UNIVERSITÉ DE STRASBOURG

École Doctorale des Sciences de la Vie et de la Santé

UMR 7021 : Laboratoire de Bioimagerie et Pathologies

THÈSE présentée par :

Antoine ABOU JAOUDE

soutenue le : **19 Décembre 2018**

Pour obtenir le grade de : **Docteur de l'université de Strasbourg**

Spécialité : **Aspects moléculaires et cellulaires de la biologie**

Rôle de ShcA dans l'athérosclérose et dans la différenciation des chondrocytes

THÈSE dirigée par :

Pr. BOUCHER Philippe	Professeur, Université de Strasbourg

RAPPORTEURS EXTERNES:

Dr. PINZANO Astrid	Chargée de de Recherche HDR, Université de Lorraine
Dr. YVAN-CHARVET Laurent	Directeur de Recherche, Université Nice Sophia Antipolis

---

AUTRES MEMBRES DU JURY :

Pr. BARBERO Andrea	Professeur, Université de Bâle
Dr. BRONNER Christian	Directeur de Recherches, IGBMC, Strasbourg
Pr. NIEMEIER Andreas	Professeur, Université de Hambourg
Dr. MATZ-WESTPHAL Rachel	Maître de Conférences, Université de Strasbourg

“We live on an island surrounded by a sea of ignorance.

As our island of knowledge grows, so does the shore of our ignorance.”

- *John Archibald Wheeler*

Acknowledgments

Firstly, I would like to express my sincere gratitude to Pr. Laurent Yvan-Charvet, Dr. Astrid Pinzano, Pr. Andrea Barbero, Pr. Andreas Niemeier and Dr. Christian Bronner for accepting to evaluate my work, I am truly honored.

I am extremely thankful to my thesis director, Pr. Philippe Boucher for welcoming me in his lab and offering continuous guidance and supervision. Our weekly meetings were indispensable and greatly appreciated.

My deepest appreciation goes to Dr. Rachel Matz-Westphal for her patience, supervision, advice, invaluable support throughout my PhD and all the time she invested on my journey. I will always remember her efforts, all the way from the beginning, her aid with Pr. Boucher to prepare me for the interview to be accepted in the IdEx program and all the times she helped me with different presentations and posters, her mentoring and teaching me most of the techniques I needed to learn, her relentless belief that I could accomplish what sometimes seemed impossible, her push to always get the best out of me up until the end, with her help in correcting the manuscript and preparing me for the defense.

I would like to thank our collaborators, Pr. Barbero and his team for offering their expertise and elevating my project, and Pr. Niemeier for welcoming me in his lab for an extended period and for his supervision, I would also like to thank the members of his team, Dr. Friederike Behler-Janbeck and Mr. Walter Tauscher.

My most-heartfelt thanks to Dr. Jérôme Terrand who constantly offered a helping hand and a sympathetic ear. I greatly appreciate how you always had time for me and my questions that constantly interrupted your experiments, your train of thought as well as your lunch breaks.

My appreciation and gratitude extend to all the members of Pr. Boucher's team, to Mrs. Hélène Justiniano, her assistance and technical advice were immensely valuable and came whenever needed and my fellow colleague Sara Awan for offering a much-

needed companionship along the journey, I wish her best of luck on completing her PhD.

To Dr. Lionel Host, it was a great pleasure to work with him at the beginning and a bigger pleasure to see him join the team again. His work with Mr. Jean François Poirier has always been reliable.

I would like to thank Dr. Emmanuel Boutant and Pr. Philippe Georgel for making my first “TP” teaching experience rewarding and unforgettable.

Thanks to Dr. Cyril Auger for accepting to be a jury member of my mid-thesis evaluation committee, his questions and insight helped me realize there are always more things to learn.

Thanks to all the interns that have come and gone, each of them left a mark on my work. I would like to thank in particular both Marjorie Courtes and Clauda Abboud, their work has tremendously advanced my project, and it was reassuring that I could rely on them on parts of my project.

To my colleagues, Dr. Marie-Cécile Mercier and Elisabete Silva, sharing the office space could not have been more comfortable. I wish them best of luck in all their endeavors.

I would also like to thank the team of Dr. Monique Dontenwill, Pr. Maxime Lehmann, Dr. Véronique Bruban-Schann, Dr. Sophie Martin, Dr. Laurence Choulier, Mrs. Fanny Noulet and Mrs. Tania Steffan. The cheerfulness and friendliness in the workplace always made working more enjoyable.

Finally, I couldn’t have done it without the inexhaustible source of compassion and understanding that is my family and friends. To my parents and sisters, thanks for always blindly believing in me.

Table of contents

List of figures

List of Abbreviations:

ABCA1, ABCG1: ATP-binding cassette transporters.

AC: Articular cartilage.

ACAN: Aggrecan.

ACI: Autologous chondrocyte implantation.

ADAMTS: A Disintegrin and Metalloproteinase with Thrombospondin Motifs.

AKT: Protein kinase B.

ALP: Alkaline phosphatase.

ApoE: Apolipoprotein E.

B-gp: beta-glycerophosphate.

BMP: Bone morphogenetic protein.

C: Chondrogenic differentiation.

CB: cytochrome c-binding motif.

CCL2: Chemokine ligand 2.

CCN/2: Connective tissue growth factor.

CD-36: Cluster of differentiation 36.

cDNA: Complementary DNA.

CH: Collagen homology.

COL I/II/X/IX/XI: Collagen types I/II/X/IX/XI.

CR: Cysteine-rich complement-type repeats.

CRISPR/Cas9: Clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9.

CT: Cycle threshold.

CTGF: Connective tissue growth factor.

DMEM: Dulbecco's Modified Eagle Medium.

DNA: Deoxyribonucleic acid.

ECL: Enhanced chemiluminescence.

ECM: Extracellular matrix.

EDTA: Ethylenediaminetetraacetic acid.

EGF: Epidermal growth factor.

EGFR: Epidermal growth factor receptor.

EGTA: Ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

EO: Endochondral ossification.

ERK1/2: Extracellular signal-regulated kinases 1/2.

FAK: Focal adhesion kinase.

FBS: Fetal bovine serum.

FGF: Fibroblast growth factor.

FGFR: Fibroblast growth factor receptor.

GAB1: GRB2-associated-binding protein 1.

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

GDF: Growth and differentiation factor.

GF: Growth factor.

GH: Growth hormone.

GP: Growth plate.

GRB2: Growth factor receptor-bound protein 2.

GTP: Guanosine triphosphate.

HA: Hyaluronic acid.

HCS 2/8: Human chondrosarcoma cell line.

HDL: High density lipoprotein.

HEK: Human embryonic kidney cell line.

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

HRP: Horse radish peroxidase.

HTRA1: High-temperature requirement A1 serine protease.

HYP: Hypertrophic differentiation.

ICAM-1: Intercellular adhesion molecule 1.

IFN-gamma: Interferon-gamma.

IGF-1: Insulin-like growth factor 1.

IGF-1R: Insulin-like growth factor 1 receptor.

IHH: Indian hedgehog.

IL: Interleukin.

IP: Immunoprecipitation.

kDA: kilo Dalton.

KO: Knockout.

LATS1/2: Large tumor suppressor kinase 1/2.

LDL: Low density lipoprotein.

LRP1: Low density lipoprotein receptor-related protein 1.

M: Meniscus.

MACT: Matrix-assisted autologous chondrocyte transplantation.

MAPK: Mitogen-activated protein kinase.

MCP-1: Monocyte chemoattractant protein 1.

M-CSF: Macrophage colony-stimulating factor.

MEF: Mouse embryonic fibroblasts cell line.

MEF-2C/D: Myocyte-specific enhancer factor 2C/D.

MMP: Matrix metalloproteinase.

mRNA: Messenger RNA.

MSC: Mesenchymal stem cells.

MSC TERT: Telomerase reverse transcriptase transfected mesenchymal stem cells.

MSX2: Muscle segment homeobox 2.

ND: Non-differentiated.

NF-KB: Nuclear factor kappa-light-chain-enhancer of activated B cells.

NLS: Nuclear localization sequence.

NO: Nitric oxide.

Nrf2: Nuclear factor erythroid-2-related factor 2.

NSAIDs: Nonsteroidal anti-inflammatory drugs.

OA: Osteoarthritis.

OARSI: Osteoarthritis Research Society International.

PBS: Phosphate buffered saline.

PCR: Polymerase Chain Reaction.

PDGF: Platelet-derived growth factor.

PDGFR: Platelet-derived growth factor receptor.

PI3K: Phosphoinositide 3-kinase.

PKC: Protein kinase C.

PMSF: Phenylmethylsulfonyl fluoride.

PPAR-gamm: Peroxisome proliferator-activated receptor gamma.

PTB: Phosphotyrosine-binding domain.

PTC-1: Patched 1.

PTH: Parathyroid hormone.

PTHRP: Parathyroid hormone-related protein.

P-tyr: Phosphorylated tyrosine.

QRT PCR: Quantitative real-time polymerase chain reaction.

RANKL: Receptor activator of nuclear factor kappa-B ligand.

RNA: Ribonucleic acid.

ROS: Reactive oxygen species.

RPM: Rounds per minute.

RT: Room temperature.

RTK: Receptor tyrosine kinase.

RunX2: Runt-related transcription factor 2.

SDS: Sodium dodecyl sulfate.

SDS-PAGE: Sodium dodecyl sulfate polyalcrylamide gel electrophoresis.

SED : Spondylepiphyseal dysplasia.

Ser: Serine.

SH: Src Homology domain.

Shc: Src homology 2 domain containing protein.

SiRNA: Small interfering ribonucleic acid.

SirT1: Sirtuin 1.

Smad2/3: Mothers against decapentalgic homolog 2/3.

SMC: Smooth muscle cell.

Smo: smoothened.

SOS: Son of sevenless.

SOX9: Sex-determining region Y-type high mobility group box 9.

SR-A: Scavenger receptor A.

STAT: Signal transducer and activator of transcription.

T4-T3: Thyroxin.

TAZ: Tafazzin.

TBP: Tata-binding protein.

TBS: Tris-buffered saline.

TBST: Tris-buffered saline with tween.

TGF-b: Transforming growth factor-beta.

TIMP-3: Metalloproteinase inhibitor 3.

TNF: Tumor necrosis factor.

VCAM-1: Vascular cell adhesion molecule 1.

VEGF: Vascular endothelial growth factor.

VEGFR: Vascular endothelial growth factor receptor.

VLDL: Very low density lipoprotein.

YAP1: Yes associated protein 1.

ZEB1: Zinc Finger E-Box Binding Homeobox 1.

Introduction

Atherosclerosis, the leading cause of death worldwide, is a multifactorial cardiovascular disease involving a series of complex processes that eventually culminate to serious outcomes, such as myocardial infarction and strokes[1].

The disease is initiated with abnormal accumulation and oxidation of low density lipoproteins (LDL) in the sub-endothelial matrix, leading to endothelial dysfunction and ensuing inflammation[2,3].

This is followed by the proliferation and migration of smooth muscle cells (SMCs) to the deeper inflamed layer which ultimately leads to vascular calcification, characterized by the expression of cartilage and bone-specific markers (Sox9, ALP1 and Osteocalcin)[4] and the presence of chondrocyte-like cells in the arterial wall[5] suggesting that vascular calcification is not merely a passive degenerative disorder, but an active process reminiscent of bone formation[6].

Pr. Philippe Boucher's team has demonstrated the crucial role of LRP1 in protecting from atherosclerosis. In fact, its conditional knockout from SMCs led to the development of advanced atherosclerotic plaques in mice fed a high-fat diet[7].

Moreover, the conditional deletion of PPAR-gamma - a potent inhibitor of chondrogenesis - in SMCs promoted vascular calcification by activating a pro-chondrogenic LRP1/Wnt pathway[8].

The aim of my PhD thesis were to better characterize the role of LRP1 and its signaling partner ShcA in atherosclerosis and chondrogenic differentiation. Once activated, LRP1 is tyrosine-phosphorylated on an NPxY consensus sequence within its cytoplasmic tail, which leads to the recruitment of the adaptor protein ShcA[9].

ShcA is not only capable of mediating LRP1 signaling but it can also integrate converging signals from various growth factor receptors, such as PDGF, IGF1 and TGF-b, from integrins, as well as cytoplasmic tyrosine kinases like Src and FAK[10,11,12,26.] This central role of ShcA is potentially crucial in controlling atherosclerosis and chondrocyte proliferation and differentiation.

ShcA.

Shc (Src Homology and Collagen) proteins were first identified in 1992 as novel transforming proteins, defined as having an SH2 (Src Homology 2) domain and deprived of any catalytic abilities, unlike other SH2 proteins such as Src. Moreover, they were shown to be bound to, and phosphorylated by activated growth factor receptors[13]. This gave Shc proteins the ability to tie these receptors to downstream pathway effectors, classifying them as adaptors.

Four related Shc proteins have been described in mammals; ShcA (Shc1), ShcB (Sck/Sli/Shc2), ShcC (Rai/N-Shc/Shc3)[14,15,16] and more recently, ShcD (Shc4/RaLP (Rai-like protein))[17].

ShcA in its three isorforms; p46, p52 and p66 *(Fig.1)* is ubiquitously expressed in all tissues of the organisms, except adult mature brains[38], and throughout all developmental stages (except for p66 which is absent in cells of hematopoietic lineage)[13]. Also, its Knock-Out leads to lethality at embryonic day E11.5 due to cardiovascular anomalies[18].
It is interesting to note that the p66 isoform plays a role in the cellular oxidative stress response, and p66 KO animals have an increased lifespan of 30%[19].
The two isoforms p46 and p52 are products of the same transcript through use of different start codons, but it's through alternative splicing that p66 is obtained, endowed with an N-Terminal extension of 110 amino acids[20].

ShcB only exists in one isoform of 68kDa, and ShcC in two isoforms of 64kDa and 52kDa. The absence of ShcB and ShcC in animals in not lethal, however KO animals do exhibit fewer sensory nociceptor neurons[15].

Finally, ShcD is mainly expressed in precursors of the central nervous system, muscle, epithelia and bone tissues[21].

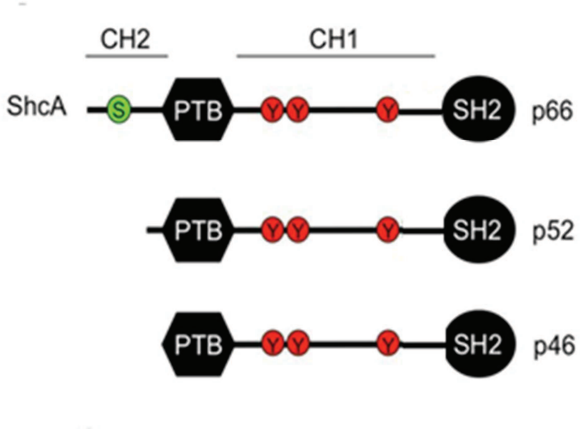


Figure 1: ShcA isoforms and domains.
All three isoforms have an N-terminal PTB domain, a C-terminal SH2 domain and three central tyrosine residues (red circles).
Uniquely for the p66 isoform, a serine residue is key for oxidative stress signaling (green circle) and an additional N-Terminal CH2 domain[22] *(modified)*.

Shc signaling.

Growth factors (GF) exercise a potent effect on cell behavior, a lot of these GFs act on transmembrane tyrosine kinase receptors (RTKs).
Binding to an extracellular ligand prompts the dimerization and autophosphorylation of the RTK on specific tyrosine residues. Thanks to both the C-terminal SH2 domain and the N-terminal PTB (phosphotyrosine-binding domain), Shc proteins, via their PTB domains, are able to bind to these P-tyr residues within the consensus sequence Φ-X-N-P-X-pY and via their SH2 domains to the residues within this sequence pY-Φ-X-I/L where Φ represents any large hydrophobic amino acid[23].
It is important to note that binding through the PTB domain is dominant over the SH2 domain[24,25]. The two domains are connected by a CH1 (collagen homology 1) region that also contains consensus tyrosine residues (Positions 239/240 and 317) that will be subsequently phosphorylated and recognized by downstream signaling effectors[22,23]. The subsequent enzymatic cascade ultimately leads to the regulation of gene transcription[26].

These effectors include mainly cellular kinases such as Src, Fyn and Lyn[24] and the Ras/MAPK adaptor Grb2 (Growth factor receptor bound protein 2)[23], that all have the SH2 domain able to bind to the CH1 domain of Shc.

Found only in the longer isoforms (p66 for ShcA) is an N-terminal CH2 domain that contains a functional CB (cytochrome c-binding) motif allowing the generation of ROS

(Reactive Oxygen Species) and giving p66 a central role in controlling the cellular oxidative stress[27].

The (CH2)–PTB–CH1–SH2 orientation is only found in Shc adaptors, it's true that the PTB and SH2 domains, individually, are very common in signaling proteins, however, Shc proteins are the only adaptor proteins that possess both the SH2 and PTB for p-tyr recognition[28].

ShcA is classically known for its canonical pathway involving the recruitment of Grb2, which in turn forms a complex with the GTP-exchange factor SOS (Son of Sevenless). This complex converts Ras into its active GTP-bound form, thus activating c-Raf, MEK1 and finally Erk1/2 which phosphorylates numerous transcription factors involved in cell proliferation and differentiation[29].

The ShcA pathway is defined as both redundant, since it is not the only pathway leading to MAPK activation, and dominant, since the cell has a preference for this pathway over others[30,31]. It was observed in hepatocytes, as cells undergo senescence, the interaction between Shc and the Epidermal Growth Factor Receptor (EGFR) weakens, leading to a defective Erk1/2 activation and costing the cells their ability to proliferate[32].

Apart from the MAPK pathway, ShcA is known to activate the PI3K/Akt (phosphatidylinositol-3 kinase/Protein Kinase B) pathway. Also by recruiting Grb2, ShcA was found to form a complex with Gab1 (Grb2-associated binding protein 1), leading to the engagement of the p85 subunit of PI3K to the activated receptor, thus promoting this pathway[33] *(Fig 2)*.

ShcA is, to a great degree, regarded as a cytosolic adaptor, nevertheless, it has been reported in some cases of carcinomas, that p46 was found in the nucleus[34,35], also, an interaction between p52 and numerous nuclear import factors has been established[36] even though none of the ShcA isoforms contains an NLS (Nuclear Localisation Sequence) making the role of ShcA in the nucleus to this day a mystery.

ShcA is also able to integrate signals from different stimuli simultaneously, for example, during angiogenesis, a cell survival response is induced only when ShcA is activated by both the VEGF (Vascular Endothelial Growth Factor) Receptor and converging signals from the ECM through integrins[10].

Genetic alterations in Shc protein expressions can lead from embryonic lethality to a lifespan prolonged by 30%. One can only imagine the diversity of the effects produced and the molecular mechanisms involved when ShcA activity is affected.

It is still mysterious how different ligands, each binding to particular receptors, end up recruiting the same ShcA, which in turn will activate the ubiquitous MAPK pathway leading, somehow, to a precise cellular response, unique to each type of receptor activated. However, more and more factors involved in maintaining the signal identity in different contexts are beginning to come to light[37,38].

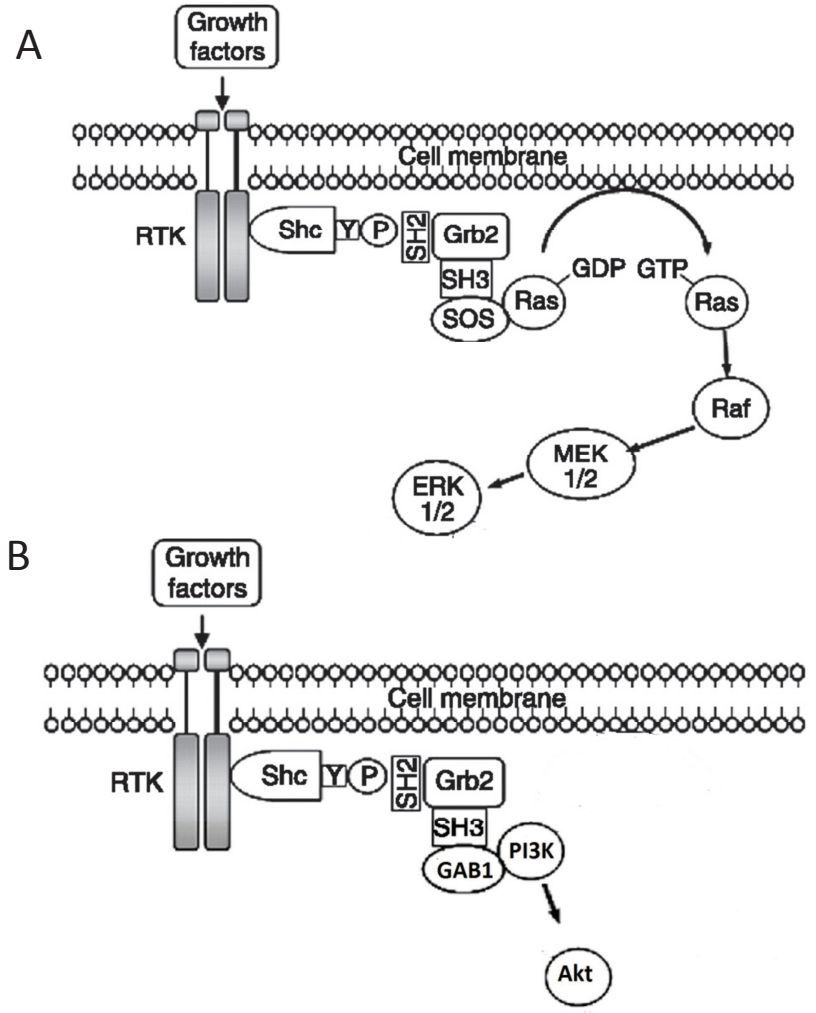


Figure 2: ShcA Signaling Cascades.
(A). Following receptor activation and recruitment of ShcA, downstream signaling leads to the induction of the MAPK pathway and activation of Erk1/2. **(B)** ShcA recruitment leads to recruitment of the PI3K and activation of Akt.

LRP1.

LRP1 or low density lipoprotein (LDL) receptor related protein 1 is a member of the LDL receptor family that includes 7 other members. Its germ-line deletion leads to lethality in mice suggesting a still undefined role for LRP1 during development[39].

All members share a common structure *(Fig 3)*:

_ An extracellular domain (of 515 kDa in size in the case of LRP1), comprised of clusters of cysteine-rich complement-type repeats (CR) or ligand-binding repeats (in the case of LRP1, there are 4 clusters), EGF repeats, and beta-propeller domains.

_ A single-pass trans-membrane domain.

_ A cytosolic domain (of 85 kDa in size for LRP1).

LRP1 can bind to a wide variety of ligands, such as lipoproteins, growth factors and ECM proteins, thus it is involved in numerous signaling pathways[9,40].

LRP1 is mainly known for its role in endocytosis of lipoproteins, proteases, leading to their degradation and the activation of lysosomal enzymes.

It can interact with cell surface integrins, thus regulating their activation, internalization and subsequent degradation[41]. Multiple growth factors are also ligands for LRP1, such as the connective tissue growth factor (CTGF), where LRP1 plays a crucial role in its hepatic clearance[42].

For LRP1, the cytosolic domain contains 2 dileucine motifs and 2 NPxY motifs. The second NpXY motif is tyrosine phosphorylated once LRP1 is activated either by direct binding to one of its ligands, or by activation by growth factor receptors. This phosphorylation is recognized by a number of adaptor proteins, including ShcA, that bind to the cytoplasmic tail of the receptor and launch a signaling cascade. Thus, LRP1 is linked to numerous pathways, the MAPK pathway, the PKC (protein kinase C) mediated pathway, regulating proliferation and differentiation, Src activation, and NO synthase activation to cite a few[9].

In different animal models of autoimmune diseases, neuroinflammatory diseases and macrophage specific LRP1-KO models, LRP1 has been attributed a role in inhibiting the NF-kB pathway that leads to the expression of inflammatory mediators such as IL-6[43,44].

Our lab has recently demonstrated the involvement of LRP1 in cholesterol metabolism by stimulating the canonical Wnt5a/b-catenin pathway[8,45].

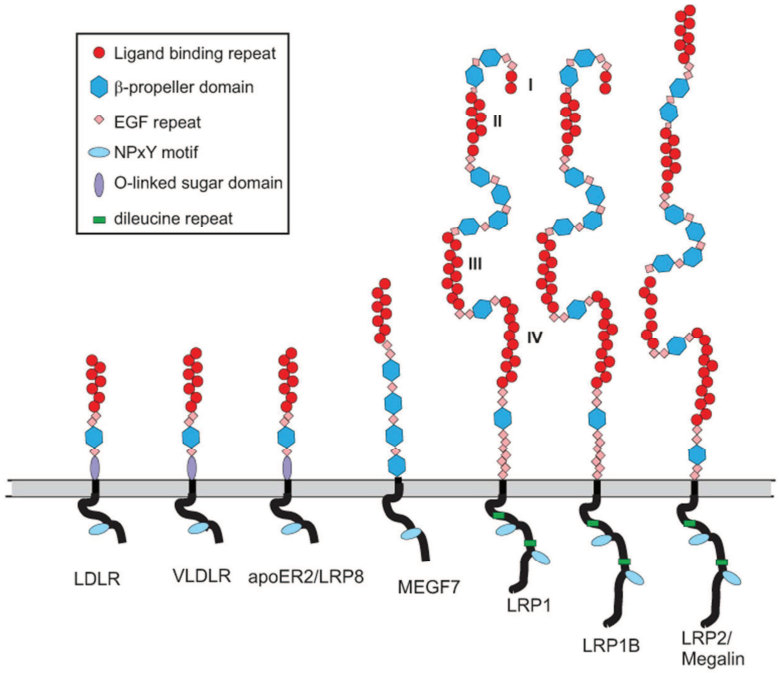


Figure 3: Illustration of the LDL receptor family members[9].

Chapter I:
Role of ShcA and LRP1 in chondrocyte differentiation.

1. Chondrogenesis during embryonic development.

The shape, size and function of the bones at the base of the skull differ greatly from those of long bones, yet they are both formed through the same process, endochondral ossification. That is why the sequence of events leading up to their formation is finely regulated at every step in order to ensure a symmetric body with paired limbs of the same length endowed with coordinated movement[47,51].

And the first checkpoint is during embryological development where the control of the size of pre-cartilage condensations formed by mesenchymal stem cells occurs[51].

The cells at the core of the condensation differentiate into chondrocytes[46] while the thin coat of cells at the periphery form the perichondrial cells. Then the chondrocytes proliferate in a rapid pace and the ones in the middle of the condensation stop dividing, become hypertrophic and induce the mineralization of their matrix. Adjacently, perichondrial cells form the bone collar.

Blood vessels attracted to the mineralized matrix bring along osteoblasts to mold the primary spongiosa (or ossification center) at this spot. A secondary ossification center takes place when chondrocytes in new locations repeat the same process. Between the primary spongiosa and the secondary ossification center, is where chondrocytes will form the **growth plates**, and at the end of the long bones is where the **articular cartilage** is formed[47] *(Fig 4)*.

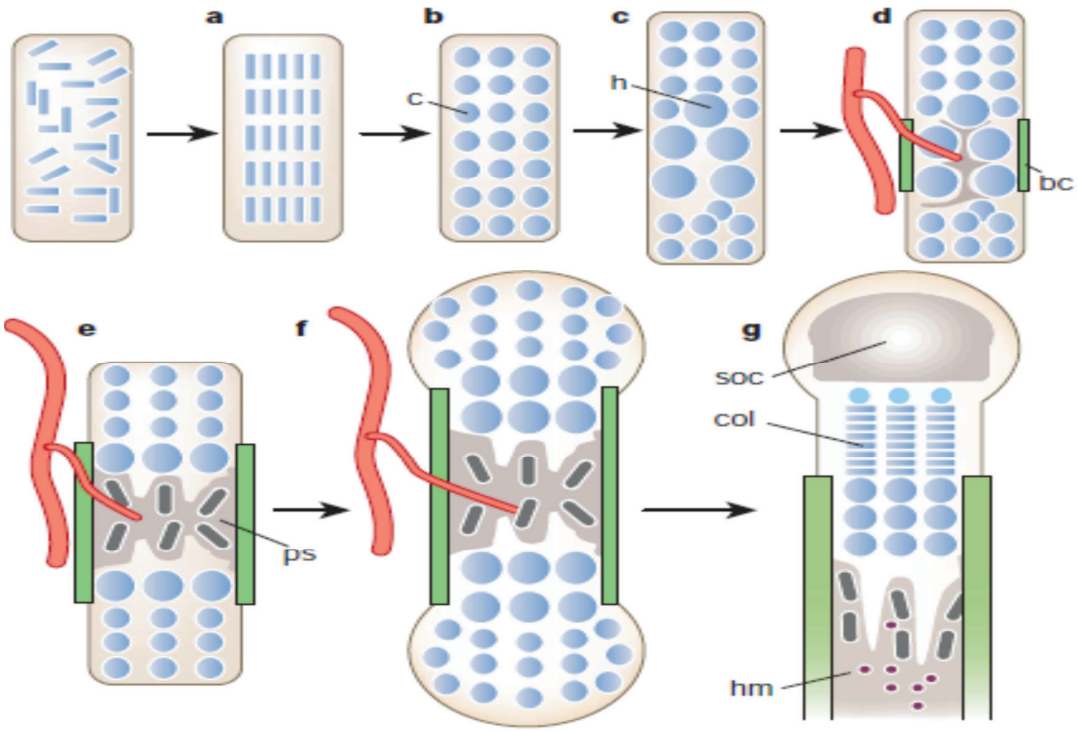


Figure 4: Endochondral bone formation.

a. Mesenchymal cells form pre-chondral condensations. **b.** Cells of condensations become chondrocytes. **c.** Chondrocytes. **h.** Hypertrophic chondrocytes at the center of the condensation. **d.** Perichondrial cells become osteoblasts and form bone collar. **bc.** Bone collar. **e.** Hypertrophic chondrocytes orchestrate matrix mineralization, attract blood vessels and undergo apoptosis, to be replaced by osteoblasts. **ps.** Primary spongiosa. **f.** Proliferating chondrocytes contribute to increasing bone length. **g.** Repetition of the same cycle of chondrocyte proliferation and differentiation leads to the formation of the secondary ossification centre **(soc)**. **col.** Column of proliferating chondrocytes forming the growth plate. **hm.** Haematopoietic marrow[47].

2. Post-natal development.

During this period, the growth plate is responsible for the linear growth of the bone[51].

2.1. Morphology of the growth plate.

The growth plate is divided in three zones, each reflecting a different state of chondrocyte differentiation *(Fig 5)*:

The resting zone, or germinal zone, is where the cells are in a relatively quiescent state and the extracellular matrix (ECM) is abundant. In this region, chondrocytes provide the stock or reserve of future proliferating chondrocytes, they are responsible for their orientation as well, thus ensuring a unidirectional growth of the bone[48].

The proliferative zone is where chondrocytes are flattened, begin to divide, and are organized into columns. Proliferating chondrocytes along with the dense ECM they produce, increase the volume of the growth plate cartilage.

The ECM of these zones is rich in collagens, predominantly Col II (Collagen type II) that forms a fibril network along with other less-expressed collagens (types IX and XI), that bind col II to the rest of the ECM. Other components include proteoglycans, mainly aggrecan, that provide osmotic resistance to compressive forces, and other non-collagenous proteins like Thrombospondin 1-3 and 5 and fibronectin[49,50,47].

Apart from paracrine/autocrine and endocrine regulation, the high proliferative activity is tightly regulated by many other factors such as circadian rhythm and age[51].

Finally, the zone of maturation is where the synthesis of ECM allows the recently divided cells to separate from each other and undergo terminal maturation or hypertrophy.

Chondrocytes leaving the proliferative pool cease to divide, become pre-hypertrophic in a zone called “transition zone”, then they undergo a 5 to10 fold increase in cell volume, as they ultimately progress to the terminal differentiation stage, or hypertrophy[52]. Their whole genetic program is altered, hypertrophy is associated with a halt in the expression of Col II and aggrecan, and a marked increase in the production of Col X (Collagen type X)[47].

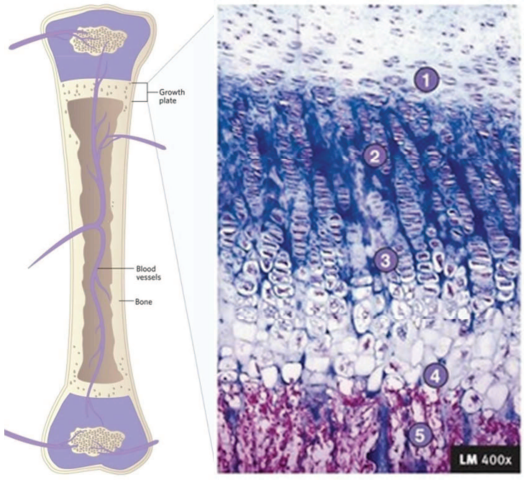


Figure 5: The growth plate.

1. Resting zone.
2. Proliferation zone. Chondrocytes are organized in columns
3. Hypertrophic zone.
4. Calcification zone. Chondrocytes undergo apoptosis.
5. Ossified bone. The calcified matrix is replaced by bone tissue[53].

2.2. From cartilage to bone: the central role of hypertrophic chondrocytes.

Hypertrophic chondrocytes orchestrate the process of bone growth and initiate the sequence of events leading to the ossification of the cartilage matrix; their enlargement is due mainly to an increase in the number of mitochondria and endoplasmic reticulum reflecting an active metabolism[54], and this increase in volume by itself contributes to the growth of the bone. Also, these cells prepare their ECM for mineralization by releasing matrix vesicles, which are particles containing significant amounts of alkaline phosphatase whose role is to increase the concentration of phosphate ions necessary to create calcium hydroxyapatite nucleation sites leading to the calcification of the matrix[55,56]. The ECM at this point undergoes drastic remodeling that necessitates the breakdown of its protein components, a process involving a wide range of proteases, also secreted by the matrix vesicles. First, several MMPs (matrix metalloproteinase) including MMP13, MMP8 and MMP14, endowed with a collagenolytic activity are able to cleave Col II and other fibrillar collagens. The resulting fragments are further cut down by MMPs with gelatinase activity such as MMP2 and MMP9[57]. In addition to other members of the MMP family, members of the ADAMTS family (A Disintegrin and Metalloproteinase with Thrombospondin Motifs)[58], namely ADAMTS1, ADAMTS4 and ADAMTS5 are able to fragment aggrecan thus increasing the rate of ECM degradation[59,60,61].

Additionally, through the expression of VEGF (Vascular endothelial growth factor), hypertrophic chondrocytes[62,63,64,65] attract blood vessels as well as chondroclasts and osteoclasts that play a role in the resorption of the hypertrophic cartilage. VEGF might also have an autocrine role on hypertrophic cells since they express its receptor (VEGFR)[62].Subsequently, these chondrocytes in the mineralized matrix undergo apoptotic cell death, and their removal leaves space for invading blood vessels and matrix-degrading osteoclasts and matrix-forming osteoblasts, that will use the mineralized ECM as scaffold to begin forming a bone matrix[66].

Any disruption or abnormality during any of the stages results in a number of conditions linked to dwarfism. For example, aberrant chondrocyte proliferation is linked to achondroplasia, mutations in the Col II gene to SED (Spondylepiphyseal dysplasia), mutations in the Col X gene are linked to the Schmid metaphyseal chondrodysplasia[51] and mice that lack the expression of MMP13 showed similar defects as the ones seen in human chondrodysplasias[67].

After puberty, or as sexual maturation approaches accompanied by skeletal maturation, fewer and fewer chondrocytes continue to proliferate, meaning fewer and fewer chondrocytes undergo hypertrophy, eventually reducing the size of the growth plate[68].
The growth plate at the final stages is completely resorbed followed by physeal closure (fusion of the epiphysis with the metaphysis). This process is under the control of estrogen in both males and females[69].

Growth plate cartilage is considered to be a temporary or transient hyaline (*hyalos,* Greek for glassy) cartilage that serves as a precursor of bone tissue during the endochondral ossification. Another type of hyaline cartilage is the articular cartilage, which in contrast is a permanent one with a unique structure and physiology, whose integrity is crucial for a functional joint[70].

3. The Articular cartilage.

Articular cartilage (AC) is a highly specialized connective tissue of the joint. It provides the articulation with a smooth, lubricated surface ranging between 2 to 4 mm in thickness that offers protection, allows movement and reduces friction. The tissue is not vascularized, not innervated, alymphatic and hypocellular thus leaving the only cells resident in the cartilage, the chondrocytes, in anaerobic conditions. These cells comprise no more than 2-5% of the total volume of the tissue, their role is to produce the dense, gel-like ECM they reside in, thus contributing to the formation, maintenance and repair of the cartilage. The cells are somewhat isolated and are rarely found to form cell-to-cell contact or communicate, however they do respond to other types of stimuli, such as growth factors and mechanical pressure. Chondrocytes have a low potential for proliferation, thus restricting the intrinsic healing capacity of cartilage in response to injury[71,72,73].

3.1. ECM composition.

The ECM consists mainly of collagens, where Col II makes up 90-95% of total collagen content composed of over 15 different collagen types (such as types I,IV,V,VI,IX and XI) that form a fibril network to confer to the AC its strength and stability[74,77]. The half-life of Col II is somewhere between 100 and 400 years[75], that is why, in normal conditions, there is little turnover of matrix collagens. Another important component are proteoglycans that consist of a protein monomer that forms the anchor on which one or more linear glycosaminoglycan chains are covalently attached. Aggrecan is the most extensively studied proteoglycan in cartilage composition given that it is the largest in size and most abundantly present. It contains more than 100 chondroitin sulfate and keratin sulfate chains, each containing maybe more than a 100 monosaccharides. Aggrecan, by interacting with hyaluronan (HA), forms large proteoglycan aggregates. Due to its global negative charge, it provides the cartilage with osmotic or water-retention properties, critical to its ability to resist compression[76,77]. Its half-life ranges between 3 to 24 years, which is why it is more frequently replaced than collagens[78].

Other, smaller proteoglycans rather interact with the collagens in the ECM, namely Col II and contribute to the formation of the fibril network, where the interactions between fibrils confer to the cartilage its tensile strength.
Last but not least, contributing to about 80% of the total weight of the cartilage is water along with the dissolved inorganic ions. Its presence and flow through the pores of the cartilage help to transport and distribute nutrients, growth factors and cytokines to chondrocytes, in addition to providing lubrication[79].

3.2. Structure of the articular cartilage.

Apart from synthesizing the ECM and regulating the metabolism of the whole tissue, the distribution of chondrocytes across the cartilage divides it to different zones *(Fig 6)*. The thin superficial zone constitutes up to 20 % of the overall thickness of the cartilage. It serves to protect the deeper zones, also, since it is the only zone in contact with the synovial fluid, it permits the diffusion of nutrients. The chondrocytes within this region are abundant and mostly flattened and the collagen fibrils are tightly packed and horizontally aligned, parallel to the articular surface[72].
The middle or transitional zone connects the superficial and deep zones and constitutes the first line of resistance to compression. It represents 40% to 60% of the total cartilage volume. In this layer, the collagen fibrils are much thicker and are organized obliquely. Also, the chondrocytes are spherical and at a lower density.
However the greatest resistance to compressive forces comes from the deep zone due to its architecture where the collagen is arranged perpendicular to the articular surface and to the fact that it contains the highest proteoglycan content and the lowest water concentration (only 65% compared to 80% in the superficial zone)[72]. The chondrocytes are typically arranged in columnar orientation. The deep zone represents the remaining 30% of articular cartilage volume[77], it forms the tidemark, the border between the AC and the calcified cartilage where there are hypertrophic chondrocytes that express Col X and alkaline phosphatase.

In this tissue, chondrocytes are required to accomplish many tasks: matrix synthesis, maintaining a metabolic homeostasis by providing a perpetual balance between

anabolic (synthesis of ECM component) and catabolic activities (synthesis of matrix degrading enzymes) and matrix degradation. These tasks are normally performed by more than one cell type in other tissues, for example, during the process of bone development, chondroclasts resorb the mineralized cartilage template, osteoclasts resorb bone tissue whereas osteoblasts deposit the bone matrix.

Any deregulation in any of the steps is likely to set off a chain reaction that will ultimately compromise the whole joint. That is why the cartilage is susceptible to many pathologies, most commonly the degenerative disease, osteoarthritis (OA).

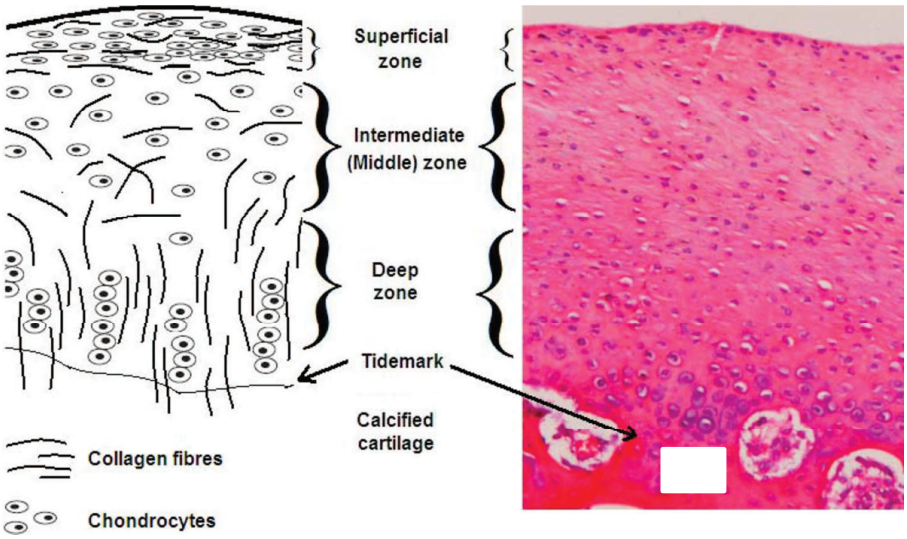


Figure 6: The articular cartilage.
Schematic representation of the structural organization of the articular cartilage from the superficial zone to the deep zone and the calcified cartilage[80] *(rabbit model)*.

4. Osteoarthritis: Relevance and Impact

OA affects over 25% of the population aged more than 45, and in 2032, at least an additional 26,000 individuals per 1 million of the population aged ≥45 years are estimated to be affected by OA[81]. Probably many others are still in stages of the disease too early to be detected by imaging techniques[82], thus earning OA the title of the most common joint disease and the leading cause of disability after cardiovascular diseases. These findings reflect a need to develop new effective OA treatments.

It is a debilitating disease, especially for the elderly and a huge economic burden. The costs are as high as 408 to 817 billion €/year in Europe[83.] Knee pain, morning stiffness and impaired mobility prevent most patients from accomplishing the simplest of usual activities[84].

Risk factors that affect the progression of OA are numerous and becoming more and more common in our modern society, they include: increasing age, hereditary predisposition, such as polymorphisms or mutations in the genes that encode for the ECM components or for signaling molecules involved in growth factor signaling, obesity, which also implies a sedentary lifestyle or physical inactivity.
In contrast, intense physical activity may also lead to OA if trauma ensues. Malalignement or instability of the joint, previous knee injury and knee surgery are considered important factors[85,86].
It was shown recently that environmental factors play an important role since the incidence of OA has doubled since the mid 20th century[87].

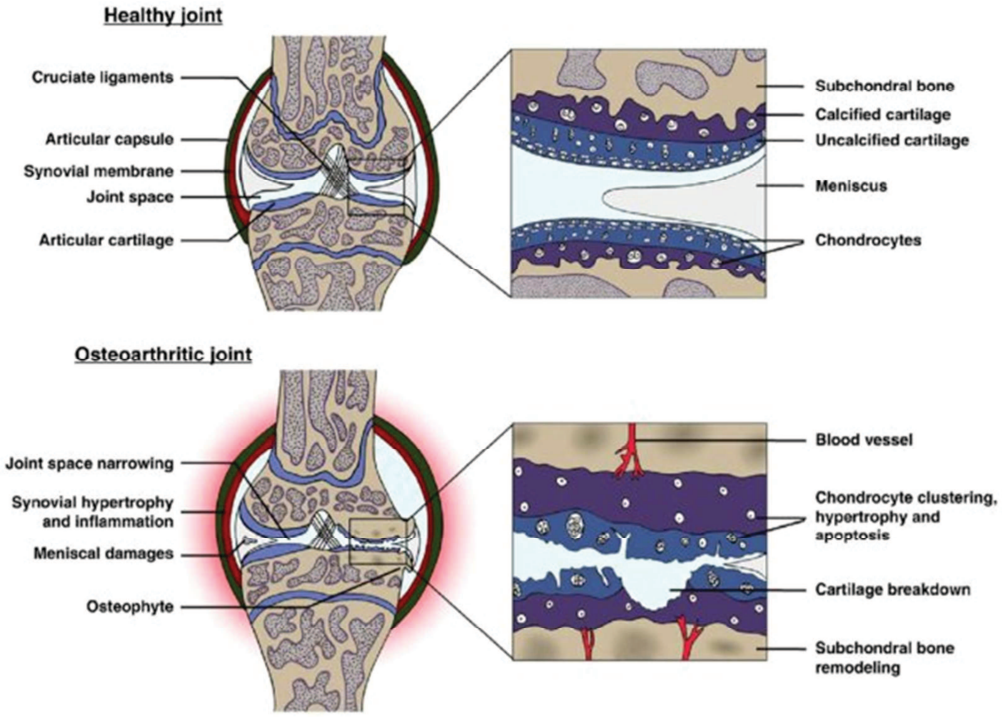


Figure 7: Normal and osteoarthritic knee. On the top, representation of a normal knee joint. On the bottom, a knee joint with OA. In the OA joint, the cartilage covering the ends of the bones is degraded and thinned, accompanied by synnovitis. Osteophytes form, and the space between the two bones becomes narrow[88].

Progression of OA and the central role of hypertrophic chondrocytes.

The joint with all its structures is affected by OA, this is why it is considered a multifactorial disease. It is mainly characterized by loss or degradation of the hyaline articular cartilage, which is, at later stages, accompanied by bony remodeling with the formation of osteophytes *(Fig 7)*, inflammation of the synovial membrane (synovitis), hypertrophy of the joint capsule and menisci and weakness of periarticular muscles[89].

Until recently OA has just been considered a “wear and tear” disease, but it has become evident now that it is much more than that. We observe abnormal remodeling of the joint tissue with ectopic bone tissue development and it is now considered a low-grade inflammatory disease. Pain is mainly due to the inflammation of the synovial membrane and it may be the first clinical manifestation of the disease, thus hindering its detection at early stages, but what really leads to disability, loss of function of the joint and an impaired quality of life is the gradual destruction of the cartilage[103].

Cartilage lesions at first occur in localized areas, which could disrupt the load transfer and put a strain on the rest of the joint, leading to the progression of cartilage loss

across the organ. At some point, the joint loses its symmetry or becomes tilted, this ensuing malalignment further aggravates the rate at which the cartilage is degraded[89,82].

Articular chondroytes, characterized by low metabolic activity and extremely limited capacity to regenerate are the only cells resident in the cartilage, they are responsible for maintaining the structural integrity of the tissue and sensing any damage done to the tissue and attempt to fix it. However, their response is not usually the most adequate.
For example, it is true that non-traumatic and constant compression is healthy and stimulates the synthesis of ECM components by chondrocytes in their resting states, but in response to traumatic injury, or simulated injurious compressions *in-vitro,* chondrocytes on the surface of cartilage become activated, proliferate and form clusters that tend to produce inflammatory cytokines and matrix-degrading enzymes (MMPs and ADAMTS) and only moderate levels of Col II and aggrecan[90,91]. This stress response is mediated mainly by integrins that act as receptors to fibronectin and Col II fragments[92,93]. This abnormal response is due to an aberrant chondrocyte phenotype evident by the presence of molecules that are not normally present in a healthy cartilage. In OA cartilage, there's presence of Col X and other hypertrophy markers (alkaline phosphatase, osteopontin, osteocalcin, Indian Hedgehog, Runx2, VEGF, HtrA1 and transglutaminase-2) involved in the formation of a non-functional "repair" cartilage also called fibrocartilage that cannot withstand strong compressive forces, indicating that the chondrocytes tend to undergo irreversible differentiation in these conditions and re-employ mechanisms that highly resemble what usually occurs in the hypertrophic zone of the growth plate and lead to cartilage degradation and bone development[94,95]. Chondrocytes, in response to trauma, tend to aggravate its severity, which is why even the tiniest lesions in the articular cartilage tend to become fully developed OA through slow but progressive stages. OA is to be considered as an active response to damage rather than a passive process of degeneration.

By studying OA mouse models, it has become evident that OA development correlates with high expression of Col X and MMP13 meaning that the disease and chondrocyte hypertrophy are interrelated phenomena. Moreover, experiments with transgenic mice

whose gene mutations induce chondroctyte hypertrophy show that these genetic alterations increase the risk of developing OA or accelerate its progression or even increase its severity[96,97,105].

It is important to note that in OA, the inflammatory environment accelerates chondrocyte hypertrophy. The presence of Interleukins and the activation of the NF-kB pathways are all factors involved in the expression of hypertrophic markers[98,99].

But in the absence of traumatic injury, it is not clearly understood what initiates chondrocyte hypertrophy. Chondrocytes do appear to show weaker anabolic activity with age, their capacity to repair and remodel the cartilage is attenuated[100,101,102]. Also, the ECM composition and structure are completely altered, there is accumulation of advanced glycation end-products which leads to an increase in the production of ROS (Reactive Oxygen Species). Aggrecan is expressed in a smaller molecular weight, and there's more col II cross-linking making the cartilage more fragile which could be a potent inducer of chondrocyte hypertrophy and favoring the balance for catabolic activities on the expense of anabolic activities[103,104]. Moreover, the ECM is a reservoir for growth factors, alterations in its structure could cause a depletion of these factors, thus altering chondrocyte biology[105].

5. Treatment options for OA.

Treatment of OA aims mainly to alleviate pain and reduce inflammation. There is no existing treatment to prevent, slow down or reverse the progression of the disease.

Current available treatment options include:

Nonsteroidal antiinflammatory drugs (NSAIDs) for pain relief through systemic administration, but due to their gastric toxicity, they should be coupled with proton pump inhibitors. Corticosteroids could be used but local corticosteroid injections are only effective for up to three weeks.

Injections of Hyaluronic Acid (HA) are successful in no more than 57% of treated patients[106].

Non-pharmacological treatments include physical therapy and exercise, weight loss to reduce the load on the joint and realignment when necessary by using shoe inserts and in some cases braces.

Finally, in the most advanced stages of OA, joint replacement with prosthetics through surgery is required. Successful surgeries still implicate a major impairment in the quality of life and the average lifespan of joint prosthesis in 10-15 years[82,107,108].
Basically, all these options are not suitable for young patients or for patients at early stages of OA where joint replacement is too drastic, and simply treating pain means condemning them to aggravated OA at some point in their lives. Luckily, there exist some surgical alternatives, although their outcomes leave much to be desired. Microfracture, autologous mosaicplasty and osteochondral plug transplantation to cite a few. The cartilage ensuing from these techniques is alas fibrocartilage and follow up surgeries are required most of the time[109,110]. ACI (Autologous Chondrocyte Implantation) emerges as the technique that will save the day.

6. Alternative Surgical Option: ACI and MSCs.

The notion that an injured cartilage cannot be fixed has survived more than 250 years, ever since William Hunter stated it in 1743[111]. Finally, we are closer than ever to proving the opposite and it is long overdue. Autologous Chondrocyte Implantation (ACI) is a cell-based therapy for OA developed by Lars Peterson and Matts Brittberg in 1987. It requires the removal of a small piece of healthy cartilage from a non-bearing zone, isolation of chondrocytes, expanding them *in-vitro* to obtain a sufficient number of cells and using them to reconstruct artificial cartilage that will be then implanted in areas affected by lesions in the hope that this neo-cartilage will restore the functionality and healthy properties of hyaline cartilage *(Fig 8)*.
Its main advantage is the low risk of rejection of the transplant and transmission of diseases. Some studies have followed up until 2 years after surgery and showed that the tissue still appeared to be healthy and expressing hyaline cartilage markers, Col II

and aggrecan, however, it also showed expression of Col I meaning it did not remain as stable as hoped for and it is expressing characteristics of fibrocartilage[112,113]. The limitations of this technique are due to the fact that the phenotype of quiescent chondrocytes is short-lived, unstable and quickly lost during monolayer expansion. This approach requires a step of monolayer amplification of chondrocytes which leads to their dedifferentiation, ultimately the reconstructed cartilage is not stable and hyaline in characteristics, but instead a more brittle, less sturdy, or in other words, non-functional fibrocartilage[114,115].

Many attempts in ACI have been made, taking this technique through 4 generations, and with each one, new improvements and new limitations appear.

The second generation involved using a collagen membrane instead of a periosteum membrane (MACT; Matrix-assisted chondrocyte transplantation) for the transplantation procedure, thus establishing the use of 3D cultures for this method instead of introducing a cell suspension[116].

Mesenchymal stem cells (MSCs) have shown more promising results. In-vitro, there was no evidence of matrix calcification or expression of hypertrophy markers by MSC-derived chondrocytes, however, matrix calcification was observed in-vivo along with vascularization. It would appear that once they undergo a chondrogenic differentiation program, these cells are fated to go through the entire developmental program until they reach ossification, with no possibility of locking them in a quiescent state for a sufficiently long period[117,118,119,120]. Much improvement is still required before considering them for clinical applications.

An important variable is the source of MSCs. The ideal source should procure large amounts of cells that could be collected in a way that is not very invasive. What we learnt so far is that bone marrow, Wharton's Jelly and umbilical cord MSCs showed the most successful chondrogensis[242,244,245].

More studies are being conducted to optimize the use of these cells for cartilage regeneration and recently, Pr. Barbero's team successfully managed to develop a human MSC-based model that refrained from progressing to bone remodeling in-vivo[121].

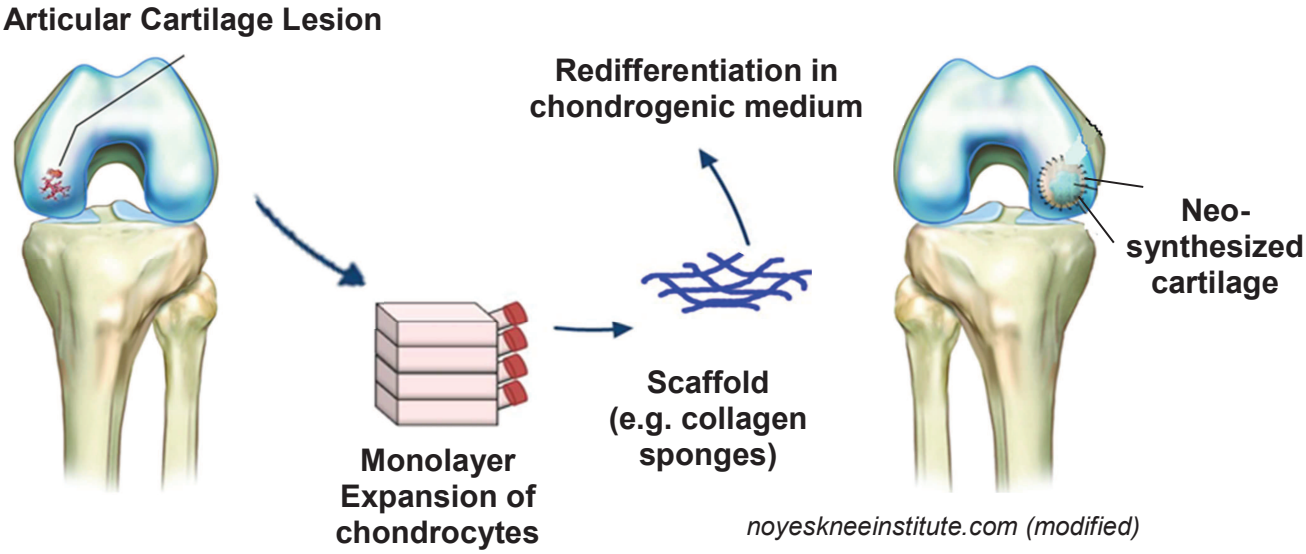


Figure 8: ACI.
Illustration of the Autologous Chondrocyte Implantation (ACI) procedure.

Controlling chondrocyte differentiation is crucial in order to obtain better outcomes in cartilage repair techniques and to better understand the physiopathology of OA. More and more studies aim to uncover the involvement of new key molecules involved.

7. Master regulators of chondrocyte proliferation and differentiation.

The factors that control chondrogenesis and chondrocyte differentiation are too many to count. A simple search on pubmed for “chondrocyte” and “differentiation” revealed more than 500 papers published in 2017 alone. I will only mention here the factors that are most-understood or at least, most-studied and how they contribute to chondrocyte biology from the early stages of mesenchymal condensation all the way to the end-stage terminal hypertrophy.

7.1. Sox9

Mesenchymal stem cells can differentiate into a variety of cell types: osteoblasts, chondrocytes, myocytes and adipocytes. As is for the other cell types, chondrogenesis

is regulated by transcription factors that control the expression of many genes, most essentially the genes encoding the proteins of the ECM.

Col II, Col IX and Col XI, collagens found in stable hyaline cartilage, are under the control of Sox9 (sex-determining region Y-type high mobility group box 9), which acts in collaboration with L-Sox5 and Sox6 to activate the transcription of these fibrillar collagens as well as aggrecan[122,123].

Some studies have also found that Sox9 may also be responsible for the expression of adhesion molecules essential for the mesenchymal condensation process, since mesenchymal stem cells that do not express Sox9 fail to differentiate into chondrocytes as seen in conditional Sox9 KO mice[124].

Transgenic Sox9-floxed mice that express the Cre recombinase under the control of the Col II promoter show a prematurely terminated proliferation of growth plate chondrocytes as well as an early conversion to hypertrophy[125].

7.2. PTHrP/Ihh

For the stages following mesenchymal condensation, a master coordinator of chondrocyte proliferation is Ihh (Indian Hedgehog), a member of the hedgehog family of secreted ligands. Ihh binds to its receptor Patched-1 (Ptc-1) which would activate Smoothened (Smo), a membrane protein which in turn is responsible for the activation of target genes[126].

Experiments with Ihh and Smo KO mice show that in the absence of either one of these two proteins, chondrocytes pre-maturely stop proliferating and undergo early hypertrophic differentiation, which was successfully salvaged by transgenic re-expression of Ihh or Smo[127,128].

The main role of Ihh in controlling chondrocyte proliferation, although the mechanisms are still poorly understood, is through the synthesis of PTHrP (parathyroid hormone-related protein), which activates the same receptors as PTH (parathyroid hormone) to keep the chondrocytes in the proliferative pool[47].

PTHrP KO mice and PTHrP-receptor KO mice as well as transgenic mice overexpressing PTHrP display a phenotype of dwarfism. In the first two cases, it is due

to prematurely induced hypertrophy, and in the third case, it is due to a delay in hypertrophy[129,130].

In fact, Ihh and PTHrP form a negative feedback loop, chondrocytes at the ends of the long bones with a low proliferation rate secrete PTHrP to keep dividing chondrocytes in a proliferative state, to delay their own hypetrophy and to suppress the expression of Ihh. In the absence of PTHrP stimulus, cells leaving the proliferative pool (pre-hypertrophic/hypertrophic cells) start to secrete Ihh in order to increase the proliferation rate of dividing chondrocytes and to stimulate the expression of PTHrP[47].

7.3. TGF-b

TGF-b (Transforming Growth Factor – beta) also induces the expression of PTHrP and by binding to its type II serine/threonine receptor, stimulates a Smad 2/3-dependant signaling pathway, that has been proven to play a pivotal role in regulating the proliferation of growth plate cells[131,132,47].

Mice that do not express Smad 3 show premature hypertrophy of both growth plate and articular chondrocytes, and a reduced longitudinal growth of their bones[133].

In-vitro, blocking Smad 3 accelerates hypertrophic differentiation[134] which is why TGF-b, is used as an effective inhibitor of hypertrophy, col X expression and alkaline phosphatase activity[135,136,137].

7.4. IGF-1

Another autocrine factor that enhances chondrocyte proliferation is IGF-I (Insulin-like Growth Factor I). To illustrate its potency, GH (Growth Hormone) increases the secretion of IGF-I in growth plates to induce cell division[138]. And GH KO mice that show a reduced bone growth can be rescued by administering IGF-I[139]. Moreover, it is able to enhance the pro-proliferation effects of TGF-b[140] thus making its way in regulating the PTHrP/Ihh feedback loop.

However, IGF1 signaling is sensitive to the intracellular environment and differentiation state of the cell, since it is also known for its role as inducer of chondrocyte hypertrophy and bone growth by activating the Wnt pathway[141]. And it has been shown to activate

either the PI3k/Akt pathway or the MAPK pathway leading to sometimes opposite effects[142,143,144].

7.5. FGFs

The FGFRs (Fibroblast Growth Factor Receptors) are made up of four tyrosine kinase receptors that can bind more than 18 members of the FGF family; FGFR3 is the most extensively studied. It can bind at least nine different ligands and it exerts its effects mainly through the transcription factor STAT1 and Erk 1/2(Signal transducer and activator of transcription 1)[145,146]. Its absence in transgenic mice leads to prolonged proliferation of growth plate cells and expanded hypertrophic zones and its over-expression results in growth arrest and subsequent apoptosis[147]. FGFR1, expressed mainly in hypertrophic cells induces its effects through the induction of Erk1/2, studies have shown that this pathway is essential for the FGF-induced hypertrophic engagement and secretion of ECM degrading enzymes[148,149].

7.6. BMPs

In-vitro, isolated chondrocytes tend to undergo hypertrophic maturation spontaneously if no inhibitory signals have been added. BMP (Bone Morphogenetic Protein) or GDF (Growth and Differentiation Factor) signaling is one of the main pathways involved since it has been shown that its inhibition prevents chondrocyte maturation[150] and the presence of BMP antagonists (noggin or chordin) *in-vivo* negatively affects the onset of hypertrophy and the expression of hypertrophy markers[151].

Different members of the family induce different effects, for example, BMP2, mostly expressed in hypertrophic chondrocytes exhibits contrary effects to BMP7 expressed in proliferating chondrocytes. This could be due to the fact that they exhibit higher affinity to different receptors[152,153].

Thyroxin (T4), converted into T3 in the cartilage, and retinoic acid, both favor chondrocyte maturation through the activation of BMP signaling by inducing Smad1 and Smad 5 to launch the transcription of Col X and alkaline phosphatase[154].

7.7. RunX2

Just as Sox9 is the most valuable transcription factor in the onset of chondrogenesis, the same can be said about RunX2 (Runt-related transcription factor 2), also known as CBF-alpha-1 (Core-binding Factor subunit alpha-1) when it comes to hypertrophic differentiation[155,156,157]. The Col X promoter contains a core RunX2 regulated element[158]. And the loss of RunX2 expression in KO mice severely delays and even blocks chondrocyte hypertrophy in a number of developing bones[159] and these mice lack all types of osteoblasts, neither endochondral nor intramembranous.

Many factors have been shown to affect RunX2 activity, for example, the effectors of BMP signaling, Smad1, Smad5 and Smad 8 are able to interact with RunX2 at the Col X promoter to induce its transcription[160].

MEF2C and MEF2D (myocyte enhancer factors) are essential in promoting chondrocyte hypertrophy by promoting Runx2 expression[161,162].

Moreover, RunX2 is a potent inducer of angiogenesis[130]. Interestingly, it has been shown that the PTHrP/PTH receptor delays hypertrophy to some extent in a RunX2 dependent manner[163].

7.8. Integrins

Integrins play a crucial role in chondrocyte biology and maintaining the integrity of the growth plate by ensuring a constant communication and anchoring of chondrocytes within their ECM[164,165]. Quiescent chondrocytes express different types of integrins than hypertrophic cells. The α5β1 integrin, overly expressed in hypertrophic chondrocytes, can bind fragments of degraded fibronectin and Col II from the ECM and subsequently induce the activation of the MAPK pathway to activate the expression of MMP13 by hypertrophic chondrocytes[166].

In osteoblasts, mechanical stress stimulates integrins which activate downstream MAPK pathways, most notably Erk1/2 through ShcA, thus regulating matrix gene expression, particularly promoting catabolic signaling leading to matrix degradation[167,168].

Defining a role for integrins however can be a challenge. Most studies conducted have only been *in-vitro*, because *in-vivo* disruption of integrin expression, could lead to severe pathological effects on the tissue due to altered cell-matrix interactions.

7.9. MAPK and PI3K/Akt pathways

Two prominent and ubiquitous pathways involved in chondrocyte maturation are the MAPK (Mitogen-activated Protein Kinase) especially the Erk1/2 pathway (Extracellular signal-regulated kinase) and PI3k/Akt pathways (phosphoinositide 3-kinase)[169,170].

Studies of PI3k signaling show that it plays a role in both chondrocyte proliferation as well as hypertrophic differentiation. For example, treatment with its inhibitor blocked the proliferative effects of PTH and IGF1[171,172] but also showed a reduction in the size of hypertrophic zones. Both these pathways need to be induced in order for chondrocytes activated by Osteopontin to express VEGF[173].

Moreover, results with transgenic mice showed that the expression of a constitutively active form of Akt in the cartilage induced hypertrophy and the expression of a dominant negative form showed the opposite effect[174].

Erk1/2 has been shown to play an important role even in the early stages of chondrogenesis. Mesenchymal cells cultured in a micromass model and treated with MEK1 inhibitors (PD98059 or U0126) exhibited an increase in the expression of Col II and proteoglycans, as well as Sox9[175].

It has also been proven that Erk1/2 functions downstream of activated FGFRs. In fact, FGF treatment of mesenchymal micromass cultures inhibits chondrogenesis, and the reversal of this effect is possible either by blocking the FGFRs, or by blocking the MAPK pathway[176]. Similarly, Erk1/2 activation after EGF (epidermal growth factor) treatment, shows the exact same effects[177].

Actually, Erk1/2, shown to be uniquely expressed in the hypertrophic zone of tibial growth plates[178], is indispensable for the action of multiple cytokines to induce the expression of matrix degrading enzymes[179]. Moreover, a recent study showed that the absence of Erk1/2 in hypertrophic chondrocytes of transgenic mice led to an impaired terminal differentiation[180].

8. Preliminary Results

ShcA is recruited by a multitude of regulators of chondrocyte biology, such as growth factor receptors (PDGF (platelet-derived growth factor), IGF1, TGF-b) and integrins. It is also an upstream activator of at least two pathways crucial for chondrocyte proliferation and maturation (MAPK and PI3k/Akt)[26,29,33,38].

Also, LRP1, once activated, recruits ShcA to it its cytoplasmic tail. In these conditions, our lab showed that the LRP1/ShcA axis is in fact responsible for deciding cellular fate between proliferation or differentiation by regulating the IGF-1R-induced signaling[181].

Our lab has also shown that the deletion of PPAR (Peroxisome Proliferator-activated Receptor) gamma in smooth muscle cells (SMCs) promotes vascular calcification and the emergence of chondrocyte-like cells in the vascular wall. In fact, PPAR gamma is a potent inducer of adipogenesis, and this study demonstrated that it is capable of inhibiting chondrogenesis by blocking the pro-chondrogenic LRP1/Wnt pathway[8].

This is why our lab was interested in studying the role of LRP1 and its partner ShcA in the process of chondrocyte differentiation and our hypothesis is that an LRP1/ShcA axis is involved, making them both potential target genes for cartilage repair.

To directly test the role of ShcA in chondrocyte differentiation *in-vivo*, the host laboratory used the Cre-lox system to generate conditional mice that do not express ShcA (ShcA KO) in chondrocytes. ShcA was specifically deleted in chondrocytes by mating mice expressing the Cre recombinase under the control of the Twist2 promoter (Jax Laboratories) with ShcA floxed mice (generated by Dr. R. Matz). Twist2 is expressed in mesenchymal condensations giving rise to the chondrocyte and osteoblast lineage.

Pr. Andreas Niemeier's team (Institute of molecular and cellular biology, Eppendorf University Hospital, Hamburg-Germany) generated conditional LRP1 KO mice. They used the RunX2 promoter as a driver for Cre expression, leading to a knockout of LRP1 in hypertrophic chondrocytes and osteoblasts, at a later stage compared to Twist2-driven Cre recombination *(Fig12)*.

1. ShcA KO mice exhibit a dwarfism phenotype.

Mice that lack cartilage-specific ShcA (ShcA KO) exhibit a dwarfism phenotype with increased cartilage-to-bone ratio. They also show reduced longitudinal growth of their long bones.

These observations suggest a disruption in the bone formation process *(Fig 9)*.

Figure 9: ShcA KO mice exhibit dwarfism compared to wild type mice (WT).
[A]. Alcian blue (cartilage in blue) and Alizarin Red (bone in pink) stainings demonstrate increased cartilage-to-bone ratio in ShcA KO mice (right) **[B]** and reduced longitudinal growth of long bones (right) **[C]**.

2. The articular cartilage of ShcA KO mice shows weaker Col X expression.

1-year old ShcA KO mice exhibit a thick AC as seen with the Col II staining, that contains fewer hypertrophic chondrocytes, accompanied by decreased Col X staining compared to WT mice *(Fig10)*.

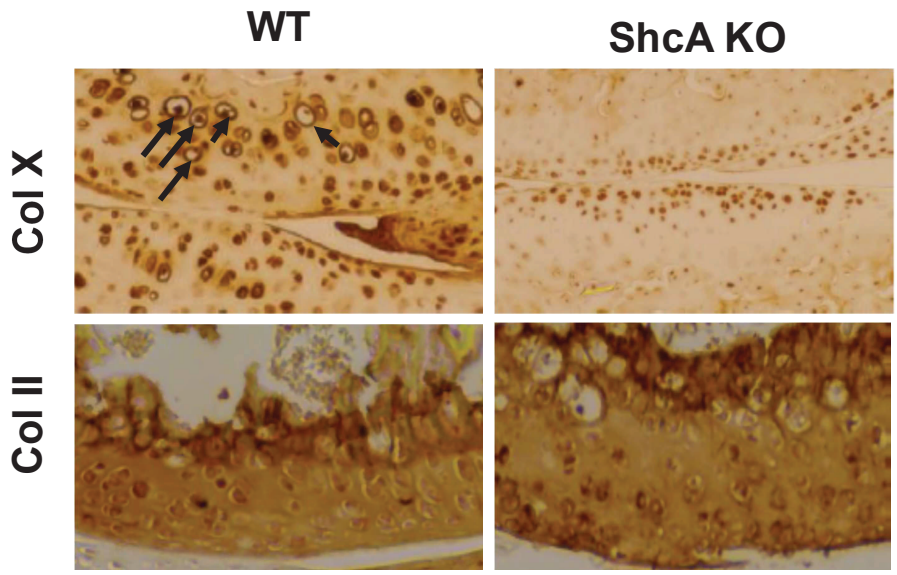


Figure 10: Immunohistological analysis of the articular cartilage of one year old WT and ShcA KO mice.

Staining with Col II and Col X. ShcA KO mice show a decreased Col X staining and a reduced number of enlarged hypertrophic chondrocytes associated with a thicker AC. Black arrows represent enlarged hypertrophic chondrocytes.

3. LRP1 KO mice exhibit a dwarfism phenotype.

LRP1 KO mice exhibit a dwarfism phenotype similar to the one seen in ShcA KO mice *(Fig 11)*.

Figure 11: LRP1 KO mice exhibit dwarfism compared to wild type mice (WT).

These results suggested a role for ShcA and LRP1 in regulating chondrocyte hypertrophy and oriented my project in that direction.

9. Objectives of Chapter I:

During my thesis, we aimed to:

1. Characterize the phenotype of ShcA KO mice and validate the implication of ShcA in chondrocyte hypertrophy.
2. Explore the signaling pathways regulated by ShcA that control chondrocyte hypertrophy and the expression of Col X.
3. Characterize the physiopathology of age-induced OA in Twist2 ShcA KO mice.
4. Develop an engineered cartilage using ShcA KO primary chondrocytes and test its chondrogenic capacity as well as its capacity to undergo hypertrophic differentiation.
5. Characterize LRP1 KO mice and and establish the role of LRP1 in chondrocyte differentiation.

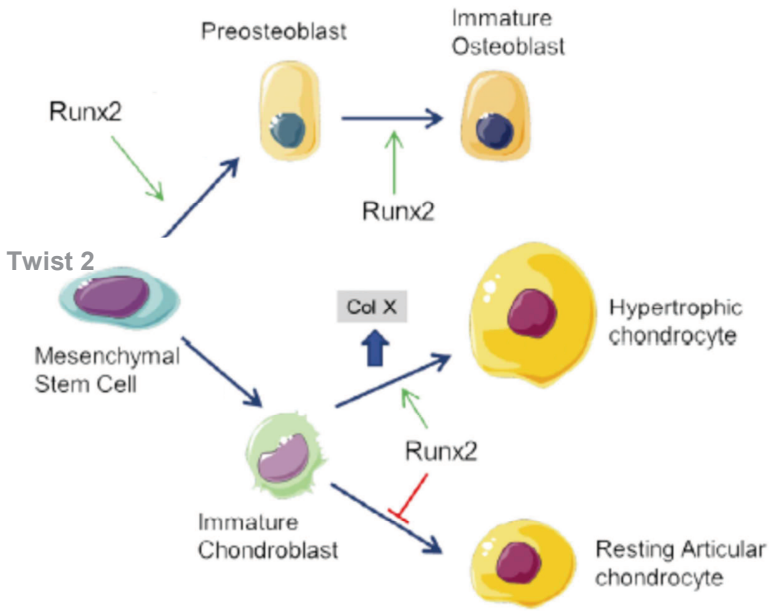


Figure 12: Schematic representation of the differential activation of Twist 2 and RunX2 during the differentiation of mesenchymal stem cells. (*Modified*[182]).

Results

Part 1: Characterization of the phenotype of ShcA KO mice and validation of the implication of ShcA in chondrocyte hypertrophy.

1. Validation of the knockout of the ShcA gene through genotyping.

The Cre-induced recombination leads to the excision of the 2kb region between exons 1 and 8, encompassing the region coding for the PTB domain of ShcA. The ensuing shortened sequence is thus detectable through PCR amplification.
The Cre recombinase sequence was also amplified through PCR as another way of verification of the genotype *(Fig13)*.

Figure 13: Determining the genotype of the mice.

- **A.** PCR amplification to detect whether the ShcA gene has been excised. A positive band reflects a KO genotype.
- **B.** PCR amplification to detect the presence of the Cre recombinase gene. A positive band reflects a KO genotype.
  H2O: Negative Control.

2. The dwarfism of ShcA KO mice is associated with a smaller growth plate hypertrophic zone.

To further characterize the dwarfism phenotype, we studied the chondrocyte hypertrophic differentiation in the growth plate. We observed a decreased size of the hypertrophic zone (WT vs ShcA KO: 61.14 ± 7,610 µm2 vs 31.54 ± 5.205 µm2) and a disorganized proliferation zone *(Fig 14)*. Through immunohistological analysis on growth

plate sections, we investigated the differential expression of both hypertrophic and quiescence markers in KO and WT mice *(Fig 15)*. Col II was markedly increased in the growth plate of KO mice. Col X expression however was much fainter in the zone characterized as hypertrophic and marking clearly showed fewer stained cells, reflecting an abnormal endochondral ossification process.

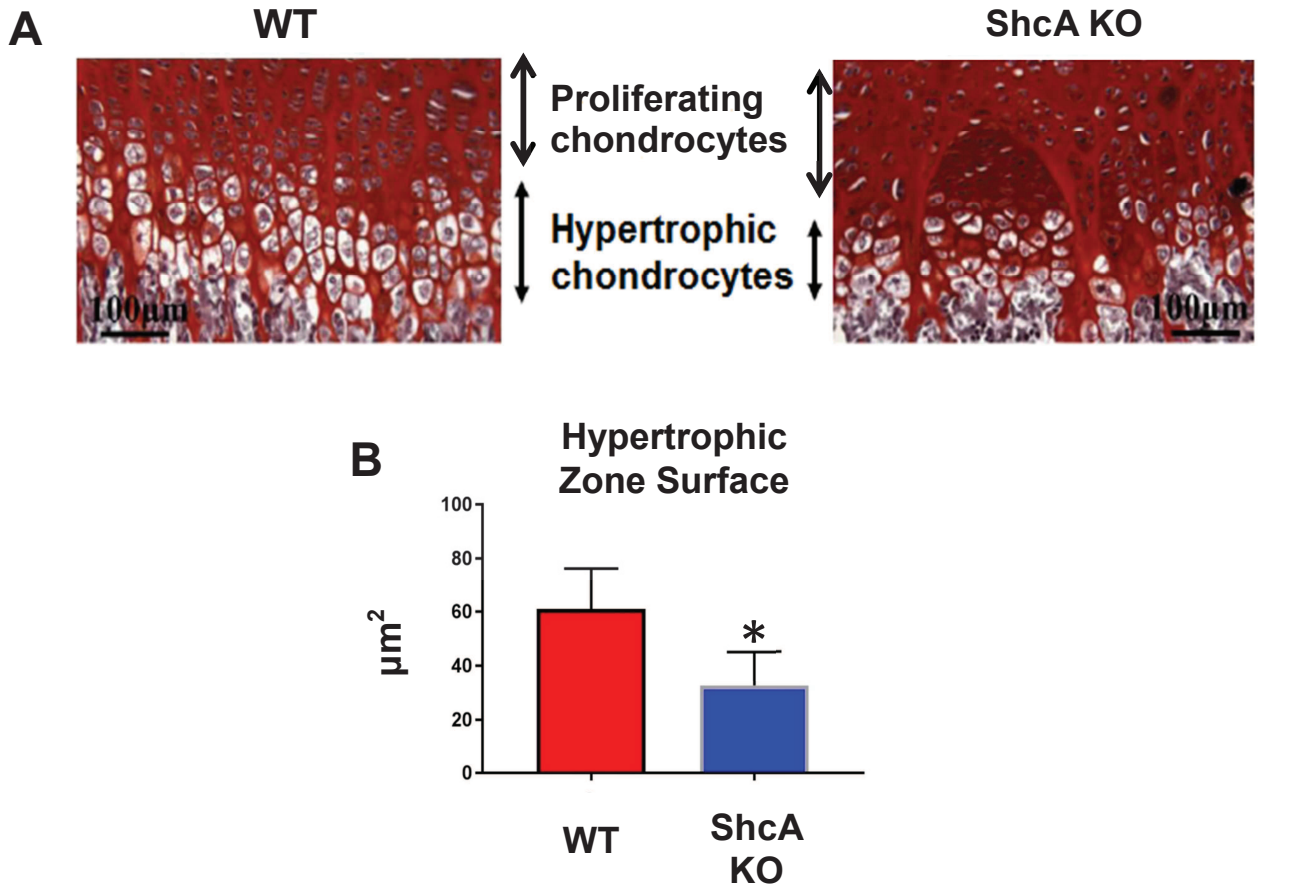


Figure 14: Safranin O staining of tibial growth plate of WT and ShcA KO mice shows altered hypertrophic maturation of chondrocytes and a disorganized proliferation zone in the absence of ShcA **[A]**. Measurements of the hypertrophic zone show a decrease in the surface of the zone: 61.14 ± 7,610 µm2 in WT mice vs 31.54 ± 5.205 µm2 in ShcA KO mice (n=4) **[B]**. Error bars, s.e.m. *$P < 0.05$.

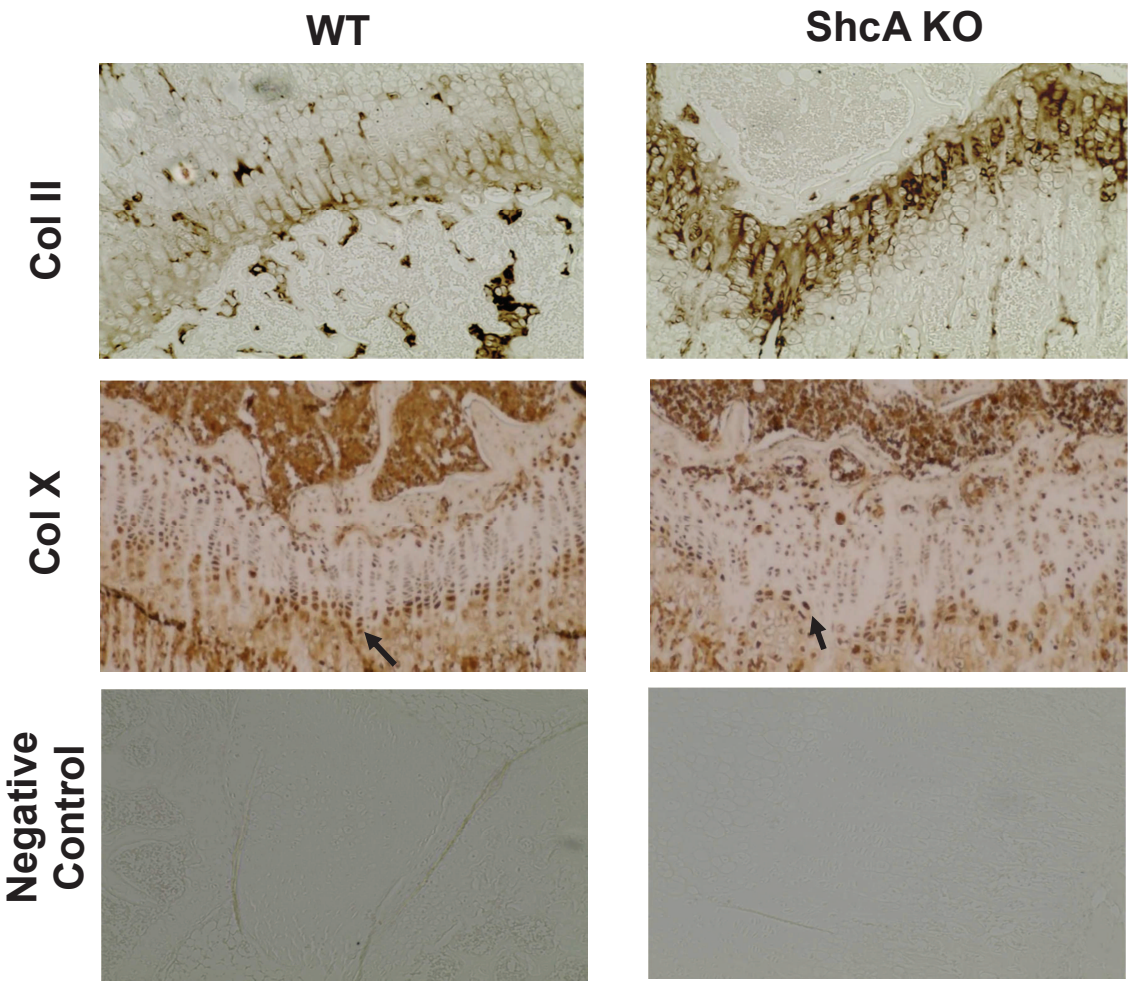


Figure 15: Immunohistological analysis of growth plate sections from one-month-old WT and ShcA KO mice.
ShcA KO mice show an increased Col II staining, associated with decreased Col X staining and a reduced number of marked hypertrophic chondrocytes (n=5). Black arrows represent Col X-positive hypertrophic chondrocytes.

These results demonstrate an *in-vivo* tendency to restrict hypertrophy in the absence of ShcA. We next wanted to validate a cell model that could mimic what we observed *in-vivo* and allows us to explore the molecular mechanism at play behind the phenotype.

3. Primary chondrocytes refrain from undergoing hypertrophy in the absence of ShcA.

Freshly isolated chondrocytes from WT and ShcA KO mice were cultured in monolayers[249]. Monolayer cultures are reported to lead to cellular dedifferentiation associated with an increase in Col I expression and a decrease in the expression of cartilage-specific markers such as Col II and aggrecan. Also, the cells lose their round or cubic shapes and adopt a polymorphic, fibroblast-like form along with an increase in cell volume as passages progress[183,184].

Col X expression is observed when cells are cultured in monolayers[185],[186], and studies done on human articular chondrocytes show that this expression is later down-regulated when these cells are re-differentiated back to their chondrocyte phenotype using 3D culture models[187].

We examined cultured chondrocytes and analyzed the expression of chondrogenic markers (Col II) and hypertrophy markers (Col X, ALP1, RunX2 and MMP13) both at the mRNA and protein levels *(Fig 16,17)*.

We observed that as the cells undergo dedifferentiation, chondrocytes isolated from ShcA-KO mice showed an 80% higher expression of Col II at the protein level even after up to 6 passages compared to cells from WT mice (WT vs ShcA KO: 1 vs 1.8±0.1873. p=0.0028. n=5). This accompanied a significantly lower expression of the following hypertrophy markers: a 40% decrease in Col X expression at the protein level (WT vs ShcA KO: 1 vs 0.511±0.026. p<0.0001. n=11), a 60% decrease in ALP1 expression at the mRNA level (WT vs ShcA KO: 1 vs 0.43±0.112. p=0.0106. n=3) and a 25% decrease in RunX2 expression (WT vs ShcA KO: 1 vs 0.77±0.05. p=0.0299. n=3). Western Blot analysis showed that there was no significant difference in the expression of MMP13 between WT and ShcA KO cells (WT vs ShcA KO: 1 vs 1.191±0.236. p=0.4483. n=4).

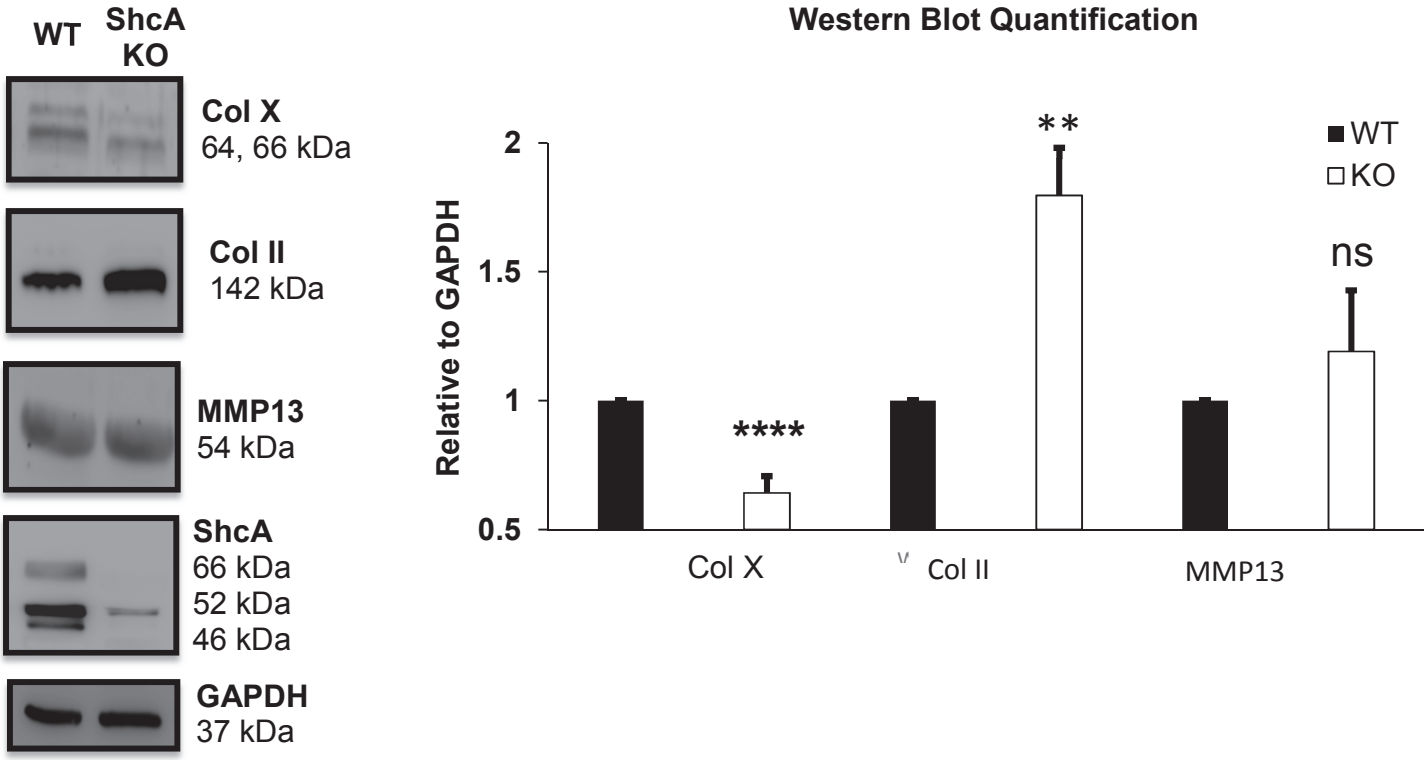


Figure 16: Western Blot analysis of WT ShcA KO primary chondrocytes.

Primary chondrocytes express higher levels of the stability marker Col II and lower levels of hypertrophy markers in the absence of ShcA. Western Blot analysis and quantification of the expression of collagen II (n=5), collagen X (n=11), MMP13 (n=4), ShcA and GAPDH in ShcA KO and WT chondrocytes.

Error bars, s.e.m. **P<0.01, ns = non-significant, (****) p<0.0001.

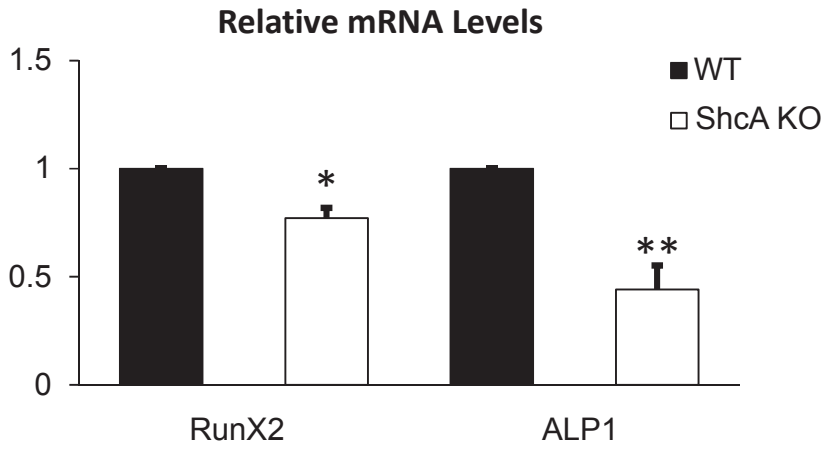


Figure 17: Gene expression analysis of primary WT and ShcA KO chondrocytes.
Primary chondrocytes express lower levels of hypertrophy markers in the absence of ShcA. RT–PCR of the indicated genes in ShcA KO and WT primary chondrocytes (n=3)
Error bars, s.e.m. *$P < 0.05$ **P<0.01.

Protein expression of MMP13 may not completely reflect the presence of matrix-degrading activity, that is why we next sought to assess gelatinase activity.

During the process of cartilage remodeling, a whole set of enzymes come into play, from metalloproteinases with collagenase or gelatinase activity, to aggrecanases from the ADAMTS family. We used gelatin-gel zymography to measure the activity of various ECM-degrading enzymes *(Fig 18)*. Primary chondrocytes were cultured in monolayer throughout different passages, and since most of these enzymes are secreted, we used the culture media supernatant to assess total gelatinase activity. We observed several bands corresponding to different gelatinases, one band in particular at around 55-65 kDa exhibited significantly lower activity in ShcA KO-derived cells (WT vs ShcA KO: 1 vs 0.6±0.106. p=0.0068. n=5).

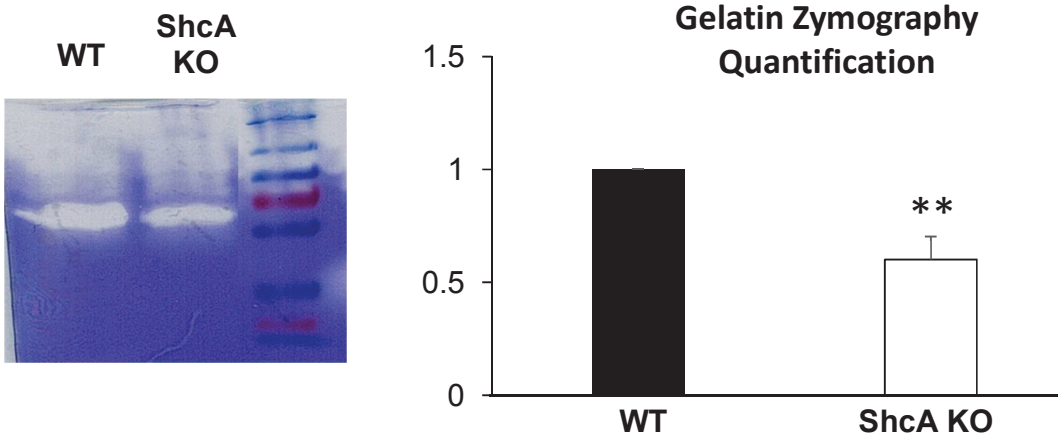


Figure 18: Gelatin-gel zymography.
Primary chondrocytes from ShcA KO mice exhibit weaker gelatinase activity. Gelatin-gel zymography analysis and quantification (n=5).
Error bars, s.e.m. **P<0.01.

These results establish a role for ShcA in the chondrocyte hypertrophic differentiation since ShcA depleted primary chondrocytes conserve markers of a stable chondrocyte phenotype in conditions favoring dedifferentiation and they have a weaker tendency to undergo hypertrophy.

4. ShcA is downregulated in pro-chondrogenic culture conditions.

Primary chondrocytes grow too slowly, and we need a large number of mice to have a sufficient number of cells to conduct experiments. These are some of the technical reasons why we used the ATDC5 cell line for some experiments.

The ATDC5 cell line derived from mouse teratocarcinoma cells has been characterized as a chondroprogenitor cell line that goes through each of the chondrogenic differentiation steps observed during endochondral bone formation[188]. We cultured these cells either in their maintenance medium without supplementary differentiation factors, or in a pro-chondrogenic medium or in a pro-chondrogenic medium for 1 week followed by a hypertrophy-inducing medium for up to 6 weeks[189]. We observed that ShcA is downregulated when these cells are cultured in a chondrogenic medium and re-expressed when these cells undergo forced hypertrophic differentiation *(Fig 19)*

suggesting an active role for this gene in the transition from a stable chondrocyte phenotype to a hypertrophic phenotype (n=3).

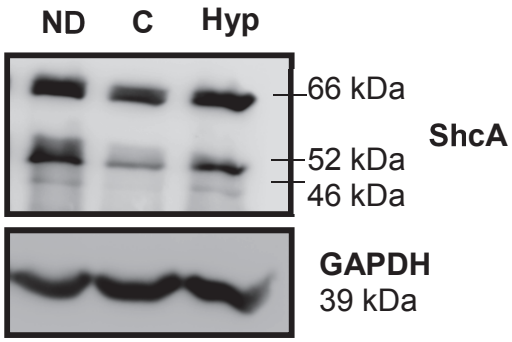


Figure 19: Western blot analysis of differentiated ATDC5 cells.

ATDC5 cells cultured in chondrogenic medium conditions tend to downregulate the expression of ShcA. They re-express ShcA when they are cultured in chondrogenic conditions followed by culture in hypertrophic condition. Western Blot analysis (n=3).
ND: Non-differentiated ATDC5 cells. C: ATDC5 cultured in chondrogenic conditions for up to 6 weeks. Hyp: ATDC5 cultured in chondrogenic conditions for 1 week, followed by up to 6 weeks of culture in hypertrophic conditions.

Next, we sought to determine the downstream signaling effectors, whose function was altered by the absence of ShcA.

Part II: Identification of the signaling pathways regulated by ShcA that control chondrocyte hypertrophy and the expression of Col X.

Col X is expressed in the hypertrophic cartilage[190], cells begin to express it as they transform from pre-hypertrophic to hypertrophic chondrocytes and maintain its expression throughout their differentiated state. Many studies focus on understanding the mechanisms controlling the expression of Col X as a way to peer into the process of differentiation.

1. ShcA induces RunX2 nuclear translocation in a MAPK-Erk1/2 dependent manner

RunX2, as previously discussed, is a crucial regulator of chondrocyte hypertrophy and a potent activator of Col X expression, as well as the expression of other hypertrophy markers like MMP13 and VEGF[156,191,192]. RunX2 mRNA expression showed a slight decrease in the absence of ShcA, however as it had been reported, its activity could be assessed by its nuclear translocation[193]. We conducted cell fractionation and immunofluorescence assays on isolated primary chondrocytes *(Fig 20)*. We observed that RunX2's nuclear expression was reduced in the absence of ShcA (WT vs KO: 1 vs 0.45±0.125. p=0.0023. n=5) and it showed more of a cytoplasmic distribution across the cell.

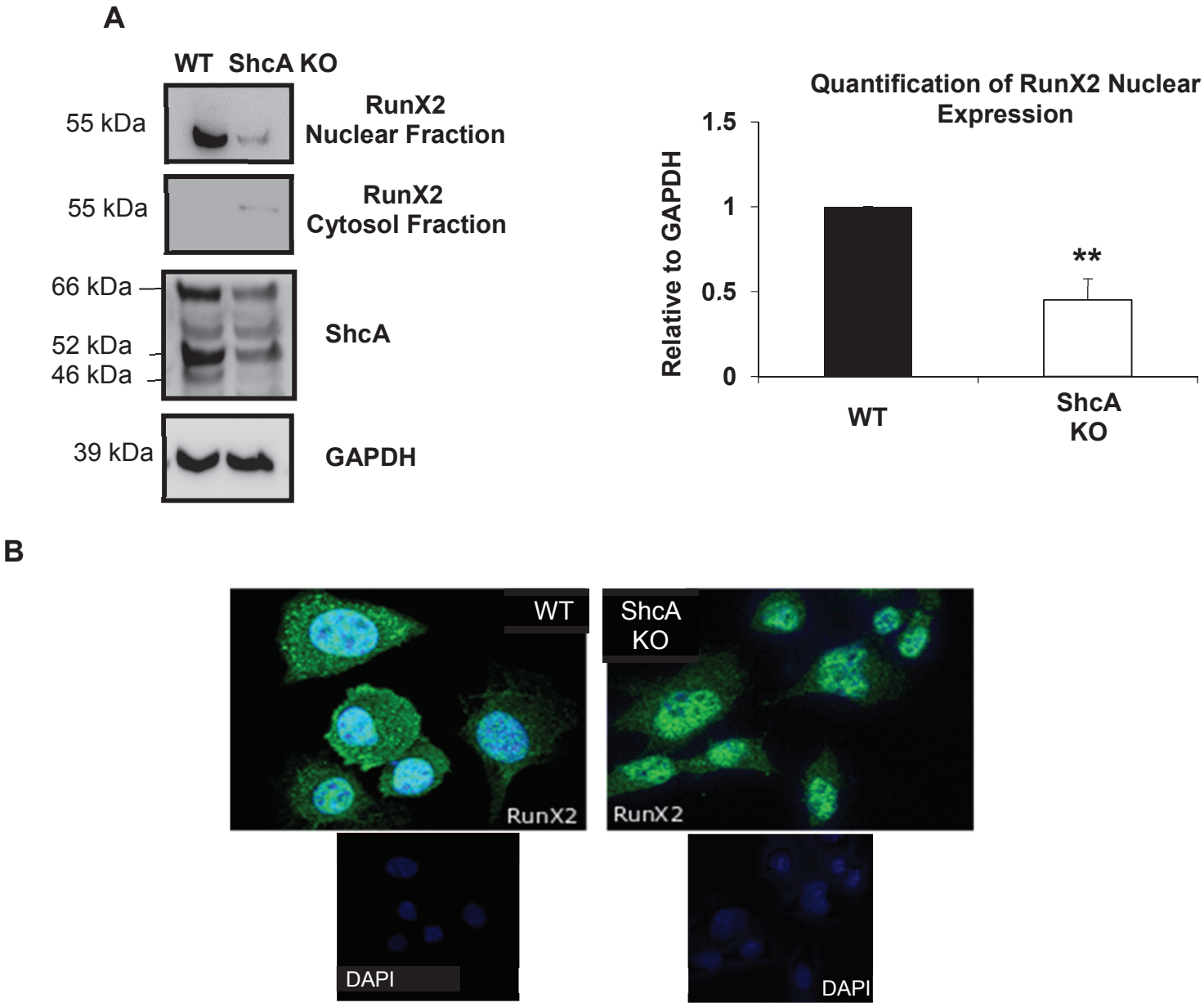


Figure 20: ShcA-deficient primary chondrocytes express weaker RunX2 nuclear localization.

Cell fractionation analysis for RunX2 on the left. Quantification of levels of RunX2 nuclear expression (n=5) **[A].** Immunofluorescence assay on primary chondrocytes showing a strong nuclear RunX2 signal in WT cells vs a dispersed cytoplasmic expression in KO cells **[B]**. Error bars, s.e.m. **P<0.01.

RunX2 activation and nuclear translocation ultimately lead to its binding to the Col X promoter and the activation of Col X transcription[194]. We next sought to identify the actors recruited by ShcA and working upstream of RunX2 leading to its activation. Our first candidate was Erk1/2, it is after all the most documented effector of ShcA signaling. Moreover, it is able to phosphorylate RunX2 on two serine residues (S301 and 319) which results in the up-regulation of the DNA-binding and transactivation potential of RunX2[195,196,197]. Erk1/2 is also involved in the acetylation and subsequent stabilization of RunX2[198,199].

It has been previously reported that in MEF cells (Mouse Embryonic Fibroblast), the deletion of ShcA did not affect MAPK activation post EGFR stimulation, implying that the cells can take an alternative route towards MAPK activation, a route that is independent of ShcA[18]. So we decided to investigate if Erk1/2 activation is altered in isolated primary chondrocytes in the absence of ShcA *(Fig 21)*. What we saw is a significant decrease of 50% (WT vs KO: 1 vs 0.509±0.0559. p<0.0095. n=14) in Erk1/2 phosphorylation in the absence of ShcA suggesting an imperative role for ShcA in the induction of the MAPK pathway.

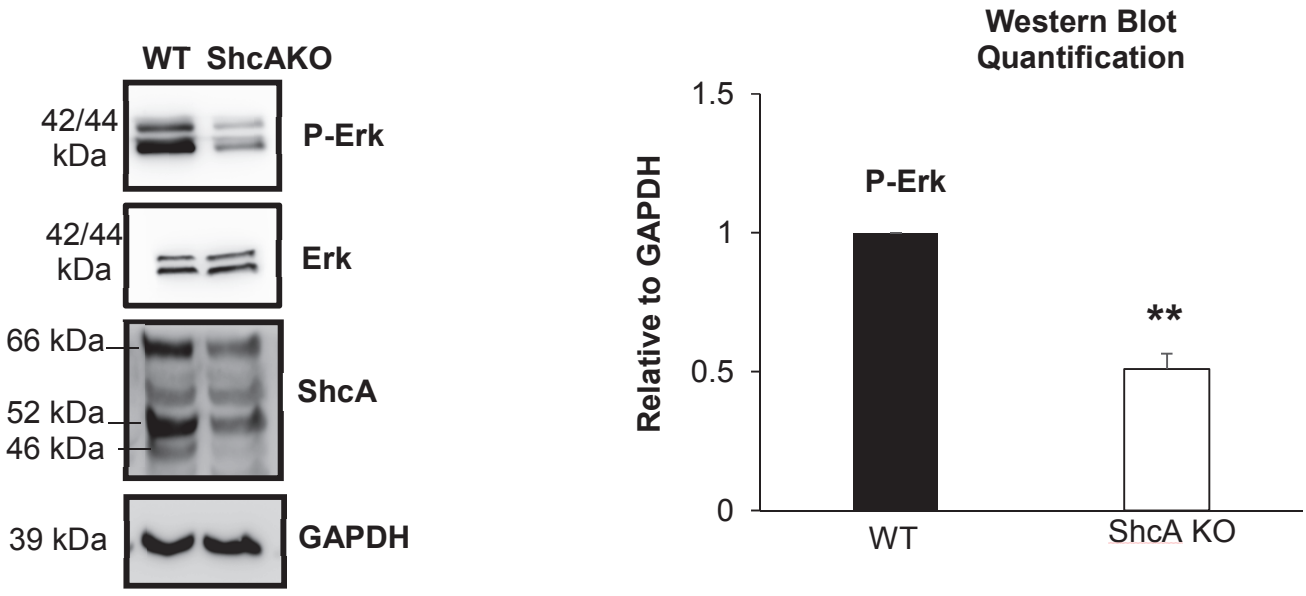


Figure 21: ShcA-deficient primary chondrocytes express less p-Erk.
Western Blot analysis and for p-Erk on the left. Quantification of levels of p-Erk in whole cell lysats (n=5). Error bars, s.e.m. (**) p<0.01.

Furthermore, after treating the cells with the MEK1 inhibitor PD98059 (50µM), thus inhibiting Erk1/2 activation *(Fig 22)*, RunX2 nuclear translocation was drastically diminished in WT cells (Treated vs Non-treated: 1 vs 0.23±0.0379. p=0.0004. n=3) confirming the need for the MAPK pathway to induce RunX2 nuclear translocation in our cell model.

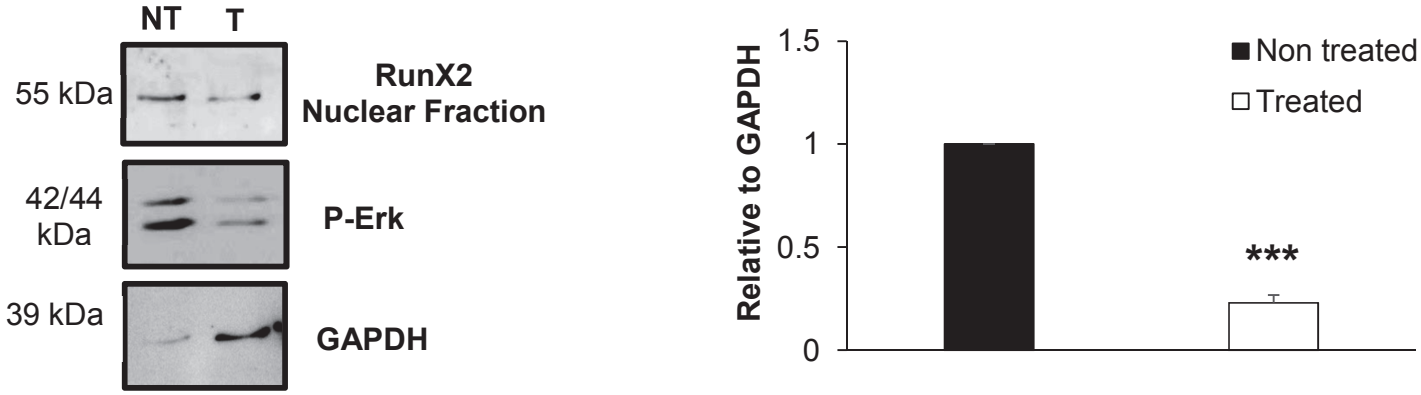


Figure 22: Primary chondrocytes express weaker RunX2 nuclear localization following treatment with 50µM PD98059.

Cell fractionation assay on WT chondrocytes on the left. Quantification of RunX2 nuclear expression on the right. NT: Non treated WT cells, T: WT cells treated with 50µM PD98059 (n=3). Error bars, s.e.m. (**) p<0.001.

These results validate that ShcA, through the Mek1-Erk1/2 pathway, induces the nuclear translocation of RunX2, most probably by an Erk1/2 activating phosphorylation.

However, RunX2 activity is not only regulated by post-translational modifications or its cellular localization. Many transcription factors can act as either transcription co-activators or repressors once they bind to RunX2 on the Col X promoter. We investigated two of these factors in the following.

2. ShcA affects the Hippo pathway by regulating the cellular localization of its effector, YAP1.

It's not until recently that numerous studies have started to shed the light on the role of the Hippo pathway in regulating both chondrogenic and hypertrophic differentiation.
First discovered in Drosophila more than 20 years ago, it has been attributed central roles in controlling cell fate[200]. Not unlike most pathways, the main components consist of protein kinases, transcription factors and DNA-binding proteins. However, this list keeps on expanding and adding more complexity to the Hippo signaling network which includes now more than 30 components.
Classically, the kinase cascade leads to the phosphorylation and activation of the large tumor suppressor 1/2 (LATS1/2) responsible for restricting the activity of the two transcription co-activators YAP1 (Yes-Associated Protein 1) and TAZ (Tafazzin). When the Hippo pathway is not induced, YAP1 is found in the nucleus in its active form. But once the pathway is initiated, LATS1/2 phosphorylates YAP1 on its Ser127 residue leading to its inactivation, nuclear export, cytoplasmic retention and eventually triggering its proteosomal degradation[201].

We wanted to study how the Hippo pathway was involved in our cellular models. When we cultured ATDC5 cells in pro-chondrogenic vs hypertrophic conditions, we observed a higher expression of inactive, phosphorylated YAP1 in hypertrophic conditions *(Fig 23)*, suggesting that YAP1 activity is down-regulated for a proper induction of hypertrophy.

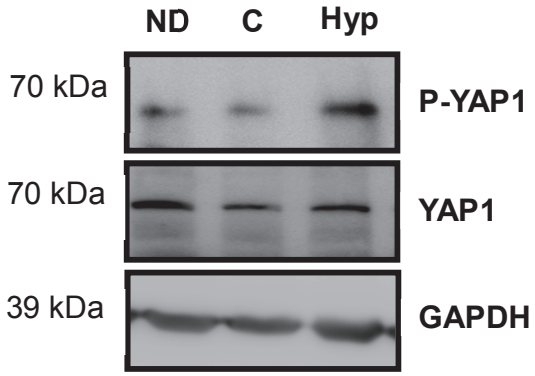


Figure 23: YAP1 activity is down-regulated in hypertrophic conditions.
Western Blot analysis on differentiated ATDC5 cells and quantification showing YAP1 is found in its inactive cytoplasmic form during hypertrophy. ND: Non-Differentiated, C: Chondrogenic differentiation, Hyp: Hypertrophic differentiation. (n=3).

In fact, several studies have found that YAP1, via its WW domain, can bind to RunX2 and inactivate it, thus leading to a decrease in Col X expression[202,203,204].We next wanted to investigate whether ShcA had any effect on the Hippo pathway and its RunX2-regulating activities. We observed in both primary chondrocytes and in ATDC5 cells treated with either scramble siRNAs, or siRNAs targeting ShcA, a higher expression of YAP1 in its inactive, phosphorylated cytoplasmic form when ShcA is present (WT vs KO: 1 vs 0.54±0.13. p=0.0065. n=5). We also observed an increase in the nuclear translocation of YAP1 in the absence of ShcA, suggesting a role for ShcA in the inactivation and cytoplasmic retention of YAP1 *(Fig 24)* (SiCtrl vs SiShcA: 1 vs 2.008±0.3108. p=0.0183. n=3).

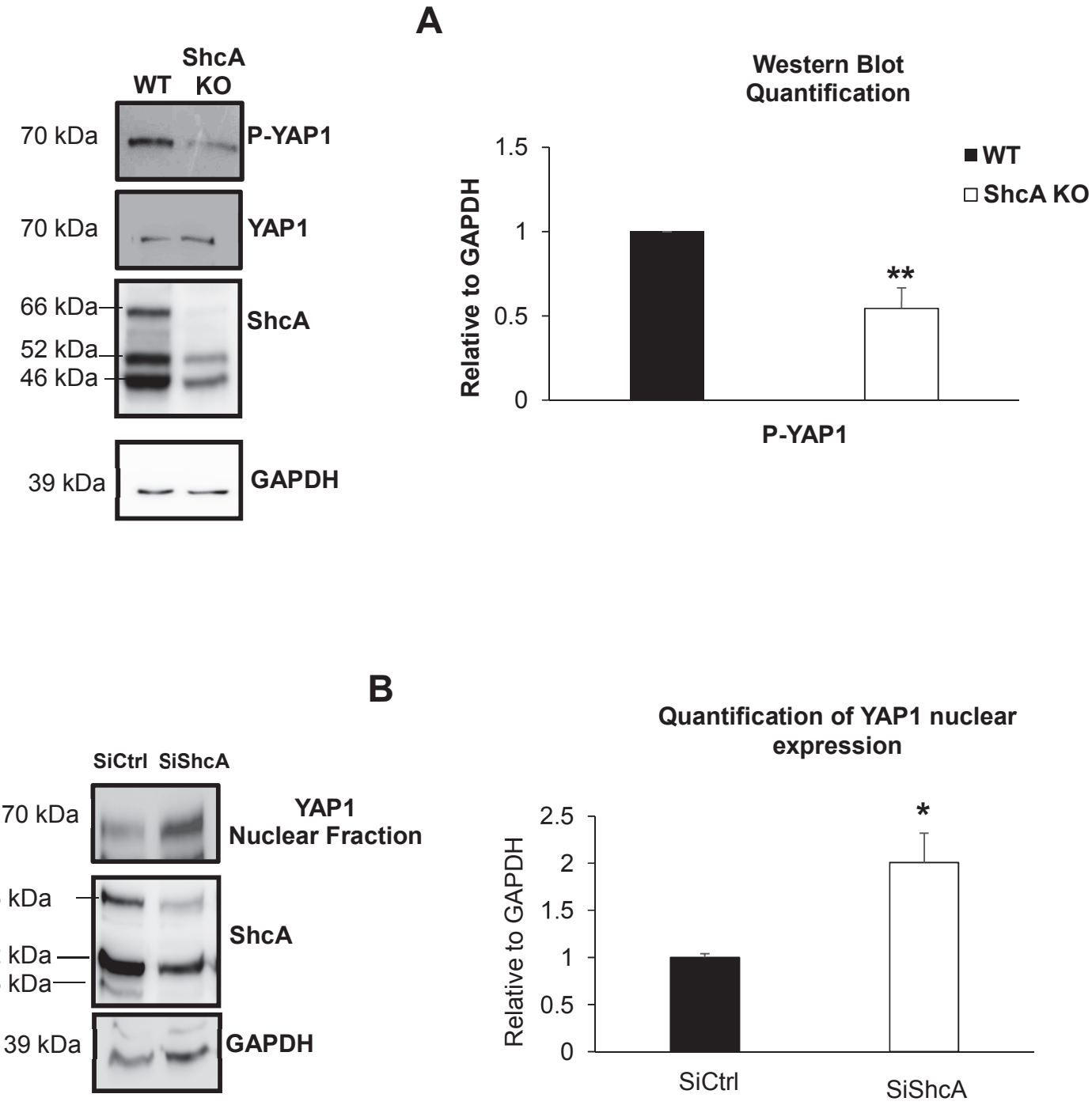


Figure 24: ShcA-deficient cells exhibit stronger nuclear expression of YAP1 and a weaker expression of its cytoplasmic inactive phosphorylated form.

Western Blot analysis and quantification on WT and ShcA KO cells showing an elevated expression of inactive phosphorylated YAP1 in the presence of ShcA (n=5)**[A]**. Cell Fractionation assay and its quatification on ATDC5 transfected with either non-targeting siRNAs (SiCtrl) or SiShcA, showing an elevated nuclear localization of YAP1 in the absence of ShcA (n=3) **[B]**.

Error bars, s.e.m. * p<0.05, (**) p<0.001.

Moreover, by conducting immunoprecipitation experiments on ATDC5 cultured in chondrogenic or hypertrophy-inducing differentiation mediums, we found that YAP1's more pronounced cytopolasmic expression is associated with its interaction with ShcA in hypertrophic conditions *(Fig 25A)*.

We finally aimed to validate the involvement of ShcA in impeding the regulatory effects that YAP1 exerts on RunX2. We used HEK293 cells, their culture conditions are simple, they proliferate rapidly and their transfection is easy and efficient. Cells transfected to overexpress RunX2 showed a stronger interaction between RunX2 and YAP1 when ShcA was downregulated *(Fig 25B)*.

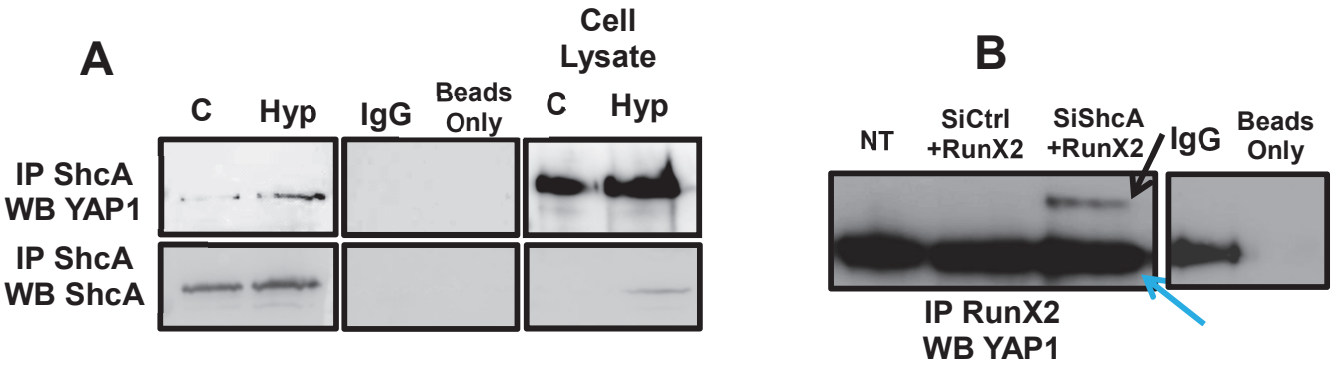


Figure 25: ShcA binds to YAP1 in hypertrophic conditions and the absence of ShcA favors the interaction between YAP1 and RunX2.

Coimmunoprecipitation assay on differentiated ATDC5 cells showing a strong interaction between ShcA and YAP1 in hypertrophic conditions (n=3) **[A]**.
Coimmunoprecipitation assay on HEK293 cells transfected to over-express RunX2 and downregulate the expression of ShcA, showing a strong interaction between RunX2 and YAP1 in the absence of ShcA. Black arrow represents YAP1. Blue arrow represents the heavy chain IgG bands (n=1)**[B]**.

Chondrocyte hypertrophy is a process associated with the development of OA[94]. After having validated a role for ShcA in chondrocyte terminal hypertrophy and highlighted several of the molecular mechanisms behind it, we next sought to investigate whether ShcA plays a role in the physiopathology of the disease.

Part III: Characterization of the physiopathology of age-induced OA in ShcA KO mice.

1. Validation of a spontaneous age-related OA model

The Twist2 ShcA KO mice and the WT mice used in this study were generated on a C57BL/6 genetic background that has been previously described in literature to develop spontaneous OA lesions with aging[205,206]. We have made histological analysis of knee joint cartilage from both young (1-month-old) and old (19 to 25-month-old) mice with safranin O/ Fast Green staining to validate the model of age-related OA *(Fig 26)*. Knee joints from young WT mice show histologic features characteristic of normal AC such as the presence of a thick cartilage with a smooth surface where the chondrocytes and ECM are organized in appropriately oriented and ordered zones (superficial zone, mid zone, deep zone, calcified cartilage, and articular bone plate), whereas knee joints from aged WT mice demonstrate erosion (loss of AC tissue including superficial and at least portions of deeper cartilage layers), denudation (matrix loss extending to calcified cartilage interface), and articular plate fracture (discontinuity through subchondral bone plate), as a result subchondral bone is exposed at articular surface.
These results validate the development of OA with aging in this mouse model.

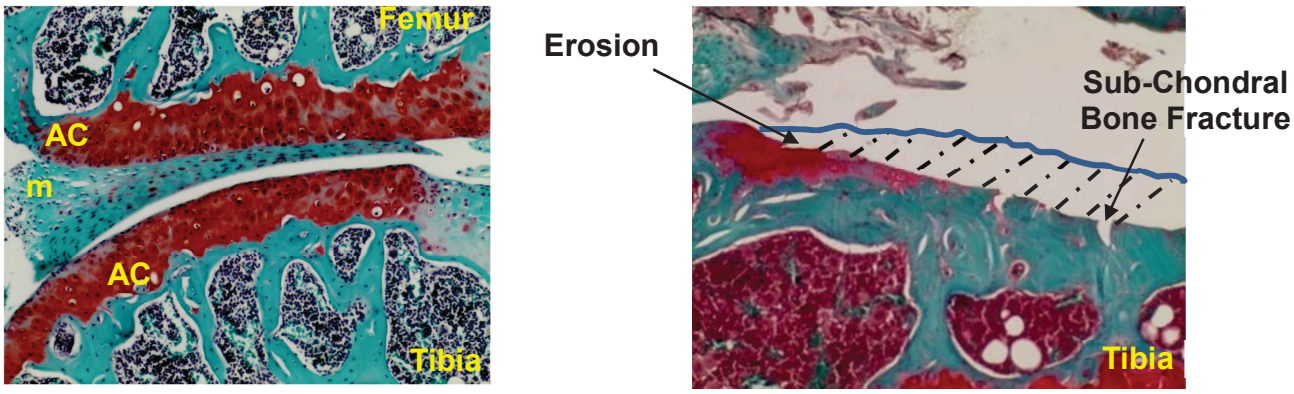
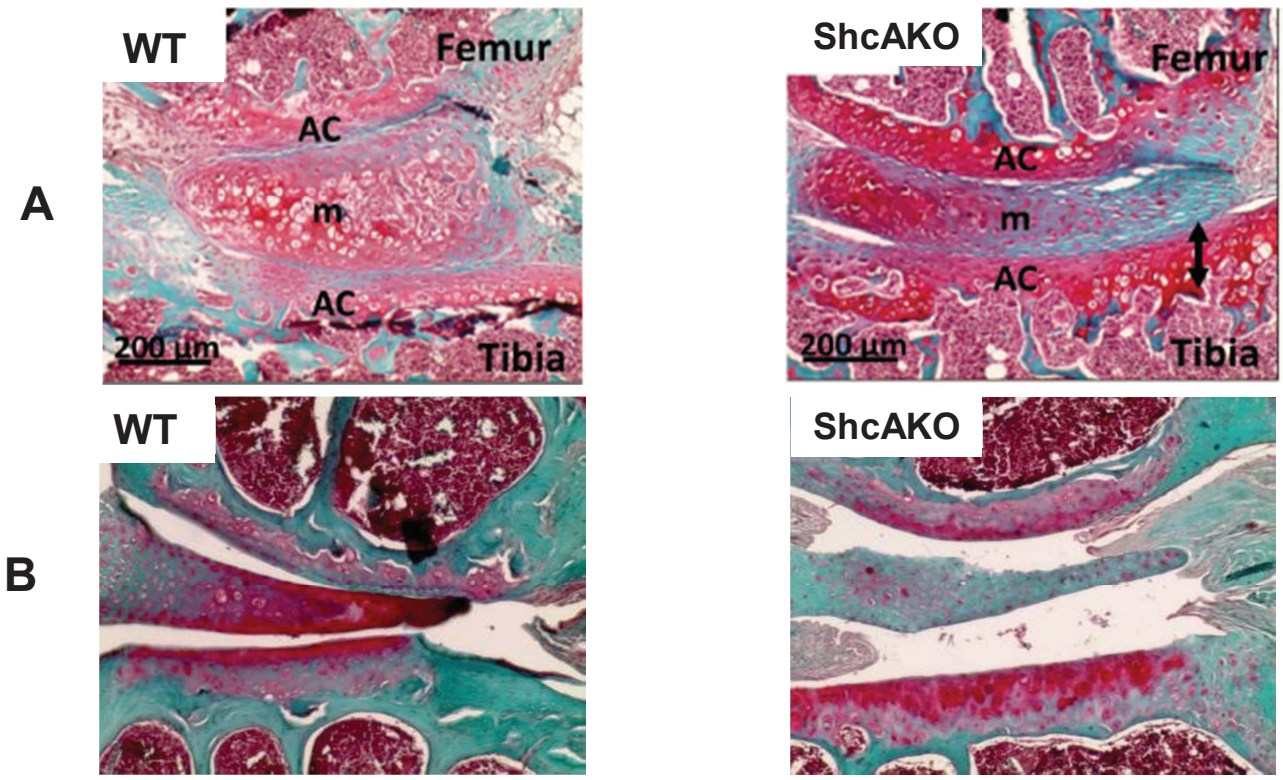


Figure 26: The mouse model develops spontaneous OA with age.
On the left, SafraninO/Fast Green Staining of the knee section of a 3-month old (young) WT mouse. On the right, SafraninO/Fast Green Staining of the Knee section of a 24-month old (old) WT mouse. Aged WT mice exhibit erosion and denudation thus validating our age-related OA animal model (n=5). AC: Articular Cartilage, m: meniscus.

2. ShcA KO mice are protected from developing severe age-related OA

To test the influence of ShcA deletion on OA development using the above described model of age-related OA, we compared safranin O/ Fast Green stained knee joints from aged WT mice to aged ShcA KO mice *(Fig 27 A,B)*. The aged ShcA KO mice exhibit an increase in matrix thickness and increased matrix proteoglycan compared to aged WT mice. The increased AC thickness observed in aged Twist2 ShcA KO mice already exists in young ShcA KO mice. Furthermore, cartilage histopathology was assessed and scored using the OARSI[207] and the modified Mankin[208] scoring systems *(Fig 27 C)*. The modified Mankin scoring system assigns grades to histological features characteristic of OA independent of the location or extent, more specifically, we looked at changes in pericellular staining, background staining, AC structure and arrangement of chondrocytes within the AC. As mice develop arthritis, pericellular staining is increased with the appearance of chondrocyte clusters whereas overall AC staining becomes fainter.
The OARSI however attributes stage to the horizontal extent and grades to the vertical depth within the cartilage reflecting the aggressiveness of the lesions.

1-month old mice are given a score of 0 for all the criteria, and aged WT and KO mice are compared to their respective young controls.
Aged ShcA KO mice exhibit a decrease in the mean Mankin and OARSI scores compared to aged WT mice: 7 ± 0.89 versus 3.6 ± 1.08. p=0.0403. (n=5) and 12.5 ± 2.24 versus 4.1 ± 1.2. p=0.0114 (n=5).
We have also analyzed knee samples by immunohistology using Col II, Col X and MMP13 antibodies. Both young *(Fig 28)* and old ShcA KO *(Fig 29)* mice exhibit an increase of Col II and a decrease of Col X compared to WT mice. However, no difference was seen in MMP13 expression between aged ShcA KO and WT mice.
These results indicate that the deletion of ShcA can slow down the development of age-related OA and decrease its severity.



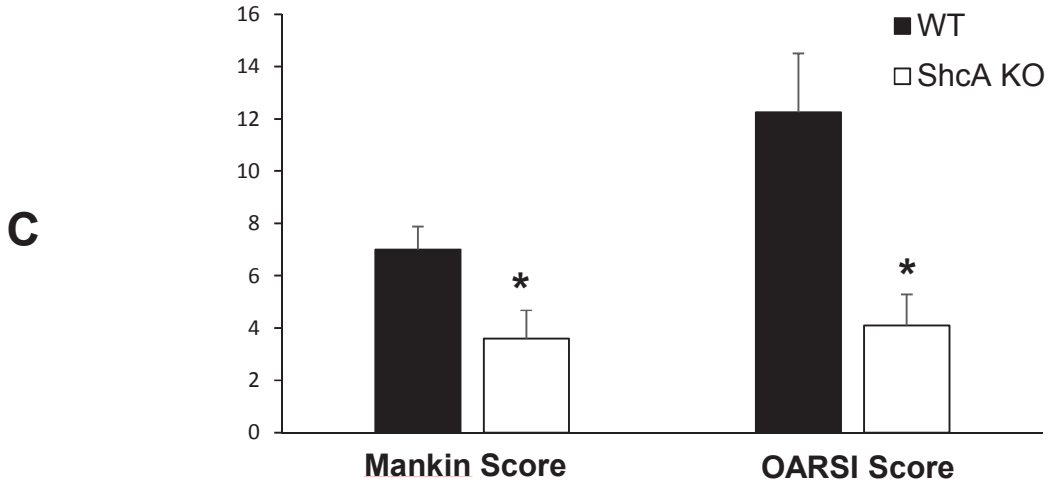


Figure 27: ShcA KO mice are protected from developing sever OA.
Safranin O/Fast Green staining of knee joints from 1 month old **[A]** and 21-month-old **[B]** WT and ShcA KO mice. AC: Articular Cartilage; m: meniscus. Black double headed arrow represents AC thickness. At both a young and old age, ShcA KO mice exhibit a thicker AC with a more prounouced Safranin O staining. MANKIN and OARSI scores on n=5 aged mice **[C].**

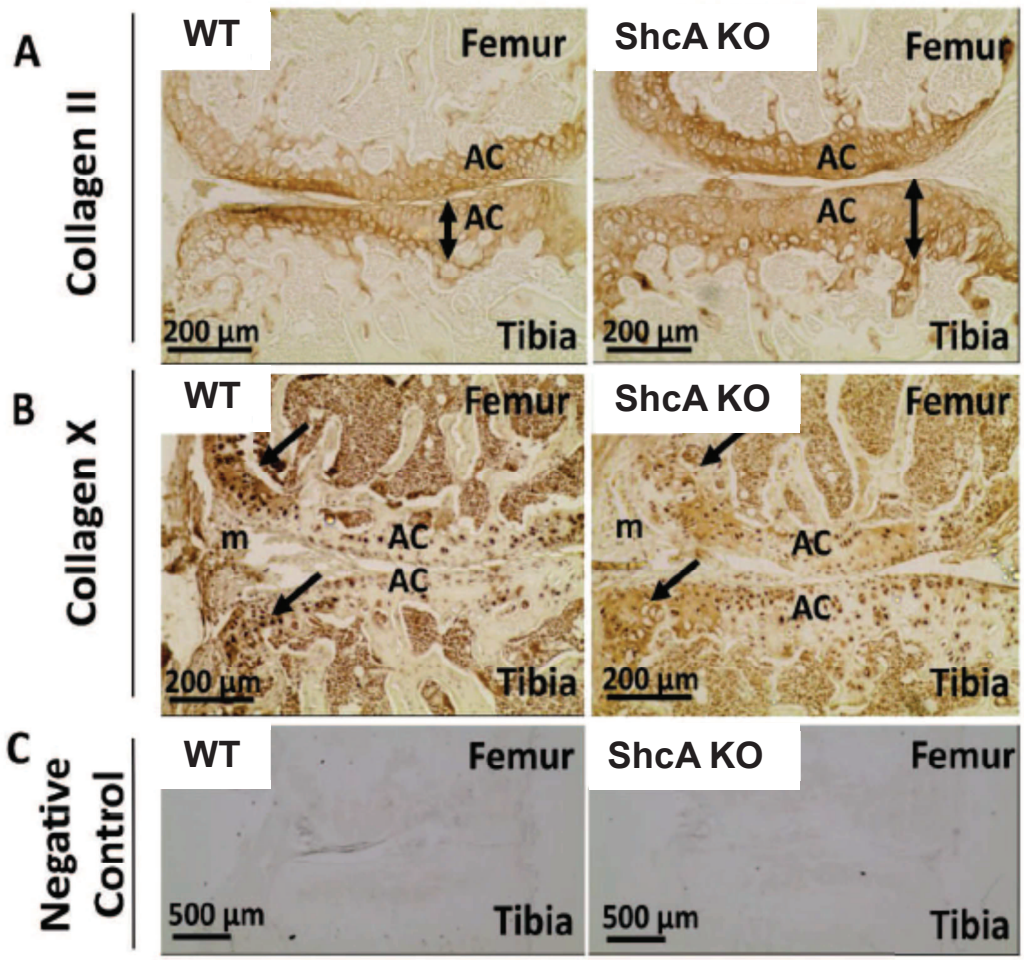


Figure 28: Immunohistological stainings of knee joint from young 1-month-old WT and ShcA KO mice.

Stained with Col II **[A]**, Col X **[B]** and control **[C]**. AC: Articular cartilage; m: meniscus. Arrows represent Col X-positive hypertrophic cells. Double headed arrows represent the thickness of AC. Results are representative of n=5 mice.

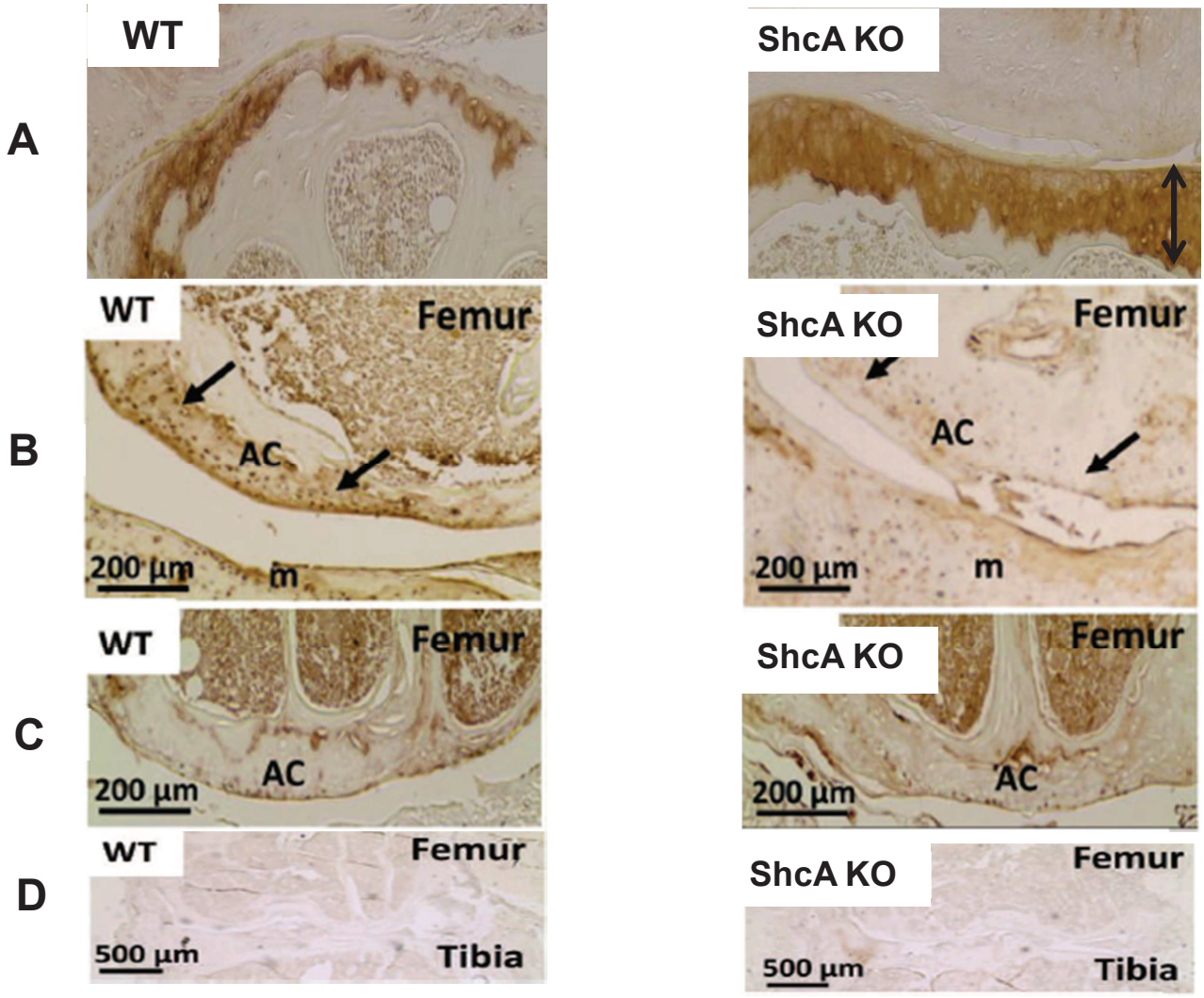


Figure 29: Immunohistological stainings of knee joint from aged 2-year-old WT and ShcA KO mice.

Stained with Col II **[A]**, Col X **[B],** MMP13 **[C]** and control **[D]**. AC: Articular cartilage; m: meniscus. Arrows represent Col X-positive hypertrophic cells. Double headed arrows represent the thickness of AC.
Results are representative of n=5 mice.

After witnessing the drastic effect ShcA has on chondrocyte hypertrophy and OA development, we next aimed to assess the chondrogenic potential of ShcA deficient artificial cartilage and whether it would demonstrate a higher restraint in hypertrophy than WT-derived cartilage.

Part IV: Developing an engineered cartilage using ShcA KO primary chondrocytes.

ShcA deletion promotes a stronger chondrogenic differentiation and hinders hypertrophy in a 3D cartilage model.

This part of the project benefited from the collaboration with Pr. Andrea Barbero's team (Ivan Martin's lab, Basel Stem Cell Network, Basel - Switzerland) and their interdisciplinary expertise in cartilage tissue engineering.

His group integrates cell biology, biomedical engineering and materials science to generate human cell-based three-dimensional (3D) tissues. The engineered constructs developed are used as grafts for the treatment of traumas/diseases of the musculoskeletal system, and/or as 3D model systems to investigate fundamental aspects of cell differentiation and tissue development under controlled and defined conditions[209,210,211,212,213,214.] Using their established protocols, we sought out to engineer and characterize a cartilage tissue made of ShcA-/- cells. We used chondrocytes of knee articular cartilage isolated from 10-day-old Twist2 ShcA KO mice and controls. These chondrocytes were expanded in order to amplify them. Then, the cells were cultured in collagen sponges either in chondrogenic medium or in chondrogenic medium followed by culture in hypertrophic conditions to test both the chondrogenic and bone formation capacity of these cells. The obtained neo-cartilage samples were analyzed by immunohistology to characterize chondrocyte morphology, ECM composition and chondrocyte phenotype.

Unfortunately, cells seeded at 135000 cells per construct, i.e. of the first experiment and half of the samples of the second experiment failed to colonize the scaffold and we

encountered difficulties analyzing them. But samples seeded at a higher cell density were successfully characterized.

The obtained ShcA-deficient neo-cartilage showed a tendency to deposit higher amounts of cartilaginous material as observed by the more intense Safranin O staining, consistent with higher levels of Col II staining in both chondrogenic *(Fig 30)* and hypertrophic medium *(Fig 31)*. Also, WT-derived cells expressed higher Col X expression demonstrating that ShcA-deficient cells showed features of a stable cartilage which is refrained from hypertrophy. To confirm our observations more experiments are needed, with higher cell density seeding.

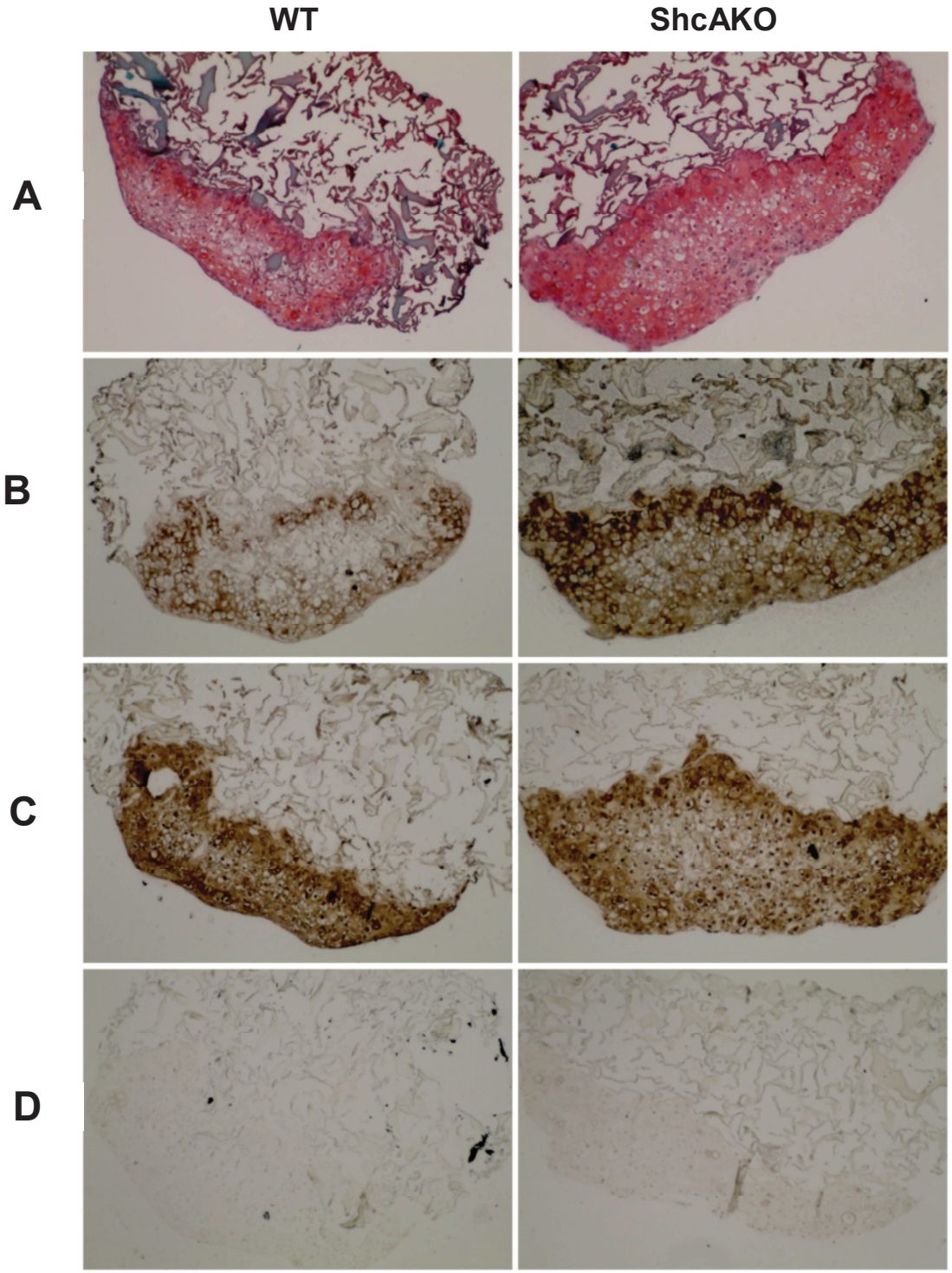


Figure 30: **ShcA KO neo-synthesized cartilage expresses markers of stable phenotype.**
Safranin O staining and immunohistoligical analysis of 3D cartilage model constructed with dedifferentiated WT or ShcA KO chondrocytes seeded on collagen type I scaffolds that were next put through chondrogenic differentiation. Safranin O/Fast Green staining **[A]**. Col II **[B]**. Col X**[C]**. Negative Control **[D]**.

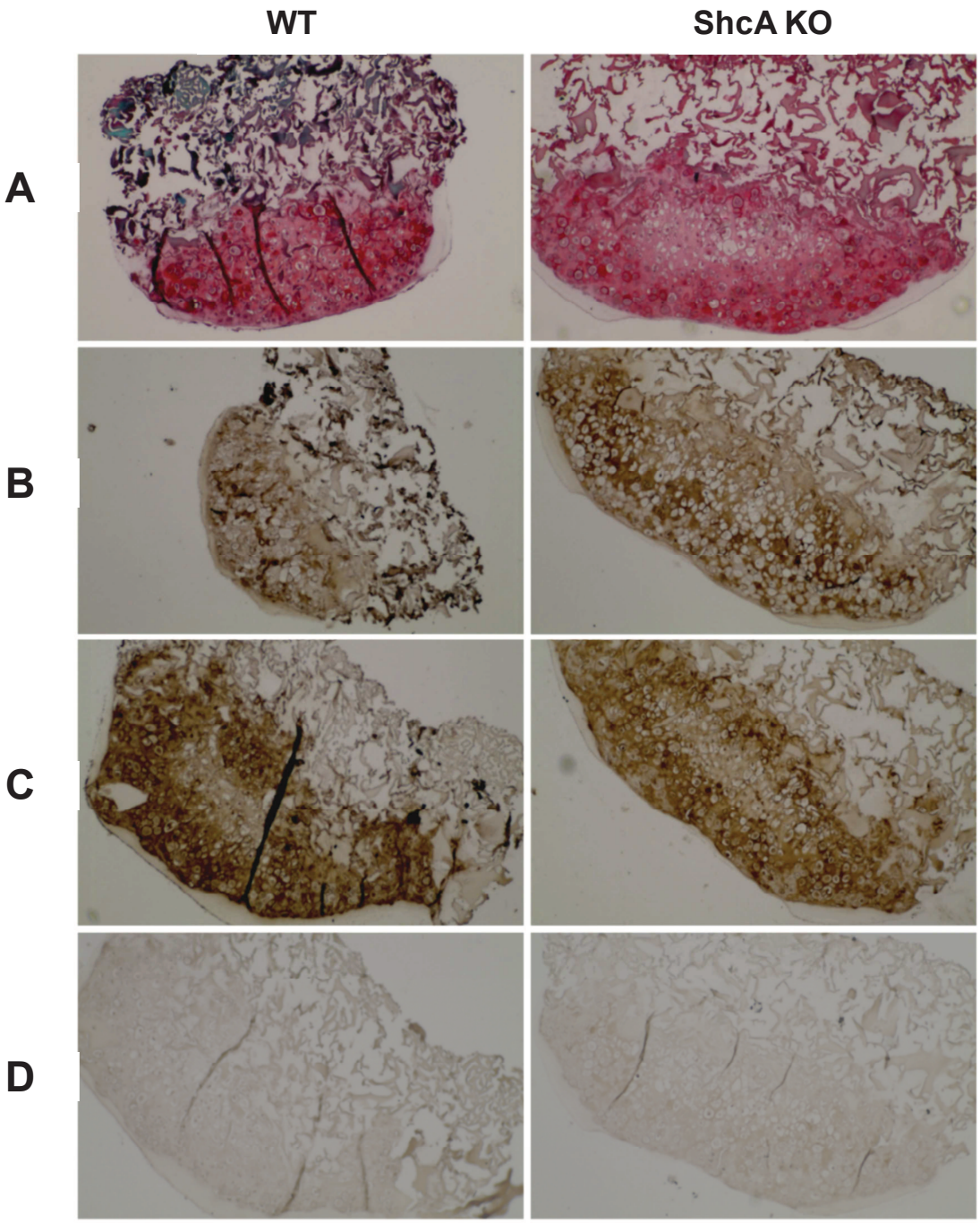


Figure 31: **ShcA KO neo-synthesized cartilage shows weaker expression of hypertrophy markers.** Safranin O staining and immunohistoligical analysis of 3D cartilage model constructed with dedifferentiated WT or ShcA KO chondrocytes seeded on collagen type I scaffolds that were next put through hypertrophic differentiation. Safranin O/Fast Green staining **[A]**. Col II **[B]**. Col X**[C]**. Negative Control **[D]**.

ShcA is an established binding partner to LRP1, a type I transmembrane cell surface receptor that can bind over 40 ligands[9]. LRP1 plays a key role in maintaining cartilage homeostasis by endocytosing not only matrix-degrading aggrecanases like ADAMTS-4/5[215,216], but also matrix metalloproteinase inhibitors like TIMP-3 (Tissue Inhibotor of Metalloproteinases)[217]. It was also shown to inhibit chondrocyte hypertrophy by blocking the activity of the connective tissue growth factor (CCN-2)[218].

In this part of the project, conduct at Pr. Niemeier's lab, we aimed to investigate LRP1 in the cartilage and establish his function.

Part V: Role of LRP1 in chondrocyte differentiation.

Pr. Niemeier's team have generated a conditional knockout mouse model where LRP1 expression was attenuated in hypertrophic chondrocytes and osteoblasts through RunX2-driven Cre recombination[219,225].

1. Validation of the knockout of the LRP1 gene through genotyping.

The Cre-induced recombination leads to the excision of the region between the first lox site at 240pb upstream of the transcription initiation site and the second site downstream of exon 2[219].
To verify the genotype, the Cre recombinase sequence was amplified through PCR *(Fig 32)*.

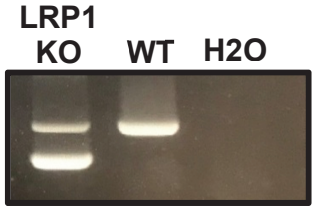


Figure 32: Determining the genotype of the mice.
PCR amplification to detect the presence of the Cre recombinase gene. A positive band reflects a KO genotype. H2O: Negative Control.

2. LRP1 KO mice exhibit a dwarfism phenotype associated with an increase in the size of the growth plate hypertrophic zone.

Given the dwarfism phenotype, we suspected aberrant endochondral ossification as well, so we decided to investigate the growth plate. Unlike ShcA KO mice, our results indicate that in the absence of LRP1, the hypertrophic zone of 4-week old LRP1 KO mice appears to be larger in size and immunohistological analysis reveals an increased Col X staining *(Fig 33)*.

Moreover, unlike WT mice where MMP13 expression was uniquely observed in the hypertrophic and calcified zone, in KO mice, it seemed to extend to all the regions of the growth plate.

Proliferating chondrocytes of the growth plate showed columnar organization in both WT and KO mice with no noticeable difference in Col II staining, suggesting a role for LRP1 in preventing hypertrophy rather than promoting chondrogenesis.

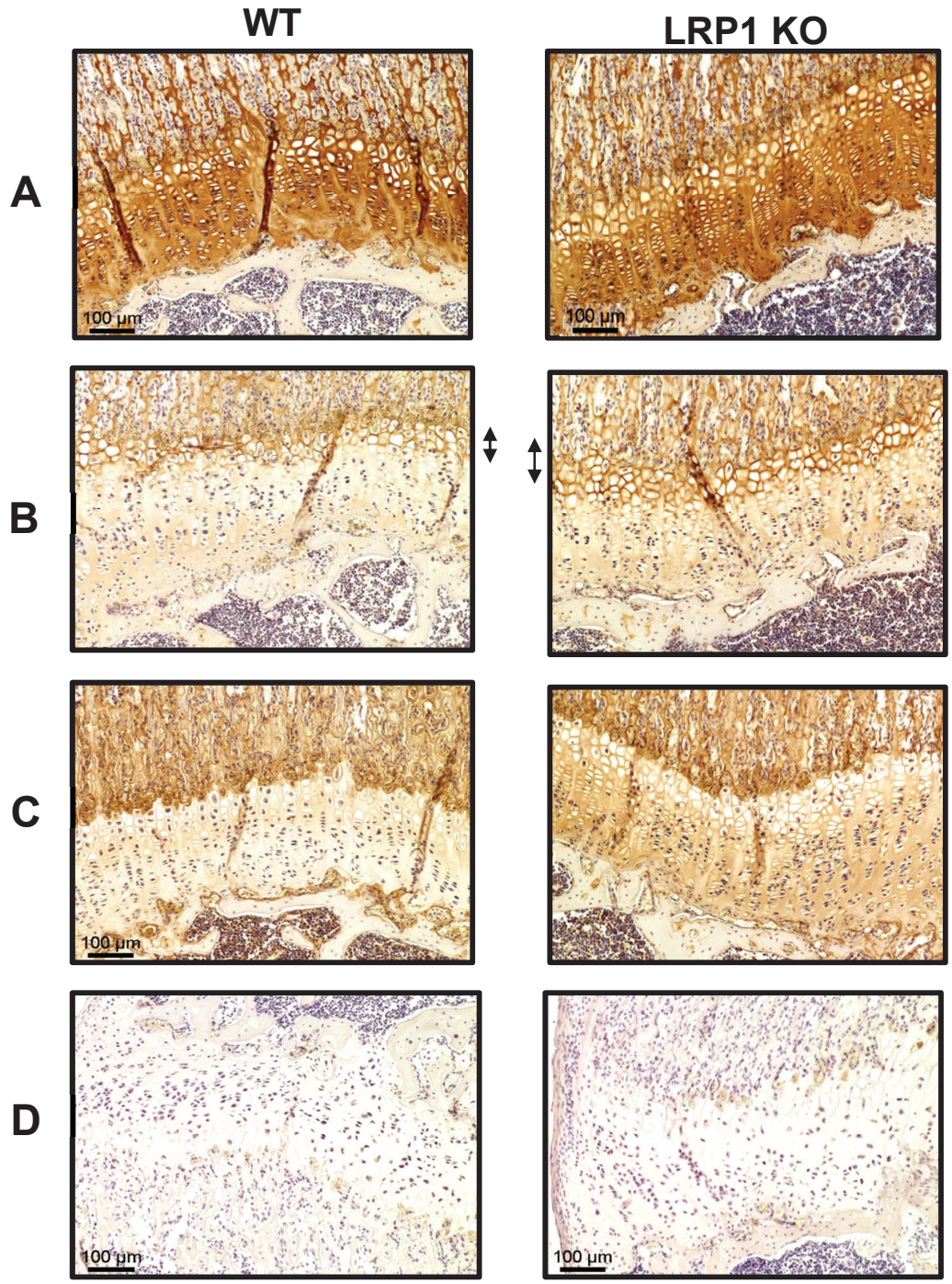


Figure 33: **Immunohistological analysis of growth plate sections from one-month-old WT and LRP1 KO mice**.
Stained with Col II **(A)** and Col X **(B)** and MMP13 **(C).** Negative control **(D)**. LRP1 KO mice show an increased Col X staining and a higher number of marked hypertrophic chondrocytes associated with an increase in the size of the hypertrophic zone. Col II staining showed no important difference between WT and LRP1 KO mice. MMP13 staining showed an extensive staining that is not uniquely restricted to the hypertrophic zone in LRP1 KO mice (n=2). Double-headed arrow represents the Col X-positively stained hypertrophic zone.

3. Chondrocytes express higher levels of MMP13 in the absence of LRP1.

We isolated primary chondrocytes from WT and LRP1 KO mice to investigate the gene expression of several markers of quiescence and hypertrophy *(Fig 34)*.

LRP1 mRNA expression was knocked down in our primary culture, confirming our genotyping and our cellular model (WT vs LRP1 KO: 1 vs 0.5244±0.0761, p=0.0008, n=4).

We observed no difference in the expression of Aggrecan (Acan) (WT vs LRP1 KO: 1 vs 0.97725±0.0563, p=0.4678, n=4), Col II (WT vs LRP1 KO: 1 vs 0.9923±0.0843, p=0.7252, n=4), and, contrary to our *in-vivo* results, Col X (WT vs LRP1 KO: 1 vs 1.0444±0.105, p=0.8455, n=4). Also, the downregulated expression of LRP1 did not majorly affect ShcA mRNA levels (WT vs LRP1 KO: 1 vs 0.9245±0.020, p=0.0211, n=4). Sox9 expression was slightly downregulated in the absence of LRP1 (WT vs LRP1 KO: 1 vs 0.86333±0.02744, p=0.0173, n=3). However, we observed a two-fold increase in MMP13 expression in LRP1-deficient cells (WT vs LRP1 KO: 1 vs 2.152±0.217, p=0.0021, n=4).

These results indicate a role for LRP1 in regulating MMP13 expression.

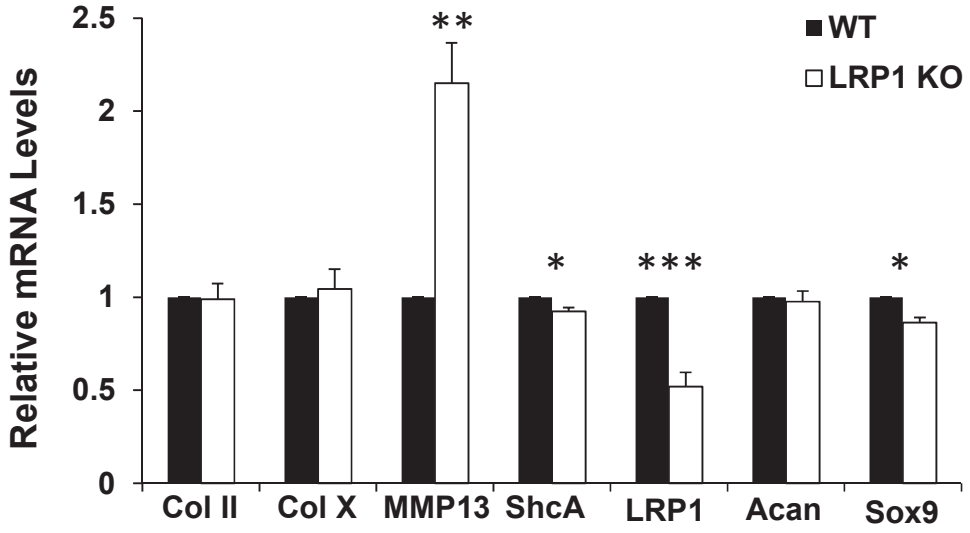


Figure 34: Primary chondrocytes express lower levels of MMP13 in the absence of LRP1.

RT– PCR of the indicated genes in LRP1 KO and WT primary chondrocytes (n=4). Error bars, s.e.m. *P < 0.05 **P<0.01, **P < 0.01, ***P < 0.001.

4. Activation of the MAPK, NF-kB and canonical Wnt pathways in the absence of LRP1.

We next aimed to investigate which mechanisms were regulated by LRP1, leading to the inhibition of MMP13 expression.

The expression of members of the MMP family, particularly MMP13 is under the control of the MAPK effectors. Erk1/2, once activated, can phosphorylate a key transcription factor, c-Jun, member of the activating protein 1 (AP-1) family[220]. In fact, on multiple MMP promoters, c-Jun binds to Ets1 (member of the erythroblastosis twenty-six family) whose activity is also regulated by Erk1/2[221,222].

Pr. Boucher's team have demonstrated that the lack of LRP1 expression in smooth muscle cells lead to elevated P-Erk1/2 expression in a PDGF-R dependent manner[7].

Another crucial pathway is the NF-kB pathway. Once initiated, it ultimately leads to the phosphorylation, activation and nuclear translocation of the p65 and p50 subunits[223],

which can bind to MMP promoters to induce transcription[224]. Pr. Niemeier's team have recently demonstrated that in primary osteoblasts extracted from the same RunX2-Cre mice, the NF-kB pathway was activated in the absence of LRP1 also in a PDGF-R dependent manner[225]. Similarly, we observed an increase in the levels of phosphorylated Erk1/2 and active phosphorylated NF-kB in the absence of LRP1 (n=3) *(Fig 35)*.

LRP1 is involved in multiple signaling pathways, one of the most prominent ones in chondrocyte biology is the Wnt/b-catenin pathway. It has been shown to be involved in all the stages of chondrogenesis, from mesenchymal condensation, to proliferation, maintenance of the quiescence phenotype and hypertrophy[226]. Pr. Boucher's team have previously demonstrated that LRP1 in fact regulates this pathway to control cholesterol metabolism[45]. Moreover, a 2009 study revealed that siRNA-mediated LRP1-knock down in the chondrocytic cell line HCS-2/8, led to the activation of the canonical Wnt pathway responsible for the appearance of the hypertrophic phenotype[227].

Indeed, our results were coherent with the previous studies and we observed an increase of active dephosphorylated b-catenin levels in the absence of LRP1 *(Fig 35)*.

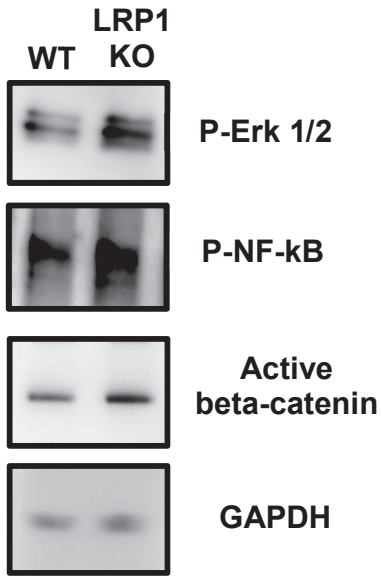


Figure 35: Primary chondrocytes express higher levels of P-Erk1/2, P-NF-kB and active b-catenin in the absence of LRP1.
Western Blot analysis (n=3).

Discussion and Conclusion

Endochondral ossification is a physiological event that occurs in the growth plates and ensures longitudinal bone growth and lasts until the individual reaches sexual maturation. It is a multistep process that requires proliferating chondrocytes to transition into large hypertrophic cells[47,228], an irreversible transition that doesn't only affect growth plate cartilage. Healthy articular cartilage is the home of quiescent or post-mitotic chondrocytes that at some point undergo the hypertrophy program, a transition that has come to be known as a crucial step in the pathogenesis of OA[105]. Last but not least, hypertrophy is a sure sign of failure when it comes to cartilage tissue engineering. Cell-based therapy for OA consists of the replacement of damaged AC through transplantation of a neo-synthesized cartilage, and recent advances aim to obtain an artificial cartilage with a long-lasting, stable chondrogenic phenotype without any expression of the dreaded hypertrophic markers[229],[213].

In all three cases, hypertrophic chondrocytes have a specific role, to remodel the cartilage into a calcified matrix, made up of Col X, devoid of Col II and its pressure-resistant fibrillar network and lacking proteoglycans. Moreover, by secreting a matrix-degrading cocktail of enzymes (MMPs and ADAMTS), they orchestrate firstly the destruction of the once-hyaline ECM, and at later stages the resorption of the mineralized matrix. Finally, by secreting VEGF, they recruit blood vessels that bring along osteoblast progenitor cells that will take over the cartilage by replacing the apoptotic hypertrophic chondrocytes and producing a bone matrix. These events will culminate into a) an increase in bone length during EO, b) the degeneration of the AC and development of OA and c) obtaining an artificial cartilage made up of non-functional fibrocartilage[46,228,105.]

That is why a better understanding of the molecular mechanisms involved in chondrocyte hypertrophic differentiation will identify new potential therapeutic targets that could lock chondro-induced cells in a desired differentiation stage to develop new cartilage tissue engineering options.

ShcA is an adaptor protein recruited by growth factor receptors, integrins and other elements that have been extensively described in literature to play a major role in chondrocyte biology. Furthermore, our lab has established a central role for ShcA in

alternating cellular fate between proliferation and differentiation by regulating signals converging upstream from LRP1 and IGF1-R[45,181].

These reasons made ShcA worth investigating in the context of chondrogenesis and chondrocyte hypertrophy.

Preliminary results revealed abnormal hypertrophic differentiation in ShcA KO mice, evidenced by the reduced size of the hypertrophic zone in the growth plates of these mice, which led to the manifestation of their characteristic dwarfism phenotype *(fig 9)*. Add to that a lower expression of the hypertrophy marker Col X in the AC of 1-year-old KO mice compared to WT mice of the same age *(fig 10)*. These findings oriented my project towards exploring in depth the exact role of ShcA in regulating hypertrophy.

Our first objective was to validate a role for ShcA in chondrocyte hypertrophy.

In-depth analysis of the growth plate revealed a more restricted hypertrophic zone with a weaker staining of Col X and fewer hypertrophic cells in the growth plates of mice lacking the expression of ShcA. However, it also revealed a much stronger Col II staining in ShcA KO mice along with a disorganized proliferation zone which suggests that lack of ShcA not only actively suppresses hypertrophy but also actively promotes pro-chondrogenic mechanisms associated with the production of Col II and it might regulate chondrocyte proliferation *(fig 14,15)*. It would be interesting to analyze the proliferation zone by immunostaining the growth plate with BrdU to quantify the number of cells actively dividing.

Primary chondrocytes isolated from WT and ShcA KO mice, cultured in monolayers to induce what is described in literature as an OA-like dedifferentiation, revealed that cells extracted from ShcA KO mice maintained a stable chondrocyte phenotype with a sustained high expression of Col II even after several passages, associated with weak expression of hypertrophy markers like Col X and ALP1 compared to WT-derived cells *(fig 16,17)*. These results suggest the involvement of ShcA in the control of Col X and Col II expressions. MMP13 expression however was not affected. It has been previously reported that the expression of Col X and of MMP13 can be uncoupled. In fact, chondrocyte terminal hypertrophy may not be the only driver of MMP13 expression, studies have indicated that its expression is considered constitutive even in healthy

cartilage[233], and it is the cells cultured in pro-inflammatory conditions that tend to over express it in an NF-kB-dependent manner[105,230,231,232]. Nonetheless, overall gelatinase activity from cell culture medium supernatant was significantly reduced in ShcA-deficient cells *(fig 18)*. The mechanisms regulating the activity of matrix-degrading enzymes are complex, they could implicate a differential expression of other members of the MMP family whose role is to activate the gelatinases and collagenases[230], or, even more likely, it could involve an LRP1/ShcA axis. ShcA is the binding partner of LRP1, a well-documented membrane receptor involved in the endocytosis of secreted MMPs and ADAMTS[233].

We also observed that ShcA was downregulated when ATDC5 cells were cultured in pro-chondrogenic conditions but was expressed normally in hypertrophic conditions *(fig 19)*. This shows that cells tend to actively regulate the expression of ShcA with respect to the required phenotype. This further validated the role of ShcA in controlling both chondrogenesis and hypertrophy. It would be of interest to study the exact mechanisms involved in suppressing and activating ShcA expression, they could reflect active mechanisms that control the switch in chondrocyte phenotype.

The three isoforms of ShcA are more or less ubiquitously expressed in a constitutive manner[38]. Given the role of p66 in the oxidative cellular response[19], it is implicated in a wide range of biological pathologies involving cancer, immunity, diabetes and cardiovascular diseases, to cite a few and thus has been the most studied. Its transcript is controlled by the downstream promoter, characterized by a Cpg-rich region, and regulated by a number of transcription factors (STAT4 and Nrf2)[234,235,236,237,238]. The p52 and p46 isoforms haven't been as extensively studied. Their transcripts are generated from the upstream promoter through the use of alternating starting codons.

It would be interesting to investigate the epigenetic signature in chondrogenic and hypertrophic cells in regards to the control of the expression of ShcA. Moreover, we have shown that ZEB1 affects the expression of all three ShcA isoforms in endothelial cells[284], and its role in chondrocyte biology has yet to be determined.

These findings allowed us to move on to the next objective. ShcA seemed to be a driver of chondrocyte hypertrophy, which in turn is a main driver of the development of OA.
Several animal models of OA have been described. They are divided into spontaneous and induced OA. Induced OA models such as the surgical induction model, DMM (destabilization of the medial meniscus), induces rapidly progressing OA (after 8 weeks). However, it is more reflective of a post-traumatic OA. Spontaneous age-dependent OA model does not require any surgical intervention and is representative of idiopathic (primary) OA[239].
After validating our animal model of spontaneous age-related development of OA *(fig 26)*, we sought to characterize the role of ShcA in OA pathogenesis.
Aged ShcA KO mice are characterized by an increase in AC thickness. The increased AC thickness observed in aged ShcA KO mice already exists in young ShcA KO mice which points out a role of ShcA in the quantitative production of ECM already in young mice. AC was scored using two scoring systems: the OARSI and the Mankin scores *(fig 27)*[207,208].
These scores reflect the quality of AC and severity of OA. The mean Mankin and OARSI scores are decreased in aged ShcA KO mice compared to WT mice of the same age. These results indicate that ShcA is involved in the maintenance of cartilage homeostasis and the deletion of ShcA can slow down the development of age-related OA.
The protective role of ShcA deletion is corroborated by immunohistological analysis of the ECM protein composition. Immunohistological analysis mirrored what we observed *in-vitro*, there was an increase of Col II, a decrease of Col X and no difference in MMP13 staining in knee joint AC of ShcA KO mice compared to WT mice *(fig 28,29)*.

One of our objectives was to directly test the mechanisms involved in ShcA-induced control of Col X expression. We investigated the implication of the Runx2 signaling pathway. Runx2, a master transcription factor involved in chondrocyte hypertrophy, has been described as a transactivator of Col X. And Erk1/2, the main effector of ShcA signaling, can phosphorylate Runx2 which results in upregulation of the DNA-binding and transactivation potential of Runx2 in osteoblasts and Runx2 activation leads to its

translocation to the nucleus[182,194,195,197,157]. Our results demonstrated that ShcA deletion impedes Erk1/2 phosphorylation, a necessary factor for the translocation of Runx2 to the nucleus *(fig 20,21,22)*. The only thing missing to complete the puzzle of RunX2 activation would be to test whether RunX2 phosphorylation is in fact diminished in the absence of ShcA, but unfortunately there aren't any antibodies specific for the phosphorylated form of RunX2 marketed for research purposes. We have tried to conduct immunoprecipitation assays using anti-RunX2 antibodies and check a specific band for phosphorylated serine/threonine residues, but we were unsuccessful because RunX2 has the same size as the heavy IgG band, and obtaining an interpretable and specific signal was impossible.

Another factor that has recently been implicated in the regulation of RunX2 activity, is the Hippo pathway effector YAP1. By directly binding to RunX2, YAP1 is a validated repressor of RunX2 activity and subsequent expression of Col X[203].

The mechanism we propose based on our observations is that during hypertrophy, ShcA binds YAP1 *(fig 25)*, most probably in the cytoplasm, leading to an upregulated cytoplasmic retention of YAP1, as evidenced by a marked serine phosphorylation of YAP1 *(fig 24)*, which in most cases leads to YAP1's degradation. This results in a diminished nuclear translocation of YAP1, as seen with our cell fractionation assays *(fig 24)*, thus freeing RunX2 of the protein complex it forms with YAP1 and its YAP1-induced inhibition, thus launching Col X transcription *(fig 36)*.

This mechanism could explain the decreased expression of Col X in ShcA-deleted chondrocytes due to defective transactivation of Col X.

There are however some gaps to fill in order to completely validate the mechanism. For instance, the experiment in which we observed the RunX2/YAP1 complex in the absence of ShcA was only done once and we need to replicate it before we can confirm the result.

The role of ShcA in regulating RunX2's DNA-binding potential could be assessed by studying the interaction between RunX2 and the Col X promoter in our primary chondrocytes through chromatin immunoprecipitation assays.

And to determine whether ShcA can activate Col X transcriptional activity, performing a luciferase assay on our primary chondrocytes could put an end to all doubts and confirm

our findings once and for all. By cloning the Col X promoter region in a pGL3 plasmid, and transfecting this plasmid into ShcA KO and WT cells, Col X transcriptional activity can be tested. But the Col X promoter sequence is very complex and different studies have used different regions of this sequence, making the choice of the right region to study a bit difficult. Based on previous attempts, and published works of this technique, primary chondrocytes are not the ideal cell type for transfection, this implies that an extra step for ShcA knockdown is required if another cell type is to be used. Also, for a proper induction of the luciferase signal, RunX2 needs to be over-expressed, making the experimental conditions less and less a reflection of true biological events that take place during hypertrophic differentiation.

Another way ShcA could be controlling the expression of Col X is by regulating the stability of its mRNA. To study the role of ShcA on collagen X mRNA, the half-life of collagen X mRNA can be measured in ShcA KO and WT cells.

It is true that our first findings revealed also a role for ShcA not just in hypertrophy but also in healthy chondrocyte biology, the expression of Col II and in cell proliferation. In the future, it would be interesting to investigate whether ShcA could control the activity of the master transcription activator of Col II expression and chondrogenesis, Sox9[124], as well whether it is involved in pathways crucial to cell proliferation and chondrogenic differentiations such as the PTHrP pathway[130].

Also, epigenetic modifications are important factors in deciding the fate of chondrocytes. For example, the histone deacetylase SirT1 has long been known to control the expression of genes involved in many age-related diseases. *In-vitro*, the knock down of SirT1 by siRNA led to chondrocyte hypertrophy as evidenced by an elevated expression of Col X[240].

One of the aims of my thesis was achieved thanks to the collaboration with Pr. Andrea Barbero's team (Basel Stem Cell Network, Basel-Switzerland), specialists in cartilage tissue engineering[209].

We developed an engineered cartilage using ShcA KO primary chondrocytes expanded through monolayer culture, then re-differentiated on collagen scaffolds either in a pro-chondrogenic medium or a hypertrophy inducing medium. We then tested its

chondrogenic capacity as well as its capacity to evade hypertrophic differentiation through immunohistological analysis.

It is important to note that we couldn't execute many assays, due to the time-consuming nature of the experiment. For this type of construct, established protocols require a cell density of 500000 cells per construct[212]. The results obtained reflect a mere tendency and more experiments should be conducted if we were to make a proper conclusion, however they were encouraging, we did observe higher Col II expression and weaker Col X expression in ShcA-deficient scaffolds cultured in both pro-chondrogenic and hypertrophic culture conditions *(fig 30,31)*.

Murine primary chondrocytes are not the ideal cell-type for this experiment. After a long period of monolayer culture (exceeding 4 passages), chondrogenic re-differentiation have been described in some studies as rather impossible[241].

In cell-based therapies for cartilage regeneration, mesenchymal stem cells have shown successful chondrogenic differentiation *in-vitro*[242,243,244,245]. And unlike autologous chondrocytes, their harvest does not lead to donor site morbidity issues. According to our results, ShcA might represent a novel therapeutic target for this therapeutic strategy. In the future, we plan on using human MSC TERT, that are immortalized stem cells.

ShcA will be invalidated using the CRISPR/Cas 9 technology. These cells will be chondro-induced, cultured in a matrix scaffold and submitted to a hypertrophic medium to test their potential to avoid hypertrophy.

Next, this neo-synthesized cartilage will be transplanted sub-cutaneously in nude mice. Ectopic transplantation rather than transplantation in the joint promotes the ossification of the transplant[246]. The lack of hypertrophy-suppressing signaling existing in the joint, and the exposure to nearby blood vessels, lead to spontaneous hypertrophy of transplanted cells. If the ShcA-deficient cartilage exhibits a successful refrain from expressing hypertrophy markers, it will be an exciting new breakthrough.

The last step would be to test whether this cartilage could repair surgically induced OA cartilage lesions in an established animal model. This test will assess the ability of the transplanted cartilage not only to repair the surgically-induced lesions in the joint of mini-pigs, but also to integrate in the tissue and restore its weight-bearing properties.

If deemed successful, these tests will validate the relevance of ShcA as a potential therapeutic target in human cartilage tissue engineering.

Finally, the last objective of my thesis was conducted in Pr. Niemeier's lab (Institute of molecular and cellular biology, Eppendorf University Hospital, Hamburg-Germany) where we aimed to characterize the role of LRP1 in chondrocyte differentiation. The team have used this RunX2-Cre LRP1 KO model to demonstrate a protective role for osteoblastic LRP1 in osteoporosis by inhibiting RANKL signaling[225].

Similarly, LRP1 seems to play an inhibiting role in chondrocyte differentiation. By studying the growth plates, we observed a thicker hypertrophic zone in LRP1 KO mice accompanied by a stronger Col X staining. Moreover, in WT mice, MMP13 staining seems to be restricted to the calcified and hypertrophic regions, whereas in the absence of LRP1, it seems to be more extensive reaching all the zones of the growth plate *(fig 33)*.

LRP1's role in the endocytosis and regulation of MMP13 is well documented[247] and the observed unrestricted MMP13 staining could very possible be due to the absence of an LRP1-mediated endocytosis.

Isolated primary chondrocytes from LRP1 KO mice were then expanded through monolayer culture.

By analyzing gene expression in these cells, we observed no significant alterations in the expression of many quiescence and hypertrophy markers, like Col II, Col X and aggrecan, suggesting that the increase of Col X staining we observed in the growth plates of LRP1 KO could be simply due to the increased number of Col X-producing hypertrophic chondrocytes. However, MMP13 expression was increased by two-fold in LRP1 KO-derived cells which could possible imply a role for LRP1 as a molecular sensor for MMP13 levels and in the absence of an LRP1-dependant MMP13 internalization, the cellular response seems to induce an overexpression of the metalloproteinase *(fig 34)*.

Studies on chondrocytic cell lines have also shown that LRP1 can inhibit the expression of hypertrophic markers by inhibiting the canonical Wnt/b-catenin pathway[227]. In our cellular model we were able to make the same observation and we detected higher

levels of activated dephosphorylated b-catenin in the absence of LRP1 *(fig 35)*. It would be interesting to examine the cellular localization of b-catenin as well to further validate the involvement of this pathway in the LRP1-mediated cellular effects.

It has been previously reported that *in-vitro* knockdown of LRP1 in primary rat chondrocytes by using lentiviral vectors led to an NF-kB pathway-dependent upregulation of MMP13 expression[248]. Similarly, and as seen in LRP1-deficient osteoblasts[225] the NF-kB pathway was indeed upregulated in our primary LRP1-deficient chondrocytes *(fig 35)*. In fact, the induction of the RANKL pathway in osteoblasts was due to the activation of the PDGF receptor[225], and Pr. Boucher's team have also previously made the observation that LRP1 can regulate PDGF signaling and inhibit the activation of PDGF-MAPK signaling[7]. In our LRP1 deficient chondrocytes, the MAPK was upregulated with an increase in the levels of phosphorylated Erk1/2 *(fig 35)*. LRP1 is known to endocytose a number of growth factors and binds to and regulates the activity of numerous growth factor receptors involved in chondrocyte biology (e.g. IGF1 receptor) and whether LRP1 inhibits both NF-kB and MAPK signaling in chondrocytes by regulating PDGF-receptor activity is a matter that requires further investigation. Also, could ShcA be involved in this upregulation of the MAPK pathway?

A defect in any of the processes of endochondral ossification leads to some form of chondrodysplasia and dwarfism features[50]. And an intensified hypertrophy, may it be premature or not, since our data so far cannot lead to a satisfactory conclusion, could very well be the cause of the dwarfism phenotype in LRP1 KO mice.

Pr. Niemeier's lab use a surgically-induced OA animal model (DMM), and our results so far suggest that the absence of LRP1 would exacerbate experimental OA, and testing that would validate a protective role for LRP1 in OA by inhibiting chondrocyte hypertrophy.

In conclusion, LRP1 and ShcA deletion in chondrocytes seem to have different effects. LRP1 also does not seem to directly affect ShcA expression, however it can affect its activity by regulating the activation of growth factor receptors that recruit ShcA to exert

their effects. Our findings reveal that both proteins are therapeutic targets for OA, and are key regulators of chondrocyte hypertrophy.

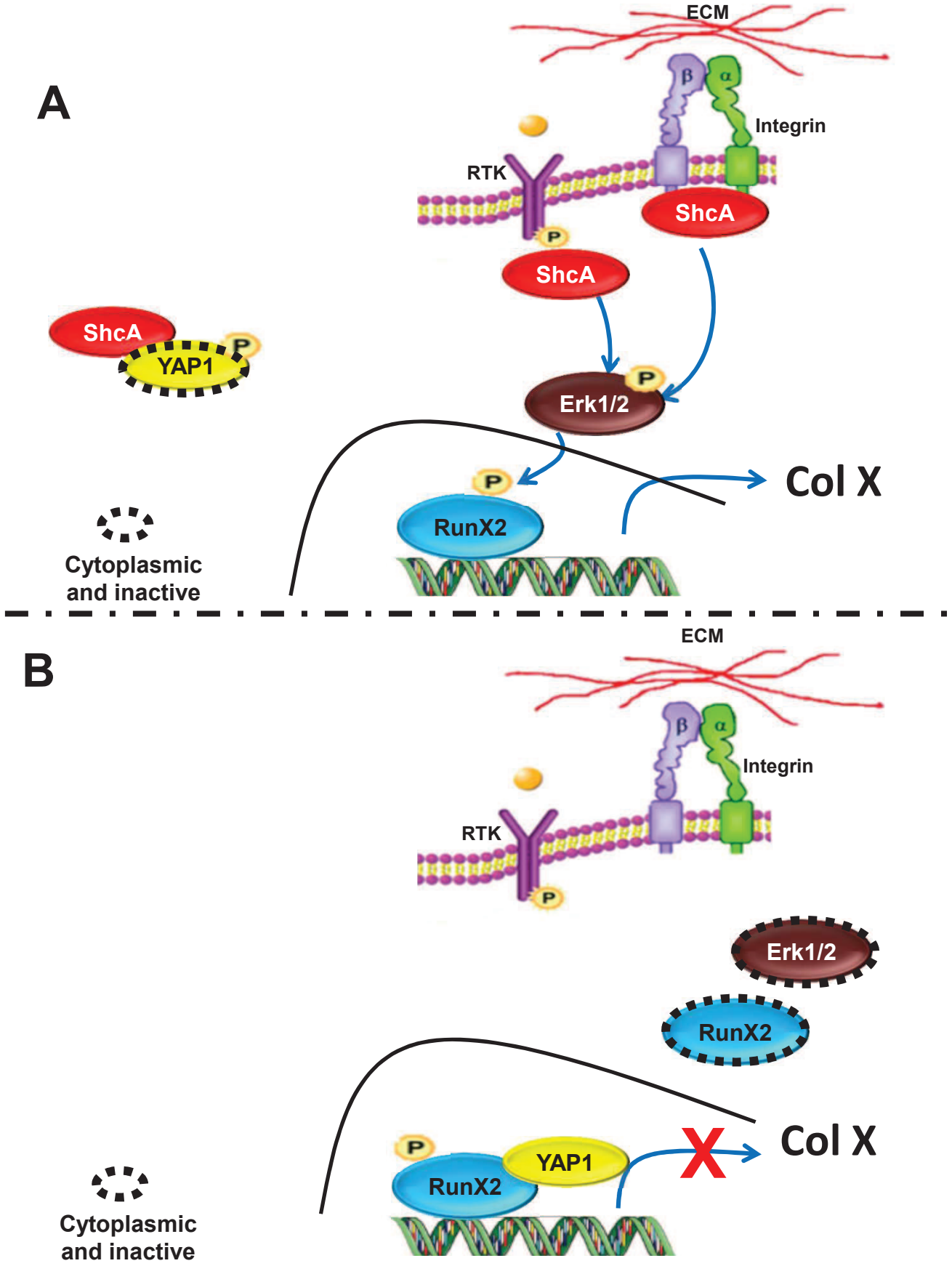


Figure 36: Suggested mechanism based on our findings.
When ShcA is expressed **(A)**.
In KO cells **(B)**.

Materials and Methods

1. Animal models.

Twist2 ShcA KO mice.

The host laboratory generated conditional mice lacking ShcA in chondrocytes using the Cre-lox system.

ShcA was specifically deleted in chondrocytes by mating:

_ ShcA floxed mice, generated by Dr. Matz-Westphal by gene targeting in embryonic stem cells.

ShcA comprises 12 exons and the lox sites were introduced upstream of exons 2 and downstream of exon 7, encompassing a sequence of 2kb that encodes the PTB domain of ShcA required for binding to phosphorylated receptors and for signaling activity.

_ Mice expressing the Cre recombinase under the control of the Twist2 promoter (bought at Jackson Laboratories)[283].

Twist2 is expressed in mesenchymal condensations, thus the Cre-mediated recombination is observed as early as embryonic day 9.5 in condensed mesenchyme-derived chondrocytes and osteoblasts.

RunX2 LRP1 KO mice.

Pr. Niemeier's team generated conditional LRP1 KO mice as previously described[219].

Also, by using the Cre-lox system, these mice were obtained by mating:

_LRP1 floxed mice with the flox sites being 240 bp upstream of the transcription initiation site and downstream of exon 2.

_Mice expressing the Cre recombinase under the control of the RunX2 promoter.

2. Genotyping.

Mice are accorded numbers and identified by tattoos on their limbs.

A sample from their tails is recuperated for genotyping.

DNA extraction.

Tail samples are digested overnight in 1.5ml Eppendorf tubes at 55^0C in a total volume of 250 µl of digestion buffer (Tris 50mM, EDTA pH8.5 5mM, SDS 1%, NaCl 0.2mM and 1.6mg/ml K proteinase). The following day, an equal volume of phenol/chloroform is added per sample. After vigorous vortexing, the tubes are centrifuged at 12000g for 15 minutes. Only the aqueous phase on the surface is transferred to a new 1.5ml Eppendorf tube containing 2v Ethanol 100% and 1/10v Sodium Acetate 3M. Tubes are left for 1 hour at -20^0C to better precipitate the DNA and then centrifuged for 20 minutes at 12000g. The supernatant is eliminated and the pelleted DNA is washed with Ethanol 70%, dried and finally dissolved in 100µl of sterile water.

PCR.

2 PCR programs were used to identify the genotype of ShcA KO mice.

The first one is to check whether they express the Cre Recombinase.
The primers used are:
GCTGCCACGACCAAGTGACAGCAATG
GTAGTTATTCGGATCATCAGCTACAC

The second is to check whether the ShcA was excised.
The primers used are:
GCCCAAGCACATCTGAGGCTTTCTG
GGGCTGTAACTCCACCCTAGG

LRP1 KO mice were genotyped using the same Cre PCR program.

The PCR is then performed following manufacturer recommendations using the DreamTaq kit (Thermo Scientific).

3. Isolation and culture of primary murine chondrocytes.

Chondrocytes were isolated from 7 to 10-day-old C57BL/6 Twist2 ShcA KO and RunX2 LRP1 KO and WT mice. Mice were anesthetized with 0.8 mg/mL Xylazine and 10 mg/mL Ketamine, injected intraperitoneally, and euthanized. Using scissors and a pincer, the skin and soft tissues are removed. Then, the femoral heads, condyles and tibial plateau are isolated, and the ribs are cut out individually using a scalpel and a pincer. For this step, it is recommended to use a microscope for a better and cleaner separation of cartilage fragments from the surrounding tissue to avoid contaminating the chondrocyte culture with fibroblasts. Also, it makes it easier to differentiate between the translucent cartilage tissue and the brown bone tissue that should be excluded. The isolated costal and knee cartilage fragments are then washed with PBS and digested 2x45 minutes at 37˚C with a collagenase solution (Sigma-Aldrich) at 3 mg/ml followed by overnight digestion with collagenase at 0.3 mg/ml. The following day, digested cartilage was filtered through a sterile 40 µm cell strainer then centrifuged for 10 minutes at 400g. Isolated chondrocytes were cultured in DMEM medium (Dulbecco's Modified Eagle Medium, [-] L-glutamine, [+] 4.5g/L glucose, 110 mg/L sodium pyruvate, Life technologies, GIBCO) with 10% (v/v) FBS (Fetal Bovine Serum, Dominique Dutscher), 1% glutamin and 50 IU/ml penicillin/streptomycin and then amplified through monolayer culture[249], incubated at 37^0C and 5% CO2 in a humidified atmosphere.
Primary chondrocytes were treated or not for 1 hour with 50µM of the MEK1 inhibitor PD98059 before the cell fractionation assay.

4. ATDC5 cell culture and differentiation.

Adenocarcinoma-derived ATDC5 cells were maintained in DMEM/F-12 (1:1) containing 5% FBS and 1% sodium pyruvate. Cells that remained throughout experiments in this basic maintenance medium were termed ND or non-differentiated cells.

ATDC5 cells show a sequential transition of phenotype that highly resembles the process of endochondral ossification. Over 300 studies have been published with this cellular model. To initiate chondrogenic differentiation, cells were seeded at a density of 6000 cells/cm2 and then their maintenance medium was supplemented with 1% ITS (Insulin, Transferrin, Selenium) (Corning). Those cells were termed C, for differentiated in a pro-chondrogenic medium. The medium was changed every other day, and cells in these conditions have been evidenced to produce Col II and other ECM components, and it's not before 5 weeks of culture before they start expressing ALP1 and Col X, reflecting a hypertrophic phenotype[250].

6 days after differentiation initiation, when cells reached confluence, the medium C was supplemented with 10 mM βGP (beta-glycerophosphate) and 50µg/ml L-ascorbate-2-phosphate (ascorbic acid)[189] to accelerate matrix mineral deposition. These cells, termed Hyp (for differentiated in a hypertrophy-inducing medium) have been shown to undergo all stages of differentiation at a faster rate than C cells. After 4 weeks of differentiation, when C cells are still expressing high amounts of Col II and haven't even started mineralizing their matrix, Hyp cells have already exited the chondrogenic phase (with highest amounts of Col II produced at day 13) and started expressing high amounts of Col X and ALP1[189].

Cells were at all times incubated at 37^0C, 5% CO2 in a humidified atmosphere.

5. Cell transfection.

HEK293 cells (Human Embryonic Kidney), cultured in DMEM medium supplemented with 10% FBS and 1% glutamin, and ATDC5 cells in there maintenance medium were transfected for subsequent experiments.

For transfection, ATDC5 cells were seeded in P100 dishes at a concentration of 1.10^6cells/dish. 24 hours later, they were transfected with either scrambled siRNA or siRNA against p66, p52 and p46 isoforms of ShcA (Dharmacon) at a final concentration of 100 nM using lipofectamine 3000. 48 hours later, the cells were used for analysis.

HEK293 cells were seeded in the same manner, transfected for 24 hours with siRNAs followed directly by a second transfection, also for 24 hours with an expression vector (pCMV6-Entry) to over-express RunX2 (Origene).

6. Gene expression analysis.

RNA extraction.

Primary murine chondrocytes were seeded in 6-well plates, and once they have reached 80% confluence, they were washed once in PBS, then 300µl of TRI Reagent (Sigma) was added. Cells were scraped in the trizol, transferred in 1.5ml Eppendorf tubes, and left for 5 minutes at room temperature (RT). 80µl of chloroform was then added for each sample, tubes were inverted multiple times, then centrifuged at 12000g for 15 minutes at 4^0C. The superficial aqueous layer is then transferred to a new 1.5ml Eppendorf tube containing 500µl of isopropanol. RNA is then precipitated and pelleted by centrifuging for 10 minutes at 12000g at 4^0C. The RNA pellet is then washed twice with Ethanol 70%, left to dry and then dissolved in 20-30µl of sterile RNAse-free water.

For LRP1 KO mice, total RNA extraction was done using NucleoSpin RNA II kit (Macherey & Nagel).

Reverse transcription.

1µg of RNA per sample was then used for reverse transcription in a total volume of 20µl as per the manufacturer's instructions (High-capacity cDNA Archive kit (Applied Biosystems). cDNA (complementary DNA) samples were then stored at -20^0C.
For LRP1 KO cells, 400 µg of RNA were used for complementary DNA synthesis using SuperScript III Reverse Transcriptase (Invitrogen).

Quantitative Real Time PCR.

PCR amplification was performed using SYBRGreen PCR master mix (Kappa Biosystems) according to manufacturer's instructions. Briefly, cDNA samples were diluted 4 times and added in a total volume of 20µl of PCR mix. Samples were analyzed in duplicates using the Step One system and software (Applied Biosystems). Cycle thresholds (Cts) were normalized to 18S house keeper levels.
The primers used are the following:

For LRP1 KO cells, quantitative real-time PCR was performed on a 7900HT sequence detector (Applied Biosystems) using TaqMan Assay-on-Demand primers supplied by Applied Biosystems. Gene of interest cycle thresholds (Cts) were normalized to *TATA-box binding protein* (*Tbp*) house keeper levels

7. **Immunoprecipitation assays.**

Immunoprecipitation assays were performed on ATDC5 cells after 4 weeks of chondrogenic and hypertrophic differentiation, and HEK293 cells transfected with SiRNAs to knockdown ShcA expression with the expression vector to over-express RunX2.
Total proteins extracted using a 10mM HEPES lysis buffer (pH 7.4) containing 1.5mM Na_2HPO_4 , 150mM NaCl, 2mM EDTA, 1% Triton X-100, 0.1% SDS, 50mM Sodium Fluoride, 2mM Na Vo_4 , 0.1% Sodium Deoxycholate and 1mM phenylmethylsulfonyl fluoride (PMSF, Sigma, Cat#P7626-5G).
2µg of specific IP antibody or non-immunized rabbit serum were incubated for 2 hours at room temperature with 20µl of protein A/G agarose beads (Santa Cruz) per sample. After 3 washes with the HEPES lysis buffer, the mixture was then incubated overnight at 4°C with 500µg of the protein extracts. The beads were washed then boiled in Laemmli Buffer and eluted proteins were analyzed by Western Blot.

8. Cell Fractionation Assays.

The cells were fractionated by differential centrifugation as previously described[251]. The sequence of increasing gravitational forces of each centrifuge leads to the separation of each cellular component according to its density. Briefly, cells were scraped in their own culture medium, quickly pelleted by centrifugation, then washed with cold PBS and resuspended in a 1M HEPES buffer pH7.4, containing KCl 2M, EDTA 1mM, EGTA 1mM, sucrose 250mM and $MgCl_2$ 1.5mM.

Cells are then homogenized in this buffer by passing them through a 22G needle and centrifuged. The supernatant contains membrane and cytosolic fraction while the pellet contains the nuclear fraction and cell debris.

The pellet is resuspended and left for 2 hours to homogenize in a 1M HEPES buffer pH7.4 containing 2.5% v/v glycerol, NaCl 5M, $MgCl_2$ 100mM, EDTA 1mM and EGTA 1mM, while the supernatant undergoes ultracentrifugation at 45000 RPM for 45 minutes. This step separates the cytosolic proteins subsequently suspended in the supernatant from pelleted membrane proteins, that are then resuspended in a 500mM Tris buffer pH 7.4, containing NaCl 5M, SDS 1%, EDTA 1mM and EGTA 1mM.

The nuclear fraction is then submitted to ultracentrifugation at 45000 RPM for 45 minutes, which results in pelleting cell debris and the nuclear proteins are left suspended in the supernatant.

9. Western Blot.

Isolated chondrocytes or ATDC5 cells were washed with PBS, then lysed using 100 µL RIPA buffer (Tris HCl 50mM pH7.8, NaCl 150mM, 1mM EDTA, 1% NP-40, 0.25% deoxycholate) supplemented with a protease inhibitor cocktail and phosphatase inhibitors (1mM NaF, 1mM Na3VO4). The lysate was centrifuged at 12000 g for 30 minutes and the remaining supernatant was used for protein dosage. Protein concentration was estimated using the Bradford assay. 30 to 50 µg of proteins were mixed with Laemmli 4x buffer, containing 1/20 volume of β-mercaptoethanol. Samples were denatured 10 min at 95°C and then subjected to SDS-PAGE in a 10%

polyacrylamide gel. Protein migration was carried out at 110 volts. Proteins were transferred to a nitrocellulose membrane previously impregnated in a transfer buffer. The transferred membrane was then incubated for 1 h at room temperature in blocking solution of TBS-Tween containing 5% milk. The membrane was further incubated in blocking solution with the primary antibodies (1:2000 dilution for collagen II; 1:2500 dilution for collagen X; 1:2000 dilution for MMP13; 1:5000 for GAPDH, 1:1000 for P-Erk, Erk, P-YAP1, YAP1 and ShcA) overnight at 4˚C. The membrane was washed with TBST and then incubated in blocking solution with 1:10000 dilution of horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Promega) for 1 h at room temperature. The membrane was then washed in TBST. The signal was visualized by exposing the membrane to ECL (Enhanced Chemiluminescence), a chemiluminescent substrate to detect Horseradish Peroxidase (HRP) conjugates on western blot (Clarity TM Western ECL substrate, Bio-Rad). Clarity western ECL substrate allowed visualization of protein expression using ImageQuant™ LAS 4000 Imaging System (Amersham). Optical densitometry was performed with Adobe PhotoshopCS and Image J normalizing bands intensity for GAPDH.
The running buffer consists of 1,5 M Tris-HCL, pH 8,8, glycine 250mM ; SDS 0,1% and the transfer buffer is Tris-HCl 25mM pH 8,5 ; glycine 250mM ; ethanol 20%

A. Primary Antibodies.

The following antibodies were used:

Anti-ShcA (06-203, Rabbit polyclonal, Millipore), anti-GAPDH (MAB374, Mouse monoclonal, Millipore), anti-collagen X (ab58632, Rabbit polyclonal, abcam), anti-collagen II (ab34712, Rabbit polyclonal, abcam), anti-MMP13(ab39012, Rabbit polyclonal, abcam), anti-P-Erk1/2 (Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody, Rabbit polyclonal #9101, Cell Signaling Technologies), Anti-Erk (p44/42 MAPK (Erk1/2) Antibody, Rabbit polyclonal #9102, Cells Signaling Technologies), anti-P-YAP1(Phospho-YAP (Ser127) Antibody, Rabbit Polyclonal #4911, Cell Signaling Technologies), anti-YAP1 (YAP Antibody, Rabbit polyclonal #4912, Cell Signaling Technologies), anti-P-NFkB (Phospho-NF-kB p65 (Ser 536) (7F1), mouse monoclonal

antibody #3036, Cell Signaling Technologies), anti-active b catenin (Anti-ABC, mouse monoclonal antibody, Cat No 05-665, Merck Millipore), anti-Runx2 used for western blots (sc-10758, Rabbit polyclonal, SANTA CRUZ), anti-RunX2 used for immunoprecipitation assays (RunX2 (D1H7) Rabbit mAb, #8486, Cell Signaling Technologies).

10. Gelatin-gel zymography.

Sample preparation.

Primary chondrocytes were grown in monolayer for 24 hours before harvesting 1ml of cell culture medium.

Protein concentration was estimated by the Bradford assay and 20µg were used for the zymography. Similarly to sample preparation for western blotting, the samples were diluted with Laemmli buffer, however they were not boiled nor treated with B-mercaptoethanol and they were used on the same day.

Gel preparation.

The gel is of the same composition as western blot gels, a 10% polyacrylamide gel with SDS, containing 2mg/ml of gelatin. The gelatin was dissolved in the running buffer, heated to a maximum temperature of 50ºC. Samples migration was done at 4ºC for 1 hour at 110V. 30 minutes after the start of the migration, heparin was added in each well at a concentration of 0.3mg/ml per sample.

Activation of the gelatinases.

Once the migration was terminated, the gel was washed twice for 30 minutes each time in a solution of 2.5% Triton X-100 and then incubated overnight at 37^0C in a solution of Tris-HCl 50 mM CaCl2 5 mM, pH 8.

Gel staining.

The next day, the gel was stained in a solution containing Coomassie Blue at 2,5 %, acetic acid 10 %, methanol 50 % and then de-stained in the same buffer that does not

contain Coomassie Blue. The coloration was finally fixed using a buffer of 10% acetic acid.

11. Whole-mount skeletal staining.

Euthanized mice are sprayed with 70% ethanol, dissected, and using scissors and forceps their skin is gently removed by pealing it. Eyes, visceral organs, fatty tissue and other tissues are eliminated to the best extent. The sample is dehydrated and fixed overnight in Ethanol 95%. Excess adipose tissue is eliminated by incubating the sample for 2 days in Acetone 100% followed by a 3-day staining in Alcian Blue (0.03% w/v dissolved in a solution of 80% Ethanol and 20% Glacial Acetic Acid) to color the cartilage tissue. The specimen is then washed twice in Ethanol 70% and left overnight in Ethanol 95% to fix the coloration. Samples are next pre-cleared for 4 hours in 1% KOH and stained in Alizarin Red (0.005 % (w/v) in 1 % (w/v) KOH solution) for 5 days to give the bone tissue a pink coloration. The skeletal tissue is then destained in 1% KOH solution[252].

12. Histological analysis.

Preparation of samples.

Samples of knee from ShcA KO and WT mice of the same age were isolated and fixed with 10% paraformaldehyde (PFA) for 5 days (1-month-old mice) and 7 days (12, 24 and 25- month-old mice), decalcified using 1M NaOH solution containing 10% EDTA disodium for 8 days (1-month-old mice) and 15 days (12, 24 and 25-month-old mice), embedded in paraffin and sectioned at 5 µm. Sections were deparaffinazed using Histosol Plus (SHANDON), rehydrated through a series of decreasing alcohol concentration (100% - 50%) and finally water.

Samples from LRP1 KO mice were treated with a different protocol. The knee samples were fixed after dissection in 4% PFA overnight, washed with PBS and then decalcified for 24 hours in a 33% formic acid decalcifying buffer containing 13.5% tri Sodium Acetate. Samples were then left under running water for 24 hours, then embedded in paraffin.

Safranin O/Fast Green staining.

Safranin O stains the AC in pink. Sections were stained in Weigert's iron hematoxylin solution (Sigma) for 10 minutes, washed with water for 4x2 minutes and stained in 1% acid alcohol for 2 seconds. Then sections were washed with water for 3x2 minutes, stained in 0.1% Fast Green solution (Sigma) for 5 minutes and 0.1% Safranin O solution (Sigma) for 5 minutes. Sections were dehydrated in 95% ethanol, 100% ethanol and finally Histosol Plus.

For quantitative analysis of the hypertrophic zone of the growth plate, images taken through the microscope were processed using ImageJ.

Assessment of cartilage histopathology

Morphological changes of AC were assessed and scored using the OARSI and the Mankin scoring systems[207,208]. Scoring of OA cartilage allows a complete assessment of cartilage histopathology. The Mankin scoring system assigns grades to histologic features characteristic of OA (the major feature is Safranin O staining depletion) independent of the location or extent of lesions whereas OARSI attributes stage to the horizontal extent and grades to the vertical depth within cartilage reflecting the aggressiveness of the lesions.

Immunohistology.

Sections were incubated in trypsin for 10-20 minutes at 37 ˚ C, washed with PBS 1X and then incubated for 30 minutes in 3% hydrogen peroxide to inhibit endogenous peroxidase. Sections were washed with TBST (Tris Buffered Saline with Tween 20 - 0.05%, Sigma) and incubated in 5% normal serum blocking solution (Goat, VECTASTAIN ABC Kit, Vector Laboratories) for 1 hour at room temperature in a humidified chamber. Excess of serum was discarded; sections were incubated with the primary antibodies (1:1000 dilution for collagen II; 1:2000 dilution for collagen X; 1:100 dilution for MMP13) overnight at 4˚C, washed with TBST, and then incubated with biotinylated secondary antibody (1:200 dilution, Goat, VECTASTAIN ABC Kit, Vector

Laboratories) for 1 hour at room temperature followed by 30 minutes incubation with avidin-biotin peroxidase complex (VECTASTAIN ABC Kit, Vector Laboratories). Visualization was performed using 3.3 diaminobenzidine.

13. Immunofluorescence microscopy.

Isolated chondrocytes (5000 cells per 6-well plate well) were grown on glass cover slips during 2 days, fixed with 3% formaldehyde, permeabilized with 0.5 % Triton X-100, blocked with 1% BSA for 1 hour and incubated for 2 hours with primary antibody at RT (1:200 dilution) against Runx2 (Santa Cruz). Alexa Fluor 488 conjugated to goat anti-rabbit antibody (1/200) (A-11008, InvitrogenTM) was used to visualize the localization of Runx2. Fluorescence was detected using the Leica TSC SPE laser confocal microscope.

14. Generation of ShcA deficient 3D cartilage model.

Primary murine chondrocytes were expanded in monolayers for 6 passages, then transferred to Pr. Barbero's lab (Department of Biomedicine, University Hospital Basel, Basel – Switzerland) where they were seeded onto a 4-mm in diameter, 1-mm thick type I collagen-based cylindrical scaffold (Ultrafoam, Davol, Warwick)[212].

First experiment, cells were seeded at a density of 135000 cells per construct:

1. Constructs were maintained for 2 weeks in serum-free medium containing TGF-β1, dexamethasone, ascorbic, and ITS to induce chondrogenic differentiation.
2. Constructs were treated as in (1), followed by implantation in subcutaneous pounches of nude mice (4 samples per mouse) for 4 weeks.
3. Constructs were treated as in (1), followed by implantation for 8 weeks.
4. Constructs were cultured in chondro-inductive medium for 3 weeks, followed by culture for 2 weeks in a hypertrophic medium, containing b-glycerophosphate and Thiroxin, and that doesn't contain TGF-b1 or dexamethasone.
5. Constructs were treated as in (4), followed by subcutaneous implantation in nude mice for 4 weeks.
6. Constructs were treated as in (4), followed by implantation for 8 weeks.

Second experiment:

1. Cells were seeded at a density of 135000 cells per construct and cultured in chondro-inductive medium for 2 weeks.
2. Cells were seeded at a density of 135000 cells per construct and cultured in chondro-inductive medium for 3 weeks followed by culture in hypertrophic medium for 2 weeks.
3. Cells were seeded at a density of 250000 cells per construct and treated as in (1).
4. Cells were seeded at a density of 250000 cells per construct and treated as in (2).

15. Statistical analysis.

Values are reported as mean ± SEM of at least triplicate determinations. Statistical significance was determined using an unpaired Student's t test (GraphPad Prism, Abacus Concepts, Berkeley, CA). (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$ and (****) $p<0.0001$.

Chapter II:
The Role of ShcA in Atherosclerosis

1. Atherosclerosis: impact and risk factors.

Atherosclerosis, the major cause of cardiovascular disease, is characterized by the slow yet progressive formation of plaques. Atherosclerotic plaques are formed due to abnormal lipid accumulation and inflammatory responses within the intima-the arteries' most inner layer (*figure 37*). Eventually, the narrowing of the arterial lumen and subsequent restriction of the blood flow lead to an impairment of downstream tissue function. Another scenario would be that atherosclerotic plaques could suddenly rupture thus triggering thrombosis[253,254].

Many risk factors contribute to atherosclerosis and its related afflictions, the most important of which is elevated plasma levels of LDL and VLDL[255]. In fact, for two decades now, the mice models used to study the development of atherosclerotic lesions are mice deficient in genes encoding the apolipoprotein E (ApoE) or encoding the low-density lipoprotein (LDL) receptor, since they are susceptible to atherosclerosis and develop advanced lesions[253]. Thus, any factor that may contribute to the abundance of plasma lipoproteins is considered a risk factor, may it be of genetic component (e.g. mutations in the ApoE[256] or lipoprotein lipase genes[257]), or environmental factors (e.g. high-fat diet, a sedentary lifestyle)[255]. Other risk factors include the increasing lifespan, hypertension, diabetes and obesity (metabolic syndrome), smoking, gender (males are at a higher risk) and low plasma levels of high-density lipoproteins (HDL)[255,258,259,260,261]. In fact, the protective role of HDL can be attributed to its ability to induce cholesterol efflux in macrophages through ABCA1 and ABCG1(ATP-binding cassette transporters), resulting in anti-inflammatory and anti-apoptotic effects[262].

Atherogenesis is a multistep process that involves endothelial dysfunction, low-density lipoproteins oxidation, inflammation, and oxidative stress *(Fig 38)*.

2. The process of atherogenesis.

Lesion initiation.

The regions where the arteries form curvatures and branching are particularly vulnerable to fluid shear stress, a powerful physical force that can affect the permeability of endothelial cells of the intima to lipoproteins and it is most commonly in these regions that atherosclerotic lesions are initiated with the accumulation of LDL in the subendothelial matrix[263].

LDL Oxidation and Endothelial Dysfunction

Next, accumulated LDL within the arterial wall undergo different modifications, among which, oxidation has been shown to be the most responsible for lesion formation[264,265]. These oxidized LDL species can then induce chronic inflammation by activating the adjacent endothelial cells to produce pro-inflammatory molecules. Endothelial dysfunction is also associated with the decrease of the bioavailability of the nitric oxide (NO), a vasodilator and an anti-atherogenic molecule[266].

Inflammation

Among the expressed pro-inflammatory molecules, adhesion molecules like P- and E-Selectins, ICAM1 (InterCellular Adhesion Molecule 1) and VCAM1 (Vascular Cell Adhesion Molecule 1), selectively expressed in areas prone to lesion formation, are crucial for the firm adhesion of monocytes to the inflamed endothelium, their slow rolling across its surface and finally their entry into the arterial wall by diapedesis between endothelial cells at their junctions[267,268]. Chemotactic factors such as MCP1 (Monocyte Chemoattractant Protein 1, also known as CCL2 for Chemokine Ligand 2) recruit circulating monocytes to the vessel wall, more specifically to the atherosclerotic lesions by providing a chemoattractant gradient[269]. Finally, secreted cytokine M-CSF (Macrophage Colony-Stimulating Factor) stimulates monocyte-macrophage differentiation and subsequent proliferation, leading the way to the next step of atherogenesis[270].

Oxidative Stress and Foam Cell Formation

Oxidative stress and inflammation share an intimate link and activated endothelial cells and macrophages perpetuate a pro-oxidative environment by continuously secreting ROS (Reactive Oxygen Species), that further oxidize LDL. In turn, modified LDL binds to macrophage scavenger receptors, the two main receptors being SR-A and CD36, which leads to LDL uptake and retention by the macrophage, transforming it into a foam cell. Eventually, all that is left of foam cells after their death are cell debris and a growing mass of extracellular lipids[271].

SMC Proliferation and Migration

Many cytokines and growth factors play a crucial role in the progression from early atherosclerotic lesions to atherosclerotic plaques, like TNF (Tumor Necrosis Factor), that assures a constant influx of inflammatory cells (B cells, T cells and macrophages) to the lesion[272] or IFN-gamma (interferon-gamma)[273] secreted by intimal macrophages, that stimulates the proliferation and migration of smooth muscle cells (SMC) from the media to the intima where they secrete their extracellular matrix, giving rise to a fibrous cap where the proliferated SMCs and their derived matrix enrobe a necrotic core of foam cells and a mass of acellular lipid debris[274,275].

Fibrous Plaque Rupture

One of the causes of atherosclerotic plaque instability is the degradation of the SMC-derived matrix by macrophage-secreted proteinases (e.g. collagenases, gelatinases, stromolysin, MMPs) which results in a thin fibrous cap (<200µm), making the plaques more vulnerable to rupturing. This is especially atherogenic since it permits a contact between the circulating blood and the macrophage-secreted pro-coagulant protein tissue factor, leading to thrombosis and its related cardiovascular accidents[273,275,276,277].

Calcification and Neovascularization

In advanced lesions, pericyte-like cells in the intima secrete a calcified matrix scaffold, along with hydroxyapatite accumulation. Differentiation of SMCs into osteoblast-like

cells and the expression of osteogenic proteins like osteocalcin is also observed, a process reminiscent of the process of bone formation[278].

Alongside calcification, neovascularization is also associated with an advanced thrombogenic lesion. However, these newly formed blood vessels in the plaque are fragile and susceptible to micro-hemorrhaging which is a potent inducer of SMC proliferation and migration[275].

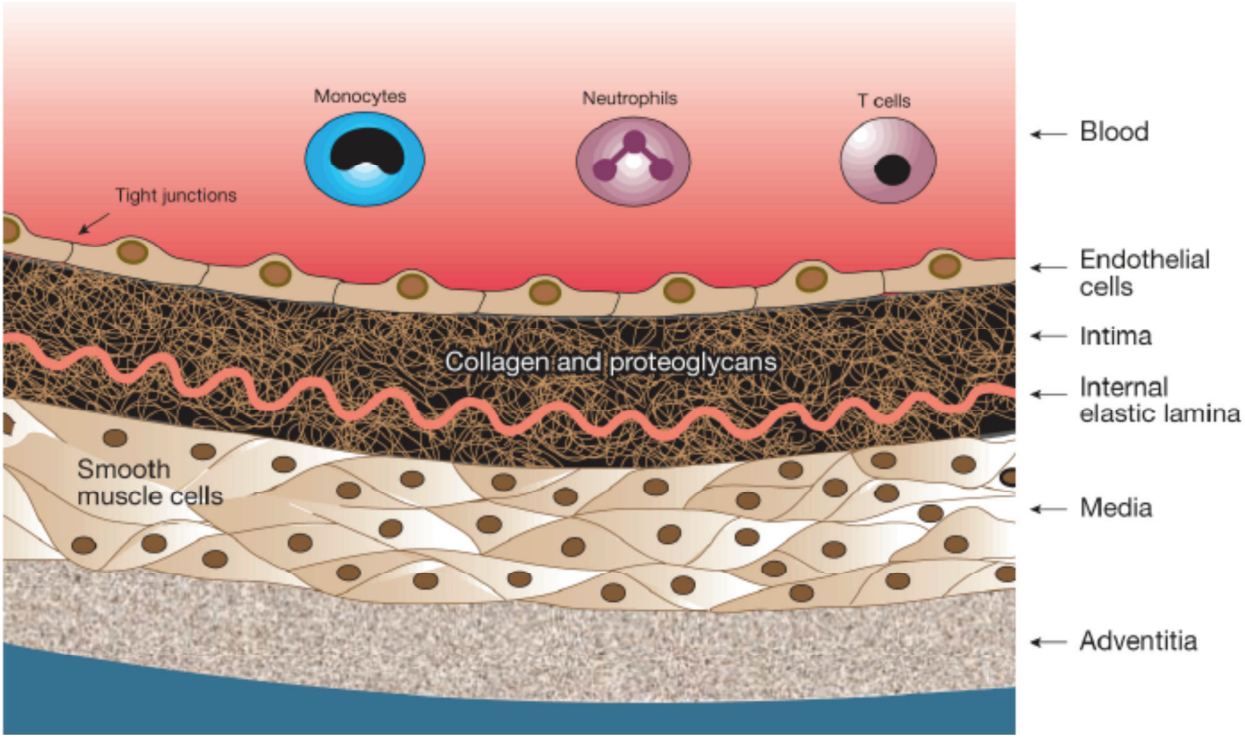


Figure 37: Structure of a normal artery.

The inner-most layer, the intima consists of a monolayer of endothelial cells on the luminal side based on the internal elastic lamina.
The lamina consists of collagen and proteoglycans.
The media, as its name suggests, is the middle layer that consists of SMCs.
The adventitia, the outer layer, consists of connective tissues made up of fibroblasts and SMCs[275].

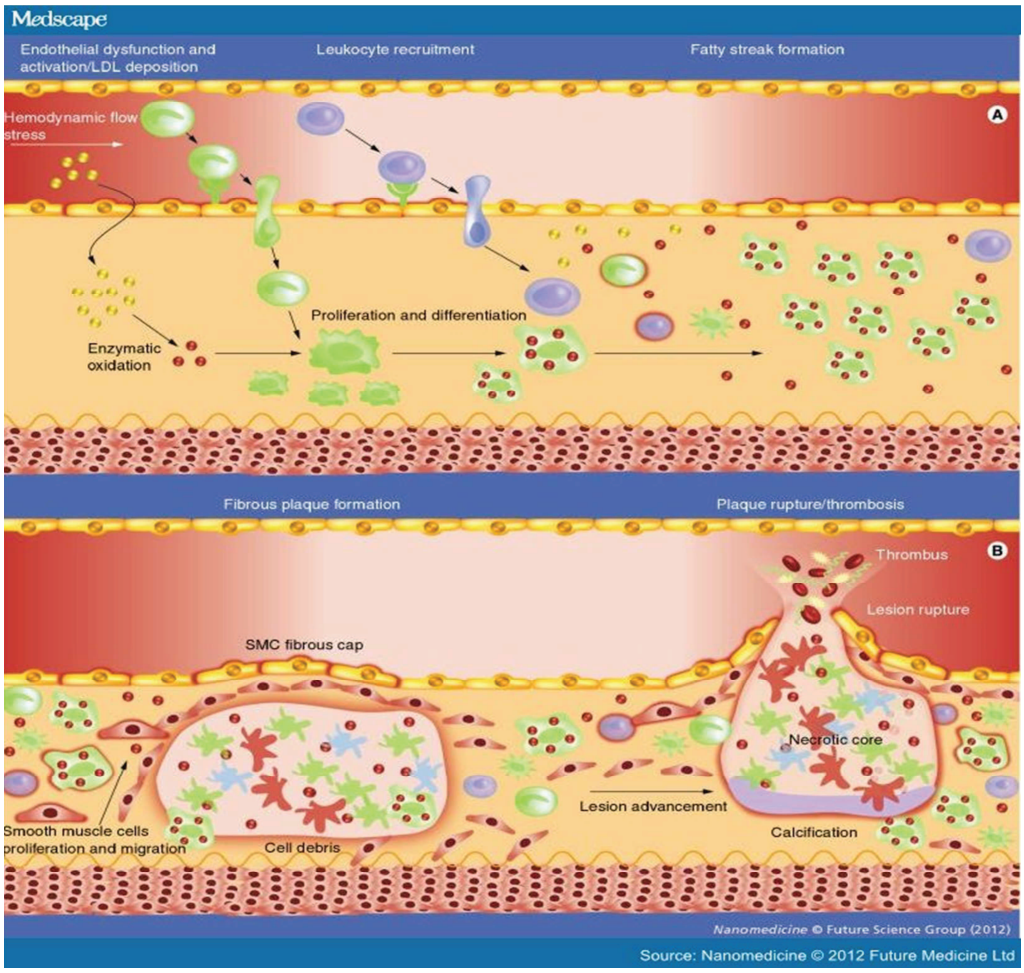


Figure 38: Stages of Atherogenesis.

(A). Increased endothelium permeability leads to low-density lipoprotein accumulation and subsequent oxidation. This is followed by endothelial cell activation with upregulated expression of proinflammatory cytokines, cellular adhesion molecules (VCAM-1, ICAM-1, P-and E-selectin) and chemokines involved in the recruitment of monocytes like MCP1. To a lower extent, lymphocytes are also recruited and internalized. The monocytes differentiate into macrophages, ingest the oxidized low-density lipoprotein and transform into foam cells. **(B)** Accumulated foam cells undergo apoptosis. Smooth muscle cells migrate from the media and proliferate thus forming a smooth muscle cell fibrous cap over the necrotic core of apoptotic foam cells and acellular lipids. The continuous inflammation and oxidative environment leads to lesion advancement, calcification below the necrotic core, release of matrix degrading enzymes and finally lesion rupture releasing the thrombus into the blood compartment[279].

3. Objectives of Chapter II:

Thrombosis leads to the release of thrombin and the activation of platelets. Activated platelets secrete both TGF-b (Transforming growth factor) responsible for collagen expression by SMCs and PDGF(Platelet-derived growth factor) that stimulates SMC proliferation and migration[253].

Our lab has previously demonstrated that the specific knock out of LRP1 in SMCs, increased the susceptibility to develop atherosclerotic plaques by upregulating both PDGF and TGF-b signaling[280,7]. A study in 2009 has shown that LRP1 also regulates ABCA1 expression and thus plays a central role in cholesterol efflux[281].

ShcA is a well-documented LRP1 binding partner[9] whose germ line deletion is lethal at embryonic day 12 due to severe cardiovascular dysfunction, demonstrating a crucial role for ShcA during development[18].

Moreover, the pro-oxidative environment drives the progression of atherosclerotic plates and the p66 isoform of ShcA is central in the cellular oxidative stress response[19].

This is further validated by the fact that mice lacking the p66 isoform exhibited reduced formation of atherosclerotic lesions when fed a high fat diet[282]. However, it remained unknown which vascular cell type was responsible for this atheroprotective effect.

Our lab has shown that ShcA deletion in SMCs did not have a substantial effect on atherosclerotic lesion development[283], in this part of the project, we aimed to investigate the role of endothelial ShcA in atherogenesis.

Using the Cre/lox system where the Cre Recombinase was put under the control of the Tie2 promoter, our lab generated KO mice that do not express ShcA specifically in endothelial cells (endoShcA-/-).

Our objectives were to:

_ Characterize the phenotype of EndoShcA-/- mice.

_ Determine the molecular mechanisms implicated in the development of atherosclerotic lesions.

4. Summary of the obtained results.

Atherosclerotic lesions, observed by Soudan IV staining, were two times smaller in endoShcA− mice fed a cholesterol-rich diet than in age-matched control mice (endoShcA+) and histological analysis showed almost no CD68-positive macrophage-foam cells accumulated in the aortas of endoShcA− mice, as well as less ICAM-1 expression in vascular endothelial cells than controls.

In-vitro, the deletion of the three isoforms of ShcA in endothelial cells leads to a decrease in the expression of the adhesion molecules ICAM1, VCAM1 and E-Selectin. This is concomitant with a decrease in the uptake of oxidized LDL by co-cultured stimulated macrophages and the subsequent formation of foam cells, as evidenced by Oil-Red-O staining.

The absence of ShcA induced the phosphorylation of the p65 subunit of NF-kB which suggests an alternate pathway controlling the expression of the adhesion molecules. When we investigated the involvement of ZEB1, an inhibitor of epithelial genes like E-cadherin, we found a marked increase in the nuclear translocation of ZEB1 when ShcA expression was repressed. Interestingly, down regulation of ZEB1 decreased both ShcA and ICAM-1 protein levels, demonstrating a feedback loop between ShcA and ZEB1.

Finally, in endothelial cells down regulated for ShcA we found an increase in p-Akt protein levels accompanied by a marked increase in eNOS phosphorylation. Immunohistochemistry analysis also showed that endoShcA- mice fed a cholesterol-rich diet expressed a larger amount of p-eNOS than control mice.

Article

SCIENTIFIC REPORTS

Correction: Author Correction

OPEN

Loss of the adaptor protein ShcA in endothelial cells protects against monocyte macrophage adhesion, LDL-oxydation, and atherosclerotic lesion formation

Received: 9 May 2017
Accepted: 6 December 2017
Published online: 14 March 2018

Antoine Abou-Jaoude[1], Lise Badiqué[1], Mohamed Mlih[1], Sara Awan[1], Sunning Guo[1], Alexandre Lemle[1], Clauda Abboud[1], Sophie Foppolo[1], Lionel Host[1], Jérôme Terrand[1], Hélène Justiniano[1], Joachim Herz[2], Rachel L. Matz[1] & Philippe Boucher[1]

ShcA is an adaptor protein that binds to the cytoplasmic tail of receptor tyrosine kinases and of the Low Density Lipoprotein-related receptor 1 (LRP1), a trans-membrane receptor that protects against atherosclerosis. Here, we examined the role of endothelial ShcA in atherosclerotic lesion formation. We found that atherosclerosis progression was markedly attenuated in mice deleted for ShcA in endothelial cells, that macrophage content was reduced at the sites of lesions, and that adhesion molecules such as the intercellular adhesion molecule-1 (ICAM-1) were severely reduced. Our data indicate that transcriptional regulation of ShcA by the zinc-finger E-box-binding homeobox 1 (ZEB1) and the Hippo pathway effector YAP, promotes ICAM-1 expression independently of p-NF-κB, the primary driver of adhesion molecules expressions. In addition, ShcA suppresses endothelial Akt and nitric oxide synthase (eNOS) expressions. Thus, through down regulation of eNOS and ZEB1-mediated ICAM-1 up regulation, endothelial ShcA promotes monocyte-macrophage adhesion and atherosclerotic lesion formation. Reducing ShcA expression in endothelial cells may represent an obvious therapeutic approach to prevent atherosclerosis.

Atherosclerosis involves multiple processes such as endothelial dysfunction, inflammation and cell proliferation. It coincides with subendothelial low-density lipoprotein (LDL) accumulation. The pro-oxidative environment favors oxidation of LDL and oxidized LDL (oxLDL) activate endothelial cells which overexpress adhesion molecules E-selectin, VCAM-1 and ICAM-1[1]. Thus, activation of these signaling pathways in endothelial cells is a key mechanism in the development of atherosclerotic lesions, and controlling endothelial dysfunction could reduce the progression of the disease.

ShcA is a cytosolic adaptor protein[2] that binds to the cytoplasmic tail of receptor tyrosine kinases (RTKs). Germ line deletion of the ShcA gene in mice leads to lethality at embryonic day 12, demonstrating an essential, but still undefined, role during development[3]. In adults and in embryos, ShcA regulates several important physiological processes. For instance, it signals in pathways such as IGF-I or PDGFβ, which are involved in proliferation/differentiation decisions[2,4–6]. These signals converge to Ras/MAP kinase and Akt/mTOR pathways. ShcA also binds to the tyrosine-phosphorylated form of the second NPxY motif within the tail of Low-density lipoprotein (LDL) receptor–Related Protein-1 (LRP1), an ubiquitously expressed transmembrane receptor that belongs to the LDL receptor gene family[7]. LRP1 is involved in lipoproteins endocytosis and in the control of intracellular signaling pathways. Mice lacking LRP1 in vascular smooth muscle cells (vSMCs) are characterized by a susceptibility to develop atherosclerosis. The lesions are associated with increased PDGFβ and TGFβ signaling that activate

[1]CNRS, UMR 7213, University of Strasbourg, 67401, Illkirch, France. [2]Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, TX, USA. Antoine Abou-Jaoude and Lise Badiqué contributed equally to this work. Philippe Boucher and Rachel L. Matz jointly supervised to this work. Correspondence and requests for materials should be addressed to R.L.M. (email: rachel.matz-westphal@unistra.fr) or P.B. (email: philippe.boucher@unistra.fr)

vSMCs proliferation[8], and decreased Wnt5a signaling that stimulates foam cell formation[9,10]. The PDGF receptor and LRP1 co-immunoprecipitate and LRP1 is a substrate for PDGF-dependent tyrosine kinases[8,11,12]. Thus, by binding to LRP1, ShcA might play an important role in atherosclerotic lesions development.

ShcA is expressed in the cardiovascular system early during embryogenesis and in adults, and controls heart development[3]. In the heart, by binding to integrins or dystrophin, it links the extracellular matrix (ECM) to the cytoskeleton and the contractile apparatus[2,13]. In the vascular wall, the role of ShcA is not well defined. The mammalian ShcA protein has 3 isoforms of 46, 52 and 66 kDa and previous studies showed that mice lacking the p66 isoform had reduced tissue oxidative stress, foam cell and early atherosclerotic lesion formation when fed a high fat diet[14]. However, the molecular and cellular mechanisms of this phenotype remain largely unknown. In particular, it does not indicate in which vascular cell type ShcA deletion would be atheroprotective. We previously reported that the deletion of ShcA in vSMCs did not modify the development of atherosclerotic lesions in mice fed an atherogenic diet[13]. Here, to study the role of ShcA in atherosclerosis and vascular remodeling, we suppressed its expression specifically in endothelial cells using the Cre/lox system.

Results

Specific deletion of ShcA in endothelial cells protects from atherosclerosis. We generated Tie2Cre+/ShcA$^{flox/flox}$ mice, in which ShcA is selectively ablated in endothelial cells, by inter-crossing Tie2Cre transgenic mice with floxed ShcA animals[13] (ShcA$^{flox/flox}$). To increase atherosclerosis susceptibility, Tie2Cre+/ShcA$^{flox/flox}$ animals were maintained on a LDL receptor-deficient background (LDLR−), fed an atherogenic diet, and are hereafter referred to as endoShcA-. Western blot analysis of ShcA in endothelial cells isolated from aortas of endoShcA- and endoShcA+ (control) mice confirmed the deletion of ShcA (Fig. 1A). Absence of ShcA expression in endothelial cells had no significant effect on plasma cholesterol (48.3 ± 10.1 mmol/l in endoShcA− mice vs 41.2 ± 3.3 mmol/l in controls) or triglyceride levels (2.5 ± 0.1 mmol/l in endoShcA− mice vs 2.9 ± 0.9 mmol/l in controls), in mice fed an atherogenic diet for 24 weeks. However, when fed an atherogenic diet atherosclerotic lesions were two times smaller in endoShcA− mice than in age-matched control mice (endoShcA+) as demonstrated by Soudan IV staining and *en face* analysis of the whole aortas (Fig. 1B,C), and histological analysis (Fig. 1D, top panels). The reduced atherosclerotic lesion size was similar in male and female mice (data not shown). During atherosclerosis, infiltration of foamy macrophages and vascular smooth muscle cells plays a crucial role. Whereas histological analysis of the arterial wall in large vessels such as thoracic aortas revealed an accumulation of CD-68-positive macrophage foam cells within the core of the atherosclerotic plaques in control mice (Fig. 1D, bottom panels), almost no CD68-positive macrophage-foam cells accumulated in endoShcA− aortas (Fig. 1D, bottom panels). This decreased number of macrophage foam cells in the atherosclerotic plaques indicates that ShcA expression in endothelial cells promotes CD-68-positive macrophage cell infiltration and/or foam cell formation.

Deletion of ShcA in endothelial cells protects from intracellular lipid accumulation and foam cell formation. To study the role of endothelial ShcA on intracellular lipid accumulation and foam cell formation, human endothelial cells (EA.hy 926 cell line) down regulated for p66, p52 and p46 ShcA isoforms and control cells were co-cultured in presence of THP-1 monocyte-derived macrophages stimulated with oxidized low-density lipoprotein to induce foam cell formation. Deletion of three isoforms of ShcA in endothelial cells significantly decreases the ox LDL uptake of macrophages and foam cell formation as evidenced by Oil-Red-O staining (Fig. 2A). Quantification analysis upon Oil-Red-O staining showed half neutral lipid accumulation in the absence of endothelial ShcA (Fig. 2B). These data indicate that endothelial ShcA promotes lipid accumulation in macrophages and foam cell formation.

Decrease of ICAM-1 expression in the absence of ShcA. Recruitment of monocytes and their endothelial cell adhesion occurs through intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin, secreted by inflamed or damaged endothelium. Among these, the key molecule ICAM-1, a member of the adhesion immunoglobulin super family[15], displays an important role in the development of atherosclerosis. For instance, deficiency in ICAM-1 was shown to disable monocyte–endothelial cell adhesion leading to reduce atherosclerotic lesion size in apoE−/− mice[16]. To test whether decreased accumulation of CD-68-positive foam cells was due to decreased expression of ICAM-1 in endothelial cells, we measured its mRNA and protein levels in EA.hy 926 endothelial cells down regulated for p66, p52 and p46 ShcA isoforms and in control cells. We found a marked decrease of ICAM-1 protein (Fig. 2C,D) and mRNA levels (Fig. 2E) in the absence of ShcA. VCAM-1 mRNA, and E-selectin mRNA and protein levels were also decreased (Fig. 2C–E).

Deletion of the p66 isoform of ShcA in endothelial cells is not sufficient to protect from intracellular lipid accumulation and foam cell formation. Since genetic deletion of the p66 isoform of ShcA reduces oxLDLuptake and early atherosclerotic plaque formation in apolipoprotein E-/- fed a high fat diet[14,17], we next wanted to test whether deletion of the p66 isoform in endothelial cells is sufficient to protect from intracellular lipid accumulation and foam cell formation. When EA.hy 926 endothelial cells down regulated for the p66 isoform of ShcA were co-cultured in presence of THP-1 monocyte-derived macrophages stimulated with oxidized low-density lipoprotein to induce foam cell formation, we found a modest decrease in foam cell formation as evidenced by Oil-Red-O staining (Fig. 3A). Quantification analysis showed a 20% decrease in neutral lipid accumulation in the absence of p66 ShcA (Fig. 3B). These results suggest that three isoforms of ShcA are required for efficient intracellular lipid accumulation and foam cell formation. We also tested whether deletion of the p66 ShcA isoform decreased expression of adhesion molecules. We found a marked decrease of ICAM-1 protein (Fig. 3C,D) and mRNA levels (Fig. 3E) in EA.hy 926 endothelial cells down regulated for the p66 isoform of ShcA compared to control cells. However, VCAM-1 protein levels were only moderately decreased, whereas VCAM-1

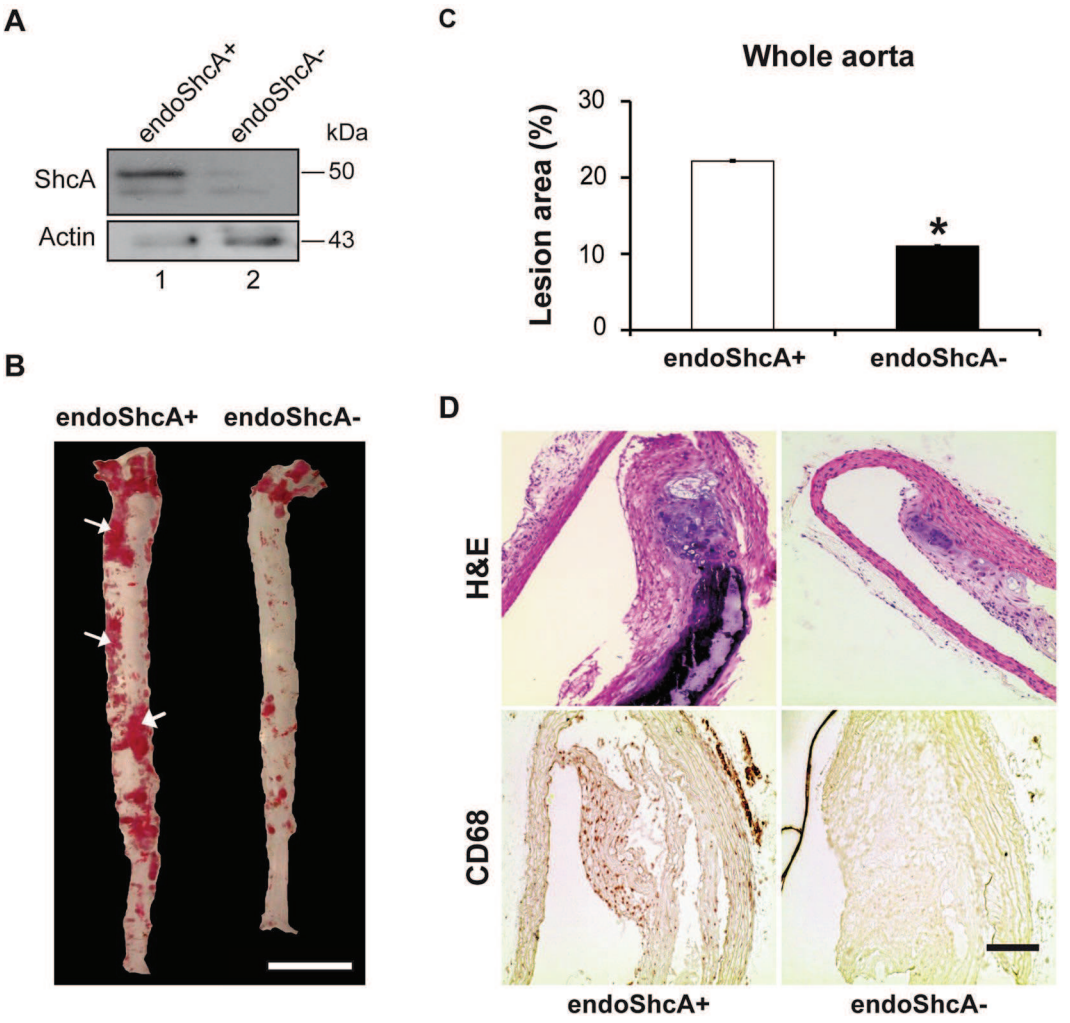


Figure 1. Absence of ShcA in endothelial cells protects from atherosclerotic lesion formation. Atherosclerosis in mice lacking ShcA in vascular endothelial cells and fed a cholesterol-rich diet. Western blotting of ShcA in endothelial cells isolated and pooled from aortas of endoShcA− and endoShcA+ mice (n = 5 mice for each genotype) **(A)**. Opened and Sudan IV-stained aortas from endoShcA− mice and controls (endoShcA+). Arrows show lipid-laden (Sudan-positive) atherosclerotic lesions; Scale bar, 0.5 cm **(B)**. Quantification of atherosclerotic lesion size in whole aortas from endoShcA− ($n = 5$) and control ($n = 5$) mice **(C)**. Hematoxylin and eosin (H&E) and CD68 staining of the lesions in thoracic aortas from endoShcA− and endoShcA+ mice. Scale bar, 20 μm **(D)**. Data are represented as mean ± SEM. *$P < 0.05$, two-tailed unpaired Student's t-test.

mRNA as well as E-selectin mRNA and protein levels remained unchanged (Fig. 3D,E). Thus, in endothelial cells p46, p52 and p66 ShcA play an important role in expression of adhesion molecules and in the interaction between endothelial cells and monocytes, leading to monocyte recruitment and subsequent development of atherosclerosis.

Increase in p-NF-κB expression in ShcA−/− endothelial cells. We next wanted to determine how ShcA regulates ICAM-1 expression in endothelial cells. The nuclear form of the NF-kappa B transcription factor (NF-κB) binds to DNA as a heterodimer of a 50 kDa (p50) and 65 kDa (p65) polypeptide[18]. Once phosphorylated, the transcription factor NF-κB is the major driver of *VCAM-1*, *ICAM-1* and *E-selectin* expression[18]. Surprisingly, treatment of endothelial cells with siRNA against ShcA triggered the phosphorylation of the p65 subunit of NF-κB (Fig. 4A,B) and increases the expression of its endogenous activator IKKβ (Fig. 4A,C)[19]. To determine whether NF-κB is activated in the absence of ShcA, we measured its nuclear translocation and activation of its target gene MCP1[20,21]. We found that in EA.hy 926 endothelial cells down regulated for ShcA (siShcA), p-NF-κB accumulated in the nucleus (Fig. 4D) and activated MCP1 (Fig. 4E) when compared to control cells (siCtrl). This suggests that, in the absence of ShcA regulation of adhesion molecules expression levels is independent of NF-κB.

Decrease in ShcA expression activates nuclear translocation of the transcription factor ZEB1 and decreases the nuclear translocation of YAP. It was previously reported that the transcription factor zinc-finger E-box-binding homeobox 1 (ZEB1) bound the ZEB1-binding sites of ShcA promoter in epithelial cells[22]. In addition, ZEB1 has at least one putative binding site on the p46/p52 isoforms promoter at the position

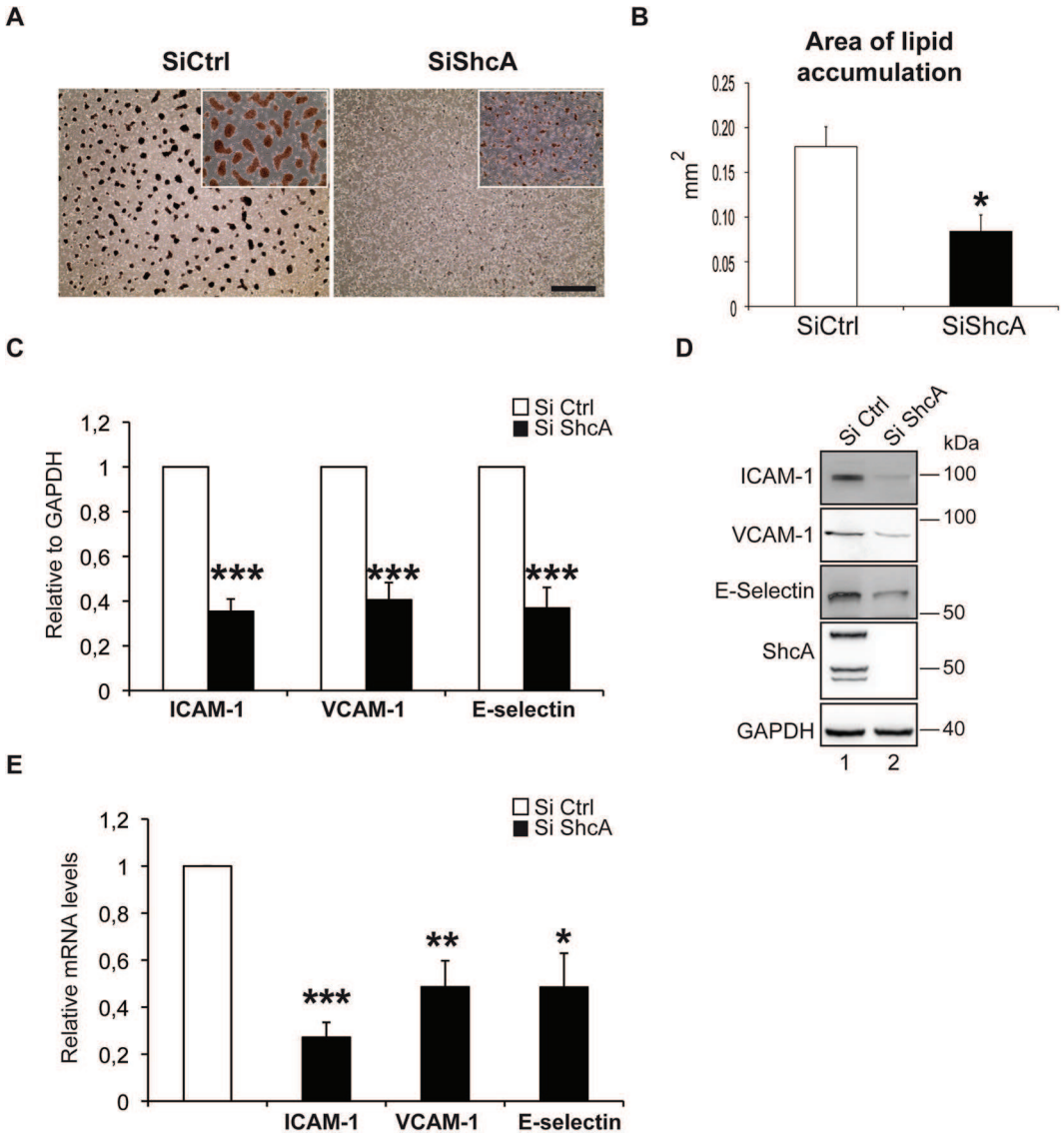


Figure 2. Expression of the three isoforms of ShcA in endothelial cells is required for foam cells formation, macrophage adhesion, and ICAM-1 expression. Accumulation of lipids in THP1-derived macrophages treated with oxidized LDL in the absence or presence of the three isoforms of ShcA in EA.hy 926 endothelial cells. Cells were stained with Oil/RedO. Representative microscopic fields. The subpanels on the right are higher magnification (2.5×) images. SiCtrl and SiShcA panels are same magnifications, and scale bare is 5 µm (**A**). Quantification of lipid accumulation upon Oil/RedO staining in THP1-derived macrophages treated with oxidized LDL in the absence (n = 5) or presence (n = 5) of ShcA in EA.hy 926 endothelial cells. Oil/RedO positive regions were manually outlined and the quantification of outlined regions was determined with Image J as described in the methods section (**B**). Quantification by western blot of indicated genes in EA.hy 926 endothelial cells down regulated for ShcA (siShcA) (n = 3) and in control cells (siCtrl) (n = 3) (**C**). Western blot analysis of ICAM-1, VCAM-1, E-Selectin, ShcA, and GAPDH expressions in EA.hy 926 endothelial cells down regulated for ShcA (siShcA) and in control cells (siCtrl) (**D**). mRNA of the indicated genes measured by Real Time PCR in EA.hy 926 endothelial cells down regulated for ShcA (siShcA) (n = 3) and in control cells (n = 3) transfected with siControl (siCtrl) (**E**). All data are represented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed unpaired Student's t-test.

−830 (Eukaryote promoter and GP miner databases). Thus, we next thought to assess the nuclear translocation of ZEB1 in EA.hy 926 endothelial cells treated with siShcA and in control cells. Using immuno-fluorescence (Fig. 5A) and cell fractionation experiments (Fig. 5B), we found, a marked increase in cytosolic expression and nuclear translocation of ZEB1 in siShcA-treated endothelial cells *versus* control cells indicating that ShcA is required for down regulation of ZEB1 expression. To evaluate the impact of reducing ZEB1 levels on ShcA and ICAM-1 expressions, we measured ShcA and ICAM-1 mRNA levels upon siZEB1 treatment. Interestingly, down regulation of ZEB1 decreased both ShcA and ICAM-1 mRNA and protein levels (Fig. 5C–D). Knockdown of ZEB1 similarly decreased p66 ShcA mRNA levels (data not shown). These data indicate that ShcA and ZEB1

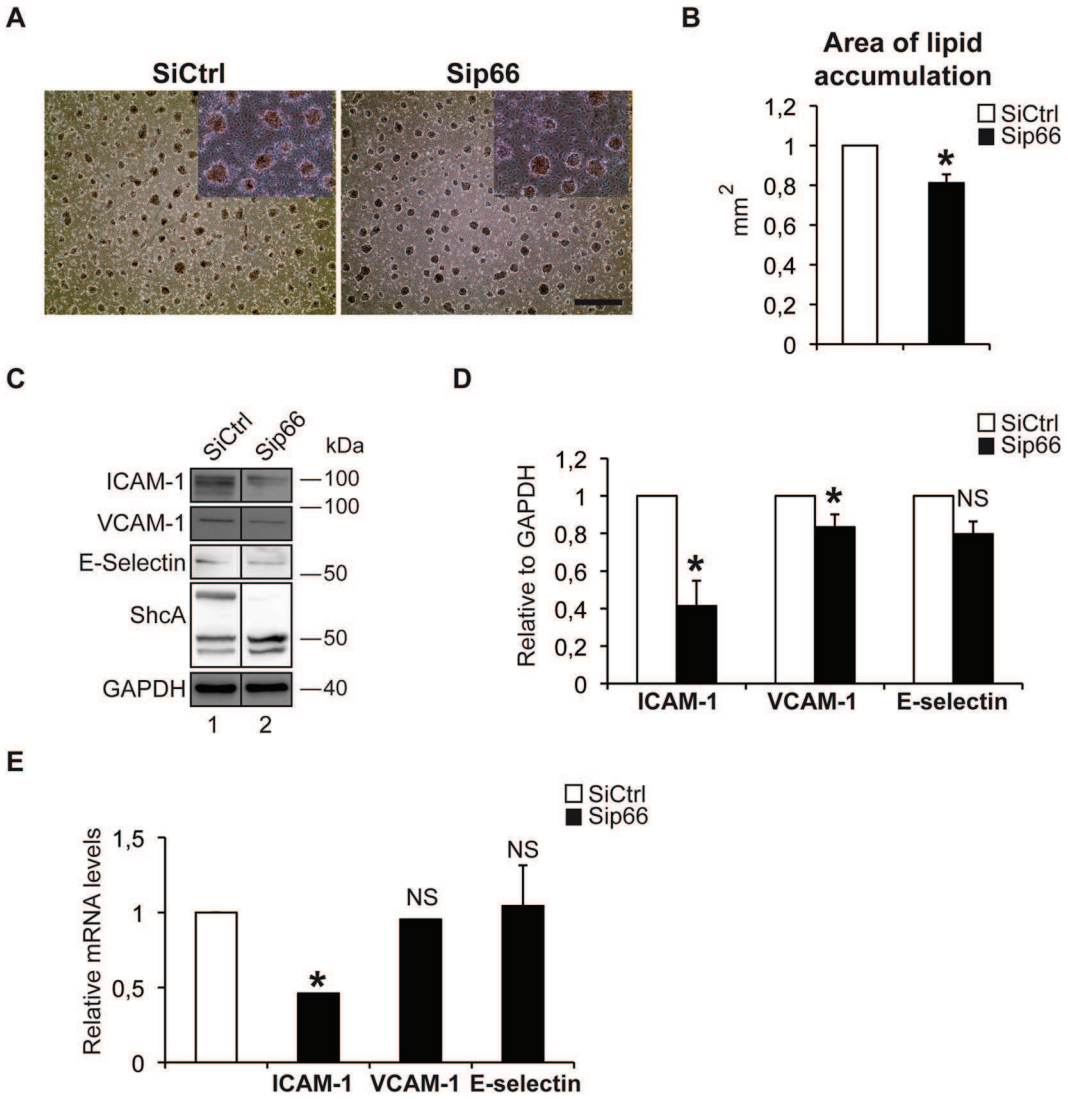


Figure 3. Deletion of p66ShcA down regulates ICAM-1 expression in endothelial cells, but does not decrease ox LDL uptake of THP-1 monocyte-derived macrophages and foam cell formation. Accumulation of lipids in THP1-derived macrophages treated with oxidized LDL in the absence or presence of p66ShcA in EA.hy 926 endothelial cells. Cells were stained with Oil/RedO. Representative microscopic fields. The subpanels on the right are higher magnification (2.5×) images. SiCtrl and p66SiShcA panels are same magnifications, and scale bare is 5 µm (**A**). Quantification of lipid accumulation upon Oil/RedO staining in THP1-derived macrophages treated with oxidized LDL in the absence (n = 5) or presence (n = 5) of p66ShcA in EA.hy 926 endothelial cells. Oil/RedO positive regions were manually outlined and the quantification of outlined regions was determined with Image J as described in the methods section (**B**). Western blot analysis (**C**) and quantification of western blot analysis of ICAM-1 in EA.hy 926 endothelial cells down regulated for p66ShcA and in controls (**D**). RT–PCR of the indicated genes in EA.hy 926 endothelial cells down regulated for p66ShcA and in controls (**E**). Error bars, s.e.m. $^*P < 0.05$, NS = non significant.

are required for ICAM-1 transcriptional regulation, whereas ShcA down regulates ZEB1 expression through a negative feedback mechanism. Mechanistically, ZEB1 function as a transcriptional repressor[23]. However, depending upon the recruitment of a different set of co-factors, direct transcriptional activation by ZEB1 has also been reported for a few target genes[24,25]. For instance, ZEB1 directly binds to YAP to stimulate transcription[24]. Since ShcA is required for nuclear translocation of YAP[26], ShcA might recruit YAP in the nucleus to switch ZEB1 from a repressor to a transcriptional activator, thereby up-regulating ICAM-1 expression. To test this, we performed cell fractionation assays in EA.hy 926 endothelial cells and found a decrease in nuclear expression of YAP in ShcA knockdown cells compared to controls (Fig. 5B). This strongly suggests that a ZEB1/YAP complex, most likely through binding to ShcA promoters, promotes ShcA-mediated up regulation of ICAM-1 expression.

Increased p-Akt that activates eNOS, decreases LDL oxidation and recruitment of monocytes. In endothelial cells, Akt can phosphorylate and activate endothelial nitric oxide synthase (eNOS) which

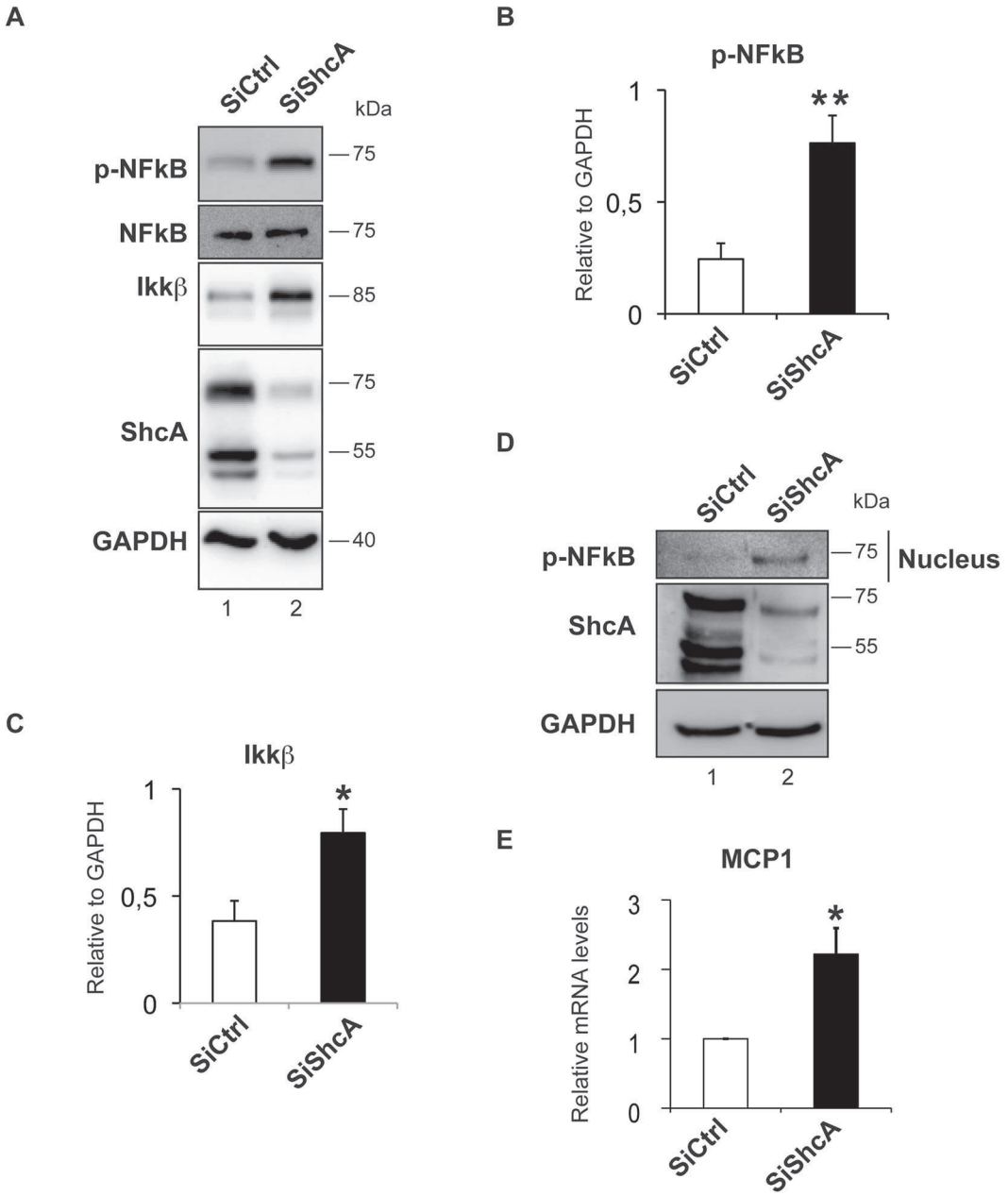


Figure 4. ShcA in endothelial cells triggers ICAM-1 expressions independently of NF-kB. Western blot analysis of the p65 subunit of NF-kB phosphorylated at the Ser536 residue (p-NF-kB), total NFkB p65 (NF-kB), IKKβ, ShcA and GAPDH in whole cell lysate from EA.hy 926 endothelial cells down regulated for the three isoforms of ShcA (siShcA) and in controls cells (siCtrl) **(A)**. Quantification by western blot of the p65 subunit of p-NF-kB protein levels ($n = 6$) **(B)** and of IKKβ protein levels ($n = 7$) in whole cell lysate **(C)**. Western blot measurement of p-NFkB nuclear accumulation in EA.hy 926 endothelial cells down regulated for ShcA (siShcA) compared to controls cells (siCtrl) (representative from n = 3 separated experiments) **(D)**. The increase in nuclear translocation of p-NFkB in EA.hy 926 endothelial cells down regulated for ShcA is accompanied by an increase in its target gene mRNA levels, MCP1 (n = 5 for each genotype) **(E)**. All data are represented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, two-tailed unpaired Student's t-test.

inhibits LDL oxidation[27]. As loss of eNOS activity is an established contributor to endothelial dysfunction[28] and endothelium-derived NO plays a vital role in the prevention of atherosclerosis[29], we explored whether absence of ShcA increased eNOS activation. In EA.hy 926 endothelial cells down regulated for ShcA we found an increase in p-Akt protein levels (Fig. 6A,B) and its target gene p-mTOR (Fig. 6A)[30], accompanied by a marked increased in eNOS phosphorylation (Fig. 6A,B). Densitometric analysis confirmed these data (Fig. 5B). Interestingly, immunohistochemistry analysis show that mice lacking ShcA in vascular endothelial cells (endoShcA−) and fed a cholesterol-rich diet expressed a larger amount of p-eNOS than control mice (Fig. 6C). Furthermore, they also expressed less ICAM-1 in vascular endothelial cells than controls (Fig. 6C). Thus, through inhibition of eNOS,

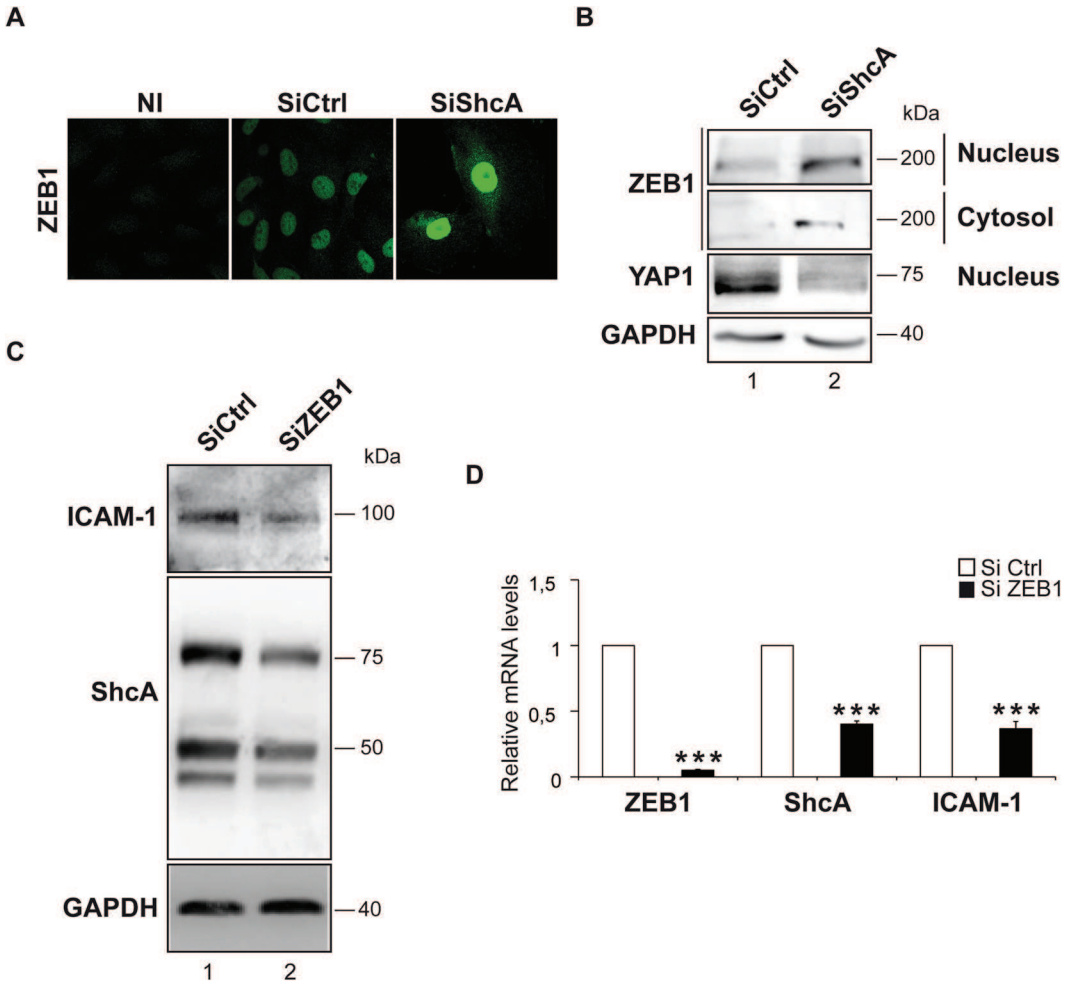


Figure 5. The absence of ShcA increased ZEB1 nuclear translocation and decreased YAP nuclear translocation. To follow accumulation of ZEB1 in the nucleus, EA.hy 926 endothelial cells down regulated for the three isoforms of ShcA (siShcA) and control cells (siCtrl) were labeled with anti-ZEB1 and analyzed by confocal images **(A)**. Western blot analysis of the indicated genes in cytosol and nuclear fractions from EA.hy 926 endothelial cells down regulated for the three isoforms of ShcA (siShcA) and control cells (siCtrl) ($n = 4$) **(B)**. Western blot analysis of ShcA and ICAM1 in whole cell lysates from EA.hy 926 endothelial cells down regulated for ZEB1 (siZEB1) ($n = 3$) and control cells ($n = 3$) (siCtrl) **(C)**. ZEB1, ShcA and ICAM1 mRNA levels analyzed by Real Time PCR in EA.hy 926 endothelial cells down regulated for ZEB1 (siZEB1) ($n = 3$) and control cells ($n = 3$) (siCtrl) **(D)**. NI, non-immun antibody. Data are represented as mean ± SEM. $^{***}P < 0.001$, two-tailed unpaired Student's t-test.

increased expression of ICAM-1, and increased LDL oxidation, and by promoting maladaptive monocyte-derived macrophage adhesion, endothelial ShcA contributes to atherosclerotic lesion formation.

Discussion

Our data indicate that three isoforms of endothelial ShcA protein play a pivotal role in the pathophysiology of atherosclerosis by triggering monocyte-derived macrophages infiltration and LDL oxidation. Previous genetic ablation studies suggested an important role for ShcA in the regulation of fat accumulation[31]. Other studies reported that constitutive mutation of p66Shc gene, one of the three isoforms encoded by the mammalian ShcA locus, decreased atherosclerotic lesion size in mice fed a high-fat diet[14,32]. Interestingly, lesions from p66 (Shc−/−) mice had less macrophage-derived foam cells than wild type mice, and decreased proatherogenic factors, including oxLDL, but, the mechanism was unknown. *In vitro*, p66 Shc is involved in oxLDL uptake[17]. Here, we report that all three isoforms of ShcA are required to decrease eNOS expression levels, to increase ICAM-1 expression, and promote cell adhesion molecules and atherosclerosis.

Our study also reveals a complex interaction between ShcA and ZEB1 that promotes adhesion molecules expression in EA.hy 926 endothelial cells. In absence of ShcA, ICAM-1 and other adhesion proteins are down regulated. Interestingly, we found that NF-κB, the primary driver of adhesion protein expression is upregulated in the absence of ShcA. This would suggest that ShcA upregulated adhesion protein expression through a mechanism independent of NF-κB. Since ZEB1 binds to the E-box consensus sequence CACCT present in the p66

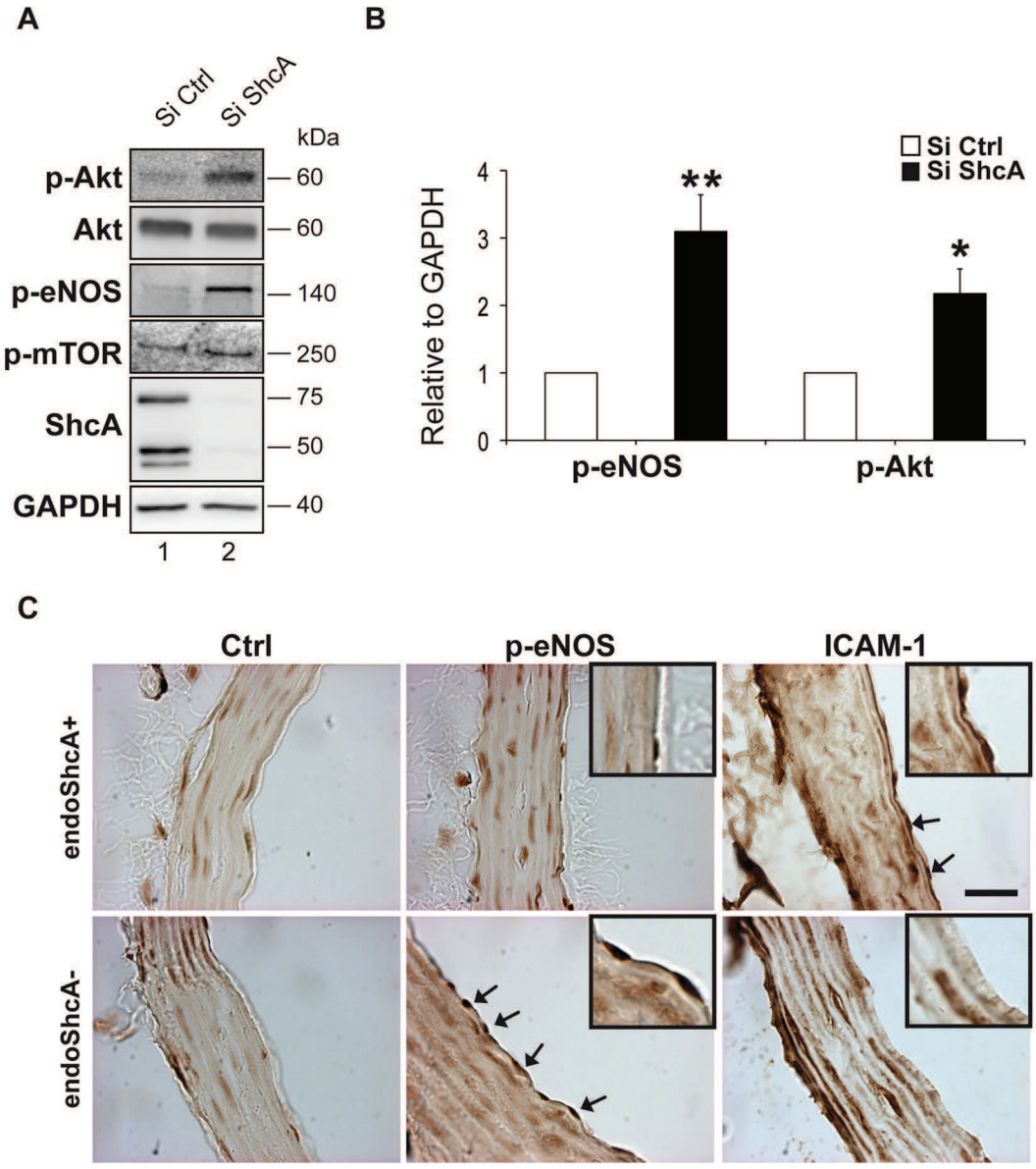


Figure 6. The decrease in ICAM-1 protein expression correlates with increased p-Akt and p-eNOS protein levels in endothelial cells deficient for ShcA. Western blot analysis of whole cell lysates from EA.hy 926 endothelial cells down regulated for the three isoforms of ShcA and in control cells using using phospho-eNOS (Ser1177) (p-eNOS), phospho-Akt (Ser473) (p-Akt), phospho-mTOR (Ser2448) (p-mTOR), Akt, ShcA, and GAPDH antibodies (**A**). Quantification of western blot analysis of p-eNOS and p-Akt in EA.hy 926 endothelial cells down regulated for ShcA and in controls ($n = 8$ separated experiments) (**B**). Representative immunohistochemistry experiment for expression of p-eNOS and ICAM-1 in mice lacking ShcA in vascular endothelial cells (endoShcA−) (n = 2) or control mice (endoShcA+) (n = 2) fed a cholesterol-rich diet as described in the method section. Decreased ShcA expression in endothelial cells of mice (endoShcA−) greatly increased expression of p-eNOS protein (arrows), as well as decreased expression of ICAM-1 (arrows) in these cells, compared to controls (endoShcA+). Non-immune antibodies (Ctrl), the subpanels on the right are higher magnification (2.5×) images of vascular endothelial cells. Scale bar, 20 μm (**C**). Data are represented as mean ± SEM. $*P < 0.05$, $**P < 0.01$, two-tailed unpaired Student's t-test.

promoter[22], and that such sequences are also present on the p46/p52 isoforms promoter, we tested whether ZEB1 activated ShcA in EA.hy 926 endothelial cells. We found that upon siZEB1 treatments, ShcA and ICAM-1 are markedly down regulated indicating that ZEB1 induces ShcA expression. In addition, ShcA knockdown is accompanied by an increase in ZEB1 expression levels and its nuclear localization in endothelial cells. This would represent an adaptive response to ShcA loss and a negative feedback loop between ShcA and ZEB1 in endothelial cells. Such a negative feedback mechanism was already described in lung epithelial cancer cells where p66Shc deficiency enhanced the expression of ZEB1 and consequently decreased E-cadherin[22].

ZEB1 is a transcriptional repressor of epithelial genes such as E-cadherin[23]. However, ZEB1 can also behave as an activator depending on its interaction with co-factors[24]. Indeed, by binding to the Hippo-pathway effector YAP (Yes-associated protein), ZEB1 switches from a repressor to an activator of transcription[24]. Here we found that in the absence of ShcA, YAP protein expression decreased in the nucleus. This strongly suggests that ShcA participate in the dual function of ZEB1. By promoting YAP nuclear translocation ShcA switches ZEB1 function from a repressor to an activator of transcription, thereby promoting ICAM-1 expression. However, we cannot exclude that interactions with other transcription factors may also confer the transcriptional co-activation by ZEB1.

Here, we also found that upon ShcA knockdown, the transcription factor NF-κB is induced and translocated to the nucleus. This would stimulate inflammation, recruitment of multiple immune cells including macrophages, and promote atherosclerosis. Since NF-κB increases ZEB1 nuclear translocation, which in turn behaves as a repressor of E-cadherin[33], similarly, in the absence of ShcA, NF-KB might repress ICAM-1 expression through increased ZEB1 nuclear translocation. Thus, whereas NF-κB stimulates inflammation, the increase in NF-κB can be not only limited to negative effects on atherosclerosis.

Previous studies reported that LDL cholesterol upregulates the expression of human endothelial p66Shc via hypomethylation of CpG dinucleotides in the p66Shc promoter[34], and that epigenetic upregulation of the p66Shc isoform of ShcA mediates increase in ICAM-1 expression[34]. This suggest that at least two independent mechanisms up regulate ShcA and the corresponding increase in ICAM-1 expression in endothelial cells, one involving hypomethylation of the promoter, another one involving transcriptional up regulation and nuclear translocation of ZEB1.

Endothelium-derived NO has also a crucial role in the local regulation of vascular homeostasis. A decrease in the bioavailability of NO aggravates the development of atherosclerotic lesions[35]. NO *per se* suppresses LDL oxidation and macrophage accumulation[36]. eNOS is an Akt target and the activation of eNOS by Akt is one of the most important physiological effects of Akt on cell attachment in endothelial cells[27]. Here we found that p-Akt is decreased when ShcA is expressed. This results by decreased eNOS levels, LDL oxidation, inflammation and atherosclerosis in mice.

In conclusion, we found that ShcA promotes a ZEB1-mediated increase of ICAM-1 expression, favor monocyte-derived macrophages adhesion, intracellular lipid accumulation and foam cell formation while simultaneously decreasing vascular NO production, events that would contribute to endothelial dysfunction commonly seen during atherosclerosis.

Methods

Mice. All animal experimentations and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of University of Strasbourg, France, and performed conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. C57/B6 mice carrying a ShcA allele into which loxP sites are integrated have been generated by gene targeting in embryonic stem cells. LoxP sites have been introduced upstream of exon 2 and downstream of exon 7 ($ShcA^{flox/flox}$)[13]. Cre-mediated recombination resulted in deletion of a 2-kb fragment containing the sequence encoding the PTB domain required for binding to phosphorylated receptors and for signaling activity. Endothelial cells specific p66, p52 and p46 ShcA inactivation was achieved by crossing transgenic mice carrying the Tie2-Cre transgene (a kind gift from Masashi Yanagisawa, University of Texas Southwestern Medical Center, at Dallas) with $ShcA^{flox/flox}$ mice. In order to increase susceptibility to spontaneous atherosclerotic lesion development, these animals were crossed to LDL receptor knock out mice (LDLR −/−). Genotyping of the wild type and ShcA mutant mice by polymerase chain reaction (PCR) was performed as described[37] using primers specific for ShcA (Primers available upon request). Animals were maintained on a 12-h light/12-h dark cycle. For *in vivo* analysis, 4 males and 1 female of three to four months old were used for each genotype, fed a Paigen diet during six months, and analyzed for atherosclerotic lesions as described previously[8]. For the isolation of tissue for further analysis, the agents used for euthanasia were ketamine (750 mg/kg) and xylazine (50 mg/kg), intraperitoneally.

Purification of lipoproteins. LDLs were isolated from South-American sourced Fetal Bovine serum by density gradient centrifugation. LDLs were dialyzed for 48 hours in a solution of NaCl 150 mM and 0.24 mM disodium EDTA, pH 7.4. LDLs were then centrifuged for 30 minutes, 4 °C at 10,000 RPM and filtered through a 0.22 µm filter. Concentrations were determined by the Lowry method.

Oxidation of LDLs. LDLs were added at a concentration of 600 µg/ml in RPMI 1640 medium supplemented with 10% South-American sourced FBS. Copper sulfide (SIGMA) was dissolved in RPMI medium, filtered through a 0.22 µm filter and added to the LDL solution to a final concentration of 10 µM. The resulting solution was incubated at 37 °C for 48 hours.

Cells. Aortic endothelial cells isolation: Thoracic and abdominal aortas were carefully dissected from 3 months old control and Tie2Cre+/ShcAflox/flox mice. The endothelial layer was removed immediately after dissection by intraluminal perfusion with 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1 propane sulphonate (CHAPS) in Physiological salt solution (PSS) for 20 s followed by repeated washing with PSS. The washed endothelial layers from 5 mice were collected, pooled and submitted to SDS-polyacrylamide gel electrophoresis and immunoblot analysis according to standard procedures.

Human EA.hy 926 endothelial cells (a kind gift from Dr Sophie Martin, UMR CNRS 7213, University of Strasbourg) were seeded in 6-well-plates at 120.000 cells/well in DMEM medium supplemented with 10% FBS and 2 mM L-Glutamine, and were placed in a 37 °C incubator with humidified atmosphere containing 5% CO2 as described[38,39]. 24 hours after seeding, cells were transfected with either scrambled siRNA, siRNA against p66, p52 and p46 isoforms of ShcA (Dharmacon) or siRNA against the p66 isoform of ShcA[40] (Dharmacon, custom siRNA,

5′-GAAUGAGUCUCUGUCAUCGUU-3′) at a final concentration of 100 nM using lipofectamine 3000. 48 hours post-transfection, DMEM medium was replaced with the LDL oxidation solution containing THP-1 (a kind gift from Prof Florence Toti, UMR CNRS 7213, University of Strasbourg) Macrophage cells at 2.10^6 cells/well. Phorbol myristate acetate (PMA) was added at a concentration of 50 ng/ml to induce macrophage differentiation. Cells were then incubated at 37 °C, 5%CO_2 for 48 hours.

Control EA.hy 926 endothelial cells transfected with scrambled siRNAs or siShcA were incubated in the same conditions but in a medium that did not contain oxidized LDLs. At the same time, 2.10^6 cells/well THP-1 cells were incubated in the same conditions for 48 hours and used as positive control. 48 hours later, wells were stained using Oil/RedO staining. We controlled SiRNA transfection efficiency using Western Blotting and/or Real Time PCR. Each experiment was repeated 5 times for statistical significance. For quantitative analysis, images taken through the microscope were processed using ImageJ. Regions formed by the accumulation of foam cells were manually outlined and the quantification of outlined region was determined by ImageJ.

Lipid Staining. The cells were fixed with 10% Paraformaldehyde for 30 minutes, washed twice with phosphate-buffered saline (PBS) buffer, pH = 7.4, and stained with a saturated concentration of oil red O for a minimum of 30 minutes. Cells were then washed twice with PBS buffer and LDL accumulation by THP1 cells was observed under microscopy; representative images of whole wells were taken for further analysis.

Gene expression analysis. RNA was isolated using TRIzol reagent (Sigma, St Louis, Mo) according to the manufacturer's instructions. 50 ng of RNA were converted to cDNA using the High-capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). PCR amplification was performed using SYBRGreen PCR master mix (Kappa biosystems, Wilmington, MA) according to the manufacturer's instructions. Primers sequences are available upon request.

Histology experiments. For immunostaining and histology experiments, mice were transcardially perfused with a 4% paraformaldehyde solution in phosphate buffered saline. Entire aortas were fixed with 4% paraformaldehyde in phosphate-buffered saline, embedded in paraffin, and cut in 5 µm slices as described[37]. Sections were stained with hematoxylin and eosin as described[37].

Western blot. Whole cell extracts were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane using a transfer apparatus according to the manufacturer's protocols (Bio-Rad). After incubation with 5% BSA TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 60 min at room temperature, the membrane was incubated at 4 °C overnight with primary antibodies directed against ShcA (Upstate), ICAM-1 (Abcam), VCAM-1 (Abcam), CD62E (E-selectin) (Abcam), NFkB p65 (Cell Signaling Technology), p-NFkB p65 (S536) (Cell Signaling Technology), Ikkbeta (Cell Signaling Technology), p-eNOS (S1177) (Cell Signaling Technology), P-mTOR (S2448) (Cell Signaling Technology), AREB6 (ZEB1) (Abcam), YAP1 (Santa Cruz), p-Akt (S473) (Cell Signaling Technology), Akt (Cell Signaling Technology), Actin (Sigma, St Louis, Mo) or GAPDH (Sigma, St Louis, Mo). Membranes were then washed five times for 5 min and incubated with a 1:10000 dilution of horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Santa Cruz) for 1 h at room temperature. Blots were washed with TBST five times and developed with the ECL system according to the manufacturer's protocols (BIO RAD Clarity™ Western ECL Substrate). Clarity™ western ECL substrate allowed visualization of protein expression using ImageQuant™ LAS 4000 Imaging System (Amersham). Optical densitometry was performed with Adobe PhotoshopCS and Image J normalizing bands intensity for GAPDH.

Cell fractionation. For cell fractionation, monolayers of human endothelial cells (EAhy) were transfected with either scramble siRNA or siRNA against ShcA (Dharmacon) (100 nM). 48 hours post-transfection, cells were then fractionated as previously described[41].

Confocal Microscopy. EAhy cells were seeded on glass slides, 24 hours later, they were treated with either scramble siRNA or siRNA against ShcA. 48 hours post-transfection, the cells were fixed with paraformaldehyde, and incubated with anti-ZEB1 or anti-IgG control primary antibodies and Alexa Fluor 488 secondary antibodies. Immunofluorescence-labeled cells were analyzed using a Leica TSC SPE confocal microscope with the ×63 oil immersion objective.

Statistical analysis. Values are reported as mean ± SEM of at least triplicate determinations. Statistical significance ($P < 0.05$) was determined using an unpaired Student's *t* test (GraphPad Prism, *Abacus Concepts, Berkeley, CA*). P-values < 0.05, < 0.01, and < 0.001 are identified with 1, 2, 3 asterisks, respectively. ns: $p > 0.05$.

References

1. Mestas, J. & Ley, K. Monocyte-endothelial cell interactions in the development of atherosclerosis. *Trends in cardiovascular medicine* **18**, 228–232 (2008).
2. Wills, M. K. & Jones, N. Teaching an old dogma new tricks: twenty years of Shc adaptor signalling. *The Biochemical journal* **447**, 1–16 (2012).
3. Lai, K. M. V. & Pawson, T. The ShcA phosphotyrosine docking protein sensitizes cardiovascular signaling in the mouse embryo. *Gene Dev* **14**, 1132–1145 (2000).
4. Demoor, M. *et al*. Cartilage tissue engineering: molecular control of chondrocyte differentiation for proper cartilage matrix reconstruction. *Biochimica et biophysica acta* **1840**, 2414–2440 (2014).
5. Wang, Y. *et al*. IGF-1R signaling in chondrocytes modulates growth plate development by interacting with the PTHrP/Ihh pathway. *Journal of bone and mineral research: the official journal of the American Society for Bone and Mineral Research* **26**, 1437–1446 (2011).
6. Jonquoy, A. *et al*. A novel tyrosine kinase inhibitor restores chondrocyte differentiation and promotes bone growth in a gain-of-function Fgfr3 mouse model. *Human molecular genetics* **21**, 841–851 (2012).

7. van de Sluis, B., Wijers, M. & Herz, J. News on the molecular regulation and function of hepatic low-density lipoprotein receptor and LDLR-related protein 1. *Curr Opin Lipidol* (2017).
8. Boucher, P., Gotthardt, M., Li, W. P., Anderson, R. G. W. & Herz, J. LRP: Role in vascular wall integrity and protection from atherosclerosis. *Science* **300**, 329–332 (2003).
9. Woldt, E. *et al*. The nuclear hormone receptor PPAR gamma counteracts vascular calcification by inhibiting Wnt5a signalling in vascular smooth muscle cells. *Nat Commun* **3** (2012).
10. Terrand, J. *et al*. LRP1 Controls Intracellular Cholesterol Storage and Fatty Acid Synthesis through Modulation of Wnt Signaling. *Journal of Biological Chemistry* **284**, 381–388 (2009).
11. Loukinova, E. *et al*. Platelet-derived growth factor (PDGF)-induced tyrosine phosphorylation of the low density lipoprotein receptor-related protein (LRP) - Evidence for integrated co-receptor function between LRP and the PDGF. *Journal of Biological Chemistry* **277**, 15499–15506 (2002).
12. Boucher, P. *et al*. Platelet-derived growth factor mediates tyrosine phosphorylation of the cytoplasmic domain of the low density lipoprotein receptor-related protein in caveolae. *Journal of Biological Chemistry* **277**, 15507–15513 (2002).
13. Mlih, M. *et al*. The Src Homology and Collagen A (ShcA) Adaptor Protein Is Required for the Spatial Organization of the Costamere/Z-disk Network during Heart Development. *Journal of Biological Chemistry* **290**, 2419–2430 (2015).
14. Napoli, C. *et al*. Deletion of the p66Shc longevity gene reduces systemic and tissue oxidative stress, vascular cell apoptosis, and early atherogenesis in mice fed a high-fat diet. *Proc Natl Acad Sci USA* **100**, 2112–2116 (2003).
15. Hopkins, A. M., Baird, A. W. & Nusrat, A. ICAM-1: targeted docking for exogenous as well as endogenous ligands. *Adv Drug Deliv Rev* **56**, 763–778 (2004).
16. Nakashima, Y., Raines, E. W., Plump, A. S., Breslow, J. L. & Ross, R. Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. *Arterioscl Throm Vas* **18**, 842–851 (1998).
17. Shi, Y. *et al*. Oxidized low-density lipoprotein activates p66Shc via lectin-like oxidized low-density lipoprotein receptor-1, protein kinase C-beta, and c-Jun N-terminal kinase kinase in human endothelial cells. *Arterioscler Thromb Vasc Biol* **31**, 2090–2097 (2011).
18. Chen, C. C., Rosenbloom, C. L., Anderson, D. C. & Manning, A. M. Selective inhibition of E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 expression by inhibitors of I kappa B-alpha phosphorylation. *J Immunol* **155**, 3538–3545 (1995).
19. Israel, A. The IKK complex, a central regulator of NF-kappaB activation. *Cold Spring Harb Perspect Biol* **2**, a000158 (2010).
20. Ishikado, A. *et al*. Soy phosphatidylcholine inhibited TLR4-mediated MCP-1 expression in vascular cells. *Atherosclerosis* **205**, 404–412 (2009).
21. Xing, L. & Remick, D. G. Promoter elements responsible for antioxidant regulation of MCP-1 gene expression. *Antioxid Redox Signal* **9**, 1979–1989 (2007).
22. Li, X. *et al*. Negative feedback loop between p66Shc and ZEB1 regulates fibrotic EMT response in lung cancer cells. *Cell Death Dis* **6**, e1708 (2015).
23. Vandewalle, C., Van Roy, F. & Berx, G. The role of the ZEB family of transcription factors in development and disease. *Cell Mol Life Sci* **66**, 773–787 (2009).
24. Lehmann, W. *et al*. ZEB1 turns into a transcriptional activator by interacting with YAP1 in aggressive cancer types. *Nat Commun* **7**, 10498 (2016).
25. Gheldof, A., Hulpiau, P., van Roy, F., De Craene, B. & Berx, G. Evolutionary functional analysis and molecular regulation of the ZEB transcription factors. *Cell Mol Life Sci* **69**, 2527–2541 (2012).
26. Wu, R. F. *et al*. p66Shc couples mechanical signals to RhoA through FAK-dependent recruitment of p115-RhoGEF and GEF-H1. *Mol Cell Biol* (2016).
27. Dimmeler, S. *et al*. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* **399**, 601–605 (1999).
28. Heitzer, T., Schlinzig, T., Krohn, K., Meinertz, T. & Munzel, T. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. *Circulation* **104**, 2673–2678 (2001).
29. Knowles, J. W. *et al*. Enhanced atherosclerosis and kidney dysfunction in eNOS(−/−)Apoe(−/−) mice are ameliorated by enalapril treatment. *J Clin Invest* **105**, 451–458 (2000).
30. Wullschleger, S., Loewith, R. & Hall, M. N. TOR signaling in growth and metabolism. *Cell* **124**, 471–484 (2006).
31. Woldt, E. *et al*. Differential signaling by adaptor molecules LRP1 and ShcA regulates adipogenesis by the insulin-like growth factor-1 receptor. *The Journal of biological chemistry* **286**, 16775–16782 (2011).
32. Martin-Padura, I. *et al*. p66Shc deletion confers vascular protection in advanced atherosclerosis in hypercholesterolemic apolipoprotein E knockout mice. *Endothelium* **15**, 276–287 (2008).
33. Chua, H. L. *et al*. NF-kappaB represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. *Oncogene* **26**, 711–724 (2007).
34. Kim, Y. R. *et al*. Epigenetic upregulation of p66shc mediates low-density lipoprotein cholesterol-induced endothelial cell dysfunction. *Am J Physiol Heart Circ Physiol* **303**, H189–196 (2012).
35. Moroi, M. *et al*. Interaction of genetic deficiency of endothelial nitric oxide, gender, and pregnancy in vascular response to injury in mice. *J Clin Invest* **101**, 1225–1232 (1998).
36. Ahmed, A. *et al*. Angiopoietin-2 confers Atheroprotection in apoE−/− mice by inhibiting LDL oxidation via nitric oxide. *Circ Res* **104**, 1333–1336 (2009).
37. Boucher, P., Gotthardt, M., Li, W. P., Anderson, R. G. & Herz, J. LRP: role in vascular wall integrity and protection from atherosclerosis. *Science* **300**, 329–332 (2003).
38. Schmitz, B. *et al*. Increased monocyte adhesion by endothelial expression of VCAM-1 missense variation *in vitro*. *Atherosclerosis* **230**, 185–190 (2013).
39. Wang, Y. *et al*. An extract from medical leech improve the function of endothelial cells *in vitro* and attenuates atherosclerosis in ApoE null mice by reducing macrophages in the lesions. *Biochem Biophys Res Commun* **455**, 119–125 (2014).
40. Kisielow, M., Kleiner, S., Nagasawa, M., Faisal, A. & Nagamine, Y. Isoform-specific knockdown and expression of adaptor protein ShcA using small interfering RNA. *Biochem J* **363**, 1–5 (2002).
41. El Asmar, Z. *et al*. Convergent Signaling Pathways Controlled by LRP1 (Receptor-related Protein 1) Cytoplasmic and Extracellular Domains Limit Cellular Cholesterol Accumulation. *The Journal of biological chemistry* **291**, 5116–5127 (2016).

Acknowledgements

We are grateful to Daniel Metzger (IGBMC, University of Strasbourg) for critical reading of the manuscript, Véronique Bruban (UMR CNRS 7213, University of Strasbourg) for technical assistance, Masashi Yanagisawa (University of Texas Southwestern Medical Center, at Dallas) for kindly providing us with the Tie2Cre mice, Sophie Martin (UMR CNRS 7213, University of Strasbourg) for Human endothelial cells (EAhy), Florence Toti (UMR CNRS 7213, University of Strasbourg) for providing us with THP-1 cells, and Irwin Davidson (IGBMC, University of Strasbourg) for YAP antibodies. This work was supported by grants from Fondation de France, Fondation pour la Recherche Médicale (FRM), the Agence Nationale de la Recherche (ANR-06-Physio-032-01

and ANR-09-BLAN-0121-01), National Institutes of Health Grants HL063762, NS093382 and AG053391 (to J.H.), the Consortium for Frontotemporal Dementia Research, and the Bright Focus Foundation.

Author Contributions

P.B. and R.L.M. designed the project and supervised the entire study. P.B. wrote the manuscript. A.A.J. and L.B. conducted most of the *in vivo* studies and all the *in vitro* studies, gene expression and immunostaining assays, and revised the manuscript, R.L.M. created the colony of endoShcA mice, conducted the Paigen *in vivo* study, and with J.H. participated in the discussion of the results and revised the manuscript, M.M., L.H., S.A., S.G., A.L., C.A., and H.J. performed the quantification of lipids in the plasma, the immunostaining, RT-PCR and western blots analysis, J.T. performed some immunostaining assays and gene expression assays and contributed to the design of the project, S.F. participated in the *in vitro* studies and J.H. critically revised the manuscript.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-22819-3.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Résumé de la thèse en français

Rôle de ShcA dans l'Athérosclérose et dans la Différenciation des Chondrocytes

L'athérosclérose implique différents processus, tels que le dysfonctionnement endothélial, l'inflammation, la prolifération cellulaire et, au cours des stades avancés, la calcification vasculaire. L'équipe du Pr. Philippe Boucher a montré que LRP1 (low density lipoprotein receptor-related protein-1) protège contre l'athérosclérose[156]. De plus, la délétion de PPAR gamma dans les cellules musculaires lisses (CML) active une voie de signalisation LRP1/Wnt pro-chrondrogénique et favorise la calcification vasculaire[8]. Afin de mieux comprendre comment LRP1 protège contre l'athérosclérose et participe à la différentiation chondrocytaire, le laboratoire d'accueil s'intéresse à ses partenaires protéiques. Lorsqu'il est phosphorylé sur sa partie cytoplasmique au niveau de son deuxième motif NPxY, LRP1 recrute la protéine adaptatrice ShcA[285]. ShcA joue un rôle essentiel dans l'activation de plusieurs voies de signalisation notamment les récepteurs tyrosine kinases (RTKs) comme les récepteurs à l'IGF1, TGF-beta, PDGF, FGF, EGF… les intégrines, Src, et FAK. Toutes ces voies sont impliquées dans les processus de prolifération/différenciation cellulaire et dans la biologie des chondrocytes. Ces signaux convergent notamment vers les voies Ras/MAP kinase et Akt/mTOR[26, 147, 286]. Les objectifs de mon projet de thèse étaient de caractériser le rôle de ShcA dans l'athérosclérose et de ShcA et LRP1 dans la différenciation chondrocytaire.

Rôle de ShcA dans l'athérosclérose

La suppression du gène ShcA chez la souris est létale au 12e jour d'embryogenèse, démontrant ainsi un rôle essentiel de ce gène au cours du développement[18]. ShcA régule plusieurs processus physiologiques importants dont le développement cardiaque. Dans le coeur, en se liant aux intégrines ou à la dystrophine, ShcA relie la matrice extracellulaire (ECM) au cytosquelette et à l'appareil contractile[283]. Cependant, dans la paroi vasculaire, le rôle de ShcA n'est pas clairement défini.

L'athérosclérose est notamment caractérisée par la formation de lésions lipidiques initiée par l'accumulation sous-endothéliale des LDL (Low Density Lipoproteins).

L'oxydation des LDL accumulés induit l'activation des cellules endothéliales qui expriment des gènes pro-inflammatoires ainsi que des molécules d'adhésion. Parmi ces molécules, E-sélectine, VCAM-1 et ICAM-1 ont un rôle primordial dans le recrutement et l'accumulation de monocytes au niveau sous-endothélial[254]. L'un des objectifs de ma thèse était d'étudier le rôle de ShcA dans le développement de l'athérosclérose.

Le laboratoire a supprimé l'expression de ShcA spécifiquement dans les cellules endothéliales en utilisant le système Cre/lox dans lequel la Cre recombinase est sous le contrôle du promoteur Tie2. Pour augmenter la sensibilité à l'athérosclérose, ces animaux étaient croisés avec des animaux KO pour les récepteurs aux LDL (LDLR−) et ont été soumis à un régime pro-athérogène.

Les premiers résultats ont montré que les lésions d'athérosclérose étaient deux fois plus petites dans les aortes des souris KO que dans celles des souris témoins. Les résultats des tests d'immunohistochimie ont montré que cela était corrélé à une diminution de l'infiltration des macrophages CD68-positifs.

Au cours de mon doctorat, nous avons mis en place un modèle *in-vitro* qui mimait le phénomène d'accumulation de macrophages et la formation de cellules spumeuses. Nous avons constaté que la suppression des trois isoformes de ShcA dans les cellules endothéliales protège contre l'accumulation lipidique intracellulaire et la formation de cellules spumeuses. Les cellules n'exprimant pas ShcA présentent également une diminution de l'expression d'ICAM-1, de VCAM-1 et d'E-sélectine. Similairement *In-vivo,* les souris KO nourries avec un régime riche en cholestérol présentent une diminution de l'expression d'ICAM- 1 dans les cellules endothéliales vasculaires que les contrôles. Ainsi, en induisant l'expression de ces molécules d'adhésion, ShcA favorise l'accumulation des macrophages. Pour comprendre comment ShcA régule l'expression d'ICAM-1, nous nous sommes intéressés au facteur de transcription NF-κB, un acteur majeur dans l'expression de VCAM-1, ICAM-1 et E-sélectine[287]. De façon surprenante, le traitement des cellules endothéliales avec des siRNA dirigés contre ShcA induit la phosphorylation de la sous-unité p65 de NF-κB et son accumulation dans le noyau. Ceci suggère que, en l'absence de ShcA, la régulation des niveaux d'expression des molécules d'adhésion est indépendante de NF-κB. Nous avons ensuite étudié l'implication de ZEB1, un répresseur de transcription de molécules d'adhésion dont la

localisation nucléaire peut être induite par NF-κB[288, 289]. Nous avons constaté une augmentation marquée de l'expression cytosolique et de la translocation nucléaire de ZEB1 dans les cellules endothéliales traitées avec siShcA.

Il a été montré que ZEB1 pouvait passer d'une fonction de répresseur à une fonction d'activateur de transcription lorsqu'il interagissait avec d'autres co-facteurs tels que l'effecteur de la voie Hippo, YAP1 (Yes associated protein)[290]. L'augmentation de la translocation nucléaire de ZEB1 que nous avons observée dans les cellules déficientes en ShcA coïncide avec une diminution de l'expression nucléaire de YAP1. Aussi, l'utilisation de siZEB1 diminue les taux d'ARNm et protéiques de ShcA et ICAM-1. Nos résultats suggèrent fortement que le complexe ZEB1/YAP1 favorise la régulation de l'expression d'ICAM-1 par l'intermédiaire de ShcA, très probablement en se liant aux promoteurs de ShcA.

Un des mécanismes protecteurs contre le développement de l'athérosclérose est l'inhibition de l'oxydation des LDL par la eNOS (NO-synthase endothéliale) dont la perte d'activité est un indicateur de dysfonctionnement endothélial[291]. eNOS est phosphorylée et activée par Akt, un des plus importants acteurs dans la signalisation de ShcA.

Effectivement, dans les cellules endothéliales EA.hy 926 qui n'expriment pas ShcA, nous avons constaté une augmentation de l'expression de p-Akt et de son gène cible p-mTOR, accompagnée d'une augmentation marquée de la phosphorylation d'eNOS. Ces résultats ont également été validés *in-vivo*, car les souris KO présentaient une expression accrue d'eNOS phosphorylée dans les cellules endothéliales par rapport aux souris contrôles.

Nos données indiquent que l'expression au niveau endothélial des trois isoformes de la protéine ShcA joue un rôle central dans la physiopathologie de l'athérosclérose. Par l'intermédiaire d'un complexe ZEB1/YAP1, ShcA est capable d'induire l'expression d'ICAM1 ainsi que d'autres molécules d'adhésion, favorisant ainsi l'adhésion des macrophages, l'accumulation de lipides intracellulaires et la formation de cellules spumeuses tout en diminuant simultanément la production de NO endothélial. Ainsi, ShcA contribue au dysfonctionnement endothélial et à la formation de lésions d'athérosclérose.

Rôle de ShcA dans la différenciation des chondrocytes

L'équipe de Pr. Boucher avait précédemment montré que le complexe LRP1/ShcA pouvait orienter la signalisation induite par l'IGF1 de l'activation de Akt/mTOR et de la différenciation adipogénique vers l'activation de Erk1/2 et la prolifération cellulaire[181]. Comme les adipocytes et les chondrocytes ont une origine cellulaire commune, nous avons formulé l'hypothèse que l'interaction LRP1/ShcA jouait un rôle dans la différenciation des chondrocytes. Afin de tester directement cela, le laboratoire d'accueil a généré des souris dépourvues de ShcA dans les chondrocytes en utilisant le système Cre-lox (Twist 2 ShcA KO). Les souris dépourvues de ShcA présentent un phénotype de nanisme associé à un rapport os-cartilage réduit. Ce phénotype est dû à une diminution de la maturation hypertrophique des chondrocytes de la zone de croissance. Ces résultats mettent en évidence un rôle crucial de ShcA dans la différentiation hypertrophique terminale.

La différenciation hypertrophique est l'un des facteurs clés impliqués dans la maladie articulaire la plus répandue, l'arthrose. Cette pathologie implique la perte progressive du cartilage articulaire (CA), composé d'un seul type de cellule, le chondrocyte. Les chondrocytes synthétisent une matrice extracellulaire (ECM) abondante constituée principalement de collagène de type II (col II) et d'aggrécane. Pendant le développement de l'arthrose, il a été montré que les chondrocytes articulaires passent d'un phénotype quiescent à un phénotype hypertrophique et synthétisent du collagène de type X (col X) et des enzymes qui dégradent l'ECM, entraînant une diminution de la production de col II[72]. Cette hypertrophie est également une cause majeure d'échec dans les approches d'ingénierie tissulaire du cartilage lorsque des cellules souches mésenchymateuses (CSM) autologues différentiées en chondrocytes sont implantées pour réparer des lésions arthrosiques[113]. Pour éviter cela, une meilleure compréhension du contrôle moléculaire de la différenciation et de l'hypertrophie des chondrocytes est nécessaire.

L'objectif de cette partie de mon projet était de mieux comprendre la physiopathologie de l'arthrose en étudiant le rôle de ShcA et de tester ce gène comme cible thérapeutique potentielle dans l'ingénierie tissulaire du cartilage.

La première étape de mon projet a été de valider le rôle de ShcA dans la maturation hypertrophique des chondrocytes *in-vitro*. Nous avons isolé des chondrocytes articulaires de souris contrôles et de souris KO âgées de 10 jours. Les cellules issues de souris KO présentent une augmentation d'expression de col II et une diminution d'expression de de col X, au niveau de l'ARNm et au niveau protéique. La synthèse de l'ARNm de la phosphatase alcaline est aussi diminuée. Ces résultats montrent que la différenciation hypertrophique des chondrocytes et le processus de minéralisation sont freinés en l'absence de ShcA.

Nous avons ensuite voulu caractériser par quels mécanismes moléculaires ShcA contrôlait l'hypertrophie et l'expression du marqueur d'hypertrophie, Col X. Nous avons étudié Runx2, un facteur de transcription primordial dans l'hypertrophie des chondrocytes. Runx2 est également un transactivateur indispensable de col X[156]. Erk1/2, le principal effecteur de la signalisation de ShcA, est capable de phosphoryler RunX2, entraînant ainsi son activation et induisant sa liaison à l'ADN et la transcription de col X[292].

Nous avons observé que dans les cellules déficientes en ShcA, la phosphorylation d'Erk1/2 était significativement réduite, entravant ainsi la capacité de cette voie à activer RunX2.

Nous avons par ailleurs observé grâce à des expériences de fractionnement cellulaire et d'immunofluorescence, que la translocation nucléaire de RunX2 n'était pas aussi efficace dans les cellules déficientes en ShcA que dans les cellules contrôles. Ces données indiquent que, en absence de ShcA, une activation réduite de Erk freine la translocation nucléaire de RunX2, ce qui pourrait diminuer la transcription de col X.

L'activité de RunX2 peut être régulée par différentes voies de signalisation. Par exemple, il a été démontré que YAP1, un effecteur de la voie Hippo, inhibe la maturation des chondrocytes en se liant à RunX2 inhibant ainsi l'expression de col X[203]. Cependant, une fois phosphorylé en Sérine, YAP1 est séquestré dans le cytoplasme et ensuite dégradé[201]. La lignée cellulaire chondroprogénitrice ATDC5 est un modèle établi pour étudier la différenciation chondrogénique ainsi que la différenciation hypertrophique des chondrocytes et la minéralisation de leur ECM[189]. Nous avons

observé une diminution de l'expression de ShcA quand ces cellules étaient cultivées dans un milieu pro-chondrogénique, alors que dans un milieu hypertrophique, ces cellules montraient une expression plus élevée. Ceci suggère un rôle actif pour ShcA dans l'induction de l'hypertrophie. Par contre, quand ces cellules étaient induites dans un milieu hypertrophique, YAP1 était exprimé en sa forme phosphorylée, cytoplasmique ce qui suggère une atténuation de l'activité de YAP1 dans ces conditions.
De plus, en conditions hypertrophiques, des expériences d'immunoprécipitation nous ont montré que ShcA se lie à YAP1. Ces résultats concordaient avec une phosphorylation plus importante de YAP1 sur son résidu Ser127 dans les cellules exprimant ShcA, démontrant ainsu que ShcA, en se liant à YAP1, favorise sa localisation cytoplasmique dans les cellules hypertrophiées. Ceci permetterait donc à RunX2 d'agir comme un transactivateur de Col X sans être inhibé par YAP1.

Après avoir validé l'implication de ShcA dans l'hypertrophie des chondrocytes et étant donné que la différentiation hypertrophique est un des processus impliqué dans la physiopathologie de l'arthrose, nous avons testé si l'absence de ShcA dans le cartilage pouvait protéger contre le développement de cette maladie. Ces expériences ont été réalisées sur des souris âgées de 1 mois et de 24 mois pour étudier l'évolution du cartilage articulaire avec l'âge[206]. On a analysé des sections articulaires tibio-fémorales pour calculer les scores Mankin et OARSI[207] qui servent à l'évaluation qualitative du développement de l'arthrose. Ces coupes ont également été utilisées pour des tests d'immunohistochimie pour évaluer l'expression des marqueurs d'hypertrophie, col X, MMP13 et des marqueurs de quiescence, col II. Nous avons pu observer que les cartilages articulaires de genou de souris KO présentent une plus grande teneur en protéoglycanes et en col II, et une expression moindre du collagène X, et sont protégés du développement grave de l'arthrose liée à l'âge.

L'équipe du Dr Andrea Barbero (laboratoire d'Ivan Martin, Basel Stem Cell Network, Bâle, Suisse) spécialiste de l'ingénierie tissulaire du cartilage, nous a permis de bénéficier de son expérience[213]. Nous avons utilisé des chondrocytes de cartilage articulaire du genou isolés chez des souris Twist2 ShcA KO âgées de 10 jours et de

souris contrôles. Ces chondrocytes ont été amplifiées au cours des passages en culture en monocouche et ensuite cultivées dans des éponges de collagène en milieu chondrogénique suivi ou non d'un milieu induisant l'hypertrophie pour tester leur capacité de se redifférencier en chondrocytes et à s'hypertrophier. Les échantillons de néo-cartilage obtenus ont été analysés par histologie et immunohistologie pour caractériser la morphologie des chondrocytes, la composition de l'ECM et le phénotype des chondrocytes.

Nous avons obtenus des résultats encourageants mais qui nécessitent d'être affinés. Nous ne pouvons pas à ce stade dégager de conclusion définitive mais il se dégage une tendance, à savoir une diminution de la synthèse de collagène X et une augmentation de la synthèse de collagène II dans le néo-cartilage déficient en ShcA.

Rôle de LRP1 dans la différenciation des chondrocytes

Ce projet a également bénéficié d'une collaboration avec le laboratoire du Pr. Andreas Niemeier (Centre médical universitaire de Hambourg, Eppendorf - Allemagne). En utilisant une approche de KO conditionnel (RunX2-LRP1 KO), l'équipe du Pr. Niemeier a révélé que les souris n'exprimant pas LRP1 présentent également un phénotype de nanisme. Notre hypothèse était que LRP1 était impliqué d'une façon similaire à ShcA, dans le processus d'hypertrophie, possiblement aussi par un mécanisme où ShcA pourrait jouer un rôle central.

J'ai réalisé une partie de mon travail de thèse au sein du laboratoire du Pr. Niemeier afin de caractériser le rôle de LRP1 dans la différentiation hypertrophique du chondrocyte.

Les analyses histologiques et d'immunohistochimie de la zone de croissance des souris RunX2-LRP1 ont montré une zone d'hypertrophie plus large et qui contenait un nombre plus élevé de chondrocytes hypertrophiés positifs pour Col X chez les souris RunX2-LRP1 KO.

De plus, l'immunomarquage de la métalloprotéinase MMP13 a montré son expression était plus diffuse et répandue tout au long la surface de la zone de croissance chez les

souris LRP1 KO. Ces observations suggèrent que l'absence de LRP1 dans les chondrocytes favorisait l'hypertrophie et possiblement l'induisait d'une manière précoce. L'équipe du Pr. Niemeier avait déjà validé le rôle de LRP1 dans le tissu osseux[225], et en utilisant le même modèle murin, ont pu isoler des ostéoblastes primaires et ont démontré le rôle que jouait LRP1 dans l'activation et le recrutement des ostéclastes.
Similairement aux souris Twis2 ShcA, LRP1 semblait être impliqué dans la différentiation hypertrophique des chondrocytes bien que son rôle soit plutôt inhibiteur de cette différentiation. En isolant des chondrocytes primaires des souris Runx2-LRP1, nous avons pu étudier l'expression des marqueurs d'hypertrophie et de quiescence. L'expression de MMP13 était 2 fois plus élevée en absence de LRP1, ce qui mimait nos observations *in-vivo*. Parmi les plusieurs voies de signalisation qui contrôlent l'expression de pas seulement MMP13 mais de nombreux membres de la famille des MMPs, sont la voie de la MAPK et son effecteur Erk1/2, la voie de NF-kB et la voie Wnt et son effecteur la b-catenine. Nos analyses de Western Blot ont montré que ces 3 voies étaient induites en absence de MMP13, ce qui pourrait expliquer l'augmentation de son expression.

Conclusion

Au cours de ma thèse j'ai pu caractériser le rôle de LRP1 dans la différentiation hypertrophique du chondrocyte ainsi que le rôle de son partenaire ShcA. On était capables aussi de démontrer l'implication de ShcA dans la pathologie de l'athérosclérose et dans et le développement de l'arthrose.
Nous avons montré que ShcA était nécessaire pour la différentiation hypertrophique du chondrocyte, qu'il permettait la synthèse de collagène X et inhibait la synthèse de collagène II.
ShcA contrôle l'expression de collagène X en activant la voie de Erk1/2, en favorisant la translocation nucléaire de RunX2 et en favorisant la localisation cytoplasmique de YAP1. Chez la souris la suppression de l'expression de ShcA freine le développement de l'arthrose. ShcA représente donc une cible thérapeutique intéressante pour la génération de cartilage à base de cellules souches mésenchymateuses. Par contre

LRP1, étant un récepteur endocytaire des MMPs semble jouer le rôle de régulateur de leurs concentration extracellulaire, et en son absence, on a observé l'activation des mécanismes cellulaires responsables de la surexpression de MMP13.

Dans les thérapies de régénération du cartilage à base de cellules, l'utilisation de CSM a donné des résultats prometteurs et, contrairement aux chondrocytes, leur disponibilité n'est pas entravée par leur disponibilité limitée. Dans la suite de ce projet, il est envisagé d'utiliser des CSM humaines TERT (cellules transduites avec le gène de la transcriptase inverse de la télomérase humaine, qui prolonge leur durée de vie prolifération et maintient leur phénotype de cellules souches et leur capacité à se différencier en plusieurs lignées, y compris les chondrocytes). Par la technologie CRISPR/Cas9, l'expression de ShcA pourrait être supprimée et celle de LRP1 suractivée dans ces cellules qui pourraient être utilisées pour synthétiser un néo-cartilage (collaboration avec Dr. Barbero, Bâle). Nous espérons que ce néo-cartilage posséderait les caractéristiques d'un cartilage sain et non hypertrophique.

Bibliography

1 Barquera S., Pedroza-Tobias A., Medina C., Hernandez-Barrera L., Bibbins-Domingo K., Lozano R., Moranc A.E. (2015). Global Overview of the Epidemiology of Atherosclerotic Cardiovascular Disease. Archives of Medical Research, 46, 328–338.

2 Lusis A.J. (2000). Atherosclerosis. Nature 407, 233–241.

3 Libby P. (2002). Inflammation in Atherosclerosis. Nature 420, 868-74.

4 Durham A.L., Speer M.Y., Scatena M., Giachelli C.M., Shanahan C.M. (2018). Role of smooth muscle cells in vascular calcification: implications in atherosclerosis and arterial stiffness. Cardiovascular Research 114, 590–600.

5 Rattazzi M., Bennett B.J., Bea F., Kirk E.A., Ricks J.L., Speer M., Schwartz S.M., Giachelli C.M., Rosenfeld M.E.(2005). Calcification of advanced atherosclerotic lesions in the innominate arteries of ApoE-deficient mice: potential role of chondrocyte-like cells. Arterioscler Thromb Vasc Biol. 25, 1420-5.

6 Demer L.L., Tintut Y. (2008). Vascular calcification: pathobiology of a multifaceted disease. Circulation. 117, 2938-48.

7 Boucher P., Gotthardt M., Li W.P., Anderson R.G., Herz J. (2003). LRP: role in vascular wall integrity and protection from atherosclerosis. Science. 300, 329-32.

8 Woldt E., Terrand J., Mlih M., Matz R.L., Bruban V., Coudane F., Foppolo S., El Asmar Z., Chollet M.E., Ninio E., Bednarczyk A., Thierse D., Schaeffer C., Van Dorsselaer A., Boudier C., Wahli W., Chambon P., Metzger D., Herz J., Boucher P. (2012). PPARγ counteracts LRP1-induced vascular calcification by inhibiting a WNT5A signaling pathway. Nat Commun. 3, 1077.

9 Lillis A.P., Van Duyn L.B., Murphy-Ullrich J.E., Strickland D.K. (2008). The low density lipoprotein receptor related protein 1: Unique tissue-specific functions revealed by selective gene knockout studies. Physiol Rev. 88, 887–918.

10 Sweet D. T., Chen Z., Wiley D. M., Bautch V. L., Tzima, E. (2012). The adaptor protein shc integrates growth factor and ECM signaling during postnatal angiogenesis. Blood 119, 1946–1955.

11 Sato K., Nagao T., Kakumoto M., Kimoto M., Otsuki T., Iwasaki T., Tokmakov A.A., Owada K., Fukami Y. (2002). Adaptor protein Shc is an isoform-specific direct activator of the tyrosine kinase c-Src. J Biol Chem. 277, 29568-76.

12 Hecker T.P., Grammer J.R., Gillespie G.Y., Stewart J. Jr, Gladson C.L. (2002). Focal adhesion kinase enhances signaling through the Shc/extracellular signal-regulated kinase pathway in anaplastic astrocytoma tumor biopsy samples. Cancer Res. 62, 2699-707.

13 Pelicci G., Lanfrancone L., Grignani F., McGlade J., Cavallo F., Forni G., Nicoletti I.,Grignani F., Pawson T., Pelicci P. G.. (1992). A Novel Transforming Protein (SHC) with an SH2 Domain is Implicated in Mitogenic Signal Transduction. Cell 70, 93-104.

14 Kojima T., Yoshikawa Y., Takada S., Sato M., Nakamura T., Takahashi N., Copeland N. G., Gilbert D. J., Jenkins N. A., Mori, N. (2001). Genomic organization of the shc-related phosphotyrosine adapters and characterization of the full-length sck/ShcB: specific association of p68-sck/ShcB with pp135. Biochem. Biophys. Res. Commun. 284, 1039–1047

15 Sakai R., Henderson J. T., O'Bryan J. P., Elia A. J., Saxton T. M., Pawson, T. (2000). The mammalian ShcB and ShcC phosphotyrosine docking proteins function in the maturation of sensory and sympathetic neurons. Neuron 28, 819–833.

16 Pelicci G., Troglio F., Bodini A., Melillo R. M., Pettirossi V., Coda L., De Giuseppe A., Santoro M., Pelicci P. G. (2002). The neuron-specific rai (ShcC) adaptor protein inhibits apoptosis by coupling ret to the phosphatidylinositol 3-kinase/akt signaling pathway. Mol. Cell. Biol. 22, 7351–7363.

17 Fagiani E., Giardina G., Luzi, L., Cesaroni M., Quarto M., Capra M., Germano G., Bono M., Capillo M., Pelicci P., Lanfrancone, L. (2007). RaLP, a new member of the src homology and collagen family, regulates cell migration and tumor growth of metastatic melanomas. Cancer Res. 67, 3064–3073.

18 Lai K. M., Pawson T. (2000). The ShcA phosphotyrosine docking protein sensitizes cardiovascular signaling in the mouse embryo. Genes Dev. 14, 1132–1145.

19 Migliaccio E., Giorgio M., Mele S., Pelicci G., Reboldi, P., Pandolfi, P. P., Lanfrancone, L. and Pelicci, P. G. (1999). The p66shc adaptor protein controls oxidative stress response and life span in mammals. Nature 402, 309–313.

20 Migliaccio, E., Mele, S., Salcini, A. E., Pelicci, G., Lai, K. M., Superti-Furga, G., Pawson, T., Di Fiore, P. P., Lanfrancone, L. and Pelicci, P. G. (1997). Opposite effects of the p52shc/p46shc and p66shc splicing isoforms on the EGF receptor–MAP kinase–fos signalling pathway. EMBO J. 16, 706–716.

21 Hawley S. P., Wills M. K., Rabalski A. J., Bendall A. J., Jones, N. (2011). Expression patterns of ShcD and shc family adaptor proteins during mouse embryonic development. Dev. Dyn. 240, 221–231.

22 Martin C.E., Petersen K.A., Aoudjit L., Tilak M., Eremina V., Hardy WR., Quaggin S.E., Takano T., Jones N. (2018). ShcA Adaptor Protein Promotes Nephrin Endocytosis and Is Upregulated in Proteinuric Nephropathies. J Am Soc Nephrol. 1, 92-103.

23 Hardy W.R., Li L., Wang Z., Sedy J., Fawcett J., Frank E., Kucera J., Pawson T. (2007). Combinatorial ShcA docking interactions support diversity in tissue morphogenesis. Science. 317, 251-6.

24 Ravichandran K. S. (2001). Signaling via shc family adapter proteins. Oncogene 20, 6322–6330.

25 Sakaguchi K., Okabayashi Y., Kido, Y., Kimura S., Matsumura Y., Inushima K. and Kasuga M. (1998). Shc phosphotyrosine-binding domain dominantly interacts with epidermal growth factor receptors and mediates ras activation in intact cells. Mol. Endocrinol. 12, 536–543.

26 Wills M.K., Jones N. (2012). Teaching an old dogma new tricks: twenty years of Shc adaptor signalling. Biochem J. 447, 1-16.

27 Giorgio M., Migliaccio E., Orsini F., Paolucci D., Moroni M., Contursi C., Pelliccia G., Luzi L., Minucci S., Marcaccio M., Pinton P., Rizzuto R., Bernardi P., Paolucci F., Pellici P.G. (2005). Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. Cell 122, 221–233.

28 Luzi L., Confalonieri S., Di Fiore P. P., Pelicci, P. G. (2000). Evolution of shc functions from nematode to human. Curr. Opin. Genet. Dev. 10, 668–674.

29 Qi M., Elion E.A. (2005). MAP kinase pathways. J Cell Sci. 118, 3569-72.

30 Trampont P., Zhang L., Ravichandran K.S. (2006). ShcA mediates the dominant pathway to extracellular signal-regulated kinase activation during early thymic development. Mol Cell Biol. 26, 9035-44.

31 Gong Y., Zhao X. (2003). Shc-dependent pathway is redundant but dominant in MAPK cascade activation by EGF receptors: a modeling inference. FEBS Lett. 554, 467–472.

32 Hutter D., Yo Y., Chen W., Liu P., Holbrook N. J., Roth, G. S., Liu Y. (2000). Age-related decline in ras/ERK mitogen-activated protein kinase cascade is linked to a reduced association between shc and EGF receptor. J. Gerontol. A Biol. Sci. Med. Sci. 55, B125–B134.

33 Besset V., Scott R. P., Ibanez C. F. (2000). Signaling complexes and protein–protein interactions involved in the activation of the ras and phosphatidylinositol 3-kinase pathways by the c-ret receptor tyrosine kinase. J. Biol. Chem. 275, 39159–39166.

34 Yoshida S., Masaki T., Feng H., Yuji J., Miyauchi Y., Funaki T., Yoshiji H., Matsumoto K., Uchida N., Watanabe S., Kurokohchi K., Kuriyama S. (2004). Enhanced expression of adaptor molecule p46 Shc in nuclei of hepatocellular carcinoma cells: study of LEC rats. Int J Oncol. 25, 1089-96.

35 Yukimasa S., Masaki T., Yoshida S., Uchida N., Watanabe S., Usuki H., Yoshiji H., Maeta T., Ebara K., Nakatsu T., Kurokohchi K., Kuriyama S. (2005). Enhanced expression of p46 Shc in the nucleus and p52 Shc in the cytoplasm of human gastric cancer. Int J Oncol. 26,905-11.

36 George R., Chan H.L., Ahmed Z., Suen K.M., Stevens C.N., Levitt J.A., Suhling K., Timms J., Ladbury J.E. (2009). A complex of Shc and Ran-GTPase localises to the cell nucleus. Cell Mol Life Sci. 66, 711-20.

37 Brown, M. D., Sacks, D. B. (2009). Protein scaffolds in MAP kinase signalling. Cell. Signalling 21, 462–469.

38 Ahmed S.B.M., Prigent S.A. (2017). Insights into the Shc Family of Adaptor Proteins. Journal of Molecular Signaling, 12, 1–17.

39 Herz J., Clouthier D.E., Hammer R.E.(1992). LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation. Cell. 71,411-21.

40 Boucher P., Herz J. (2011). Signaling through LRP1: Protection from atherosclerosis and beyond. Biochem Pharmacol. 81, 1-5.

41 Wujak L., Böttcher R.T., Pak O., Frey H., El Agha E., Chen Y., Schmitt S., Bellusci S., Schaefer L., Weissmann N., Fässler R., Wygrecka M. (2018). Low density lipoprotein receptor-related protein 1 couples β1 integrin activation to degradation. Cell Mol Life Sci. 75,1671-1685

42 Gerritsen K.G.F., Bovenschen N., Nguyen T.Q., Sprengers D., Koeners M.P., van Koppen A.N., Joles J.A., Goldschmeding R., KokRapid R.J. (2016). Hepatic clearance of full length CCN-2/CTGF: a putative role for LRP1-mediated endocytosis. J Cell Commun Signal. 10, 295-303

43 Gonias S.L., Campana W.M. (2014). LDL Receptor Related Protein-1 A Regulator of Inflammation in Atherosclerosis, Cancer, and Injury to the Nervous System. Am J Pathol 184, 18-27,

44 Gaultier A., Arandjelovic S., Niessen S., Overton C.D., Linton M.F., Fazio S., Campana W.M., Cravatt B.F., Gonias S.L. (2008). Regulation of tumor necrosis factor receptor-1 and the IKK-NF-kappaB pathway by LDL receptor-related protein explains the antiinflammatory activity of this receptor. Blood 111 , 5316-5325.

45 Terrand J., Bruban V., Zhou L., Gong W., El Asmar Z., May P., Zurhove K., Haffner P., Philippe C., Woldt E., Matz R.L., Gracia C., MetzgerD.,Auwerx J., Herz J., Boucher P. (2009). LRP1 controls intracellular cholesterol storage and fatty acid synthesis through modulation of Wnt signaling. J Biol Chem. 284, 381-8.

46 Hall, B. K., Miyake, T. (2000). All for one and one for all: condensations and the initiation of skeletal development. BioEsssays 22, 138–147.

47 Kronenberg, H.M. (2003). Developmental regulation of the growth plate. Nature 423,332-336.

48 . Abad V, Meyers JL, Weise M, Gafni RI, Barnes KM, Nilsson O, Bacher JD, Baron J (2002). The role of the resting zone in growth plate chondrogenesis. Endocrinology 143,1851–1857.

49 Roughlet P.J. (2001). Articular Cartilage and changes in arthritis Noncollagenous proteins and proteoglycans in the extracellular matrix of cartilage. Arthritis Res 3, 342-347.

50 Ballock, R.T., O'Keefe, R.J. (2003). Physiology and Pathophysiology of the Growth Plate. Birth Defects Research 69,123–143.

51 Staines K.A., Pollard A.S., McGonnell I.M., Farquharson C., Pitsillides A.A. (2012). Cartilage to bone transitions in health and disease. Journal of Endocrinology 219, R1–R12.

52 Farnum C.E., Lee R., O'Hara K., Urban J.P. (2002). Volume increase in growth plate chondrocytes during hypertrophy: the contribution of organic osmolytes. Bone 30, 574–581.

53 *The scientist magazine, May 2011.*

54 Hunziker E.B., Schenk R.K., Cruz-Orive L.M. (1997). Quantitation of chondrocyte performance in growth-plate cartilage during longitudinal bone growth. J Bone Joint Surg 69A, 162–173.

55 Anderson H.C., Hsu H.H., Morris D.C., Fedde K.N., Whyte M.P. (1997). Matrix vesicles in osteomalacic hypophosphatasia bone contain apatite- like mineral crystals. Am J Pathol 151, 1555–1561.

56 Anderson H.C. (1995). Molecular biology of matrix vesicles. Clin Orthop Relat Res 314:266–280.

57 Werb Z. (1992). The biological role of metalloproteinases and their inhibitors. In: Kuettner K, Schleyerbach R, Peyron JG, Hascall VC, editors. , eds.Articular Cartilage and Osteoarthritis. New York, NY: Raven Press; 295-304.

58 Troeberg L., Nagase H. (2012). Proteases involved in cartilage matrix degradation in osteoarthritis. Biochim Biophys Acta. 1824, 133–145.

59 Tortorella M., Pratta M., Liu R.Q., Abbaszade I., Ross H., Burn T., Arner E (2000). The thrombospondin motif of aggrecanase-1 (ADAMTS-4) is critical for aggrecan substrate recognition and cleavage. 275, 25791-7.

60 Kuno K., Okada Y., Kawashima H., Nakamura H., Miyasaka M., Ohno H., Matsushima K. (2000). ADAMTS-1 cleaves a cartilage proteoglycan, aggrecan. FEBS Lett. 478, 241-5.

61 Shindo T., Kurihara H., Kuno K., Yokoyama H., Wada T., Kurihara Y., Imai T., Wang Y., Ogata M., Nishimatsu H., Moriyama N., Oh-hashi Y., Morita H., Ishikawa T., Nagai R., Yazaki Y., Matsushima K. (2000). ADAMTS-1: a metalloproteinase-disintegrin essential for normal growth, fertility, and organ morphology and function. J Clin Invest. 105, 1345-52.

62 Carlevaro M.F., Cermelli S., Cancedda R., Descalzi Cancedda F. (2000). Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. J Cell Sci. 113, 59-69.

63 Garcia-Ramirez M., Toran N., Andaluz P., Carrascosa A., Audi L. (2000). Vascular endothelial growth factor is expressed in human fetal growth cartilage. J Bone Miner Res. 15, 534-40.

64 Gerber H.P., Vu T.H., Ryan A.M., Kowalski J., Werb Z., Ferrara N. (1999). VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med. 5, 623-8.

65 Horner A., Bishop N.J., Bord S., Beeton C., Kelsall A.W., Coleman N., Compston J.E. (1999). Immunolocalisation of vascular endothelial growth factor (VEGF) in human neonatal growth plate cartilage. J Anat. 194, 519-24.

66 Erenpreisa J., Roach H.I. (1998). Aberrant death in dark chondrocytes of the avian growth plate. Cell Death Differ. 5, 60-6.

67 Inada M., Wang Y., Byrne M.H., Rahman M.U., Miyaura C., Lopez-Otin C., Krane S.M. (2004). Critical roles for collagenase-3 (Mmp13) in development of growth plate cartilage and in endochondral ossification. Proc Natl Acad Sci U S A. 101, 17192–17197.

68 Weise M., De-Levi S., Barnes K.M., Gafni R.I., Abad V., Baron J. (2001). Effects of estrogen on growth plate senescence and epiphyseal fusion. Proc Natl Acad Sci U S A. 98, 6871-6.

69 Van Der Eerden B.C.J., Karperien M., Wit J.M. (2003). Systemic and Local Regulation of the Growth Plate. Endocrine Reviews 24(6):782–801.

70 Michigan Medical School. Michigan Histology and Virtual Microscopy Learning Resources.

71 Buckwalter J.A., Hunzinker E., Rosenberg L., et al. (1988). Articular cartilage: composition and structure. In: Woo SLY, Buckwalter JA, eds. Injury and Repair of the Musculoskeletal Soft Tissues. Park Ridge, IL: American Academy of Orthopaedic Surgeons; 405-425.

72 Buckwalter J.A., Mankin H.J. (1997). Articular cartilage, part 1: tissue design and chondrocyte-matrix interaction. J Bone Joint Surg Am. 79:600-611.

73 Alford J.W., Cole B.J. (2005). Cartilage restoration, part I: basic science, historical perspective, patient evaluation and treatment options. Am J Sports Med. 33:295-306.

74 Maroudas A. (1979). Physiochemical properties of articular cartilage. In: Freeman MAR, editor. , ed. Adult Articular Cartilage. Kent, United Kingdom: Cambridge University Press, 215-290.

75 Verzijl N., DeGroot J., Thorpe S.R., Bank R.A., Shaw J.N., Lyons T.J., Bijlsma J.W., Lafeber F.P., Baynes J.W., TeKoppele J.M. (2000). Effect of collagen turnover on the accumulation of advanced glycation end products. J Biol Chem. 275, 39027-31.

76 Buckwalter J.A., Rosenberg L.A., Hunziker E.B. (1990). Articular Cartilage and Knee Joint Function: Basic Science and Arthroscopy. New York, NY: Raven Press.

77 Fox A.J.S., Bedi A., Rodeo S.A. (2009). The Basic Science of Articular Cartilage: Structure, Composition, and Function. Sports Health, Orthopaedics. 5,371.

78 Maroudas A., Bayliss M.T., Uchitel-Kaushansky N., Schneiderman R., Gilav E. (1998). Aggrecan turnover in human articular cartilage: use of aspartic acid racemization as a marker of molecular age. 350,61-71.

79 Maroudas A., Wachtel E., Grushko G., Katz E.P., Weinberg P. (1991). The effects of osmotic and mechanical pressure on water and partitioning in articular cartilage. Biochem Biophys Acta. 1073:285-294.

80 Matsiko A., Levingstone T.J., O'Brien F.J. (2013). Advanced Strategies for Articular Cartilage Defect Repair. Materials (Basel). 6, 637-668.

81 Turkiewicz A., Petersson F., Björk J.,Hawker G., Dahlberg L.E., Lohmander L.S., Englund M. (2014). Current and future impact of osteoarthritis on health care: a population-based study with projections to year 2032. Osteoarthritis and Cartilage 22, 1826-1832.

82 Felson D.T. (2006). Osteoarthritis of the knee. N Engl J Med 354, 841-8.

83 Salmon J.H., Rat A.C., Sellam J., Michel M., Eschard J.P., Guillemin F., Jolly D., Fautrel N. (2016). Economic impact of lower-limb osteoarthritis worldwide: a systematic review of cost-of-illness studies. Osteoarthritis and Cartilage 24, 1500-1508.

84 . Hiligsmann M., Cooper C., Arden N., Boers M., Branco J.C., Luisa Brandi M., Bruyere O., Guillemin F., Hochberg M.C., Hunter D.J., Kanis J.A., Kvien T.K., Laslop A., Pelletier J.P., Pinto D., Reiter-Niesert S., Rizzoli R., Rovati L.C., Severens J.L., Silverman S., Tsouderos Y., Tugwell P., Reginster J.Y. (2013). Health economics in the field of osteoarthritis: an expert's consensus paper from the European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO). Semin Arthritis Rheum 43, 303-313.

85 Loeser F., Collins J., Diekman B. (2016). Ageing and the pathogenesis of osteoarthritis. Nature Reviews Rheumatology 12, 412–420.

86 Allen K.D., Golightly Y.M. (2015). Epidemiology of osteoarthritis: state of the evidence. Curr Opin Rheumatol 27, 276–283.

87 Wallace I.J., Worthington S., Felson D.T., Jurmain R.D., Wren K.T., Maijanen H., Woods R.J., Lieberman D.E. (2017). Knee osteoarthritis has doubled in prevalence since the mid-20th century. PNAS 114 , 9332-9336.

88 *Mével E., Monfoulet L.E., Merceron C., Coxam V., Wittrant Y., Beck L., Guicheux, J. (2014). Nutraceuticals in joint health: animal models as instrumental tools. Drug Discovery Today, 19, 1649–1658.*

89 Loeser R.F., Goldring S.R., Scanzello C.R., Goldring M.B. (2012). Osteoarthritis: A Disease of the Joint as an Organ Arthritis Rheum. 64, 1697–1707.

90 Guilak F., Fermor B., Keefe F.J., Kraus V.B., Olson S.A., Pisetsky D.S., Setton L.A., Weinberg J.B. (2004). The role of biomechanics and inflammation in cartilage injury and repair. Clin Orthop Relat Res. 423, 17-26.

91 Kurz B., Lemke A.K., Fay J., Pufe T., Grodzinsky A.J., Schünke M. (2005). Pathomechanisms of cartilage destruction by mechanical injury. Ann Anat. 187, 473-85.

92 Millward-Sadler S.J., Salter D.M. (2004). Integrin-dependent signal cascades in chondrocyte mechanotransduction. Ann Biomed Eng. 32, 435-46.

93 Pulai J.I., Chen H., Im H.J., Kumar S., Hanning C., Hegde P.S., Loeser R.F. (2005). NF-kappa B mediates the stimulation of cytokine and chemokine expression by human articular chondrocytes in response to fibronectin fragments. J Immunol. 174, 5781-8.

94 Sandell L.J., Aigner T. (2001). Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. Arthritis Res. 3, 107-13.

95 Tchetina E.V., Squires G., Poole A.R. (2005). Increased type II collagen degradation and very early focal cartilage degeneration is associated with upregulation of chondrocyte differentiation related genes in early human articular cartilage lesions. J Rheumatol. 32 ,876-86.

96 Kamekura S., Hoshi K., Shimoaka T., Chung U., Chikuda H., Yamada T. (2005). Osteoarthritis development in novel experimental mouse models induced by knee joint instability. Osteoarthritis Cartilage 13, 632-41.

97 Kamekura S., Kawasaki Y., Hoshi K., Shimoaka T., Chikuda H., Maruyama Z. (2006). Contribution of runt-related transcription factor 2 to the pathogenesis of osteoarthritis in mice after induction of knee joint instability. Arthritis Rheum 54, 2462-70.

98 . Olivotto E., Borzi R.M., Vitellozzi R., Pagani S., Facchini A., Battistelli M. (2008). Differential requirements for IKKalpha and IKKbeta in the differentiation of primary human osteoarthritic chondrocytes. Arthritis Rheum 58, 227-39.

99 Cecil D.L., Rose D.M., Terkeltaub R., Liu-Bryan R. (2005). Role of interleukin-8 in PiT-1 expression and CXCR1-mediated inorganic phosphate uptake in chondrocytes. Arthritis Rheum 52, 144-54.

100 Dudhia J. (2005). Aggrecan, aging and assembly in articular cartilage. Cell Mol Life Sci. 62, 2241-56.

101 Aigner T., Söder S., Gebhard P.M., McAlinden A., Haag J. (2007). Mechanisms of disease: role of chondrocytes in the pathogenesis of osteoarthritis--structure, chaos and senescence. Nat Clin Pract Rheumatol. 3, 391-9.

102 Aigner .T, Haag J., Martin J., Buckwalter J. (2007). Osteoarthritis: aging of matrix and cells--going for a remedy. Curr Drug Targets. 8, 325-31.

103 Loeser R.F. (2006). Molecular mechanisms of cartilage destruction: mechanics, inflammatory mediators, and aging collide. Arthritis Rheum. 54,1357-60.

104 Mobasheri A., Rayman M.P., Gualillo O., Sellam J., van der Kraan P.,Fearon U.(2017). The role of metabolism in the pathogenesis of osteoarthritis. NATURE REVIEWS RHEUMATOLOGY.

105 van der Kraan P.M., van den Berg W.B. (2012). Chondrocyte hypertrophy and osteoarthritis: role in initiation and progression of cartilage degeneration? Osteoarthritis and Cartilage 20, 223-232.

106 Bowman E.N., Hallock J.D., Throckmorton T.W., Azar F.M. (2018). Hyaluronic acid injections for osteoarthritis of the knee: predictors of successful treatment. Int Orthop. 42, 733-740.

107 Bijlsma J.W., Berenbaum F., and Lafeber F.P. (2011). Osteoarthritis: an update with relevance for clinical practice. Lancet 377, 2115-2126.

108 Zhang W., Nuki G., Moskowitz R.W., Abramson S., Altman R.D., Arden N.K., Bierma-Zeinstra S., Brandt K.D., Croft P., Doherty M., Dougados M., Hochberg M., Hunter D.J., Kwoh K., Lohmander L.S., Tugwell P. (2010). OARSI recommendations for the management of hip and knee osteoarthritis: part III: Changes in evidence following systematic cumulative update of research published through January 2009. Osteoarthritis Cartilage 18, 476-499.

109 Schinhan M., Gruber M., Vavken P., Dorotka R., Samouh L., Chiari C., Gruebl-Barabas R., Nehrer S. (2012). Critical-size defect induces unicompartmental osteoarthritis in a stable ovine knee. J Orthop Res 30, 214–220.

110 Johnstone B., Alini M., Cucchiarini M., Dodge G.R., Eglin D., Guilak F., Madry H., Mata A., Mauck R.L., Semino C.E., Stoddart M.J. (2013). Tissue engineering for articular cartilage repair–the state of the art. Eur Cell Mater 25, 248–267.

111 Hunter W. (1743). Of the structure and disease of articulating cartilages. Philos Trans R Soc Lond. 42b, 514−521.

112 Marlovits S., Singer P., Zeller P., Mandl I., Haller J., Trattnig S. (2006). Magnetic resonance observation of cartilage repair tissue (MOCART) for the evaluation of autologous chondrocyte transplantation: determination of interobserver variability and correlation to clinical outcome after 2 years. Eur J Radiol. 57, 16–23.

113 Caldwell, K.L., and Wang, J. (2015). Cell-based articular cartilage repair: the link between development and regeneration. Osteoarthritis Cartilage 23, 351-362.

114 Benya P.D., Padilla S.R., Nimni M.E. (1978). Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. Cell 15, 1313–1321.

115 van Osch G.K.V.M., Brittberg M., Dennis J.E., Bastiaansen-Jenniskens Y.M., Erben R.G., Konttinen Y.T., Luyten F.P. (2009). Cartilage repair: past and future – lessons for regenerative medicine. J Cell Mol Med. 13, 792–810.

116 Behrens P., Bitter T., Kurz B., Russlies M. (2006). Matrix-associated autologous chondrocyte transplantation/implantation (MACT/MACI)—5-year follow-up. The Knee 13, 194 – 202.

117 Pelttari K., Winter A., Steck E., Goetzke K., Hennig T., Ochs B.G., Aigner T., Richter W. (2006). Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. Arthritis Rheum. 54, 3254-66.

118 Pelttari, K., Steck, E., Richter, W. (2008). The use of mesenchymal stem cells for chondrogenesis. Injury 39, S58–S65.

119 Steinert A.F., Ghivizzani S.C., Rethwilm A., Tuan R.S., Evans C.H., Nöth U. (2007). Major biological obstacles for persistent cell-based regeneration of articular cartilage. Arthritis Res. Ther. 9, 213.

120 Gaut C., Sugaya K. (2015). Critical review on the physical and mechanical factors involved in tissue engineering of cartilage.Regen Med 10, 665–679.

121 Occhetta P., Pigeot S., Rasponi M., Dasen B., Mehrkens A., Ullrich T., Kramer I., Guth-Gundel S., Barbero A., Martin I. (2018). Developmentally inspired programming of adult human mesenchymal stromal cells toward stable chondrogenesis. PNAS 115, 4625-4630.

122 De Crombrugghe B., Lefebvre V., Behringer R.R., Bi W., Murakami S., Huang W. (2000). Transcriptional mechanisms of chondrocyte differentiation. Matrix Biol.19, 389-94.

123 Lefebvre V, Li P, de Crombrugghe B. (1998). A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. EMBO J. 17, 5718-33.

124 Bi W., Deng J.M., Zhang Z., Behringer R.R., de Crombrugghe B. (1999). Sox9 is required for cartilage formation. Nat Genet. 22, 85-9.

125 Akiyama, H., Chaboissier, M. C., Martin, J. F., Schedl, A. & de Crombrugghe, B. (2002). The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev. 16, 2813–2828.

126 Joy A., Yu Z., Markus N. (2000). Posttranscriptional Regulation of Smoothened is Part of a Self-Correcting Mechanism in the Hedgehog Signaling System. Molecular Cell. 6, 457–65.

127 Long F., Zhang X. M., Karp S., Yang, Y., McMahon, A. P.(2001). Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. Development 128, 5099–5108.

128 St-Jacques B., Hammerschmidt M., McMahon A. P. (1999). Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes Dev. 13, 2072–2086.

129 St-Jacques B., Hammerschmidt M., McMahonA.P. (1999). Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes Dev. 13, 2072–2086.

130 Schipani E., Lanske B., Hunzelman J., Luz A., Kovacs C.S., Lee K., Pirro A., Kronenberg H.M., Jüppner H. (1997). Targeted expression of constitutively active receptors for parathyroid hormone and parathyroid hormone-related peptide delays endochondral bone formation and rescues mice that lack parathyroid hormone-related peptide. Proc Natl Acad Sci U S A. 94, 13689-94.

131 Serra R., Karaplis A., Sohn P. (1999). Parathyroid hormone-related peptid (PTHrP)-dependent and independent effects of transforming growth factorbeta (TGF-b) on endochondral bone formation. J Cell Biol 145, 783–794.

132 Alvarez J., Horton J., Sohn P., Serra R. (2001). The perichondrium plays an important role in mediating the effects of TGF-_1 on endochondral bone formation. Dev Dyn 221, 311–321.

133 Yang X, Cao X. (2001). Smad interactors in bone morphogenetic protein signaling. Methods Mol Biol.177, 163-78.

134 Kim K., Sampson E.R., Maynard R.D., O'Keefe R.J., Chen D., Drissi H., Rosier R.N., Hilton M.J., Zuscik M.J. (2012). Ski Inhibits TGF-β/phospho-Smad3 Signaling and Accelerates Hypertrophic Differentiation in Chondrocytes, Cell Biochem. 113, 2156–2166.

135 Ballock R.T., Heydemann A., Wakefield L.M., Flanders K.C., Roberts A.B., Sporn M.B. (1993). TGF-beta 1 prevents hypertrophy of epiphyseal chondrocytes: regulation of gene expression for cartilage matrix proteins and metalloproteases. Dev Biol. 158, 414-29.

136 Ferguson C.M., Schwarz E.M., Reynolds P.R., Puzas J.E., Rosier R.N., O'Keefe R.J. (2000). Smad2 and 3 mediate transforming growth factor-beta1-induced inhibition of chondrocyte maturation. Endocrinology. 141, 4728-35.

137 Pateder D.B., Ferguson C.M., Ionescu A.M., Schwarz E.M., Rosier R.N., Puzas J.E., O'Keefe R.J. (2001). PTHrP expression in chick sternal chondrocytes is regulated by TGF-beta through Smad-mediated signaling. J Cell Physiol.188, :343-51.

138 Laron Z. (2001). Insulin-like growth factor 1 (IGF-1): a growth hormone. Mol Pathol. 54, 311–316.

139 SimsN.A., Clément-Lacroix P., Da PonteF., Bouali Y., Binart N., Morigg I.R., Goffin V., Coschigano K., Gaillard-Kelly M., Kopchick J. (2000). Bone homeostasis in growth hormone receptor-null mice is restored by IGF-I but independent of Stat5. Journal of Clinical Investigation 106, 1095–1103.

140 O'Keefe R.J., Crabb I.D., Puzas J.E., Rosier R.N. (1994). Effects of transforming growth factor-β1 and fibroblast growth factor on DNA synthesis in growth plate chondrocytes are enhanced by insulin-like growth factor 1. Journal of Orthopaedic Research 12, 299-310.

141 Ahmed S.F., Farquharson C. (2010). The effect of GH and IGF1 on linear growth and skeletal development and their modulation by SOCS proteins. Journal of Endocrinology 206, 249–259.

142 Zhang M., Zhou Q., Liang Q.-Q., Li C.G., Holz J.D., Tang D. (2009). IGF-1 regulation of type II collagen and MMP-13 expression in rat endplate chondrocytes via distinct signaling pathways, Osteoarthr. Cartil. 17, 100–106.

143Phornphutkul C., Wu K.-Y., Yang X., Chen Q., Gruppuso P.A. (2004). Insulin-like growth factor-I signaling is modified during chondrocyte differentiation, J. Endocrinol. 183, 477–486.

144 Qiao M., Shapiro P., Kumar R., Passaniti A. (2004). Insulin-like Growth Factor-1 Regulates Endogenous RUNX2 Activity in Endothelial Cells through a Phosphatidylinositol 3-Kinase/ERK-dependent and Akt-independent Signaling Pathway. THE JOURNAL OF BIOLOGICAL CHEMISTRY 279, 42709–42718.

145 Mansukhani A., Bellosta P., Sahni M., Basilicoa C. (2000). Signaling by Fibroblast Growth Factors (Fgf) and Fibroblast Growth Factor Receptor 2 (Fgfr2)–Activating Mutations Blocks Mineralization and Induces Apoptosis in Osteoblasts. J Cell Biol. 149, 1297–1308.

146 Krejci P., Bryja V., Pachernik J., Hampl A., Pogue R., Mekikian P., Wilcox WR. (2004). FGF2 inhibits proliferation and alters the cartilage-like phenotype of RCS cells. Exp Cell Res. 297, 152-64.

147 Jonquoy A., Mugniery E., Benoist-Lasselin C., Kaci N., Le Corre L., Barbault F., Girard A.L., Le Merrer Y., Busca P., Schibler L., Munnich A., Legeai-Mallet L. (2012). A novel tyrosine kinase inhibitor restores chondrocyte differentiation and promotes bone growth in a gain-of-function Fgfr3 mouse model. Hum Mol Genet. 21, 841-51.

148 Yan D., Chen D., Im H.J. (2012). Fibroblast growth factor-2 promotes catabolism via FGFR1-Ras-Raf-MEK1/2-ERK1/2 axis that coordinates with the PKCδ pathway in human articular chondrocytes. J Cell Biochem. 113, 2856-65.

149 Karolak M.R., Yang X., Elefteriou F. (2015). FGFR1 signaling in hypertrophic chondrocytes is attenuated by the Ras-GAP neurofibromin during endochondral bone formation. Hum Mol Genet. 24, 2552-64.

150 Grimsrud C.D., Romano P.R., D'Souza M., Puzas J.E., Schwarz E.M., Reynolds P.R., Roiser R.N., O'Keefe R.J. (2001). BMP signaling stimulates chondrocyte maturation and the expression of Indian hedgehog. J Orthop Res. 19, 18-25.

151 Zhang D., Ferguson C.M., O'Keefe R.J., Puzas J.E., Rosier R.N., Reynolds P.R. (2002). A role for the BMP antagonist chordin in endochondral ossification. J Bone Miner Res. 17, 293-300.

152 Caron M.J., Welting T.J., Surtel D.A., Cremers A., Coolsen M.M., van Rhijn L.W., Emans P.J. (2012). BMP-2 and BMP-7: differential regulation of chondrogenic differentiation. Osteoarthritis and Cartilage. 20, 151.

153 Caron M.M.J., Emans P.J., Cremers A., Surtel D.A.M., Coolsen M.M.E., van Rhijn L.W., Welting T.J.M. (2013). Hypertrophic differentiation during chondrogenic differentiation of progenitor cells is stimulated by BMP-2 but suppressed by BMP-7. Osteoarthritis and Cartilage 21, 604-613.

154 Karl A., Olbrich N., Pfeifer C., Berner A., Zellner J., Kujat R., Angele P., Nerlich M., Mueller M.B. (2014). Thyroid Hormone-Induced Hypertrophy in Mesenchymal Stem Cell Chondrogenesis Is Mediated by Bone Morphogenetic Protein-4. TISSUE ENGINEERING 20, 178–188.

155 Takeda S., Bonnamy J.P., Owen M.J., Ducy P., Karsenty G. (2001). Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. Genes Dev. 15, 467–481.

156 Zheng Q., Zhou G., Morello R., Chen Y., Garcia-Rojas X., and Lee B. (2003). Type X collagen gene regulation by Runx2 contributes directly to its hypertrophic chondrocyte-specific expression in vivo. J Cell Biol 162, 833-842.

157 Kim I.S.. Otto F., Zabel B., Mundlos S. (1999). Regulation of chondrocyte differentiation by Cbfa1. Mechanisms of Development 80, 159-170.

158 Higashikawa A., Saito T., Ikeda T., Kamekura S., Kawamura N., Kan A. (2009). Identification of the core element responsive to runt-related transcription factor 2 in the promoter of human type X collagen gene. Arthritis Rheum 60,166-78

159 Yoshida C.A., Yamamoto H., Fujita T., Furuichi T., Ito K., Inoue K., Yamana K., Zanma A., Takada K., Ito Y., Komori T. (2004). Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. Genes Dev. 18, 952–963.

160 Phimphilai M., Zhao Z., Boules H., Roca H., Franceschi R.T. (2006). BMP Signaling Is Required for RUNX2-Dependent Induction of the Osteoblast Phenotype. J Bone Miner Res. 21, 637–646.

161 Nishimura R., Hata K., Takahata Y., Murakami T., Nakamura E., Yagi H. (2017). Regulation of Cartilage Development and Diseases by Transcription Factors. J Bone Metab. 24, 147-153.

162 Arnold M.A., Kim Y., Czubryt M.P., Phan D., McAnally J., Qi X., Shelton J.M., Richardson J.A., Bassel-Duby R., Olson E.N. (2007). MEF2C transcription factor controls chondrocyte hypertrophy and bone development. Dev Cell. 12, 377-89.

163 Guo J., Chung U.I., Yang D., Karsenty G., Bringhurst F.R., Kronenberg H.M. (2006). PTH/PTHrP receptor delays chondrocyte hypertrophy via both Runx2-dependent and -independent pathways. Dev Biol. 292, 116-28.

164 Grashoff C., Aszódi A., Sakai T., Hunziker E.B., Fässler R. (2003). Integrin-linked kinase regulates chondrocyte shape and proliferation. EMBO reports 4.
165 Ruoslahti E. (1991). Integrins. J Clin Invest 87, 1–5.

166 Loeser R.F. (2014). Integrins and chondrocyte–matrix interactions in articular cartilage. Matrix Biol. 39, 11–16.

167 Lee D., Yeh C., Chang S., Lee P., Chien S., Cheng C., Chiu J. (2008). Integrin-Mediated Expression of Bone Formation–Related Genes in Osteoblast-Like Cells in Response to Fluid Shear Stress: Roles of Extracellular Matrix, Shc, and Mitogen-Activated Protein Kinase. JOURNAL OF BONE AND MINERAL RESEARCH 23, 1140-1149.

168 Perera P.M., Wypasek E., Madhavan S., Rath-Deschner B., Liu J.,Nam J., Rath B., Huang Y., Deschner J., Piesco N., Wu C., Agarwal S. (2010). Mechanical signals control SOX-9, VEGF, and c-Myc expression and cell proliferation during inflammation via integrin-linked kinase, B-Raf, and ERK1/2-dependent signaling in articular chondrocytes. Arthritis Res Ther. 12, 106.

169 Bobick B.E., Kulyk W.M. (2008). Regulation of Cartilage Formation and Maturation by Mitogen-Activated Protein Kinase Signaling. Birth Defects Research 84,131–154.

170 Beier F., Loeser R.F. (2010). Biology and Pathology of Rho GTPase, PI-3 Kinase-Akt and MAP Kinase Signaling Pathways in Chondrocytes. Cell Biochem. 110, 573–580.

171 Harrington E.K., Coon D.J., Kern M.F., Svoboda K.K. (2010). PTH stimulated growth and decreased Col-X deposition are phosphotidylinositol-3,4,5 triphosphate kinase and mitogen activating protein kinase dependent in avian sterna. Anat Rec (Hoboken). 293, 225-34.

172 Ulici V., Hoenselaar K.D., Gillespie J.R., Beier F. (2008). The PI3K pathway regulates endochondral bone growth through control of hypertrophic chondrocyte differentiation. BMC Dev Biol. 8, 40.

173 Xu J., Yi Y., Li L., Zhang W., Wang J. (2015). Osteopontin induces vascular endothelial growth factor expression in articular cartilage through PI3K/AKT and ERK1/2 signaling. Mol Med Rep. 12, 4708-4712.

174 Rokutanda S., Fujita T., Kanatani N., Yoshida C.A., Komori H., Liu W., Mizuno A., Komori T. (2009). Akt regulates skeletal development through GSK3, mTOR, and FoxOs. Dev Biol. 2009 328, 78-93.

175 Bobick B.E. Kulyk W.M. (2004). The MEK-ERK signaling pathway is a negative regulator of cartilage-specific gene expression in embryonic limb mesenchyme. J Biol Chem. 279, 4588-95.

176 Bobick B.E., Thornhill T.M., Kulyk W.M. (2007). Fibroblast growth factors 2, 4, and 8 exert both negative and positive effects on limb, frontonasal, and mandibular chondrogenesis via MEK-ERK activation. J Cell Physiol. 211, 233-43.

177 Yoon Y.M., Oh C.D., Kim D.Y., Lee Y.S., Park J.W., Huh T.L., Kang S.S., Chun JS. (2000). Epidermal growth factor negatively regulates chondrogenesis of mesenchymal cells by modulating the protein kinase C-alpha, Erk-1, and p38 MAPK signaling pathways. J Biol Chem. 275, 12353-9.

178 Provot S., Nachtrab G., Paruch J., Pin Chen A., Silva A., Kronenberg H.M. (2008). A-Raf and B-Raf Are Dispensable for Normal Endochondral Bone Development, and Parathyroid Hormone-Related Peptide Suppresses Extracellular Signal-Regulated Kinase Activation in Hypertrophic Chondrocytes. MOLECULAR AND CELLULAR BIOLOGY 28, 344–357.

179 Mengshol J.A., Vincenti M.P., Coon C.I., Barchowsky A., Brinckerhoff C.E. (2000). Interleukin-1 induction of collagenase 3 (matrix metalloproteinase 13) gene expression in chondrocytes requires p38, c-Jun N-terminal kinase, and nuclear factor kappaB: differential regulation of collagenase 1 and collagenase 3. Arthritis Rheum.43, 801-11.

180 Chen Z., Yue S.X., Zhou G., Greenfield E.M., Murakami S. (2015). ERK1 and ERK2 regulate chondrocyte terminal differentiation during endochondral bone formation. J Bone Miner Res.30, 765–774.

181 Woldt E., Matz R.L., Terrand J., Mlih M., Gracia C., Foppolo S., Martin S., Bruban V., Ji J., Velot E., Herz J., Boucher P. (2011). Differential Signaling by Adaptor Molecules LRP1 and ShcA Regulates Adipogenesis by the Insulin-like Growth Factor-1 Receptor. THE JOURNAL OF BIOLOGICAL CHEMISTRY 286, 16775–16782.

182 Bruderer M., Richards R.G., Alini M., Stodda M.J. (2014). Role and Regulation of RunX2 in Osteogenesis. European Cells and Materials 28, 269-286.

183 Salvat C., Pigenet A., Humbert L., Berenbaum F., Thirion S. (2005). Immature murine articular chondrocytes in primary culture: a new tool for investigating cartilage. Osteoarthritis and Cartilage. 13, 243-249.

184 Ma B., Leijten J.C.H., Wu L., Kip M., van Blitterswijk C.A., Post J.N., Karperien M. (2013). Gene expression profiling of dedifferentiated human articular chondrocytes in monolayer culture. Osteoarthritis Cartilage. 4, 599-603.

185 Mueller A.J. (2012). Global gene expression of dedifferentiated chondrocytes relative to tissues of mesenchymal origin. Osteoarthritis and Cartilage 20, 195-196.

186 Hamada T., Sakai T., Hiraiwa H., Nakashima M., Ono Y., Mitsuyama H., Ishiguro N. (2013). Surface markers and gene expression to characterize the differentiation of monolayer expanded human articular chondrocytes. Nagoya J Med Sci. 75, 101–111.

187 Tallheden T., Karlsson C., Brunnerz A., van der Lee J., Hagg R., Tommasini R., Lindahl A. (2004). Osteoarthritis and Cartilage. 12, 525-535.

188 Yao Y., Wang Y. (2013). ATDC5: an excellent in vitro model cell line for skeletal development. J Cell Biochem. 114, 1223-9.

189 Newton P.T., Staines K.A., Spevak L., Boskey A.L., Texeira C.C., Macrae V.E., Canfield A.E., Farquharson C. (2012). Chondrogenic ATDC5 cells: An optimised model for rapid and physiological matrix mineralization. Int J Mol Med. 30, 1187–1193.

190Reichenberger E., Aigner T., Von Der Mark K., Stobe H., Bertling W. (1991). In Situ Hybridization Studies on the Expression of Type X Collagen in Fetal Human Cartilage. Developmental Biology, 148, 562–572

191 Wang X., Manner P.A., Horner A., Shum L., Tuan R.S., Nuckolls G.H. (2004). Regulation of MMP-13 expression by RUNX2 and FGF2 in osteoarthritic cartilage. Osteoarthritis Cartilage. 12, 963-73.

192 Kwon T.G., Zhao X., Yang Q., Li Y., Ge C., Zhao G., Franceschi R.T. (2011). Physical and functional interactions between Runx2 and HIF-1α induce vascular endothelial growth factor gene expression. J Cell Biochem. 112, 3582-93.

193 Prowse P.D.H., Elliott C.G., Hutter J., Hamilton D.W. (2013). Inhibition of Rac and ROCK Signalling Influence Osteoblast Adhesion, Differentiation and Mineralization on Titanium Topographies. PLoS ONE 8, 58898.

194 Li F., Lu Y., Ding M., Napierala D., Abbassi S., Chen Y., Duan X., Wang S., Lee B., Zheng Q. (2011). Runx2 Contributes to Murine Col10a1 Gene Regulation Through Direct Interaction With Its Cis-Enhancer. J Bone Miner Res. 26, 2899–2910.

195 Ge C., Xiao G., .Jiang D., Yang Q., Hatch N.E., Roca H., Franceschi R.T. (2009). Identification and Functional Characterization of ERK/MAPK Phosphorylation Sites in the Runx2 Transcription Factor. J Biol Chem. 284, 32533–32543.

196 Ge C., Xiao G., Jiang D., Franceschi R.Y. (2007). Critical role of the extracellular signal–regulated kinase–MAPK pathway in osteoblast differentiation and skeletal development. The Journal of Cell Biology, 176, 709–718.

197 Ren, D., Wei, F., Hu, L., Yang, S., Wang, C., & Yuan, X. (2015). Phosphorylation of Runx2, induced by cyclic mechanical tension via ERK1/2 pathway, contributes to osteodifferentiation of human periodontal ligament fibroblasts. Journal of Cellular Physiology, 230, 2426–2436

198 *Park O., Kim H., Woo K., Baek J., Ryoo H. (2009). FGF2-activated ERK-mitogen activated protein kinase enhances RunX2 acetylation and stabilization. The Journal of Biological Chemistry. 285, 3568-3574.*

199 Jun J.H., Yoon W.J., Seo S.B., Woo K.M., Kim G.S., Ryoo H.M., Baek J.H. (2010). BMP2-activated Erk/MAP kinase stabilizes Runx2 by increasing p300 levels and histone acetyltransferase activity. J Biol Chem. 285, 36410-9.

200 Justice R.W., Zilian O., Woods D.F., Noll M., Bryant P.J. (1995). The Drosophila tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. Genes & Dev. 9, 534-546.

201 Meng Z., Moroishi T., Guan K. (2016). Mechanisms of Hippo pathway regulation. Genes Dev. 30, 1–17.

202 Salah Z., Aqeilan R.I. (2011). WW domain interactions regulate the Hippo tumor suppressor pathway. Cell Death Dis. 2, 172.

203 Deng Y., Wu A., Li P., Li G., Qin L., Song H., Mak K.K. (2016). Yap1 Regulates Multiple Steps of Chondrocyte Differentiation during Skeletal Development and Bone Repair. Cell Rep. 14, 2224-2237.

204 *Zaidi S.K., Sullivan A.J., Medina R., Ito Y., van Wijnen A.J., Stein J.L., Lian J.B., Stein G.S. (2004). Tyrosine phosphorylation controls Runx2-mediated subnuclear targeting of YAP to repress transcription. The EMBO Journal 23, 790–799*

205 Christiansen B., Guilak F., Lockwood K., Olson S., Pitsillides A., Sandell L., Silva M., van der Meulen M., Haudenschild D. (2015) Non-Invasive Mouse Models of Post-Traumatic Osteoarthritis. Osteoarthritis Cartilage. 23, 1627–1638.

206 Van der Kraan P.M., Stoop R., Meijers T.H., Poole A.R., van den Berg, W.B. (2001). Expression of type X collagen in young and old C57Bl/6 and Balb/c mice. Relation with articular cartilage degeneration. Osteoarthritis Cartilage 9, 92-100.

207 Glasson S.S., Chambers M.G., Van Den Berg W.B., Little k C.B. (2010). The OARSI histopathology initiative e recommendations for histological assessments of osteoarthritis in themouse. Osteoarthritis and Cartilage 18, S17-S23.

208 Xu L., Polur I., Lim C., Servais J.M., Dobeck J., Li Y., and Olsen B.R. (2009) Early-onset osteoarthritis of mouse temporomandibular joint induced by partial discectomy. Osteoarthritis Cartilage 17, 917-922.

209 Fulco I., Miot S., Haug M.D., Barbero A., Wixmerten A., Feliciano S., Wolf F., Jundt G., Marsano A., Farhadi J. (2014). Engineered autologous cartilage tissue for nasal reconstruction after tumour resection: an observational first-in-human trial. Lancet 384, 337-346.

210 Scotti C., Piccinini E., Takizawa H., Todorov A., Bourgine P., Papadimitropoulos A., Barbero A., Manz M.G., and Martin I. (2013). Engineering of a functional bone organ through endochondral ossification. Proc Natl Acad Sci U S A 110:3997-4002.

211 Sarem M., Arya N., Heizmann M., .Neffe A.T., Barbero A., Gebauer T.P., Martin I., Lendlein A., Shastri V.P. (2018). Interplay between stiffness and degradation of architectured gelatin hydrogels leads to differential modulation of chondrogenesis in vitro and in vivo. Acta Biomaterialia 69, 83-94.

212 Scotti C., Tonnarelli B., Papadimitropoulos A., Scherberich A., Schaeren S., Schauerte A., Lopez-Rios J., Zeller R., Barbero A., Martin I. (2010). Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering. Proc Natl Acad Sci U S A. 107, 7251-6.

213 Centola M., Tonnarelli B., Scharen S., Glaser N., Barbero A., and Martin I. (2013). Priming 3D cultures of human mesenchymal stromal cells toward cartilage formation via developmental pathways. Stem Cells Dev 22:2849-2858.

214 Sesia S.B., Duhr R., Medeiros da Cunha C., Todorov A., Schaeren S., Padovan E., Spagnoli G., Martin I., and Barbero A. (2015). Anti-inflammatory/tissue repair macrophages enhance the cartilage-forming capacity of human bone marrow-derived mesenchymal stromal cells. J Cell Physiol 230, 1258-1269.

215 Yamamoto K., Owen K., Parker A.E., Scilabra S.D., Dudhia J., Strickland D.K., Troeberg L., Nagase H. (2014). Low density lipoprotein receptor-related protein 1 (LRP1)-mediated endocytic clearance of a disintegrin and metalloproteinase with thrombospondin motifs-4 (ADAMTS-4): functional differences of non-catalytic domains of ADAMTS-4 and ADAMTS-5 in LRP1 binding. J. Biol. Chem. 289, 6462–6474.

216 Yamamoto K., Troeberg L., Scilabra S.D., Pelosi M., Murphy C.L., Strickland D.K., Nagase H. (2013). LRP-1-mediated endocytosis regulates extracellular activity of ADAMTS-5 in articular cartilage. FASEB J. 27, 511–521.

217 Scilabra S.D., Troeberg L., Yamamoto K., Emonard H., Thogersen I., Enghild J.J., Strickland D.K., Nagase H. (2013). Differential regulation of extracellular tissue inhibitor of metalloproteinases-3 levels by cell membrane-bound and shed low density lipoprotein receptor-related protein 1. J. Biol. Chem. 288, 332–342.

218 Kawata K., Eguchi T., Kubota S., Kawaki H., Oka M., Minagi S., Takigawa M. (2006). Possible role of LRP1, a CCN2 receptor, in chondrocytes. Biochem. Biophys. Res. Commun. 345, 552–559.

219 Rohlmann A., Gotthardt M., Willnow T. E., Hammer R. E., & Herz J. (1996). Sustained somatic gene inactivation by viral transfer of Cre recombinase. Nature Biotechnology, 14, 1562–1565.

220 Karin M. (1995). The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem. 270, 16483-6.

221 Janknecht R., Ernst W.H., Nordheim A. (1995). SAP1a is a nuclear target of signaling cascades involving ERKs. Oncogene. 10, 1209-16.

222 Vincenti M.P., Brinckerhoff C.E. (2002). Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: integration of complex signaling pathways for the recruitment of gene-specific transcription factors. Arthritis Res. 4, 157–164.

223 Karin M., Ben-Neriah Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol. 18, 621-63.

224 Mengshol J.A., Vincenti M.P., Coon C.I., Barchowsky A., Brinckerhoff C.E. (2000). Interleukin-1 induction of collagenase 3 (matrix metalloproteinase 13) gene expression in chondrocytes requires p38, c-Jun N-terminal kinase, and nuclear factor kappaB: differential regulation of collagenase 1 and collagenase 3. Arthritis Rheum. 43, 801-11.

225 Bartelt A., Behler-Janbeck F., Beil F.T., Koehne T., Müller B., Schmidt T., Heine M., Ochs L., Yilmaz T., Dietrich M., Tuckermann J.P., Amling M., Herz J., Schinke T., Heeren J., Niemeier A. (2018). Lrp1 in osteoblasts controls osteoclast activity and protects against osteoporosis by limiting PDGF–RANKL signaling. Bone Res. 6, 4.

226 Yuan X., Liu H., Huang H., Liu H., Li L., Yang J., Shi W., Liu W., Wu L. (2016). The Key Role of Canonical Wnt/β-catenin Signaling in Cartilage Chondrocytes. Curr Drug Targets. 17, 475-84.

227 Kawata K., Kubota S., Eguchi T., Moritani N.H., Shimo T., Kondo S., Nishida T., Minagi S., Takigawa M. (2010). Role of the Low-density lipoprotein receptor-related protein-1 in regulation of chondrocyte differentiation. J. Cell. Physiol. 222, 138-148.

228 Chung U. (2004). Essential role of hypertrophic chondrocytes in endochondral bone formation. Endocrine Journal 51, 19-24.

229 Madry H., Orth P., Cucchiarini M. (2011) Gene therapy for cartilage repair. Cartilage 2, 201–225.

230 Vincenti M.P., Brinckerhoff C.E. (2002). Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: integration of complex signaling pathways for the recruitment of gene-specific transcription factors. Arthritis Res. 4, 157–164.

231 Aigner T., Zien A., Gehrsitz A., Gebhard P.M., McKenna L. (2001). Anabolic and catabolic gene expression pattern analysis in normal versus osteoarthritic cartilage using complementary DNA-array technology. Arthritis Rheum, 44, 2777-2789.

232 Brew C.J., Clegg P.D., Boot-Handford R.P., Andrew J.G., Hardingham T. (2010). Gene expression in human chondrocytes in late osteoarthritis is changed in both fibrillated and intact cartilage without evidence of generalised chondrocyte hypertrophy. Ann Rheum Dis, 69, 234-240.

233 Yamamotoa K., Okanoa H., Miyagawaa W., Vissea R., Shitomi Y., Santamariaa S., Dudhiac J., Troeberga L., Stricklandd D.K., Hirohata S., Nagasea H. (2016). MMP-13 is constitutively produced in human chondrocytes and co-endocytosed with ADAMTS-5 and TIMP-3 by the endocytic receptor LRP1. Matrix Biol. 56, 57–73.

234 Du W., Jiang Y., Zheng Z., Zhang Z., Chen N., Ma Z., Yao Z., Terada L., Liu Z.(2013). Feedback loop between p66(Shc) and Nrf2 promotes lung cancer progression. Cancer Lett. 337, 58-65.

235 Paneni F., Costantino S., Volpe M., Lüscher T.F., Cosentino F. (2013). Epigenetic signatures and vascular risk in type 2 diabetes: a clinical perspective. Atherosclerosis. 230, 191-7.

236 Ventura A., Luzi L., Pacini S., Baldari C.T., Pelicci P.G. (2002). The p66Shc longevity gene is silenced through epigenetic modifications of an alternative promoter. J Biol Chem.277, 22370-6.

237 Pezzicoli A., Ulivieri C., Capitani N., Ventura A., Pelicci P., Baldari C.T. (2006). Expression in T-cells of the proapoptotic protein p66SHC is controlled by promoter demethylation. Biochem Biophys Res Commun. 349, 322-8.

238 Capitani N., Lucherini O.M., Sozzi E., Ferro M., Giommoni N., Finetti F., De Falco G., Cencini E., Raspadori D., Pelicci P.G., Lauria F., Forconi F., Baldari C.T. (2010). Impaired expression of p66Shc, a novel regulator of B-cell survival, in chronic lymphocytic leukemia. Blood. 115, 3726-36.

239 McCoy A.M. (2015). Animal Models of Osteoarthritis: Comparisons and Key Considerations. Vet Pathol. 52, 803-18.

240 Gagarina V., Gabay O., Dvir-Ginzberg M., Lee E.J., Brady J.K., Quon M.J. (2010). SirT1 enhances survival of human osteoarthritic chondrocytes by repressing protein tyrosine phosphatase 1B and activating the insulin-like growth factor receptor pathway. Arthritis Rheum 62, 1383-92.

241 Schulze-Tanzil G., Mobasheri A., de Souza P., John T., Shakibaei M. (2004). Loss of chondrogenic potential in dedifferentiated chondrocytes correlates with deficient Shc-Erk interaction and apoptosis. Osteoarthritis Cartilage. 12, 448-58.

242 A. Marmotti, S. Mattia, F. Castoldi, A. Barbero, L. Mangiavini, D. E. Bonasia, M. Bruzzone, F. Dettoni, R. Scurati, G. M. Peretti. (2017). Allogeneic Umbilical Cord-Derived Mesenchymal Stem Cells as a Potential Source for Cartilage and Bone Regeneration: An In Vitro Study. Stem Cells Int. 1732094.

243 Orth P., Rey-Rico A., Venkatesan J.K., Madry H., Cucchiarini M. (2014). Current perspectives in stem cell research for knee cartilage repair. Stem Cells Cloning 7, 1–17.

244 Reppel L., Schiavi J., Charif N., Leger L., Yu H., Pinzano A., Henrionnet C., Stoltz J., Bensoussan D., Huselstein C. (2015). Chondrogenic induction of mesenchymal stromal/stem cells from Wharton's jelly

embedded in alginate hydrogel and without added growth factor: an alternative stem cell source for cartilage tissue engineering. Stem Cell Res Ther. 6, 260.

245 Cucchiarini M., Henrionnet C., Mainard D., Pinzano A., Madry H. (2015). New trends in articular cartilage repair. J Exp Orthop. 2, 8.

246 Scott M.A., Levi B., Askarinam A., Nguyen A., Rackohn T., Ting K., Soo C., James A.W. (2012). Brief review of models of ectopic bone formation. STEM CELLS AND DEVELOPMENT 21, 655-667.

247 Yamamoto K., Okano H., Miyagawa W., Visse R., Shitomi Y., Santamaria D., Dudhia J., Troeberg L., Strickland D.K., Hirohata S., Nagase H. (2016). MMP-13 is constitutively produced in human chondrocytes and coendocytosed with ADAMTS-5 and TIMP-3 by the endocytic receptor LRP1. Matrix Biol. 56, 57–73.

248 Yang E., Zheng H., Peng H., Ding Y. (2015). Lentivirus-induced knockdown of LRP1 induces osteoarthritic-like effects and increases susceptibility to apoptosis in chondrocytes via the nuclear factor-κB pathway. Experimental and Therapeutic Medicine, 10, 97-105.

249 Gosset M., Berenbaum F., Thirion S., Jacques C. (2008). Primary culture and phenotyping of murine chondrocytes. Nature Protocols, 3, 1253–1260.

250 Shukunami C., Ishizeki K., Atsumi T., Ohta Y., Suzuki F., Hiraki Y. (1997). Cellular hypertrophy and calcification of embryonal carcinoma-derived chondrogenic cell line ATDC5 in vitro. J Bone Miner Res. 12, :1174-88.

251 El Asmar Z., Terrand J., Jenty M., Host L., Mlih M., Zerr A., Justiniano H., Matz R.L., Boudier C., Scholler C., Garnier J., Bertaccini1, Thierse D., Schaeffer C., Van Dorsselaer A., Herz J., Bruban V., Boucher P. (2016). Convergent signaling pathways controlled by LRP1 cytoplasmic and extracellular domains limit cellular cholesterol accumulation. Journal of Biological Chemistry. 291, 5116-5127.

252 Rigueur D., Lyons K.M. (2014). Whole-Mount Skeletal Staining. Methods Mol Biol. 1130, 113–121.

253 Libby P. (2002). Inflammation in Atherosclerosis. Nature 420, 868-74.

254 Mestas, J., Ley K. (2008). Monocyte-Endothelial Cell Interactions in the Development of Atherosclerosis. Trends Cardiovasc Med. 18, 228–232.

255 Assmann G., Cullen P., Jossa F., Lewis B., Mancini M. (1999). Coronary heart disease: reducing the risk. Arterioscl. Thromb. Vasc. Biol. 19, 1819–1824.

256 Xu M.., Zhao J., Zhang Y., Ma X., Dai Q., Zhi H., Wang B., Wang L. (2016). Apolipoprotein E Gene Variants and Risk of Coronary Heart Disease: A Meta-Analysis. Biomed Res Int. 3912175.

257 Xie L., Li Y. (2017). Lipoprotein Lipase (LPL) Polymorphism and the Risk of Coronary Artery Disease: A Meta-Analysis. Int J Environ Res Public Health. 14, 84.

258 Gordon D.J., Rifkind B.M. (1989). High-density lipoprotein—the clinical implications of recent studies. N. Engl. J. Med. 321, 1311–1316

259 Luft F.C. (1998). Molecular genetics of human hypertension. J. Hypertens. 16, 1871–1878.

260 Nathan L., Chaudhuri G. (1997). Estrogens and atherosclerosis. Annu. Rev. Pharmacol. Toxicol. 37, 477–515.

261 Lusis A.J., Weinreb A., Drake T.A. (1998). In: Textbook of Cardiovascular Medicine. Topol EJ, editor. Lippincott-Raven; Philadelphia: pp. 2389–2413.

262 Yvan-Charvet L., Ranalletta M., Wang N., Han S., Terasaka N., Li R., Welch C., Tall A.R. (2007). Combined deficiency of ABCA1 and ABCG1 promotes foam cell accumulation and accelerates atherosclerosis in mice. J Clin Invest. 117, 3900–3908.

263 Gimbrone M.A. Jr. (1999). Vascular endothelium, hemodynamic forces, and atherogenesis. Am. J. Pathol 155, 1–5.

264 Goldstein J.L., Ho Y.K., Basu S.K., Brown M.S. (1979). Binding sites on macrophages that mediate uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc. Natl Acad. Sci.76, 333–337.

265 Cyrus T. et al. (1999). Disruption of 12/15-lipoxygenase diminishes atherosclerosis in apoE-deficient mice. J. Clin. Invest 103, 1597–1604.

266 Knowles J.W. et al. (2000). Enhanced atherosclerosis and kidney dysfunction in eNOS(–/–) apoE(–/–) mice are ameliorated by enalapril treatment. J. Clin. Invest 105, 451–458.

267 Dong Z.M. et al. (1998). The combined role of P- and E-selectins in atherosclerosis. J. Clin. Invest 102, 145–152.

268 Collins R.G. et al. (2000). P-selectin or intercellular adhesion molecule (ICAM-1) deficiency substantially protects against atherosclerosis in apolipoprotein E-deficient mice. J. Exp. Med 191, 189–194.

269 Gu L. et al. (1998). Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein-deficient mice. Mol. Cell 2, 275–281.

270 Smith J.D. (1995). Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. Proc. Natl Acad. Sci. 92, 8264–8268.

271 Podrez E.A. et al. (2000). Macrophage scavenger receptor CD36 is the major receptor for LDL modified by monocyte-generated reactive nitrogen species. J. Clin. Invest 105, 1095–1108.

272 Zhou X., Hansson G. (1999). Detection of B cells and proinflammatory cytokines in atherosclerotic plaques of hypercholesterolaemic apolipoprotein E knockout mice. Scand J Immunol. 50, 25-30.

273 Whitman S.C., Ravisankar P., Elam H., Daugherty A. (2000). Exogenous interferon-γ enhances atherosclerosis in apolipoprotein E−/− mice. Am J Pathol. 157, 1819-24.

274 Abdolmaleki F., Hayat S.M.G, Bianconi V., Johnston T.P., Sahebkar A. (2018). Atherosclerosis and immunity: a perspective. Trends in Cardiovascular Medicine.18, 1050-1738.

275 Lusis A.J. (2000). Atherosclerosis. Nature 407, 233–241.

276 Jackson A.O., Regine M.A., Subrata C., Long S. (2018). Molecular mechanisms and genetic regulation in atherosclerosis. IJC Heart & Vasculature 21,36-44.

277 Mury P., Chirico E.N., Mura M., Millon A., Canet‐Soulas E., Pialoux V. (2018). Oxidative Stress and Inflammation, Key Targets of Atherosclerotic Plaque Progression and Vulnerability: Potential Impact of Physical Activity. Sports Med.

278 Durham A.L., Speer M.Y., Scatena M., Giachelli C.M., Shanahan C.M. (2018). Role of smooth muscle cells in vascular calcification: implications in atherosclerosis and arterial stiffness. Cardiovascular Research 114, 590–600.

279 Kanwar R.K., Chaudhary R., Tsuzuki T., Kanwar J.R. (2012). Emerging Engineered Magnetic Nanoparticulate Probes for Molecular MRI of Atherosclerosis. Nanomedicine. 7, 899-916.

280 May P., Woldt E., Matz R.L., Boucher P. (2007). The LDL receptor-related protein (LRP) family: an old family of proteins with new physiological functions.Ann Med. 39, 219-28.

281 Zhou L., Choi H.Y., Li W.P., Xu F., Herz J. (2009). LRP1 controls cPLA2 phosphorylation, ABCA1 expression and cellular cholesterol export. PLoS One. 4, 6853.

282 Napoli C., Martin-Padura I., de Nigris F., Giorgio M., Mansueto G., Somma P., Condorelli M., Sica G., De Rosa G., Pelicc P. (2003). Deletion of the p66Shc longevity gene reduces systemic and tissue oxidative stress, vascular cell apoptosis, and early atherogenesis in mice fed a high-fat diet. Proc Natl Acad Sci U S A. 100, 2112–2116.

283 *Mlih M., Host L., Martin S., Niederhoffer N., Monassier L., Terrand J., Messaddeq N., Radke M., Gotthardt M., Bruban V., Kober F., Bernard M., Canet-Soulas E., Abt-Jijon F., Boucher P., Matz R.L. (2015). The Src Homology and Collagen A (ShcA) Adaptor Protein Is Required for the Spatial Organization of the Costamere/Z-disk Network during Heart Development. Journal of Biological Chemistry 290, 2419–2430.*

284. Abou-Jaoude A., Badiqué L., Mlih M., Awan S., Guo S., Lemle A., Abboud C., Foppolo S., Host L., Terrand J., Justiniano H., Herz J., Matz R.L., Boucher P. (2018). Loss of the adaptor protein ShcA in endothelial cells protects against monocyte macrophage adhesion, LDL-oxydation, and atherosclerotic lesion formation. Scientific Reports 8, 4501.

285. Barnes, H., Ackermann, E.J., and van der Geer, P. 2003. v-Src induces Shc binding to tyrosine 63 in the cytoplasmic domain of the LDL receptor-related protein 1. Oncogene 22:3589-3597.

286. Wang, Y., Cheng, Z., Elalieh, H.Z., Nakamura, E., Nguyen, M.T., Mackem, S., Clemens, T.L., Bikle, D.D., and Chang, W. (2011). IGF-1R signaling in chondrocytes modulates growth plate development by interacting with the PTHrP/Ihh pathway. J Bone Miner Res 26:1437-1446.

287. Chen, C. C., Rosenbloom, C. L., Anderson, D. C. & Manning, A. M. (1995). Selective inhibition of E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 expression by inhibitors of I kappa B-alpha phosphorylation. J Immunol 155, 3538–3545.

288. Vandewalle, C., Van Roy, F. & Berx, G. (2009). The role of the ZEB family of transcription factors in development and disease. Cell Mol Life Sci 66, 773–787.

289. Chua, H. L. et al. (2007). NF-kappaB represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. Oncogene 26, 711–724.

290. Lehmann, W. et al. (2016). ZEB1 turns into a transcriptional activator by interacting with YAP1 in aggressive cancer types. Nat Commun 7, 10498.

291. Dimmeler, S. et al. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 399, 601–605.

292. Jessop, H.L., Rawlinson, S.C., Pitsillides, A.A., and Lanyon, L.E. (2002). Mechanical strain and fluid movement both activate extracellular regulated kinase (ERK) in osteoblast-like cells but via different signaling pathways. Bone 31:186-194.

Résumé

ShcA (Src Homology and Collagen A) est une protéine adaptatrice qui se lie à la partie cytoplasmique de LRP1 (Low Density Lipoprotein-related receptor 1), un récepteur transmembranaire qui protège contre l'athérosclérose. La calcification vasculaire est une complication majeure de cette maladie et ses mécanismes ressemblent au processus d'ostéochondrogenèse. Nous avons étudié le rôle de ShcA endothélial dans la formation des lésions d'athérosclérose ainsi que les rôles de ShcA et LRP1 dans la chondrogenèse. ShcA endothélial participe à la formation des lésions d'athérosclérose *in-vivo*. En inhibant la NOS endothéliale et activant l'expression de ICAM-1 via ZEB1, ShcA favorise l'adhésion des monocytes.
La suppression de ShcA dans les chondrocytes a conduit au développement de souris présentant un phénotype de nanisme par une inhibition de la différenciation hypertrophique des chondrocytes. Ceci conduit également à une diminution du développement de l'arthrose liée au vieillissement. La suppression de LRP1 dans les chondrocytes conduit également à un phénotype de nanisme chez la souris, mais par des mécanismes différents.

Mots clés : ShcA, LRP1, arthrose, athérosclérose, hypertrophie des chondrocytes.

Abstract:

ShcA (Src Homology and Collagen A) is an adaptor protein that binds to the cytoplasmic tail of the Low Density Lipoprotein-related receptor1 (LRP1), a trans-membrane receptor that protects against atherosclerosis. Vascular calcification is a major complication of this disease and its mechanisms highly resemble the process of osteochondrogenesis. We studied the role of endothelial ShcA in atherosclerotic lesion formation as well as the roles of ShcA and LRP1 in chondrogenesis.
Endothelial ShcA participates in the formation of atherosclerotic lesions *in-vivo*. By inhibiting endothelial NOS and activating the expression of ICAM-1 via ZEB1, ShcA enhances monocyte adhesion.
The deletion of ShcA in chondrocytes led to the development of mice with a dwarfism phenotype by inhibiting chondrocyte hypertrophic differentiation. This also led to a decrease in the development of age-related osteoarthritis.
The deletion of LRP1 in chondrocytes also led to a dwarfism phenotype in our mouse model, but trough different mechanisms.

Mots clés : ShcA, LRP1, osteoarthritis, atherosclerosis, chondrocyte hypertrophy.