM

N-Agrin (top) and C-Agrin (bottom) protein detection in the joint of 129/Sv mice 7days and 8 weeks post osteoarthritis induced by DMM surgery. Sham operated served as positive control (n=3).

to explore whether there is any evidence of proteolytic cleavage of Agrin in OA I stained sections from the knee joints of mice subjected to DMM and killed at different time points utilizing 2 independent antibodies, one for the N terminal portion of Agrin, predicted to remain attached to the cells due to laminin binding, and another one recognizing the C-terminal domain, predicted to be released by MMP3. The results show that although all portions of Agrin are ultimately lost at the 8 week time period, C-Agrin is reduced much earlier at the 7day time point where MMP3 levels are likely higher. This was consistent with proteolytic cleavage

similar to that described following traumatic nerve injury (Chao, et al., 2013) because the N-terminal portion of Agrin is anchored to the pericellular ECM through laminin binding.

These data show that, although MMP3 is unlikely to be the only contributing factor to the loss of Agrin in end stage OA, it is likely the main contributor to the proteolytic cleavage of Agrin as early as 7days following cartilage injury.

MMP3 cleavage of Agrin in vitro

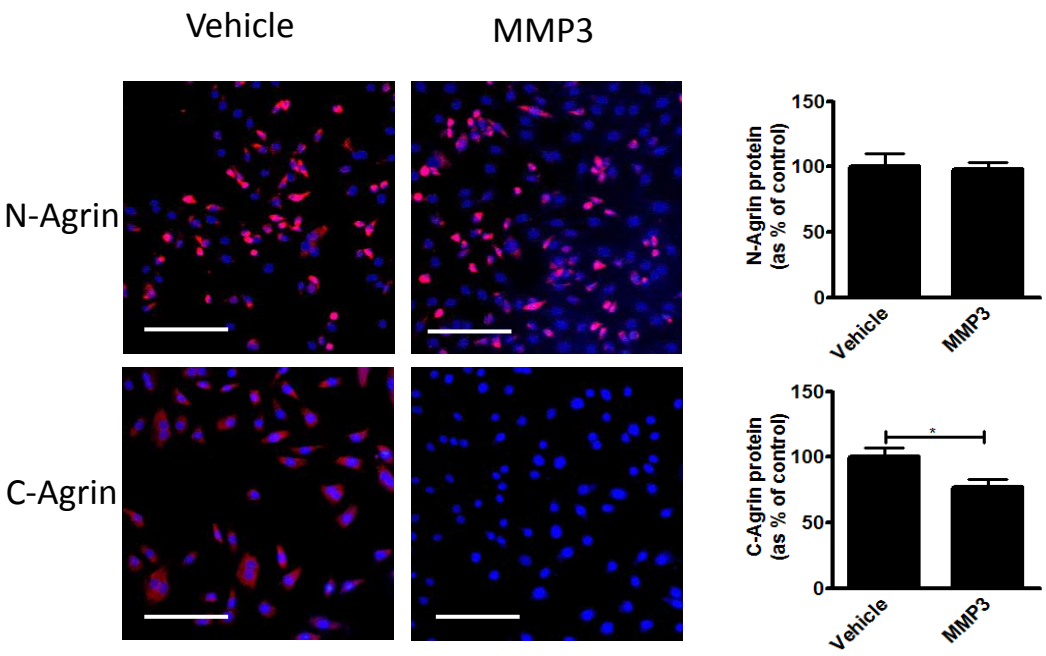


Figure 75. N-Agrin and C-Agrin protein detection in bovine chondrocytes treated with MMP3

Primary bovine chondrocytes were plated in monolayer on multi-well glass slides and allowed to attach for 24hrs before 24hrs treatment with 0.5ug/ml MMP3. Chondrocytes were stained by immunofluorescence for N-Agrin and C-Agrin protein and quantified using images J (n=4). Bar, 50µm.

To investigate whether a similar staining pattern with these two antibodies could be reproduced by treatment with MMP3, bovine primary chondrocytes were cultured in monolayer on coverslips and treated with MMP3 or vehicle for 24hrs. The data show that the N-Agrin protein levels remain unaffected in the presence of MMP3, however, there is significant reduction in the amount of C-Agrin bound to the chondrocytes following treatment, verifying C-Agrin is cleaved by MMP3 in articular chondrocytes (Figure 75).

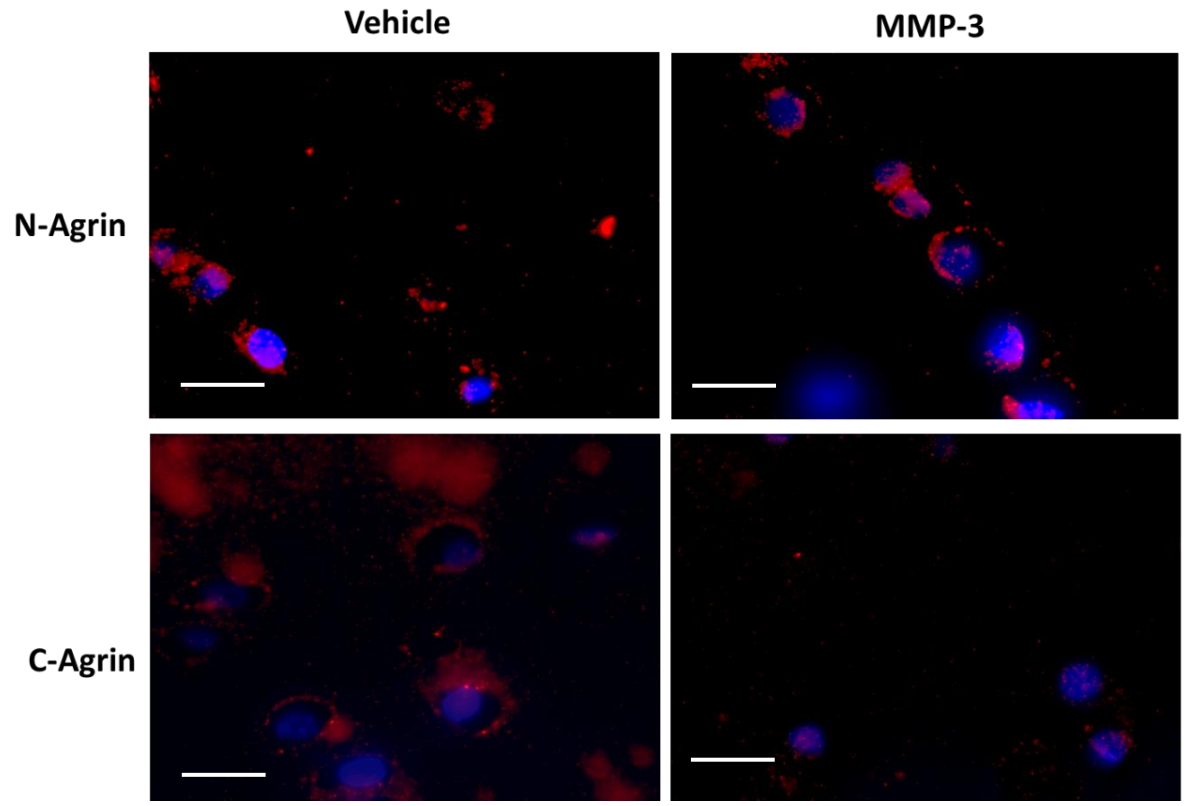


Figure 76. N-Agrin and C-Agrin protein detection in bovine cartilage explants treated with MMP3

Full thickness paired bovine cartilage explants were harvested and allowed to rest for 7days before 24hrs treatment with 0.5ug/ml MMP3. Sections were stained by immunofluorescence for N-Agrin and C-Agrin protein (n=4). Bar, 10µm.

To confirm that MMP3-induced Agrin cleavage does not only take place in monolayer cultures but also within the complex cartilage extracellular matrix, further verification was carried out using full thickness cartilage explants harvested from bovine metatarsal joints (Figure 76). This ex vivo model enabled the verification that the results seen *in vitro* are representative of the effects of MMP3 of chondrocytes within the cartilage matrix itself.

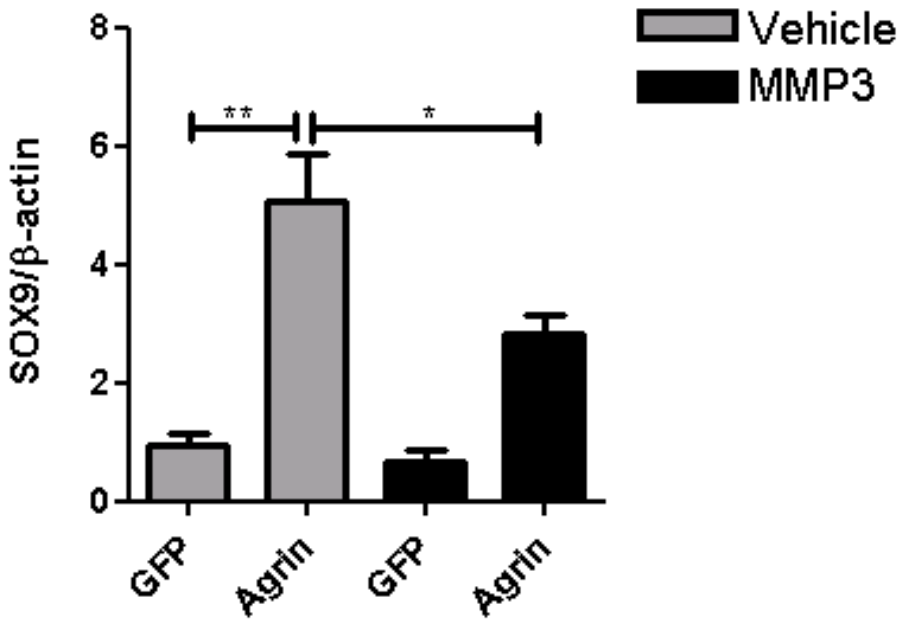


Figure 77. Gene expression analysis of SOX9 in bovine chondrocytes treated with MMP3

Bovine primary chondrocytes were transfected with GFP or Agrin and cultured in micromass in the presence of MMP3 or vehicle for 5 days. Real-time qPCR was used to measure SOX9 gene expression levels (n=4).

Since the MMP3 cleavage site is located within the first globular domain of C-Agrin, it was possible that this cleavage either inactivated Agrin or released a biologically active C-terminal portion. The above data showed that MMP3 treatment reduced the capacity of Agrin to induce SOX9 expression. This suggests that the cleavage may result in inactivation of Agrin, however it cannot be excluded that MMP3 might inactivate other homeostatic mechanisms independent of Agrin. To further explore this possibility, future experiments will focus on the creation and utilisation of an MMP3-cleavage resistant mutant of Agrin, which is hypothesised to have a greater potency within chondrocytes due to its insensitivity to MMP3 treatment.

MMP3 inhibition by Agrin

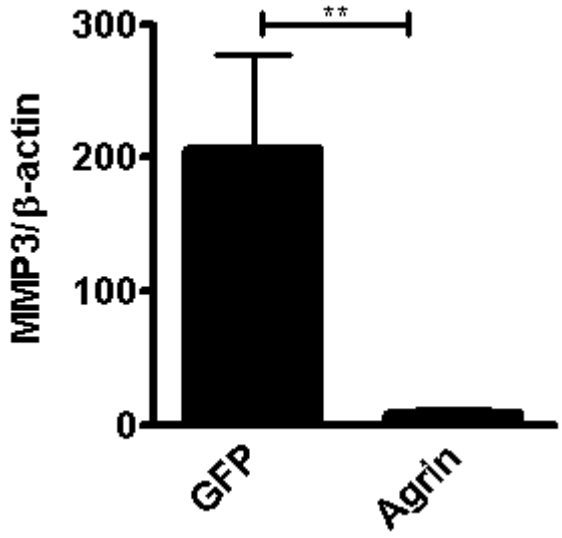


Figure 78. Agrin inhibits MMP3 expression in bovine primary chondrocytes

Bovine primary chondrocytes were transfected with GFP or Agrin and cultured in micromass for 5days. RNA was extracted and gene expression analysis of SOX9 was carried out using real time qPCR (n=4).

Finally, whether Agrin itself has any effect on MMP3 expression was explored. Strikingly, Agrin overexpression resulted in near total suppression of MMP3 expression (Figure 78). It could be speculated that this mechanism may exist to ensure that the anabolic function Agrin is self-limiting: Agrin would be expressed following injury and for a certain time would support anabolism, while through the suppression of MMP3 transcription, protect itself from MMP3-driven cleavage and suppress MMP3-driven cartilage breakdown. However, after injury and in OA, persistently high levels of MMP3 could progressively reduce Agrin protein levels, further exacerbating MMP3 expression. . At this stage this model is highly speculative (Figure 79).

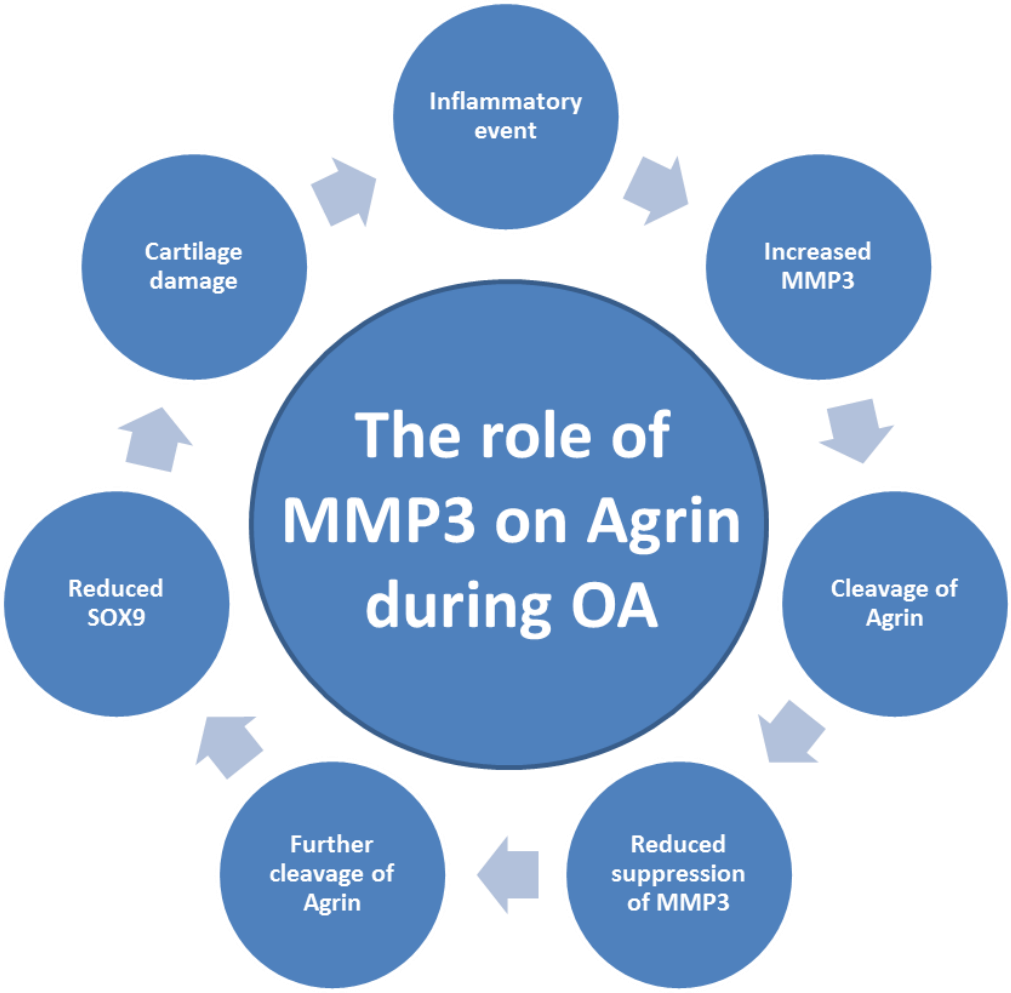


Figure 79. The hypothetical role of MMP3 on Agrin during osteoarthritis

Summary of hypothesised cyclical molecular events occurring following cartilage damage.

Discussion

Following cartilage injury and in early phases of OA the chondrocytes, in an attempt to repair the defect, increase the amount of matrix synthesis. This in turn also increases the amount of matrix degradation enzymes until ultimately an imbalance of pro and anti-inflammatory molecules is reached. MMP3 is known to be one of the persistently upregulated enzymatic, catabolic molecules both in injury (Figure 73) and in early and chronic OA (Glasson, et al., 2007; Tchetverikov, et al., 2005).

MMP3 is interesting in the context of Agrin biology as it is known to cleave the final 60kDa at the C-terminal end of the protein and is used a biomarker (VanSaun, et al., 2003). For example, C-Agrin increases have been measured and correlated with renal failure and also within serum levels of sarcopenia patients following hip fractures (Drey, et al., 2015; Hettwer, et al., 2013; Marzetti, et al., 2014; Steubl, et al., 2013) further suggesting MMP3 maybe be a contributing factor to C-Agrin cleavage in OA.

Although the data shown in this chapter strongly suggest that MMP3, and possibly other proteinases, contribute to loss of Agrin detection in OA, it is not clear whether MMP3-driven Agrin cleavage inactivates Agrin or rather releases a biologically active portion. In fact, it is known from **Error! Reference source not found.**Figure 59 and Figure 88 that the C-terminal portion of Agrin is sufficient to support cartilage differentiation *in vitro*. However, this C-Agrin plasmid encodes for a protein of approximately 95-110kDa and comprises the full three globular domains containing both the α-dystroglycan and LRP4 binding sites. This, whereas the MMP3-cleaved Agrin product is known to be around 60kDa (Chao, et al., 2013), raising the question of whether this MMP3-cleaved product is active. Figure 77 demonstrates that MMP3 reduces the capacity of Agrin to induced SOX9 regulation, however not to basal levels. This also could be explained by the ability of Agrin to inhibit the expression of endogenous MMP3 mRNA (Figure 78).

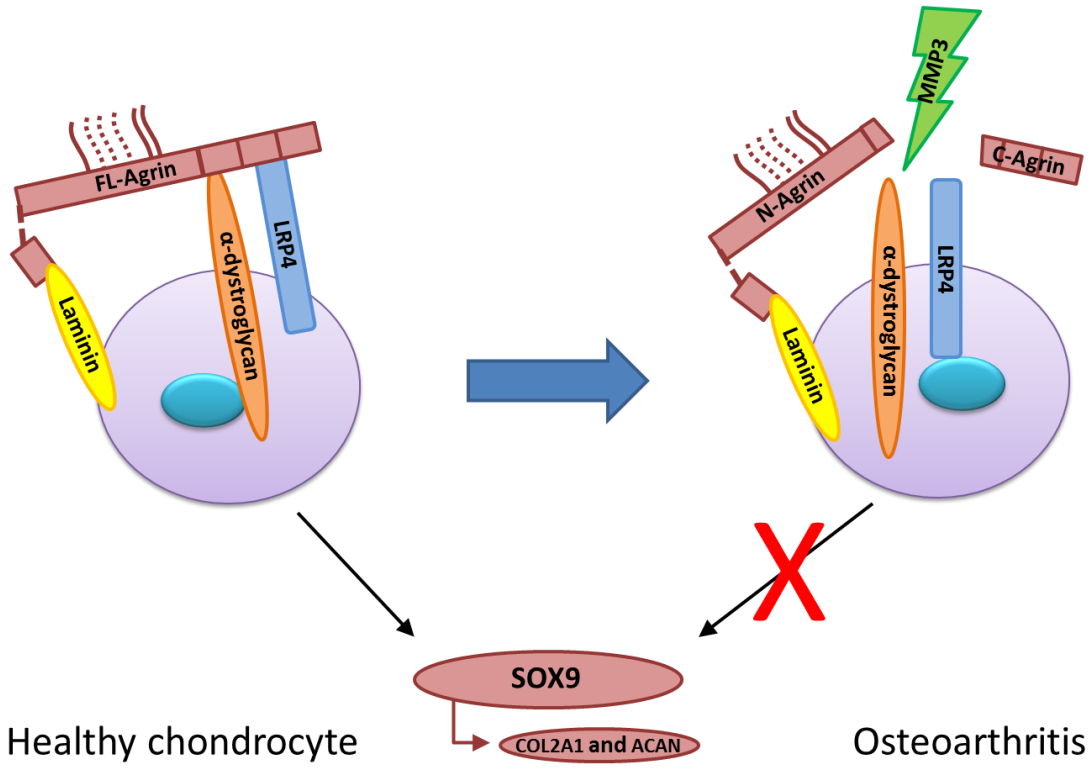


Figure 80. Hypothesised effect of Agrin cleavage by MMP3 in articular chondrocytes

Model for possible cleavage of C-Agrin by MMP3 resulting in the disruption of the LRP4 and/or α-dystroglycan binding sites leading to inhibition of Agrin-induced SOX9 upregulation in OA.

The MMP3 cleavage site is between the LRP4 and the α-dystroglycan binding sites of Agrin (Figure 72) is in keeping with the loss of anabolic activity and suggests that the same Agrin molecule has to bind both receptors. We propose the hypothesis that MMP3 cleaves the final 60kDa of the C-terminal portion of Agrin and disrupts the α-dystroglycan and/or the LRP4 binding domain in chondrocytes which ultimately inhibits the Agrin-induced SOX9 upregulation (see Figure 80) rendering Agrin inactive.

CHAPTER 10: Discussion

Discussion

This thesis reveals the discovery of the (y0, z0) isoform of Agrin is expressed in the adult articular cartilage and is downregulated in OA. We showed that Agrin has an anabolic function in maintaining chondrocyte differentiation and enhancing ECM production *in vitro* and *in vivo* without inducing chondrocyte hypertrophy or endochondral bone formation. We have found that Agrin-induced chondrocyte differentiation requires a unique receptor/co-receptor repertoire including both α-dystroglycan and LRP4 in articular chondrocytes.

Agrin function at the neuromuscular synapse requires the binding of LRP4 and, indirectly, MuSK (Choi, et al., 2013; Kim, et al., 2008; Zhang, Bin, et al., 2008), whereas the function of Agrin in muscle cells is mediated by its binding to α-dystroglycan and laminin. The differential binding to these different receptor complexes is dependent on the presence of the spliced inserts y and z, which confer high affinity for LRP4 (Gesemann, et al., 1998; Yumoto, et al., 2012). In particular, structural data of the Agrin-LRP4-MuSK complex revealed that the z8 loop of rat neuronal Agrin was responsible for the primary Agrin-LRP4 interaction, preceding the formation of the tetramer in which two Agrin-LRP4 heterodimers interact and for MuSK-dependent signalling at the neuromuscular junction (Zong, et al., 2012).

However, the engagement of the co-receptors APP and APLP2 can independently interact with the z0 form of Agrin as well as with LRP4 (Choi, et al., 2013), to either stabilize the interaction of Agrin with LRP4 or indirectly recruit LRP4 into a larger Agrin signalling complex. Both APP and APLP2 are expressed in cartilage (Figure 28) and this may explain the unexpected requirement of LRP4 in the cartilage homeostatic function of Agrin. It is tempting to speculate that α-dystroglycan may have a similar function as a co-receptor. This is probably not the case in the context of the NMJ, because in a derivative antisense C2 muscle cell line with reduced α-dystroglycan expression, the number of AChR aggregates is greatly reduced, but the AChR phosphorylation is not (Jacobson, et al., 1998) therefore suggesting that, at least at the NMJ, α-

dystroglycan participates to the formation of the AChR aggregates but not as a co-receptor, and perhaps in the stabilization of the complexes. In addition Agrin fragments that cannot bind α-dystroglycan can still activate AChR aggregation (Gesemann, et al., 1996; Hopf, & Hoch, 1996) and overexpression of α-dystroglycan in xenopus embryos caused a decrease, not an increase of AChR aggregates in keeping with a competition of α-dystroglycan, outside of the LRP4 receptor complex (Heathcote, et al., 2000). These data however, are relevant to MUSK signalling and its function in phosphorylating AChR at the NMJ. MuSK is not expressed or expressed at very low levels in cartilage (Figure 28) and therefore it is likely that the function of Agrin in cartilage may be independent of MuSK (although this awaits experimental confirmation. In this context, therefore, α-dystroglycan might still be a co-receptor for Agrin. Physical interaction studies are needed by immunoprecipitation.

One interesting aspect of LRP4 signalling is its capacity to modulate signalling pathways important for chondrogenesis including BMP and WNT signalling (Ohazama, et al., 2010). Taken together, these data suggest that an extended cell surface receptor repertoire regulates the affinity of Agrin for different receptors and the interplay with other signalling pathways including BMPs and WNTs to fine-tune biological specificity in the maintenance of articular cartilage (Figure 81). This possibility is supported by data not shown in this thesis that Agrin can prevent the upregulation of the TOPFlash reporter assay induced by WNT-3A.

LRP4 binds and mediates the function of DKK1 (a WNT inhibitor) and SOST (which is a soluble, secreted inhibitor of WNT and BMPs). Both these pathways play a role in chondrocyte differentiation. In my experiments I demonstrated that although overexpression of LRP4 alone is sufficient to induce chondrocyte differentiation, this is dependent on endogenous Agrin expression, because when Agrin was simultaneously silenced, LRP4 overexpression was inconsequential. This, of course does not exclude that, in this context, Agrin does not interfere with WNT and BMP signalling. In fact, while both these pathways are essential for the

homeostasis and integrity of cartilage throughout life (Corr, 2008; Decker, et al., 2014; Loughlin, J, 2011; Rountree, et al., 2004; Schett, et al., 2008), their excessive/inappropriate activation, drives cartilage breakdown (Davidson, et al., 2014; van der Kraan, et al., 2010). In humans, single nucleotide polymorphisms in genes involved in the WNT and BMP pathways are associated with predisposition to develop osteoarthritis (Loughlin, J, 2011; Loughlin, et al., 2004). This modulation/coordination may take place through a multitude of potential mechanisms including LRP4 acting as a receptor for the WNT and BMP inhibitors sclerostin (Choi, et al., 2009)and Wise (Ohazama, et al., 2010), as well as the WNT inhibitor DKK1 (Choi, et al., 2009), competing with the WNT co-receptors LRP5 and LRP6 and with the intracellular WNT machinery. The overexpression of LRP4 alone in primary articular chondrocytes was sufficient to increase the expression of SOX9 in primary chondrocytes, supported chondrogenic differentiation and inhibited WNT signalling in the ATDC5 cell line (Asai, et al., 2014), and , *in vivo*, mice carrying a hypomorphic LRP4 allele (Choi, et al., 2009; Johnson, et al., 2005) developed a significant skeletal phenotype. In this context, LRP4 may represent a hub where these signalling pathways converge and are modulated. The identification of a specific co-receptor repertoire and of a specific agonist, Agrin, capable of promoting the formation of phenotypically stable hyaline-like cartilage, resistant to vascular invasion and endochondral bone formation *in vivo*, is not only a unique therapeutic opportunity in cartilage regenerative medicine and in the field of osteoarthritis, but opens a new exciting avenue of investigation to understand how high specificity of a biological effect is achieved by modulating otherwise pleiotropic signalling pathways.

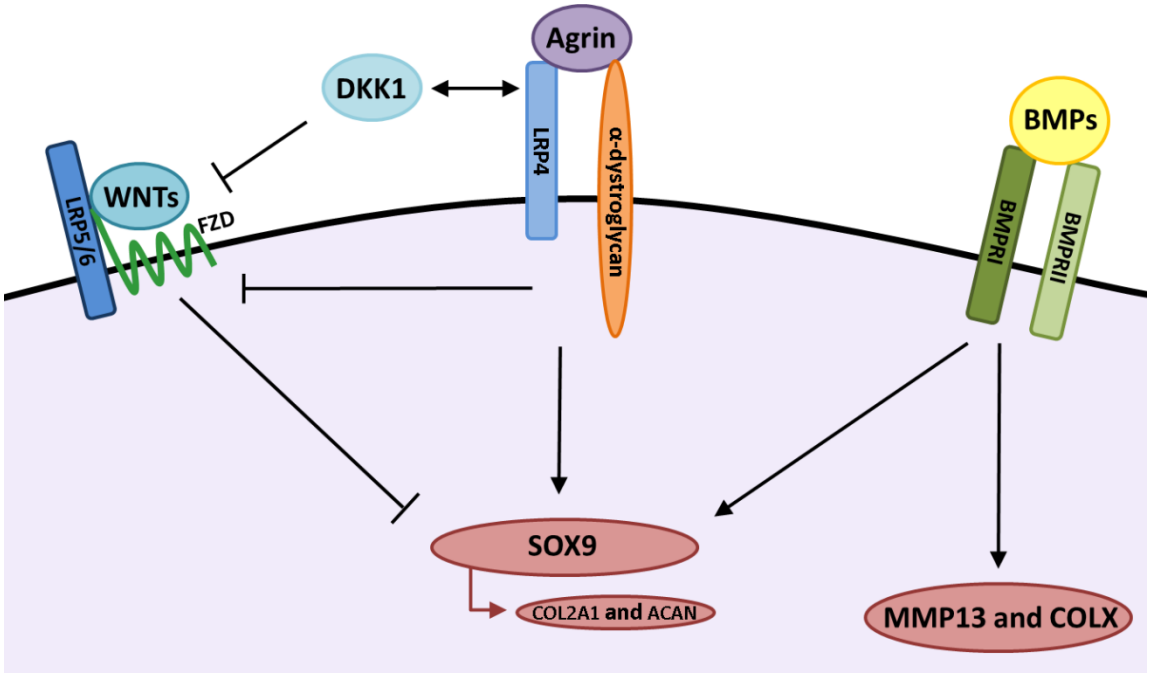


Figure 81. Agrin and LRP4 are central to a chondrogenic signalling network

Agrin is a paracrine signal which supports chondrocyte differentiation through a pathway requiring both LRP4 and α-dystroglycan which upregulates SOX9 and its transcriptional targets COL2A1 and ACAN. Agrin-induced chondrocyte differentiation is not associated with chondrocyte hypertrophy. LRP4 interacts with other pathways regulating chondrocyte differentiation including WNTs and BMPs.

Future Perspectives

Agrin as a therapeutic tool in OA and cartilage regeneration

The findings in this thesis revealed a novel action and mechanism by which Agrin is able to signal in adult skeletal tissue. One of the most interesting discoveries is that Agrin is able to increase SOX9, COL2A1 and ACAN mRNA expression levels without upregulating COL10A1, MMP13 *in vivo* and by inhibiting MMP3 *in vitro*. These data alone provide evidence that Agrin may have more favourable properties than other molecules currently tested in clinical trials for the treatment of cartilage damage and osteoarthritis such as BMPs. The data in this thesis have allowed me to secure a Rosetrees Trust personal fellowship and our group to obtain substantial funding from the UK Regenerative Medicine Platform (joint funded by the MRC, ARUK and Reumafonds) to pursue investigations into the use of Agrin for cartilage repair. This grant is funding my current research. The future of the project will be focussed on delivering Agrin to the site of the joint defect/cartilage injury in the form of an acellular device. Initial proof of concept funding from the Rosetrees Trust is aimed at optimising the delivery device, whether it be a hydrogel or collagen scaffold or other etc. The main project (funded by UKRMP) will develop a clinical grade device loaded with Agrin and to test its efficacy and safety in small and large animal models of OA and cartilage injury. In parallel to this we are aiming to determine which portion of the Agrin protein is responsible for its chondrogenic properties. To address this we seek to create deletion mutants of Agrin to isolate the smallest region possible to induce SOX9 upregulation which will enable us to further pursue the use of Agrin as therapeutic in joint defect repair.

Unravelling Agrin signalling

To fully understand the role Agrin plays in the articular cartilage in terms of other molecules, there are a number of smaller projects being carried out in our lab. Important outstanding questions include the following:

How does Agrin interact with its receptors?

Circumstantial evidence suggests that Agrin needs to bind to LRP4 and α-dystroglycan in order to support chondrocyte differentiation; however this evidence is only based on loss of function experiments. This issue must be addressed by demonstrating physical interaction. This will be likely done by co-immunoprecipitation using overexpression of tagged LRP4 and α-dystroglycan.

What is the molecular mechanism downstream of LRP4 and leading to SOX9 upregulation?

In some contexts LRP4 can function as a WNT inhibitor, and some preliminary experiments not included in this thesis confirm that Agrin induces suppression, or at least modulation of WNT signalling. How is this achieved and how this results in SOX9 upregulation is the subject of a new project.

In the final results chapter it was revealed not only that MMP3 cleaves a portion of Agrin, but also that this cleavage by MMP3 was able to negate the chondrogenic properties induced by Agrin. Further studies, currently ongoing in our lab, are aimed at mutating this MMP3

cleavage site to determine if this cleavage is responsible for the loss of the chondrogenicity or whether MMP3 is affecting the chondrocytes in an alternative way. In addition to these data sets, this MMP3-cleaved portion of Agrin has been identified as biomarker in conditions such as renal function and sarcopenia (Drey, et al., 2015; Marzetti, et al., 2014; Steubl, et al., 2013). Therefore the assessment of MMP3 mediated Agrin cleavage as a biomarker in early OA is another important research direction. It is also interesting to notice that sarcopenia is a frequent and disabling feature of OA. Therefore MMP3 mediated Agrin cleavage may have a pathogenic role in OA-associated sarcopenia and the development of MMP3-resistant Agrin may have therapeutic value.

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Appendix

Agrin sequencing results in human chondrocyte cDNA

Sequence obtained from putative y z region in human articular chondrocyte cDNA using primer shown in methods (see Methods - Sequencing):

5’CATGGGTGCCCTGCGTGTGGGCGACGGCCCCCGTGTGTTGGGGGAGTCCCCGGTTCCGCACACCGTCCTCAACC
TGAGGAGCCGCTCTACGTAGGGGGCGCTCCCGACTTCAGCAAGGTGGCCCGTGCTGCTGCCGTGTCCTCTGGCTTC
GACGGTGCCATCCAGCTGGTCTCCCTCGGAGGCCGCCAGCTGCTGACCCCGGAGCACGTGGTGCGGCAGGTGGAC
GTCACGTCCTTTGCAGGTCACCCCTGCACCCGGGCCTCAGGCCACCCCTGCCTCAATGGGGCCTCCTGCGTCCCGAG
GGAGGCTGCCTATGTGTGCCTGTGTCCCGGGGGATTGTCAGGACCGCACTGCGAGAAGGGGCTGGTGGAGAAGT
CAGCGGGGGACGTGGATACCTTGGCCTTTGACGGGCGGACCTTTGTAGAGTACCTCAACGCGGGCAGAGAGACG
AAGAGCCGTGCTG 3’

Translated into protein:

MGALRVGDGPRVLGESPVPHTVLNLKEPLYVGGAPDFSKVARAAAVSSGFDGAIQLVSLGGRQLLTPEHVVRQVDVTS
FAGHPCTRASGHPCLNGASCVPREAAYVCLCPGGLSGPHCEKGLVEKSAGDVDTLAFDGRTFVEYLNAGRETKSRA

Full Rat Agrin protein sequence (NP_786930.1); (y and z **region: bold**; **inserts: orange**):

MPPLPLEHRPRQEPGASMLVRYFMIPCNICLILLATSTLGFAVLLFLSNYKPGIHFTPAPPTPPDVCRGMLCGFGAVCEPS

VEDPGRASCVCKKNACPATVAPVCGSDASTYSNECELQRAQCNQQRRIRLLRQGPCGSRDPCANVTCSFGSTCVPSAD

GQTASCLCPTTCFGAPDGTVCGSDGVDYPSECQLLSHACASQEHIFKKFNGPCDPCQGSMSDLNHICRVNPRTRHPEM

LLRPENCPAQHTPICGDDGVTYENDCVMSRIGATRGLLLQKVRSGQCQTRDQCPETCQFNSVCLSRRGRPHCSCDRVT

CDGSYRPVCAQDGHTYNNDCWRQQAECRQQRAIPPKHQGPCDQTPSPCHGVQCAFGAVCTVKNGKAECECQRVCS

GIYDPVCGSDGVTYGSVCELESMACTLGREIQVARRGPCDPCGQCRFGSLCEVETGRCVCPSECVESAQPVCGSDGHTY

ASECELHVHACTHQISLYVASAGHCQTCGEKVCTFGAVCSAGQCVCPRCEHPPPGPVCGSDGVTYLSACELREAACQQ

QVQIEEAHAGPCEPAECGSGGSGSGEDDECEQELCRQRGGIWDEDSEDGPCVCDFSCQSVPRSPVCGSDGVTYGTEC

DLKKARCESQQELYVAAQGACRGPTLAPLLPVAFPHCAQTPYGCCQDNFTAAQGVGLAGCPSTCHCNPHGSYSGTCDP

ATGQCSCRPGVGGLRCDRCEPGFWNFRGIVTDGHSGCTPCSCDPRGAVRDDCEQMTGLCSCRPGVAGPKCGQCPDG

QVLGHLGCEADPMTPVTCVEIHCEFGASCVEKAGFAQCICPTLTCPEANSTKVCGSDGVTYGNECQLKAIACRQRLDIST

QSLGPCQESVTPGASPTSASMTTPRHILSKTLPFPHNSLPLSPGSTTHDWPTPLPISPHTTVSIPRSTAWPVLTVPPTAAAS

DVTSLATSIFSESGSANGSGDEELSGDEEASGGGSGGLEPPVGSIVVTHGPPIERASCYNSPLGCCSDGKTPSLDSEGSNC

PATKAFQGVLELEGVEGQELFYTPEMADPKSELFGETARSIESTLDDLFRNSDVKKDFWSVRLRELGPGKLVRAIVDVHF

DPTTAFQASDVGQALLRQIQVSRPWALAVRRPLQEHVRFLDFDWFPTFFTGAATGTTAAMATARATTVSRLPASSVTP

RVYPSHTSRPVGRTTAPPTTRRPPTTATNMDRPRTPGHQQPSKSCDSQPCLHGGTCQDQDSGKGFTCSCTAGRGGSV

CEKVQPPSMPAFKGHSFLAFPTLRAYHTLRLALEFRALETEGLLLYNGNARGKDFLALALLDGRVQFRFDTGSGPAVLTSL

VPVEPGRWHRLELSRHWRQGTLSVDGETPVVGESPSGTDGLNLDTNLYVGGIPEEQVAMVLDRTSVGVGLKGCIRML

DINNQQLELSDWQRAAVQSSGVGECGDHPCLPNPCHGGALCQALEAGMFLCQCPPGRFGPTCADEKSPCQPNPCHG

AAPCRVLSSGGAKCECPLGRSGTFCQTVLETAGSRPFLADFNGFSYLELKGLHTFERDLGEKMALEMVFLARGPSGLLLY

NG**QKTDGKGDFVSLALHNRHLEFCYDLGKGAAVIRSKEPIALGTWVRVFLERNGRKGALQVGDGPRVLGESP**KSRK**V**

PHTMLNLKEPLYIGGAPDFSKLARGAAVSSGFSGVIQLVSLRGHQLLTQEHVLRAVDVSPFADHPCTQALGNPCLNG

GSCVPREATYECLCPGGFSGLHCEKGLVEKSVGDLETLAFDGRTYIEYLNAVIESELTNEIPAPETLDSRALFS**EKALQSN**

HFELSLRTEATQGLVLWIGKAAERADYMALAIVDGHLQLSYDLGSQPVVLRSTVKVNTNRWLRIRAHREHREGSLQVG

NEAPVTGSSPLGATQLDTDGALWLGGLQKLPVGQALPKAYGTGFVGCLRDVVVGHRQLHLLEDAVTKPELRPCPTP

Human articular chondrocyte cDNA converted into protein and aligned with rat Agrin protein using protein BLAST (Altschul, 1997):

HcDNA 1742 DGPRVLGESP----VPHTVLNLKEPLYVGGAPDFSKLARAAAVSSGFDGAIQLVSLGGRQ 1797
DGPRVLGESP VPHT+LNLKEPLY+GGAPDFSKLAR AAVSSGF G IQLVSL G Q
rAGRN 1633 DGPRVLGESPKSRKVPHTMLNLKEPLYIGGAPDFSKLARGAAVSSGFSGVIQLVSLRGHQ 1692

HcDNA 1798 LLTPEHVLRQVDVTSFAGHPCTRASGHPCLNGASCVPREAAYVCLCPGGFSGPHCEKGLV 1857
LLT EHVLR VDV+ FA HPCT+A G+PCLNG SCVPREA Y CLCPGGFSG HCEKGLV
rAGRN 1693 LLTQEHVLRAVDVSPFADHPCTQALGNPCLNGGSCVPREATYECLCPGGFSGLHCEKGLV 1752

HcDNA 1858 EKSAGDVDTLAFDGRTFVEYLNAVTES----------EKALQSNHFELSLR 1898
EKS GD++TLAFDGRT++EYLNAV EKALQSNHFELSLR
rAGRN 1753 EKSVGDLETLAFDGRTYIEYLNAVIESELTNEIPAPETLDSRALFSEKALQSNHFELSLR 1812

Additional figures

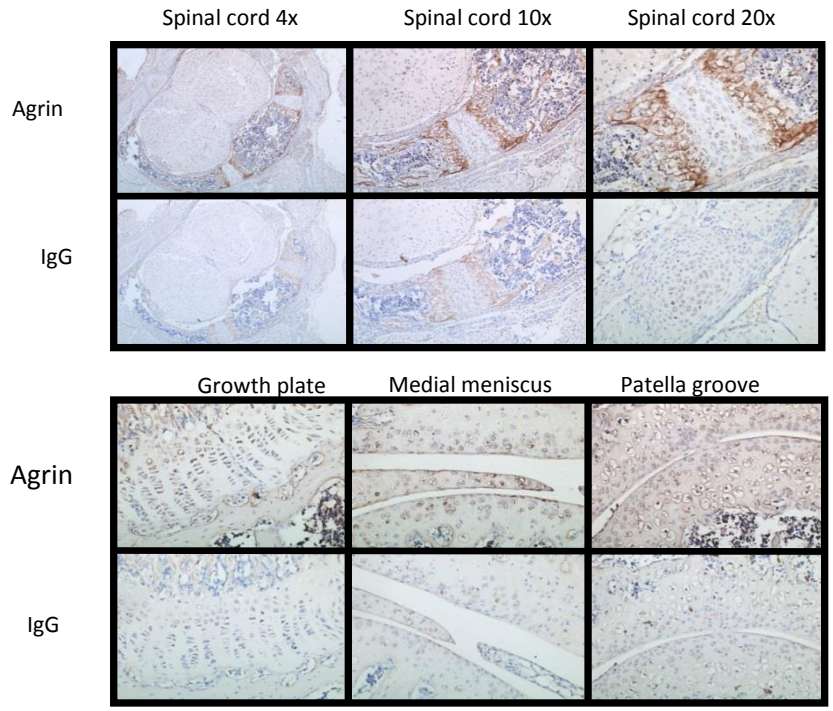

Figure 82. Agrin protein detection in the murine spinal cord and articular joint

Immunohistochemistry of Agrin protein in murine BALB/C spinal cord (above); growth plate, medial meniscus and the patella groove (below).

As Agrin is most commonly known for its role in neurons, sections of murine spinal cord were used as positive control for identifying Agrin at protein level (above) by immunohistochemistry. The growth plate, medial meniscus and patellar groove also revealed the presence of Agrin at protein level in the knee joint (below).

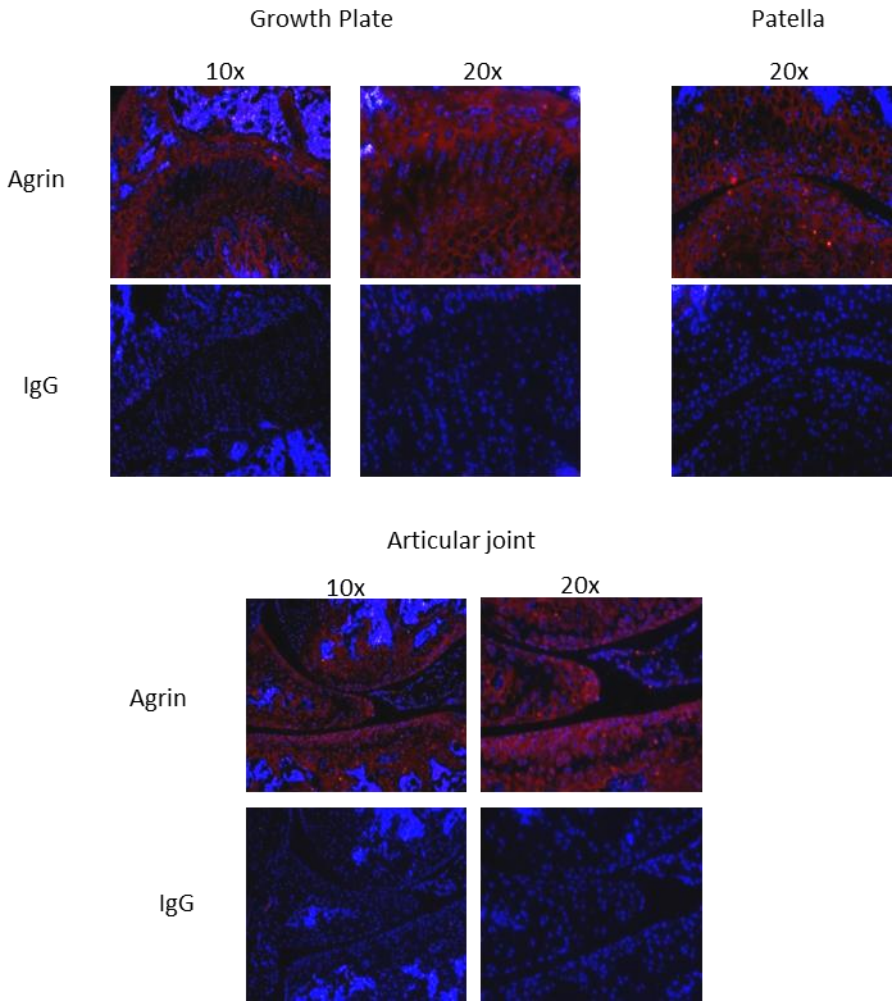

Figure 83. Agrin protein detection in the murine articular joint

Immunofluorescence detection of Agrin protein in healthy adult 129/Sv murine growth plate and articular joint.

Human osteoarthritis

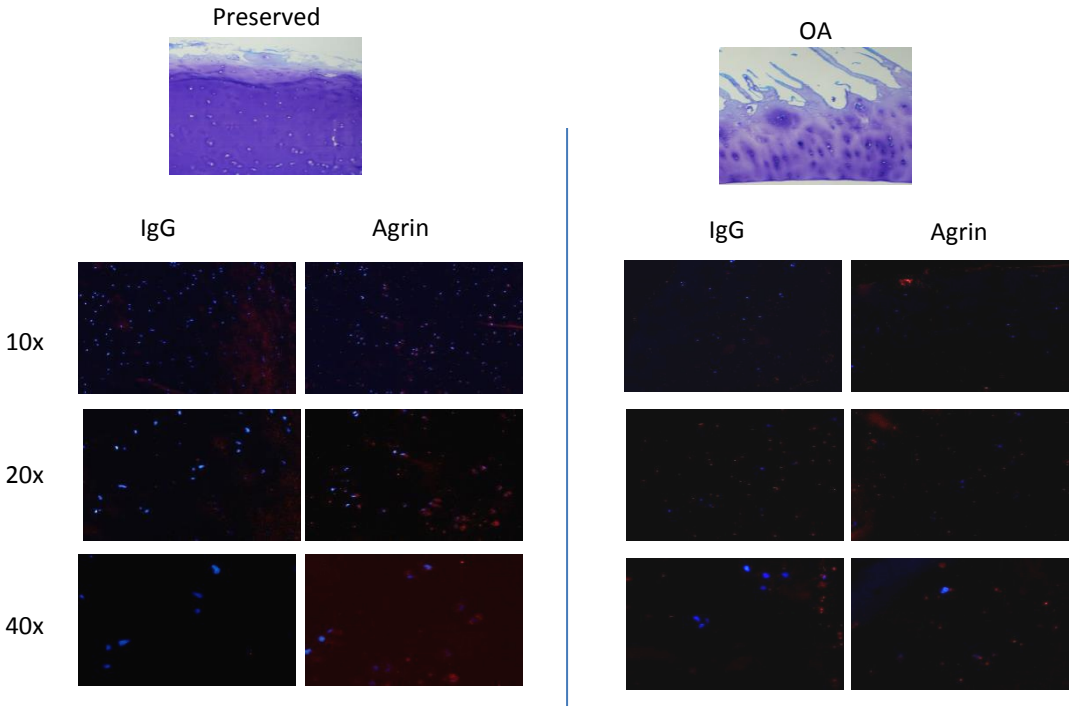

Figure 84. Comparison of Agrin in mild and severe OA in humans

Toluidine blue staining of 'preserved' (Mankin score >4) and 'osteoarthritic' (Mankin score <8) full thickness human articular cartilage. Agrin immunofluorescence at 10x, 20x and 40x. IgG served as negative control antibody.

A paired analysis of cartilage removed from two areas of the femoral condyle which were considered to be structurally distinct from the same patient by visual analysis. Cartilage samples were stained with toluidine blue and graded according to the Mankin scoring system and considered >4 or <8; mild or severe osteoarthritis. Sections stained for Agrin showed that even within the cartilage from the same patient, a distinct reduction in Agrin protein levels from the sections classed as severely osteoarthritic.

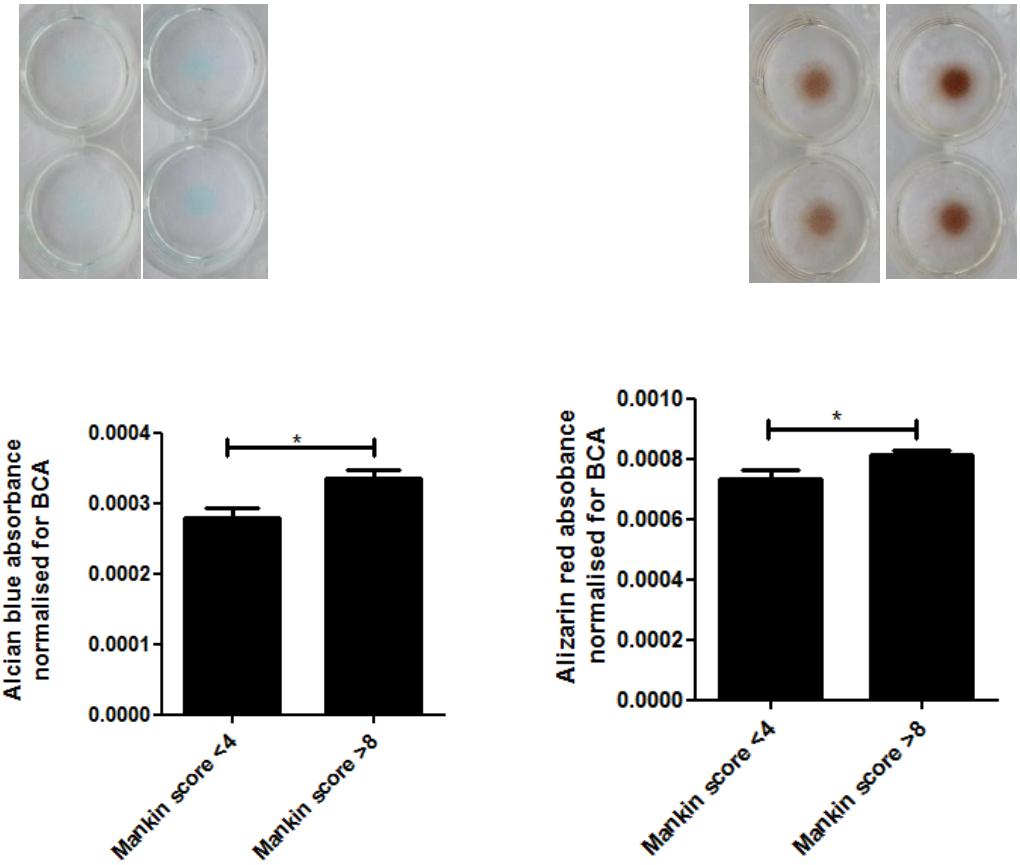

Figure 85. Comparison of GAGs andmineralisation in paired human chondrocyte cultures

Alcian blue and Alizarin red levels of staining from micromasses created with chondrocytes from a preserved area of a donor compared to a severely affected area measured by spectrophotometric quantification and normalised for protein content (n=1).

Chondrocytes isolated from these respective 'mild' or 'severe' areas were cultured in micromass and assessed by alcian blue and alizarin red staining. Despite and increased amount of sulphated proteoglycan production in the severely affected chondrocytes (as shown by alcian blue), this matrix was found to contain moremineralisation (alizarin red) and is off less articular chondrocyte quality. This increased matrix production may be due to the absence of catabolic factors from external sources such as the synovial fluid combined with the chondrocytes attempting to replace the lost matrix by increasing GAG synthesis.

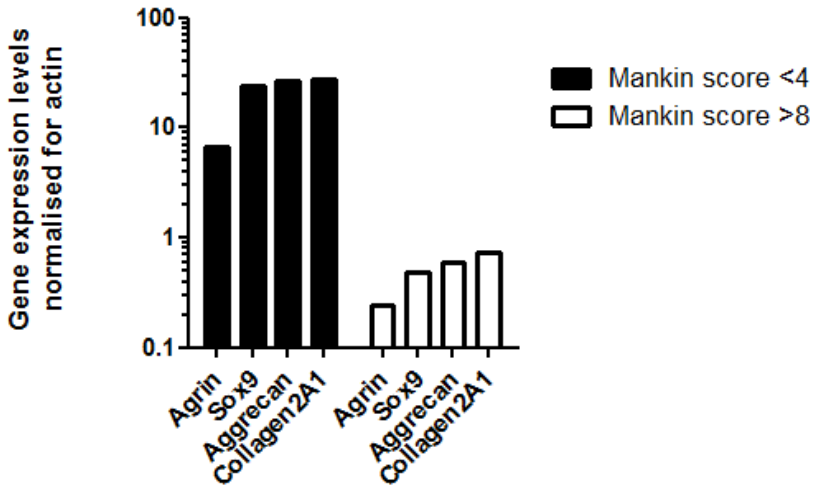

Figure 86. Gene expression analysis of paired osteoarthritic human chondrocytes

Analysis of AGRN, SOX9, ACAN and COL2A1 gene expression levels with chondrocytes obtained from different severity of OA within the same joint (n=1).

RNA extracted from these paired micromasses showed that mRNA levels of Agrin, the key chondrogenic transcription factor SOX9 and its downstream targets ACAN and COL2A1 were all expressed at much lower levels in the severely affected area. These data further corroborate what has been shown before (Yagi, et al., 2005). However, these data are preliminary and more donors with mild and severe osteoarthritic areas would be required for substantial evidence of these results.

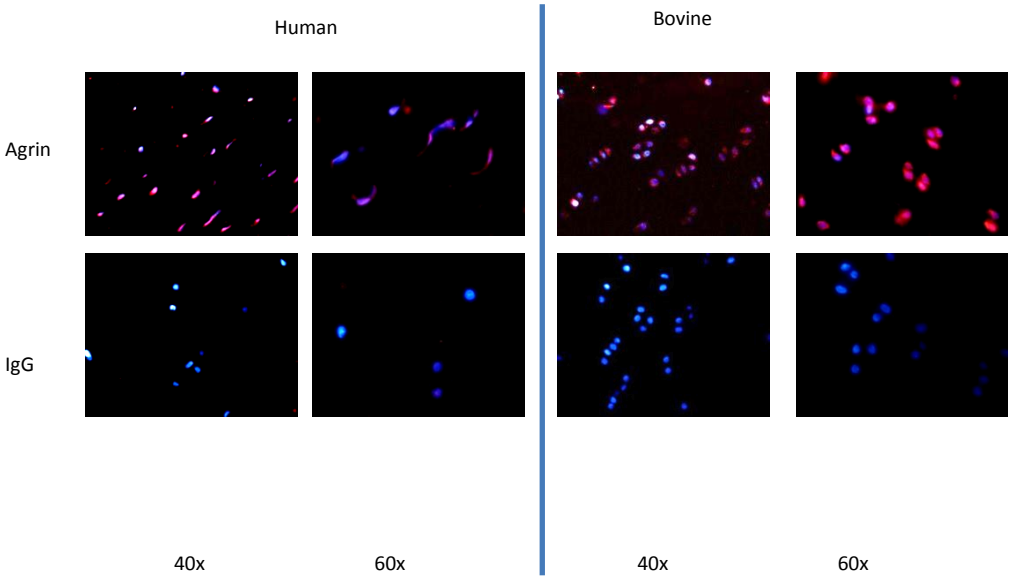

Figure 87. Agrin protein detection in healthy human and bovine cartilage explants

Agrin protein was detected in healthy adult human (left) and bovine (right) articular cartilage explants. Original fluorescence images are shown.

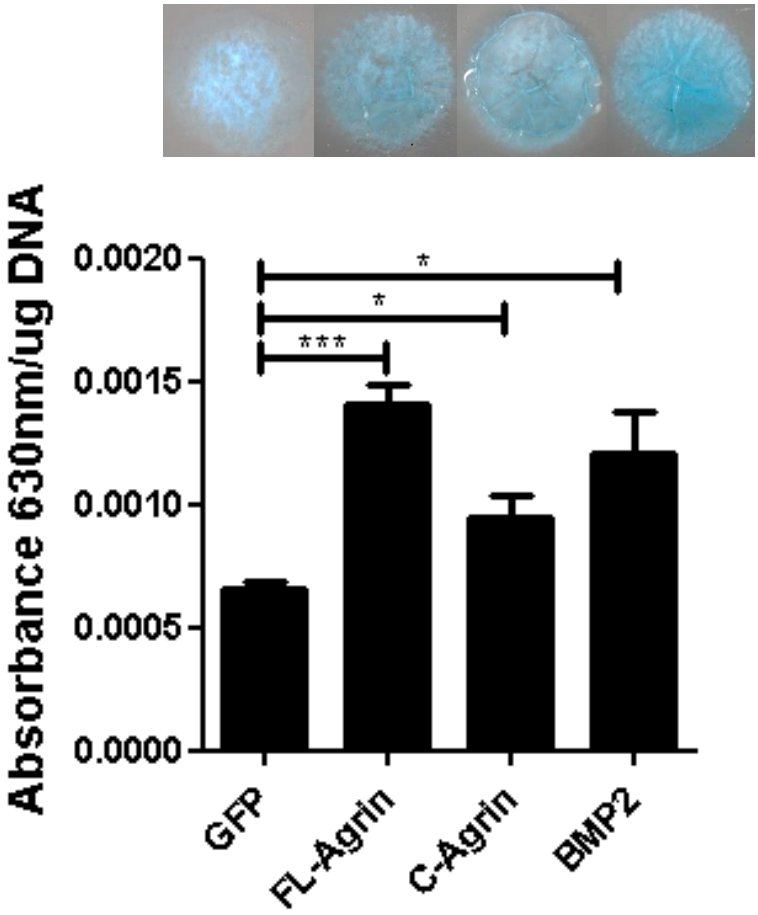

Figure 88. GAG analysis of C28/I2 cells transfected with FL-Agrin or C-Agrin

C28/I2 cells were transfected with GFP, FL-Agrin, C-Agrin or BMP and cultured in micromass for 4days and stained with Alcian blue. Alcian blue was quantified spectrophotometrically following guanidine extraction and normalised for DNA content (n=4).

C28/I2 cells were transfected with full length (FL-Agrin) or just the C-terminal portion of Agrin (C-Agrin) which, at the NMJ, is known to be sufficient to support aggregation AChRs. Micromasses were cultured and stained with Alcian blue and under these conditions, FL-Agrin increased GAG production more potently than C-Agrin, and thus was used in the majority of experiments.

MMP3 data

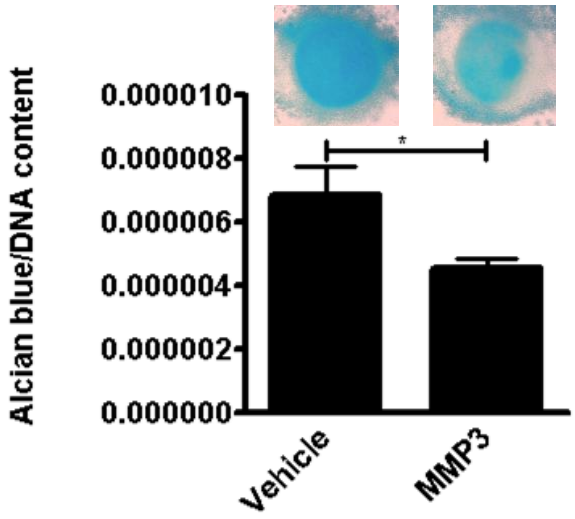

Figure 89. GAG analysis of MMP3 treated C28/I2 cells

C28/I2 cells were cultured in micromass for 3days. On the fourth day 0.5ug/ml MMP3 or vehicle was added to the cultured for a further 24hrs before being stained with Alcian blue. Alcian blue was quantified spectrophotometrically following guanidine extraction and normalised for DNA content (n=4).

Here we show that C28/I2 cells cultured in micromass in the presence of MMP3 have reduced ECM production or increased degradation. This outcome verified this model as a reliable *in vitro* model of what has previously been described *in vivo*.

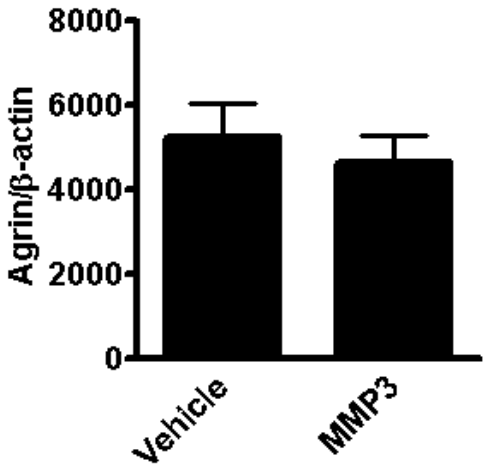

Figure 90. Gene expression level of Agrin in MMP3 treated C28/I2 cells

C28/I2 cells were cultured in micromass for 3days. On the fourth day 0.5ug/ml MMP3 or vehicle was added to the cultured for a further 24hrs. Gene expression analysis of Agrin mRNA levels was quantified by real time qPCR (n=4).

Due to the reduced ECM, lack of production or degraded, it was important to ensure MMP3 was not able to influence the regulation of Agrin. Here we show C28/I2 cells cultured in micromass in the presence of MMP3 do not have altered expression levels of Agrin at mRNA level.

Publications

- Nalesso*, G. and Thomas*, B.L., Yu, J., Addimanda, O., Sherwood, J.C., **Eldridge, S**., Dale, L., Schett, G., Zwerina, J., Eltawil., N., Pitzalis, C. and Dell'Accio F. (2016). WNT16 antagonizes excessive canonical WNT activation and protects cartilage in osteoarthritis. *Ann.Rheum.Dis.*
- **Eldridge, S.,** Nalesso, G., Ismail, H., Vicente-Greco, K., Kabouridis, P., Ramachandran, M., Niemeier, A., Herz, J., Pitzalis, C., Perretti, M. and Dell'Accio, F. (2015). Agrin mediates chondrocyte homeostasis and requires both LRP4 and α-dystroglycan to enhance cartilage formation *in vitro* and *in vivo*. *Ann.Rheum.Dis.*
- Sherwood, J. C., Bertrand, J., **Eldridge, S. E.,** & Dell'Accio, F. (2014). Cellular and molecular mechanisms of cartilage damage and repair. *Drug Discovery Today*, *19*(8), 1172–1177.