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WNT SIGNALLING IN JOINT REPAIR AND HOMEOSTASIS

By Giovanna Nalesso

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY AT THE UNIVERSITY OF LONDON

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ABSTRACT

Disruption of the Wnt canonical signalling leads to the development of osteoarthritis both in human and in mice but the underlying molecular mechanisms are poorly understood. Both forced activation and blockade of Wnt/β catenin signalling lead to cartilage breakdown. This study attempts to unravel the mechanisms leading to such paradox and is based on the hypothesis that ***WNT-3A triggers multiple signalling pathways simultaneously, with distinct outcomes.***

WNT-3A-stimulation induced activation of Wnt/β-catenin pathway in articular chondrocytes and promoted proliferation and loss of chondrocytes phenotype markers, such as *COL2A1*, *Aggrecan* and *SOX9* mRNA. However, whereas the inhibition of the Wnt/β-catenin pathway by DKK1 rescued the proliferative effect of WNT-3A, it did not rescue the loss of chondrocyte phenotype but, paradoxically, it further enhanced it. Therefore I tested the possibility that WNT-3A-induced chondrocyte de-differentiation could be mediated by other WNT pathways independent of β-catenin. Indeed, in AHAC WNT-3A induced intracellular calcium mobilization and phosphorylation and nuclear localization of CaMKII in a G-protein dependent manner, suggesting the activation of the Wnt/CaMKII pathway.

Inhibition of the Wnt/CaMKII pathway rescued the loss of the phenotypic markers *SOX9* and *COL2A1* induced by WNT-3A, indicating that this pathway drives WNT-3A-induced chondrocyte de-differentiation. Finally, my data show that the Wnt/β-catenin and the Wnt/CaMKII pathways are mutually inhibitory, explaining why both exogenous WNT-3A and its inhibitor DKK1 lead to chondrocyte de-differentiation: the first through direct activation of CaMKII, and the second indirectly by removal of the inhibition of CaMKII exerted by the β-catenin pathway.

My results show for the first time that a single WNT ligand can simultaneously activate at least two different pathways in the same cells with different outcomes. These findings highlight the possibility to therapeutically target individual outcomes of Wnt signalling, for instance to prevent chondrocyte de-differentiation without affecting crucial anabolic processes such as cell proliferation.

Once upon a time, there was a little girl named Goldilocks. […]
At the table in the kitchen, there were three bowls of porridge. Goldilocks was hungry. She tasted the porridge from the first bowl.

"This porridge is too hot!" she exclaimed.

So, she tasted the porridge from the second bowl.

"This porridge is too cold," she said.

So, she tasted the last bowl of porridge.

"*Ahhh, this porridge is **JUST RIGHT***," she said happily and she ate it all up.

From "The story of Goldilocks and the three bears"

Any intelligent fool can make things bigger and more complex... It takes a lot of courage to move in the opposite direction

Albert Einstein

AKNOWLEDGMENTS

I will always be grateful to my supervisor, Dr Francesco Dell'Accio, who has been my mentor, who taught me the important lesson that "a good scientist knows that 2+2 almost never is equal to 4", and who more importantly has a matchless and contagious enthusiasm and passion for science.

I owe a huge thanks to my lovely husband, who shared my happiness for my little discoveries and successes during my scientific journey, and more often supported me in the moments of discomfort and frustration.

A big thank to Prof. Pitzalis and all the colleagues at the EMR for the big scientific support they gave me during these years.

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Thanks to Alessandra, Fabia, Olga and Eli, not only colleagues, from whom I learned and I with whom I shared experiences, but real friends who know me as a person and not only as a scientist.

I thank MRC and AR UK for funding the projects I have been involved so far.

And finally, thanks to the United Kingdom: this country gave me the opportunity to become a scientist, and for this I will always be in debt.

DECLARATION

The work presented in this thesis is less than 100,000 words and was performed and analysed by the candidate except for the calcium mobilization assay done with PTX and with sFRP1 which were performed respectively by Miss Joanna Sherwood and Dr. Jessica Bertrand, the FACS analysis, who was performed in collaboration with Dr. Fulvio d'Aquisto and the His-tag staining who was performed by Dr. Noha Eltawil. The microarray analysis on cartilage explants cited in Fig. 19 was performed by Dr. Francesco Dell'Accio (Dell'Accio et al., 2008). Dr. Francesco Dell'Accio closely supervised the project providing scientific guidance and advice regarding experimental design and planning as well as interpretation of the results and critical revision of the manuscript.

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SUPERVISORS
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Prof. Costantino Pitzalis

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ABBREVIATIONS

AHAC	adult human articular chondrocytes
ADAMTS	A-disintegrin and metalloproteinases with thrombospondin motifs
AER	Apical Ectodermal Ridge
AP	Alkaline Phosphatase
BMP	Bone morphogenetic protein
BSA	Bovine Serum Albumin
CAMKII	Calcium calmodulin kinase II
CKA	Chemokine alpha 3
ColI	collagen type I
ColII	collagen type II
ColVI	collagen type VI
ColX	collagen type X
CMV	Cytomegalovirus
COMP	cartilage oligomeric protein
CS	chondroitin sulphate
Cys	Cysteine
DMEM	Dulbecco's modified Eagle's medium
DMM	Destabilization of the medial meniscus
DS	dermatan sulphate
DVL	Dishevelled
ECM	Extracellular matrix
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FZD	Frizzled
GAG	Glycosaminoglycans
GCP2	Granulocyte chemotactic protein 2
GDF	Growth differentiation factors
GPCR	G-protein coupled receptors
GSK3	Glycogen Synthase Kinase 3
HA	Hyaluronic acid
HS	Heparan sulphate
HSPG	Heparan sulphate proteoglycans
Hyp	Hydroxyproline
IGD	Intraglobular domain
IGF1	Insulin growth factor 1
Ihh	Indian Hedgehog
IL-1	Interleukin 1
KS	Keratan sulphate
LB medium	Luria-Broth medium
LEF	Lymphoid enhancer binding factor
LiCl	Lithium chloride
LRP5/6	Low density lipoprotein receptor-related receptor

OA	Osteoarthritis
OM	Optimem
MMP	Metalloproteinase
N-CAM	Neural cell adhesion molecule
PCP	Planar cell polarity
PI3K	Phosphoinositol-3-kinase
PBS	Phosphate Buffer Solution
PMA	Phorbol-12-myristate-13-acetate
Pro	Proline
PTHrP	Parathyroid hormone related peptide
o/n	Overnight
RT	Room Temperature
Shh	Sonic Hedgehog
SOX9	SRY (sex determining region Y)-box 9
TCF	T-cell factor
TNFα	Tumor Necrosis Factor α
VEGF	Vascular endothelial growth factor
ZPA	Zone of Polarizing Activity

BACKGROUND

Activation of Wnt-mediated signalling cascades is associated with many biological processes, from embryo development to the maintenance of the homeostasis of adult tissues.

In particular, modulation of the Wnt/β-catenin signalling has been demonstrated to be of paramount importance for joint formation (Guo et al., 2004;Hartmann and Tabin, 2001;Koyama et al., 2008) and for chondrocyte differentiation (Church et al., 2002;Goldring, 2006;Yamaguchi et al., 1999).

The concept that WNT/β-catenin signalling plays an important role in osteoarthritis (OA) is now established not only for the abundant data demonstrating activation of this pathway in OA cartilage (Dell'Accio et al., 2008;Zhu et al., 2009) but also for the genetic association of mutations that result in disregulation of Wnt signalling with OA predisposition (Lories et al., 2006;Loughlin et al., 2004;Lories et al., 2006;Loughlin et al., 2004) and several functional studies both *in vitro* and *in vivo* (Akiyama et al., 2004;Koyama et al., 2008;Ryu et al., 2002;Ryu and Chun, 2006).

However, paradoxically, both activation and repression of this signalling cascade result in OA (Chen et al., 2008;Koyama et al., 2008;Lories et al., 2007;Zhu et al., 2008;Zhu et al., 2009).

The aim of this study was to understand the molecular basis of this paradox and to test the hypothesis that individual Wnt ligands can modulate chondrocyte phenotype through multiple signalling pathways each with individual outcomes.

Before describing my work, I will provide an overview of general cartilage biology, osteoarthritis and Wnt signalling.

CHAPTER 1: INTRODUCTION

LIMB DEVELOPMENT:

The appendicular skeleton develops from the lateral plate mesoderm within the limb buds which are constituted by a core of mesenchymal cells covered by a layer of ectoderm (Karsenty, 2003). The limb buds are first visible in the human embryo after 4 weeks from the beginning of the gestation. According to a well-established model (Tickle, 1994;Fallon and Kelley, 1977) the morphogenesis of the limb bud is regulated by two signalling centres: the apical ectodermal ridge (AER), which, through the secretion of fibroblast growth factor 8 (FGF8) regulates the proximo-distal axis acting within a specialized area of the limb bud mesenchyme called the progress zone (Fallon et al., 1994;Niswander and Martin, 1992;Sun et al., 2002;ten Berge et al., 2008) and the zone of polarizing activity (ZPA), which regulates the anterior-posterior axis through the formation of a gradient of the morphogen Sonic Hedgehog (Shh) (Niederreither et al., 1999;Drossopoulou et al., 2000). Finally, the secretion of WNT-7A by the dorsal ectoderm determines the dorso-ventral axis (Kengaku et al., 1997;Parr and McMahon, 1995)(Fig. 1).

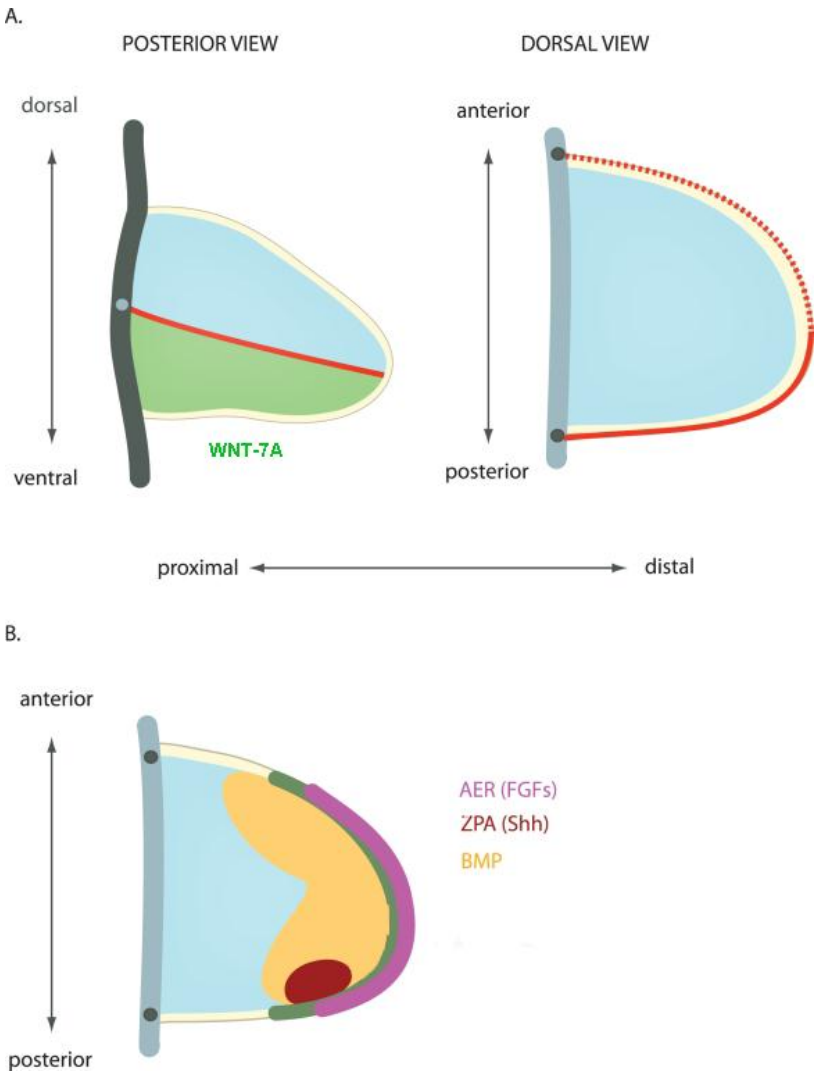


Figure 1: Limb patterning

A) Geometry of the limb bud. white: ectodermal layer, blue: dorsal mesenchyme, green: ventral mesenchyme. Red line indicates the dorso-ventral boundary (solid - posterior, dashed - anterior). The thick grey lines represent the flank, with the dots indicating the points of cross-section between the posterior and the dorsal view. B) Expression domains of patterning signals. The AER, expresses FGF encoding genes (such as *Fgf8*). The ZPA, is the source of Sonic Hedgehog (Shh). Figure modified from Kicheva and Briscoe (Kicheva and Briscoe, 2010).

Positional information along all the axes needs to integrate with each other in order to ensure the proper limb pattern. How the different structures of a limb are determined during limb development, is still under debate. A theory proposes that the amount of time the undifferentiated cells spend within the progress zone specifies the position and the identity that these cells will acquire in the developed limb (Summerbell et al., 1973). According to a

more recent theory (Dudley et al., 2002), cells in the progress zone would acquire their positional identity immediately after limb bud formation and they would retain it during the growth of the limb (Dudley et al., 2002)(Fig. 2).

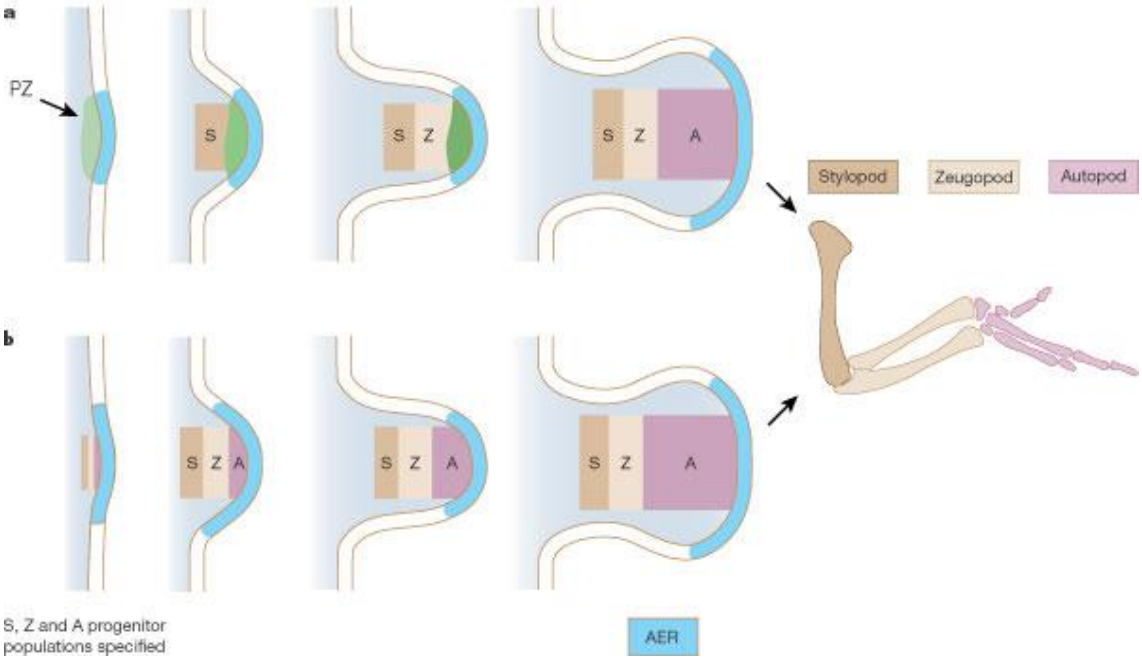


Figure 2: Progress zone model vs early specification model

a) The progress zone (PZ) model proposes that the final position of a cell in the developed limb is determined as the cell leave the progress zone (in green) at the edge of the limb bud. **b**) The early specification model proposes that the positional information is acquired already in the early limb bud. The cells then undergo considerable expansion before becoming determined to form the different skeletal elements, as indicated (Mariani and Martin, 2003).

SKELETOGENESIS

Different types of tissues originate from the limb bud mesenchyme, including cartilage, perichondrium, tendons, connective tissue and dermis (Pearse et al., 2007;SEARLS, 1965;Stark and SEARLS, 1973). Myogenic cells migrate from the somite into the limb bud, differentiating following a pattern determined by the interaction of these precursors with the connective tissue (Kieny et al., 1979;Kieny and Chevallier, 1979;Chevallier et al., 1978;Chevallier et al., 1977). Soon after the formation of the limb bud, the mesenchymal cells condensate in the centre to form a chondrogenic core (Fig. 3a) (Kronenberg, 2003). Mesenchymal cell condensation is mediated by cell-cell and cell-matrix interactions. N-cadherin and Neural Cell

Adhesion Molecule (N-CAM) mediate cell-cell adhesion and are important for the initiation and maintenance of condensation (Widelitz et al., 1993;DeLise and Tuan, 2002a;DeLise and Tuan, 2002b;Molenaar et al., 1996;Oberlender and Tuan, 1994b;Oberlender and Tuan, 1994a). Cell-matrix interactions are instead mediated by fibronectin, cartilage oligomeric protein (COMP), tenascin, and syndecans (DeLise et al., 2000).

The cells at the periphery of the condensation core become flattened and elongated and start forming the perichondrium (Goldring, 2006).

Soon after condensation, the cells in the centre of the condensation (diaphysis) undergo chondrogenesis and deposit large amounts of cartilage-specific extracellular matrix. Chondrocyte differentiation is associated with the expression of the transcription factor SRY (sex determining region Y)-box 9 (Sox9) (Bi et al., 2001;Akiyama et al., 2004). Sox9 is strictly required for chondrogenesis and directly drives the expression of collagen type 2 (ColII), the hallmark of cartilage tissue (Akiyama et al., 2007).

Afterwards, the cartilage anlagen become segmented, and, within the individual skeletal elements, chondrocytes undergo a coordinated process of proliferation and maturation which culminates in hypertrophy. Hypertrophic chondrocytes undergo terminal differentiation, driving the secretion of mineralized cartilage and the invasion of the cartilage anlagen with blood vessels, after which they undergo apoptosis. With vascular invasion, several cell types populate the cartilage anlagen including chondroclasts/osteoclasts, which degrade the matrix, and osteoblasts, which form bone at the expense of the cartilaginous template. This process is known as endochondral bone formation (Goldring, 2006;Shimizu et al., 1997)(Fig. 3).

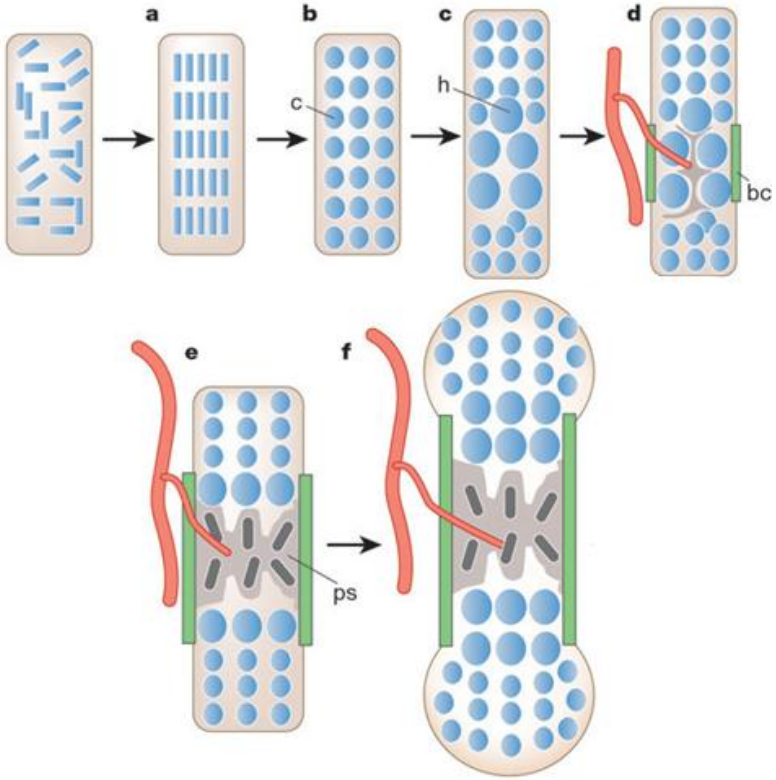


Figure 3: Endochondral bone formation

a) Mesenchymal cell condensation **b)** Differentiation of mesenchymal cells into chondrocytes (c) **c)**, Hypertrophic maturation starts from the centre of condensation (h) **d)** Perichondrial cells adjacent to hypertrophic chondrocytes differentiate into osteoblasts, forming bone collar (bc). Hypertrophic chondrocytes direct the formation of mineralized matrix, attract blood vessels, and undergo apoptosis **e)** Osteoblasts accompany vascular invasion, forming the primary spongiosa (ps) **f)** Chondrocytes continue to proliferate, lengthening the bone. Osteoblasts of primary spongiosa are precursors of eventual trabecular bone; osteoblasts of bone collar become cortical bone (Kronenberg, 2003)

SYNOVIAL JOINT FORMATION

As mentioned previously, the appendicular skeletal elements originate from segmentation of continuous anlagen of condensed mesenchymal cells. Such process of segmentation is at the basis of synovial joint formation. The first step in joint formation is the condensation of mesenchymal cells in structures called joint interzones. Joint interzones are constituted by densely packed flattened cells, oriented with their major axis perpendicular to the major axis of the condensation, connected by gap junctions, clearly demarcating the future site in which the articular surfaces of two adjacent bones will face each other (Fig. 4). The cells of the joint interzone have been demonstrated to give origin to the permanent articular cartilage,

synovium, tendons, ligaments and joint capsule (Koyama et al., 2008;Pacifici et al., 2006). Interzone cells express several molecular markers including GDF5/CDMP1 (Chang et al., 1994;Storm and Kingsley, 1999), Noggin, a BMP antagonist (Brunet et al., 1998), CD44, receptor for hyaluronic acid (Edwards et al., 1994), autotoxin, lubricin, the COL2A1-A splicing isoform (Gao et al., 2011;Koyama et al., 2008;Sandell et al., 1991;Sandell et al., 1994) and are known to differentiate into chondrocytes when cultured *in vitro* (Pacifici et al., 2006). In addition, some members of the Wnt family – WNT-4, WNT-14 and WNT-16 - are early and specific markers of the joint interzones (Guo et al., 2004;Hartmann and Tabin, 2001) and mediate the activation of the Wnt/β-catenin pathway at the site of joint formation (Guo et al., 2004;Hartmann and Tabin, 2001;Koyama et al., 2008).

The joint interzones are formed by three layers of cells. The middle one is composed of very packed cells believed to give rise to the articular cartilage, in which cells express increasing levels of hyaluronan (HA), promoted by mechanical stimulation of the developing limb (Dowthwaite et al., 1998;Dowthwaite et al., 1999;Osborne et al., 2002;Pitsillides et al., 1995). High concentrations of HA leads to the saturation of the hyaluronan receptor CD44, followed by cell apoptosis and consequent joint cavitation (Toole, 1990). The outer layers of the cells in the interzone participate in the growth of the anlagen (Ito and Kida, 2000) and further differentiate into the cells of the different tissues of the joint (Koyama et al., 2008).

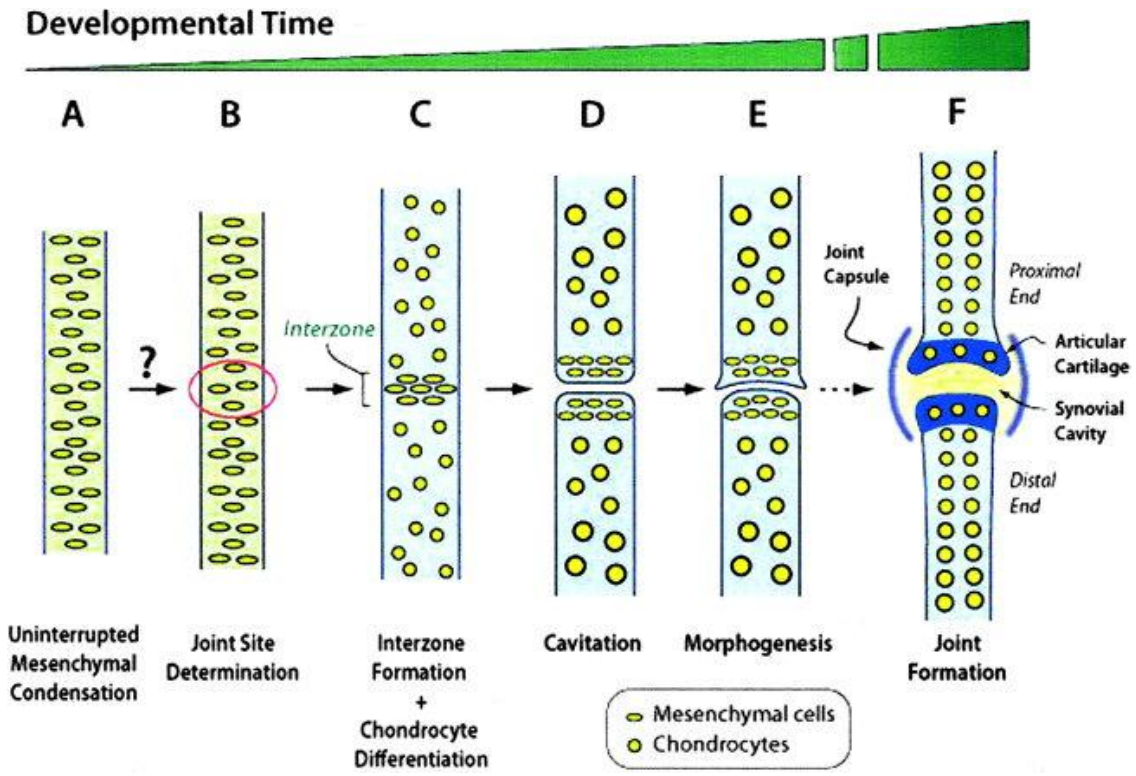


Figure 4: Synovial joint formation

A: Condensation of mesenchymal cells **B:** Partially understood mechanisms determine the site of synovial joint formation **C:** Formation of the joint interzone. In the meantime, the rest of the condensed mesenchymal cells differentiate into chondrocytes and establish the cartilage elements. **D:** Beginning of the cavitation process which will eventually lead to physical separation of the adjacent skeletal anlagen and formation of a synovial cavity. **E:** Morphogenetic processes begin to mold the opposing sides of the joint into reciprocally-shaped and interlocking structures. **F:** Differentiation of the mesenchymal cells in the distinct components of the mature synovial joint tissues (Pacifici et al., 2005).

THE ARTICULAR CARTILAGE

Articular cartilage is the avascular connective tissue covering the surfaces of the diarthrodial joints. Its function is to allow a frictionless motion and to support and distribute loads on the underlying bone.

Articular cartilage is mainly constituted by a highly hydrated extracellular matrix (ECM), composed by collagens and proteoglycans which surround the only cell type present in the tissue, the chondrocyte.

The articular cartilage can be divided into four different zones, in which ECM and chondrocytes differ respectively in composition and in the expression of biochemical markers (Fig. 5). The most superficial zone is characterized by the presence of flattened chondrocytes expressing lubricin, an O-linked glycosylated protein first identified in the synovial fluid (Swann et al., 1985), important for the lubrication of the articular surface and inhibiting the overgrowth of synovial cells at the same time (Flannery et al., 1999;Rhee et al., 2005). Chondrocytes in this zone are surrounded by collagen fibres running parallel to the surface of the tissue. Chondrocytes of the middle or transitional zone have a more rounded shape and collagen fibres are organized in arches in which tangential fibres from the superficial zone become perpendicularly orientated and are in continuity with those in the deep zone. In the deep zone, chondrocytes are organized in columns and here the concentration of proteoglycans is the highest. In the calcified zone chondrocytes express type X collagen (ColX) and alkaline phosphatase (AP) and have a hypertrophic phenotype (Linsenmayer et al., 1986).

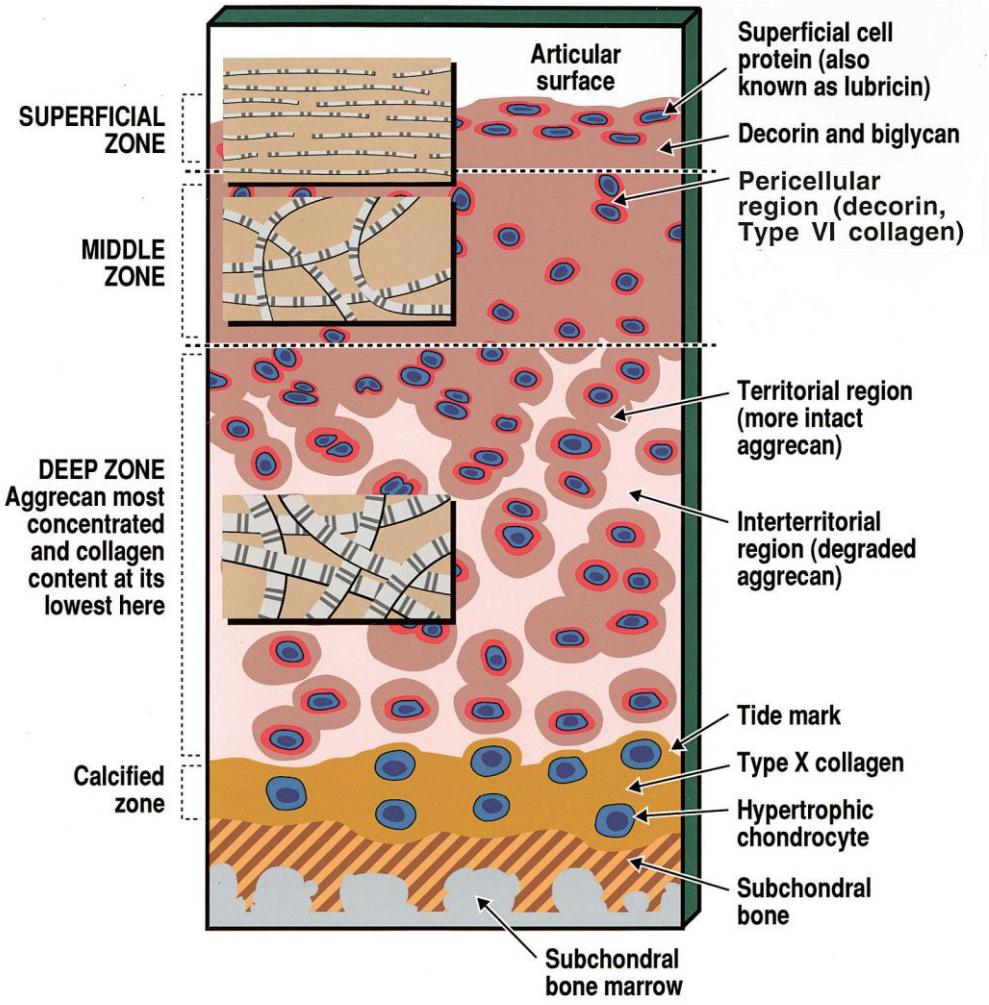


Figure 5: Organization of the articular cartilage

Articular cartilage can be divided in 4 different morphological and functional areas, as indicated by the figure (Poole et al., 2001).

The composition of the ECM varies depending on their proximity to the chondrocytes and therefore three different regions can be distinguished in the healthy articular cartilage. The ECM immediately surrounding the chondrocyte is called pericellular matrix and it is enriched in collagen type VI (ColVI) (Poole et al., 1988) and perlecan (Costell et al., 1999), along with membrane-associated proteins such as fibronectin, allowing the anchorage of chondrocytes to the ECM. The areas surrounding clusters of chondrocytes or with the higher cellular density are instead defined as intraterritorial matrix. It is mainly constituted by collagen type II (ColII) and chondroitin sulphate proteoglycans forming a tight framework. Finally the remaining part

of the ECM is called interterritorial matrix, and it is manly acellular. Here collagens and proteoglycans are arranged as described for each layer in Fig. 5.

THE CHONDROCYTE

The only cell type present in the articular cartilage is the chondrocyte. Chondrocytes are round shape cells of approximately 13µm of diameter sitting in matrix cavities called lacunae. They represent between the 5 and the 10% of the total cartilage volume. Their role is to support and maintain the trophism of the cartilage matrix (Lin et al., 2006). Articular cartilage is an aneural, avascular and alymphatic tissue, therefore chondrocytes must rely on diffusion of metabolites and nutrients for their sustenance.

As opposed to the epiphyseal chondrocytes which are responsible for the formation of the cartilage anlagen and are replaced by bone during endochondral bone formation, articular chondrocytes are phenotypically stable through their life, are resistant to vascular invasion and endochondral bone formation (Dell'Accio et al., 2001). From a topological point of view it might be tempting to speculate that articular chondrocytes originate from the growth plates and escape terminal differentiation. However, recent lineage tracking studies using the Cre-LoxP technology have confirmed that articular chondrocytes differentiate from interzone cells and therefore have a distinct embryological origin acquired very early on during skeletogenesis (Koyama et al., 2008)

Articular chondrocytes are able to grow in anchorage independent conditions (Benya and Shaffer, 1982;Benya et al., 1978) and a have remarkable phenotypic stability that allows them to retain their phenotype *in vitro* for some passages (Benya and Shaffer, 1982) and even to form ectopic stable cartilage following intramuscular implantation in immunodeficient mice (Dell'Accio et al., 2001). Late passage chondrocytes can be partially and transiently re-differentiated in anchorage independent culture, but do not re acquire their phenotypic stability and when implanted into damaged muscle, tend to differentiate into cells of the muscular lineage (Dell'Accio et al., 2003a).

THE EXTRACELLULAR MATRIX:

COMPOSITION:

COLLAGENS:

Fibrillar collagens are polymers composed by three α-polypeptide chains arranged triple helix. The mechanism of synthesis of collagen is described in Fig. 6. Depending on the collagen type, the three α-chains may either be identical or differ. The characteristic amino acidic sequence of all the types of collagens is constituted by the repetition of Gly-X-Y triplets, where X is very often a Proline (Pro) and Y a 4-hydroxyproline (Hyp), a post-translationally modified form of Pro catalysed by prolyl hydroxylase. The presence of Hyp gives a high stability to collagen, due to the higher formation of intramolecular hydrogen bonds, involving the interaction with molecules of water. The organization in fibres and the high stability of collagen make it responsible for the high resistance to tensile strength of articular cartilage (Voet and Voet, 1995). The type of collagen mainly expressed in the articular cartilage ColII. ColII consists of 3 α1 chains and is cross-linked and stabilised by fibril-associated collagens, type IX and type XI. ColVI is found within the pericellular matrix of cartilage where it contributes to chondrocyte attachment and ECM interactions (Poole et al., 2001;Guilak et al., 2006). ColX is expressed by hypertrophic chondrocytes and is hence found within the calcified layer (Eyre, 1991).

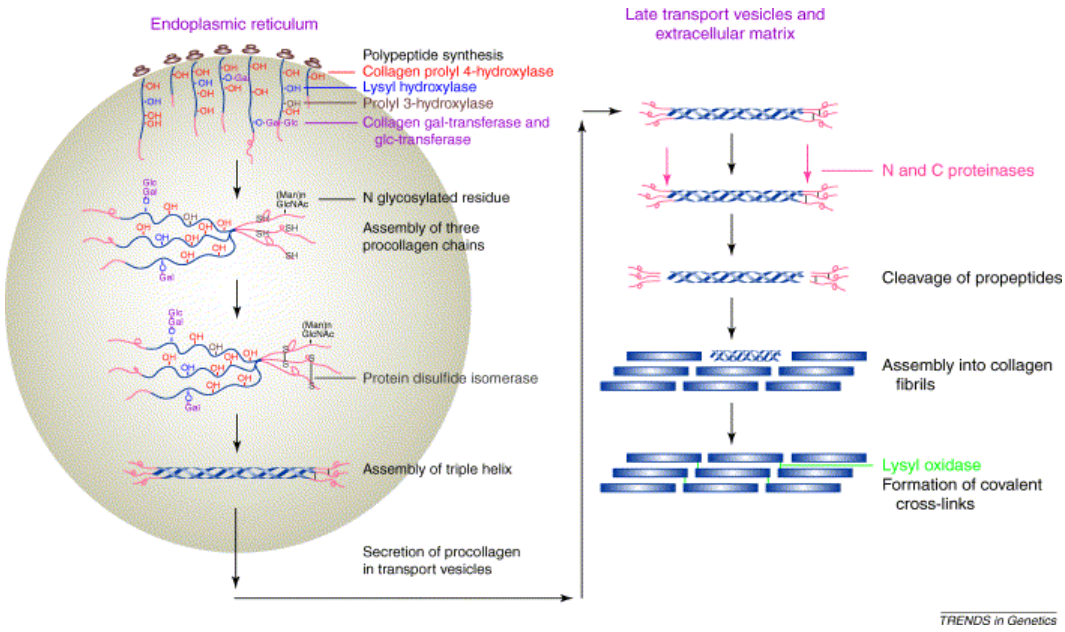


Figure 6: Synthesis of collagen

Collagen synthesis: a) The polypeptide chains are synthesized in the ribosomes and secreted into the lumen of the endoplasmic reticulum, where they are subjected to different post-translational modification such as hydroxylation of Pro and Lys and glycosylation of other amino acidic residues. b) The C-pro-peptides are then assembled in the characteristic triple helix and secreted in the extracellular space. The subsequent steps are c) cleavage of the N and C pro-peptides, d) spontaneous self-assembly of the resulting collagen molecules into fibrils, and e) formation of covalent crosslinks. Figure from (Myllyharju and Kivirikko, 2004)

PROTEOGLYCANS:

Proteoglycans are heavily glycosylated proteins, constituted by a protein core to which one or more glycosaminoglycan (GAG) chains are covalently attached. GAGs are long polysaccharide chains made by repetitions of disaccharide groups, each carrying an amino sugar and either a carboxylate or a sulphate group, conferring to these molecules a high negative charge. This negative charge attracts cations including Na^{2+}, which increase the osmolarity of the cartilage. Due to this property, water molecules attracted into the tissue are retained within the macromolecular network of GAG chains amongst the collagen network, providing articular cartilage with its high resistance to compressive loads.

Different GAGs define the pool of proteoglycans present in the articular cartilage, the most expressed being chondroitin sulphate (CS), heparan sulphate (HS), keratan sulphate (KS), dermatan sulphate (DS) proteoglycans and hyaluronic acid (HA).

The proteoglycans mostly expressed in the articular cartilage is aggrecan (Fig. 7).

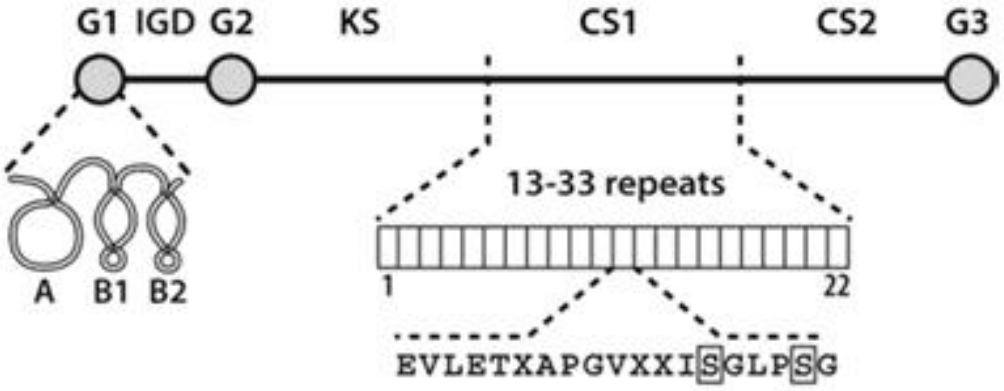


Figure 7: Structure of Aggrecan

Aggrecan is composed by three globular domains (G1, G2, G3), and interglobular domain (IGD) and multiple KS and CS units (Doege et al., 1991).

The protein core of aggrecan is constituted of three globular domains (G1, G2 and G3), each having cysteines which can participate to the formation of disulphide bonds (Doege et al., 1991). G2 and G3 regions are separated by a long GAG chain made of KS and CS units. G1 is situated at the N-terminus of the protein and can be divided in three subdomains, A, B1 and B2. B domains are responsible for the interaction with HA (Watanabe et al., 1998). A molecule of HA may interact with as many as 100 molecules of aggrecan, giving origin to massive aggregates. Each of this interaction is stabilized by link proteins (Morgelin et al., 1988). The G3 domain is at the C-terminus end, it is responsible for the proper post-translational modification of the molecule and its secretion (Roughley, 2006).

In addition to aggrecan, other smaller leucine-rich proteoglycans are expressed in the hyaline cartilage including the dermatan sulphate proteoglycans biglycan and decorin, the keratan sulphate proteoglycans fibromodulin and lumican (Roughley, 2006) and the heparan sulphate proteoglycan perlecan, abundant in the pericellular matrix directly surrounding the chondrocytes (Melrose et al., 2006). These proteoglycans account only for a small percentage of the total volume of the ECM and they do not contribute in a substantial way to the mechanical properties of the tissue. Nevertheless, besides contributing to stabilize the ECM (Knudson and Knudson, 2001), they play important roles in regulating cartilage homeostasis by binding and modulating the activity of growth factors, by establishing functional morphogenetic gradients, and by directly acting as signalling molecules (Echtermeyer et al., 2009).

The absence of genes involved in the synthesis or post-translational modification of heparan sulphate proteoglycans (HSPG) have been shown to lead to aberrant joint phenotypes, such as joint fusions and bone growth delay both in mouse and in zebrafish (Mundy et al., 2011;Wiweger et al., 2010). In addition Syndecan-3, another HPSG, can bind bone morphogenetic protein 2 (BMP2) and modulate its activity during cartilage differentiation (Fisher et al., 2006).

In human cartilage, HSPG perlecan has been shown to bind and sequester FGF2 (Vincent et al., 2007) and FGF18/FGFR3 (Chuang et al., 2010), suggesting that this proteoglycan might be involved in chondrocyte response to mechanotransduction and in the modulation of proliferative signalling. Decorin, biglycan and fibromodulin bind the chondrogenic factor transforming growth factor β (TGFβ) (Hildebrand et al., 1994) and the same small proteoglycans also bind and modulate the activity of matrix metalloproteinase 13 (MMP13) (Zhang et al., 2010) an enzyme involved in matrix remodelling and OA pathogenesis (Mitchell et al., 1996). Recent studies also demonstrate that syndecan 4 (Syn 4) is involved in the activation of A-disintegrin and metalloproteinases with thrombospondin motifs 5 (ADAMTS5)(Echtermeyer et al., 2009), a proteinase largely involved in cartilage degradation in

OA (Glasson et al., 2005;Glasson et al., 2004), and mice deficient of Syn 4 are protected from the development of the disease (Echtermeyer et al., 2009).

Finally, HPSGs are also required to maintain the solubility (Fuerer et al., 2010) and activity (Han et al., 2005) of WNT proteins, morphogens involved in joint formation (Guo et al., 2004;Hartmann and Tabin, 2001) and in the maintenance of adult tissue homeostasis (Dell'Accio et al., 2008;Eltawil et al., 2009;Nalesso et al., 2011).

NON-COLLAGENOUS STRUCTURAL PROTEINS

A small group of non-collagenous proteins and glycoproteins concur to promote the trophism of the ECM and the interactions between chondrocytes and the ECM. Cartilage oligomeric protein (COMP) binds to collagen type I (ColI) and ColII through zinc-dependent interactions and promotes collagen crosslinking (Shen et al., 1995). Chondronectin is a glycoprotein mediating the adherence of articular chondrocytes to the ECM, binding to CS, HA and ColII (Hewitt et al., 1982).

Fibronectin is a heterodimeric molecule particularly abundant in the superficial layer of the articular cartilage and in the pericellular matrix. It has been shown to provide attachment to cartilage progenitor cells within the superficial layer, and support their phenotype (Dowthwaite et al., 2004;Williams et al., 2010) and therefore may play a role in the guidance and regulation of these important cells in homeostatic processes and in cartilage healing.

Fibronectin expression, limited to the superficial layer in normal cartilage, is increased in the deeper layer in OA (Homandberg, 1999). Fragments of this protein have been shown to activate catabolic pathways, including upregulation of MMPs and aggrecanases (Xie and Homandberg, 1993).

ECM TURNOVER

Chondrocytes are responsible both for the synthesis and the degradation of the extracellular matrix. A fine-tuned homeostatic equilibrium between catabolic and anabolic activities maintains the integrity of healthy articular cartilage.

CATABOLIC FACTORS

METALLOPROTEINASES

The main players in the ECM turnover are the different classes of metalloproteinases, the synthesis and the activity of which is modulated by chondrocytes. In the following paragraph I will limit the description of these enzymes to their biochemical structure and I will give an overview of their function and activity in the section concerning the pathogenesis osteoarthritis, since the major role that these enzymes play in this process.

Metalloproteinases are divided in 5 families: the serralysins, the astacins, the ADAMTS, the MMPs and the pappalysins (Barrett, 1979). They are mainly responsible for the turnover of the ECM, but they are also responsible for the cleavage of other proteinases, proteinase inhibitors, growth factor binding proteins, chemotactic molecules, cell surface receptors and cell-cell adhesion molecules (Rengel et al., 2007).

The most expressed metalloproteinases in the joint are the MMPs and the ADAMTS. The activity of both these classes of enzymes is modulated by their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs).

MMPS

From a structural point of view (Rannou et al., 2006), MMPs are composed by three different domains: a pre-domain, responsible for enzyme maturation and its release from the cell, a pro-domain, which maintain the enzyme in an inactive status (zymogen), and the catalytic domain, containing a zinc atom, which is required for the proteolytic activity of MMPs. MMPs are produced in an inactive form and require the cleavage of the pro-domain by other proteinases to become active. MMPs have been divided in 5 different classes: collagenases (MMP-1, MMP-8 and MMP-13), capable to cleave collagens at neutral pH, gelatinases (MMP-2 and MMP-9), able to further degrade collagens fragments generated by collagenases, stromelysins (MMP-3, MMP-10 and MMP-11), which can cleave many substrates such as proteoglycans and fibronectin, matrilysins, and membrane type MMPs (MT-MMPs)(MMP-14; -15, -16, -17,-24 and -25).

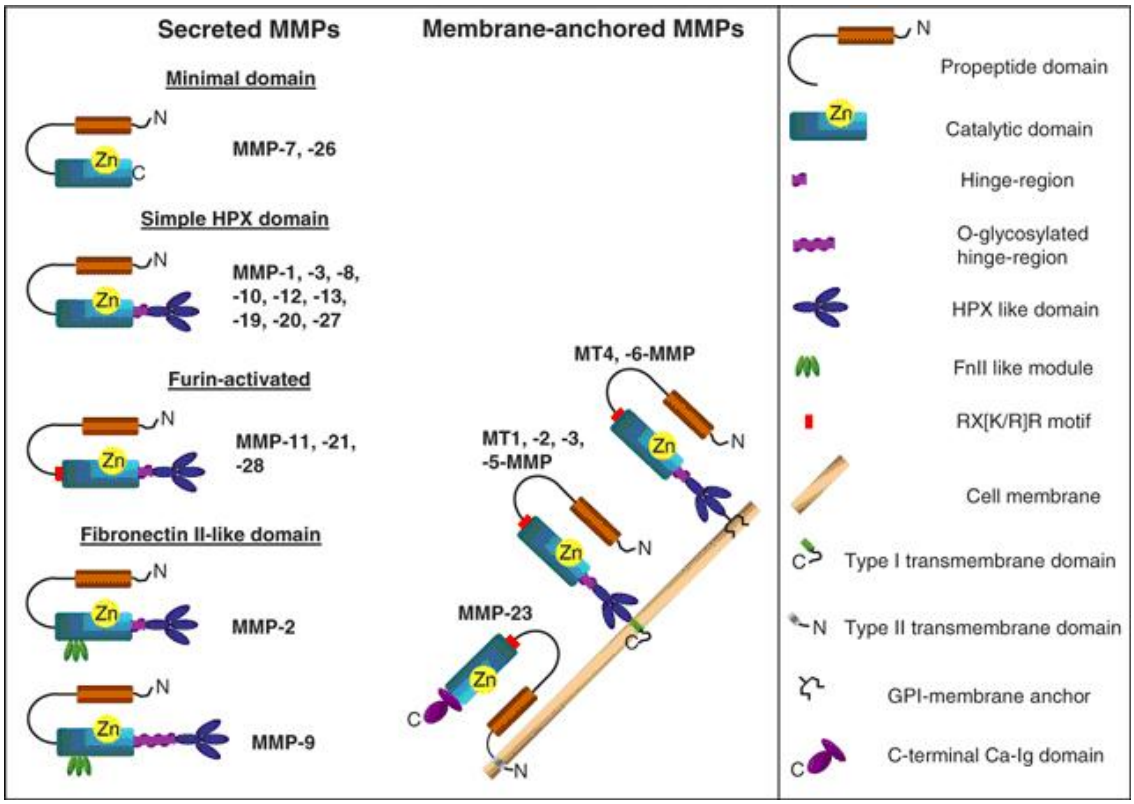


Figure 8: MMPs

Domain structure of secreted and membrane-anchored MMPs. Most MMPs contain a pro-peptide domain, a catalytic domain, a linker (hinge-region) and a C-terminal hemopexin (HPX)-like domain. Figure from (Hadler-Olsen et al., 2011)

ADAMTS

The ADAMTS are a family of 19 proteins sharing a number of structural features as outlined below and in Fig. 9 (Jones and Riley, 2005). Like MMPs, they contain a zinc ion within the active site, but differently from them, they exhibit narrower substrate specificity. From a structural point of view, ADAMTS possess a signal peptide, a pro-domain, which is cleaved within the cell to activate the enzyme, a metalloproteinase domain, a disintegrin domain, two or more thrombospondin motifs (TS) and several repeats at the C-terminus (Nagase and Kashiwagi, 2003) (Fig. 9).

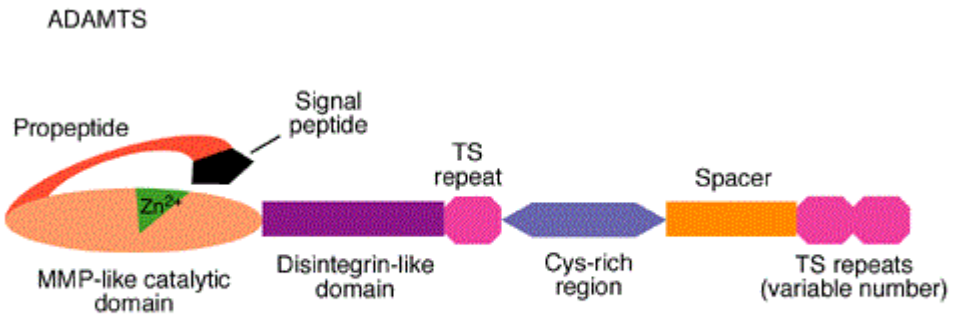


Figure 9: Molecular structure of ADAMTS

Figure from (Jones and Riley, 2005).

Two different subgroups of ADAMTS are expressed in articular cartilage: pro-collagen N-propeptidases (ADAMTS-2, -3 and -14) are responsible for the removal of the telopeptides from ColI and ColII allowing fibrils formation (Fernandes et al., 2001). The other subgroup, composed by ADAMTS-1, -4, -5, -8, -9, -15 and -20, are also called aggrecanases: they in fact cleave aggrecan at a specific site between the G1 and G2 domains, at the Glu^{373}-Ala^{374} bond in the IGD and at 4 additional sites located in the CS-enriched region (Tortorella et al., 2000). MMPs can cut aggrecan at the Asn^{341}-Phe^{342} (Flannery et al., 1992;Sandy et al., 1992).

TIMPS

Metalloproteinases and aggrecanases activity is modulated at different levels: at a the transcriptional level, in response to inflammatory cytokines (such as, IL-1β, TNF-α) and growth factors (EGF, PDGF, FGFs and TGF-β) (Varghese, 2006); at the a post-translational level, through the activation of proteases which cleave MMP determining their activation, and finally, through their inhibition by specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs).

Four TIMPs have been identified in humans, and they are more effective in blocking MMPs than ADAMTS activity (Baker et al., 2002). In homeostatic conditions, metalloproteinases and TIMP activity is tightly balanced. This equilibrium is disrupted in pathologic conditions such as OA, in favour of a higher catabolic activity (Dean et al., 1989). TIMP-3 in particular has been

shown to have a protective role in cartilage homeostasis, since TIMP-3 KO mice spontaneously develop OA (Sahebjam et al., 2007).

ANABOLIC FACTORS

Different growth factors and cytokines have been shown to have an anabolic effect in the articular cartilage. Insulin Growth Factor 1 (IGF1) and TGF-β have been shown to induce synthesis of highly sulphated GAGs in several in vitro systems (Morales, 1994;van Osch et al., 1998;Luyten et al., 1988). *In vivo*, BMPs have the unique capacity to promote ectopic cartilage and bone formation (Urist, 1965;Wozney, 1995), can stimulate ECM synthesis and are thought to be involved in the regenerative process following cartilage damage (Blaney Davidson et al., 2007;Dell'Accio et al., 2006;Volek-Smith and Urist, 1996).

BMP and TGFβ signalling are important for the maintenance of cartilage homeostasis in physiologic conditions: mice deficient of BMP receptor 1 (BMPRI) spontaneously develop articular cartilage loss (Rountree et al., 2004) and absence of a TGFβ receptor II (TGFbRII) also results in cartilage damage through promotion of terminal differentiation and hypertrophy of articular chondrocytes (Serra et al., 1997). In humans, genetic polymorphisms in the members of the TGFβ superfamily-such as GDF5 and SMAD3- have been shown to be linked to a predisposition to develop OA, highlighting the importance of these molecules/signalling pathways to maintain cartilage integrity.

Intra-articular injection of TGF-β in the mouse joint promotes proteoglycan synthesis (van Beuningen et al., 2000) and injection of TGF-β3 alone in rabbits efficiently induced the regeneration of severely injured articular cartilage (Warnke, 2010). Injection of mesenchymal stem cells isolated from perichondrium and infected with adenovirus encoding for BMP2 and IGF1 were also seen to have a reparative effect in the articular cartilage of rats in which partial thickness defect was performed (Gelse et al., 2003). In addition these two molecules together can block cytokine-induced ECM destruction and induce the expression of MMPs inhibitors (Hui et al., 2001).

Finally, mechanical loading, within physiological values, has been shown to promote the maintenance of cartilage health. Cyclical loading promote ECM synthesis (Larsson et al., 1991;Valhmu et al., 1998) whereas immobilization of the joints is proved to be detrimental for the maintenance of the proteoglycans content in the ECM (Setton et al., 1995).

Despite the knowledge of several signalling cascades and molecules involved in potential reparative processes after cartilage damage, few molecules are currently under clinical trial for treatment of cartilage diseases (TGF-β1 and BMP-7 are in phase 1 of clinical trials sponsored respectively by TissueGene and Stryker Biotech). This is due to the fact that some anabolic factors such as the members of the TGF-β superfamily, in addition to their potential regenerative properties, are also involved in endochondral bone remodelling after damage (Blaney Davidson et al., 2006;Scharstuhl et al., 2003;van Beuningen et al., 2000), and might bring to joint deformity if not opportunely modulated. Therefore, further studies are needed to have a better inside in the modulation of these pathways and how they cross-interact with each other, in order to plan an effective therapeutic strategy.

CARTILAGE HOMEOSTASIS AND OSTEOARTHRITIS

CARTILAGE HOMEOSTASIS

Cartilage homeostasis is the maintenance of cartilage integrity and function through a fine-tuned modulation of catabolism and anabolism. In physiologic conditions, the chondrocyte metabolism and matrix turnover are very slow. When injurious events, such as trauma, mechanical stress or inflammation challenge the articular tissue, a rapid anabolic response follows the initial catabolic events, in order to maintain homeostasis (Dell'Accio and Vincent, 2010;Buckwalter and Mankin, 1998).

When this equilibrium is broken and the anabolic activity cannot compensate anymore the injurious factors, cartilage is progressively lost, ultimately resulting in the development of OA (Bay-Jensen et al., 2010).

OSTEOARTHRITIS

OA is a leading cause of disability worldwide, affecting up to 1/3 of the population over the age of 50 (Lawrence et al., 1998;Lawrence et al., 2008) and costing £5.7 billion annually only in the UK (Arthritis care report, 2007).

The aetiology of primary OA is unknown: the incidence of the disease increases with age (primary osteoarthritis) but it can also develop following a joint injury or as a consequence of developmental, metabolic or neurological disorders (secondary osteoarthritis) (Buckwalter and Mankin, 1998).

Cartilage breakdown is the hallmark of OA but cartilage is not the only tissue involved. In OA, virtually every tissue is involved in the pathological process. The changes in the subchondral bone include an initial phase of bone resorption, and a subsequent phase culminating in a characteristic bone sclerosis (Buckwalter and Mankin, 1998) meniscal and ligament lesions can be either primary and themselves driving OA progression (Englund et al., 2009) or secondary (Buckwalter and Mankin, 1998). The synovial membrane may also play an important role either supporting the inflammatory process (Sellam and Berenbaum, 2010) and then contributing to a pathogenic mechanism, or possibly can produce progenitor cells important for reparative mechanisms (De Bari et al., 2001;Karystinou et al., 2009). Finally, the geometry of the joint is also altered by the presence of osteophytes and by bone remodelling events and this is known to further contribute to cartilage loss (Wluka et al., 2005).

In the interest of brevity and focus, in this thesis, I will limit this introduction to describe the pathogenic processes within the cartilage.

From a histological point of view, the first signs of OA are the fibrillation of the superficial layers of the articular cartilage, followed by a progressive loss of proteoglycan content (Buckwalter and Mankin, 1998). In the majority of the cases, as the disease progresses, fibrillations become clefts and fissures that can reach the calcified areas and the subchondral bone.

During the early phases of OA the articular cartilage can show hypercellularity, resulting from the increased proliferation of chondrocytes (Mankin, 1974a;Mankin, 1974b), especially near the damaged areas (clustering). In the later stage of the disease, when the ability of chondrocytes to counterbalance the catabolic processes is compromised, there is a decline in their anabolic response, followed by cell death and areas of hypocellularity (Mankin, 1974a;Mankin, 1974b).

Unfortunately, no treatment for OA is currently available, except for pain control and physiotherapy, until prosthetic joint replacement is required.

Understanding the pathogenetic mechanisms leading to the disruption of homeostasis and cartilage breakdown is of paramount importance to develop effective pharmacological treatments.

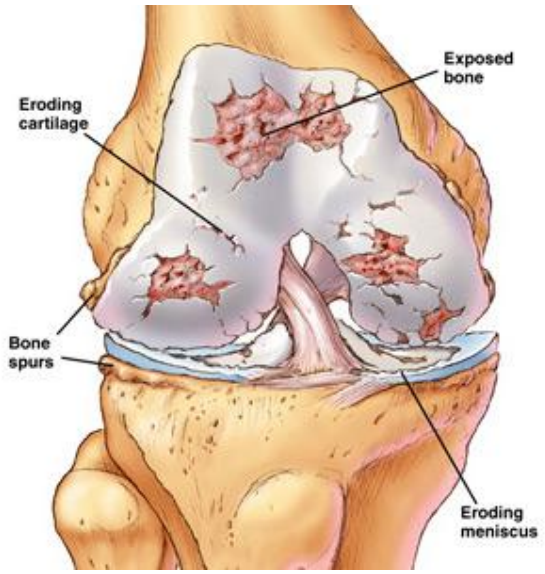


Figure 10: Graphic representation of an osteoarthritic knee

Main features of advanced osteoarthritis are the degradation of the articular cartilage, with consequent exposure and damage of the subchondral bone, bone remodelling, with formation of bone spurs and osteophytes and degeneration of other articular tissues, such as the synovium and the menisci (Figure from the NHS website).

PHENOTYPIC MODULATION OF CHONDROCYTES IN OA

In healthy cartilage, chondrocytes support and stabilize the ECM, favouring a low rate turnover of the matrix components. After injury and in the early phases of OA, chondrocytes increase their anabolic activity, in a regenerative attempt to restore the lost homeostasis.

In this phase chondrocytes produce and secrete an increased amount of matrix components, such as ColII, ColVI Col IX and ColXI as well as Aggrecan (Lorenz et al., 2005).

In OA, articular cartilage chondrocytes change their phenotype in a hypoxia induced factor (HIF)2α-dependent manner and acquire features of hypertrophy, expressing COLX, VEGF, and MMP 13 (Saito et al., 2010), and such phenotypic change has pathogenetic importance, since the deletion of HIF2α gene resulted in halting the hypertrophic differentiation of articular chondrocytes following DMM and in resistance to OA cartilage damage (Saito et al., 2010;Yang et al., 2010).

During OA progression, the equilibrium between anabolic and catabolic activity is disrupted in favour of the latter with increased expression and activity of MMPs and ADAMTS, with a consequent degradation of the ECM.

MATRIX TURNOVER IN OA

ROLE OF MMPS IN OA PATHOGENESIS

The degradation of the ECM by MMP activity is required for many homeostatic mechanisms, such as cell migration (Goetzl et al., 1996), tissue remodelling during development and after damage (Page-McCaw et al., 2007), release of proteoglycan-bound cytokines and morphogens (Dierker et al., 2009). Consequently, disruption of the modulation of MMP activity has been shown to be involved in different pathologies, from cancer, where tissue degradation promoted by MMPs leads to tumour invasiveness (Gialeli et al., 2011) to osteoarthritis, where they contribute to cartilage destruction. The expression of several MMPs is increased in cartilage from OA patients (Kevorkian et al., 2004). Constitutive expression of MMP-13 under control of ColII-promoter, resulted in increased cartilage degradation in a murine model of osteoarthritis (Neuhold et al., 2001) in comparison to wild type littermates. Conversely, MMP13 deficient mice were protected from instability induced OA (Little et al., 2009). In the SRT/Ort mice, a strain of mice which spontaneously develop OA (Walton, 1977;Walton, 1978;Mason et al., 2001), expression of MMPs is higher in comparison to CBA mice which do not develop the disease. Indeed, oral administration of a selective inhibitor of MMP1, MMP8 and MMP13 protected cartilage from damage in these mice (Brewster et al., 1998). On the contrary, MMP-3 knock-out mice develop more severe articular damage OA in comparison to wild type controls (Chambers et al., 2001). Furthermore, a mutation in the gene encoding for Aggrecan that makes the protein resistant to MMP cleavage, not only does not protect these mice from the development of OA but actually make them more susceptible to the development of the disease (Little et al., 2007). These data suggest that a tightly regulated MMP activity is required for cartilage homeostasis, but, in pathology, uncontrolled MMPs activity may become a pathological mechanism. The requirement of MMP activity for the

maintenance of cartilage homeostasis might explain why inhibition of MMPs activity failed to provide an efficacious treatment for OA (Tu et al., 2008) and warrants further research to seek a greater level of specificity and control for MMP inhibition in OA.

Role of ADAMTS in OA pathogenesis

Aggrecan fragments generated by ADAMTS can be detected using antibodies specific for the proximal and distal stubs (Calabro et al., 1992) and are increased in the cartilage and in the synovial fluid of osteoarthritic and rheumatoid arthritis patients (Struglics et al., 2006;Sandy et al., 1992). Mice harbouring a mutation in the intraglobular domain of aggrecan, which makes the molecule resistant to ADAMTS cleavage, are protected from cartilage damage in the early onset of osteoarthritis in the destabilization of the medial meniscus (DMM) model (Little et al., 2007). In arthritis, at least in mouse, ADAMTS5 is the main aggrecanase, since mice deficient in the *ADAMTS5* gene are protected from cartilage destruction in an experimental model of OA (Glasson et al., 2005) and inflammatory arthritis (Stanton et al., 2005). In the human cartilage however, *ADAMTS4* may have a more important role (Yang et al., 2010;Bondeson et al., 2008;Davidson et al., 2006). In addition, although ADAMTS5 function is well established in OA, it is not clear whether it has any function in the normal remodelling of the ECM in healthy cartilage, since *ADAMTS5* deficient mice are phenotypically normal, and, in physiological conditions, ADAMTS5 is not even expressed in cartilage (McCulloch et al., 2009).

Role of inflammation in OA

The role of inflammation in osteoarthritis is not as prominent as in inflammatory arthritides such as rheumatoid arthritis (RA) (Buckwalter et al., 2005). However, several pro-inflammatory molecules such as interleukin-1 (IL-1) and tumour necrosis factor α (TNF-α) are abundantly expressed in the synovial fluid of OA patients (Saklatvala, 1986;Rowan et al., 2001;Bondeson et al., 2006) suggesting that at least in the initial phases, inflammation might play an important role in OA pathogenesis.

OA chondrocytes have an increased expression of IL-1β, IL-1β converting enzyme, IL-1β receptor type I, TNFα and IL-6 (Guerne et al., 1990;Kobayashi et al., 2005;Moos et al.,

1999;Umlauf et al., 2010). IL-1 has been shown to promote MMPs and ADAMTS5 activity (Tetlow et al., 2001) and to induce rapid degradation of the ECM (Saklatvala, 1987). Recently, in a murine model of osteoarthritis, Syndecan-4 deficient mice have been shown to be protected by OA development, through a mechanism in which IL-1 seems to have a protagonist role (Eichtermeyer et al., 2009). IL-1β can suppress the expression of ColII, to increase that one of hypertrophic markers such as MMP13 (unpublished material) and finally to promote the expression of other pro-inflammatory cytokines such as IL-6 and IL-8 (Goldring and Berenbaum, 2004).

Finally, both IL-1 and TNF-α can stimulate nitric oxide (NO) and prostaglandin production in articular cartilage (Palmer et al., 1993). Nitric oxide synthase (iNOS) is up-regulated in the cartilage of OA patients (Amin et al., 1995;Melchiorri et al., 1998) and its activation results in persistent biological effects including post-translational modification of the collagen network (Hughes et al., 2010).

Despite there is evidence showing the negative role of inflammatory cytokines in OA development, some contrasting data show that basal levels of some of these molecules might also have a homeostatic role in the maintenance of articular cartilage homeostasis. The use of mouse genetics, in this field has in fact led to contrasting results. Clements et al. (Clements et al., 2003) reported that IL-1 deficient mice develop more severe OA than wild type controls in a surgical model of instability-induced OA. In contrast, IL-1β KO mice have been shown to be protected by articular cartilage damage in a different model of arthritis (collagenase induced OA) (Glasson, 2007). Blockade of NO production, as well as its IL-1-stimulated synthesis both result in an increased ECM degradation (Abramson, 2008;Stefanovic-Racic et al., 1996).

The pharmacological blockade of IL-1 activity with the competitive antagonist of the IL-1 receptor Anakinra, has been demonstrated to be effective in the reduction of proteolytic activity and osteophyte formation in a canine model of OA in comparison to control (Caron et al., 1996). On the contrary, a multi-centre clinical trial testing the beneficial of the use of Anakinra in humans, failed in decreasing pain and matrix degradation in OA patients (Chevalier

et al., 2009). Of course, this might be due to differences in the responsivity to the drug across species, but the fact that also the complete absence of IL-1, IL-1 converting enzyme, iNOS or MMP1 genes has been shown to be detrimental in the development of OA (Clements et al., 2003) suggests that actually a basal activity of these molecules and enzymes is required for the maintenance of cartilage health.

WNT SIGNALLING

WNTs are a family of 19 morphogens known to regulate many developmental processes, such as tissue patterning, cell proliferation and migration (Logan and Nusse, 2004) and to maintain tissue homeostasis in the adult (Nusse, 2008). Given the multitude of processes in which they are involved, disregulation of Wnt pathways is associated with a variety of different diseases, from cancer (Clevers, 2006) to osteoarthritis (Lawrence et al., 1998;Lawrence et al., 2008;Loughlin et al., 2004;Luyten et al., 2009;Zhu et al., 2008;Zhu et al., 2009). Being morphogens, Wnts are produced and secreted by cells, and form a concentration gradient which spread in the tissues (Neumann and Cohen, 1997;Zecca et al., 1996) and let the cells in the proximity to respond to it in a concentration-dependent manner, by promoting the transcription of cell specific target genes. Traditionally, Wnt ligands have been divided into two different groups depending on their ability to promote or not axis duplication in *Xenopus* embryos (McMahon and Moon, 1989). Wnts promoting axis duplication are known as “canonical Wnts” and activate the Wnt/β-catenin signalling pathway, also called for this reason “canonical” pathway. Among the members of this group there are WNT-1, WNT-3A and WNT-8A. Non canonical Wnts-such as WNT-4, WNT-5A, WNT-5B, WNT-7A and WNT-11- do not promote axis duplication nor activate the Wnt/β-catenin pathway but other, less characterized, signalling cascades, grouped under the name of “non-canonical” signalling pathways. Recent overexpression experiments have questioned this rigid distinction proposing that, in some cases, the receptor repertoire may influence the downstream signalling pathway to the same ligand (Medina et al., 2000;Mikels and Nusse, 2006a;Tao et al., 2005;Heisenberg

et al., 2000), however the supra-physiological expression levels of the overexpressed gene in this type of experiments is such that "off target" or non-specific effects, which are not relevant at physiological expression levels cannot be excluded. Therefore the physiological significance of these findings remains uncertain.

WNT LIGANDS

On a structural point of view, all Wnts share common features including a signal sequence for secretion, several highly charged amino acidic residues and many glycosylation and lipid modification sites (Nusse and Varmus, 1992). They all share a sequence of 22 cysteine residues which are believed to be important for the protein folding (Mikels and Nusse, 2006b).

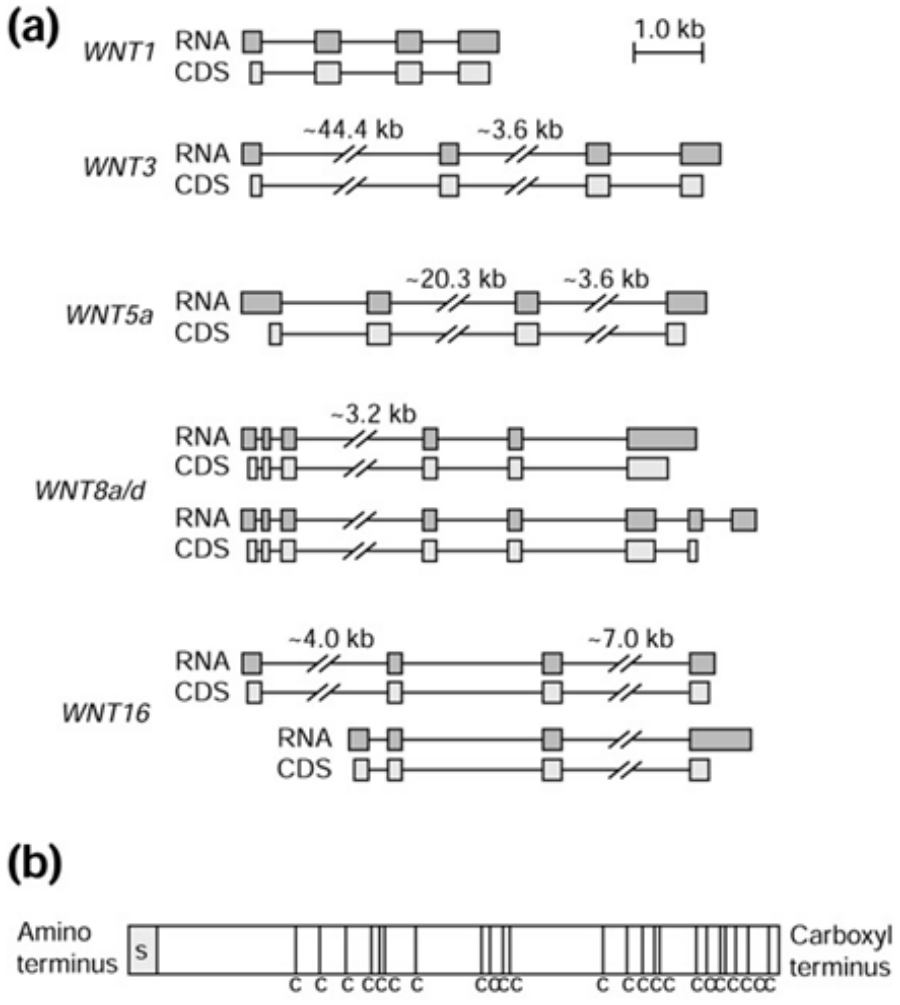


Figure 11: Gene and protein structure of Wnts

a) Gene structure of some members of the Wnt family. Exons are represented as boxes, and introns as lines. WNT-8 is an example of a gene which can be subjected to alternative splicing at the 3'terminus and WNT-16 can be subjected to different splicing at the 5'. CDS=Coding sequence b) at protein level all WNTs possess a signal sequence (S) for extracellular secretion, and 23/24 Cys residues (Miller, 2002).

The sequence of Wnts is preserved across species: when compared, any two different WNTs normally share between the 30 and the 60% of similarity at aminoacid level, regardless of the species (Nusse and Varmus, 1992). The evolutionary preservation of these molecules underlines their important role.

Glycosylation and lipid modification have been shown to be essential for their signalling properties and the stability of these proteins. Mutation of the glycosylation sites has been reported to compromise secretion (Kurayoshi et al., 2007;Komekado et al., 2007) whereas lipid

modification are thought to anchor them to the cell membrane and to be important both for secretion and signalling (Willert et al., 2003;Franch-Marro et al., 2008;Takada et al., 2006).

Wnts can travel up to 20 cell diameters away from the cell in which they are produced (Zecca et al., 1996;Neumann and Cohen, 1997). The mechanism of diffusion and gradient formation of such hydrophobic molecules has not been clarified yet. Current hypotheses include that, upon translocation to the membrane, lipase or metalloproteinases may cleave the hydrophobic lipid modification of the proteins allowing the release outside of the cells, or that WNTs may be secreted inside micelle-like aggregates or on membranous particles (Port and Basler, 2010). Intriguingly, HSPGs have been shown to bind Wnts and to be involved in Wnt gradient formation in Drosophila (Han et al., 2005) and be important for their stability in human (Fuerer et al., 2010). Considering the high content of HSPGs in the articular cartilage, these molecules are very likely to play an important role in the formation of Wnt gradients in this tissue.

FRIZZLED (FZD) RECEPTORS

Wnts are known to interact with FZD receptors to mediate their signalling cascades. The basic structure of all FZD is that of seven transmembrane domain receptor, with an extracellular N-terminus and an intracellular C-terminus. At the N-terminus all frizzled possess a Cys-rich domain (CRD) which probably represents the binding site for Wnt ligands (Xu and Nusse, 1998). The transmembrane domain of FZD contains some similarities with G-protein-coupled receptors (GPCR) (Malbon, 2004) and indeed much evidence suggest that FZDs are GPCRs (Koval and Katanaev, 2011;Slusarski et al., 1997a). At the C-terminus, FZD share a KTxxxW domain (PDZ domain) which allows the interaction with Dishevelled (DVL), an adaptor protein important for the mediation of both canonical and non canonical pathways (Grumolato et al., 2010).

Several single transmembrane domain receptors have been shown to act as co-receptors for FZDs and to mediate different signalling cascades. Low density lipoprotein receptor-related

receptors 5 and 6 (LRP5/6) interact with FZD receptors after binding of Wnts to FZDS and this interaction promotes their phosphorylation in the intracellular domain (Zeng et al., 2008). The formation of this ternary complex-Wnt/FZD/LRP-is essential for the activation of the Wnt-β-catenin pathway (Nusse, 2005). Ten LRPs have been identified in human and have been shown to have important functions in endocytosis, cell-cell communication, embryonic development, and diseases (Li et al., 2001;May et al., 2007).

ROR1/2 and RYK tyrosine kinases receptors heterodimerize with FZD receptor and can mediate Wnt non-canonical signalling autonomously, as FZD co-receptors, or both (Schulte, 2010). Abnormalities in ROR1/2 receptors have been associate with skeletal disorders (Minami et al., 2010). Binding of Wnts to RYK trasduces important signalling involved in axon guidance and neurite autgrowth (Yoshikawa et al., 2001;Lu et al., 2004). However, the mechanism of activation and the signalling cascades mediated by these receptors are still not-well characterized and will require further investigation.

WNT SIGNALLING PATHWAYS

WNTs signal through multiple pathways (Semenov et al., 2007;Macdonald et al., 2007) the best characterized of which is the Wnt/β-catenin-dependent pathway. In this signalling cascade, in the absence of ligands, the constitutively active kinase glycogen synthase kinase (GSK)-3β phosphorylates β-catenin within a destruction complex, addressing it for degradation through the proteasome pathway (Logan and Nusse, 2004). The engagement of some members of the Wnt family, such as WNT-1 and WNT-3A (Logan and Nusse, 2004;Shimizu et al., 1997) with Frizzled (FZD) receptors and the co-receptors LRP5/6 results in the disruption of the destruction complex, accumulation of β-catenin in the cytosol, and its migration in the nucleus, where β-catenin interacts with T-cell factor (TCF)/ Limphoid enhancer binding-factor (LEF) transcription factors and supports the transcription of cell-specific target genes (Molenaar et al., 1996) involved in different biological processes, such as cell proliferation, apoptosis and cell determination (Moon et al., 2002).

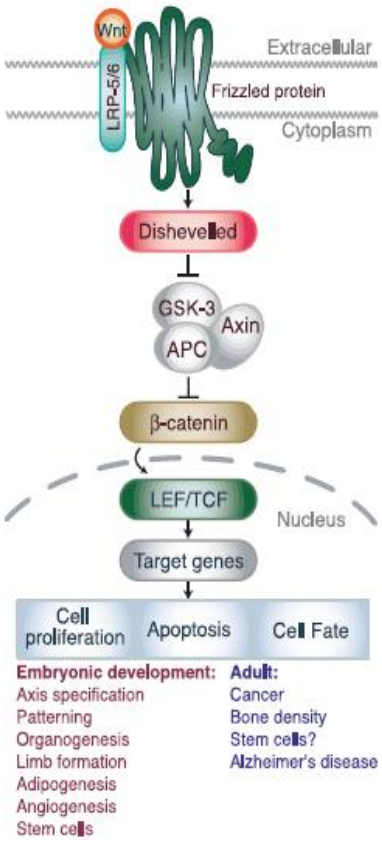


Figure 12: Wnt/β-catenin dependent pathway

Upon engagement with Wnt ligands, FZD receptors heterodimerize with LRP5/6 co-receptor and recruit axin to the cell membrane. This in turn promotes the destabilization of the destruction complex in which β-catenin is retained, allowing its accumulation in the cytoplasm. β-catenin then transmigrates in the nucleus and it interacts with TCF/LEF transcription factors, promoting the transcription of cell-specific genes (Moon et al., 2002).

Other WNTs, such as WNT-5A and WNT-11 (Kuhl et al., 2000) can instead activate other β-catenin independent signalling cascades, generally referred as “non-canonical” pathways. The best characterized of them is the planar cell polarity (PCP) pathway, which has been extensively studied in Drosophila, and which has been proved to modulate different biological processes such as the orthogonal polarity of the cells (Fanto and McNeill, 2004) and convergent extension movements, hair follicle orientation, and neural tube formation (Wallingford and Harland, 2001;Wang et al., 2006b;Wang and Nathans, 2007).

Some FZD receptors have been shown to mediate intracellular calcium accumulation in response to WNT stimulation (Kuhl et al., 2000;Slusarski et al., 1997a;Slusarski et al., 1997b) a

mechanism which is G-protein mediated because it can be blocked by pertussis toxin (Kuhl et al., 2000;Sheldahl et al., 1999;Slusarski et al., 1997a). The increase in intracellular calcium has then been associated with the activation of different Ca^{2+}-dependent pathways, such as the protein kinase C (PKC), calmodulin kinase II (CaMKII) and calcineurin (CNA) pathways. In particular the Ca^{2+}/CamKII pathway has been shown to be important during development, driving ventral cell fate in *Xenopus* (Kuhl et al., 2000) and modulating differentiation and proliferation in the epiphyseal cartilage both in chicken (Taschner et al., 2008) and in mouse (Li et al., 2011). Finally, activation of CaMKII pathway has been shown to antagonize the β-catenin- dependent pathway in different systems (Topol et al., 2003;Ishitani et al., 2003).

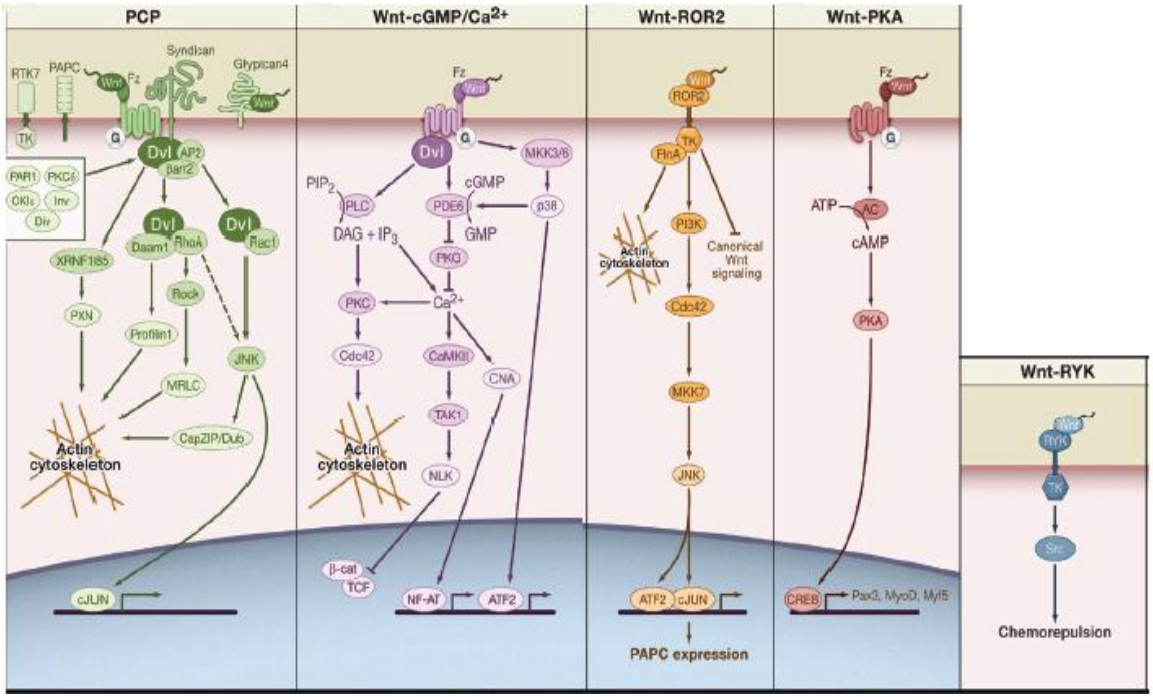


Figure 13: Non canonical Wnt signalling pathways

In addition to the Wnt/β-catenin signalling, some Wnt ligands have been shown to activate several other signalling cascades which probably require the interaction of FZDs with co-receptor other than LRPs or are mediated by FZDs alone. The figure gives an overview of some of the pathways that have been described so far (Semenov et al., 2007).

WNT INHIBITORS

Wnt signalling can be antagonized at different levels. Of particular interest, in this thesis, are the secreted inhibitors of Wnt signalling. Different classes of soluble antagonists have been identified: among these Dikkopfs (DKK) and secreted frizzled related proteins (sFRPs) are the best characterized. Dkks - especially Dkk1 and 4 - bind to LRP5/6 and promote their heterodimerization with Kremen receptors (Mao et al., 2001). This coupling, in turn, promotes LRP internalization via a clathrin-mediated mechanism (Yamamoto et al., 2008). The removal of LRPs from the cell surface prevents the formation of the ternary complex Wnt/LRP/FZD and the activation of the canonical pathway (Bafico et al., 2001). Secreted frizzled related proteins (sFRPs) are a class of soluble inhibitors composed by 5 members with distinct expression patterns (Jones and Jomary, 2002). They bind to Wnts through their frizzled-related N-terminal domain and act as scavenger molecules, preventing the interaction with FZD receptors.

However, the role of these molecules is still not completely understood because they are also been proposed to be WNT agonists, acting as carriers for Wnts or modulating the concentration of Wnts in the cellular environment (Kawano and Kypta, 2003).

WNT IN LIMB FORMATION AND SKELETAL PATTERNING

A temporal and spatial finely tuned expression of WNTs has been shown to be crucial for limb development. In chicken, WNT-3A is expressed in the AER and promotes the expression of FGF8, through the activation of the β-catenin dependent pathway, sustaining the maintenance of the AER and the limb outgrowth (Kengaku et al., 1997;Kawakami et al., 2001). Similarly, in the mouse, inactivation of both TCF1 and LEF1, ultimate mediators of β-catenin signalling, prevents limb outgrowth (Galceran et al., 1999).

Later in development, activation of the WNT/canonical pathway has been demonstrated to be required and sufficient for synovial joint formation (Guo et al., 2004;Hartmann and Tabin, 2000) and abrogation of this signalling cascade results in joint fusions (Koyama et al., 2008). Conversely, inactivation of the canonical pathway is required for chondrocyte differentiation, since the overexpression of β-catenin in the limb mesenchyme represses the expression of Sox9 (Akiyama et al., 2004).

Also the activation of the non-canonical pathways, plays a role in limb development: mice lacking of WNT-5A, which activates the Ca^{2+}/CaMKII pathway both in Zebrafish and *Xenopus* (Slusarski et al., 1997b), have shorter limbs and a global retardation in development due to a delay in chondrocyte maturation (Yamaguchi et al., 1999). However, also overexpression of WNT-5A results in a similar phenotype of chondrodysplasia making of difficult interpretation its mechanism of action (Yang et al., 2003).

Finally WNT-7A controls dorso-ventral patterning: the absence of WNT-7A promotes ventralization of several skeletal structures (Parr et al., 2001) a phenotype which is similar to that of LRP6-/- mice, leading to the hypothesis that WNT-7A requires LRP6 to mediate its signalling.

WNT SIGNALLING IN TISSUE HOMEOSTASIS AND DISEASE

In adult life, modulation of Wnt signaling has an important role in cartilage and bone homeostasis. Our group demonstrated activation of the canonical Wnt pathway in osteoarthritic cartilage in humans (Dell'Accio et al., 2006) and following cartilage injury in mice (Dell'Accio et al., 2008;Eltawil et al., 2009). Genetic studies revealed that loss of function polymorphisms in the *SFRP3* and *LRP5* gene (Gong et al., 2001;Mizuguchi et al., 2004;Riancho et al., 2011) are linked respectively to an increased susceptibility to OA (Loughlin et al., 2004;Lories et al., 2007) and osteoporosis (Gong et al., 2001). Overexpression of Dkk1 has been shown to enhance bone erosion in a model of inflammatory arthritis (Diarra et al., 2007) and to mediate chondrocyte apoptosis in osteoarthritic cartilage (Weng et al., 2010). These data, together with other *in vivo* and *in vitro* experimental evidence suggest that the β-catenin-dependent/Wnt pathway is a master regulation of joint remodelling (Luyten et al., 2009;Schett et al., 2008).

However, results of functional studies using mouse genetics are contrasting and of difficult interpretation. For instance, both repression and forced activation of the canonical Wnt signalling resulted in chondrodysplasia in embryonic life (Akiyama et al., 2004) and in OA postnatally (Koyama et al., 2008;Lories et al., 2007;Zhu et al., 2009;Chen et al., 2008;Zhu et al., 2008). Therefore further analysis is required to better clarify the role of this pathway in cartilage disease.

CHAPTER 2: HYPOTHESIS AND AIMS

HYPOTHESIS

This work tests the hypothesis that the maintenance of a fine-tuned equilibrium of Wnt-mediated signalling cascades is required for the maintenance of chondrocyte phenotype.

AIMS

1) To evaluate the effect of the activation and repression of the Wnt/β-catenin signalling pathway on articular chondrocyte *in vitro*

    - Evaluation of the effect of the stimulation of human articular chondrocytes (AHAC) with a well characterized activator the Wnt/β-catenin signalling pathway -WNT-3A- in presence or absence of a WNT/β-catenin inhibitor-DKK1

2) To confirm these data in an in model of cartilage formation in vivo (Dell'Accio et al., 2001).

CHAPTER 3: MATERIALS AND METHODS

For all the recipes of media and buffers please refer to the end of the "Materials and Methods" session.

CARTILAGE HARVEST AND CHONDROCYTE ISOLATION

Human adult articular cartilage was obtained from 10 female and 4 male patients who underwent joint replacement for knee osteoarthritis after obtaining consent. All procedures have been approved by the East London and The City Research Ethics Committee 3 (REC 07/Q0605/29).

Surgical samples containing bone and cartilage were immediately placed in a closed sterile jar to maintain moisture and sterility, and processed within 4 hours from the operation. Full thickness cartilage explants were dissected from the femoral condyles and the patellar groove in sterile conditions, excluding the mineralized cartilage and the subchondral bone. From each sample, one full thickness portion was processed for histological scoring. The sample was then cut and stained with toluidine blue and scored for features of OA as described below. The rest of the cartilage was washed twice in high-glucose Dulbecco's modified Eagle medium (D-MEM/F-12 1:1 plus GlutaMax, Invitrogen) containing 10% v/v foetal bovine serum (FBS), 1mM sodium pyruvate (NaPyr), and 2% v/v antibiotic-antimycotic solution (Gibco, Invitrogen) for 10 minutes at RT under rotation. Cartilage was then digested under slow agitation for 30 minutes at 37°C in 1mg/ml pronase (Roche) diluted in complete medium (same composition, with 1X antibiotic/antimycotic solution) and then overnight at 37°C in 1mg/ml collagenase P (Roche).

CELL COUNTING AND TRYPAN BLUE EXCLUSION ASSAY.

Ten µl of cell suspension was mixed 1:1 with a 0.5% of Trypan blue and immediately counted using a Neubauer haemocytometer. Cell viability as evaluated by trypan blue exclusion was routinely ≥ 95%. The cell suspension recovered from the isolation procedure was then resuspended in complete medium at 37^{o}C in a humidified atmosphere with 5% CO_2, seeded at a density of 10^4 cells/cm^2, and allowed to attach for 7 days. At this point, non-attaching cells were removed by changing the medium.

Porcine chondrocytes were isolated from the metatarsal joints of pigs obtained from a local abattoir within 24 hours from the death of the animal, isolated and cultured with the same procedure.

CELL EXPANSION

Chondrocytes were cultured at 37°C in humidified atmosphere containing 5% CO_2. The medium was replaced two times per week.

For cell expansion, 80% confluent chondrocytes were split 1:4 upon attainment of confluency.

To this end, cultures were washed once in Ca^{2+}-, Mg^{2+}- free PBS, incubated for 5 minutes with pre-warmed 0.025% Trypsin EDTA (Gibco) at 37^{o}C, and detached by gentle tapping. Cells were immediately pelletted by centrifugation at 2000 rpm and re-plated.

All the experiments were performed on freshly isolated or P0 cells as indicated within 7 days from the attainment of confluence.

Only chondrocytes deriving from preserved cartilage areas (Mankin score ≤ 4) were used. Previous studies in our laboratory showed that such score is associated with a gene expression profile and responsivity to injury and Wnts very comparable to normal cartilage (Dell'Accio et al., 2008).

HISTOLOGICAL SCORING

The severity of OA in human cartilage explants was evaluated using Modified Mankin score (Mankin et al., 1971;van der Sluijs et al., 1992). The score goes from 0-normal cartilage- to 13-severe cartilage lesions. This system assesses different aspects of the articular cartilage morphology and structure (Table 1). Staining for scoring was performed with Toluidine blue (see below).

TABLE 1: MANKIN SCORE

Category	**score**
Structure	
Normal	**0**
Slight disorganization (cellular row absent, some superficial clusters)	**1**
Irregular surface, including fissures into the radial layer	**2**
Pannus	**3**
Superficial cartilage layers absent	**4**
Fissures into the calcified cartilage layer	**5**
Sever disorganization (chaotic structure)	**6**
Cellular abnormalities	
Normal	**0**
Hypercellularity	**1**
Clusters	**2**
Hypocellularity	**3**
Matrix staining (Metachromasia)	
Normal	**0**
Staining reduced I radial layer	**1**
Staining reduced in inter-territorial matrix	**2**
Only present in pericellular matrix	**3**
Absent	**4**
Total Maximum score	**13**

CELL PROLIFERATION

Freshly isolated porcine articular chondrocytes were seeded in a 96 well plate at a density of 10000 cells/cm^2 and left attaching overnight in 200 µl of complete medium. One day after seeding, the medium was changed and cells were treated for 6 hours with fresh medium

containing 100ng/ml of recombinant WNT-3A (R&D system) resuspended in 0.1% BSA w/v in PBS or vehicle alone (0.1%BSA w/v in PBS) diluted in complete medium at 37°C (100µl/well). Two µCu/µl/cm^2 of ^{3}H-thymidine were then added to each well and the incubation was prolonged for additional 12 hours. At the end of the incubation the cells were thoroughly washed, lysed in trichloracetic acid and the radioactivity (recorded as count per minutes-CPM) was measured using a 1900 Liquid Scintillation analyser (Packard). The same procedure was repeated at 2 and 3 day time points.

MICROMASS CULTURE AND ALCIAN BLUE STAINING

Freshly isolated human articular chondrocytes were resuspended at a density of 2.0x10^7 cells/ml in complete medium and micromass cultures were obtained by pipetting 20µl drops of cell suspension per each well of a 24 well plate. The cells were left attaching for 3 hours and then 1 ml of complete medium was added. Twenty four hours later, micromasses were exposed to treatment as described in the individual experiments. Finally, micromasses were fixed for 30 minutes in acetone at -20°C and whole-mount stained with alcian blue GS 0.5% as described before (De Bari et al., 2001). Proteoglycan content was extracted by incubating the micromasses for 6 hours with 6M guanidine HCl and determined measuring the adsorbance of the alcian blue at 630nm. Data were then normalized for protein content (determined by BCA assay). Absorbance was measured by using a GENios spectrophotometer (Tecan).

BACTERIAL CULTURES

AMPLIFICATION OF PLASMID DNA

PREPARATION OF LB-AGAR PLATES

After being sterilized, LB-Agar medium was added with Ampicillin or Kanamycin to a final concentration of 150µg or 50µg respectively, mixed gently, and approximately 15 ml of solution was poured in each individual 300mm bacterial plate. The plates were allowed to set for 2 hours at room temperature, inverted, and allowed to dry overnight. The plates were then either used immediately, or stored at 4°C.

BACTERIAL TRANSFORMATION

One or more aliquots (30µl each) of thermo-competent DH5α bacteria were defrosted on ice. One µg of plasmid DNA was then added to each aliquot and the cells were left resting on ice for 30 minutes. For transformation, the bacteria were heat shocked for 20 seconds in a 42°C pre-warmed water bath. The bacteria were then put on ice for 2 minutes. Nine hundred seventy µl of pre-warmed LB medium were added to each aliquot and the tubes were incubated at 37°C for 1 hour at 300 rpm.

200µl of the transformed cells were then spread under sterile conditions in a pre-warmed selective plate and incubated overnight at 37°C. To control for possible contamination of the agar plates, one plate was incubated without the addition of bacteria and one plate was seeded with untransformed bacteria (heat shocked but without addition of plasmids). As a positive control, one plate was spread with a bacterial stock from a previous successful transformation.

The rest of the bacteria was stored until the following day at 4°C.

PLASMID PURIFICATION

The day following transformation one or more colonies were removed from the plate by using a sterile tip and immersed in 2ml of pre-warmed LB medium containing 0.05-0.15 mg/ml of the selective antibiotic solution. The tubes were incubated at 37°C for 14-16 hours with shaking (300rpm).

Following the 14 hours incubation, 1.4ml of each bacterial culture were transferred into one Eppendorf tube and centrifuged at maximum speed for 20 seconds. The supernatant was discarded and the pellet placed on ice. The bacteria were resuspended by vortexing in 300 µl of buffer P1 (Qiagen). Immediately afterwards, 300 µl of buffer P2 (Qiagen) were added and the samples were mixed by inversion and put back at room temperature. This resulted in the lysis of the cells and release of the amplified plasmid DNA. Finally, 300 µl of buffer P3 (Qiagen) were added to each sample and the tubes were placed again on ice for 10-15 minutes. The tubes were centrifuged at maximum speed for 10 minutes in a 19°C pre-cooled centrifuge.

After spinning, each sample appears to be divided in three parts: a precipitate, a liquid and clear part containing the plasmid DNA, and a top precipitated layer. Eight hundred µl of the clear part were removed and mixed with 560 µl of ice-cold isopropanol. The tubes were incubated on ice for 20 minutes and centrifuged again at 4°C for further 20 minutes to precipitate plasmid DNA. The supernatant was discarded and the pellet was washed with 1ml of ice-cold 70% v/v ethanol. The samples were eventually centrifuged at maximum speed for 5 minutes at 4°C. The excess of ethanol was carefully removed without disturbing the pellet. The pellet was air dried for 15 minutes at room temperature and resuspended in 20 µl of ultrapure, sterile water (Baxter). The concentration of the plasmid DNA was measured by using the Nanodrop and adjusted to a final concentration of 1µg/µl. Plasmid stocks were stored at -20°C until further use.

Generation and preservation of transformed bacterial stocks

Following transformation, the remaining part of the bacterial cell suspension was preserved by adding pure sterile glycerol to a final concentration of 10% v/v, mixing, and storing at -80°C. This bacterial stock was subsequently used to re-amplify the plasmid DNA. The frozen bacterial stock was gently touched with a sterile toothpick, which was subsequently used to streak a new LB-agar plate and start a new culture from a single colony.

Transfections and reporter assay

Cells were plated into 24 or 6 well well plates and left growing until the attainment of 50-60% confluency. The protocols outlined below represent the optimized versions, but different proportions of reagents were used in the optimization steps as described in the “Results” section.

Lipofectamine

The volumes and the concentrations of the reagents used in the following protocol are for a single well of a 24 well plate.

Before transfection, the cells were washed once with pre-warmed PBS and the medium replaced with antibiotic- and antimycotic-free complete medium. Five hundred µl of medium were added to each well. 1µg of plasmid DNA were added to 50ul of Optimem (OM) (Gibco). In the meantime, 2ul of Lipofectamine 2000 (Invitrogen) were incubated for 5 minutes in 50ul of pre-warm OM (Gibco) and then added to the medium containing the plasmidic DNA. Lipofectamine and DNA were incubated together for 20 minutes at room temperature and then added to the cells for 8 hours. The medium was replaced with complete medium without washing.

FUGENE AND FUGENE HD

The volumes and concentrations of the following protocol are sufficient for the transfection of a well of a 6-well plate.

Before transfection the medium of the cells to be transfected was changed with antibiotic- and antimycotic-free complete medium (2ml/well). Three µl of Fugene 6 or Fugene HD were mixed to 97µl of OM (Gibco) and incubated at RT for 5 minutes. Afterwards, 1 µg of plasmid DNA was added to the tube containing the Fugene solution and incubated at room temperature for 15 minutes. The mix of Fugene and DNA was finally added to the cells. The medium was then replaced after 24 hours.

NUCLEOFECTION (AMAXA)

Freshly isolated chondrocytes were cultured for 48 hours in complete medium. The culture medium was removed from the cells 3 hours before nucleofected and the cells were washed in PBS. In order to detach the cells, chondrocytes were incubated for 3 hours at 37°C with a solution of complete medium containing 1mg/ml of collagenase P (Roche) and Pronase 1mg/ml (Roche). After detachment the cells were washed once in PBS and centrifuged for 8-10 minutes at 200 rpm. This passage was repeated one more time. In the meantime the supplemented Human Chondrocyte Nucleofector Solution and the complete medium containing 20% FBS were equilibrated to room temperature. One ml of the pre-warmed medium was then added to each well of a 6 well plate and left in the incubator. After

centrifugation the chondrocytes were re-suspended to a final concentration of 10^6 cells/100µl in the pre-warmed Human Chondrocyte Nucleofector Solution. The samples were finally mixed with 2 µg of plasmid and transferred into an Amaxa cuvette, avoiding bubbles. The cells were then nucleofected by using program U28 in the Amaxa nucleofector. The cells were then added with 500 µl of 20% FBS-containing medium and transferred into the 6 well plate previously filled with pre-warmed medium and incubated overnight in standard culture conditions.

ATELOCOLLAGEN*:*

Atelocollagen-mediated transfection of chondrocytes was tested with two different conditions.

1) P0 chondrocytes were resuspended in atelocollagen 0.005%, 0.01%, 0.03%, 0.05%, 0.1% v/v diluted in complete medium in presence of 1µg plasmid/10000cells and seeded.

2) P0 chondrocytes were seeded into plates pre-coated with atelocollagen 1% diluted in complete medium and containing 1 µg of plasmidic DNA.

The medium was changed after 24 hours from transfection.

TRANSFECTION EFFICIENCY AND CYTOTOXICITY

The efficiency of transfection was estimated by the proportion of cells that displayed GFP fluorescence 24 hours following transfection with the CMV-GFP plasmid. To this end, bright field and fluorescent images were taken 24 hours after transfection using a fluorescence microscope and fluorescent and non-fluorescent cells were counted in two random fields of each well. Transfection efficiency was defined as the percentage of fluorescent to total cells.

In some experiments the transfection efficiency was determined by FACS analysis as the percentage of green fluorescent cells.

The survival rate was determined by trypan blue exclusion test or by FACS analysis. FACS analysis was performed by Dr Fulvio D'Aquisto. In the FACS plot in Fig. 51 and 54, SSH represents the granularity of the cells and the FSH the dimension of the cells. Gated dots represent the number of cells which identified as viable chondrocytes.

REPORTER ASSAY

In all the reporter assays described in this thesis transfection was performed with Lipofectamine as described previously.

SUPER8XTOP/FOP REPORTER ASSAY

After reaching 50%-60% confluency, human articular chondrocytes were co-trasfected with SUPER8XTopFlash –or its mutagenized form SUPER8XFopFlash- reporter vector (kind gift of Prof. Moon, University of Washington, USA), and CMV-Renilla luciferase vector (Promega), in a ratio 1:10. The SUPER8XTopFlash plasmid encodes for the Firefly (*Photinus pyralis*) luciferase gene under control of 7 TCF/LEF binding sequences whereas the SUPER8XFOPFlash is the control vector for theSUPER8XTOP, containing 6 mutated and not functional TCF/LEF binding sequences. The CMV-Renilla plasmid encodes for the Renilla (*Renilla reniformis*) luciferase gene under control of the CMV promoter. After overnight incubation at 37°C, the medium was changed and the cells were incubated for 24 hours with 100ng/ml of recombinant WNT-3A (R&D System) diluted in complete medium. In some experiments conditioned medium obtained by WNT-3A overexpressing L-cells was also used. The cells were subsequently lysed in passive lysis buffer 1X (Promega) for 30 minutes at room temperature under slow agitation and then scraped with the help of a pipette tip. Twenty µl of each lysate were successively added to 100µl of the Firefly Luciferase Assay Substrate (part of the Dual Luciferase Reporter Assay System kit, Promega) and gently mixed. The luminescence was recorded for 20 seconds by mean of TD-20/20 Luminometer (Turner Designs). Firefly luciferase luminescence was then quenched by adding 100µl of the Stop & Glo reagent (Dual Luciferase Reporter Assay System kit, Promega), containing also the substrate for the Renilla luciferase. The activity of renilla luciferase was measured with the same parameters used for the Firefly luciferase. Firefly luciferase activity was normalised by Renilla luciferase activity and expressed as relative luciferase units (RLU).

RNA EXTRACTION

MONOLAYER

Total RNA was extracted from fully confluent P0 chondrocytes monolayer cells seeded in 24well plates. After one wash in PBS, the cells were lysed by direct addition of TRIzol reagent (Invitrogen) (1ml/well), and each sample was gently sheered with a syringe for five times and collected in an Eppendorf tube. After a first centrifugation at 20000g for 10 minutes, 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 at least 20 seconds before being incubated on ice for 2 minutes. The samples were centrifuged for 15 minutes at 10000g. 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 30 minutes, to allow the precipitation of the RNA. If the expected amount of RNA was very low, in order to maximize the RNA yield, 0.5µl of Glycogen (Boheringer Mannheim GmbH) was added to each sample. The samples were centrifuged at maximum speed for 30 minutes at 4°C and the RNA pellet was resuspended in 70% v/v ethanol and eventually centrifuged at maximum speed for 5 minutes at 4°C. After centrifugation the ethanol was carefully removed and the pellets were allowed to air-dry for 15 minutes at room temperature, and subsequently re-dissolved in 4-12µl of ultrapure water, depending on the amount of the RNA purified. The concentration of the RNA was evaluated by reading the absorbance at 260 nm using a Nanodrop (Nanodrop) spectrophotometer and considering that pure RNA will have a 260/280 ratio between 1.8 and 2.

IMPLANTS

To extract RNA from human cartilage implants, cartilage was immediately frozen in liquid nitrogen, after the retrieval from the thigh of the $CD1^{nu/nu}$ mice. The implants were then stored in liquid nitrogen until the time of the extraction.

Before starting the procedure, a mortar and a pestle were thoroughly washed in 0.5% SDS, extensively rinsed and left air drying. They were afterwards submerged in liquid nitrogen along with a spatula. All the tools were then removed and liquid nitrogen was added to the mortar. The frozen cartilage was immediately placed in the liquid nitrogen-containing mortar avoiding thawing, and pulverised with the pestle. With the help of the pre-frozen spatula the powder was transferred into a vial containing 1ml of TRIzol. To allow a better disaggregation of the tissue, the suspension was sheared gently with a syringe and incubated on ice for 15 minutes under gentle tilting. The suspension was then moved to an Eppendorf tube and centrifuged at maximum speed for 10 minutes. The pellet was discarded and 200µl of chloroform were added to the sample which was then incubated on ice for 2 minutes. The tubes were centrifuged for 15 minutes at 10000g at 4°C and the aqueous phase containing the RNA fraction, collected. These were added with an equal volume of phenol in citrate buffer (Invitrogen) pH 4.5, mixed, and left 15 minutes on ice. After addition of an equal volume of chloroform:isoamyl alcohol 24:1, the samples were mixed, incubated on ice for 2 minutes and centrifuged again for 15 minutes at 10000g. The aqueous phase was collected and extraction with phenol/chloroform:isoamyl alcohol 24:1 was repeated once more. After a last separation with only chloroform:isoamyl alcohol 24:1, the aqueous phase obtained from the last centrifugation was mixed with an equal volume of pre-chilled isopropanol, shaken for at least 20 seconds and incubated on ice for 30 minutes. The samples were centrifuged at maximum speed for 40 minutes at 4°C. The pellet was washed with 70% v/v pre-chilled ethanol, centrifuged again at maximum speed for 5 minutes and left air drying for 15 minutes at 4°C. The RNA was eventually dissolved in 10µl of RNA-se free water, the RNA concentration measured spectrophotometrically using a Nanodrop spectrophotometer and stored at -80°C until further use.

GENE EXPRESSION ANALYSIS

Five hundred nanograms of total RNA from each sample were reverse transcribed by using Thermoscript Reverse Transcriptase Kit (Invitrogen) with oligo dT primers and quantitative polymerase chain reaction (PCR) was performed with hot-start DNA polymerase (Qiagen) in the presence of 0.1X SYBR green (Sigma-Aldrich) and 0.2X ROX dye (Invitrogen) by using a T7900 HD (Applied Biosystem) machine. For primer sequences see Table 2.

TABLE 2 HUMAN PRIMERS

Gene	Sense	Antisense	Lenght
β-actin,	5′-CACGGCTGCTTCCAGCTC-3′,	5′-CACAGGACTCCATGCCCAG-3′	134bp
ADAMTS-5	5′- GACCGATGGCACTGAATGTA -3′	5′- TGTACAGCTGGAGTTGTCTCCT -3′	145bp
Aggrecan	5′-GTTGTCATCAGCACCAGCATC-3′,	5′-ACCACACAGTCCTCTCCAGC-3;	509bp
Axin-2	5′-TACCGGAGGATGCTGAAGGC-3′	5′-CCACTGGCCGATTCTTCCTT-3′,	345bp
Col1A1,	5′-GCCCTGTCTGCTTCCTGTAA-3′,	5′-GGTTCAGTTTGGGTTGCTTG-3′,	104bp
Col2A1	5′-CTGCTCGTCGCCGCTGTCCTT-3′	5′-AAGGGTCCCAGGTTCTCCATC-3′	432bp
MMP-3	5′-CAACCGTGAGGAAAATCGATGCAG-3′	5′-CGGCAAGATACAGATTCACGCTCAA-3′	440bp
MMP-13	5′-ACGGACCCATACAGTTTGAATACAGC-3′,	5′-CCATTTGTGGTGTGGGAAGTATCATC-3′	360bp
PCNA	5′-GGAGAACTTGGAAATGGAAAC-3′	5′-CTGCATTTAGAGTCAAGACCC-3′	548bp
Sox9	5′-GAACGCACATCAAGACGGAG-3′	5′-TCTCGTTGATTTCGCTGCTC-3′	631bp
WNT-3A	5′-CCATCCTCTGCCTCAAATTC-3′	5′-TGGACAGTGGATATAGCAGCA-3′.	70bp
FZD1	5′-TTCAGCAGCACATTCTGAGG-3′	5′-CCTGCACACATTTTCCCTTT-3′	154bp
FZD2	5′-TCACGGTCTACATGATCAAA-3′	5′-GCAACCTAAAAGTGAAATGG-3′	266bp
FZD3	5′-GGATGATCAAAGAAGCAAAG-3′	5′-TTGAGCCGATGAGAACTACT-3′	186bp
FZD4	5′-GACTTTGGAAGGAACCTTTT-3′	5′-TGAAACCCCGTCTCTACTAA-3′	238bp
FZD5	5′-GGTTTGGTGCAGGTGAATTT-3′	5′-CTACAGCATGGGATAGGCACT-3′	125bp
FZD6	5′-TTCGGCAGCTCACTAGGATT-3′	5′-CATCAGAAAATCTTGCCCAA-3′	190bp
FZD7	5′-CTGGAGTTCTTTGAAATGTGCT-3′	5′-AAGGTTAGCTCCCATGATTCTC-3′	133bp
FZD8	5′-CGGTTTGGGTATTCTTAATG-3′	5′-ACAGGGGTAAGCCTCTAAAC-3′	215bp
FZD9	5′-AACACAGAGAAGCTGGAGAA-3′	5′-ACCACCAGTGACATGAAAAT-3′	251bp
FZD10	5′-AGAAACCCTTCAGTGCTACA-3′	5′-AAAGTGTCTCTGCCAACCTA-3′	205bp

PCR CONDITIONS:

Cycling was preceded by 2 minutes at 50°C and by a first denaturation step at 96°C, which was also needed 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:

COL2A1, Aggrecan: 2 steps 68 (96°C 30'', 68°C 1'30'', 40 cycles)

COL1A1: 2 steps 60 (96°C 30'', 60°C 1'30'', 40 cycles)

All the other primers: 3 steps 55 (96°C 30'', 55°C 30'', 72°C 1')

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 non-specific 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.

SDS-PAGE AND WESTERN BLOTTING

After treatment, P0 adult human articular chondrocytes (AHAC) cells seeded into 6 well plates well plate were washed once in ice-cold PBS containing phosphatase and protease inhibitors (Roche).

Total cell extracts were obtained by scraping the cells in 100µl of extraction buffer and leaving the lysates on ice for 30 minutes in total, vortexing the samples every 10 minutes. Protein

concentration of the samples was determined by BCA assay (Pierce). A total of 60µg of protein lysate per sample were added to 10 µl of Laemmli buffer 5X and the volume was adjusted up to 50µl with sterile PBS.

45 µl of each sample were loaded into 10% v/v Tris-Glycine pre-cast gel (Invitrogen) and run at 150V until the front dye reaches the bottom of the gel. The gels were then blotted by using i-Blot system (Invitrogen), a semi-dry blotting system allowing the transfer of the proteins from the polyacrilamide gels to nitrocellulose membranes.

β-catenin staining: the membranes were blocked in 5% non-fat milk prepared in PBS/0.1% v/v Tween (milk) for at least 3 hours at RT. The membranes were then incubated overnight at 4°C with anti β-catenin antibody (1:1000, Cell Signaling) diluted in milk, under slow agitation.

CaMKII and pT286CaMKII staining: the membranes were blocked in 5% BSA prepared in PBS/0.1% TWEEN (blocking solution) for at least 3h at RT. The membranes were then incubated overnight at 4°C with anti CaMKII antibody (1:2000, Abcam) or anti p286CaMKII (1:1000, Cell signalling) diluted in the blocking solution, under slow agitation.

The primary antibody was then removed and the membranes washed three times consecutively in PBS/TWEEN 0.1% (washing buffer). After these three initial washes the membranes were washed other 4 times every 15 minutes for 1 hour in total, under slow agitation. Subsequently, the membranes were incubated for 45 minutes at RT with the appropriate HRP-conjugated secondary antibody (goat anti-rabbit or goat anti-mouse from Santa Cruz) diluted 1:2000 in milk. The samples were washed again as after the primary antibody, and incubated for 1 minute and 30 seconds in the dark with home-made chemiluminescent solution.

PHOSPHO CAMKII IMMUNOFLUORESCENCE

For immunofluorescence, AHAC were seeded (10^4 cells/cm^2) on chamber slides (Lab-Tek) and treated for 24 hours with 100 ng/ml of recombinant WNT-3A in combination with 10µM KN92 or KN93 (Calbiochem). The cells were then washed in PBS and fixed in 4% buffered PFA (pH 7.4) for 5 minutes at room temperature. Auto-fluorescence was quenched by incubating the section twice in 50mM NH_4Cl for 10 minutes. After 1 hour blocking in FBS diluted 1:5 plus 0.2% Triton X, the sections were incubated overnight at 4°C with anti phospho-CaMKII rabbit polyclonal antibody (Cell Signalling) or rabbit IgG (Dako) as negative control, both diluted 1:100 in blocking solution. After washing twice for 10 minutes each in PBS/0.2%Triton X (washing solution), the cells were incubated with cy3-conjugated goat anti-rabbit antibody 1:300 (Jackson ImmunoResearch Laboratories) for 1 hour. Slides were mounted in Mowiol (Calbiochem, Merck Biosciences Ltd) and images were taken with an Olympus BX61 microscope by using 40X magnification/0.85 of numerical aperture. Images were acquired with a fluorescence microscope (Olympus BX61) by using an uplan Fluo 40x NA 0.85 objective. After acquisition and densitometric analysis (see below), the contrast of the images was enhanced for best graphic rendering using Adobe Photoshop 7.0, all with the same parameters, without altering relationship of target to control images. The fluorescence intensity was measured by using Image J software on the original photographs. The average pixel intensity profile (y-axis: 0=black, 255=white) was plotted over a linear length of a whole cell. The mean nuclear and cytoplasmic fluorescence intensity, obtained by the plot profile as shown in Fig. 35, were generated by analysing 40 cells per field in duplicate for each condition.

HIS-TAG DETECTION BY IMMUNOFLUORESCENCE

For the detection of the His Tag in the cartilage implants retrieved after the co-injection of AHAC with Cos7 cells transfected with a His-tagged vector encoding for granulocyte chemotactic protein 2/chemokine alpha 3 (GCP2/CKA) (pGCP2MycHis, map in Fig. 14), sections were equilibrated in 0.02% HCl for 15 minutes then digested in 0.25 mg/ml of pepsin (Sigma) in

0.02% v/v HCl for 45 minutes at 37°C, washed in water and allowed to air dry for 20 minutes. Next, the sections were post-fixed in 4% PFA for 10 minutes at room temperature, and washed twice with PBST (5 minutes washes). The endogenous peroxidase activity was quenched by incubating the sections with 10% v/v H_2O_2 for 15 minutes. The sections were blocked in Protein Blocking Solution (Dako) for 1 hour at room temperature, blotted, and incubated overnight at 4°C with the primary antibody Goat polyclonal to 6x His Tag HRP diluted to a final concentration of 4mg/ml (Abcam) in the protein Blocking Solution (Dako). Sections were then washed three times in PBST and developed with liquid DAB Substrate Chromogen System (DAB) as peroxidase substrate (BDH).

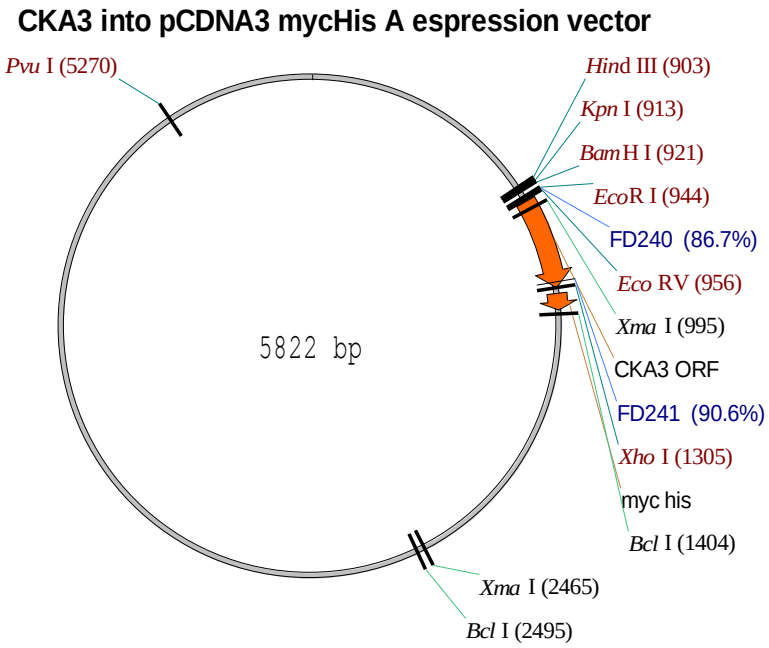


Figure 14: GCP2 (CKA) MycHis tagged encoding plasmid

Calcium Mobilization Assay

P0 AHAC were seeded at a density of 6000cells/well into a 96 well plate. One day after seeding the cells were labelled for 30 minutes at RT and for 30 minutes at 37°C with 50µl of Fluo-4-Direct kit (Invitrogen) solution containing 2.5mM Probenecid and were subjected to treatments with growth factors or inhibitors as indicated for individual experiments. After

treatments, the fluorescence emitted by the cells (excitation wavelength 494 nm, emission wavelength 516 nm) was recorded using a Fluo-Star Galaxy fluorimeter. The fluorescence was expressed as Relative Fluorescence Units (RFU).

ECTOPIC CARTILAGE FORMATION ASSAY

This assay measures the capacity of cells to form ectopic stable cartilage *in vivo* and was performed as previously described (Dell'Accio et al., 2001). Five million cells per injection were used. Cells were washed twice in PBS and resuspended in aliquots of $5X10^6$ cells in 50µl of PBS and injected in the posterior compartment of the thigh of female $CD1^{nu/nu}$ mice. After 14 days the mice were killed and the implants carefully dissected. Each implant was measured for wet weight and cut in half along the longest axis. One half was processed for total RNA extraction, and the other fixed and embedded in Optimal Cutting Temperature (OCT) or paraffin for histological examination. The sample orientation was arranged in such a way that the first section went through the cleavage plane of the explants and represented the cross-sectional largest area.

GENERATION OF GROWTH ARRESTED CELL LINES FOR GENE DELIVERY

In some experiments, chondrocytes were co-injected with growth arrested cell lines-Cos7 (monkey kidney fibroblasts) and L-cells (murine skin fibroblasts) overexpressing foreign genes. To induce growth arrest, the cells were cultured until they reached 80% confluency. 7.5 µg/ml (for L-cells) or 5 µg/ml (for Cos7 cells) of mitomicyn C where added to the culture medium and the cells returned to the incubator. After two hours, the cells were washed twice in PBS, counted and checked for viability. The cells were then either replated to control for efficacy of growth arrest, or used for co-implantation in a proportion of 1/10 growth arrested cells/chondrocytes.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

PARAFFIN EMBEDDING

a) Samples were fixed overnight in freshly made 4% buffered PFA o/n at 4°C.

b) The samples were dehydrated through a gradient of ethanol, xylene and paraffin.

c) The samples were embedded in paraffin and stored at RT until needed.

d) Sectioning was performed with 5µm slices. The sample number was recorded to allow serial staining.

OCT EMBEDDING

a) A drop of OCT was placed in a foil mould.

b) The sample was added, and oriented as required, and then covered completely with OCT.

c) A 6 well plate filled with isopentane (2-methyl butane) was placed in a box filled with liquid nitrogen, bringing the isopentane at its freezing point (-160°C).

d) The cylinder with the embedded sample was then placed within the ice-cold isopentane, until completely frozen.

e) Blocks were stored at -80°C in 24 well plates until needed.

f) The OCT blocks were sectioned serially using a cryostat with 7 µm-thick sections. All sections were serially numbered to facilitate serial staining.

PREPARATION OF PARAFFIN SECTIONS

a) Sections were heated at 55°C for 15 minutes and immediately immersed in xylene.

b) Sections were deparaffinized through 2 serial passages (5 minutes each) in xylene, and 2 passages in 100% ethanol (5 minutes each).

c) Sections were air dried for 20 minutes.

PREPARATION OF FROZEN SECTIONS

a) The OCT blocks were equilibrated overnight at -20°C

b) The cryostat was equilibrated with a room temperature of -18°C and a sample temperature of -20°C

c) Sectioning was performed at an interval of 7µm

d) Sections were air-dried overnight and then stored at -20°C until further use

e) Sections were thawed and air dried at room temperature for 30 minutes

f) For some applications including immunohistochemistry, sections were post fixed with one drop of freshly made 4% PFA for 5 minutes, and rinsed in PBS.

HAEMATOXYLIN AND EOSIN (H&E) STAINING

1) Frozen sections were gradually rehydrated in descending grades of ethanol (100%, 70% and 50% ethanol (5 minutes each)
2) Sections were washed in distilled water for 5 minutes
3) The sections were stained for 5 minutes in Mayer's hematoxylin (BDH)
4) The slides were then washed again in water and placed in Eosin (BDH) for 1 minute
5) The slides were washed again and dehydrated in ascending grades of ethanol (50%, 70% and 100% for 5 minutes each
6) Two passages in xylene (5 minutes each) and mounted with DEPEX.

TOLUIDINE BLUE STAINING

1) The slides were deparaffinised in xylene (2X5minutes) and 100% ethanol (2X5minutes)
2) The sections were washed in distilled water for 5 minutes
3) One drop of toluidine blue prepared in acetate buffer (pH 4.0) was put on each section for a maximum of 30 seconds. The excess of dye was removed by washing the slides in water and the sections were left air drying.
4) The sections were then differentiated in ethanol 100% ethanol (2X5minutes), then in xylene (2X5 minutes) and mounted with DEPEX.

SAFRANIN O STAINING

1) The slides were deparaffinised in xylene (2X5 minutes) and 100% ethanol (2X5 minutes)
2) The sections were washed for 5 minutes in and immersed in safranin O (SO) 0.2% w/v for 13 minutes.
3) The sections were rinsed thoroughly in distilled water (2X2 minutes)
4) The sections were then differentiated in ethanol 100% ethanol (2X5minutes), then in xylene (2X5 minutes) and mounted with DEPEX.

IMAGES AND DIGITAL PHOTOGRAPHY

Images were taken with an Olympus BX61 microscope. Images were acquired at 22°C, by using an Olympus F-View II (SIS) camera and Cell P software.

For fluorescent images, the acquisition parameters were set using the positive and negative control sections to achieve the maximum sensitivity and specificity as described below and were then used constant for the acquisition of all images.

a) Autogain off
b) Setting exposure time. The negative control (non-immune IgG) was placed in on the microscope and the exposure time set and fixed in such a way that the background

fluorescence was barely visible or not visible at all. This same exposure time was used to acquire all images.

c) Gain setting. The gain parameter was set using the section, within the experiment, with the strongest fluorescence. The upper limit was then set so that the brightest point was nearly completely white, but with a value ≤ 225, and then kept constant for all images. This allowed preventing saturation and consequent loss of sensitivity.

IMAGE ANALYSIS

Image analysis was performed using image J software. Original black and white images were used to quantify fluorescence. Original color images were used to quantify morphological parameters and safranin O staining.

AREA DETERMINATION

5 non-consecutive sections were chosen through the centre of the explants covering a total of 350µm in thickness. These sections therefore represented the maximum cross-sectional area. The implant area was manually drawn, eliminating the residual skeletal muscle left attached. After calibration, the area of the implant in each section was determined and the value averaged.

SAFRANIN O INTENSITY

The sample area identified in the previous paragraph was saved as a mask. The pictured were transformed in grayscale. The average gray value within each mask was averaged and represented the intensity of safranin O staining.

FLUORESCENCE INTENSITY AND CELLULAR DISTRIBUTION (CAMKII IMMUNOSTAINING)

Fluorescent images were acquired as described above. For each cell analysed a line along the maximum axis (Fig. 32) was drawn. The grayscale values along the line were plotted with a histogram using Image J.

The area under the curve was calculated and represented the total fluorescence. The area under the curve relative to the segment corresponding to the nucleus was also calculated and represented the nuclear fluorescence (Fig. 35).

STATISTICAL ANALYSIS

Parametric data were compared with student T-test. For multiple comparisons we used ANOVA analysis, with Dunnet post-test. Data in Fig. 33B were analysed with Kruskal Wallis with Dunns post-test. P values less than 0.05 were considered significant. *, $p<0.05$; **, $p<0.005$; ***, $p<0.0005$. In the graphs values are expressed as mean±SEM.

BUFFERS AND MEDIA

COMPLETE MEDIUM

1% antibiotic-antimycotic solution

1% NaPyr

10% serum

FREEZING MEDIUM

7.5ml FBS

5ml DMSO

Top to 25 ml with complete medium

Sterilize by filtration

LB MEDIUM

10g tryptone

5g yeast extract

10g NaCl

LB-AGAR

10g tryptone

5g yeast extract

10g NaCl

15g AGAR

Autoclave to solubilize

SDS-PAGE

EXTRACTION BUFFER

10 mM HEPES

1.5 mM $MgCl_2$

10 mM KCl

0.5 mM DTT

0.05% NP40

pH 7.9

TRIS-GLYCINE RUNNING BUFFER

25mM Trizma-Base

192mM Glycine

0.1% SDS

pH8.3

LAEMMLI BUFFER

63mM Tris HCl

10% Glycerol

2% SDS

0.0025% Bromophenol blue

pH 6.8

BLOCKING SOLUTION (BETA-CATENIN)

5% non-fat milk

0.1% TWEEN

PBS

BLOCKING SOLUTION (CAMKII OR PCAMKII)

5% non-fat milk

0.1% TWEEN

TBS

WASHING SOLUTION

0.1% TWEEN

PBS/TBS

ECL

Solution 1

1ml of luminol stock (250mM luminol in DMSO (0.88g/20ml))

0.44ml p-coumaric acid stock (0.29g/20ml)

10ml 1M Tris Base (pH 8.5)

Make up to 100ml with distilled water

Solution 2

64µl 30% H_2O_2

10ml Tris Base (pH 8.5)

Make up to 100ml with distilled water

Mix 1:1 and add for 1'30" on the nitrocellulose membrane prior developing.

ACETATE BUFFER

Stock solutions:

Stock A: 0.2M acetic acid

(1.2 cm^3 glacial acetic acid in 100 cm^3 of distilled water)

Stock B: 0.2M sodium acetate

(1.64g sodium acetate anhydrous in 100 cm^3 of distilled water)

For toluidine blue solution

41 cm^3 of Stock A + 9cm^3 of Stock B made up 100cm^3 with distilled water (final solution=pH 4.0)

TOLUIDINE BLUE

Add 0.2g toluidine blue powder to 100ml of the acetate buffer and filter.

Leave resting at RT for at least 1 week.

SAFRANIN O

0.2% SO in 0.2M glacial acetic acid in distilled water

HAEMATOXYLIN

For 2 liters

- 2g Haematoxylin powder
- 100g Aluminium Potassium Sulphate
- 0.4g Sodium Iodate

Heat at 85°C for 1-2 hours

Once dissolved add:

- 1g Citric Acid
- 100g Choral Hydrate

Heat again at 85ºC for 1-2 hours

EOSIN

Dissolve 10g of Eosin Y in 1l of distilled water on a warm stirrer plate. Filter the solution prior the use.

CHAPTER 4: RESULTS

BIOLOGICAL EFFECTS OF WNT-3A TREATMENT ON ARTICULAR CHONDROCYTES *IN VITRO*

EXPRESSION OF WNT-3A AND FZD RECEPTORS IN HUMAN ARTICULAR CHONDROCYTES

To test the biological effect of the activation of the Wnt/β-catenin pathway in human articular chondrocytes, we chose to stimulate these cells with WNT-3A. WNT-3A is a prototypical, well characterized activator of the β-catenin dependent canonical pathway (Shimizu et al., 1997) which an established role in arthritis (Nakamura et al., 2005). Confirming such involvement, we detected WNT-3A in normal articular cartilage and we discovered that its mRNA expression was down-regulated in OA (Fig. 15).

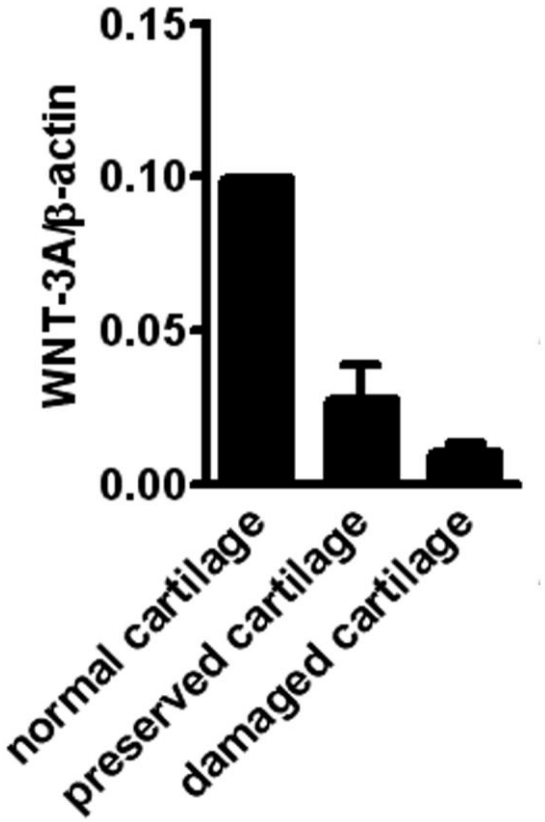


Figure 15 :WNT-3A mRNA expression in human articular cartilage

WNT-3A mRNA expression in human articular cartilage. Normal cartilage was removed from the knee of an amputee patient (n=1). Preserved cartilage was defined by a Mankin score ≤ 4; damaged cartilage had a Mankin score ≥ 6 (n=3). Preserved and damaged cartilage were removed from different areas of the tissue taken from the same patient. Gene expression values were obtained by Q-PCR and normalized for the housekeeping gene *β-actin*. The values are expressed as mean±SEM. The values are not statistically different in a significant way.

Before evaluating the effect of WNT-3A stimulation I also checked for the expression of all the Frizzled (FZD) receptors known to modulate canonical and non-canonical pathways (Fig. 16).

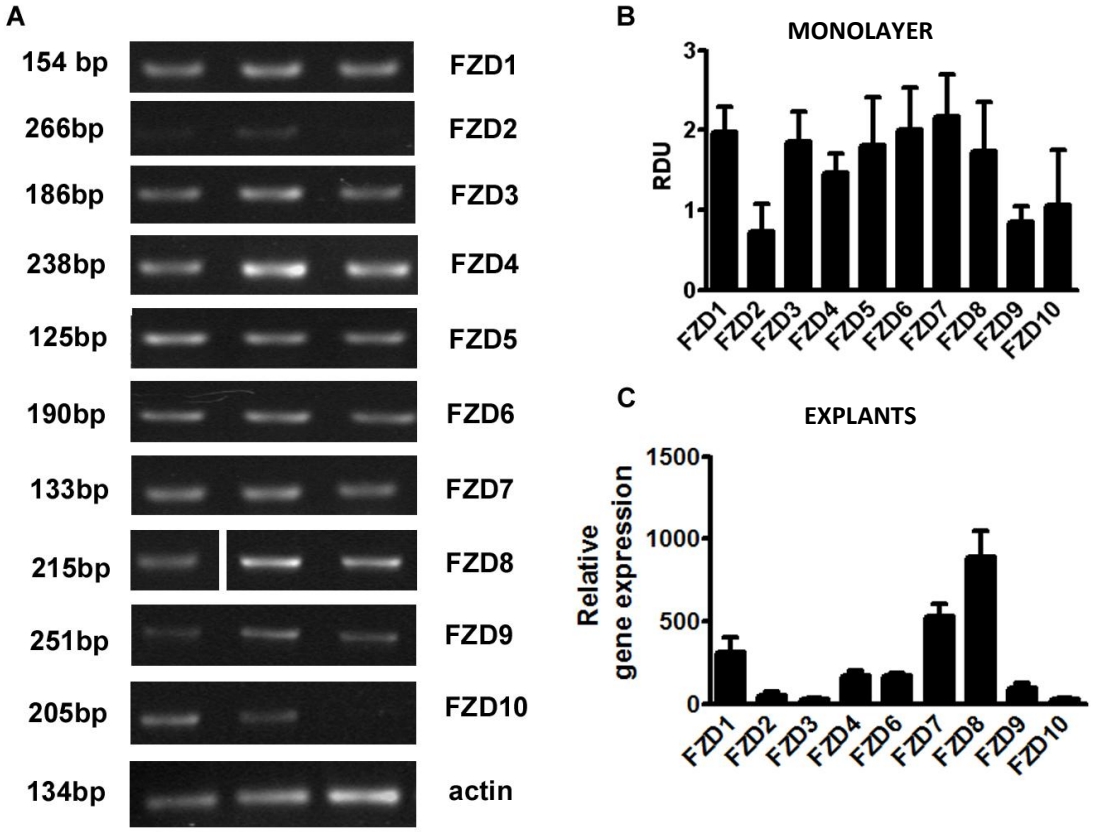


Figure 16: Gene expression analysis of FZD receptors in human cartilage explants and P0 chondrocytes

(A-C) Gene expression levels of *FZD* receptors in human cartilage explants and P0 chondrocytes. (A) Expression levels of *FZD* receptors in P0 chondrocytes were assessed by semi-quantitative PCR (40cycles). Each band correspond to cDNA extracted from a different donor. (B) The intensity of the bands was measured using densitometry by Image J software and then normalized for the intensity of β-actin (25 cycles). (C) Expression levels in human cartilage explants were obtained by a microarray analysis in cartilage explants (Dell'Accio et al., 2008). RDU= Relative Densitometry Units.

All FZD receptors were expressed at mRNA levels both in articular chondrocytes cultured in monolayer and in human cartilage explants (Fig. 16). Therefore, articular chondrocytes maintain *in vitro* the expression of FZD receptors and the capacity to respond to Wnts.

WNT-3A PROMOTES ARTICULAR CHONDROCYTE PROLIFERATION

I then started to investigate the function of this ligand on the proliferation, differentiation, and extracellular matrix production of articular chondrocytes, because all these biological outcomes are relevant to cartilage homeostasis.

Treatment of primary porcine articular chondrocytes with 100 ng/ml of recombinant WNT-3A over a three day time course resulted in a statistically significant increase of cell proliferation compared to vehicle control (Fig. 17A). The proliferative effect of WNT-3A was confirmed in human articular chondrocytes, where the proliferation marker *PCNA* was up-regulated 24 hours after stimulation with WNT-3A (Fig. 17B).

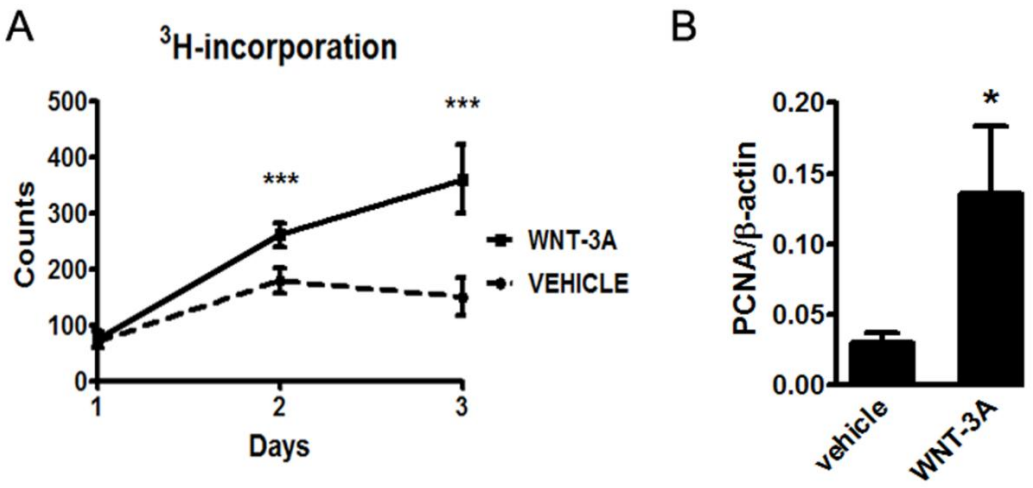


Figure 17: WNT-3A promotes articular chondrocyte proliferation

(A) Porcine articular chondrocytes were treated with 100ng/ml of recombinant WNT-3A or vehicle for three days. Incorporation of tritiated thymidine was recorded every 24 hours and the counts plotted (n=6 for each time point). (B) WNT-3A promotes up-regulation of the proliferation marker gene PCNA in AHAC. P0 AHAC were treated for 24 hours with 100 ng/ml of recombinant WNT-3A and PCNA gene expression levels were analyzed by Q-PCR and normalized for β-actin (n=4). Statistical analysis was performed with unpaired t-test.

WNT-3A PROMOTES ARTICULAR CHONDROCYTE DE-DIFFERENTIATION

WNT-3A has been shown to delay chondrogenesis in mesenchymal stem cells and to promote chondrocyte de-differentiation in articular chondrocytes (Ryu et al., 2002;Reinhold et al., 2006). To test the effect of WNT-3A stimulation in my system, I compared the expression of chondrocyte-lineage markers by confluent primary AHAC treated with either WNT-3A or vehicle control. *COL2A1*, *Aggrecan*, and *SOX9* mRNA were statistically significantly down-regulated following WNT-3A treatment (Fig. 18). In contrast with previous data (Yuasa et al., 2008), the chondrocyte hypertrophy marker *MMP13* was also down-regulated. Other genes relevant to cartilage homeostasis/biology including *COL1A1*, *MMP-3*, and *ADAMTS5* (Goldring, 2006) were unchanged (Fig. 18).

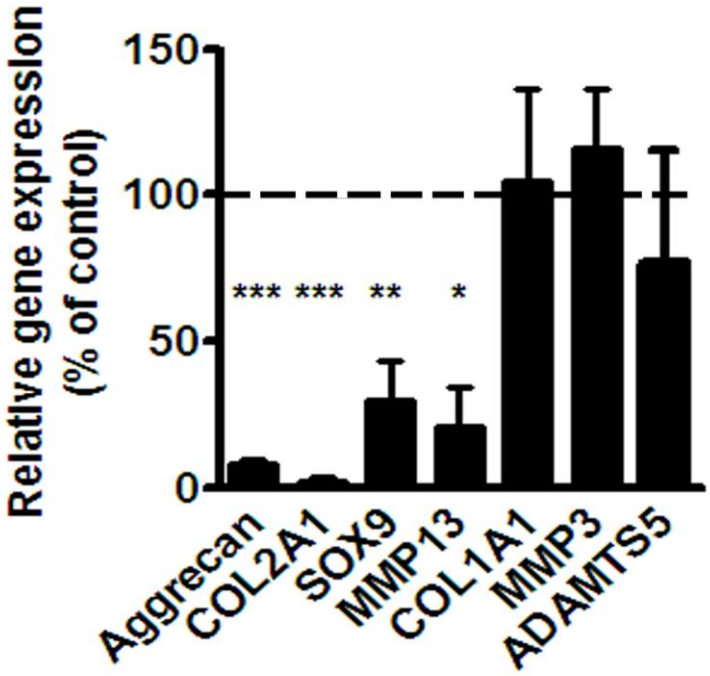


Figure 18: WNT-3A promotes articular chondrocyte de-differentiation

WNT-3A induced down-regulation of the differentiation markers *COL2A1*, *Aggrecan*, *SOX9* and *MMP13*, as evaluated by Q-PCR (n=6, 3 donors, 2 samples/donor). Gene expression values are reported as percentage of up- or down-regulation compared to control (100%-dotted line in the graph). Statistical analysis was performed by paired T-test.

The main function of articular chondrocytes is the production of a specialized extracellular matrix rich in highly sulphated GAGs, which provide the elastic biomechanical properties required for motion and weight bearing. Therefore, to test whether WNT-3A affected this important function of chondrocytes, I took advantage of the capacity of chondrocytes to produce large amounts of such matrix when cultured in 3D micromasses.

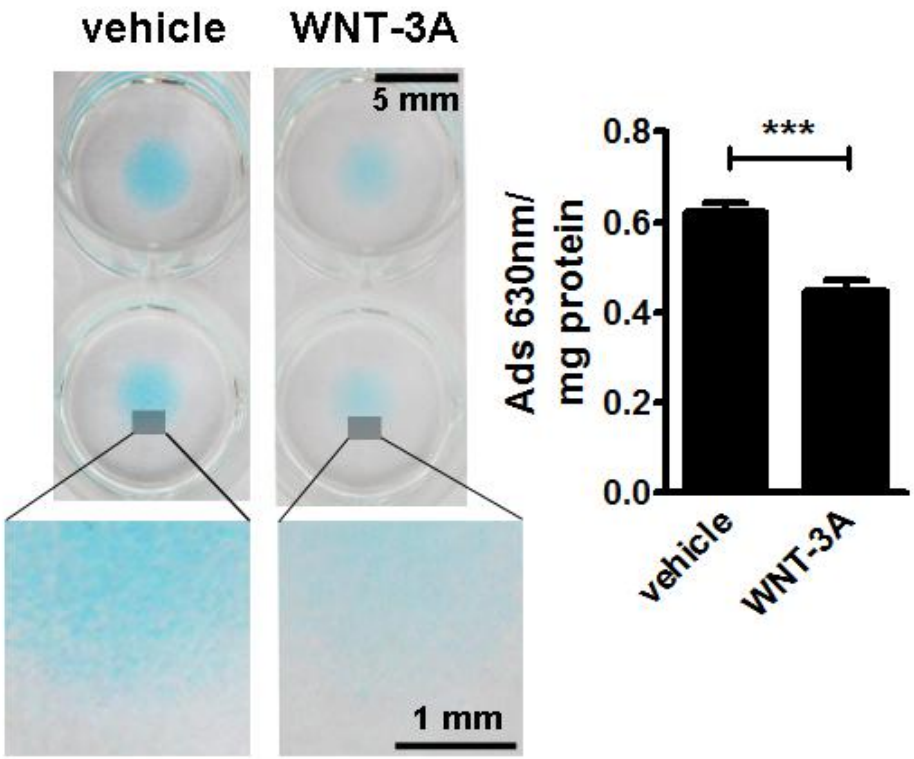


Figure 19: WNT-3A decreases ECM deposition in AHAC micromasses

P0 Chondrocyte micromasses were stimulated for four days with 100ng/ml of recombinant WNT-3A. The micromasses were then fixed in methanol and stained overnight with alcian blue. Following extraction in 6M guanidine, the amount of the Alcian blue was quantified by spectrophotometric measurement at 630nm (n=4). Statistical analysis was performed by unpaired t-test.

Treatment of human primary AHAC micromasses with 100ng/ml of WNT-3A for 4 days induced a statistically significant decrease in the accumulation of highly sulphated GAGs, as evaluated by Alcian Blue staining and spectrophotometric quantitation (Fig. 19).

On mesenchymal stem cell micromasses, the decrease of proteoglycan content due to WNT-3A stimulation was shown to be an irreversible process (ten Berge et al., 2008). To test if this was true also in micromasses obtained from fully differentiated cells, I stimulated AHAC micromasses for four days with WNT-3A and then I withdrew the stimulus for additional 4 days.

The proteoglycan content of micromasses in which the stimulus was withdrawn was similar to the ones in which WNT-3A stimulation was prolonged for 8 days; this shows that the biological effect of WNT-3A is permanent and irreversible also in this biological system (Fig. 20)

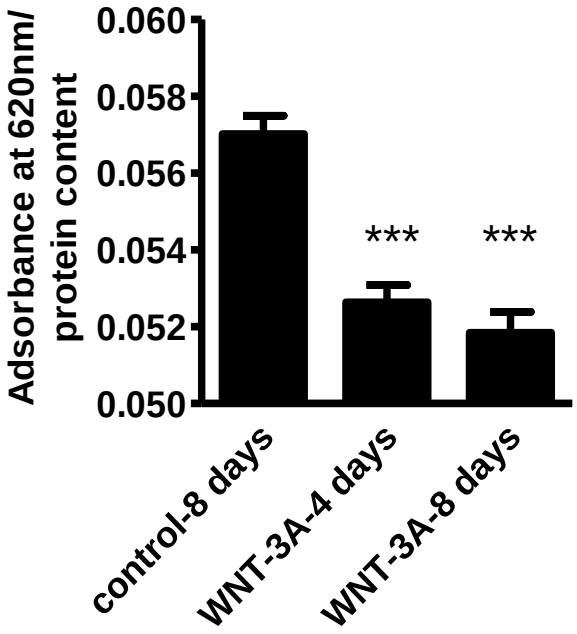


Figure 20: Articular chondrocyte de-differentiation promoted by WNT-3A is irreversible

Chondrocyte micromasses were stimulated for 8 days with conditioned medium obtained from L-cells overexpressing WNT-3A (diluted as described in the "Appendix 2" session) with control-medium. A separate group was stimulated with WNT-3A conditioned medium for 4 days before withdrawing the stimulus for other 4 days. The micromasses were stained with alcian blue overnight and proteoglycan content measured after guanidine extraction. n=4 for each condition. Statistical analysis was performed by unpaired t-test.

These data are largely in keeping with previous literature: WNT-3A treatment promoted human articular chondrocyte proliferation, but also induced down-regulation of the chondrocyte phenotypic marker genes *COL2A1*, *AGGRECAN*, and *SOX9*. In addition, WNT-3A-stimulation decreases the expression of the hypertrophic marker gene *MMP13*.

WNT-3A treatment also decreased the accumulation of GAGs in chondrocyte micromasses.

From this experiment we cannot determine if this is due to a decreased synthesis or to an increased catabolic activity. On the basis of the gene expression analysis data, we prefer the first hypothesis, since the expression of *Aggrecan* and *MMP13* mRNA is reduced upon WNT-3A stimulation as well as *MMP13* expression. However, both decreased synthesis and increased metalloproteinase activity have been demonstrated in the previous literature (Yuasa et al., 2008). To better understanding how WNT-3A influence GAGs content, ^{35}S-incorporation and metalloproteinase activity should be monitored.

BIOLOGICAL EFFECTS OF WNT-3A TREATMENT ON ARTICULAR CHONDROCYTES *IN VIVO*

To confirm the validity of the biological effects of WNT-3A in an *in vivo* system, we used a well-established model that measures the capacity of chondrocytes to form ectopic stable hyaline-like cartilage when implanted intramuscularly in nude mice (Dell'Accio et al., 2001). The ectopic cartilage formation assay has been proven to be an accurate and reproducible tool to assess if the chondrocytes have lost their phenotypic stability (Dell'Accio et al., 2001). Following serial culture expansion, chondrocytes irreversibly lose their phenotype and the ability to form cartilage *in vivo*, although they still can re-express *COL2A1* or *Aggrecan* when cultured in anchorage independent conditions (e.g. in agarose)(Benya and Shaffer, 1982). The assay was also validated to predict the successful outcome of autologous chondrocyte transplantation (ACI) in a goat model (Dell'Accio et al., 2003b) and in human (Saris et al., 2009;Saris et al., 2008).

To provide a constant supply of WNT-3A during the cartilage implant formation *in vivo* with minimal manipulation of the chondrocytes, we set up a cell-delivery system based on the co-injection of the articular chondrocytes with growth-arrested L-cells overexpressing WNT-3A or untransfected, growth arrested, L-cells as control. We then performed a thorough validation of this method demonstrating effective growth arrest, that growth arrested L-cells retain the capacity to stably express and secrete biologically active WNT-3A for at least 2 weeks, and that growth arrested cells persist alive when co-implanted in vivo. The data relative to such validation are quite large, and therefore we have reported them as a methodological chapter in "Appendix 3" at the end of this thesis.

We performed the ectopic cartilage formation assay in nude mice by co-injecting $5X10^6$ freshly isolated porcine chondrocytes with $5X10^5$ L-WNT3-A cells or wild type L-cells as control. We performed 12 co-implantations per group. Two weeks following implantation, the mice were killed, the explants carefully dissected and measured for wet weight. Subsequently the explants were fixed, cut through their major axis, sectioned, and the sections through the centre were utilized for histomorphometrical assessment as described in detail in the "Materials and Methods" section.

To compare the degree of chondrocytic differentiation and extracellular matrix production in the two groups of implants we used Safranin O staining. The staining was statistically significantly weaker in the implants obtained by the co-injection of the chondrocytes with the WNT-3A overexpressing L-cells (Fig. 21A-F).

The wet weight and the cross-sectional area of the cartilage implants retrieved from mice injected with chondrocytes plus L-WNT-3A cells was statistically significantly higher compared to that of implants obtained from chondrocytes co-implanted with control L-cells (Fig. 22A-B), this suggesting a higher degree of chondrocyte proliferation in the implants subjected to WNT-3A stimulation.

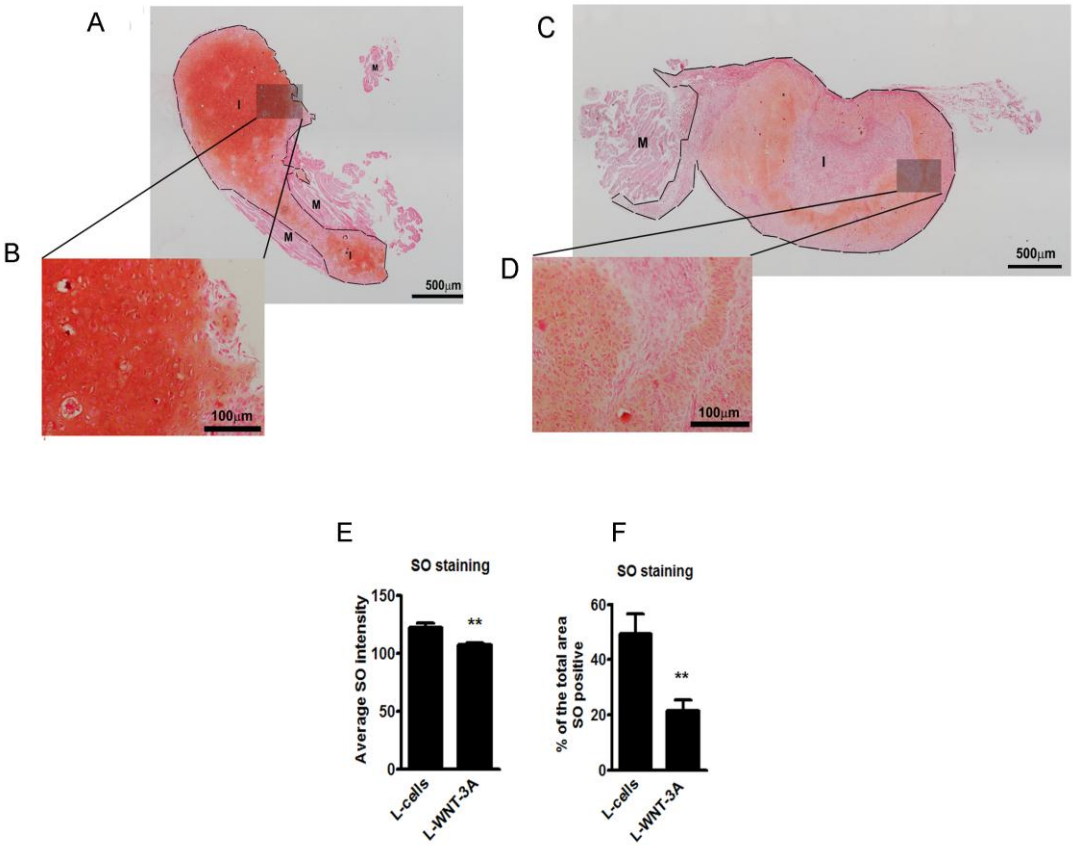


Figure 21: WNT-3A promotes de-differentiation of articular chondrocytes in vivo

Safranin O staining of cartilage implants obtained by the co-injection of porcine articular cartilage with L-cells (A-B) or L-WNT-3A cells (C-D) in nude mice. (E-F) Cartilage implants obtained from the co-injection with L-WNT-3A cells are less differentiated as shown by decreased safranin O staining. Two different parameters were evaluated by using image J software: the average of the total safranin O staining in the implant (E) or the percentage of the total area reaching an intensity of staining equal to a fixed thresholded value (F). Grayscale digital images from safranin O (SO) staining were used to calculate and compare the intensity of the staining (12 implants per conditions were evaluated; 1 slide per implant was stained and analysed). M=muscle; I=Implant. Unpaired T-test was used for statistical evaluation.

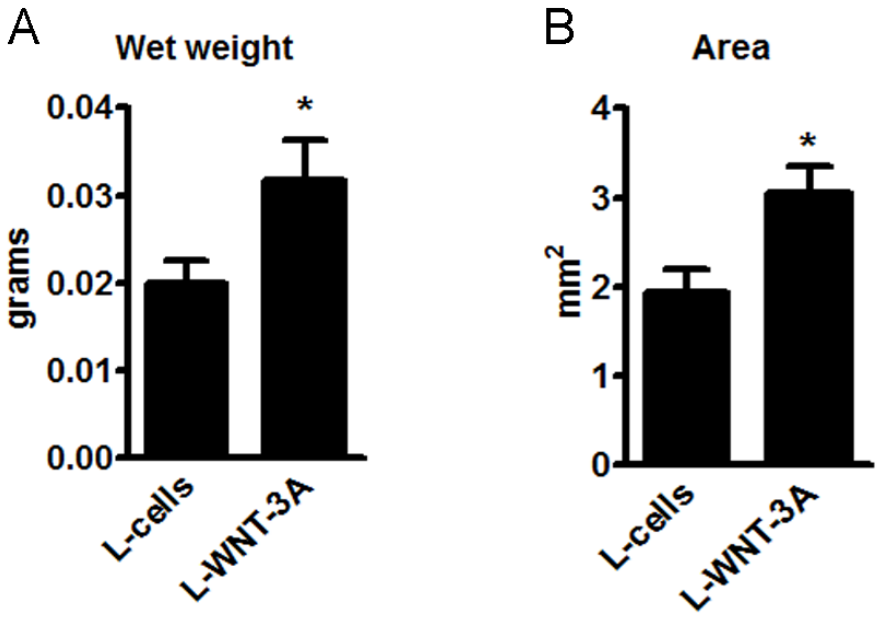


Figure 22: WNT-3A promotes the formation of larger cartilage explants in vivo.

(A) Cartilage implants retrieved from nude mice co-injected with porcine articular chondrocytes and L-WNT-3A cells had a statistically significant higher wet weight. (B) After removal from the muscle of the nude mouse, the cartilage implants were cut in two parts through the longitudinal axis and paraffin embedded. Implants were stained with safranin O and the surface area calculated with Image J software excluding any residual murine muscular tissue (12 implants per conditions were evaluated). The unpaired T-test was used for statistical evaluation.

Similar results were obtained with primary AHAC (Fig. 23), thereby confirming that this biological outcome is conserved across mammals.

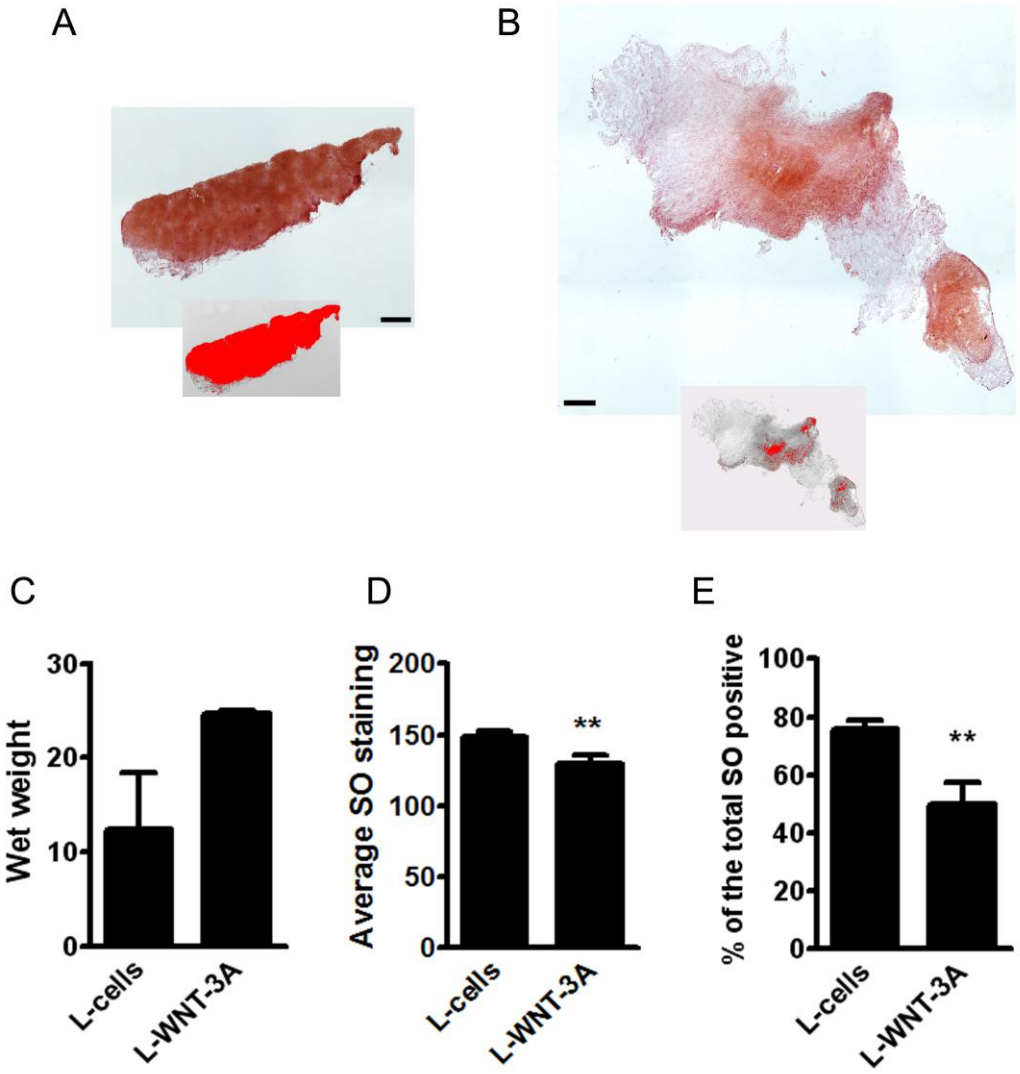


Figure 23: WNT-3A promotes the formation of larger more de-differented human articular chondrocyte implants in vivo

A-E) Cartilage implants retrieved from nude mice co-injected with human articular chondrocytes and L-WNT-3A cells had a higher wet weight and a weaker SO staining in comparison to control implants. After removal from the muscle of the nude mouse, the cartilage implants were cut in two parts through the longitudinal axis and paraffin embedded. Cartilage implants obtained from the co-injection with L-WNT-3A cells are less differentiated as shown by decreased safranin O staining. Two different parameters were evaluated by using image J software: the average of the total safranin O staining in the implant (D) or the percentage of the total area reaching an intensity of staining equal to a fixed thresholded value. (E) Grayscale digital images from safranin O (SO) staining were used to calculate and compare the intensity of the staining (3 implants per condition were analysed; five sections per implant were averaged).

The chimeric nature of these implants also allowed gene expression analysis selectively for the chondrocytes using human specific primers. The mRNA levels of the proliferation marker gene PCNA were increased, whereas *COL2A1*, *Aggrecan* and *Sox9* levels were decreased, even though this did not reach statistical significance, probably due to the small sample size (n=3 per condition) of this experiment (Fig. 24).

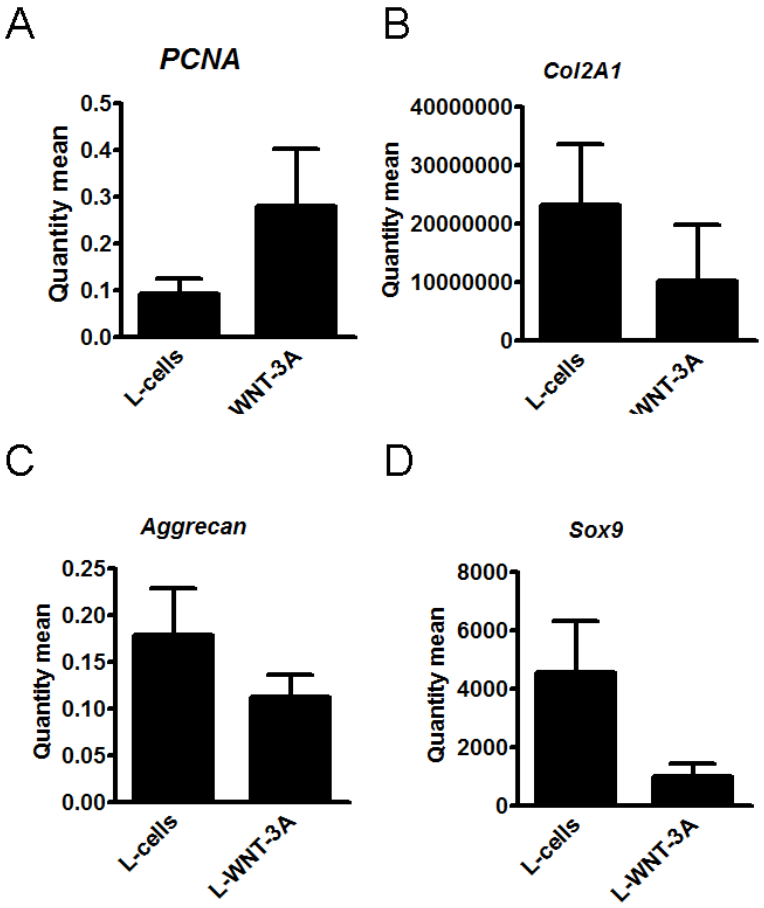


Figure 24: WNT-3A promotes human chondrocyte de-differentiation in vivo

A-D) RNA was extracted by the cartilage implants retrieved from the nude mice. After retrotranscription, the levels of *PCNA*, *COL2A1*, *Aggrecan* and *SOX9* mRNA were measured by real time PCR. n=3 for each condition.

Together, these data show that co-injection of porcine or human articular chondrocytes with L-cells over-expressing WNT-3A results in an increased proliferation of these cells and in their de-differentiation. These results confirm the biological effect of WNT-3A seen *in vitro* in an *in vivo* system of high clinical relevance.

WNT-3A ACTIVATES THE CANONICAL PATHWAY IN HUMAN ARTICULAR CHONDROCYTES

Having confirmed that WNT-3A promotes proliferation and de-differentiation, we decided to determine the molecular mechanisms of these effects. WNT-3A is known to activate the Wnt/β-catenin pathway in a variety of cells. We therefore set out to confirm the activation of this pathway by WNT-3A in AHAC. Treatment with 100 ng/ml recombinant WNT-3A resulted in accumulation of β-catenin (Fig. 25), activation of the SUPER8XTOPFlash reporter assay (Fig. 26A), and a 250 fold up-regulation of the endogenous target gene *AXIN2* (Yan et al., 2001)(Fig. 26B), indicative of activation of the β-catenin-dependent canonical pathway.

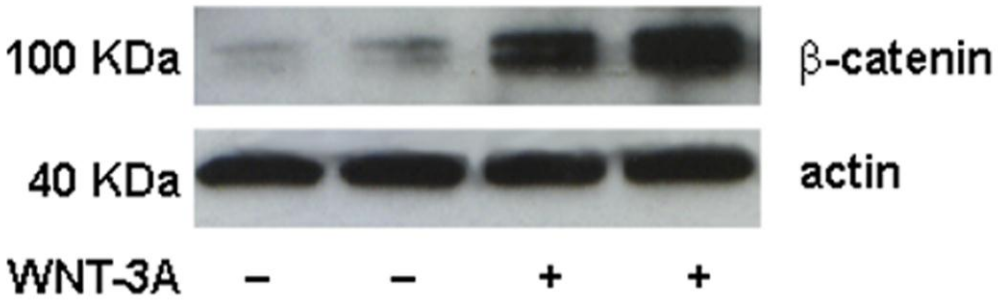


Figure 25: WNT-3A causes accumulation of β catenin in AHAC

Western blotting for β-catenin in the cytoplasmic fraction of AHAC treated for 24 hours with 100 ng/ml of recombinant WNT-3A or vehicle control.

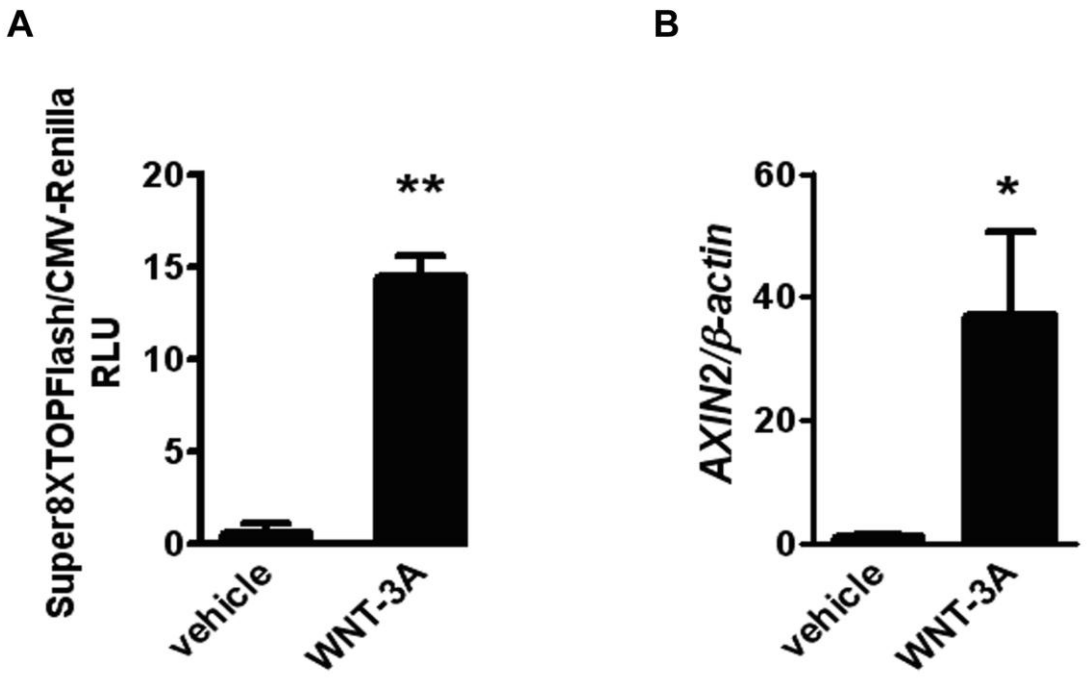


Figure 26: WNT-3A activates the LEF/TCF-dependent transcription in AHAC.

(A) Twenty four hours stimulation of AHAC with 100ng/ml of recombinant WNT-3A activated the SUPER8XTOPFlash reporter assay in P0 AHAC (n=3). (B) WNT-3A up-regulated the expression of the endogenous Wnt-canonical target gene *AXIN2* in AHAC as evaluated by Q-PCR. Values were normalized for the housekeeping gene β-actin. n=6 (3 donors; 2 samples/donor). Data were analysed with unpaired T-test.

DKK1 INHIBITS THE ACTIVATION OF THE CANONICAL PATHWAY PROMOTED BY WNT-3A BUT NOT WNT-3A-INDUCED DE-DIFFERENTIATION

To confirm that the observed AHAC phenotypic changes were due to the activation of the canonical pathway, primary AHAC were treated with either 100 ng/ml WNT-3A alone or in combination with 100 ng/ml of recombinant DKK1, which specifically inhibits the β-catenin-dependent pathway by binding to LRP co-receptors and preventing their association with FZDs (Mao et al., 2001). Co-treatment with DKK1 reverted the WNT-3A-induced up-regulation of *AXIN2*, *PCNA* and *MMP13* (Fig. 27A-C), confirming the inhibition of the canonical pathway.

Surprisingly however, DKK1 failed to rescue the down-regulation of *COL2A1*, *Aggrecan*, and *SOX9* mRNA in the samples co-treated with WNT-3A and DKK1 (Fig. 28A-C).

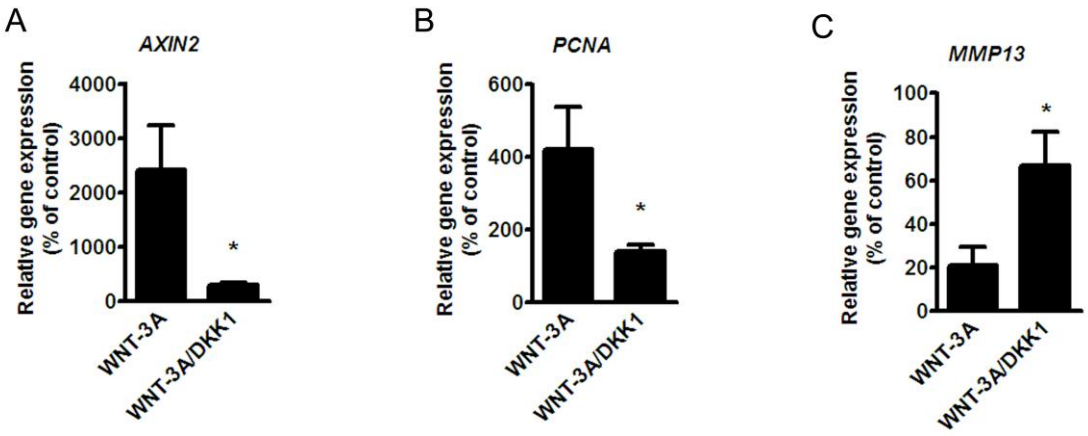


Figure 27: DKK1 inhibits the activation of the Wnt-β-catenin pathway induced by WNT-3A and rescues its effects on proliferation and MMP13 expression.

A-C) Primary AHAC were treated for 24 hours with 100 ng/ml of recombinant WNT-3A alone or in combination with DKK1, and were subjected to gene expression analysis by PCR. Blockade of the Wnt-canonical pathway by DKK1 rescued the modulation of *AXIN2, PCNA*, and *MMP13* mRNA. n=6 (3 donors, 2 samples/donor). Data were analysed by unpaired T-test. Gene expression levels are expressed as percentage of the vehicle-treated cells.

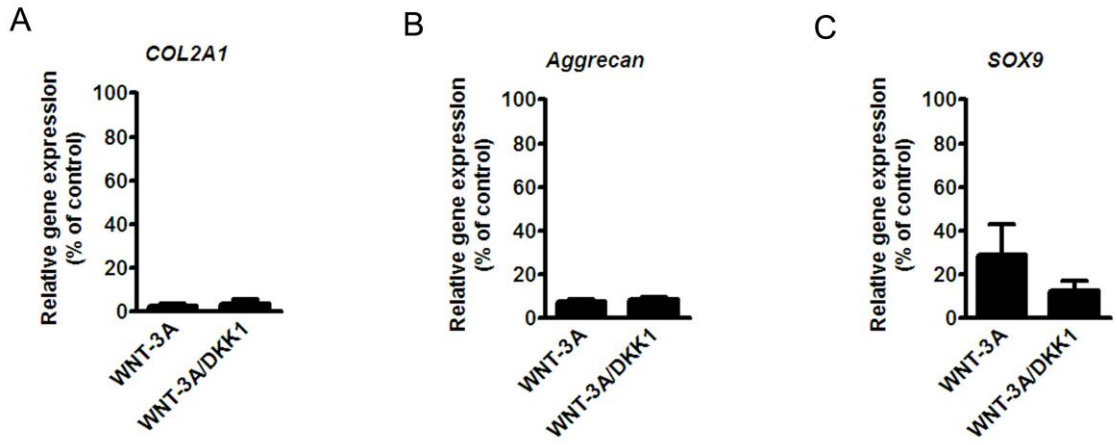


Figure 28: Figure DKK1 does not rescue the loss of chondrocyte phenotype induced by WNT-3A

A-C) Primary AHAC were treated for 24 hours with 100 ng/ml of recombinant WNT-3A alone or in combination with DKK1, and were subjected to gene expression analysis by PCR. Blockade of the Wnt-canonical pathway by DKK1 did not rescue the down-regulation of *COL2A1*, *Aggrecan,* and *SOX9* promoted by WNT-3A. n=6 (3 donors, 2 samples/donor). Data were analysed by unpaired T-test. Gene expression values are expressed as percentage of the vehicle-treated cells.

To investigate whether this was due to excessive, exogenous WNT-3A stimulation, we investigated if treatment with DKK1 alone, in the absence of exogenous WNT-3A, modified the phenotype of AHAC. Strikingly, treatment of AHAC with DKK1 in the absence of WNT-3A, promoted by itself a down-regulation of *COL2A1* and *Aggrecan*, similar to that induced by WNT-3A, and up-regulation of *MMP13* although, as expected, it did reduce *AXIN2* and *PCNA* mRNA expression (Fig. 29). Therefore, although the rescue of *AXIN2*, *PCNA* and *MMP13* demonstrated that DKK1 successfully blocked the canonical pathway, its failure to rescue *COL2A1*, *Aggrecan* and *SOX9* down-regulation suggested that these genes are regulated differently.

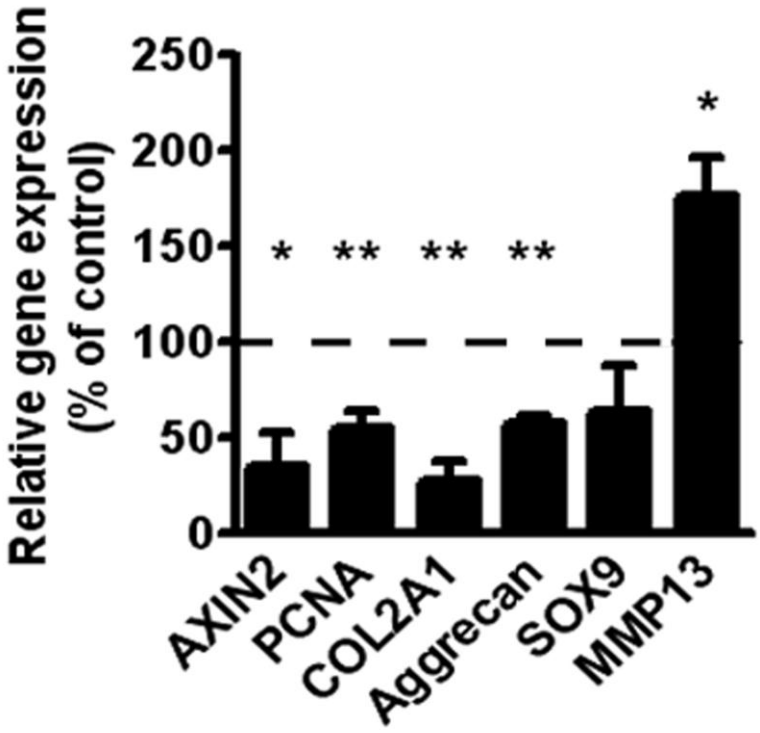


Figure 29: Stimulation of AHAC with DKK1 promotes their de-differentiation

In the absence of exogenous WNT-3A, treatment with 100 ng/ml DKK1 resulted, as expected, in down-regulation of the mRNA levels of *AXIN2* and *PCNA*, but also in a paradoxical down-regulation of *COL2A1* and *Aggrecan* similar to that induced by WNT-3A. n=6 (3 donors; 2 samples/donor). Data were analysed with paired T-test. Gene expression values are expressed as percentage of the vehicle-treated cells.

The finding that DKK1 not only did not rescue WNT-3A-induced loss of phenotypic markers but, on its own promoted it, was indeed very puzzling. Scattered reports demonstrate that, in particular circumstances, WNT-3A can activate non-canonical Wnt pathways. For instance WNT-3A has been shown to promote intracellular calcium accumulation and CaMKII activation in HUVEC cells (Samarzija et al., 2009) and to induce bone formation through a G-protein and PKC-mediated mechanism (Tu et al., 2007). In addition, Dkk1 itself has been shown to promote CamKII phosphorylation in a different biological system (Mikheev et al., 2008). Therefore we argued that a plausible explanation for our paradoxical results would be that ***while proliferation (PCNA), MMP13 expression, and AXIN2 up-regulation are driven by activation of the canonical Wnt pathway, the loss of phenotypic markers (COL2A1, SOX9) and of extracellular matrix may be regulated through a different, Wnt/non-canonical pathway which is antagonized by the canonical. This became the main hypothesis in the rest of this study.***

WNT-3A SIMULTANEOUSLY ACTIVATES THE CANONICAL AND THE Ca^{2+}/CaMKII-DEPENDENT WNT PATHWAYS IN A DOSE-DEPENDENT MANNER

To identify the signalling pathway through which WNT-3A induced chondrocyte de-differentiation, we focused on the Wnt-Ca^{2+}/CaMKII pathway because it has been shown to be active in chicken epiphyseal cartilage (Taschner et al., 2008) and to mediate Wnt signalling in Zebrafish and *Xenopus* embryos (Kuhl et al., 2000;Sheldahl et al., 1999;Slusarski et al., 1997a;Slusarski et al., 1997b) antagonizing β-catenin. As a first approach, to test whether WNT-3A activates the non-canonical Ca^{2+}/CaMKII-dependent pathway, I treated primary AHAC cultures with increasing doses of WNT-3A whilst monitoring calcium mobilization (as readout of the activation of the Ca^{2+}-dependent pathway) and *AXIN2* expression (as readout for the activation of the canonical pathway). In keeping with our hypothesis, WNT-3A elicited activation of both pathways; notably, Ca^{2+}-mobilization occurred preferably at low doses and *AXIN2* up-regulation at higher doses (Fig. 30A and B).

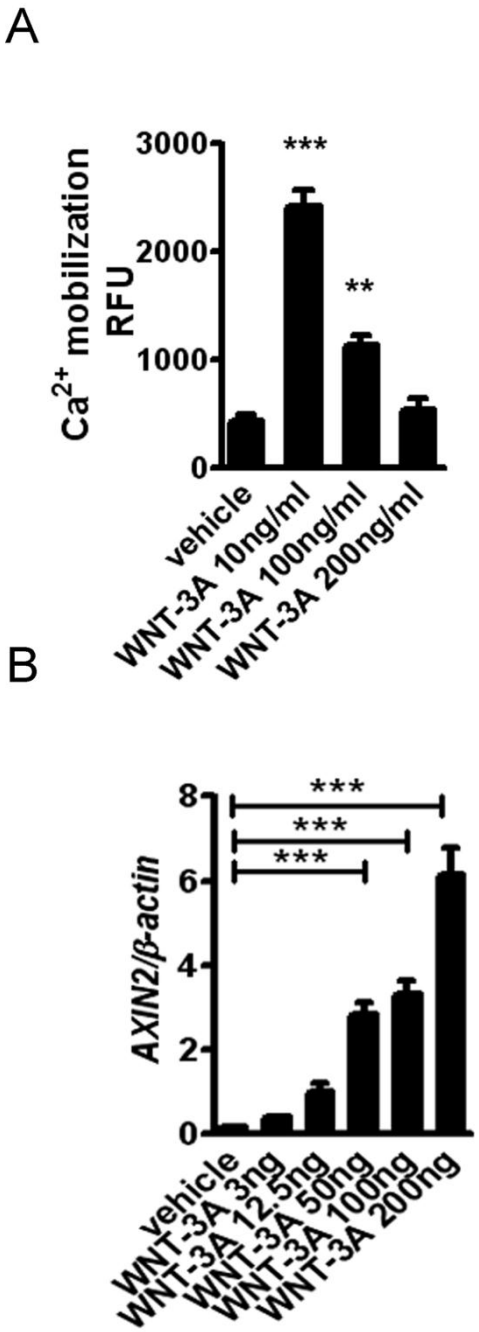


Figure 30: Dose-dependent intracellular Ca^{2+}-accumulation and activation of the Wnt/β-catenin-dependent pathway following treatment with WNT-3A

(A) WNT-3A caused calcium mobilization in AHAC, particularly at low doses. Primary AHAC were treated with different doses of recombinant WNT-3A or vehicle control for 5 minutes and then subjected to fluorometric determination of calcium mobilization (n=9; 3 donors, 3 samples/donor). (B) Q-PCR for *AXIN2* mRNA in primary chondrocytes exposed for 24 hours to different doses of recombinant WNT-3A. WNT-3A induced dose dependent up-regulation of *AXIN2* in primary AHAC (n=3). Data were analysed by unpaired t-test in (A) and with Kruskal Wallis with Dunns post-test in (B).

WNT-3A PROMOTES INTRACELLULAR CALCIUM MOBILIZATION TROUGH A G-PROTEIN DEPENDENT MECHANISM

In Zebrafish and *Xenopus* embryos, it was shown that the Ca^{2+}/CaMKII pathway signals in a G-protein dependent manner (Kuhl et al., 2000;Sheldahl et al., 1999;Slusarski et al., 1997a). Furthermore, WNT-3A has been recently proved to elicit G-protein-mediated properties of mammalians FZD receptors (Koval and Katanaev, 2011). To explore whether in articular chondrocytes, the calcium release demonstrated following WNT-3A treatment was G protein-dependent, we used pertussis toxin (PTX), a $G_{\alpha i}$, $G_{\alpha 0}$ and $G_{\alpha t}$ protein inhibitor (Sheldahl et al., 1999;Slusarski et al., 1997a). In a collaborative effort with Joanna Sherwood, PhD student in our laboratory, we demonstrated that PTX treatment resulted in an efficient blockade of WNT-3A-induced calcium mobilization (Fig. 31), thereby confirming a strong analogy between the Ca^{2+}/CaMKII pathway activated by WNT-5A in *Xenopus* and what was being observed in articular chondrocytes in response to WNT-3A.

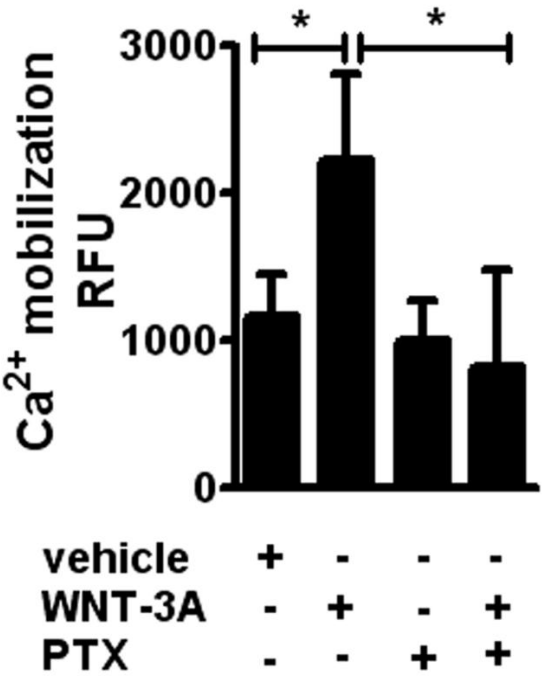


Figure 31: WNT-3A-mediated intracellular calcium accumulation is G-protein dependent

AHAC were pre-incubated overnight at 37°C with 1µg/ml of the G-protein inhibitor PTX (pertussis toxin) and then treated for 5 minutes with 100ng/ml of WNT-3A or vehicle control. PTX treatment blocked calcium mobilization induced by WNT-3A (n=6; 3 donors, 2 samples/donor). Data were analysed by unpaired t-test. Experiment performed by Joanna Sherwood.

WNT-3A PROMOTES PHOSPHORYLATION AND NUCLEAR ACCUMULATION OF CaMKII IN HUMAN ARTICULAR CHONDROCYTES

Encouraged by these results, we went on to evaluate the involvement of CaMKII. Phosphorylation in Thr286 (T286) is a key event in CaMKII activation (Griffith et al., 2003). As a first step we aimed at investigating whether WNT-3A treatment resulted in increased phosphorylation of T286. I treated AHAC with 100 ng/ml WNT-3A or vehicle control for 24 hours and stained the monolayers with an antibody to detect the activated, phosphorylated form of CaMKII (Soderling, 1999). PhosphoCaMKII staining was increased in WNT-3A-treated samples, with increased nuclear localization, confirming the activation of this pathway (Fig. 32A-F). Such phosphorylation was abrogated in presence of the selective CaMKII inhibitor KN93 (Fig. 32A).

CaMKII phosphorylation was also detected by western blotting, after 15 minutes and 1 hour from the addition of WNT-3A to the cells (Fig. 33).

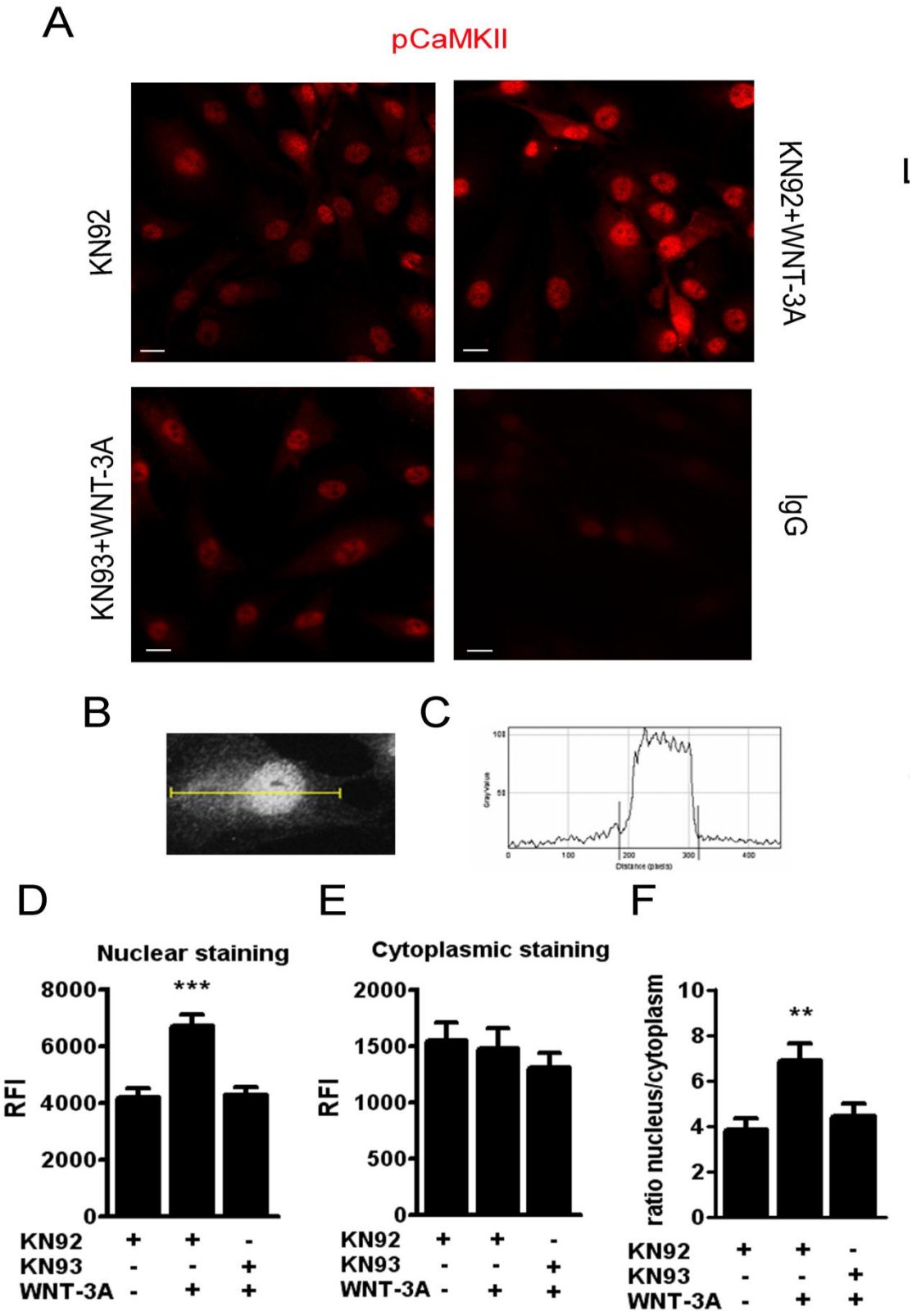

A
pCaMKII
KN92
KN92+WNT-3A
KN93+WNT-3A
IgG
B
C
D
Nuclear staining
***
8000
6000
4000
2000
0
RFI
KN92 + + -
KN93 - - +
WNT-3A - + +
E
Cytoplasmic staining
2000
1500
1000
500
0
RFI
KN92 + + -
KN93 - - +
WNT-3A - + +
F
ratio nucleus/cytoplasm
**
10
8
6
4
2
0
KN92 + + -
KN93 - - +
WNT-3A - + +

Figure 32: WNT-3A promotes CaMKII phosphorylation and nuclear translocation in AHAC

(A) WNT-3A induced nuclear accumulation of the phosphorylated form of CaMKII. Immunofluorescence staining for pCaMKII in P0 AHAC stimulated for 24 hours with WNT-3A in combination with either the CaMKII inhibitor KN93 or its inactive analogue KN92 (both 10µM) (B-F). Quantification of the pCaMKII fluorescence intensity in the nuclear and in the cytoplasmic fractions of stimulated AHAC. The pixel intensity profile was plotted over a linear section of a whole cell (B-C). The nuclear and cytoplasmic fluorescence intensity was then calculated as the area under the curve (D-F). RFI=Relative Fluorescence Intensity. Scale bar in D =20µm. Statistical analysis was performed with unpaired t-test.

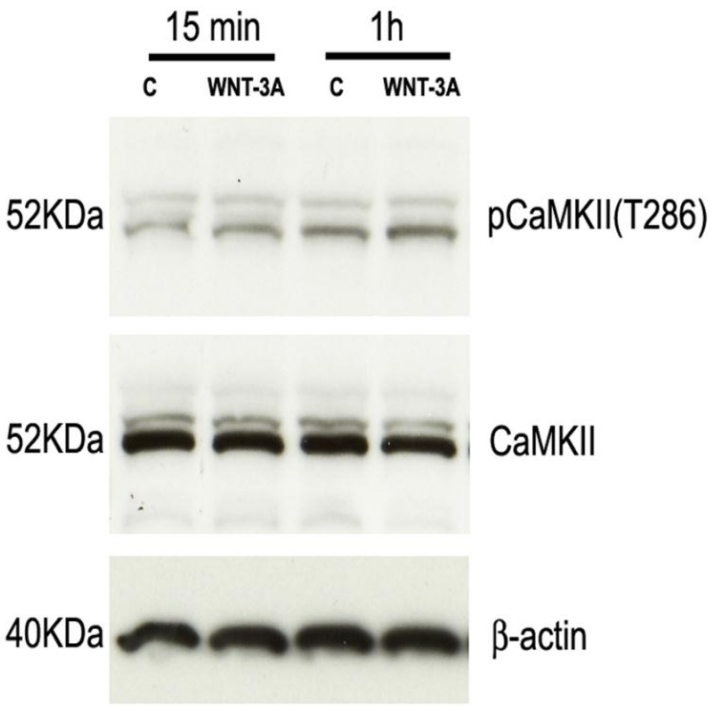


Figure 33: WNT-3A promotes CaMKII phosphorylation in AHAC

100ng/ml of recombinant WNT-3A promoted phosphorylation of CaMKII in Thr286 after 15 minutes and 1 hour from stimulation in AHAC.

Taken together, my data indicate that exogenous application of WNT-3A, in addition to the activation of the Wnt-β-catenin pathway, also promotes intracellular calcium accumulation through a G-protein dependent mechanism. This phenomenon is also associated with phosphorylation and nuclear translocation of CaMKII. Activation of the Wnt/β-catenin or of the Ca^{2+}/CaMKII pathways is dose-dependent, with low doses of WNT-3A activating the Ca^{2+}/CaMKII and high doses the β-catenin pathways.

WNT-CANONICAL AND WNT-CAMKII NON CANONICAL PATHWAYS ARE RECIPROCALLY INHIBITORY IN AHAC

In *Xenopus*, the Wnt/Ca^{2+}/CaMKII pathway was shown to inhibit the Wnt-β-catenin dependent pathway (Kuhl et al., 2000;Sheldahl et al., 1999). This is interesting because reciprocal inhibition of the two pathways would explain why DKK1 alone cause de-differentiation in our model. To investigate the interaction between the CaMKII-pathway with the Wnt-canonical pathway following WNT-3A stimulation, I monitored the activation of the canonical pathway in WNT-3A-treated P0 AHAC (100ng/ml, a dose that activates both CaMKII and the β-catenin pathways) in the presence of the selective CaMKII inhibitor KN93, or its inactive analogue KN92 (both 10μM) for 24 hours (Fig. 34).

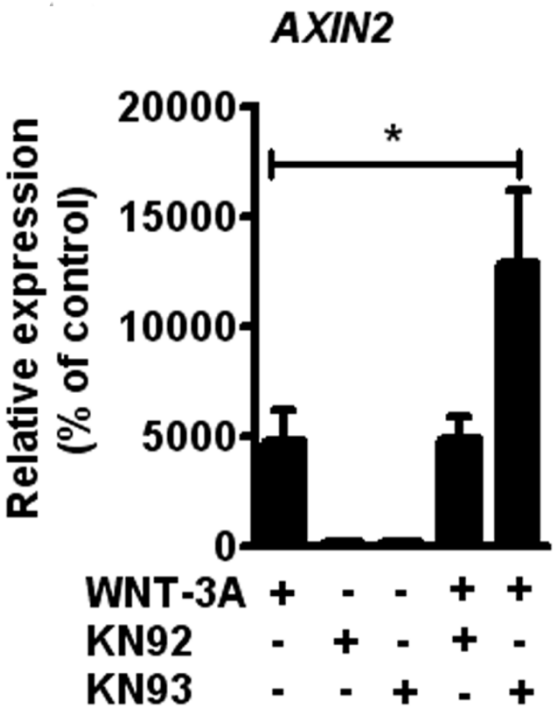


Figure 34: Wnt-Ca^{2+}/CaMKII pathway inhibits the activation of the Wnt-canonical pathway

Q-PCR for *AXIN2* of primary AHAC treated with 100ng/ml WNT-3A in the presence of the CaMKII inhibitor KN93 or its inactive analog KN92 (10µM). (n=8, 4 donors, 2 samples/donor). Statistical analysis was performed with ANOVA.

CaMKII inhibition by KN93 significantly increased the WNT-3A-induced up-regulation of *AXIN2* mRNA (Fig. 34), thereby confirming that activation of CaMKII inhibits the Wnt/β-catenin pathway. In contrast KN92, which is structurally similar to KN93 but does not inhibit CaMKII (Marley and Thomson, 1996), did not alter the expression of *AXIN2* (Fig. 34).

To study if the Wnt-β catenin pathway modulates the Wnt-Ca^{2+}/CaMKII pathway, I performed the reciprocal experiment in which Ca^{2+}-mobilization was monitored in AHAC treated with WNT-3A in the presence or the absence of DKK1 (Fig. 35).

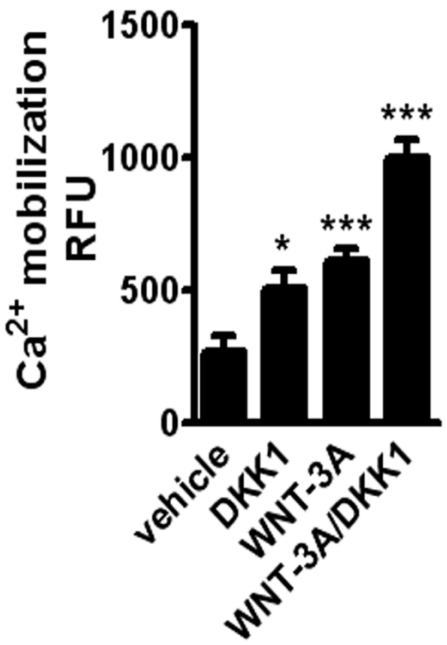


Figure 35: Wnt-canonical pathway inhibits the activation of the Ca^{2+}/CaMKII pathway

DKK1 promoted intracellular calcium accumulation in P0 AHAC. The inhibition of the canonical pathway with DKK1 enhanced intracellular calcium accumulation induced by WNT-3A. In all the experiments n=12 (4 donors, 3 samples/donor). Data were analysed by unpaired T-test.

Both WNT-3A and DKK1 stimulation resulted in statistically significant activation of Ca^{2+}-mobilization, which was further increased when DKK1 and WNT-3A were used together, thereby demonstrating that the selective inhibition of the canonical pathway enhances the activation of the Ca^{2+}-mobilization (Fig. 35). The increased Ca^{2+} mobilization induced by DKK1 in the absence of exogenous WNT-3A might be due to the inhibition of the basal Wnt activation supported by endogenous Wnt ligands.

sFRP1 DOES NOT BLOCK WNT-3A MEDIATED INTRACELLULAR CALCIUM ACCUMULATION

To gain a better insight into this mechanism and specifically if the selectivity of the blockade for the canonical pathway was essential for Ca^{2+}-mobilization or whether general out-titration of Wnts also induced Ca^{2+} mobilization, we repeated the same experiment utilizing sFRP1 as a Wnt inhibitor instead of DKK1. We chose sFRP1 because it is known to bind and inhibit several Wnts including WNT-3A (Wawrzak et al., 2007;Galli et al., 2006) preventing their interaction with FZD receptors. This experiment was performed in collaboration with Dr Jessica Bertrand in our laboratory.

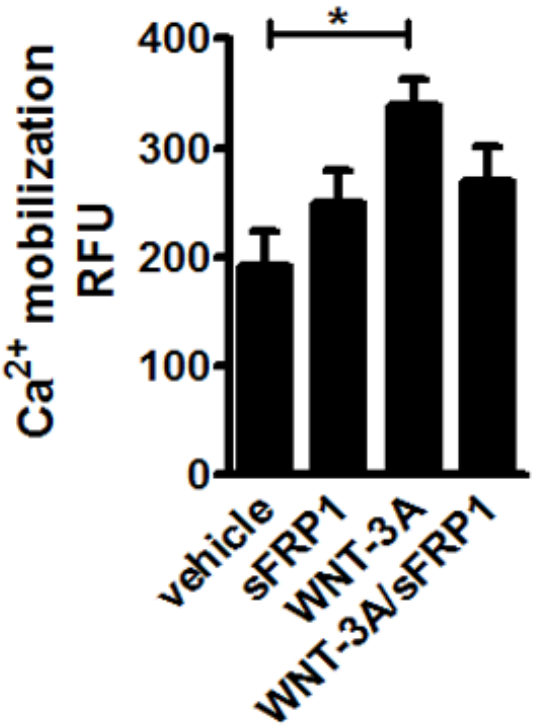


Figure 36: sFRP1 does not promote intracellular calcium accumulation

P0 AHAC were incubated with 100ng/ml of sFRP1 alone or in combination with 100ng/ml of recombinant WNT-3A for 5 minutes and intracellular calcium accumulation was measured. sFRP1 alone did not promote intracellular calcium accumulation in P0 AHAC but partially inhibited WNT-3A mediated Ca^{2+} mobilization (n=12; 4 donors, 3 samples/donor). Statistical analysis performed by unpaired t-test.

sFRP1 treatment prevented WNT-3A-induced intracellular calcium accumulation but, in contrast to DKK1, on its own, did not induce Ca^{2+}-mobilization, thereby confirming that Ca^{2+}-mobilization induced by DKK1 is due to selective inhibition of the canonical pathway and consequent de-repression of the Ca^{2+}/CaMKII pathway (Fig. 36).

Taken together, these data demonstrate reciprocal inhibition of the Wnt-β catenin and Wnt-Ca^{2+}/CaMKII pathways following WNT-3A stimulation in articular chondrocytes, and that the inhibition of one causes de-repression of the other.

WNT/β-CATENIN PATHWAY AND WNT/Ca^{2+}/CAMKII PATHWAY REGULATES DISTINCT BIOLOGICAL PROCESSES IN AHAC

Having demonstrated that WNT-3A can signal simultaneously through two intracellular pathways, we set out to discriminate CaMKII- from β-catenin-dependent transcriptional targets. To this end, I treated chondrocytes with WNT-3A in the presence of either the CaMKII inhibitor KN93, or the inactive compound KN92.

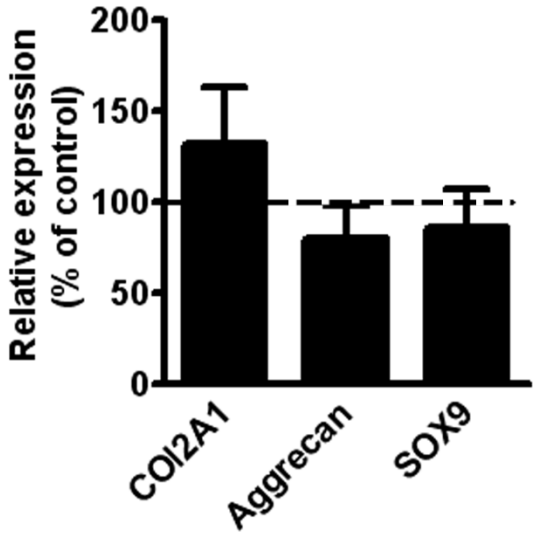


Figure 37: CaMKII inhibition does not alter chondrocyte phenotype

Primary AHAC were treated for 24 hours with 10 µM KN93. Blockade of CaMKII with KN93 did not alter the basal levels of *Aggrecan*, *COL2A1*, and *SOX9* n=6 (3 donors, 2 samples/donor). Statistical analysis was performed with paired t-test.

Treatment of P0 AHAC with KN93 alone did not have any biological effect of the cells (Fig. 37). As expected, WNT-3A induced a significant down-regulation of *COL2A1*, *Aggrecan* and *SOX9* mRNA compared to control, but CaMKII blockade with KN93 resulted in a statistically significant rescue of *COL2A1* and *SOX9* expression (Fig. 38A and C), demonstrating that WNT-3A-induced chondrocyte de-differentiation is, at least in part, CaMKII dependent. In contrast KN93 did not rescue the down-regulation of *MMP13* mRNA (Fig. 38D). Therefore WNT-3A up-regulates *AXIN2* and *PCNA* through the canonical pathway and down-regulates *SOX9* and *COL2A1* in a CaMKII-dependent manner.

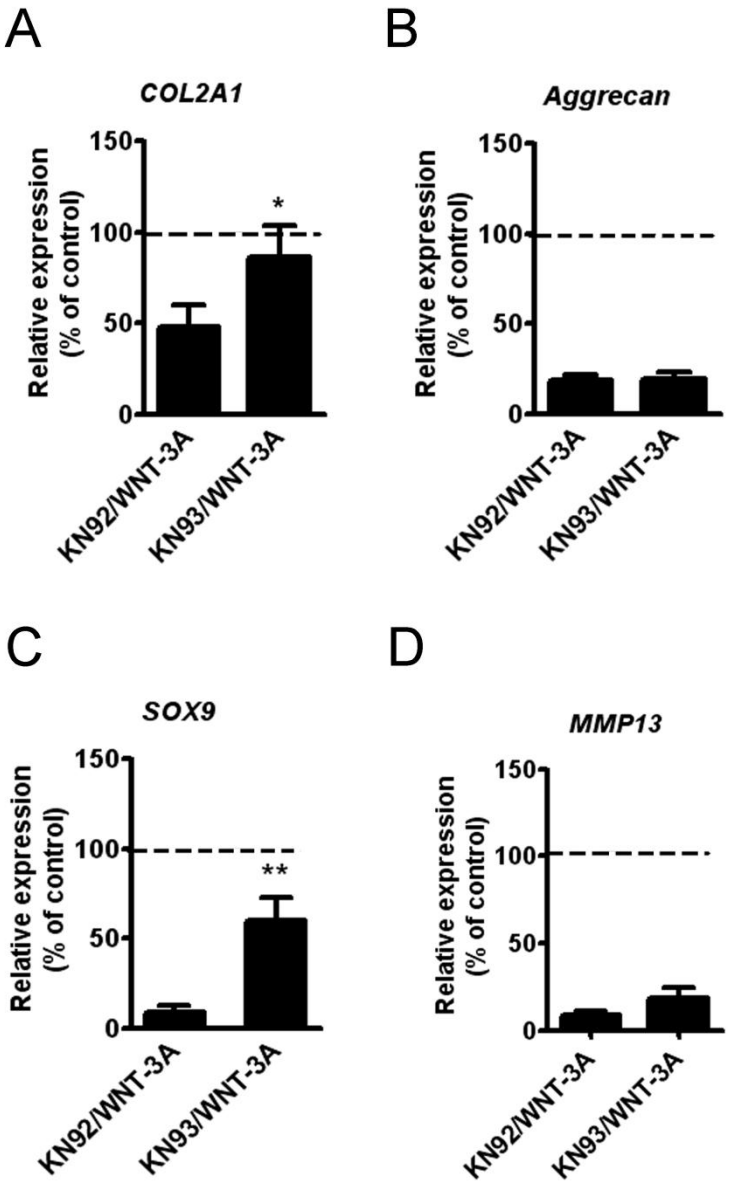


Figure 38: Inhibition of the Ca^{2+}/CaMKII pathway rescued the loss of COL2A1 and SOX9 mRNA expression in AHAC.

CaMKII blockade rescues WNT-3A- induced down-regulation of *COL2A1* and *SOX9* mRNA. Primary AHAC were cultured for 24 hours in the presence of 100ng/ml WNT-3A or vehicle control, and either the CaMKII inhibitor KN93 or the inactive control KN92 (both 10µM). Gene expression was evaluated by Q-PCR. The values were normalized for *β-actin* and expressed as a percentage of the KN92-treated group (n=6; 3 donors, 2 samples/donor). Statistical analysis performed with paired T-test.

SPECIFICITY OF CA^{2+}/CaMKII SIGNALLING: PMA-INDUCED CA^{2+}-ACCUMULATION IS NOT SUFFICIENT TO DOWN-REGULATE *COL2A1* AND *SOX9* EXPRESSION

Ca^{2+}-mobilization is a priming event for the activation of several partially independent signalling pathways, including phospho-Inositol-3-kinase (PI3K), calcineurin, PKC and CaMKII pathways. To test the specificity of the Ca^{2+}/CaMKII pathway activated by WNT-3A and resulting in *COL2A1* and *SOX9* down-regulation, we tested whether a non-specific Ca^{2+}-mobilization such as that induced by phorbol-12-myristate-13-acetate (PMA), could achieve the same result.

PMA successfully promoted intracellular calcium mobilization in articular chondrocytes (Fig. 39).

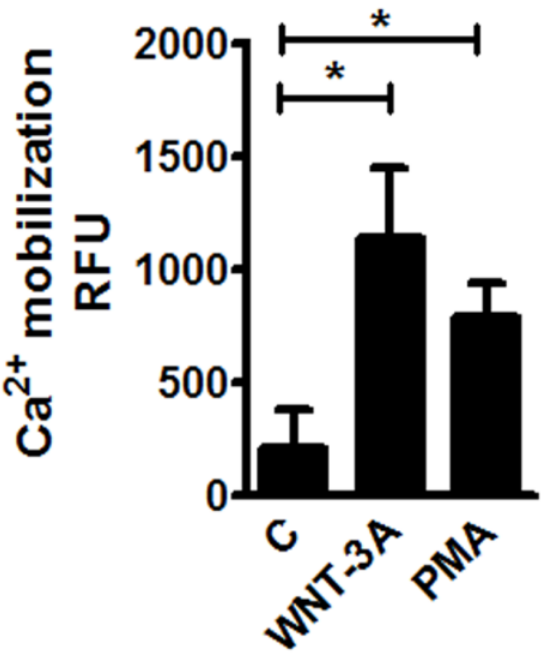


Figure 39: PMA induced Ca^{2+}-mobilization in AHAC

Treatment with 10ng/ml of PMA or 100ng/ml of WNT-3A for 5 minutes promoted intracellular calcium mobilization in AHAC (n=8, 4 donors, 2 samples/donor). Statistical analysis performed with unpaired t-test.

PMA-stimulation resulted in down-regulation of *Aggrecan* but not *SOX9* or *COL2A1* (Fig. 40). This, and the fact that instead CaMKII blockade rescued the expression of *COL2A1* as well as *SOX9*, suggests that the WNT-3A-induced effects on these three genes rely specifically on CaMKII-dependent Ca^{2+}-pathway.

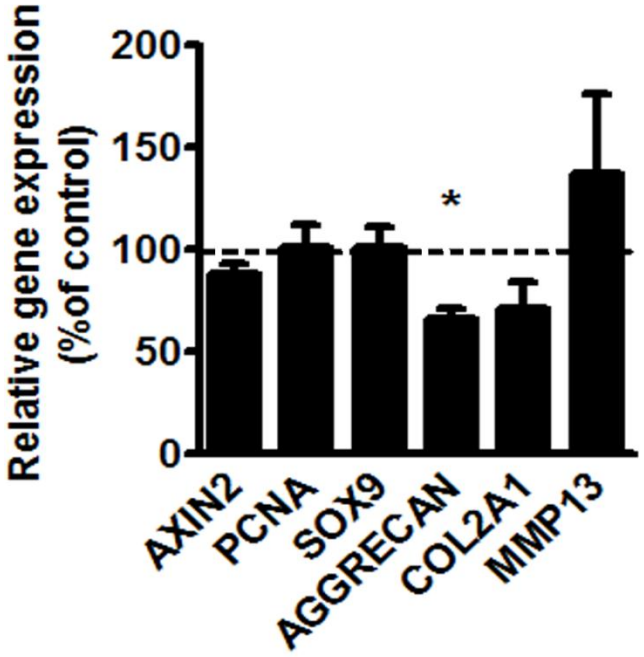


Figure 40: PMA down-regulates Aggrecan in AHAC

In contrast to WNT-3A PMA failed to down-regulate *SOX9* and *COL2A1*. *Aggrecan* was instead statistically significantly down-regulated (n=6; 3 donors, 2 samples/donor). Statistical analysis performed by paired t-test.

Blockade of the Ca^{2+}/CaMKII pathway rescued the loss of the chondrocyte markers *SOX9* and *COL2A1* induced by WNT-3A (Fig.38).

These results demonstrate that WNT-3A simultaneously activates the Wnt/β-catenin pathway and the Wnt/Ca^{2+}/CaMKII pathways with different outcomes: WNT-3A in fact mediates proliferation through β-catenin activation and de-differentiation through CaMKII.

CaMKII IS ACTIVATED IN OSTEOARTHRITIC CARTILAGE

Our data so far identify CaMKII as a key molecule mediating outcomes of Wnt signalling that can be postulated to be pathogenic in arthritis.

It is therefore tempting to hypothesize that a switch of Wnt signalling from β-catenin to CaMKII may be a pathogenic event in OA development that transforms Wnt signalling from homeostatic to catabolic. If so, we would expect that CaMKII phosphorylation should be associated with OA lesions. Therefore, I compared the expression of pCaMKII on cartilage explants isolated from normal donors and from osteoarthritic patients (Mankin Score =6) (Fig. 41).

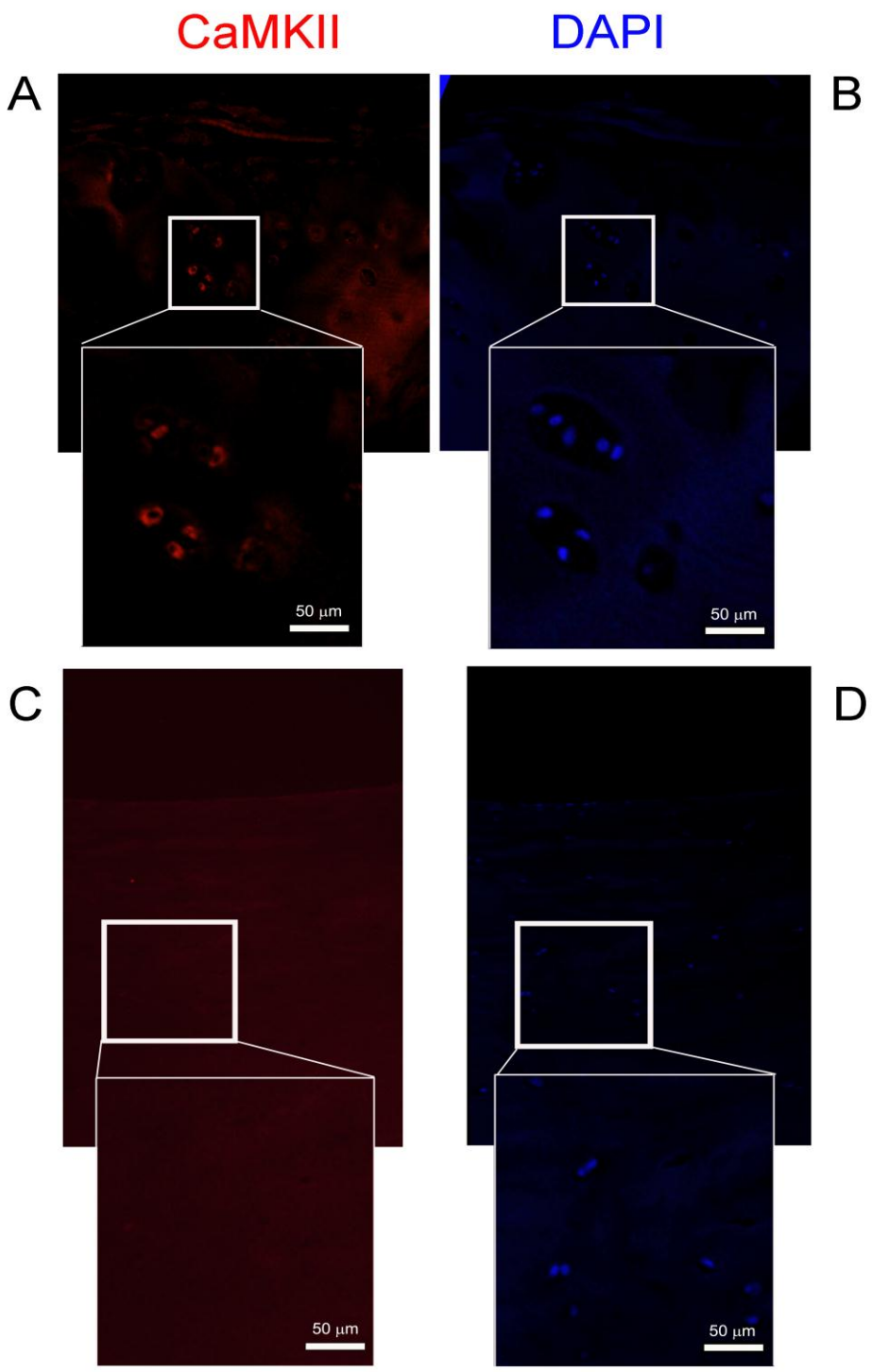


Figure 41: CaMKII is phosphorylated only in the cartilage of OA patients

Cartilage explants from OA patients (Mankin score ≥ 4) and from normal patients (n=3 for both conditions) were stained with anti pT286CaMKII. The phosphorylated and active form of the enzyme was expressed only in the cartilage from the OA patients. Images are representative of the cartilage of one OA (A-B) and one normal cartilage sample.

Indeed the phosphorylated form of CaMKII was expressed only in the cartilage of the OA patients and was completely absent in cartilage removed from normal donors.

The expression of pCaMKII only in the cartilage from OA patients suggests that CaMKII might have an active role in the pathogenesis of OA.

Previous data showed that overexpression of CaMKII in the epiphyseal cartilage in chicken can be associated with accelerated maturation and hypertrophy of the chondrocytes (Taschner et al., 2008).

This is interesting because ectopic hypertrophic maturation has been described in articular chondrocytes in OA, and recently proven to drive cartilage breakdown (Saito et al., 2010;Yates et al., 2010). Therefore it would be very interesting to see whether Wnt-dependent CaMKII signalling controls such process, because pharmacological CaMKII blockers are commercially available.

CHAPTER 5: DISCUSSION

We have demonstrated that chondrocyte exposure to exogenous WNT-3A can simultaneously activate both the β-catenin-dependent and the G-protein-mediated Ca^{2+}/CaMKII/pathways which are in equilibrium due to reciprocal inhibition (See Scheme in Fig. 42). These two pathways modulate the transcription of distinct target genes involved in different biological processes: WNT-3A promotes chondrocyte proliferation through β-catenin and differentiation through CaMKII. According to our model, blockade of the canonical/β-catenin pathway will also cause articular-chondrocyte de-differentiation, through de-repression of the CaMKII pathway (Fig. 42 and B).

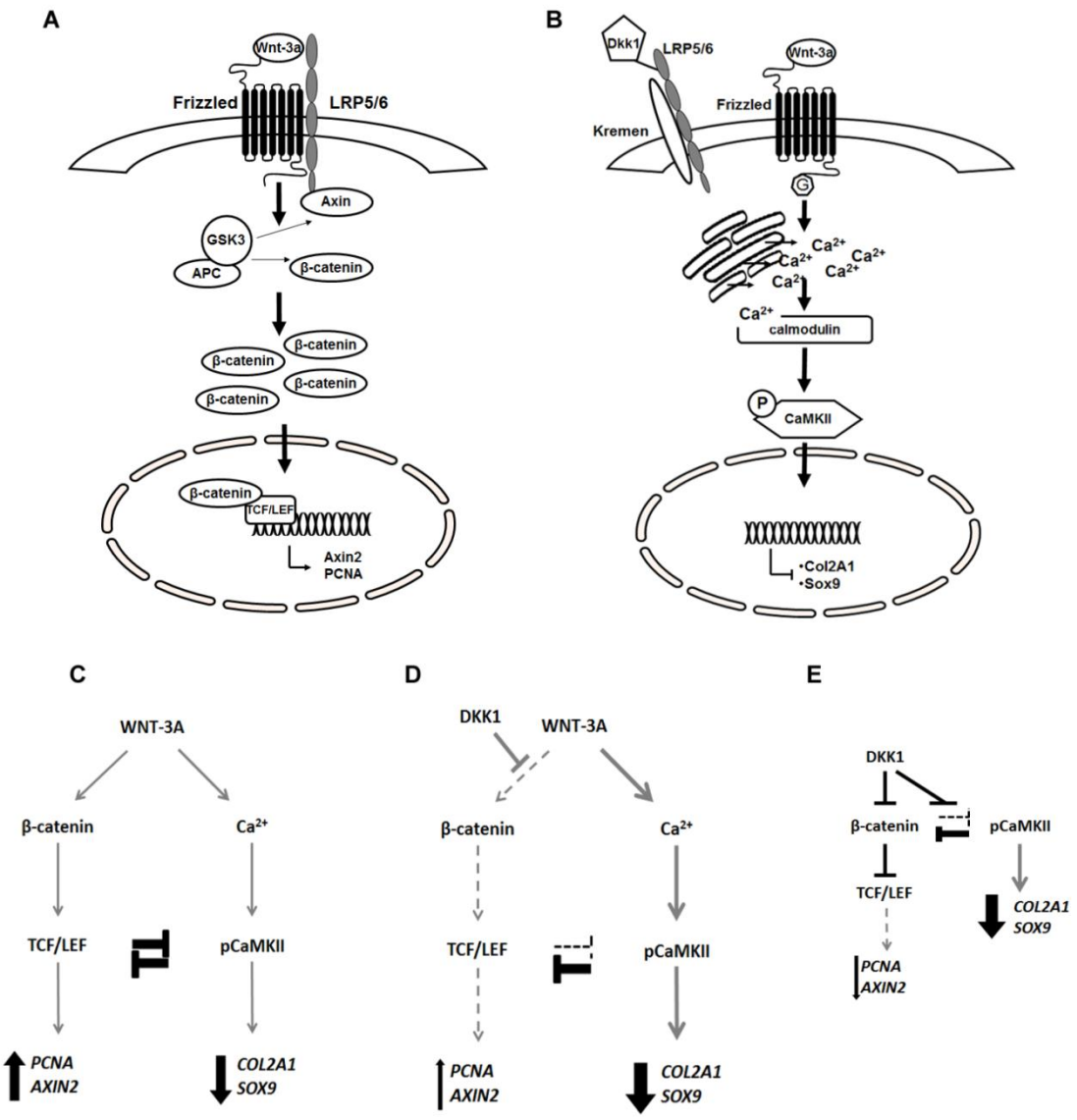


Figure 42: Simultaneous activation and reciprocal inhibition of β-catenin and CaMKII dependent pathways in AHAC

In AHAC, WNT-3A up-regulates *AXIN2* expression and proliferation through the Wnt-canonical pathway (A), but induces loss of differentiation via a G-protein-mediated Ca^{2+}/CaMKII dependent pathway (B). These two pathways are reciprocally inhibitory and in equilibrium (C). Therefore, either exogenous WNT-3A (C) or blockade of the canonical pathway with DKK1 (D-E) will both result in loss of chondrocyte phenotype.

This model may help explaining contradictions in the current literature. In particular, both activation or disruption of the canonical Wnt signalling results in chondrodysplasia with delayed maturation of chondrocytes (Akiyama et al., 2004;Enomoto-Iwamoto et al., 2002) and, in adulthood in cartilage breakdown (Chen et al., 2008;Koyama et al., 2008;Zhu et al., 2008;Zhu et al., 2009). Similarly, in the mouse embryo (Yang et al., 2003;Yamaguchi et al., 1999) both the overexpression and the knockout of WNT-5A, which in Zebrafish and *Xenopus* has been shown to activate the Ca^{2+}/CaMKII pathway (Kuhl et al., 2000;Slusarski et al., 1997b) , result in a similar phenotype of chondrodysplasia, in both cases with reduced chondrocyte proliferation and hypertrophic maturation (Yang et al., 2003;Yamaguchi et al., 1999). Although this phenotype, *in vivo*, may be partially determined by compensatory mechanisms involving modulation of the Ihh-PTHrP pathway, COL2A1 expression was up-regulated in the growth plate of WNT-5A KO mice and down-regulated in mice overexpressing WNT-5A under the transcriptional control of the collagen type 2 promoter (Yang et al., 2003). In addition, *in vitro*, WNT-5A dose-dependently down-regulated the activity of a *COL2A1* reporter in mouse chondrocytes. Importantly, this study showed that WNT-1, a well validated canonical Wnt, could also decrease *COL2A1* reporter activity, but in a TCF-independent manner, since overexpression of TCF1 failed to achieve the same result (Yang et al., 2003). This may suggests that WNT-3A may not be the only canonical Wnt to signal through multiple pathways.

At large in keeping with my data, Taschner et al. reported that forced activation of CaMKII in the chick epiphyseal cartilage, resulted in down-regulation of cell-cycle regulators and acceleration of hypertrophic differentiation (Taschner et al., 2008), which is notoriously associated with down-regulation of *COL2A1* and *SOX9* (Taschner et al., 2008;Lefebvre et al., 1998). However, Taschner's results cannot directly compared with ours, because of the intrinsically different biology of epiphyseal chondrocytes, which are destined to undergo hypertrophic differentiation and eventually replaced by bone, and the articular chondrocytes, which are resistant to hypertrophy and to endochondral bone formation. In addition, in Taschner's study, proliferation is driven by non-cell autonomous mechanisms including

secretion of Ihh and consequent PTHrP signalling. With all these limitations in comparing the experimental setups, our data, and a recent similar paper from Li et al. (Li et al., 2011) in which overexpression of constitutively active CaMKII was complemented by loss of function experiments, show the role of this kinase in the initiation of hypertrophy, a process that is associated with the loss of stable chondrocytes markers including *SOX9* and *COL2A1* (Lefebvre et al., 1998).

Our findings have been recently published in the Journal of Cell Biology and have been commented by Kestler and Kuhl in an editorial in the same issue (Nalesso et al., 2011;Kestler and Kuhl, 2011). The two authors suggested that our model can help explaining other poorly understood phenomena happening in different biological systems. Wnt11 has been shown to drive dorso-ventral axis formation in *Xenopus*, but whereas it activates the Wnt/β-catenin pathway in the dorsal side of the embryo (Tao et al., 2005), it was shown to lead the ventral side formation through activation of the Ca^{2+}/CaMKII pathway (Kuhl et al., 2000). These puzzling data can find explanation in our model, since Wnt11 is expressed at higher levels in the dorsal side and at lower concentrations in the ventral side (Schroeder et al., 1999), therefore supporting dorso/ventral axis polarization trough a gradient formation and the reciprocal inhibition of the Wnt/β-catenin and the Wnt/Ca^{2+}/CaMKII pathways.

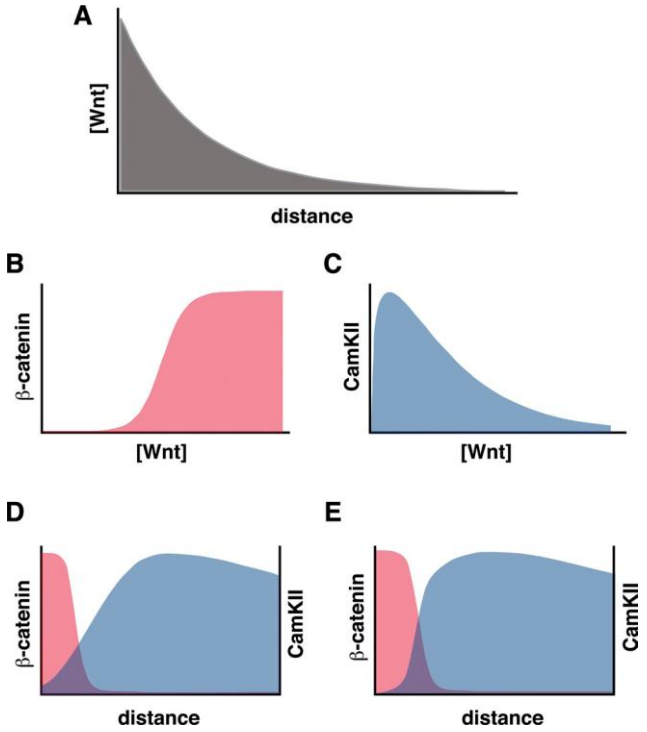


Figure 43: Different concentrations of Wnts can activate different signalling pathways

The formation of a Wnt gradient in a tissue determines the activation of different signalling cascades (A-D). E) The inhibitory cross-regulation determines a Wnt-switch

The reciprocal inhibition of the canonical and CaMKII dependent pathways activated by WNT-3A may represent a hub through which different stimuli active in cartilage and known to influence CaMKII can dramatically influence the outcome of Wnt signalling by switching between β-catenin and CaMKII dependent target genes. Such stimuli include inflammatory molecules (Racioppi and Means, 2008;Pritchard and Guilak, 2006), biomechanics (Shimazaki et al., 2006;Valhmu and Raia, 2002), or PTHrP activity (Taschner et al., 2008). We are currently investigated these aspects in our group.

Although we and others (Kuhl et al., 2000;Sheldahl et al., 1999;Slusarski et al., 1997a) have shown that Ca^{2+}/CaMKII activation is GPCR dependent, it is still unclear how Wnts activate G proteins. In a recent paper, Koval and Katanev demonstrated the activation of G protein following WNT-3A stimulation in a different biological system (Koval and Katanev., 2011). In addition, based on the similarity between FZD receptors and other GPCR (Wang et al., 2006a) it is tempting to postulate that, in the absence of DKK-engaged LRP, FZD receptors may

function as GPCRs (Wang et al., 2006a). However, we cannot exclude that in our experimental system G-protein activation may be indirect, e.g. through the activation of a different GPCR.

My data suggest that the CaMKII-dependent effects of WNT-3A are independent from LRP because they are not blocked, and rather enhanced, by DKK1. A separate issue is as to whether, in our system, individual FZD receptors can activate both pathways, or if the simultaneous activation of canonical and CaMKII pathway is due to the co-expression, on chondrocytes, of FZD receptors with different signalling specificity. Slusarski and colleagues demonstrated that in Zebrafish, expression of rat frizzled 2 receptor (Rfz-2), but not Rfz-1, increased intracellular calcium accumulation, which was further enhanced by the co-expression of *Xenopus* Wnt-5a (Xwnt-5a)(Slusarski et al., 1997a). In addition, in a mammalian cell line Xwnt-5a, but not Xwnt-8, stimulated calcium accumulation via Rfz-2 in a PTX sensitive way (Liu et al., 1999). Subsequently, Kuhl at al. have demonstrated in *Xenopus* embryos that that Wnt-5a, but not canonical Wnts, induced Ca^{2+}-mobilization, PKC and CaMKII activation, in a FZD specific manner. Only some FZD receptors were able to signal through CaMKII, and such signal was also strictly ligand-specific (Kuhl et al., 2000).

We have demonstrated that AHAC express different FZD receptors (Fig. 17) known to mediate both the canonical (Kemp et al., 2007;Gazit et al., 1999;Terasaki et al., 2002;Karasawa et al., 2002) and the Ca^{2+}-dependent pathways (Kuhl et al., 2000;Nishita et al., 2010;Robitaille et al., 2002). Therefore, it is possible that the receptor or of the co-receptor (Grumolato et al., 2010) repertoire of chondrocytes may determine the balance of the WNT-3A signalling.

Alternatively, we cannot exclude that mechanisms downstream of FZD receptors may activate CaMKII: Ryu et al demonstrated that GSK3 inhibitor LiCl also down-regulated *COL2A1* expression (Ryu et al., 2002;Ryu and Chun, 2006) and Topol and colleagues (Topol et al., 2003) showed that WNT-5A can induce degradation of β-catenin even in the presence of LiCl, which is a canonical Wnt agonist and stabilizes β-catenin in a receptor-independent manner by inhibiting GSK3β (Topol et al., 2003).

However, lithium is also an inhibitor of the inositol pathway (Berridge and Irvine, 1989), hence these data are of difficult to interpret.

Whichever the mechanism, it is unlikely that in our system the activation of the Ca^{2+}/CaMKII pathway is indirect, since Ca^{2+}-mobilization occurred as early as 5 minutes following addition of WNT-3A.

Equally, although it cannot be completely excluded, it is unlikely that exogenous WNT-3A may have caused promiscuous activation of receptors, first because Ca^{2+} release took place particularly at low doses of WNT-3A, whereas β-catenin activation was observed only at the higher doses, and, second, because DKK1 caused Ca^{2+} release and chondrocyte de-differentiation also in the absence of exogenous WNT-3A (Figure 29, 35 and 42D-E), which cannot be explained by promiscuous receptor activation by supra-physiological levels of exogenous WNT-3A. In addition in our system, WNT-3A expression was indeed down-regulated in the most damaged areas of cartilage explants, this suggesting that alteration of the basal level of expression of Wnt ligands might bias the activation of different signalling pathways and disrupt the maintenance of chondrocyte homeostasis.

In our system, activation of CaMKII was associated with its nuclear localization, which could be blocked with the CaMKII inhibitor KN93. A similar activation-dependent nuclear localization of phosphoCaMKII has been reported in smooth muscle cells (Cipolletta et al., 2010) and very recently in epiphyseal chondrocytes (Li et al., 2011) *in vivo*. WNT-3A itself has also been reported to promote nuclear localization of CaMKIV in hippocampal neurons (Arrazola et al., 2009). Nuclear localization of CaMKII is regulated by several factors including splicing of a nuclear localization signal (NLS), phosphorylation of serines downstream of the NLS, and binding to anchoring and interacting molecules (Griffith et al., 2003). The specific function of the nuclear localization in this context is not known, however subcellular localization of CaMKII has been shown to restrict/regulate substrate specificity in different cell types or in response to other signals (Griffith et al., 2003;Tsui et al., 2005). Therefore it is tempting to speculate that CaMKII may have a nuclear function, either in mediating chondrocyte de-differentiation, or

suppression of the canonical pathway. In this regard it has been shown that CaMKII activation in colon cancer cells mediates nuclear export of TCF isoforms (Najdi et al., 2009).

FUTURE PERSPECTIVES

A novel and important aspect of this study is the discovery that Wnt/β-catenin and Wnt/Ca^{2+}/CaMKII pathways have different transcriptional targets.

Wnt signalling is an attractive therapeutic target for its demonstrated role on cartilage and joint homeostasis, but because of the multitude of processes regulated by these pathways, and the lack of information regarding their reciprocal interaction, it has not been possible until now to target it pharmacologically. The discovery that single Wnts can activate simultaneously different pathways, regulating distinct and possibly cell-specific transcriptional targets, represents an opportunity to achieve a higher degree of target specificity. Therefore, thanks to the availability of molecules and drugs that selectively can inhibit either GPCR-mediated signalling cascades (Lehmann et al., 2008;Sheldahl et al., 1999;Mukai et al., 1992;Freissmuth et al., 1996), CaMKII (Slater et al., 1999;Ishida et al., 1995;Sumi et al., 1991) or the canonical Wnt pathway (Huang et al., 2009;Trosset et al., 2006;Lu et al., 2009), we have planned to interfere at different levels with the WNT-3A activated pathways and to dissect their molecular targets, which potentially could be therapeutically relevant for the treatment of acute cartilage injuries and/or osteoarthritis.

From a biochemical point of view, different aspects require a deeper analysis: at the moment we do not know whether the biochemical behaviour of WNT-3A is shared by other Wnts and/or if it is cell-context dependent. In addition, the expression of different FZD or other poorly characterized co-receptors -such as ROR1/2 or HSPGs- on the cell membrane might be different in pathologic conditions; this, in turn, could lead Wnt ligands to preferentially activate a pathway that physiologically they do not activate or they activate in a lesser extent. Of course, also the presence of cytokines or inflammatory molecules-such as IL-1 or TNFα- might influence Wnt signalling, as, for instance, they can activate Ca^{2+}-mediated pathways

(Pritchard and Guilak, 2006). All these aspects are currently under investigation in our laboratory and will help understanding wnt biology in the adult cartilage tissue.

Finally, we demonstrated that CaMKII is phosphorylated only in the articular cartilage of OA patients and not in normal cartilage: we currently do not know the biological meaning of this phenomenon, nor we know if the intracellular sub-localization of this kinase is relevant for its function and if it differs in physiologic vs pathologic conditions. Our data showed nuclear localization of CaMKII upon WNT-3A phosphorylation: it would be now interesting to evaluate whether this is necessary for the phosphorylation and consequent modulation of transcription factors -such as TCF/LEF and Sox9- and the relevance of this phenomenon for the maintenance of cartilage homeostasis.

> Too much Wnt signaling is detrimental but too little Wnt signalling has its own set of adverse sequelae. What happens if the Goldilocks principle is employed and Wnt signaling is modulated until it is “just right”?
>
> (Leucht et al., 2008)

METHODOLOGICAL APPENDICES

The data included in the following chapters are of a methodological nature. Nevertheless this relatively large amount of work has been crucial for achieving the results reported in the previous chapters, and may be of interest to other investigators. Therefore, I felt that they could be presented as methodological *Appendices*.

APPENDIX 1: TRANSFECTION OF HUMAN ARTICULAR CHONDROCYTES

Transfection of exogenous DNA is a useful tool to evaluate the biological function of a gene or to assess if and how different types of stimuli might influence its transcription.

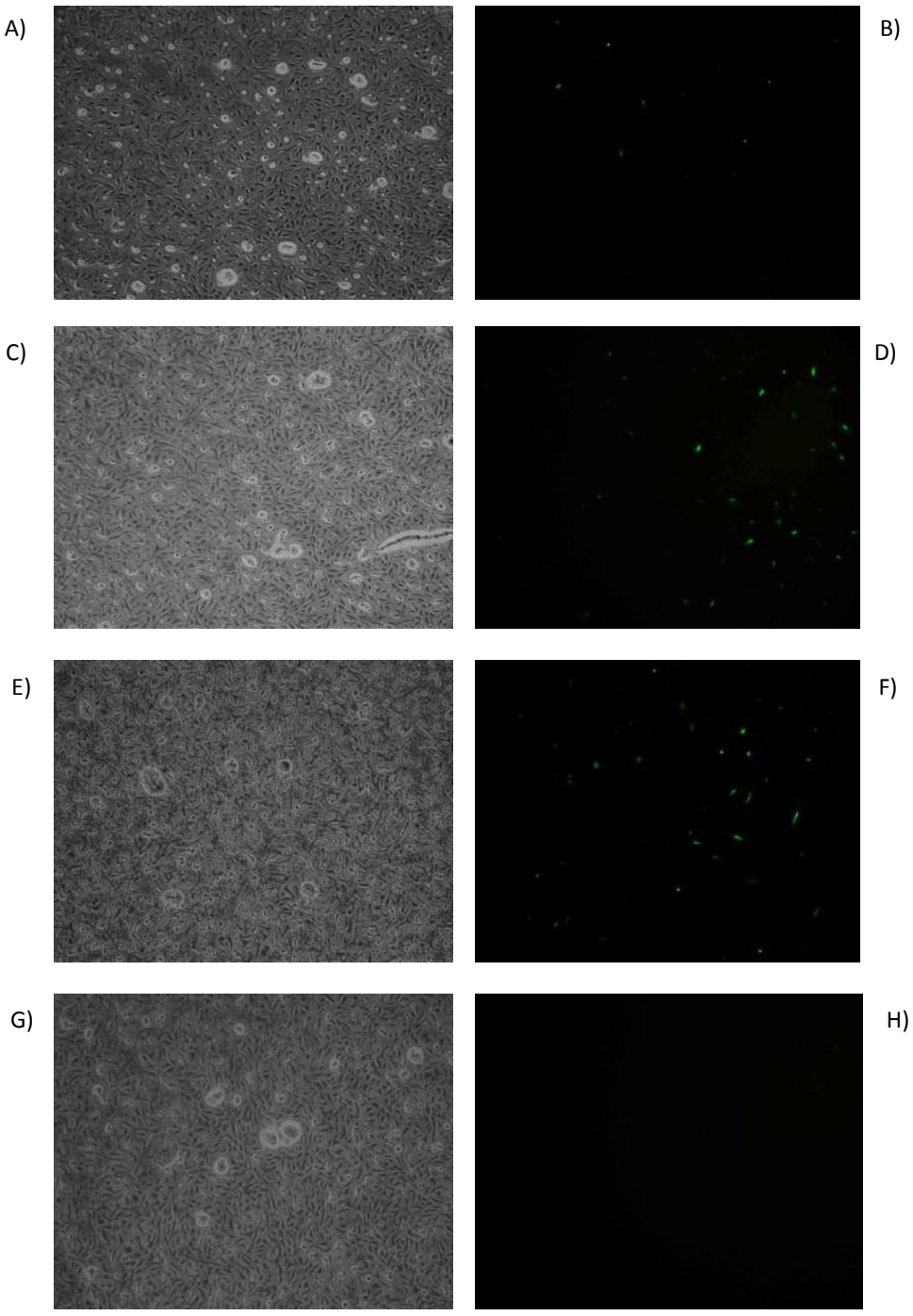
Articular chondrocytes are notoriously very difficult to transfect and, to preserve their phenotype and behaviour, one should avoid too harsh treatments. Therefore a careful optimization was necessary. In order to maximise the transfection efficiency I have tried different transfection methods and compared their efficiency and cytotoxicity.

LIPOSOMAL-BASED TRANSFECTION REAGENTS

I first compared the efficiency of lipid-based transfection reagents such as Lipofectamine 2000 (Invitrogen), Fugene 6 (Roche) and Fugene HD (Roche) by using a GFP-encoding expression vector on confluent P0 chondrocytes. The cells have been transfected with different ratios of transfection reagent/GFP-encoding vector (see Table 3 for summary) and transfection efficiency has been calculated as percentage of fluorescent cells/total number of cells.

	Trasfection reagent/GFP vector		
Lipofectamine 2000	1:2	1:1	2:1
Fugene 6	3:1	3:2	6:1
Fugene HD	3:2	5:2	8:2

TABLE 3
The plasmid/transfection reagent ratios have been expressed as volume of transfection reagent/µg plasmidic DNA



<u>Figure 44: Transfection of P0 AHAC with different ratios of Lipofectamine/GFP-encoding vector.</u>

A-B): Lipofectamine/GFP-vector ratio 1:2; C-D): ratio 1:1; E-F: ratio 2:1; G-H): lipofectamine only. The transfection efficiency has been assessed 24 hours post-transfection.

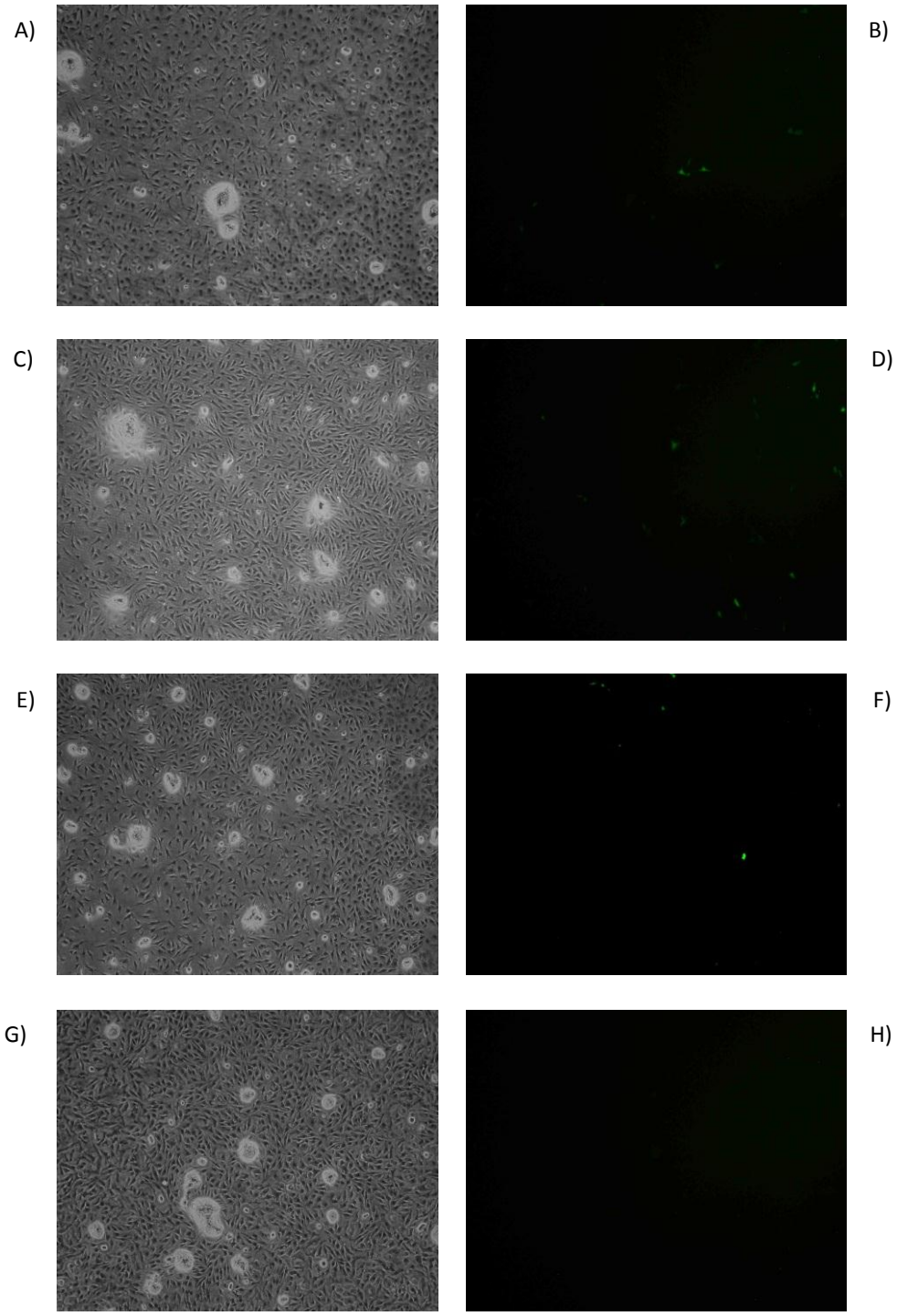


Figure 45: Transfection of P0 AHAC with different ratios of Fugene 6/GFP-encoding vector.

A-B) Fugene 6/GFP-vector ratio 3:1; C-D) ratio 3:2; E-F) ratio 6:1; G-H): Fugene 6 only. 4X magnification. The transfection efficiency has been assessed 24 hours post-transfection.

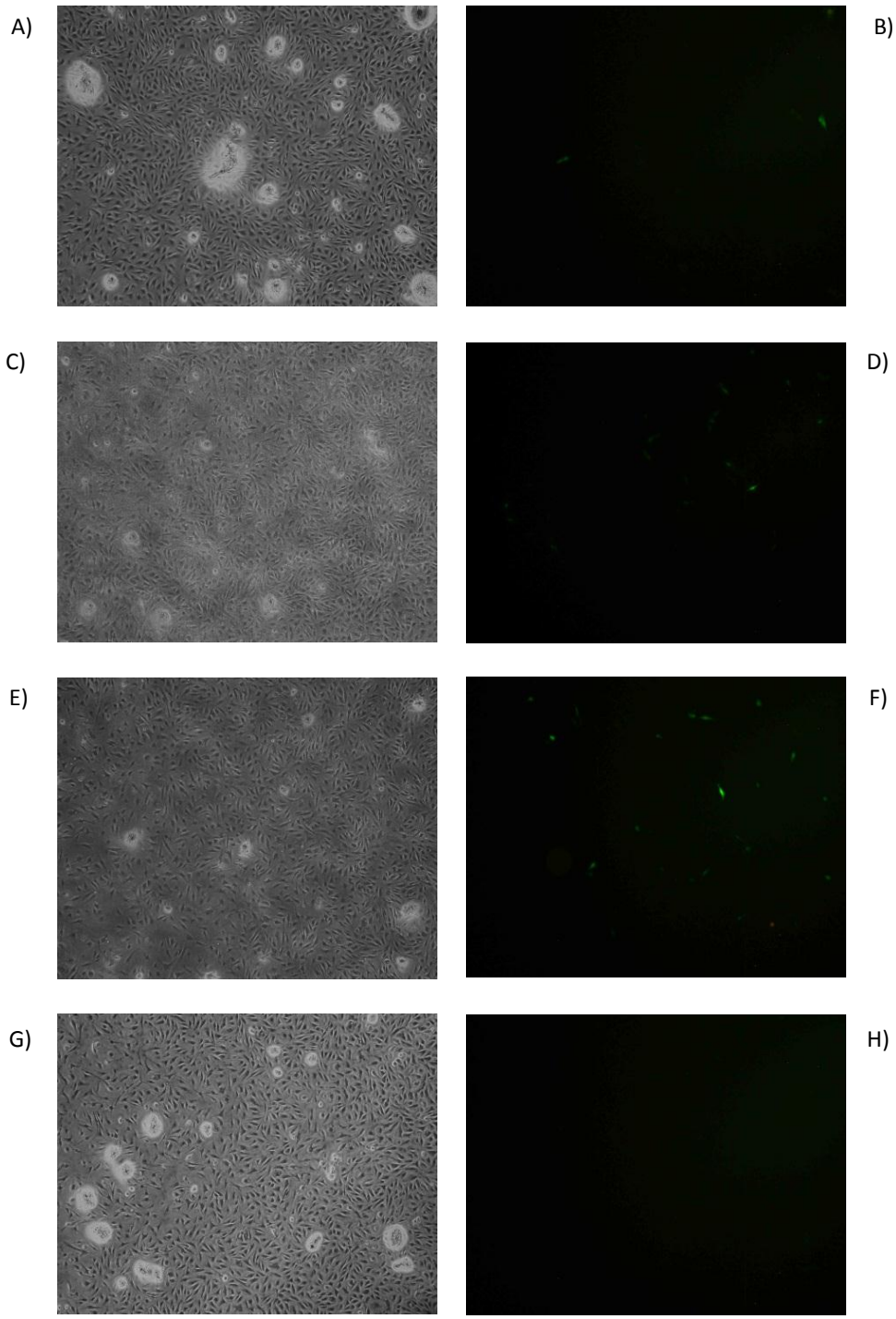


Figure 46:Transfection of P0 AHAC with different ratios of Fugene HD/GFP-encoding vector

A-B) Fugene HD/GFP-vector ratio 3:2; C-D) ratio 5:2; D-F: ratio 8:2; F-G) Fugene HD only. 4X magnification. The transfection efficiency has been assessed 24 hours post-transfection.

Lipofectamine 2000 had an average transfection efficiency equal to 7% whereas the two other transfection reagents, Fugene 6 and Fugene HD did not reach the 5% (fig.44-46). No evident toxicity was recorded after 24 hours from transfection with all the reagents as evaluated with trypan Blue exclusion assay.

TRANSFECTION OF NON-CONFLUENT AHAC WITH LIPOFECTAMINE 2000

Lipofectamine 2000 had the highest transfection rate on confluent cells. In order to improve it, the transfection was attempted on non-confluent cells, with the most efficient ratio of lipofectamine 2000/GFP-vector (Fig. 47).

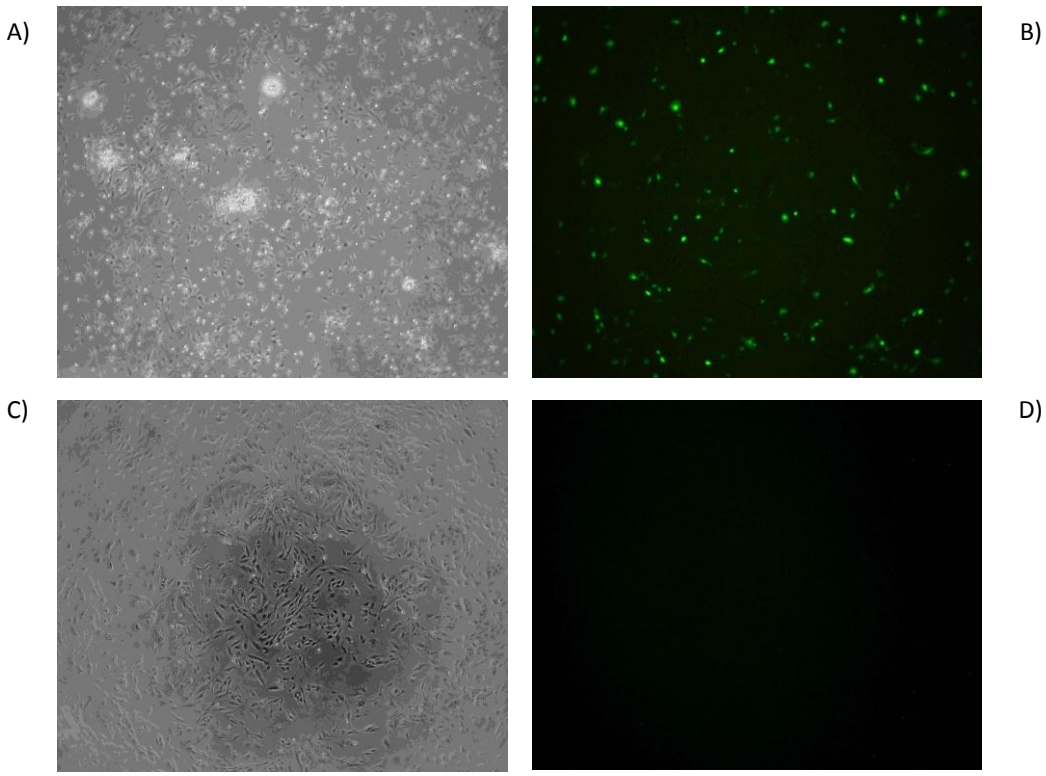


Figure 47: Transfection of 50%-confluent P0 chondrocytes with Lipofectamine 2000

A-B) Non-confluent primary P0 AHAH transfected with Lipofectamine 2000/GFP-vector, ratio 2:1, for 24h C-D) Un-transfected cells. 4X magnification. Transfection efficiency has been evaluated 24h post-transfection.

The transfection of non-confluent cells improved the transfection efficiency of lipofectamine, which reached the 20%. Unfortunately, the cytotoxicity of the reagent increased along with the efficiency, as many of the transfection cells died 24 hours post-transfection.

LIPOFECTAMINE 2000 TIME COURSE

To minimize the cytotoxic effect of Lipofectamine 2000, I incubated non-confluent chondrocytes with Lipofectamine/GFP-vector complexes for different periods of time. The transfection efficiency and cytotoxicity was then assessed by FACS analysis (Fig. 48 and 49) utilizing, as a measure of cell death, the size of the cell (See the Materials and Methods section). The FACS analysis was performed in collaboration with Dr. Fulvio d'Aquisto.

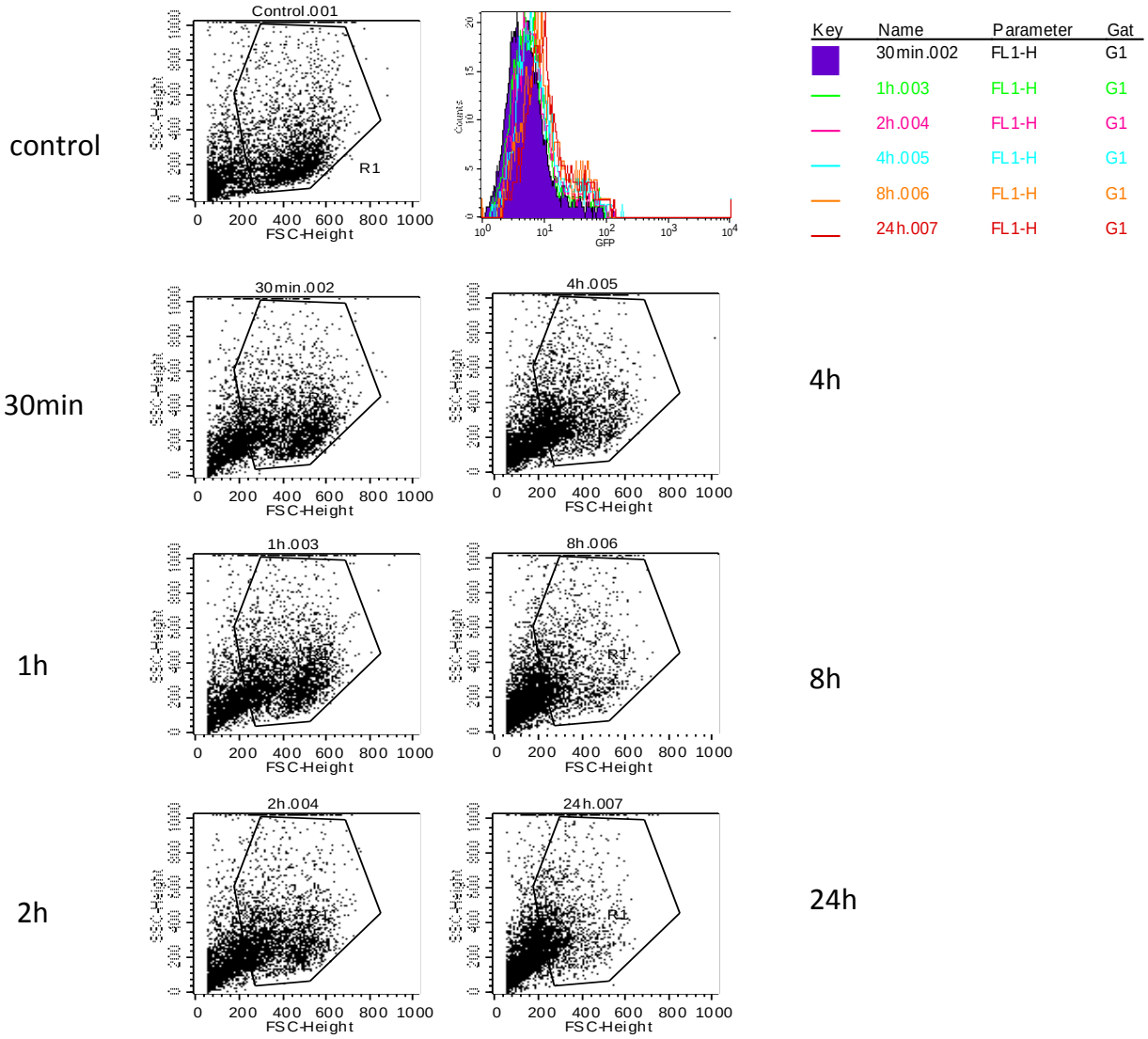


Figure 48: Time course of incubation with Lipofectamine 2000

P0 articular chondrocytes were incubated with Lipofectamine/pGFP complexes for different time points. The cells were detached by trypsin digestion and subjected to FACS analysis. The gated events at each time point represent the number of alive and GFP-positive cells. Control cells=un-transfected chondrocytes.

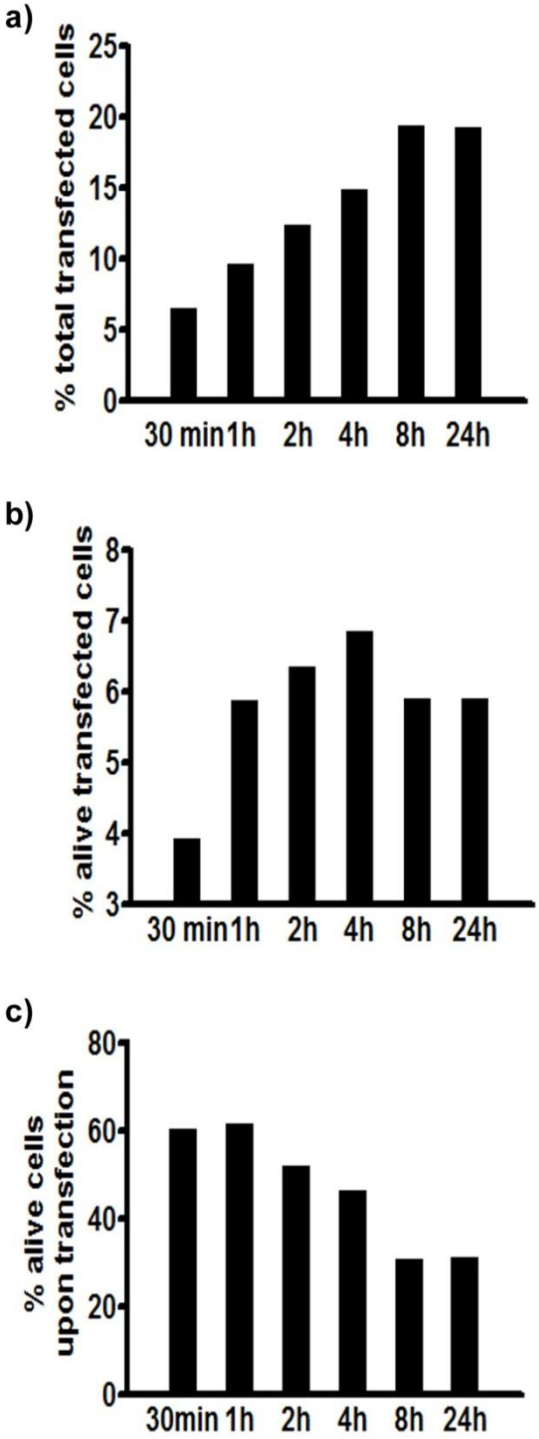


Figure 49: Transfection efficiency and cytotoxicity of lipofectamine 2000

a) Transfection efficiency of lipofectamine at different time points; b and c) chondrocyte survival rate. Data determined by FACS analysis.

The transfection efficiency of lipofectamine 2000 and its cytotoxic effect are time dependent and reached their maximum after 8 hours from transfection.

NUCLEOFECTION

In alternative to lipid-based transfection reagents an electroporation-based method was also evaluated. Electroporation consists in applying an electric field to the plasma membrane of a cell, increasing its permeability and allowing the introduction of polar molecules, such as DNA, in the cell. Nucleofection is a more efficient form of electroporation, which allows the direct introduction of the exogenous DNA, directly into the nucleus of the electroporated cells. Freshly isolated primary chondrocytes were nucleofected by using the appropriate kit for primary chondrocytes (Lonzabio). The cells have been electroporated by using both the pMAx-eGFP encoding vector (eGFP), provided with the nucleofection kit, and with the GFP-encoding vector used in the experiment with the liposome-based transfection reagents (GFP). The transfection efficiency was measured by FACS analysis. The cytotoxicity was quantified by Trypan blue exclusion test.

Nucleofection had ~40% transfection efficiency, with both eGFP and GFP encoding vectors (Fig. 51E). The lethality rate recorded after 24 hours was around 30% for the eGFP- and 40% with the GFP-vector (Fig 51F). Unfortunately, at 48 hours post-electroporation, most of the remaining cells died (data not shown) making this transfection method unsuitable.

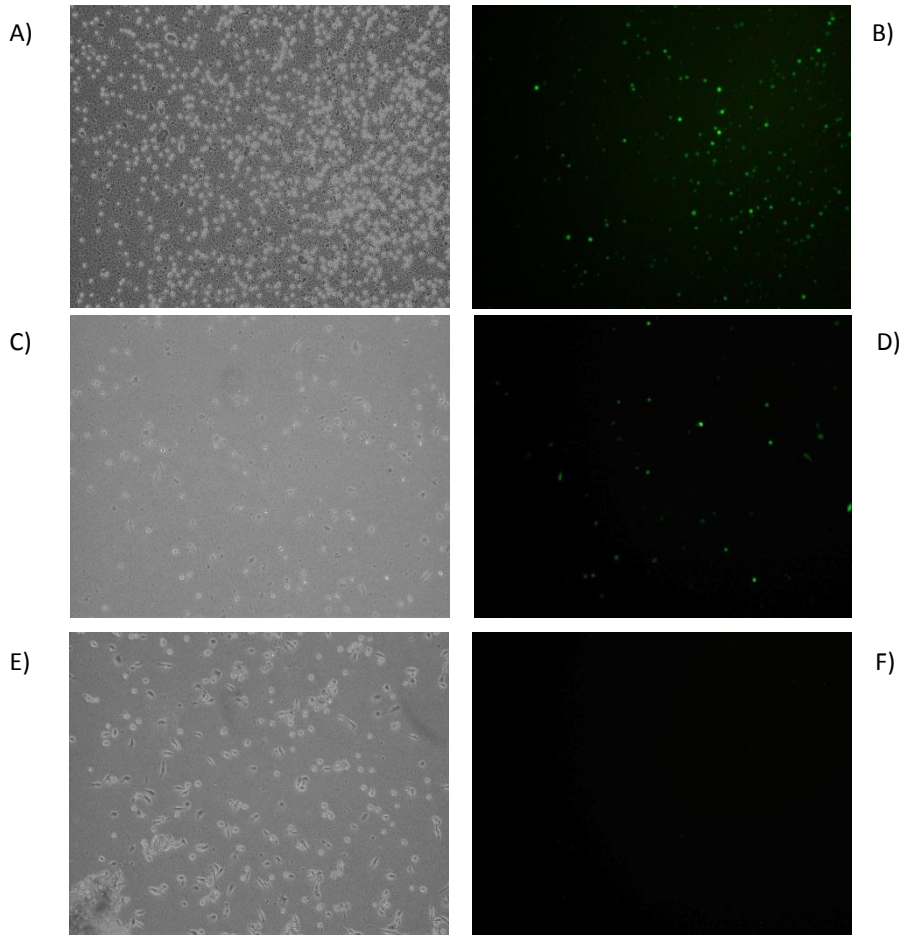


Figure 50: Nucleofection of primary chondrocytes

A-B) Freshly isolated chondrocytes nucleofected with pMAX-eGFP-encoding vector C-D) Chondrocytes nucleofected with GFP encoding vector E-F) Chondrocytes nucleofected in absence of any plasmid.

A)

Marker	Left, Right	Events	% Gated	% Tota	Mean	Geo Mear	CV	Mediar	Peak Ch
Al	1, 991	1000(	100.00	100.00	1269.03	100.17	186.6(	112.40	1
M1	1, 1	2876	28.76	28.76	4.79	4.14	55.16	4.07	3
M2	14, 858:	6261	62.61	62.61	1430.45	450.51	134.58	572.55	1625

B)

Marker	Left, Right	Events	% Gated	% Tota	Mean	Geo Mear	CV	Mediar	Peak Ch
Al	1, 991	5655	100.00	100.00	302.35	19.03	308.62	7.04	1
M1	1, 1	3000	53.05	53.05	4.47	3.90	54.51	3.82	3
M2	14, 858:	2315	40.94	40.94	654.23	201.48	165.76	192.82	18

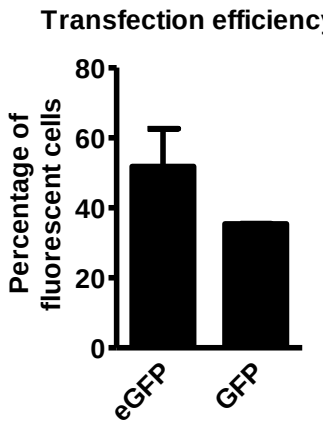
C)

Marker	Left, Right	Events	% Gated	% Tota	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991	1110	100.00	100.00	370.24	12.96	311.14	3.46	1
M1	1, 1	584	52.61	52.61	3.05	2.56	70.01	2.29	1
M2	14, 858:	394	35.50	35.50	894.20	280.73	156.80	347.55	16

D)

Marker	Left, Right	Events	% Gatec	% Tota	Mean	Geo Mea	CV	Mediar	Peak Ch
Al	1, 991	1125	100.00	100.00	386.30	13.15	330.62	3.43	1
M1	1, 1	578	51.38	51.38	3.01	2.50	74.00	2.21	2
M2	14, 858	408	36.27	36.27	849.10	258.66	173.24	261.80	39

E)



F)

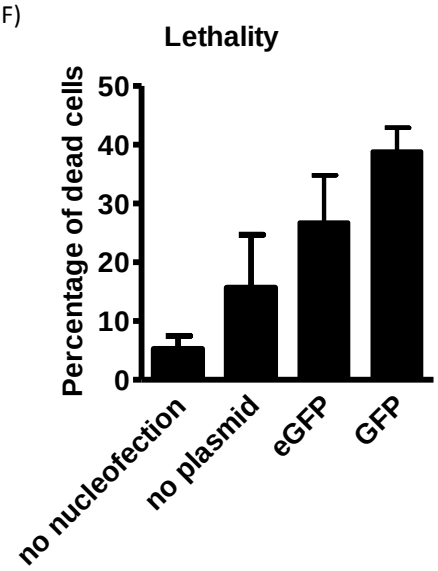


Figure 51: Quantification of the transfection efficiency of nucleofection on primary articular chondrocytes.

Five hundred thousand human articular chondrocytes were isolated from two different donors and electroporated with two different vectors encoding for GFP by using the appropriate device for nucleofection (Lonzabio). The cells were then incubated overnight in standard growth conditions. The transfection efficiency was measured as percentage of fluorescent cells by FACS analysis. A and C) Circled in red, the percentage of cells transfected with pMAX-eGFP-encoding vector in donor 1 and 2. B and D) Circled in red, the percentage of cells transfected with a GFP-encoding vector in donor 1 and 2. e) Transfection efficiency of nucleofection with the two different GFP-encoding vectors. f) Trypan exclusion test n=3

TRANSFECTION WITH ATELOCOLLAGEN

Atelocollagen (KOKEN) is highly purified calf dermis collagen type I enzimatically digested with pepsin. The digestion removes the C- and N-telopeptides from the molecule. The resulting cleaved protein has a highly conserved sequence across the species and consequently a very low immunogenicity (Honma et al., 2004).

Atelocollagen has been proven to promote the introduction of functional siRNA in vivo (Diarra et al., 2007), and it has been shown to allow the transfection of siRNA and adenoviral vector in vitro, in different systems (Honma et al., 2004).

Transfection with atelocollagen has been tried with two different approaches: in one experiment atelocollagen DNA was resuspended in atelocollagen 1% and the polymer was used to pre-coat the plate were the chondrocytes were seeded. In a second attempt, the cells were directly resuspended in different concentrations of atelocollagen diluted in complete medium. In both cases the concentration of DNA was maintained constant to 1µg/µl.

Atelocollagen alone did not promote any transfection of primary chondrocytes in our system, (see Table 4 for summary). These results indicate that atelocollagen is unsuitable for the transfection of plasmid DNA *in vitro* in primary articular chondrocytes. Taken together, these results indicate lipofectamine 2000 as the transfection reagent with the higher transfection efficiency in articular chondrocytes.

Method	Transfection efficiency	Ratio transfection reagent/DNA (v/w)	Survival rate
Lipofectamine-confluent cells	<5%	2:1	100%
Lipofectamine-non confluent cells	~7%	2:1	50%
Fugene 6	<5%	3:1	100%
Fugene 6	<5%	3:2	100%
Fugene 6	<5%	6:1	100%
Fugene HD	<5%	3:2	100%
Fugene HD	<5%	5:2	100%
Fugene HD	<5%	8:2	100%
Cells seeded on atelocollagen 1%	No transfection	n/a	n/a
Cells resuspended in atelocollagen 0.005-0.1%	Progressive increase from no transfection with atelocollagen 0.1%to <5% with atelocollagen 0.005%	n/a	n/a
AMAXA-kit for primary human chondrocytes	~40%	n/a	50%

TABLE 4

The table summarizes the efficiency of all the transfection methods/conditions tested on primary articular chondrocytes

APPENDIX 2: FUNCTIONAL VALIDATION OF WNT-3A-CONTAINING SUPERNATANT FROM STABLY TRANSFECTED L CELLS AND TCF/LEF REPORTER ASSAY

To evaluate if a 7% transfection efficiency was enough to promote a recordable biological response in primary chondrocytes, we performed a TOP-flash (TCF-optimal promoter) reporter assay (Molenaar et al., 1996) on WNT-3A stimulated cells.

The TOPFlash reporter assay consists in transfecting the cell type of interest with a vector encoding for firefly luciferase under control the transcriptional control of a TCF-promoter. Specifically, I used an optimized vector, the SUPER8XTopFlash reporter vector (kind gift of Prof. R. Moon, University of Washington, USA), containing 7 TCF binding sites on the TCF promoter, in order to maximize the biological response of the cells.

To validate the reporter assay I transfected HEK293 cells, which have a higher transfection rate that chondrocytes, with the Super8XTOPFlash reporter vector and I stimulated the cells for 24 hours with three different concentrations of recombinant WNT-3A and with LiCl 10mM as positive control (Fig. 52A).

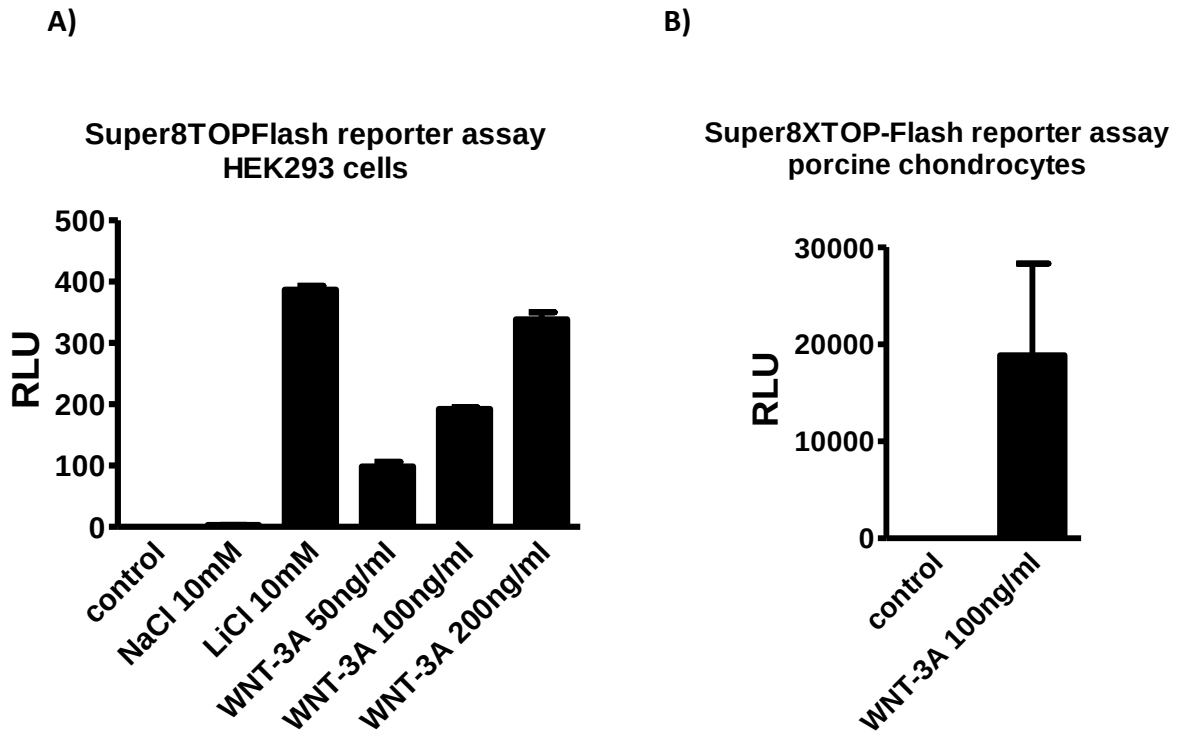


Figure 52: TCF/LEF reporter assay

A) Seventy per cent confluent HEK293 cells were co-transfected with the Super8XTOP-Flash reporter vector and a vector encoding for Renilla firefly luciferase under control of CMV promoter. Twenty four hours after transfection the cells were stimulated with different doses of recombinant WNT-3A or 10mM LiCl. 0.1%BSA in PBS was used as negative control for WNT-3A and 10mM NaCl. The cells were then lysed and luciferase activity recorded. B) Reporter assay performed on porcine articular chondrocytes. Data are expressed as firefly luciferase activity normalized for Renilla luciferase activity. n=3

Since WNT-3A stimulation promoted a dose-dependent increase in the luciferase activity in HEK293 cells (Fig. 52A), I then performed the reporter assay on porcine articular chondrocytes (Fig. 52B).

WNT-3A successfully induced the activation of the reporter assay in porcine articular chondrocytes, this demonstrating that the transfection efficiency of lipofectamine is enough to perform gain of function experiments in primary chondrocytes.

I also used this assay to titrate the content of secreted WNT-3A in a conditioned medium obtained by L-cells overexpressing WNT-3A.

We compared different dilutions of the conditioned medium with different known concentrations of recombinant WNT-3A (Fig. 53).

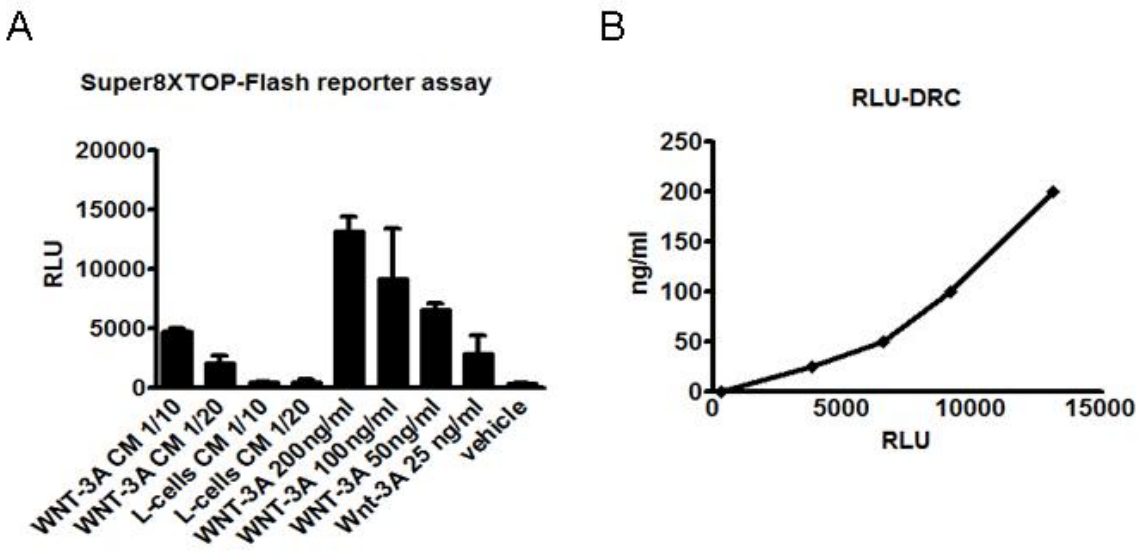


Figure 53: Determination of the concentration of secreted WNT-3A in a conditioned medium

HEK293 were transfected with the SUPER8XTOPFlash reporter vector and stimulated for 24 hours with dilutions 1/10 and 1/20 of the conditioned medium obtained by culture of L-cells stably transfected with a WNT-3A encoding vector (WNT-3A CM) or with medium removed from un-transfected cells (L-cells CM). The cells were also stimulated in parallel with different doses of recombinant WNT-3A. Data are expressed as firefly luciferase activity normalized for Renilla luciferase activity. n=3 B) The RLU of the different doses of recombinant WNT-3A were used to build-up a standard curve to determine the concentration of secreted WNT-3A in the conditioned medium. DRC= Dose Response Curve RLU=Relative Fluorescent Units

On the base of the standard curve obtained by plotting the values of Luminescence emitted by HEK293 cells stimulated with different known doses of recombinant WNT-3A, I determined the concentration of WNT-3A in the conditioned medium to be 200.70 ng/ml. I used this batch of medium diluted 1:2 for all the experiments performed with conditioned medium described in this thesis, in order to be able to compare these results with the ones obtained with the dose of recombinant protein used for all the other experiments (100ng/ml).

SUMMARY OF RESULTS FROM APPENDIX 1 AND 2

In vitro gain of function experiments are a useful tool to investigate the signalling pathways activated by the transcription of a specific gene in a particular biological context.

Introduction of exogenous DNA in primary articular chondrocytes is made difficult by the presence of the thick extracellular matrix surrounding these cells. To overcome this problem, chondrocytes can be infected with viral vectors encoding for the gene of interest. Viral transduction has been proved to provide a high rate of transgene expression (Lu et al., 2005;Li et al., 2004) and might be transient or permanent depending on the viral vector used.

Transient gene expression is less laborious, and can be also achieved with non-viral methods. Lipid-based transfection reagents such as Lipofectamine (Invitrogen), Fugene 6 and Fugene HD (Roche) exploit the ability of positively charged lipids to interact with DNA and to be up-taken by the cells through endocytosis. Atelocollagen, pepsin digested type I collagen defective of the two telopeptides, has been proved to successfully promote the introduction of exogenous nucleic acids both *in vitro* and *in vivo* (Honma et al., 2004). Finally electroporation, which transiently increases the permability of the cell membrane, has also been shown to promote chondrocyte transfection (Welter et al., 2004).

I tried all these methods in order to find the one with the best transfection efficiency and the lowest cytotoxicity. Of all the non-viral transfection methods which have been tested, Lipofectamine 2000 had the best combination of these two parameters, reaching a transfection efficiency equal to 7% and a low toxicity after 4 hours from the transfection. In

addition, this method successfully allowed performing a reporter assay on primary cells, indicating its suitability for our *in vitro* experiments.

APPENDIX 3: STIMULATION OF ARTICULAR CHONDROCYTES *IN VIVO* IN THE ECTOPIC CARTILAGE FORMATION ASSAY

To evaluate the biological effects of WNT-3A in an *in vivo* system, we exploited the unique ability of chondrocytes to form ectopic, stable, hyaline-like cartilage when implanted intramuscularly in nude mice (Dell'Accio et al., 2001).

The ectopic cartilage formation assay has been shown to predict the successful outcome of ACI in human (Saris et al., 2009;Saris et al., 2008). ACI is a surgical procedure allowing the repair of articular cartilage injuries of limited size. It consists in the removal of the cartilage from a non-bearing area of the joint, in the isolation and expansion *in vitro* of the articular chondrocytes and finally in their re-implantation in the zone of the area of the articular damage, where they contribute to the repair process. Only chondrocytes which maintain the expression of some specific biomarkers- such as *COL2A1* and *Aggrecan*- during the expansion in culture can successfully contribute to the repair. The expression of these biomarkers correlates with the success of the cartilage formation assay in nude mice (Dell'Accio et al., 2001) as well as the clinical outcome of ACI in patients (Saris et al., 2009;Saris et al., 2008).

One major problem in using this assay to evaluate the effects of WNT-3A on ectopic cartilage formation was the need to provide a constant supply of WNT-3A within the implant, with minimal manipulation of the chondrocytes, because such manipulations may *per* se influence the ectopic cartilage formation capacity.

To achieve this goal we opted for a cell delivery system based on the co-injection of chondrocytes with a small aliquot of a cell line overexpressing WNT-3A.

To test the validity of the system we first co-injected porcine articular chondrocytes with Cos7 cells co-transfected with a GFP-encoding vector and with a MycHis-tagged GCP2 expressing plasmid (Fig. 54 and 55). GCP2 is a chemokine with very mild pro-inflammatory properties (Wuyts et al., 1997) and abundantly expressed and secreted by chondrocytes (Not shown). We decided to use this vector because we could test the ability of the cells to secrete the protein

in the implants by immunohistochemistry, and we could distinguish the protein from the endogenous form expressed by chondrocytes thanks to the presence of the tag. We have chosen to use porcine chondrocytes to perform the assay for the easier availability of the cells in comparison to human samples and for the higher number of cells obtainable from the same amount of cartilage. Porcine chondrocytes have been extensively tested in this model (Dell'Accio et al., unpublished results).

In this pilot experiment we also growth-arrested the cells by treating them with mitomycin C 5µg/ml for 2 hours at 37°C, in order to avoid the formation of the tumours in the legs of the injected mice.

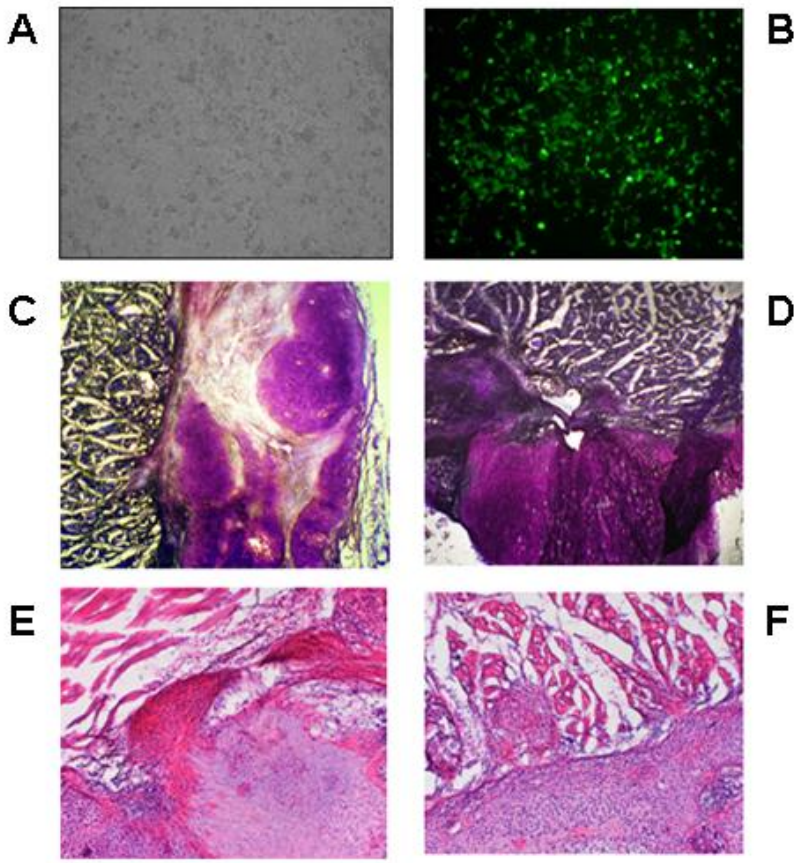


Figure 54: Porcine chondrocytes do form cartilage implants when co-injected with GFP-transfected Cos7 cells

A-B) Bright field and green fluorescence images of Cos7 cells transfected with pGFP. C-D) Toluidin blue-haematoxylin/eosin staining of hyaline-like cartilage implant formed by freshly isolated porcine-chondrocytes co-injected with transfected 10% Cos7 cells E-F) Haematoxylin/eosin staining of hyaline-like cartilage implant formed by freshly isolated pig-chondrocytes co-injected with eGFP-transfected cells. All the pictures were taken with a 10X magnification and were modified by Photoshop 7.0 for best rendering the fluorescence/staining.

Cos7 cells had a transfection efficiency of around 60% (Fig. 57A-B) and eGFP-fluorescence was detected in monolayer for two weeks after transfection (Data not shown). The treatment with Mitomycin C did not compromise their ability to express GFP (not shown).

The co-injection did not impair the ability of the chondrocytes to form cartilage *in vivo* (Fig. 57C-D). No infiltration of inflammatory cells was noticed after haematoxylin-eosin staining of the tissue (Fig. 57E-F).

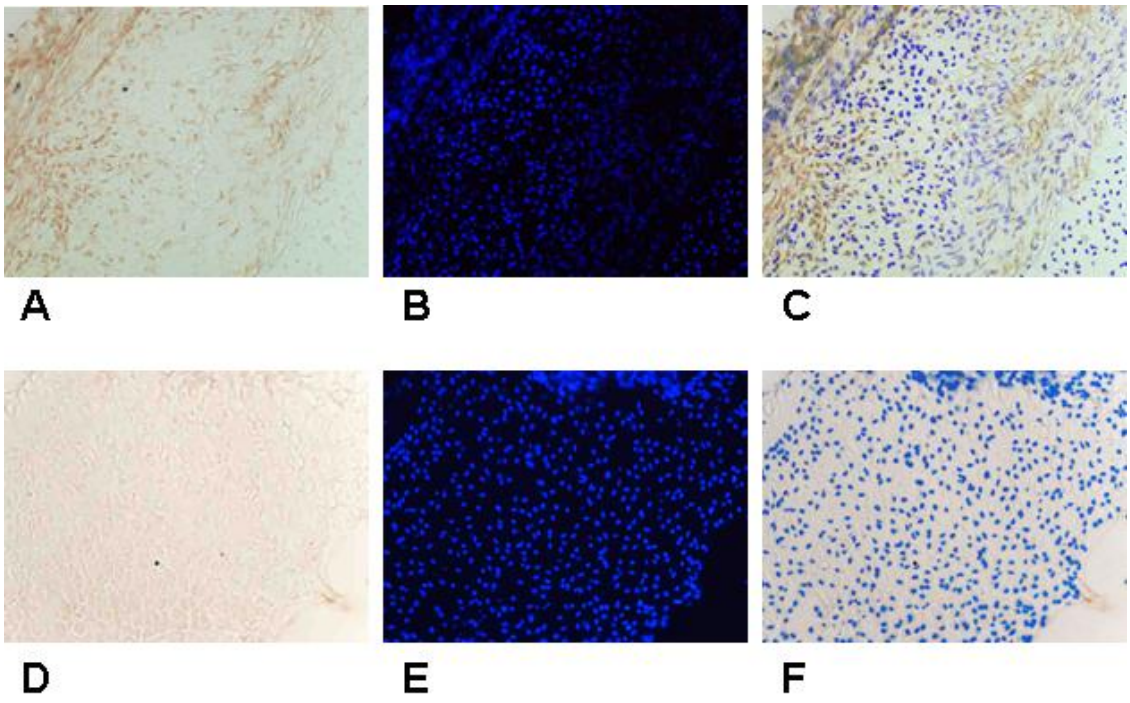


Figure 55: Anti His immunostaining of cartilage implant formed by pig chondrocytes co-injected with Cos7 cell previously transfected with GCP2 Myc-His tagged.

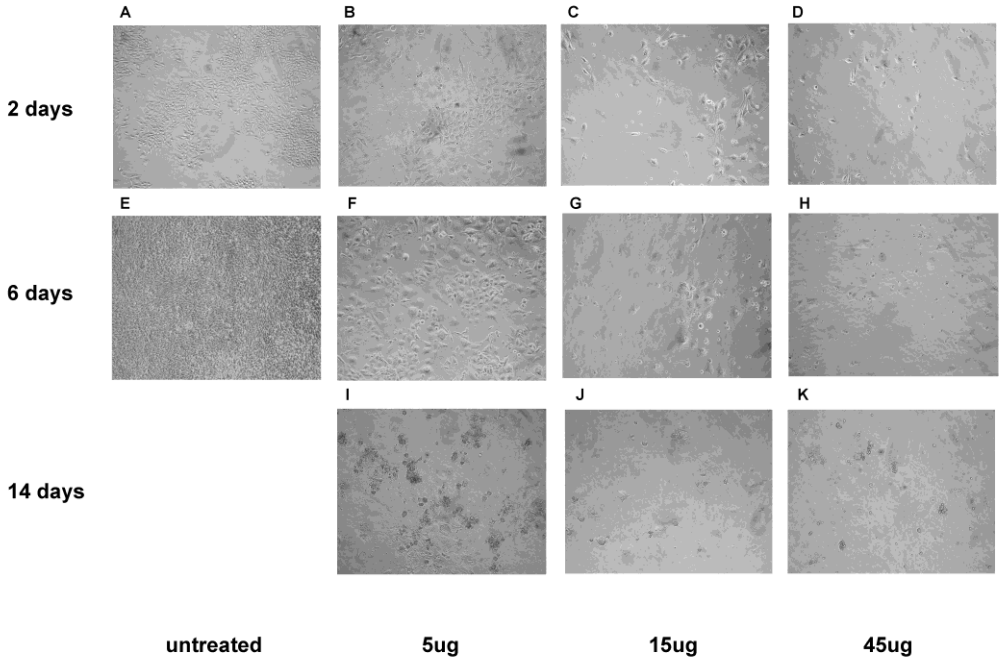
A) anti-His tag staining on frozen section of implant formed by co-injected cells B) DAPI staining on A, C) A and B merged D) Anti-His tag staining on frozen section of cartilage implant formed by pig chondrocytes injected alone E) DAPI staining of D, F) d and e merged. Images were modified by using Photoshop 7.0 for best rendering the staining

Persistence of the cells in the implant was further confirmed by anti-His immunostaining which revealed tagged GCP2 in the tissue (Fig. 58A-C). The immunostaining was performed by Dr. El Tawil, in our laboratory.

Having validated the method, we then tried to co-injected articular chondrocytes with WNT-3A overexpressing cells, to evaluate the biological effect of this ligand on chondrocyte phenotype *in vivo*.

We co-injected growth-arrested L cells (L-WNT-3A) stably transfected to express WNT-3A (Willert et al., 2003) along with porcine articular chondrocytes. Wild type L cells were used for control injections. The use of L-cells was particularly advantageous since these are murine fibroblasts, and therefore I could still selectively monitor gene expression in co-injected human or porcine chondrocytes using species-specific PCR primers. I then optimized their growth

arrest testing three different doses of Mitomycin C in order not to compromise their viability and especially their capacity to secrete biologically active WNT-3A for at least 14 days (Fig. 56). Since 5 µg/ml of the compound weren't enough to completely arrest the growth of the cells (Fig. 56F) and 15µg/ml compromised the viability of the cells (Fig. 56G) we opted for an intermediate dose (7.5ug/ml) to growth arrest the cells before the injection. Indeed this dose did not impair the viability of L-cells (Fig. 56L-M) but efficiently stopped their growth.



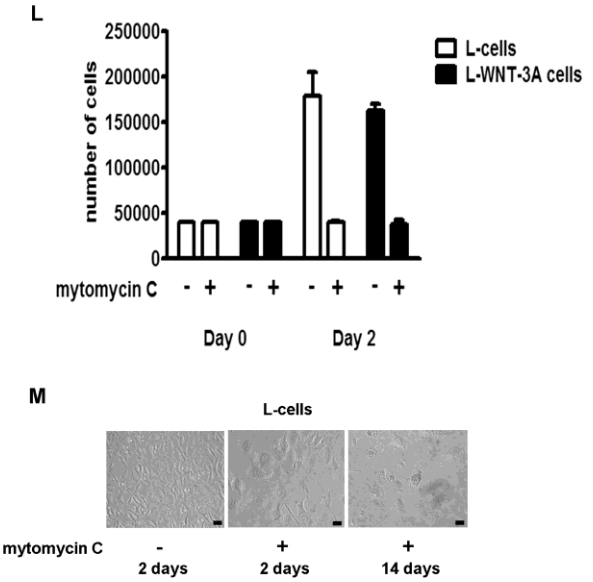


Figure 56: Mitomycin C treatment does not compromise the viability of L-cells

A-K) L-cells were treated with different doses of mitomycin C and their viability was monitored for 14 days L) 40000 L-cells were plated and treated with 7.5µg/ml of mitomycin C. After 2 days the cells were counted to evaluate the efficacy of the growth arresting treatment M) L-cells growth arrested with mitomycin C were maintained in culture for 14 days upon treatment. The treatment did not compromise their survival. Pictures were acquired at 10X magnification. Scale bars=20µm

Finally we tested if the mitomycin C could impair the ability of L-cells to secrete biologically active WNT-3A. In order to do so, we used conditioned medium from growth-arrested L-WNT-3A cells to stimulate porcine articular chondrocytes transfected with the SUPER8XTOPFlash reporter vector (Fig. 57)

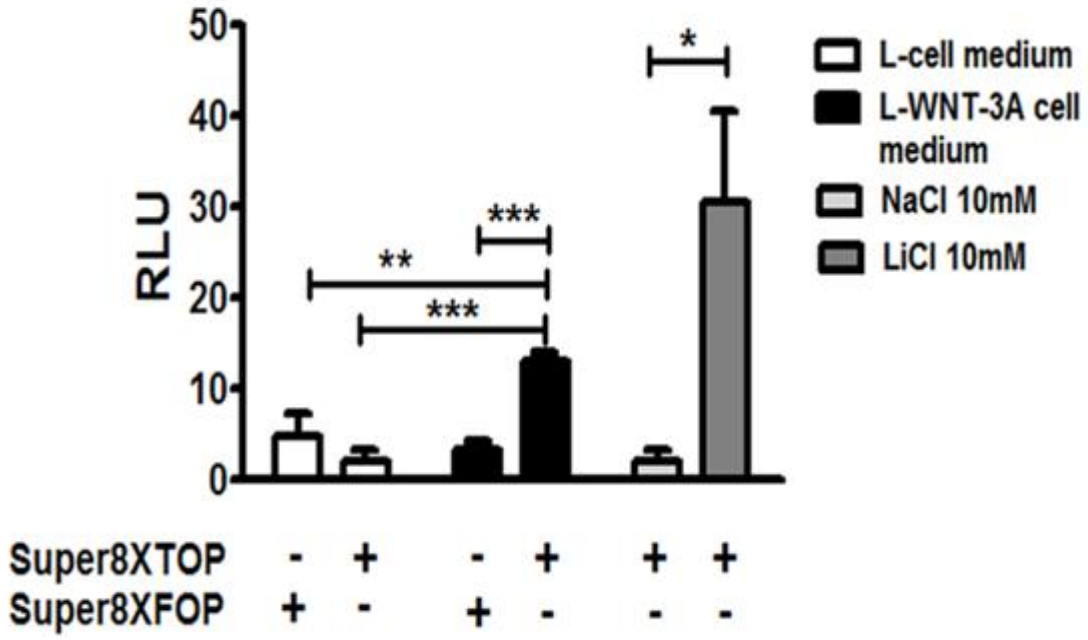


Figure 57: Mitomycin C treatment does not impair the ability of L-WNT-3A cells to secrete WNT-3A

To confirm that mitomycin C-growth-arrested L-WNT-3A cells still produced biologically active WNT-3A, the conditioned medium (from the last change of medium) obtained 14 days following mitomycin C treatment was tested for the ability to activate the SUPER8XTOPFlash reporter assay in porcine articular chondrocytes. Indeed conditioned medium from L-WNT-3A cells, but not from control cells, activated the SUPER8XTOPFlash reporter. The mutagenized SUPER8XFOPFlash vector was used to control for specificity (n=4). Unpaired t-test was used for the statistical analysis.

Mitomycin C treatment did not block the expression on secretion of biologically active WNT-3A, since the conditioned medium from growth-arrested cells was still able to activate the TCF/LEF reporter assay in chondrocytes.

Finally, we tested the ability of L-WNT-3A cells to promote chondrocyte de-differentiation in vivo when co-injected with porcine and human articular chondrocytes (See Fig. 24 and 26). As

discussed above, the experiment resulted indeed in de-differentiation of chondrocytes in the implants retrieved from mice co-injected with chondrocytes and L-WNT-3A.

SUMMARY OF APPENDIX 3

We validated a new, convenient and efficient method to test the biological effect of secreted stimuli on chondrocytes in an in vivo system.

This assay is advantageous from different points of view:

- This assay is a modified version of the validated "ectopic cartilage formation assay" which is a ***WELL ESTABLISHED***, ***REPRODUCIBLE*** and ***CLINICALLY RELEVANT*** chondrogenesis assay
- The easy access to different, commercially available cell lines, stably overexpressing and secreting biologically relevant molecules make this assay ***CONVENIENT*** and of ***QUICK*** and ***EASY EXECUTION***
- The co-injection of articular chondrocytes with the growth-arrested cell line ***AVOIDS THE MANIPULATION OF THE CHONDROCYTES*** -e.g. by viral infection- which could affect *per se* the outcome of the assay and delay the execution of the experiment, and that may require special facilities for their optimization- eg. class II rooms for the manipulation of lentiviruses-.

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