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The role of PPARγ in cartilage growth and development using cartilage-specific PPARγ knockout mice

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The role of PPARγ in cartilage growth and development using cartilage-specific PPARγ knockout mice

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RÉSUMÉ

Le cartilage est un tissu conjonctif composé d'une seule sorte de cellule nommée chondrocytes. Ce tissu offre une fondation pour la formation des os. Les os longs se développent par l'ossification endochondral. Ce processus implique la coordination entre la prolifération, la différenciation et l'apoptose des chondrocytes, et résulte au remplacement du cartilage par l'os. Des anomalies au niveau du squelette et des défauts liés à l'âge tels que l'arthrose (OA) apparaissent lorsqu'il y a une perturbation dans l'équilibre du processus de développement. À ce jour, les mécanismes exacts contrôlant la fonction et le comportement des chondrocytes pendant la croissance et le développement du cartilage sont inconnus.

Le récepteur activateur de la prolifération des peroxysomes (PPAR) gamma est un facteur de transcription impliqué dans l'homéostasie des lipides. Plus récemment, son implication a aussi été suggérée dans l'homéostasie osseuse. Cependant, le rôle de PPARγ *in vivo* dans la croissance et le développement du cartilage est inconnu. Donc, pour la première fois, cette étude examine le rôle spécifique de PPARγ *in vivo* dans la croissance et le développement du cartilage. Les souris utilisées pour l'étude avaient une délétion conditionnelle au cartilage du gène PPARγ. Ces dernières ont été générées en employant le système LoxP/Cre.

Les analyses des souris ayant une délétion au PPARγ aux stades embryonnaire et adulte démontrent une réduction de la croissance des os longs, une diminution des dépôts de calcium dans l'os, de la densité osseuse et de la vascularisation, un délai dans l'ossification primaire et secondaire, une diminution cellulaire, une perte d'organisation colonnaire et une diminution des zones hypertrophiques, une désorganisation des

plaques de croissance et des chondrocytes déformés. De plus, la prolifération et la différenciation des chondrocytes sont anormales. Les chondrocytes et les explants isolés du cartilage mutant démontrent une expression réduite du facteur de croissance endothélial vasculaire (VEGF)-A et des éléments de production de la matrice extracellulaire. Une augmentation de l'expression de la métalloprotéinase matricielle (MMP)-13 est aussi observée. Dans les souris âgées ayant une délétion au PPARγ, y est aussi noté des phénotypes qui ressemblent à ceux de l'OA tel que la dégradation du cartilage et l'inflammation de la membrane synoviale, ainsi qu'une augmentation de l'expression de MMP-13 et des néoépitopes générés par les MMPs.

Nos résultats démontrent que le PPARγ est nécessaire pour le développement et l'homéostasie du squelette. PPARγ est un régulateur essentiel pour la physiologie du cartilage durant les stades de croissance, de développement et de vieillissement.

Mots clés: Arthrose, Souris ayant une délétion au PPARγ, Croissance et le développement du cartilage, Ossification endochondral, Formation du cartilage, Vieillissement

SUMMARY

Cartilage, a connective tissue composed of chondrocytes, provides an intermediate template on which bones are formed. Long bones develop through endochondral ossification, involving coordination between chondrocyte proliferation, differentiation and apoptosis, resulting in bone replacing cartilage. Disturbances in this balance results in skeletal abnormalities, and age-related defects including osteoarthritis (OA). The exact mechanisms that control chondrocyte function and behaviour during growth and development are unknown.

Peroxisome proliferator-activated receptor (PPAR) gamma, a transcription factor involved in lipid homeostasis, has recently been suggested to be involved in bone homeostasis. However, PPARγ's role in cartilage growth and development *in vivo* is unknown. Therefore, for the first time, this study examines PPARγ's specific *in vivo* role in cartilage growth and development using cartilage-specific PPARγ knockout (KO) mice.

Conditional KO mice were generated using LoxP/Cre system. Histomorphometric analyses of embryonic and adult mutant mice demonstrate reduced long bone growth, calcium deposition, bone density, vascularity, and delayed primary and secondary ossification. Mutant growth plates are disorganized with abnormal chondrocyte shape, proliferation and differentiation, reduced cellularity, loss of columnar organization, and shorter hypertrophic zones. Isolated mutant chondrocytes and cartilage explants show decreased vascular endothelial growth factor (VEGF)-A and extracellular matrix (ECM) production product expression, and increased matrix metalloproteinase (MMP)-13 expression. Aged mutant mice exhibit accelerated OA-

like phenotypes, and enhanced cartilage degradation, synovial inflammation, MMP-13 and MMP-generated neoepitope expression.

Our data demonstrate that PPARγ is required for normal skeletal development and homeostasis, and is a critical regulator of cartilage health and physiology in early growth and development and aging.

Keywords: Osteoarthritis, Knockout mice, PPARγ, Cartilage growth and development, Endochondral ossification, Chondrogenesis, Aging

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LIST OF ABBREVIATIONS

ACH: Achondroplasia
Bps: Basepairs
BrdU: 5-bromo-2'-deoxyuridine
BSA: Bovine Serum Albumin
C1-2C: Col2-3/4C_{short}
cAMP: Cyclic Adenomonophosphate
Cbfa1: Core Binding Factor Alpha1
CDK: Cyclin-Dependent Kinase
COX-2: Cyclooxygenase-2
CR-CHUM: University of Montreal Hospital Research Centre
Dkk: Dickkopf
DMEM: Dulbecco's Modified Eagle Medium
E: Embryonic Day
ECM: Extracellular Matrix
EDTA: Ethylenediaminetetraacetic Acid
ERK: Extracellular Signal-Regulated Protein Kinase
FBS: Fetal Bovine Serum
FGF: Fibroblast Growth Factor
FGFR: Fibroblast Growth Factor Receptor
FrzB: Frizzled-Related Protein
Fz: Frizzled
GSK: Glycogen Synthase Kinase
IgG: Immunoglobulin G
IHC: Immunohistochemistry
Ihh: Indian Hedgehog
IL: Interleukin
IL-1R: Interleukin-1 Receptor
ILK: Integrin-Linked Kinase
iNOS: Inducible Nitric Oxide Synthase
JNK c-Jun N-Terminal Kinase
KO: Knockout
MAPK Mitogen-Activated Protein Kinase
MMP: Matrix Metalloproteinase
mPGES-1: Microsomal Prostaglandin E Synthase-1
NA: Numerical Aperture
NF: Nuclear Factor
NSAID: Non-Steroidal Anti-inflammatory Drug
OA: Osteoarthritis
OARSI: Osteoarthritis Research Society International
P: Post-Natal Day
PBS: Phosphate Buffered Saline
PCR: Polymerase Chain Reaction
PECAM: Platelet/Endothelial Cell Adhesion Molecule
PGE2: Prostaglandin E2
PPAR: Peroxisome Proliferator-Activated Receptor
Ptc1: Patched-1
PTEN: Phosphatase and tensin homolog
PTHrP: Parathyroid Hormone-Related Peptide
Runx2: Runt-Related Transcription Factor2
SAPK Stress-Activated Protein Kinase
SEM: Standard Error of the Mean
sFRP: Secreted Frizzled-Related Protein
Smo: Smoothened
TACE: TNF-α converting enzyme
TBS: Tris Buffered Saline
TBS-T: Tris Buffered Saline containing Tween-20
TD: Thanatophoric Dysplasia
TGF: Transforming Growth Factor
TNF: Tumor Necrosis Factor
TNFR: Tumor Necrosis Factor Receptor
VEGF: Vascular Endothelial Growth Factor
WT: Wild-Type

DEDICATION

To my parents, sisters, family, and friends for always telling me I can.

Thank you for believing in me when I don't believe in myself.

Everybody dies but not everybody lives ~ Aubrey Drake Graham

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INTRODUCTION

Cartilage Growth and Development

Cartilage is a dense connective tissue composed of only one cell type called chondrocytes, which are formed through the process of chondrogenesis. Endochondral ossification is the process through which long bones are formed and lengthened upon the replacement of cartilage with bone [1, 2]. During this transition from cartilage to bone, cartilage provides an intermediate template on which bone is laid down [3]. Chondrogenesis and endochondral ossification mark the two foundational processes of cartilage growth and development.

Chondrogenesis

The first step of chondrogenesis is the condensation of mesenchymal cells. In these condensations, mesenchymal cells differentiate to chondrocytes [4] and the density of the initial condensations is directly proportional to the level of chondrogenic differentiation [5]. Differentiated chondrocytes express specific molecular markers that undifferentiated mesenchymal cells do not, thus allowing the two cell types to be distinguishable [6]. For example, during chondrogenesis, chondrocytes become spherical and begin expressing transcription factors such as Sox9, 5, and 6, all of which regulate genes encoding for aggrecan and collagen type II, the two main components of the cartilage extracellular matrix (ECM) [7-10]. Undifferentiated mesenchymal cells remain located at the border of condensations and form the perichondrium, a layer of connective tissue surrounding the cartilage [6].

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Endochondral ossification

Chondrocytes are organized into three zones, resting, proliferating, and hypertrophic, within the epiphyses of the cartilage. The chondrocytes belonging to each zone are unique; cell shape, behaviour, and function as well as the molecular markers they express allow resting, proliferating, and hypertrophic chondrocytes to be distinguishable [2].

Resting chondrocytes are round, sparse, and sit at the top of the growth plate in a dormant state. In this region, the ratio of ECM to cell volume is high [11, 12]. The chondrocytes within the proliferating zone are flat and organized into columnar structures as they produce ECM primarily vertically. This orientation drives bone lengthening. It is within this zone that chondrocytes begin to divide [13, 14]. Both these types of chondrocytes express high levels of aggrecan and collagen type II, and a genetic program driven by Sox9 and other transcription factors. Furthermore, they control the rate of hypertrophic differentiation [4, 15].

Hypertrophic chondrocytes are divided into two subpopulations: prehypertrophic and hypertrophic. Akin to resting and proliferating chondrocytes, prehypertrophic chondrocytes express collagen type II whereas hypertrophic chondrocytes express collagen type X, the only molecular marker specific to these cells [16, 17]. These two types of chondrocytes form the growth plate, the cartilage segment on both sides of the primary ossified region, which is responsible for the longitudinal growth of long bones [13, 14, 18].

There are several aspects upon which the normal lengthening of long bones is dependent, including the rate of production of hypertrophic chondrocytes from

proliferating chondrocytes, the volume increase of hypertrophic chondrocytes, and the number of proliferative cycles a chondrocyte undergoes [4, 14, 18-20].

Upon differentiation from mesenchymal cells to chondrocytes, the newly formed chondrocytes will either remain as chondrocytes or differentiate to hypertrophic chondrocytes. It is the chondrocytes within the cartilage anlagen that proliferate while central chondrocytes differentiate to hypertrophy. Chondrocytes that do not differentiate form the cartilage on the articular surface of joints [2]. Before entering the hypertrophic zone, cells exit the cell cycle and begin to differentiate to hypertrophic chondrocytes [18]. These chondrocytes exhibit an increase in cell size and volume, and contribute to the formation of the cartilage intermediate template [2, 21]. Upon differentiation, they mineralize their surrounding ECM in the cartilage centre and undergo apoptosis [15, 22]. This mineralization process is termed primary ossification [13]. Thereafter, vascular endothelial growth factors (VEGF) facilitate blood vessel invasion in the hypertrophic zone [15, 23]. Along with the invasion of blood vessels come osteoblasts and osteoclasts. Osteoclasts play a role in mineralized cartilage degradation while osteoblasts replace it with bone tissue [1, 2, 24]. Secondary ossification centers are formed in the cartilage epiphyses postnatally [1].

Due to the many intracellular and extracellular factors involved in cartilage growth and development, tight regulation of the processes is important. Improper coordination of any of the variables or disturbances in the balance regulating endochondral bone growth results in growth-related abnormalities such as dwarfism and skeletal deformities [4, 11, 13, 25].

Essential Factors for Skeletogenesis

There are several main factors that are necessary for the proper formation of long bones during cartilage growth and development.

Cartilage Extracellular Matrix

In the joint, cartilage acts as a shock absorber. In order to exude this property, it must distribute load efficiently at the joint, which is dependent on the composition and organization of the ECM. The two main components of the cartilage ECM that contribute to the tissue's mechanical properties are aggrecan and collagen type II. Without these vital elements, the composition, organization, and overall structural integrity of the cartilage ECM is compromised, leading to cartilage dysfunction [26].

Collagen type II is the primary type of collagen expressed in the growth plate and is a fundamental component of the cartilage ECM [6]. It provides tensile strength, helps with molecular organization within the matrix, and contributes to structure maintenance with the help of its triple helical fibrils [27]. The intricate collagen framework is created during development and chondrocytes are responsible for network maintenance. Adult cartilage does not readily secrete collagen in large amounts [28, 29]. Therefore, in adult cartilage, once the collagen network is destroyed, it cannot be newly created or replaced, making collagen loss irreversible [30].

Aggrecan, a proteoglycan containing gylcosaminoglycan chains, is another macromolecule that plays a fundamental role in the structural integrity of the cartilage ECM. Aggrecan contributes to the cushioning trait that the matrix possesses as well as cartilage's compressibility and elasticity properties due to its high negative charge and

water-binding capacity. Similar to collagen type II, it aids in the organization of molecules within the ECM [31-33]. In contrast to collagen type II, however, aggrecan is somewhat replaceable as it is synthesized by chondrocytes [28, 29].

Sox9

Sox9, a transcription factor expressed in chondrocytes, is a member of the Sox gene family, a clan of genes known to be involved in various developmental processes [9, 34-36]. During chondrogenesis, chondrocytes begin expressing Sox9. Specifically, Sox9 is expressed in resting and proliferating chondrocytes within the growth plate of the cartilage, but displays maximum expression in prehypertrophic chondrocytes [4, 15]. It is essential for proliferation and differentiation of chondrocytes, and regulates the expression of genes encoding aggrecan and collagen type II [6, 27, 31]. Moreover, Sox9 has shown to be involved in the formation of the axial skeleton and animal mouse models have demonstrated its expression in mesenchymal condensations [37]. Therefore, it plays a crucial role in chondrogenesis as well as cartilage growth and development.

Collagen Type X

During endochondral ossification, hypertrophic chondrocytes within the growth plate of long bones express collagen type X. Collagen type X expression allows hypertrophic chondrocytes to be distinguishable as it is a cartilage ECM component unique to the hypertrophic zone. This collagen type serves as a marker of terminally differentiated hypertrophic chondrocytes and due to its expression in hypertrophic

chondrocytes, which are soon to undergo a transition from cartilage to bone, it is hypothesized that collagen type X is involved in various steps of endochondral ossification, such as mineralization, vascularization, and matrix stabilization [38-41]. Specifically, collagen type X facilitates endochondral ossification by regulating matrix mineralization and compartmentalizing matrix components [42]. Its principal role in endochondral ossification was demonstrated in animal models, which have shown that mice with altered collagen type X function exhibit skeletal defects, including compressed growth plates with reduced proliferating and hypertrophic zones, and trabecular bone thickness [43-45].

p57

Cyclin-dependent kinases (CDKs) are one type of kinases that are responsible for controlling cell cycle progression [46-49]. One of the several factors regulating CDK activity is the level of inhibitory proteins, namely, the Cip/Kip (p21, p27, p57) and Ink (p15, p16, p18, p19) families, otherwise knows as CDK inhibitors. By blocking phosphorylation of the retinoblastoma protein, a tumor suppressor protein, these inhibitors negatively regulate cell cycle progression and proliferation. As a result, CDK inhibitors are crucial in cell cycle exit regulation [50].

p57, a member of the Cip/Kip family, has been shown to be involved in cartilage growth and development. Specifically, studies using targeted inactivation of the gene have demonstrated that severe skeletal abnormalities occur due to delayed cell cycle progression as well as disrupted hypertrophic differentiation [51, 52]. In addition, animal models using p57 knockout (KO) mice have shown abnormalities within the

endochondral skeleton while rat chondrocytes overexpressing p57 have shown to induce cell cycle exit [53]. Overall, chondrocytes have been shown to be sensitive, when compared to other cell types, to changing levels of p57, emphasizing its necessity for normal hypertrophic differentiation and, in general, its vital role in cartilage growth and development [18].

Vascular Endothelial Growth Factor-A

The final step of endochondral ossification is the replacement of avascular cartilage by highly vascularized bone. Therefore, blood vessel invasion is a prerequisite for bone formation and angiogenesis plays a fundamental role in bone development.

VEGF is a signal protein that positively regulates angiogenesis [54, 55], and, as such, is important in bone growth [24, 56-58]. In addition to its critical role in this step of bone development, it is essential during various steps of endochondral ossification, such as chondrocyte differentiation, osteoblast differentiation, and osteoclast recruitment [59-61]. VEGF is highly expressed in hypertrophic chondrocytes, thus supporting the fact that blood vessel invasion and angiogenesis begin in the hypertrophic zone of the cartilage. Specifically, VEGF is secreted by hypertrophic chondrocytes in the upper region of the hypertrophic zone. As a result, it regulates blood vessel invasion from the perichondrium and the removal of the cartilage matrix [57]. Studies in which VEGF-A was inactivated have shown abnormalities in trabecular bone formation due to suppressed invasion of new blood vessels [24]. Other studies have shown that when VEGF-A is specifically inactivated in chondrocytes, endochondral bone and blood vessel development are abnormal [62].

Signaling Pathways

Parathyroid hormone-related peptide (PTHrP), Indian hedgehog (Ihh), and transforming growth factor (TGF)-β are signaling molecules involved in a local feedback loop. This local feedback loop is responsible for regulating the rate at which proliferating chondrocytes exit the proliferating zone and begin to differentiate to hypertrophic chondrocytes [11]. Other signaling pathways involved in the regulation of chondrogenesis, endochondral ossification, and cartilage growth and development include fibroblast growth factors (FGFs), integrins, Akt, and Wnt/β-cantenin.

Indian Hedgehog

Ihh is a mammalian homolog of the *Drosophila* Hedgehog secreted factor [63]. Specifically, it is a growth factor that plays an important role in bone development. It is involved in chondrocyte proliferation and differentiation, and osteoblast differentiation. During endochondral bone development, Ihh is synthesized by prehypertrophic and early hypertrophic chondrocytes [4]. The Ihh signaling pathway is activated by Smoothened (Smo), which is released when Ihh and its receptor, patched-1 (Ptc1), bind. In the absence of Ihh, Ptc1 represses Smo, a membrane protein required for Ihh cellular actions, thus preventing activation of the signaling pathway [64].

Ihh regulates several factors during bone development, and studies using Ihh KO mice have demonstrated its importance throughout various stages of endochondral bone ossification. During mesenchymal condensations, Ihh KO mice have normal bones. However, as bone formation continues, there is a decrease in chondrocyte proliferation, thus causing bone growth abnormalities and all cartilage elements to remain small [63].

The same conclusion was drawn in cartilage-specific Smo KO mice [65].

Another abnormality exhibited by Ihh KO mice is an increase in hypertrophic chondrocytes. This is caused by an increase of chondrocytes exiting the proliferating zone prematurely, which results from the inability of cartilage to synthesize PTHrP. PTHrP is a protein secreted by perichondrial cells and early proliferating chondrocytes. It is responsible for keeping proliferating chondrocytes in the proliferating zone [4]. PTHrP-deficient chondrocytes become hypertrophic close to the ends of bones [66, 67]. In contrast, PTHrP overexpression in chondrocytes causes hypertrophic chondrocytes to appear later, meaning proliferating chondrocytes are late to leave the proliferating zone [68]. Ihh has the ability to stimulate PTHrP production, thus delaying chondrocyte hypertrophy and increasing the rate of proliferation [69]. This proves that PTHrP signaling is compulsory in mediating the action of Ihh to delay hypertrophy. Together Ihh and PTHrP control the fate of chondrocytes to leave the proliferating zone through a negative feedback loop. When chondrocytes are no longer sufficiently stimulated by PTHrP, they stop proliferating and synthesize Ihh. The interactions of PTHrP and Ihh are important in determining the lengths of proliferative columns [4].

Ihh also regulates mesenchymal cell differentiation into osteoblast progenitors [70]. Ihh controls osteoblast differentiation and is required for osteoblast formation as demonstrated by Ihh KO mice that are osteoblast-deficient. To form osteoblasts, Ihh acts on perichondrial cells to convert them into osteoblasts [4, 63].

Finally, Ihh contributes to the transcriptional control of chondrocyte hypertrophy. Core binding factor alpha1 (Cbfa1)/Runt-related transcription factor 2 (Runx2), which is expressed in nonhypertrophic chondrocytes, is a transcription factor

involved in chondrocyte hypertrophy [71, 72]. A regulatory loop exists between Cbfa1 and Ihh. Cbfa1 regulates Ihh expression while Ihh regulates Cbfa1 expression [15]. In the absence of Cbfa1 or Ihh, osteoblasts are not formed and chondrocytes do not differentiate to hypertrophy [63]. Their interdependence demonstrates the strong relationship that exists between chondrocytes and osteoblasts [15].

Transforming Growth Factor-β

There are 35 members in the TGF-β superfamily, including TGF-βs, activins, and bone morphogenetic proteins [73]. These members play an important role in various physiological processes, and regulate cell proliferation, differentiation, apoptosis and migration [74]. TGF-β exists in three isotypes, β1, β2, and β3. Each isotype possesses different promoter sequences, and, as a result, their expression is regulated differently at the transcriptional level [75-77]. Chondrocytes secrete TGF-β in an inactive form comprising of a TGF-β dimer non-covalently associated to latency associated peptide, which in turn is covalently bound to TGF-β binding proteins. In order to bind to its receptor, TGF-β is activated from the growth plate by factors such as matrix metalloproteinases (MMPs) [78-83].

TGF-β plays a regulatory role in several mechanisms. For example, it controls fibronectin expression, which regulates chondrogenesis. It is responsible for stimulating chondrocytes in order to induce aggrecan and collagen type II production [84-87], and for initiating the first step of chondrogenesis, mesenchymal cell condensation [88-90]. In addition, the TGF-β signaling pathway is essential in postnatal joint growth and development, and for the maintenance of the articular chondrocyte phenotype [11]. For

example, TGF-β-deficient mice display a cartilage phenotype similar to the cartilage pathology in osteoarthritis (OA) [74]. Moreover, TGF-β is essential in osteoblast differentiation. For example, studies have shown that osteoblasts overexpressing TGF-β2 cause low bone mass and an increase in osteocytes [91]. Finally, TGF-β works against inflammatory cytokines such as interleukin (IL)-1, which is responsible for upregulating MMPs such as MMP-13. These proteinases cause cartilage degradation and TGF-β is able to prevent this by stimulating ECM and protease inhibitor synthesis as well as downregulating cytokine receptor and cartilage-degrading enzyme expression [74, 92].

Smad 3 is a transcription factor that mediates and regulates this pathway. Both Smad 3 and TGF-β are expressed in chondrocytes and in the perichondrium [93]. During adolescence, TGF-β inhibits chondrocyte differentiation [94, 95]. Animal mouse models have confirmed this by demonstrating that a disruption in Smad 3 causes a degenerative joint disease as a result of TGF-β's inability to inhibit chondrocyte differentiation [96]. Furthermore, in murine models of OA, a decrease in TGF-β signaling or inhibition of endogenous TGF-β leads to an increase in cartilage damage [97, 98]. This further demonstrates TGF-β's significant role in cartilage growth and development.

Additionally, TGF-β acts as an anabolic cytokine that promotes cartilage ECM synthesis by counteracting the effects of catabolic cytokines [99] that induce MMP-13 production in OA human cartilage [100].

Fibroblast Growth Factors

FGFs are a family of polypeptide growth factors that contain 22 members in vertebrates. These genes range in molecular weight from 17 to 34 kDa and most share a common core region of 28 highly conserved and six identical amino-acid residues [101, 102].

FGFs are essential for normal bone formation. They control bone growth when coupled with systemic factors such as growth hormones [4]. In primary chondrocytes and undifferentiated mesenchymal cells, FGF signaling pathways induce Sox9 expression, a transcription factor critical in chondrocyte differentiation, indicating its involvement in early stages of endochondral bone formation [103, 104]. FGF signaling accelerates terminal differentiation, induces hypertrophic differentiation, and inhibits proliferation [105, 106]. Defects in FGF signaling in mesenchymal condensations result in skeletal abnormalities [107].

In the FGF family, there exist four receptor genes. Each receptor possesses a unique role during the various stages of bone formation. FGF receptors (FGFRs) transmit mitogenic signals, which regulate cell proliferation, differentiation and survival [108]. FGFR3 is expressed in proliferating chondrocytes and its signaling enhances chondrocyte proliferation in mesenchymal condensations, and promotes chondrogenesis and cartilage production [15, 109, 110].

Animal mouse models show that FGFR3 inactivation causes an increased rate of proliferating chondrocytes, a larger proliferating zone with longer columns, and an extended endochondral bone formation process, thus creating longer bones [111, 112]. FGFR3 KO mice exhibit skeletal overgrowth whereas animal mouse models

overexpressing FGFR3 exhibit skeletal dwarfism. These cases demonstrate that FGFR3 signaling negatively regulates chondrocyte proliferation and differentiation as chondrocytes mature [113]. In contrast, it enhances proliferation of immature chondrocytes during early embryonic development [109, 114].

FGFR3 mutations present in human chondrodystrophies decrease the rate of chondrocyte proliferation and produce short and disorganized columns in transgenic mice [113]. Mutations in FGFR3 cause abnormalities such as achondroplasia (ACH) and thanatophoric dysplasia (TD). ACH is the most common form of dwarfism among humans and is identified by an absence of nonhypertrophic chondrocytes [115-118]. The mutations in FGFR3 that cause ACH and TD lead to impaired cell proliferation and, consequently, dwarfism [15].

FGFR3 KO mice display increased Ihh expression, while FGFR3 activation leads to decreased Ihh expression [108, 113]. The factors influenced by FGF signaling are mediated by suppressing Ihh expression [105]. Thus, FGF signaling shortens proliferating zone columns both by decreasing chondrocyte proliferation and by suppressing Ihh expression [4]. The fact that FGFR3 signaling inhibits Ihh expression demonstrates that the Ihh and FGF pathways are linked [113, 119].

FGF ligands are expressed in developing bones [108]. Specifically, FGF2 is expressed in osteoblasts and FGF2 KO mice exhibit decreased osteoblastogenesis [120, 121]. Furthermore, overexpression or mutations of FGF2 in chondrocytes promote apoptosis, thus demonstrating its role in cell death [122, 123]. FGF18 is expressed in the perichondrium and KO mice exhibit delayed ossification, either due to signaling to osteoblasts or hypertrophic chondrocytes or to a delay in vascular invasion of the

growth plate [107, 119]. These animal mouse models show an increase in chondrocyte proliferation, similar to FGFR3 KO mice. This indicates that FGF18 is a ligand for FGFR3 in chondrocytes [119, 124]. Finally, FGF18 also acts on another receptor, FGFR1, to delay terminal differentiation of hypertrophic chondrocytes. It acts on FGFR1 and FGFR2 together to delay osteoblast differentiation [4].

Other FGFRs involved in bone formation include FGFR1 and FGFR2. FGFR1 is expressed in loose mesenchyme, prehypertrophic and hypertrophic chondrocytes, and perichondrial cells. It regulates cell survival, cell differentiation, ECM production, and cell death [4, 107, 125, 126]. FGFR2 is expressed in condensing mesenchymal cells, perichondrial cells, the periosteum and the primary spongiosa. FGFR2 conditional KO mice develop skeletal dwarfism and exhibit a decrease in bone mineral density [127, 128]. In addition, FGF signaling regulates osteoblast function [107]. Specifically, FGFR2 signaling positively regulates osteoblastogenesis [127, 128].

Integrins

Integrins are adhesion receptors that mediate binding between cells [129]. However, because chondrocytes secrete large amounts of ECM as they mature, integrins increasingly mediate cell-matrix interactions instead [130]. Integrins are heterodimers that contain two chains, namely, alpha and beta. In mammals, there exist 19 α and 8 β subunit genes; however, the β1 chain is a component of most chondrocyte integrins [131, 132]. Chondrocytes express several integrins such as fibronectin receptors, a laminin receptor and collagen receptors [133-136]. Overall, integrins

regulate several downstream pathways and their role in cell signaling is critical in normal chondrocyte proliferation and hypertrophy [130, 132].

β1 integrin plays a role in chondrogenesis by either promoting or inhibiting the process. Tension force, which is mediated by focal adhesion kinase and integrins β1, α2, and α5, inhibits chondrogenesis and stimulates β1 integrin expression [137, 138]. When the β1 integrin pathway is blocked, chondrogenesis and chondrocyte matrix production is inhibited [139]. Cartilage-specific β1 KO mice exhibit chondrodysplasia [140]. Their chondrocytes display reduced proliferation, and deregulated expression of cell cycle proteins [2]. Specifically, proliferating chondrocytes exhibit loss of columnar structure and adhesion to collagen type II, resulting in abnormal cell shape. Together, the loss of β1 and αnβ5 integrins promote apoptosis in growth plate chondrocytes [141]. In addition, mice lacking β1 integrin demonstrate increased expression of p21 and p16, cell-cycle inhibitors, and, as a result, reduced chondrocyte proliferation [140].

Integrin signaling stimulates the formation of signaling complexes consisting of elements such as kinases, specifically, integrin-linked kinase (ILK) [142, 143]. Cartilage-specific ILK-deficiency has shown to cause chondrodysplasia, abnormal chondrocyte cell shape, defective cell adhesion, and reduced chondrocyte proliferation [144, 145]. Cartilage-specific β1 integrin-deficiency displays similar phenotypes [140].

Although the β1 integrin chain is a component of most chondrocyte integrins, there are other chains involved in cartilage growth and development. For example, proliferative and hypertrophic chondrocytes highly express the α5β1 integrin chain, which is involved in the formation of major joints. Improper expression causes reduced chondrocyte proliferation, premature formation of prehypertrophic chondrocytes, and

fusion joints [146, 147]. α10 integrin KO mice exhibit growth plate abnormalities [148]. In collagen-specific knockout models, apoptosis increases and cell shape becomes irregular. In contrast, α1 integrin KO mice exhibit OA without growth plate abnormalities [149].

Akt

Akt is a serine/threonine protein kinase, also called protein kinase B, that is highly involved in cell signaling, and regulates many cellular functions such as nutrient metabolism, transcriptional regulation, and cell growth, apoptosis and survival [150-152]. There exists a positive correlation between OA progression and chondrocyte apoptosis, and Akt is a potent inhibitory signal for apoptosis in several cell types [153-156]. Akt belongs to the cyclic adenomonophosphate (cAMP)-dependent protein kinase A/protein kinase G/protein kinase C super family of protein kinases. Members of this family share both structural homology within their catalytic domain and common mechanisms of activation. Specifically, Akt contains a conserved domain structure including a specific pleckstrin homology domain, a central kinase domain, and a carboxyl-terminal regulatory domain that mediates the interaction between signaling molecules [150, 151].

There are three genes in the Akt protein family, Akt1, Akt2, and Akt3, which are located at chromosomes 14q32, 19q13, and 1q44, respectively [150]. Akt1 and Akt2 demonstrate similar expression in most tissues and, consequently, Akt1 and Akt2 KO animal mouse models show similar phenotypes including growth retardation [157-159]. Akt2 is more highly expressed in insulin target tissue and Akt2 KO mice display severe

diabetes, thus confirming its role in inducing glucose transport and, more broadly, in the insulin signaling pathway [157, 160-162]. Both Akt1 and Akt2 are more highly expressed than Akt3, except in the brain and testis where Akt3 is more highly expressed [157, 163]. Akt3 KO mice show decreased brain and testes sizes [164, 165].

Akt1, a key signaling protein, is the most highly expressed Akt in chondrocytes and plays a critical role in these specialized cells. It is a complex kinase that promotes cartilage calcification by inhibiting pyrophosphate accumulation, which is a crucial calcification inhibitor. Therefore, it controls endochondral ossification as well as osteophyte formation in OA. Additionally, Akt1 inhibits apoptosis and induces protein synthesis pathways, thus exhibiting its function in the cellular pathways leading to skeletal muscle hypertrophy and general tissue growth [152].

Akt1 KO mouse models exhibit the gene's significant role in various processes. Firstly, Akt1 KO mice display a decrease in osteophyte formation and suppression of cartilage calcification in the growth plate, demonstrating that Akt1 positively regulates chondrocyte calcification. Moreover, the chondrocytes of Akt1-deficient mice display a decrease in total Akt protein, verifying its important role in chondrocytes. Next, Akt1 deficiency does not affect cartilage degradation and both proliferating and hypertrophic zones are normal because it does not play a role in the regulation of chondrocyte hypertrophy. Finally, Akt1 KO mice exhibit osteopenia as a result of defects in the bone remodelling process [152, 166].

Animal mouse models that are deficient in more than one Akt gene display more severe phenotypes, thus indicating specificity and redundancy among the isoforms [152]. For example, when mice are both Akt1- and Akt2-deficient, they display

abnormal bone development and die as newborns [167]. Moreover, Akt1- and Akt-3 deficient mice exhibit embryonic lethality due to placental insufficiency and display severe developmental defects [161, 168]. Both Akt1 and Akt2, and Akt1 and Akt3 KO mice display more severe defects in skeletal growth than Akt1 KO mice [157-159]. Finally, Akt2 and Akt3 KO mice are viable and demonstrate only moderate defects in skeletal growth and glucose metabolism [169]. These studies provide evidence to the fact that Akt1 plays a critical role in skeletal development and postnatal survival while Akt2 and Akt3 contribute to early endochondral ossification, a process mainly regulated by Akt1 [152].

Wnt/β-catenin

The Wnt family signals through four pathways and it is the Wnt/β-catenin signaling pathway that contributes to OA development [170]. β-catenin plays an integral role in the canonical Wnt signaling pathway and is highly involved during chondrocyte formation and maturation [171, 172]. Wnt/β-catenin signaling pathway regulates articular chondrocyte function and is regulated by modulator proteins. It is critical for joint development and maintenance, cell differentiation, proliferation and apoptosis, and bone mass regulation [170, 173]. Wnt proteins, which form a family of signaling molecules, regulate cell-cell interactions during embryonic development of cartilage and bone, cell differentiation, tissue morphogenesis and remodelling, and body patterning [174-177]. The pathway is activated in the hypertrophic zone of long bones when Wnt proteins bind to Frizzled (Fz) receptors and low-density lipoprotein receptor-related protein 5 or 6 co-receptors [178-181]. Binding is regulated and modulated by

endogenous antagonists such as secreted Frizzled-related proteins (sFRPs), Wnt-inhibitory factors, Cereberus and Dickkopf (Dkk) [170, 182].

The balance between matrix synthesis and degradation that allows the ECM to maintain its structure is disrupted upon activation of the Wnt/β-catenin signaling pathway because it stimulates MMP-13 expression, a major mediator of cartilage degradation [170, 183]. In human and animal OA, Wnt/β-catenin pathway proteins are overexpressed, thus causing an increase in bone formation, and, consequently, osteophyte formation. In addition, Wnt proteins and their inhibitors are differentially expressed in OA synovium [184-187].

There exist approximately 19 Wnt proteins and 10 Fz receptors in humans, all of which play a variety of roles in a range of physiological and pathological processes [187]. Wnts 3a, 4, 5a, 5b, 7a, 11 and 14 are differentially expressed through the various stages of mesenchymal cell condensation and chondrocyte differentiation while Wnts 4, 5a and 14 are expressed in developing joints [31, 188-192]. Wnt-2b contributes to bone formation, thus affecting chondrocytes by driving them to terminal differentiation [193]. Wnt-7a inhibits apoptosis in articular chondrocytes and chondrogenesis [194, 195]. It is also responsible for inducing dedifferentiation of articular chondrocytes by stimulating β-catenin transcriptional activity [194]. Wnt-10b initiates mesenchymal cell differentiation into osteoblasts or chondrocytes [196]. Wnt-14 is involved in the formation of the interzone in synovial joint development [188, 197, 198]. Wnt-16 contributes to synovial joint formation during embryonic development and its expression elevates in response to mechanical cartilage damage [198, 199]. Wnt-7b is

upregulated in OA cartilage and both Wnts 2b and 16 are upregulated in the synovium and cartilage of experimental OA [177, 200].

Wnt-I-induced secreted protein-1 is expressed in the synovium and cartilage, and may therefore contribute to the phenotypic change of chondrocytes. It is overexpressed in human and experimental OA, leading to the release of MMPs and aggrecanases, and, as a result, cartilage degradation [177].

Dkk-1, a Wnt inhibitor, modulates Wnt signaling by disrupting the canonical β-catenin pathway and reducing the signaling pathway's ability to induce osteoblastogenesis [176, 201]. Dkk-1 is important in OA as its serum levels may be associated with joint destruction in terms of development and progression [187]. Animal models demonstrate that inhibiting Dkk-1 results in the bone-forming pattern of OA and OA animal mouse models display a decrease in bone erosion and osteophyte formation [202]. Dkk-2 is another Dickkopf involved in OA. Dkk-2-deficient mice display osteopenia, which is caused by a decrease in osteoblast terminal differentiation and a mineralization defect [203]. Finally, Dkk-3 is upregulated in the synovium and cartilage of experimental OA [177].

Frizzled-related protein (FrzB) is a Wnt-binding protein involved in embryonic development. Irregular Wnt signaling may contribute to OA pathogenesis due to the fact that a FrzB polymorphism results in increased susceptibility to OA development. FrzB KO mice display no abnormalities as newborns; however, as they age, they lose cartilage integrity at a faster than normal rate [177, 187]. They also exhibit elevated MMP-3 levels, another MMP that contributes to cartilage destruction. The Wnt signaling pathway's involvement in OA progression is apparent through FrzB

mutations. A specific FrzB mutation reduces its affinity for Wnt molecules, making it incapable of suppressing Wnt signaling. Additionally, in an OA animal model where FrzB function is deleted, OA is worsened [204].

sFRPs competitively inhibit activation of the Wnt/β-catenin pathway, and, consequently, disrupt both canonical and non-canonical signaling pathways [187, 205]. sFRP-1 is integral in the regulation of osteoblast differentiation, proliferation, and function, and osteoblast/osteocyte apoptosis [206]. It binds to receptor activator of nuclear factor (NF)-κB ligand to inhibit osteoclastogenesis [207]. sFRP-3, encoded by FrzB, modulates chondrocyte maturation and Wnt signaling [173, 204]. As well, it plays a potential role in preventing the development or progression of cartilage loss in human and animal OA. Similar to Dkk-1, a link exists between sFRP-3 serum levels and the development and progression of joint destruction [187]. A specific sFRP-3 mutation leads to the protein's inability to antagonize Wnt signaling. This causes altered joint development, which increases the risk of developing OA [208, 209]. Finally, FrzB-2, which encodes sFRP-4, is highly expressed in human OA and is involved in chondrocyte apoptosis [210].

Osteoarthritis

OA, the most common form of arthritis, is a degenerative and progressive joint disease. It affects a large portion of the population with approximately 27 million Americans presenting with clinical symptoms and more than 50% of those aged 65 years old and older showing radiological signs [29, 211-213]. In Canada, OA is a leading cause of disability and, as a result, direct and indirect health and economical

expenses related to the disease is close to $22 billion. These high costs place a large burden on the government as well as insurance companies.

While OA is often described by articular cartilage deterioration and damage, it is in fact a disease affecting the whole joint. Other affected parts of the joint include the peri-articular and subchondral bones, synovial membrane, and adjacent supporting connective tissue elements [212, 214-216]. Currently, the pathophysiology of joint degradation leading to OA and the mechanisms that control its initiation and progression is largely unknown. Hence, the development of adequate therapeutics against the disease is limited.

OA is a complex disease with a number of underlying biochemical and physical causes. Several factors, including genetic, developmental, metabolic, mechanical, trauma, and obesity contribute to the onset of OA. Other aspects, however, such as hereditary factors and the effects of aging contribute to disease progression. The areas of the skeleton mostly affected by OA are the hands, knees, hips, and spine. OA is clinically described by joint pain, dysfunction, stiffness, and deformity, which is caused by cartilage, subchondral bone, and tendon destruction, joint space narrowing, osteophyte formation, and remodeling and sclerosis of subchondral bone [29, 212, 214, 215, 217-219]. Therefore, OA leads to chronic disability and reduced mobility, severely affecting one's quality of life.

Osteoarthritic Cartilage

Chondrocytes are said to control cartilage metabolism as they maintain the structural integrity of the ECM, a factor upon which normal cartilage function is

dependent [29, 220]. Chondrocytes secrete catabolic and anabolic cytokines that are responsible for maintaining an equilibrium between matrix synthesis and degradation. This tightly regulated balance allows the cartilage ECM to preserve its structure [221, 222]. In joints affected by OA, however, a combination of mechanical and biochemical factors break this balance. This causes the degradation process to take precedence, resulting in a loss of articular cartilage, and, subsequently, OA [223]. One of the components of the cartilage ECM, aggrecan, is relatively replaceable. Therefore, its destruction is not highly critical until a certain point. However, because collagen is not released abundantly from the tissue, its loss is essentially irreversible [28, 29].

Cartilage Metabolism

Catabolic and anabolic cytokines play a role in cartilage metabolism. Cytokines are small proteins that mediate cell-cell signaling by transmitting information from cell to cell [224]. Cytokines that stimulate protease expression are released from inflamed synovial membrane and chondrocytes [225]. During OA progression, cytokines are released depending on the cartilage ECM matrix environment. For example, during matrix degradation, otherwise known as a catabolic state, the level of catabolic cytokines are elevated, therefore stimulating the expression of degradative enzymes, including, MMPs [226, 227].

Catabolic cytokines contribute to the equilibrium required to preserve the ECM structure by driving matrix degradation. Catabolic cytokines such as IL-1β and tumor necrosis factor (TNF)-α are detrimental because they induce chondrocytes to produce inducible Nitric Oxide Synthase (iNOS) and other pro-inflammatory cytokines, such as

IL-6, -8, -17, and -18, leukemia inhibitory factor, prostaglandin E2 (PGE2), and chemokines [228]. NO contributes to inflammation and cartilage deterioration as it upregulates MMP production, inhibits collagen, aggrecan, and IL-1 receptor (IL-1R) synthesis, and promotes chondrocyte apoptosis. In addition, catabolic cytokines have proinflammatory properties.

In contrast, anabolic cytokines such as TGF-β promote cartilage ECM synthesis by counteracting the effects of catabolic cytokines [99]. In normal cartilage, a balance between catabolic and anabolic cytokines is maintained to preserve the structural integrity of the cartilage ECM. In OA, however, the expression of anabolic factors is downregulated, catabolic cytokines take precedence, and, as a result, matrix degradation occurs and OA follows [229, 230].

Interleukin-1β

IL-1β is a catabolic cytokine produced by OA synovial membrane cells and chondrocytes, and is involved in cartilage deterioration [231, 232]. IL-1β is first synthesized in an inactive state. Subsequently, it is activated by the IL converting enzyme [233]. Type I IL-1R and type II IL-1R exist, both having different affinities for IL-1β. Type I IL-1R has a higher affinity for IL-1β and is responsible for signal transduction. In addition, OA chondrocytes have shown elevated levels of Type I IL-1R [232]. IL-1β is responsible for causing chondrocytes to produce disproportionate amounts of collagen. For example, levels of collagen type I and III are increased while the level of collagen type II remains the same. As a result, the structural integrity of the cartilage ECM is compromised and cartilage is destroyed [233]. Furthermore, IL-1β

stimulates MMP expression [227]. Overall, IL-1β destroys the balance between anabolic and catabolic processes, causing catabolism to take precedence, which results in cartilage degradation. IL-1β causes these changes by activating signal transduction cascades such as extracellular signal-regulated protein kinase (ERK) 1/2, p38, and stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK), all of which influence gene expression [233].

Tumor Necrosis Factor-α

Another catabolic cytokine that contributes to OA progression is TNF-α. Similar to IL-1β, TNF-α is synthesized in an inactive pro-form and subsequently activated by TNF-α converting enzyme (TACE). Upon activation, TNF-α binds with another TNF-α molecule to form a trimer in order to subsequently bind with one of two TNF-α receptors (TNFR), TNFR55 or TNF57 [234]. TNFR55 is the main receptor of TNF-α in OA [235]. The levels of TACE have been shown to be elevated in OA, thus emphasizing the predominant role of catabolic cytokines in OA [236].

Matrix Metalloproteinase-13

In addition to factors such as mechanical stress, tissue injury, oxidative stress, degraded matrix, and joint destabilization, catabolic cytokines and pro-inflammatory enzymes, including cyclooxygenase-2 (COX-2), iNOS, and microsomal prostaglandin E synthase-1 (mPGES-1) stimulate chondrocytes to release cartilage degradative molecules such as MMPs [215, 237-241]. Other stimulants also found to play a role in

MMP upregulation through cartilage explant studies include IL-17, oncostatin M, proteolytic fragments of fibronectin, and endothelin 1 [242-246].

MMPs, metalloenzymes from the metzincin superfamily, are proteinases that contribute to pathological processes, such as facilitating inflammation and cartilage destruction by releasing growth factors from carrier proteins, inactivating proteinase inhibitors, and activating or inactivating inflammatory cytokines and chemokines [247-250]. There are 25 MMP genes that exist in the human genome [223, 251] and all share a common domain structure with a signal peptide, a pro-peptide, a catalytic domain, a hinge region, and a hemopexin C-terminal domain [247]. Based on substrate specificity, primary structure, and cellular location, MMPs are divided into functional groups [221]. MMP-13 is a collagenase and has the greatest specificity for collagen type II, a key structural protein in cartilage [252]. This MMP appears to play an important in OA pathophysiology.

In general, the expression of MMPs is low in normal articular joint tissue [253]. In OA, however, there is an elevated level of MMPs. Among them is MMP-13, which increases collagen breakdown and cartilage destruction [254, 255]. Furthermore, animal mouse models overexpressing MMP-13 in articular cartilage have shown to display OA-like characteristics [256]. In terms of anabolic cytokines, it has been shown that TGF-β induces MMP-13 production in OA human cartilage [100]. To further demonstrate the role of MMP-13 in OA, a study in which OA was surgically induced in MMP-13 KO mice demonstrated significant inhibition of cartilage structural damage, indicating that damage is dependent, at least in part, on MMP-13 activity. Furthermore,

MMP-13 deficiency inhibited cartilage erosion even in the presence of aggrecan depletion [257].

Treatment

While many clinical trials have been conducted for OA, an effective and satisfactory treatment that will stop or arrest the disease process is yet to be discovered. Some of the treatments that currently exist for OA patients, including acetaminophen, opioids, and non-steroidal anti-inflammatory drugs (NSAIDs) such as selective COX-2 inhibitors, aim at symptomatic relief. This said, there is no cure or preventative method for the underlying pathology of OA [212, 218, 223]. Hence, OA remains at the forefront of research and requires thorough attention to alleviate the growing health and economical concerns.

Osteoarthritis and Aging

OA is an age-related disease whereby the effects of aging contribute to disease progression [214, 219, 258]. Symptoms usually appear in middle age and more than 50% of Americans aged 65 years old and older show radiological signs of the disease [211]. Before the age of 55, OA is equally common in men and women while after the age of 55, OA mostly affects women. As age is a major contributor to the disease and individuals are living longer due to increased life expectancy, OA is a growing socioeconomic and clinical concern.

Studies have shown that early growth and developmental defects may lead to age-related musculoskeletal defects such as OA. For example, animal KO models for

integrin α1 and Smad3 showed that defects in early cartilage development and endochondral ossification are associated with spontaneous OA during aging. In particular, animal mouse models that are homozygote for a targeted disruption of Smad3 exon 8 display a loss of chondrocyte responsiveness to TGF-β signals during chondrogenesis. This results in ectopic chondrocyte hypertrophy and progressive degenerative cartilage disease, which resembles human OA [96, 149].

It has been suggested that skeletal deformities caused by defects in early cartilage growth and development affect joint geometry and biochemical properties, leading to accelerated and spontaneous OA-like phenotypes during aging. *Stattin et al.* [259] supported this hypothesis by demonstrating that patients with familial dysplasia develop joint malformations, and, subsequently, develop secondary OA.

Peroxisome Proliferator-Activated Receptor gamma

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily. In general, PPARs play a role in lipid and lipoprotein metabolism, and glucose homeostasis. In addition, they have been shown to be involved in cell proliferation, differentiation and death [260]. To date, three primary isoforms of this group of receptors encoded by different genes have been identified, namely, PPARα, PPARβ/δ, and PPARγ [261]. These PPAR isoforms possess a set of common functional domains including an N-terminal region, DNA-binding domain, flexible hinge region, ligand binding domain, and C-terminal region, thus sharing structural similarity [262]. However, all three subtypes play important roles in a variety of physiological processes.

For example, PPARα is involved in the catabolism of fatty acid and is highly expressed in tissues, including the liver, heart, kidney and muscle [263]. PPARβ/δ plays a vital role in several processes such as lipid homeostasis, epidermal maturation, skin wound healing, and brain development [264, 265]. Finally, PPARγ, the most studied member of the PPAR group, plays a key role in lipid and glucose homeostasis [266, 267]. Through alternative splicing and the use of different promoters, PPARγ exists in two isoforms, PPARγ1 and PPARγ2. Although both isoforms come from the same gene, PPARγ2 has 30 more amino acids at the NH_2-terminal end compared to PPARγ1, and they are expressed in different locations. PPARγ1 and PPARγ2 are primarily expressed in inflammatory and immune cells, and adipose tissues, respectively [268, 269].

As a transcription factor, PPARγ is involved in controlling gene expression. Specifically, when PPARγ binds with retinoid X receptor, it forms a heterodimer. Thereafter, this complex binds to sequence-specific PPAR response elements in the promoter region of target genes and regulates gene expression. PPARγ is activated by natural and synthetic ligands. Natural ligands include fatty acids and prostaglandin J2 [270] while synthetic ligands include antidiabetic thiazolidinediones, such as rosiglitazone and pioglitazone, which bind to and activate PPARγ [271, 272]. Upon PPARγ binding to its ligand, transcription of the target genes is either increased or decreased [266, 267].

Although PPARγ has been shown to play a key role in lipid and glucose homeostasis, recent studies have suggested that it also plays a role in bone homeostasis by contributing to osteoclastogenic and osteoblastogenic pathways. Specifically, animal mouse models where PPARγ is deleted in osteoclasts but not in osteoblasts have shown

to develop osteopetrosis as a result of impaired osteoclast differentiation and bone resorption. Therefore, PPARγ plays a role in promoting osteoclast differentiation and bone resorption [273].

In addition, PPARγ pathway activation has been demonstrated to be of benefit in the treatment of Type 2 diabetes mellitus. Specifically, synthetic ligands of PPARγ such as rosiglitazone and pioglitazone are clinically used for the treatment of insulin resistance in this disease [260, 274-276]. Furthermore, PPARγ plays a role in preadipocyte differentiation, inflammation, wound healing, angiogenesis, cancer and atherosclerosis [270, 274, 277-281].

PPARγ in Osteoarthritis

More recently, it has been suggested that the activation of PPARγ is a therapeutic target for OA. *Stanton et al.* [282] studied PPARγ2 expression in chondrocytes. Not only did they conclude that growth plate chondrocytes express PPARγ2 in a developmentally regulated pattern, but they also determined that several signaling pathways regulate PPARγ2 expression during chondrocyte differentiation. More specifically, they found that PPARγ2 expression is regulated by p38 and glycogen synthase kinase (GSK)-3β. They observed that inhibition of p38 activity results in increased PPARγ2 expression and activity whereas inhibition of GSK-3β activity has the opposite effect. This suggests that these signaling pathways are required for PPARγ expression.

PPARγ agonists can reduce the synthesis of various catabolic and inflammatory factors involved in OA pathophysiology. For example, it can reduce inflammatory

cytokines such as IL-1β, TNF-α, PGE2, IL-6, NO and MMPs such as MMP-1 and -13, and can suppress TGF-β-mediated proteoglycan production [251, 281, 283-287]. Moreover, pioglitazone can reduce *in vivo* the development of cartilage lesions in experimental OA models of dogs [288] and guinea pigs [289] as well as the synthesis of key OA mediators including MMPs and iNOS, and inhibit OA-involved signaling pathways such as mitogen-activated protein kinases (MAPKs) (ERK 1/2 and p38) and NF-κB.

Studies using the natural and synthetic agonists of PPARγ have not been able to describe the exact role of PPARγ in the pathophysiology of OA. Agonists of PPARγ inhibit inflammation, and reduce the synthesis of cartilage degradation products both *in vitro* and *in vivo*, and the development and progression of cartilage lesions in OA animal models [288-291]. However, synthetic agonists such as rosiglitazone are able to regulate other signaling pathways *in vivo* independent of PPARγ, thus resulting in serious side effects [292-295]. Therefore, studies using synthetic and natural ligands of PPARγ have not been successful in describing the exact role of PPARγ in OA pathophysiology.

PURPOSE OF STUDY

While the processes of chondrogenesis, endochondral ossification, and cartilage growth and development are known, the exact mechanisms through which chondrocyte function and behaviour are controlled during cartilage growth and development are largely unknown.

While PPARγ expression has been identified in growth plate chondrocytes [296], its specific *in vivo* function and the signaling pathways affecting its expression and activity in cartilage growth and development still remains largely unknown. In order to achieve therapeutic efficacy with potentially less side effects, it is essential to first understand its specific role in this process. Therefore, for the first time, this study will examine the specific *in vivo* contribution of PPARγ to cartilage growth and development using cartilage-specific PPARγ KO mice. Whole-genome PPARγ KO mice exhibit embryonic lethality due to major placental and cardiac defects [297] and heterozygous PPARγ KO mice have not been studied in early cartilage growth and development. We used a conditional system directed to collagen type II. Hence, the LoxP/Cre system using Col2Cre mice was used to generate conditional KO mice.

HYPOTHESES AND AIMS

It is hypothesized that cartilage-specific ablation of PPARγ in mice during embryonic and postnatal growth stages results in abnormal cartilage growth and development, and that cartilage-specific ablation of PPARγ in mice during aging results in accelerated and spontaneous OA.

The first objective of this study is to determine the *in vivo* effects of cartilage-specific ablation of PPARγ during embryonic and postnatal growth stages on:

- Skeletal growth, ossification patterns, organization of growth plates, chondrocyte proliferation and hypertrophy, cell differentiation, vascular invasion, mineralization, and bone density

- Temporal expression/production profile of VEGF-A and ECM production and degradation markers including aggrecan, collagen type II, and MMP-13 in chondrocytes and cartilage explants

The second objective of this study is to determine the *in vivo* effects of PPARγ during aging using cartilage-specific PPARγ knockout mice. During older stages (14 months old), we will determine the *in vivo* effects of cartilage-specific ablation of PPARγ on:

- Phenotypic changes in the joint, bone, and synovium
- Inflammation and presence of macrophages
- Expression of MMP-13, VDIPEN, an MMP-generated aggrecan neoepitope, and Col2-3/4C$_{short}$ (C1-2C), an MMP-generated collagen type II neoepitope

ROLE OF PPARγ IN CARTILAGE/BONE GROWTH AND DEVELOPMENT, AND OSTEOARTHRITIS *IN VIVO*

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Abstract

Defects in endochondral ossification, the process of long bone formation, results in skeletal abnormalities and age-related defects including OA. Here, we describe a critical *in vivo* role of transcription factor PPARγ in cartilage growth and development and age-dependent OA. Cartilage-specific PPARγ KO mice were generated using LoxP/Cre system. Histomorphometric analyses of embryonic and adult mutant mice demonstrate reduced long bone growth, and delayed primary and secondary ossification associated with disorganized growth plates, abnormal chondrocyte shape, proliferation, and differentiation, hypocellularity, loss of columnar organization, shorter hypertrophic zones, and reduced bone density, calcium deposition, and vascularity. Isolated mutant chondrocytes and cartilage explants show decreased expression of VEGF-A, ECM production products, aggrecan and collagen type II, and increased expression of catabolic enzyme, MMP-13. Furthermore, aged mutant mice exhibit an accelerated OA-like phenotype associated with enhanced cartilage degradation, synovial inflammation and MMP-13 expression, accompanied by increased MMP-generated aggrecan and collagen type II neoepitope expression. In summary, for the first time, our data demonstrate a critical role of PPARγ in normal skeletal development.

Introduction

Long bones are formed and lengthened through a process termed endochondral ossification whereby a cartilage anlagen grows through proliferation and hypertrophy of chondrocytes [2] after which cartilage is replaced by bone [1]. In this process, cartilage provides an intermediate template on which bone is laid down [3]. During endochondral bone growth, chondrocytes are organized into three zones within the epiphyses of the cartilage, namely, resting, proliferating, and hypertrophic zones. Resting and proliferating chondrocytes express high levels of aggrecan and collagen type II, the two main components of the cartilage ECM, while hypertrophic chondrocytes express collagen type X. Prior to entering the hypertrophic zone, cells exit the cell cycle and begin to differentiate to hypertrophic chondrocytes [18]. Subsequently, they mineralize their surrounding ECM in the cartilage centre and undergo apoptosis [15, 22]. Blood vessels then invade the hypertrophic cartilage region mainly through the production of VEGFs, bringing in osteoblasts and osteoclasts [15]. Osteoclasts degrade mineralized cartilage while osteoblasts replace it with bone tissue [1, 2, 24]. This mineralization process is termed primary ossification [13]. Secondary ossification centres are formed in the epiphyses post-natally [1].

The normal lengthening of long bones depends on the rate of production of hypertrophic chondrocytes from proliferating chondrocytes, the volume increase of hypertrophic chondrocytes, and the number of proliferative cycles a chondrocyte undergoes [4, 14, 18-20]. Disturbances of the fine balance controlling endochondral bone growth results in growth- and development-related abnormalities such as dwarfism and skeletal deformities [4, 11, 13, 25]. In addition, early growth and developmental

defects may lead to age-related musculoskeletal defects such as OA, as observed in mice deficient in integrin α1 and Smad3 [96, 149]. In particular, mutant mice homozygote for a targeted disruption of Smad3 exon 8 display a loss of chondrocyte responsiveness to TGF-β signals during chondrogenesis, which ultimately results in ectopic chondrocyte hypertrophy and progressive degenerative cartilage disease resembling human OA [96]. Several other signaling molecules, including Ihh, FGFs, Akt, and Wnt/β-cantenin have been shown to play a role in chondrogenesis, and cartilage growth and development [63, 113, 193, 298] (Reviewed in [4]). However, the exact mechanisms through which chondrocyte function and behaviour is controlled during chondrogenesis, and cartilage growth and development are largely unknown.

The transcription factor PPARγ belongs to the family of ligand-activated nuclear receptors and plays a key role in lipid and glucose homeostasis [266, 267]. It regulates gene expression by binding as a heterodimer with retinoid X receptor. This heterodimer complex acts as a transcriptional regulator upon binding to sequence-specific PPAR response elements in the promoter region of target genes [266, 267]. Recent studies suggest that PPARγ is involved in the maintenance of bone homeostasis by contributing to osteoclastogenic and osteoblastogenic pathways. Mice lacking PPARγ in osteoclasts develop osteopetrosis, which results from impaired osteoclast differentiation [273]. However, the *in vivo* role of PPARγ in chondrogenesis, and cartilage growth and development is still largely unknown. Therefore, for the first time, this study examined the specific *in vivo* contribution of PPARγ towards chondrogenesis, and cartilage growth and development using cartilage-specific PPARγ KO mice as global PPARγ KO mice exhibit embryonic lethality due to placental defects [297].

Materials and methods

Materials

C57BL/6-PPARγ$^{fl/fl}$ mice were obtained from Jackson Laboratory. C57BL/6 Col2-Cre transgenic mice were obtained from Dr. Réné St-Arnaud (Shriners Hospital for Children, Montreal, Canada). The following antibodies were used in this study: platelet/endothelial cell adhesion molecule (PECAM) #SC-1506, Sox9 #SC-20095, p57 #SC-8298, PPARγ #SC-7273, Goat-anti-rabbit #SC-2004, Goat-anti-mouse #SC-2005, Rabbit-anti-goat #SC-2768 (Santa Cruz Biotechnology); Collagen type X #C-7974, 5-bromo-2'-deoxyuridine (BrdU) #B-8434, Cre #C-7988, MMP-13 #M-4052 (Sigma-Aldrich); VEGF-A #ABS-82 (Millipore); MOMA-2 antibody #ab33451 (abcam); Dulbecco's modified eagle medium (DMEM) and trypsin-ethylenediaminetetraacetic acid (EDTA) (Wisent). Anti-VDIPEN and -C1-2C antibodies were obtained from Dr. John Mort (Shriners Hospital for Children, Montreal, Canada).

Generation of cartilage-specific PPARγ KO mice

Genetically modified mice harbouring a cartilage-specific deletion of PPARγ were generated using the Cre Lox methodology in which mice carrying Cre recombinase under the control of the collagen type II promoter were used to induce specific recombination in chondrocytes. Briefly, mice containing a PPARγ gene flanked by LoxP sites [C57BL/6-PPARγ$^{fl/fl}$, Jackson Laboratory] were mated with C57BL/6 Col2-Cre transgenic mice [145, 299, 300] to generate mice bearing Col2-Cre and a floxed allele in their germline (genotype: PPARγ$^{fl/+}$, Cre). These mice were backcrossed to homozygote floxed mice in the following cross: PPARγ$^{fl/+}$, Cre X PPARγ$^{fl/fl}$ to

generate mice with both alleles inactivated in chondrocytes (genotype: PPARγ$^{fl/fl}$, Cre). PPARγ$^{fl/fl}$, Cre mice are referred to as homozygote PPARγ KO mice, PPARγ$^{fl/+}$ Cre mice are referred to as heterozygote PPARγ KO mice, and PPARγ$^{fl/fl}$ mice without Cre transgene are referred to as control mice.

All procedures involving animals were approved by the Comité Institutionnelle de Protection des Animaux, an Animal Use Subcommittee of the Canadian Council on Animal Care at CR-CHUM, and the Animal Use Subcommittee at the University of Western Ontario. All animal studies including housing and breeding were performed as approved by the aforementioned committees. All mice were kept in a 12 hour light/dark cycle. Food and water were available *ad libitum*.

Western blotting

Freshly dissected cartilage from newborn control, and heterozygote and homozygote PPARγ KO mice was homogenized (Kinematica Polytron) in 50 mmol/L Tris-HCl pH 7.5 containing protease inhibitors. Cells were lysed in Tris-buffered saline (TBS) containing 0.1% sodium dodecyl sulfate (SDS), and the protein content of the lysates was determined using bicinchoninic acid protein assay reagent (Pierce Rockford) with bovine serum albumin (BSA) as the standard. Cell lysates were adjusted to equal equivalents of protein and then were applied to SDS-polyacrylamide gels (10–20%) for electrophoresis. Next, the proteins were electroblotted onto polyvinylidene fluoride membranes. After the membranes were blocked in 10 mM TBS containing 0.1% tween-20 (TBS-T) and 5% skim milk, the membranes were probed for 1.5 hours with the respective antibodies in TBS-T. After washing the membranes with TBS-T, the

membranes were incubated overnight with horseradish peroxidase-conjugated anti-rabbit or horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (1:10,000 dilution in TBS-T containing 5% skim milk) at 4°C. After further washing with TBS-T, protein bands were visualized with an enhanced chemiluminescence system using a Bio-Rad Chemidoc Apparatus.

Primary culture of chondrocytes

Primary chondrocytes were prepared from femurs of embryonic day (E) 16.5 control, and homozygote and heterozygote PPARγ KO mice [301]. Cartilage was dissected from femurs, rinsed in phosphate buffered saline (PBS), and incubated at 37°C for 15 minutes in trypsin-EDTA followed by digestion with 2 mg/ml collagenase P at 37°C for 2 hours in DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin under an atmosphere of 5% CO_2. The cell suspension was filtered through a 70-µm cell strainer (Falco), washed, counted and plated. At confluence, the cells were detached and passaged, and the 3–4 passage cells were used for the experiments.

RNA isolation and Real-Time Polymerase Chain Reaction (PCR)

Freshly dissected cartilage was homogenized as above. Total RNA was isolated from cartilage of E16.5 control, and homozygote and heterozygote PPARγ KO mice using TRIzol (Invitrogen) (RNeasy; QIAGEN), reverse transcribed and amplified using TaqMan Assays-on-Demand (Applied Biosystems) in a reaction solution containing two unlabeled primers and 6-carboxyfluoroscein-labelled TaqMan MGB probe [302,

303]. Samples were combined with One-Step MasterMix (Eurogentec). Amplified sequences were detected using the ABI Prism 7900HT sequence detector (Applied Biosystems). The expression values were standardized to values obtained with control GAPDH RNA primers using the ΔCt method. All primers for each target gene are available from Applied Biosystems Assay on demand. Data was normalized to GAPDH mRNA levels and represent averages and standard error of the mean (SEM) from direct comparison of KO and control mice. Statistical significance of Real-Time PCR results was determined by one-way analysis of variance.

Skeletal staining

Newborn mice were skinned, eviscerated, and dehydrated in 95% ethanol and acetone overnight. Skeletons were stained with 0.015% alcian blue, 0.05% alizarin red, and 5% acetic acid in 70% ethanol for several days. Skeletons were then cleared in 1% KOH, passed through a decreasing KOH series and stored in glycerol/ethanol (1:1) [299, 304].

Histological and immunohistochemistry (IHC) studies

Histological and IHC studies were performed using specific antibodies for target genes. Freshly dissected mouse long bones were fixed with 10% neutral buffered formalin and decalcified with 0.1 M EDTA at room temperature before paraffin embedding and sectioning at the Centre for Bone and Periodontal Research at McGill University (Montreal, Quebec), the Notre-Dame Hospital Pathology Department (Montreal, Quebec), and the Osteoarthritis Research Unit at CR-CHUM (Montreal,

Quebec). Sections (5 μm) were deparaffinised in xylene followed by a graded series of alcohol washes. Sections were stained with Safranin-O/Fast Green (Sigma). For IHC analysis, Dakocytomation (Dako) labelled streptavidin biotin + System-horseradish peroxidase kit was used. Briefly, endogenous peroxide was blocked for 5 minutes using 3% H_2O_2. Nonspecific IgG binding was blocked by incubating sections with BSA (0.1%) in PBS for 1 hour. Sections were then incubated with primary antibody in a humidified chamber and left overnight at 4°C. Next, sections were incubated with biotinylated link for 30 minutes followed by streptavidin for 1 hour. The diaminobenzidine tetrahydrochloride chromogen substrate solution was then added until sufficient color developed.

BrdU labelling

For BrdU labelling, pregnant female mice were injected intraperitoneal one day before being sacrificed with BrdU at a dose of 0.01 ml/g. BrdU was detected in paraffin sections using an anti-BrdU antibody through IHC as detailed above.

Bone mineralization

Freshly dissected mouse long bones were fixed with 10% neutral buffered formalin before plastic embedding, sectioning, and staining at the Centre for Bone and Periodontal Research at McGill University (Montreal, Quebec). Sections (5 μm) were stained with von Kossa to determine calcium deposition in the bone, Goldner to determine bone density, and Safranin-O/Fast Green to determine trabecular bone

thickness. Bone density and trabecular bone thickness were quantified using Bioquant Osteo II software [305].

Cartilage explants studies

Cartilage explants studies were conducted using femoral head cartilage from 3 week old control and homozygote PPARγ KO mice. Cartilage was extracted, rinsed in PBS and RNA was isolated and subjected to Real-Time PCR using specific primers as described above.

Aging studies

Knee joints from 14 months old control, and heterozygote and homozygote PPARγ KO mice were dissected and processed as detailed above. Histological analyses were performed using Safranin-O/Fast Green or Hematoxylin and Eosin staining, and slides were evaluated by two independent readers in a blinded fashion. To determine the extent of cartilage deterioration during aging, a histological scoring method issued by Osteoarthritis Research Society International (OARSI) was used. The OARSI scale of 0-6 signifies: 0 = Normal, 0.5 = Loss of Safranin-O/Fast Green staining without structural changes, 1 = Roughened articular surface and small fibrillations, 2 = Fibrillation down to the layer immediately below the superficial layer and some loss of surface lamina, 3 = Fibrillation/erosion to the calcified cartilage extending to < 25% of the articular surface, 4 = Fibrillation/erosion to the calcified cartilage extending to 25-50% of the articular surface, 5 = Fibrillation/erosion to the calcified cartilage extending

to 50-75% of the articular surface, and 6 = Fibrillation/erosion to the calcified cartilage extending to > 75% of the articular surface.

To evaluate the degree of inflammation in the synovium, Hematoxylin and Eosin stained sections were blindly scored on a scale of 0-3: 0 = No inflammation, 1 = Less inflammation, 2 = Moderate inflammation, 3 = High inflammation. In addition, IHC analysis using MOMA-2 antibody was performed to account for inflammation and determine the type of inflammatory cells present in the synovium of aged mice. Further, IHC studies using antibodies against MMP-13, VDIPEN and C1-2C were performed in order to determine the degree of cartilage degradation during aging.

Statistical analysis

All images were taken at room temperature with a Retiga OEM Fast camera connected to a Leica Diaplan fluorescence microscope using either 4X numerical aperture (NA) 0.12, 6.3X NA 0.20, 10X NA 0.30, 25X NA 0.60, and 40X NA 0.70 objective lenses. All primary images were acquired and analyzed using Bioquant Osteo II software. Blinded observers were used for each histomorphometric analysis. Results are expressed as mean ± SEM for all counts. Statistical analysis was evaluated by two-tailed Student's t-test. $P < 0.05$ was considered statistically significant.

Results

Characterisation of cartilage-specific PPARγ KO mice

Due to the fact that global PPARγ KO mice die as a result of embryonic lethality, the LoxP/Cre system was used to generate germ-line cartilage-specific PPARγ

conditional KO mice. Mice containing a PPARγ gene flanked by LoxP sites (PPARγ F/F) were mated with mice carrying Cre recombinase under the control of the collagen type II promoter to induce specific recombination in chondrocytes [145]. Generation of conditional KO mice was first determined by tail DNA genotyping, which confirmed the presence of the Cre transgene in heterozygote (PPARγ F/W Cre) and homozygote (PPARγ F/F Cre) PPARγ KO mice and its absence in wild-type (WT) (control) mice (Figure 1A). Loss of PPARγ expression in chondrocytes isolated from homozygote and heterozygote PPARγ KO versus control mice was confirmed by Western blotting (Figure 1B) and Real-Time PCR (Figure 1C), demonstrating that PPARγ mRNA levels were reduced by >96% in chondrocytes from homozygote KO mice. Additionally, IHC studies performed on femurs of post-natal day (P) 0 control mice demonstrated strong expression of PPARγ in resting and hypertrophic chondrocytes of the growth plate. However, PPARγ positive chondrocytes were undetectable in the resting and hypertrophic zones of the growth plate of homozygote PPARγ KO mice (Figure 1D), thus confirming that recombination occurred with high efficiency.

Cartilage-specific deletion of PPARγ results in reduced growth

We first determined the effect of cartilage-specific ablation of PPARγ on body weight, length and skeletal growth of mice. Analyses of newborn litters demonstrated growth retardation in homozygote PPARγ KO mice compared to control mice (Figure 2A). Whole-mount skeletal staining with alcian blue and alizarin red confirmed that newborn homozygote PPARγ KO mice show reduced skeletal growth compared to control mice (Figure 2B) and skeletal staining in some regions of the limbs in mutant

mice was weaker compared to control mice (Figure 2C). Measurements of growth over 42 days post-birth demonstrated that homozygote PPARγ KO mice had significantly reduced body length (Figure 2D) and weight (Figure 2E) compared to control mice. Measurements of individual bones demonstrated that homozygote PPARγ KO mice had significantly reduced length of tibiae and femurs at time of birth (Figure 2F). No significant differences in the viability of mice were observed. A consistent pattern of growth and weight retardation was observed in both genders.

PPARγ-deficient mice show delayed ossification and disorganization of growth plates

We further examined the effect of PPARγ deficiency on the organization of growth plates and ossification patterns. Histological analysis by Safranin-O/Fast Green staining demonstrated that femurs of E16.5 homozygote PPARγ KO mice exhibit reduced length and width, and delayed primary ossification compared to control mice (Figure 3A). In addition, femurs of P14 homozygote PPARγ KO mice exhibit delayed secondary ossification compared to control mice (Figure 3B).

Blinded histological analyses were also performed to examine growth plate organization on a cellular level at time of birth. Safranin-O/Fast Green staining of P0 mouse femurs demonstrated that homozygote and heterozygote PPARγ KO mice exhibit reduced length of long bones (Figure 4A) and growth plate defects in a gene-dose dependent manner, which is associated with hypocellularity in all three zones, loss of columnar organization in the proliferating zone, altered chondrocyte shape in the hypertrophic zone, and a shortened hypertrophic zone compared to control mice (Figure 4B,C).

Cartilage-specific deletion of PPARγ results in reduced chondrocyte proliferation and differentiation, and delayed vascular invasion

Subsequently, we determined if PPARγ deficiency caused aberrant chondrocyte proliferation and differentiation, and vascular invasion. IHC using antibodies against Sox9 (Figure 5A), a marker of early chondrocyte differentiation, BrdU (Figure 5B), a marker of proliferation, and p57 (Figure 5C), a cell cycle inhibitor required for normal hypertrophic differentiation, revealed that femurs of E16.5 homozygote PPARγ KO mice exhibit reduced chondrocyte differentiation and proliferation as demonstrated by a reduced percentage of positive cells compared to control mice. IHC using collagen type X, a marker of chondrocyte hypertrophy, demonstrated that the femurs of E16.5 homozygote PPARγ KO mice exhibit reduced chondrocyte hypertrophy compared to control mice (Figure 5D). IHC for PECAM, a cell surface marker for endothelial cells and a marker of new blood vessel formation, further demonstrated delayed vascular invasion in the femurs of E16.5 homozygote PPARγ KO mice compared to control mice (Figure 5E).

Cartilage-specific deletion of PPARγ causes reduced bone density

We further investigated whether the loss of PPARγ resulted in abnormal calcium deposition and bone density. Two bone mineralization staining methods, von Kossa (Figure 6A) and Goldner (Figure 6B), in combination with measurements to quantify bone density (Figure 6C) using Bioquant Osteo II software, demonstrated that E16.5 homozygote PPARγ KO mouse femurs exhibit reduced calcium deposition and bone density, respectively, compared to control mice. Measurements using Bioquant Osteo II

software to quantify trabecular bone thickness in P40 and P70 mice further demonstrated that homozygote PPARγ KO mice display significantly reduced trabecular bone thickness compared to control mice (Figure 6D).

PPARγ-deficiency results in reduced expression of VEGF-A, aggrecan and collagen type II, and increased expression of MMP-13

In order to determine the mechanisms associated with early skeletal defects associated with PPARγ KO mice, we performed expression analysis of various markers involved in the process of endochondral ossification. VEGF-A is one of the key factors involved in endochondral ossification and bone angiogenic processes [306]. Therefore, we first determined the effect of PPARγ-deficiency on the expression of VEGF-A in the growth plate of E16.5 mice using IHC. Our results showed that homozygote PPARγ KO mice exhibit reduced VEGF-A expression as demonstrated by the percentage of positive cells compared to control mice (Figure 7A).

Next, we performed *in vitro* and *ex vivo* studies using chondrocytes isolated from E16.5 control and homozygote PPARγ KO mice, and hip cartilage explants isolated from 3 week old control and homozygote PPARγ KO mice. We identified VEGF-A expression in isolated chondrocytes and cartilage explants. Quantitative PCR showed that both PPARγ-deficient mouse chondrocytes and cartilage explants exhibited significantly reduced VEGF-A expression compared to control mouse chondrocytes and cartilage explants. Furthermore, we examined the expression of ECM proteins, aggrecan and collagen type II, and the catabolic factor MMP-13 in chondrocytes and cartilage explants. Our analysis indicated that homozygote PPARγ KO mouse

chondrocytes and cartilage explants exhibit significantly reduced expression of aggrecan and collagen type II, and significantly increased expression of MMP-13 compared to control mouse chondrocytes and cartilage explants (Figure 7B,C).

Cartilage-specific PPARγ KO mice exhibit spontaneous OA-like characteristics during aging

Since we observed early endochondral bone growth defects in heterozygote and homozygote PPARγ KO mice, we next examined the effects of cartilage-specific ablation of PPARγ on the integrity of knee joints during aging. Histological and IHC analyses were performed on the knee joints of 14 months old mice to examine cartilage degradation and synovial inflammation during aging. Safranin-O/Fast Green staining demonstrated that the joints of control mice were intact with no signs of deterioration. In heterozygote PPARγ KO mice, we observed slight changes in the integrity of the cartilage (tibial plateau), including loss of Safranin-O staining accompanied by reduced chondrocyte cellularity. In homozygote PPARγ KO mice, we observed accelerated cartilage degradation, loss of chondrocytes, and fibrillations to the calcified cartilage extending up to 50% of the articular surface (Figure 8A). The average OARSI score, which scores OA severity [307], was significantly higher along with greater loss of chondrocytes in aged homozygote PPARγ KO mice compared to aged heterozygote PPARγ KO and control mice in a gene dose-dependent fashion (Table 1: OARSI Score). To examine synovial inflammation, we performed Hematoxylin and Eosin staining on 14 months old mice, which further revealed increased inflammatory cell influx in homozygote and heterozygote PPARγ KO mice versus control mice in a gene-

dose dependent fashion (Figure 8B, Table 1: Inflammation Score). To determine the type of inflammatory cells present in the synovium, we performed IHC studies using MOMA-2 antibody (macrophage marker) in 14 months old mice. Our results showed enhanced macrophage influx in the synovial membrane of homozygote and heterozygote PPARγ KO mice versus control mice in a gene dose-dependent manner (Figure 8C).

Since MMP-13 is one of the major catabolic enzymes associated with OA pathophysiology, we performed IHC studies using an anti-MMP-13 antibody. These studies demonstrated that aged homozygote PPARγ KO mice exhibited significantly increased MMP-13 expression as demonstrated by larger percentage of positive cells in the tibial plateau compared to aged control mice (Figure 9A). IHC studies for VDIPEN, an MMP-generated aggrecan neoepitope, demonstrated that aged homozygote PPARγ KO mice exhibited a significantly increased percentage of positive cells in the tibial plateau compared to aged control mice (Figure 9B). Additionally, IHC studies for the collagen type II breakdown product C1-2C demonstrated that aged homozygote PPARγ KO mice exhibited a significantly increased percentage of positive cells in the tibial plateau compared to aged control mice (Figure 9C). These results collectively suggest that genetic ablation of PPARγ leads to accelerated cartilage degradation resembling spontaneous OA.

Discussion

Our results, for the first time, demonstrate an important *in vivo* role of PPARγ in endochondral ossification, cartilage growth and development, and age-dependent OA

through the use of cartilage-specific PPARγ KO mice. We show that genetic ablation of PPARγ in cartilage *in vivo* results in marked reduction of endochondral bone growth, with specific alterations at the tissue, cellular, and molecular levels. PPARγ deficiency caused reduced growth and skeletal size, shorter length of long bones, delayed primary and secondary ossification patterns, disorganization of growth plates accompanied by hypocellularity, reduced chondrocyte proliferation, differentiation and hypertrophy, delayed vascular invasion, decreased bone density, calcium deposition and trabecular bone length, and aberrant expression of key markers involved in skeletogenesis (Sox9, p57, collagen type X and VEGF-A) as well as ECM synthesis (aggrecan and collagen type II) and degradation (MMP-13). Furthermore, aged homozygote PPARγ KO mice exhibit an accelerated, OA-like phenotype associated with enhanced cartilage degradation, synovial inflammation and increased expression of MMP-13 and its breakdown products. Collectively, our results demonstrate that PPARγ plays a pivotal role in coordinating diverse aspects of skeletal morphogenesis.

PPARγ regulates normal cartilage and endochondral bone development

As demonstrated by skeletal staining and measurements, cartilage-specific deletion of PPARγ resulted in abnormal endochondral bone growth, including reduced body size in terms of height and weight as well as reduced skeletal size and length of long bones. Histological analyses further revealed that PPARγ deficiency caused delayed primary and secondary ossification and disorganization of the growth plates. All three zones of the cartilage epiphyses of mutant mice showed signs of highly disorganized growth plates including hypocellularity and reduced chondrocyte

proliferation, differentiation and hypertrophy. Since the normal lengthening of long bones depends on the rate of production of hypertrophic chondrocytes from proliferating chondrocytes, the volume increase of hypertrophic chondrocytes, and the number of proliferative cycles a chondrocyte undergoes [4, 14, 19, 20], improper coordination of these variables results in abnormal bone length by hindering mineralization, apoptosis, vascular invasion, and thus, overall endochondral bone development.

Some of the key essential factors required for the normal ossification, ECM production and vascularization processes during early development of cartilage and bone include Sox9, p57, aggrecan, collagen type II, collagen type X and VEGF-A. During chondrogenesis, chondrocytes begin expressing Sox9, a chondrogenic transcription factor that is essential for proliferation and differentiation of chondrocytes. Within the growth plate, Sox9 is expressed in resting and proliferating chondrocytes, with maximum expression in prehypertrophic chondrocytes, and regulates the expression of genes encoding aggrecan and collagen type II, the two main components of the cartilage ECM [6, 27, 31]. Thereafter, resting and proliferating chondrocytes express a genetic program driven by Sox9, and control the rate of hypertrophic chondrocyte differentiation. p57 is a cell cycle inhibitor essential for cell cycle exit and onset of hypertrophic differentiation. Collagen type X is a cartilage ECM component unique to the hypertrophic zone and serves as a marker of terminally differentiated hypertrophic chondrocytes [41]. In the final step of endochondral bone development, chondrocytes undergo hypertrophy and produce a calcified, cartilaginous ECM and

angiogenic factors such as VEGF, which initiate and propagate vascular invasion. As a result, avascular cartilage is replaced by densely vascularized bone.

Delayed ossification and growth plate abnormalities in PPARγ mutant mice can be explained by the abnormal expression of Sox9, p57, collagen type X and VEGF-A. Our results show that the growth plates of homozygote PPARγ KO mice exhibit significantly reduced expression of Sox9 and p57, thus affecting the normal sequence of chondrocyte proliferation, differentiation and hypertrophy. In addition, the growth plates of homozygote PPARγ KO mice exhibit significantly reduced expression of VEGF-A and PECAM, indicating delayed vascularization. Hypertrophic chondrocytes express high levels of VEGF *in vivo*. In response to VEGF, capillary invasion and angiogenesis is initiated in the hypertrophic cartilage. VEGF is not only essential for angiogenic processes, but is also involved in endochondral ossification [59]. It has been shown that VEGF is secreted by cells in the upper zone of hypertrophic cartilage, which regulates the invasion of new blood vessels from the perichondrium and the removal of the cartilage matrix [57]. Inactivation of VEGF-A suppresses blood vessel invasion and impairs trabecular bone formation [24]. In addition, inactivation of VEGF-A specifically in chondrocytes results in abnormal endochondral bone formation and blood vessel development [62]. Therefore, significant reduction in the expression of VEGF-A, as observed in the growth plates of homozygote PPARγ KO mice, may contribute to reduced angiogenesis and impaired ossification patterns. In addition to these factors, growth plates of homozygote PPARγ KO mice showed reduced expression of collagen type X. Collagen type X facilitates endochondral ossification by regulating matrix mineralization and compartmentalizing matrix components [42]. Mice

with altered collagen type X function exhibit skeletal defects including compressed growth plates with reduced proliferative and hypertrophic zones, and trabecular bone thickness [43-45]. Therefore, reduced length of long bones, bone density and trabecular bone thickness may in part be dependent on decreased collagen type X expression upon genetic deletion of PPARγ.

It should be mentioned that previous studies have shown that PPARγ activation suppresses collagen type X expression and other markers of hypertrophic chondrocyte differentiation [282, 308]. At first glance, this appears to be contradictory to the delay in hypertrophic differentiation upon inactivation of PPARγ that we observed here. However, several possibilities could explain these apparent discrepancies. First, it is possible that there is an optimal level of PPARγ activity to promote hypertrophy and that both overactivation and inactivation suppress this process. Second, it is possible that reduced hypertrophy in these two settings is due to different mechanisms; PPARγ activation might directly suppress hypertrophy, while the smaller hypertrophic zone in cartilage-specific PPARγ KO mice could be secondary to reduced chondrocyte proliferation (e.g. less cells entering the hypertrophic zone). Further studies will be required to decipher the mechanisms involved.

Absence of PPARγ results in enhanced cartilage destruction and OA-like characteristics during aging

It has been previously shown that PPARγ expression is reduced in OA cartilage compared to normal cartilage [291]. This finding suggests that reduced PPARγ expression in OA cartilage may reflect increased expression of inflammatory and

catabolic factors. Indeed, treatment of human OA chondrocytes with pro-inflammatory factors including IL-1β, TNF-α, IL-17, and PGE2 suppresses PPARγ expression [291]. Simultaneously, natural and synthetic PPARγ agonists can reduce the synthesis of various catabolic and inflammatory factors implicated in OA pathophysiology; examples include inflammatory cytokines such as PGE2, IL-1β, TNF-α, IL-6, NO, and MMPs such as MMP-1 and MMP-13 [251, 283, 284, 286, 287]. A wide range of synthetic compounds bind to and activate PPARγ, including anti-diabetic thiazolidinediones, also known as glitazones, such as troglitazone, pioglitazone, ciglitazone, and rosiglitazone. Pioglitazone has been shown to reduce the development of cartilage lesions in experimental dog [288] and guinea pig [289] models of OA *in vivo* and to reduce the synthesis of key OA mediators such as MMPs. Therefore, PPARγ activation seems to be an attractive therapeutic target for counteracting OA.

Our histological analyses indicated that at 14 months of age, homozygote and heterozygote PPARγ KO mice exhibit accelerated cartilage degradation, hypocellularity, and increased synovial inflammation (increased macrophage influx) compared to control mice in a gene dose-dependent manner. These findings resembled OA and were further supported by histopathological OARSI scoring. In addition, our IHC studies demonstrated increased expression of MMP-13 and MMP-generated neoepitopes (aggrecan/VDIPEN and collagen type II/C1-2C) in the tibial plateau of aged homozygote PPARγ KO mice compared to aged control mice, thus supporting the aforementioned studies relating PPARγ with cartilage destruction associated with OA.

One can speculate that early skeletal defects observed in PPARγ KO mice could be a contributing factor towards enhanced cartilage degradation observed during aging.

For example, in KO animal models for integrin α1 and Smad3, it has been shown that defects in early cartilage development and endochondral ossification are associated with spontaneous OA during aging [96, 149]. It is hypothesized that one of the reasons for spontaneous OA-like characteristics during aging is that defects in early cartilage development cause skeletal malformations that affect joint geometry and biochemical properties predisposing to OA. As a result, cartilage degradation occurs at a higher rate as does the incidence of OA. *Stattin et al.* [259] supported this by demonstrating that patients with familial dysplasia develop joint malformations and, subsequently, develop secondary OA. At this moment, we cannot determine whether accelerated OA in our mice is secondary to the observed developmental defects or whether it is caused by the independent roles of PPARγ in articular cartilage. Specific inactivation of the PPARγ gene in adult articular cartilage (using an inducible Cre system) will be required to resolve this question. Nevertheless, our data clearly demonstrate that disruption of PPARγ function leads to earlier OA.

In closing, our results, for the first time, demonstrate that PPARγ is a critical regulator of cartilage health and physiology not only in early growth and development, but also during aging (Figure 10).

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Figure Legends

Figure 1. **Generation of cartilage-specific PPARγ KO mice.** (A) Genotyping of P0 control, and heterozygote and homozygote PPARγ KO mice confirmed the presence of the Cre transgene in heterozygote and homozygote KO mice and its absence in control mice. PPARγ KO band was detected at 230 basepairs (bps), WT band was detected at ~200 bps, and Cre band was detected at 700 bps. (B) Western blotting performed on isolated chondrocytes from control, and heterozygote and homozygote PPARγ KO mice demonstrated reduced expression of PPARγ in heterozygote and complete absence of PPARγ protein expression in homozygote PPARγ KO mice compared to control mice. (C) Real-Time PCR on isolated chondrocytes further confirmed reduction of PPARγ mRNA levels by >96% in chondrocytes from homozygote KO mice compared to control mice. Representative data from n=6 independent isolated chondrocytes per group. Bar graph shows mean ± SEM of each group. *, $P<0.05$ (D) IHC studies using P0 femurs from control and homozygote PPARγ KO mice confirmed the absence of PPARγ expression in the resting and hypertrophic chondrocytes within the growth plates of homozygote PPARγ KO mice compared to control mice. n=4 per group (bar, 100 μm). Figures show one representative experiment of at least four independent experiments.

Figure 2. **PPARγ deficiency results in reduced growth.** (A) Newborn homozygote PPARγ KO mice exhibited growth retardation compared to control mice (bar, 10 mm). Skeletal staining demonstrated that newborn homozygote PPARγ KO mice exhibited (B) reduced skeletal size (bar, 10 mm) and (C) reduced length of long bones (bar, 1

mm). (D) Body length and (E) body weight measurements in the first 42 days of life demonstrated that homozygote PPARγ KO mice exhibit delayed growth compared to control mice. (F) Measurements at time of birth demonstrated that homozygote PPARγ KO mice have reduced length of tibiae and femurs compared to control mice. *n*=6 per group. Figures show one representative experiment of six independent experiments. Bar graphs show mean ± SEM of each group. *, $P < 0.05$

Figure 3. **Lack of PPARγ results in delayed primary and secondary ossification.** (A) Safranin-O/Fast Green staining demonstrated that E16.5 homozygote PPARγ KO mice exhibit delayed primary ossification and reduced length of femurs compared to control mice. *n*=6 per group (bar, 500 μm). (B) Safranin-O/Fast Green staining showed delayed secondary ossification in the femurs of P14 homozygote PPARγ KO mice compared to control mice. *n*=4-6 per group (bar, 200 μm). Figures show one representative experiment of at least four independent experiments.

Figure 4. **Lack of PPARγ causes disorganization of growth plates.** (A) Safranin-O/Fast Green staining demonstrated that P0 homozygote and heterozygote PPARγ KO mice exhibit reduced length of femurs compared to control mice in a gene dose dependent fashion. *n*=6 per group (bar, 500 μm). (B) Growth plate organization was revealed by Safranin-O/Fast Green staining of femurs from P0 mice. Homozygote PPARγ KO growth plates showed disorganization in a gene dose-dependent manner with reduced cellularity in the resting zone, loss of columnar organization in the proliferating zone, a shorter hypertrophic zone with altered chondrocyte shape, and

hypocellularity in all three zones compared to control and heterozygote PPARγ KO mice. *n*=6 per group (bar, 100 μm). (C) Bar graphs corresponding to histological analysis show reduced cellularity in the resting, proliferating, and hypertrophic zones, and shortened hypertrophic zone in homozygote and heterozygote PPARγ KO mice compared to control mice. Figures show one representative experiment of six independent experiments. Bar graphs show mean ± SEM of each group. *,+ $P < 0.05$

Figure 5. **Genetic ablation of PPARγ results in reduced chondrocyte proliferation, differentiation and hypertrophy, and delayed vascular invasion.** Staining for (A) Sox9 (bar, 100 μm), (B) BrdU (bar, 100 μm), (C) p57 (bar, 100 μm), and (D) collagen type X (bar, 500 μm) demonstrated reduced chondrocyte proliferation, differentiation and hypertrophy in E16.5 homozygote PPARγ KO mouse femurs compared to control mice. Bar graphs corresponding to IHC analysis show reduced percentage of positive cells for Sox9, BrdU, and p57 in homozygote PPARγ KO mice compared to control mice. *n*=4 per group (E) IHC for PECAM confirmed reduced vascularity in the femurs of E16.5 homozygote PPARγ KO mice compared to control mice. *n*=4 per group (bar, 500 μm). Figures show one representative experiment of four independent experiments. Bar graphs show mean ± SEM of each group. *, $P < 0.05$

Figure 6. **PPARγ deficiency results in decreased calcium deposition, bone density and trabecular bone thickness.** (A) Von Kossa staining of E16.5 mouse femurs showed that homozygote PPARγ KO mice exhibit reduced calcium deposition compared to control mice. (B) Goldner staining and (C) bone density quantification

using Bioquant Osteo II software demonstrated that the femurs of E16.5 homozygote PPARγ KO mice show decreased bone density compared to control mice. (D) Quantification of trabecular bone thickness using Bioquant Osteo II software demonstrated that P40 and P70 homozygote PPARγ KO mice exhibit decreased trabecular bone thickness compared to control mice. *n*=4 per group (bar, 500 μm). Figures show one representative experiment of four independent experiments. Bar graphs show mean ± SEM of each group. *, $P < 0.05$

Figure 7. **Genetic ablation of PPARγ results in aberrant expression of genes involved in the process of endochondral ossification.** (A) IHC demonstrated that E16.5 homozygote PPARγ KO mice growth plate exhibit reduced VEGF-A expression compared to control mice. Bar graphs corresponding to IHC analysis show reduced percentage of positive cells for VEGF-A in homozygote PPARγ KO mice compared to control mice. *n*=4 per group (bar, 100 μm). Figures show one representative experiment of four independent experiments. (B) mRNA expression using RNA isolated from E16.5 mouse chondrocytes indicated decreased expression of VEGF-A, aggrecan, and collagen type II, and increased expression of MMP-13 in homozygote PPARγ KO mice compared to control mice. (C) mRNA expression using RNA isolated from femoral head cartilage explants of 3 week old mice also indicated decreased expression of VEGF-A, aggrecan, and collagen type II, and increased expression of MMP-13 in homozygote PPARγ KO mice compared to control mice. Representative data from *n*=4-6 independent isolated chondrocytes and cartilage explants experiments per group. Bar graphs throughout figure show mean ± SEM of each group. *, $P < 0.05$

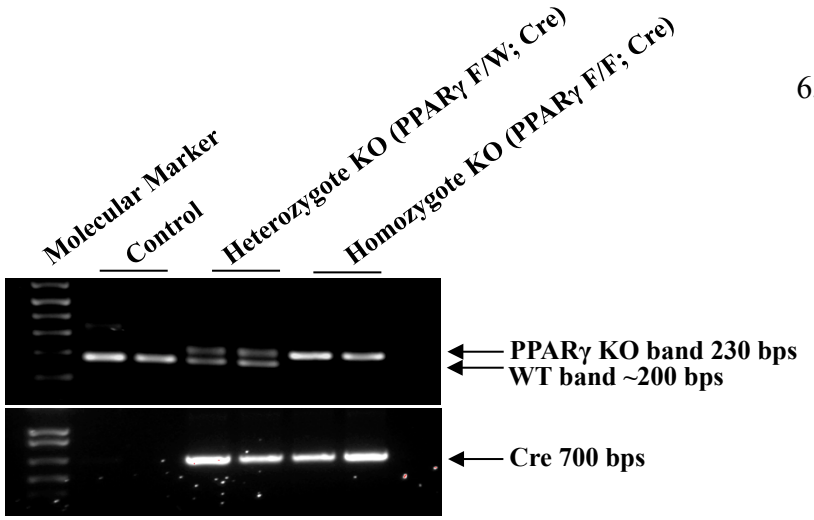
Figure 8. **Cartilage-specific deletion of PPARγ results in spontaneous OA-like characteristics during aging.** (A) Safranin-O/Fast Green staining demonstrated that 14 months old homozygote PPARγ KO mice exhibit accelerated cartilage degradation and loss of chondrocytes compared to aged control and heterozygote PPARγ KO mice (bar, 200 μm). (B) Hematoxylin and Eosin staining demonstrated that aged homozygote PPARγ exhibit enhanced inflammatory cell influx in the synovium compared to aged control and heterozygote PPARγ KO mice (bar, 200 μm, 100 μm). (C) IHC using macrophage marker MOMA-2 demonstrated that aged homozygote PPARγ KO mice exhibit increased macrophage influx in the synovial membrane compared to aged control and heterozygote PPARγ KO mice (bar, 50 μm). *n*=5-6 per group. Figures show one representative experiment of at least five independent experiments.

Figure 9. **Cartilage-specific deletion of PPARγ results in spontaneous OA-like characteristics during aging.** IHC demonstrated that aged homozygote PPARγ KO mice exhibit increased (A) MMP-13, (B) VDIPEN, and (C) C1-2C expression compared to aged control PPARγ KO mice. Bar graphs corresponding to IHC analysis show increased percentage of positive cells for MMP-13, VDIPEN, and C1-2C in aged homozygote PPARγ KO mice compared to aged control mice. *n*=5-6 per group (bar, 200 μm). Figures show one representative experiment of at least five independent experiments. Bar graphs show mean ± SEM of each group. *, $P < 0.05$

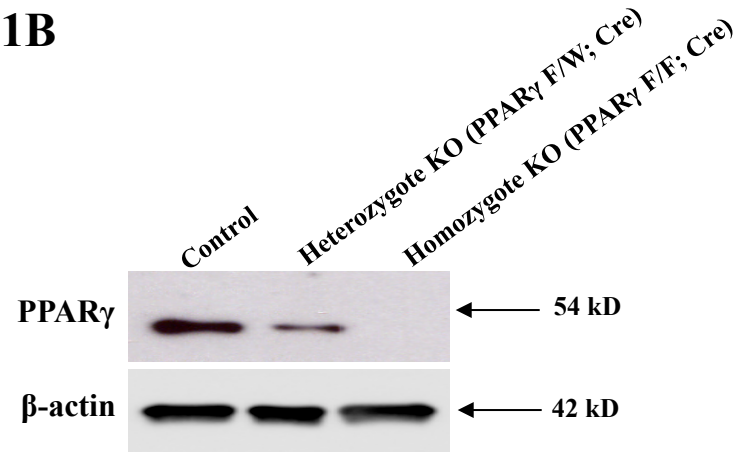
Figure 10. **Cartilage-specific deletion of PPARγ causes early cartilage developmental defects and OA-like characteristics during aging.** Cartilage-specific

PPARγ KO mice demonstrate abnormal chondrocyte function as well as dysregulated gene expression of several factors including ECM genes, aggrecan and collagen type II, and MMP-13, which causes abnormal early cartilage growth and development. During aging, lack of PPARγ facilitates increased expression of C1-2C, VDIPEN, and ECM degradation product, MMP-13, and decreased expression of ECM production products, aggrecan and collagen type II, leading to excessive cartilage destruction and enhanced synovial inflammation which resemble age-dependent OA-like characteristics.

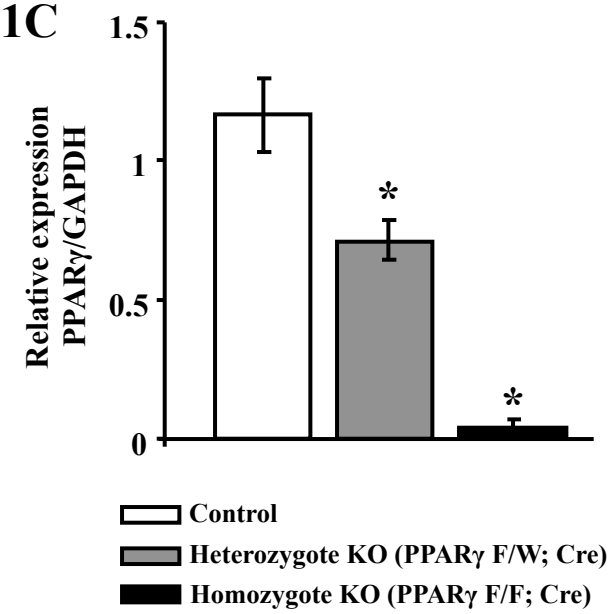
1A


Molecular Marker
Control
Heterozygote KO (PPARγ F/W; Cre)
Homozygote KO (PPARγ F/F; Cre)
PPARγ KO band 230 bps
WT band ~200 bps
Cre 700 bps

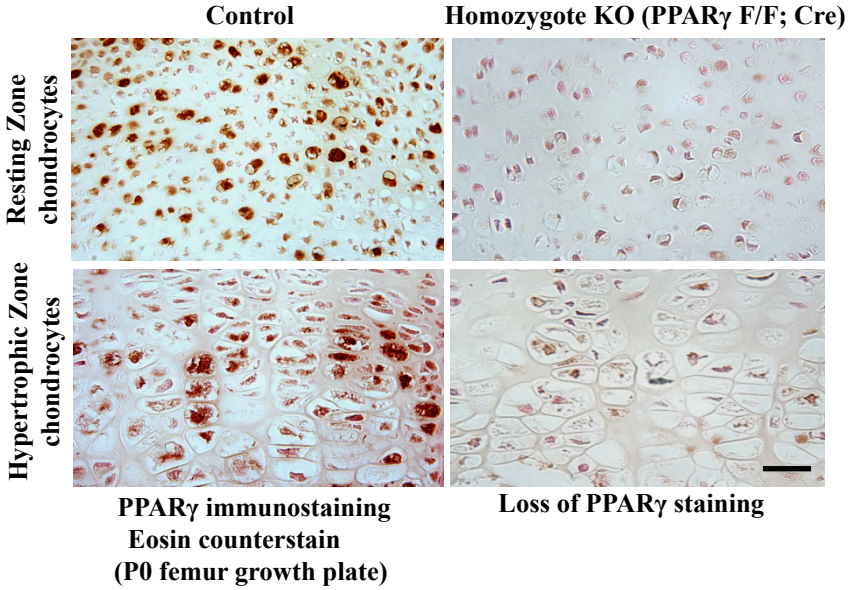
1B


Control
Heterozygote KO (PPARγ F/W; Cre)
Homozygote KO (PPARγ F/F; Cre)
PPARγ
54 kD
β-actin
42 kD

1C


Relative expression PPARγ/GAPDH
1.5
1
0.5
0
*
*
Control
Heterozygote KO (PPARγ F/W; Cre)
Homozygote KO (PPARγ F/F; Cre)

1D


Control
Homozygote KO (PPARγ F/F; Cre)
Resting Zone chondrocytes
Hypertrophic Zone chondrocytes
PPARγ immunostaining
Eosin counterstain
(P0 femur growth plate)
Loss of PPARγ staining

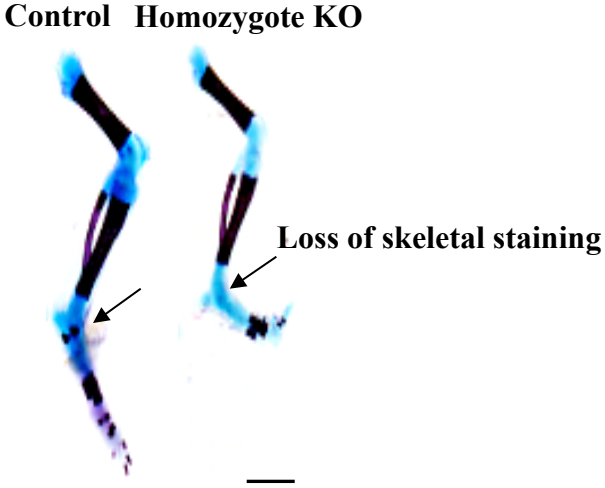
2A

P0

2B

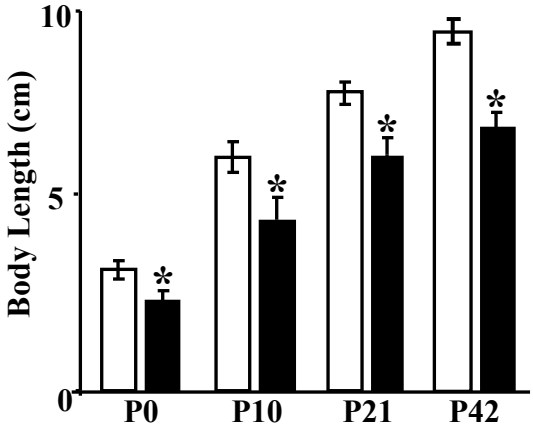
P0

2C

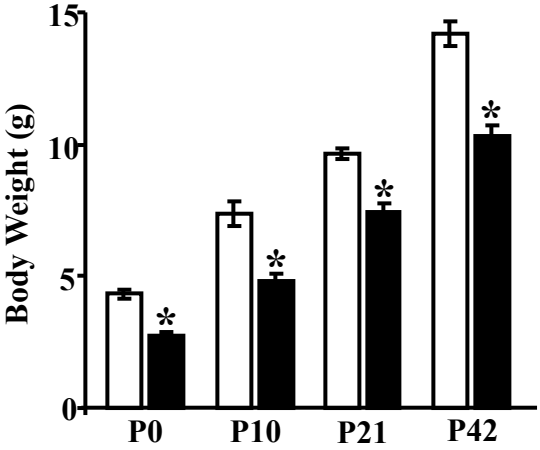


P0

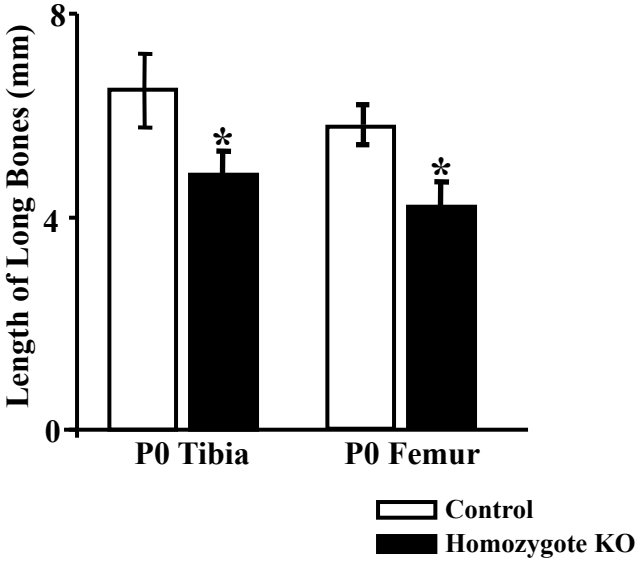
2D



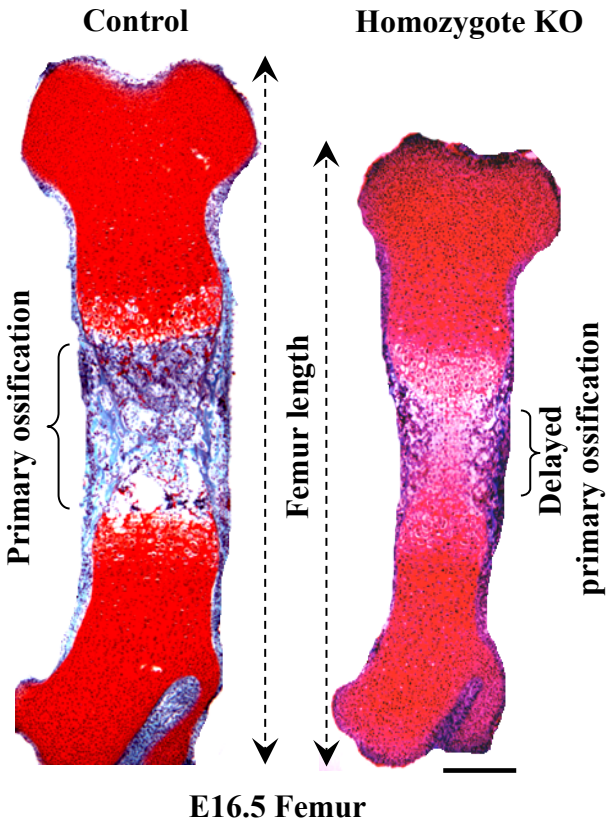
2E



2F

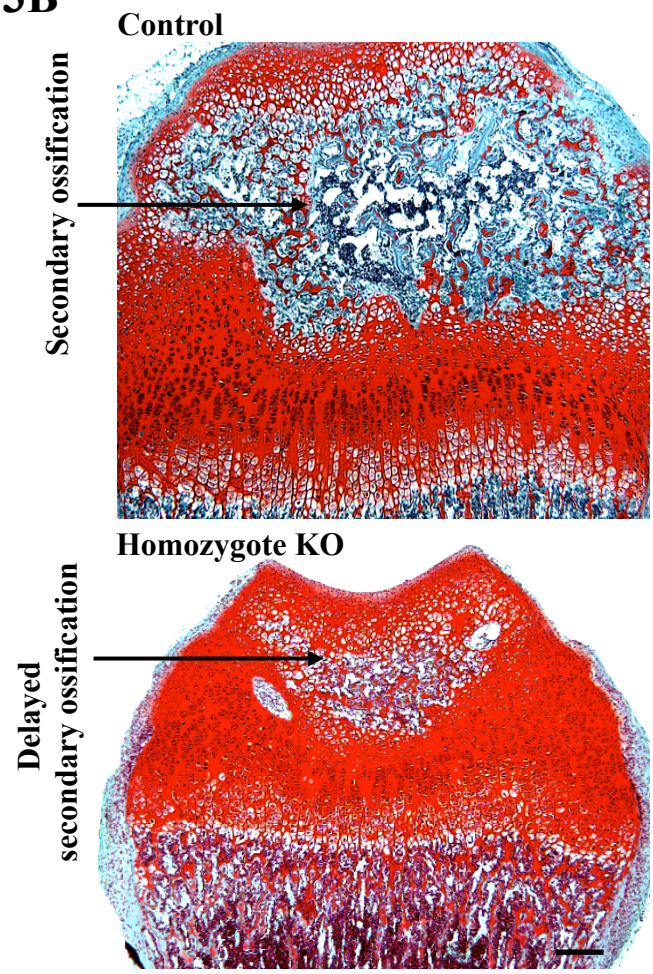


3A

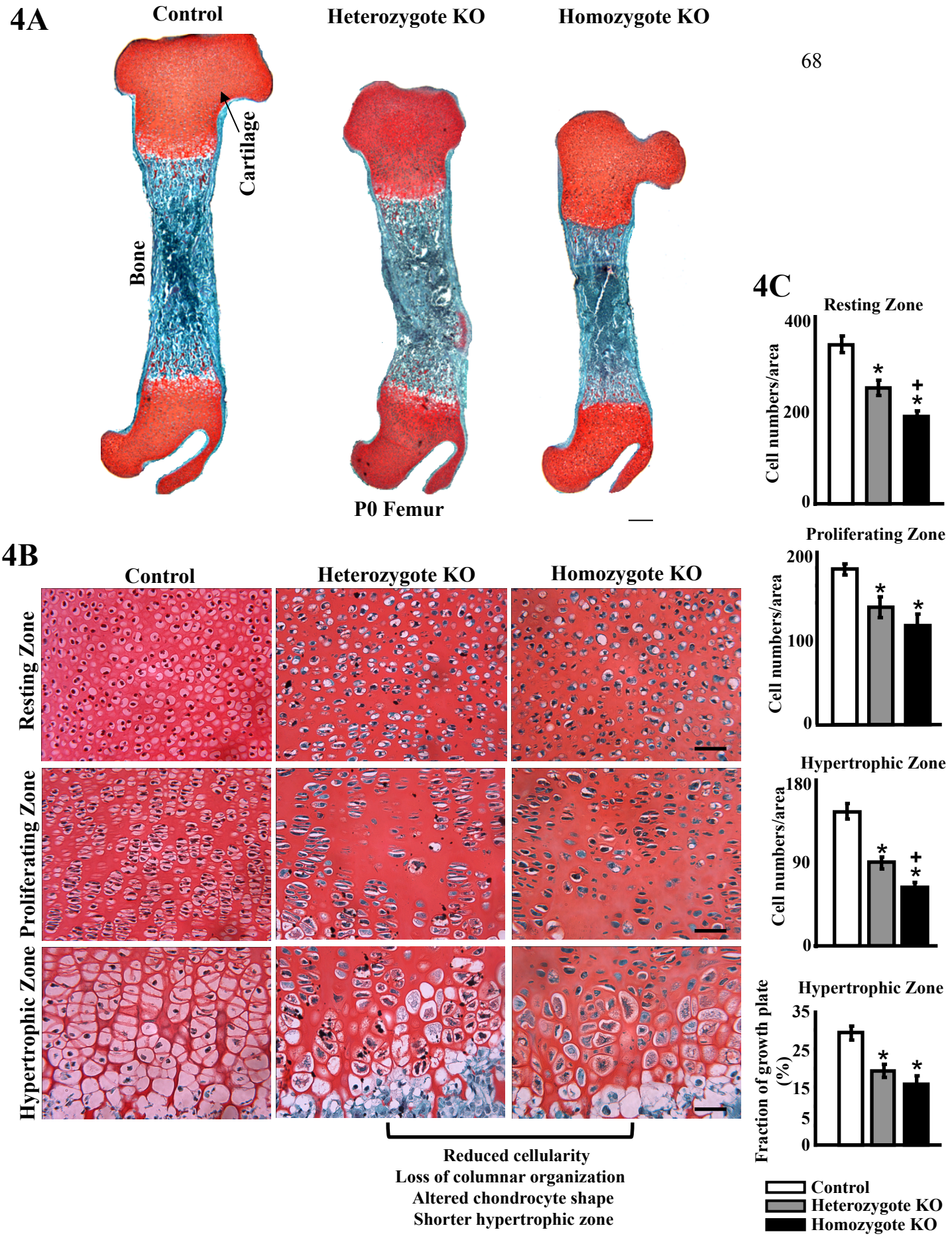


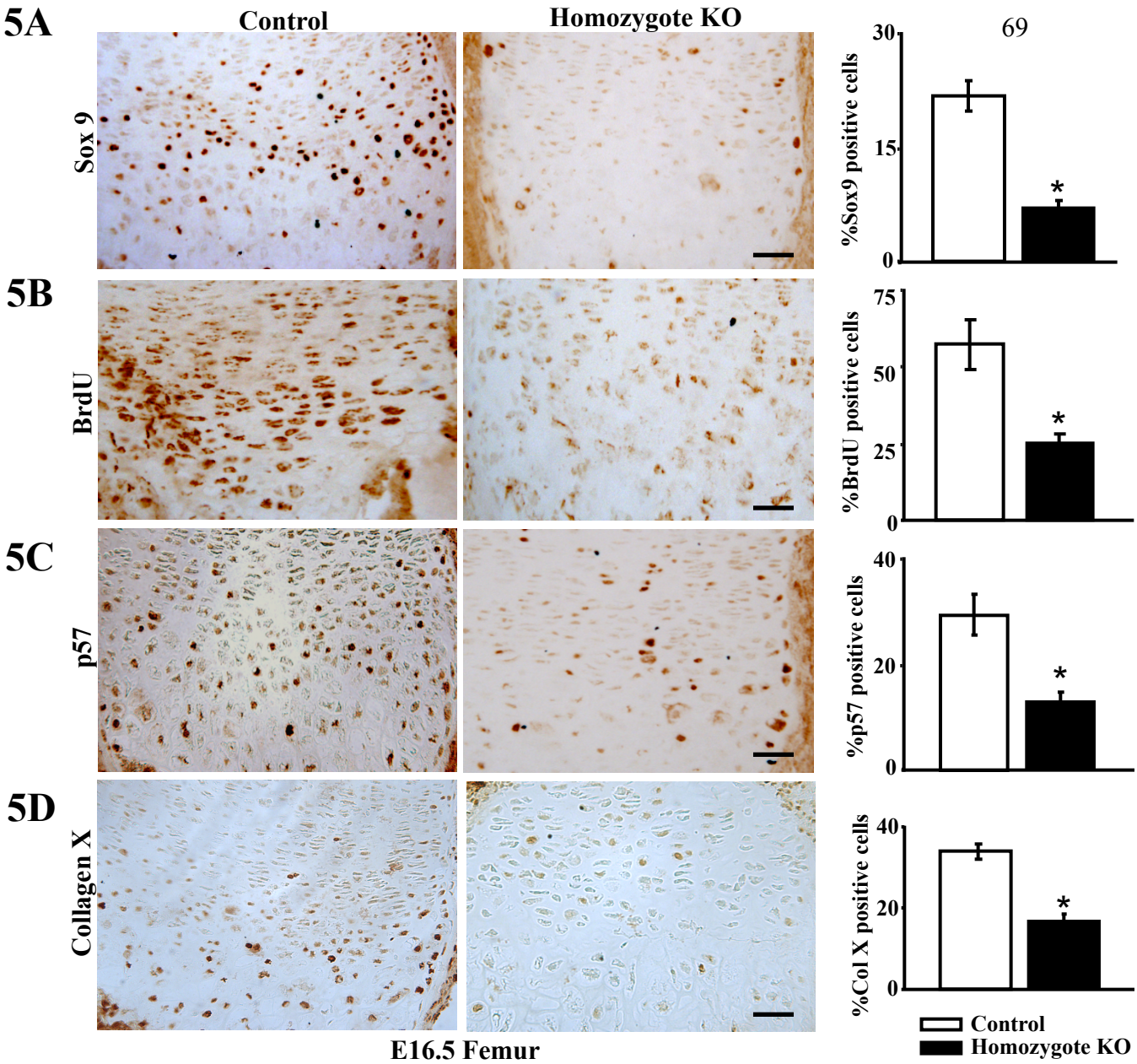
E16.5 Femur

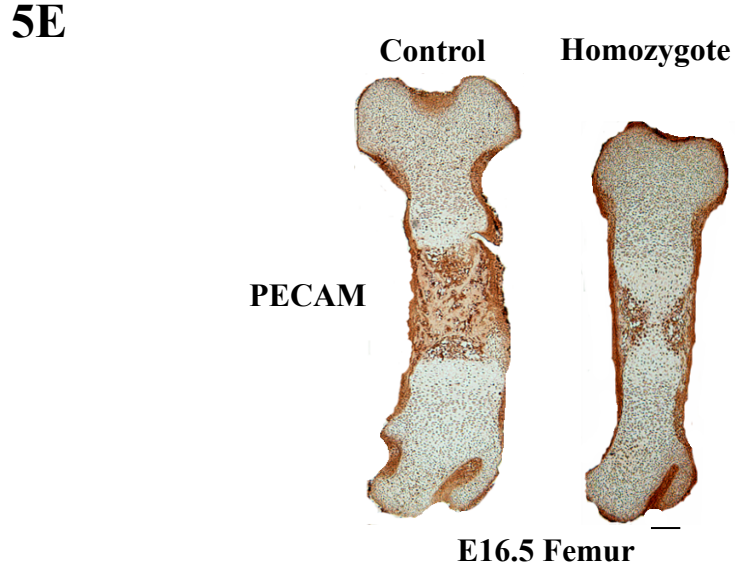
3B

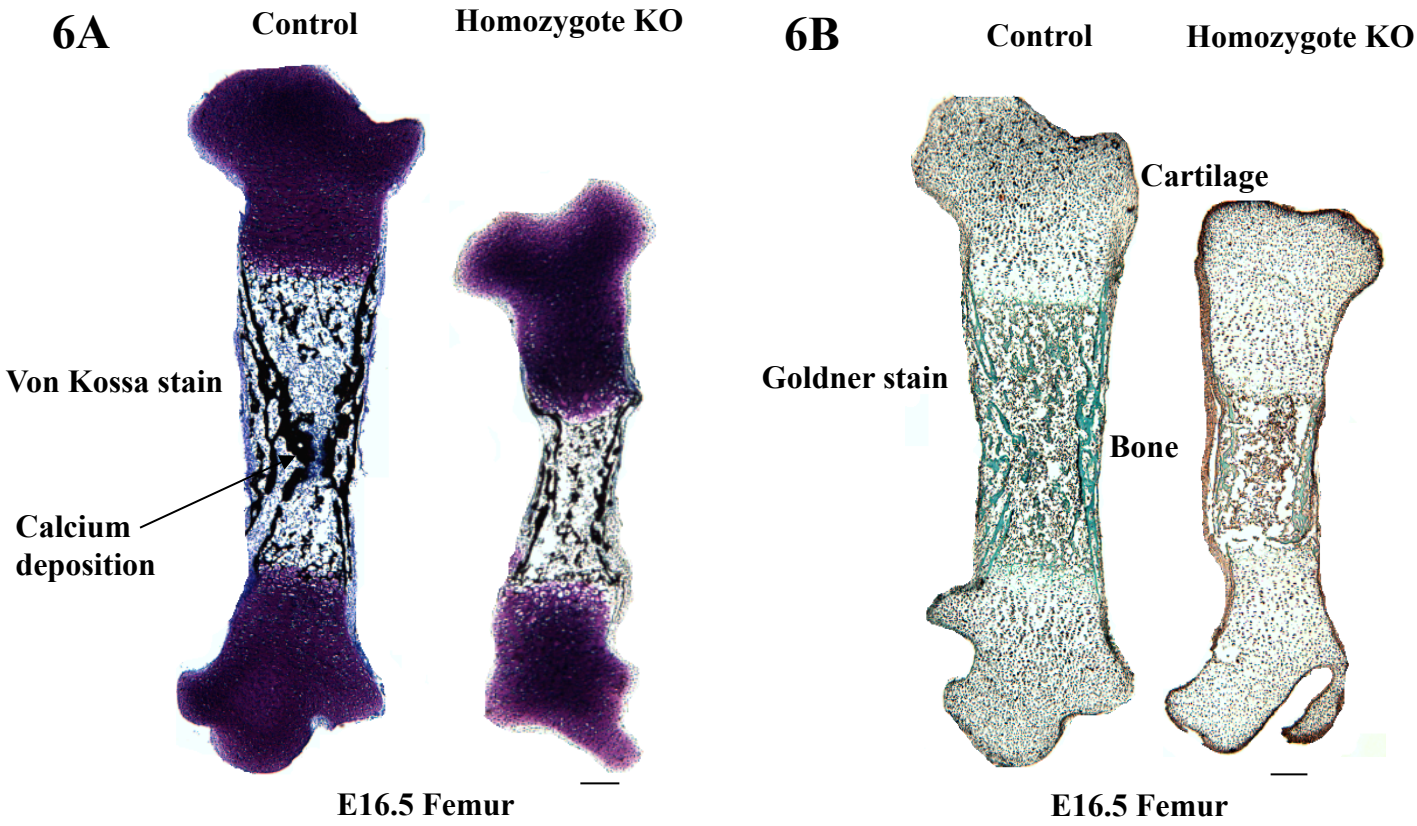


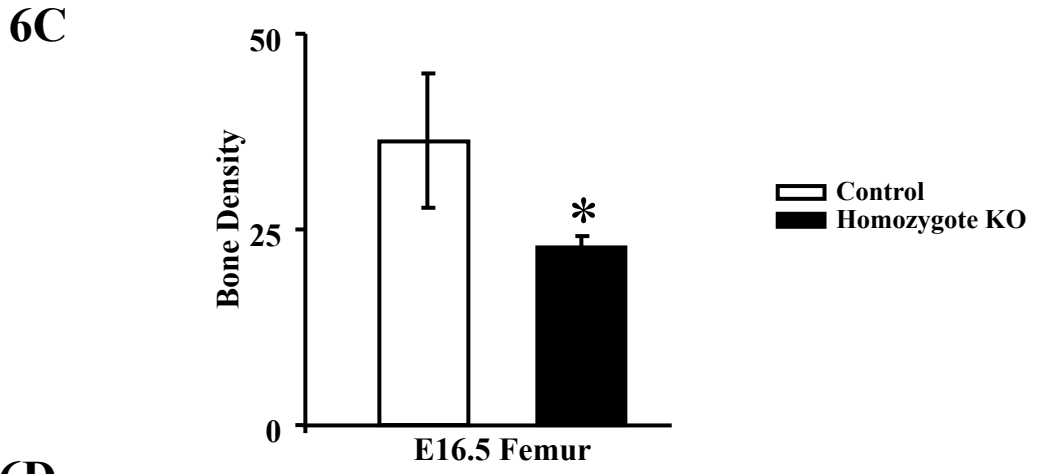
P14 Femur

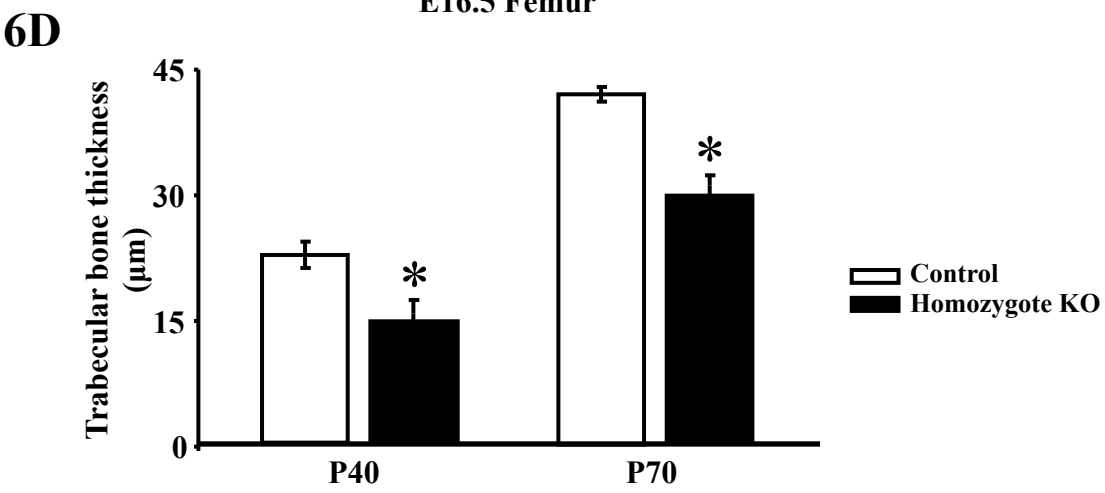

4A
Control
Heterozygote KO
Homozygote KO
Cartilage
Bone
P0 Femur
4B
Control
Heterozygote KO
Homozygote KO
Resting Zone
Proliferating Zone
Hypertrophic Zone
Reduced cellularity
Loss of columnar organization
Altered chondrocyte shape
Shorter hypertrophic zone
4C
Resting Zone
Cell numbers/area
400
200
0
Proliferating Zone
Cell numbers/area
200
100
0
Hypertrophic Zone
Cell numbers/area
180
90
0
Hypertrophic Zone
Fraction of growth plate (%)
35
25
15
5
0
Control
Heterozygote KO
Homozygote KO

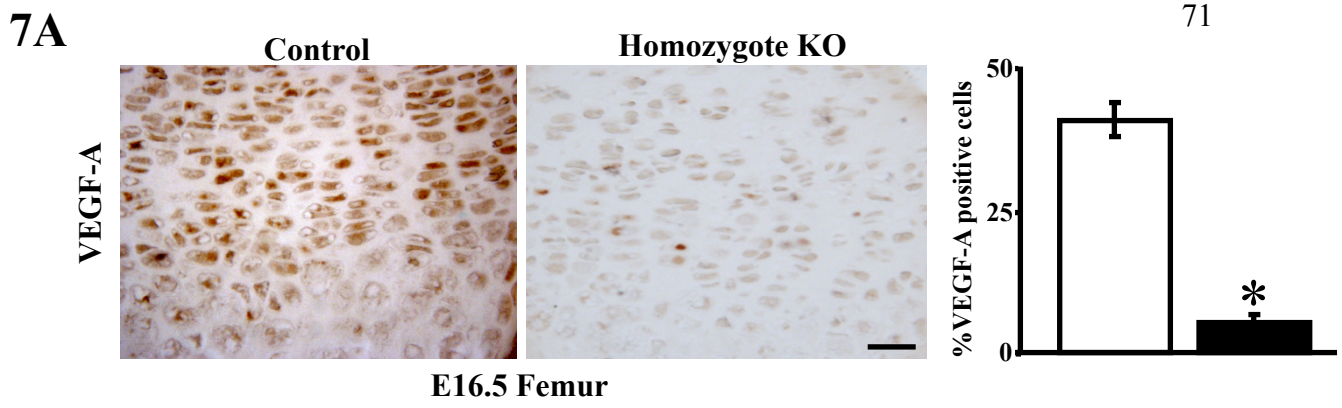

5A
Control
Homozygote KO
Sox 9
%Sox9 positive cells
30
15
0
*
5B
BrdU
%BrdU positive cells
75
50
25
0
*
5C
p57
%p57 positive cells
40
20
0
*
5D
Collagen X
%Col X positive cells
40
20
0
*
Control
Homozygote KO
E16.5 Femur

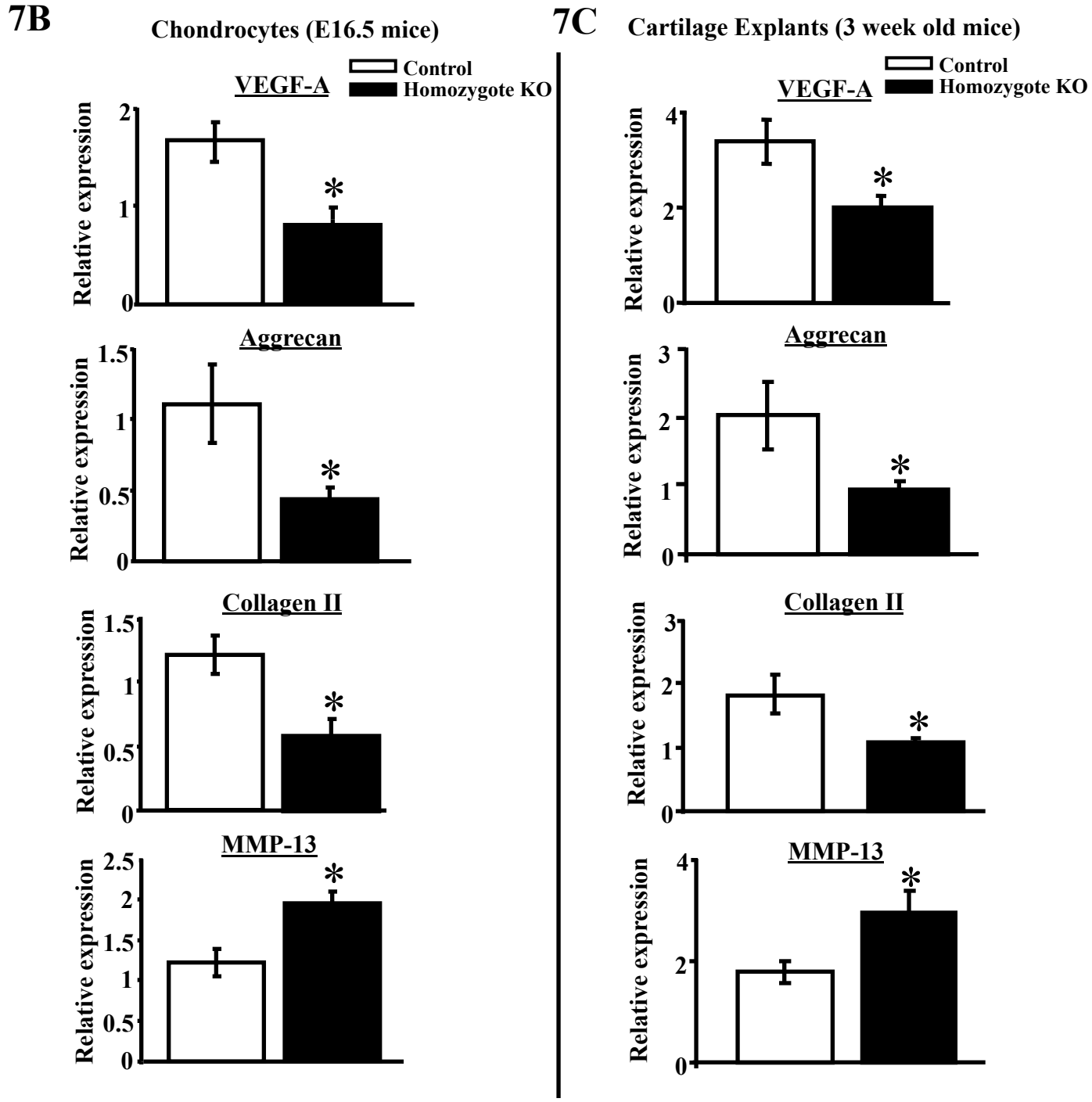

5E
Control
Homozygote KO
PECAM
E16.5 Femur


6A
Control
Homozygote KO
Von Kossa stain
Calcium deposition
E16.5 Femur
6B
Control
Homozygote KO
Cartilage
Goldner stain
Bone
E16.5 Femur

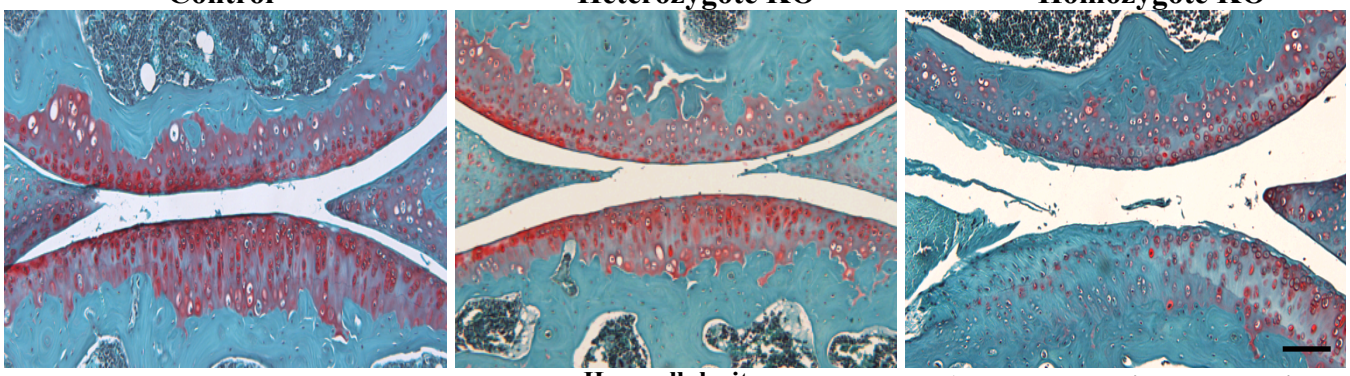

6C
50
25
0
Bone Density
*
Control
Homozygote KO
E16.5 Femur


6D
45
30
15
0
Trabecular bone thickness (μm)
*
*
Control
Homozygote KO
P40
P70


7A
Control
Homozygote KO
VEGF-A
%VEGF-A positive cells
50
25
0
*
E16.5 Femur


7B
Chondrocytes (E16.5 mice)
7C
Cartilage Explants (3 week old mice)
Control
Homozygote KO
VEGF-A
Aggrecan
Collagen II
MMP-13
Relative expression
*

8A

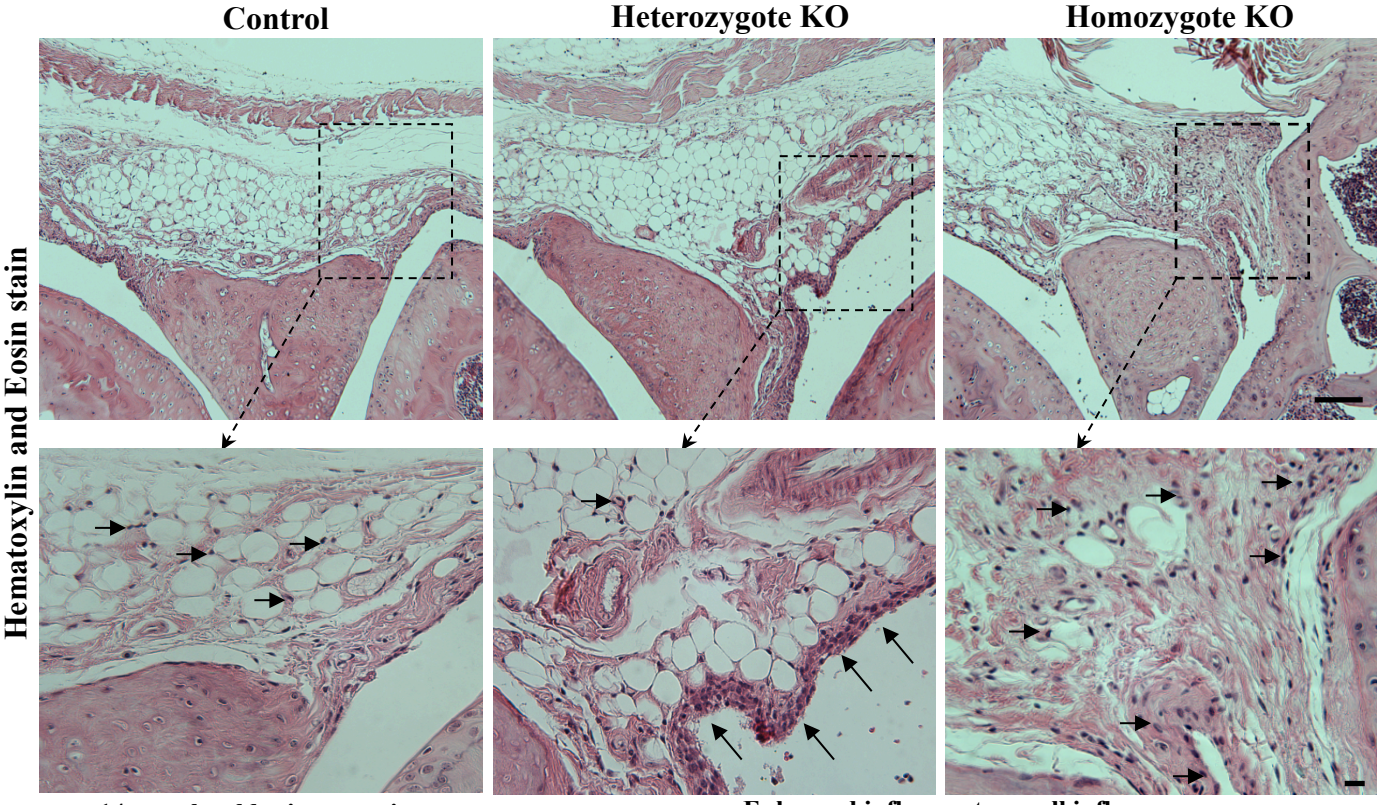


14 months old mice knee joint

Hypocellularity
Loss of Safranin-O/Fast Green staining

Accelerated cartilage destruction
Hypocellularity
Loss of Safranin-O/Fast Green staining

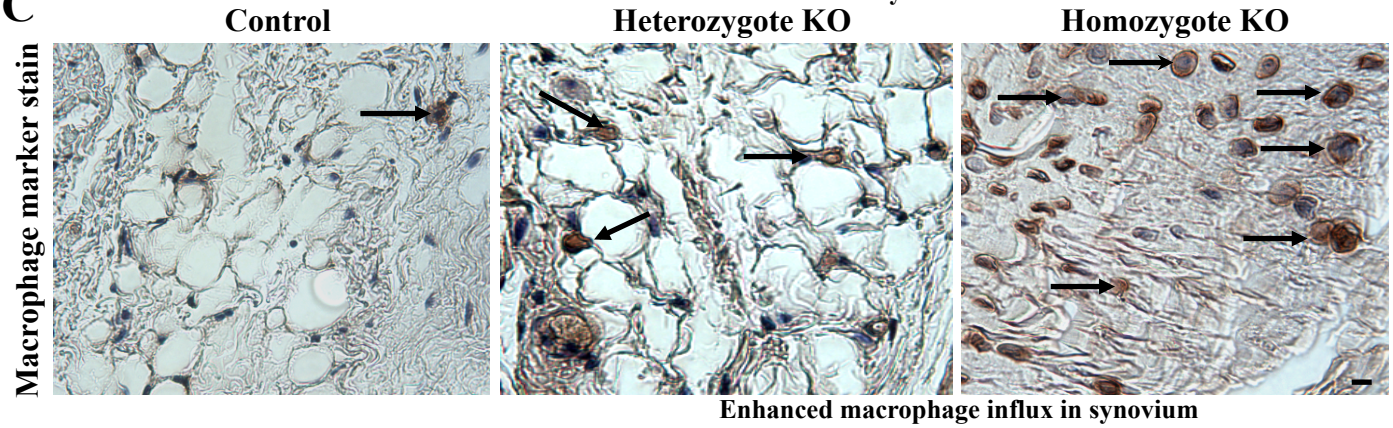
8B



14 months old mice synovium

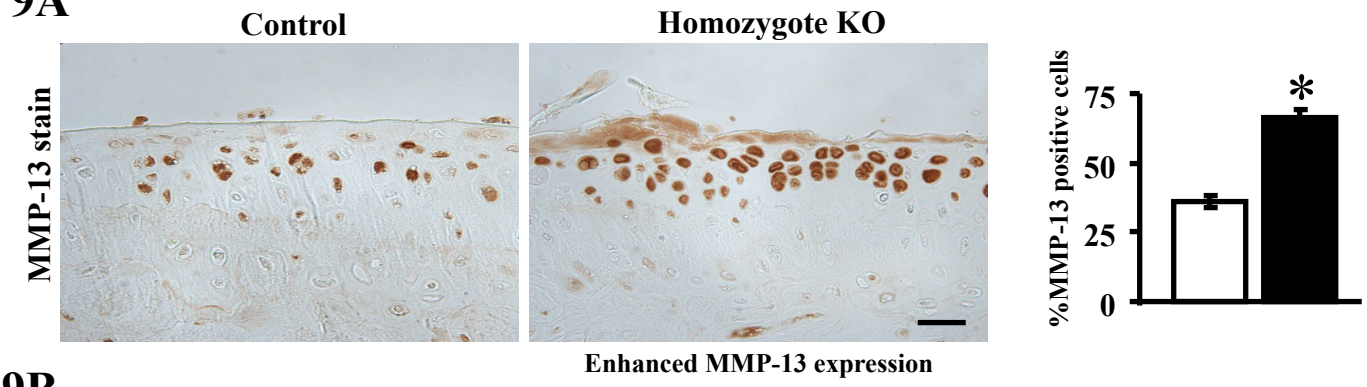
Enhanced inflammatory cell influx
Enhanced synovial inflammation

8C

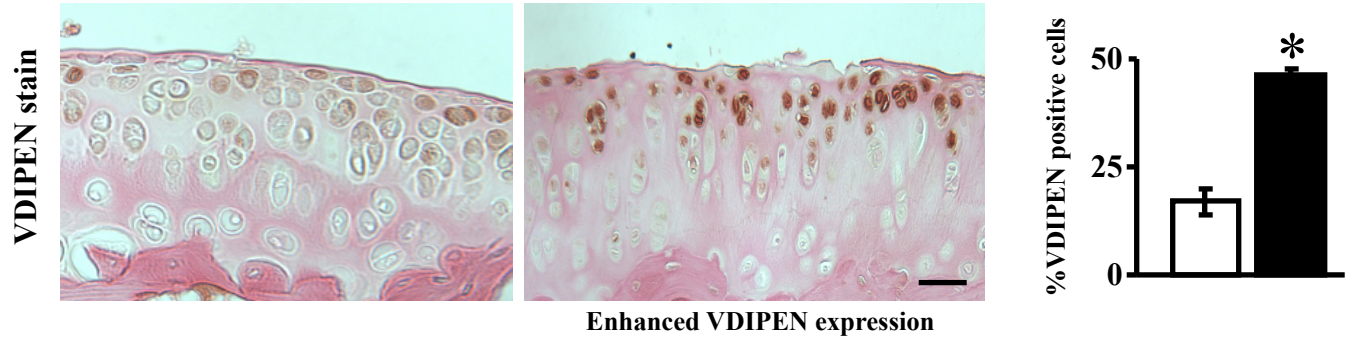


Enhanced macrophage influx in synovium

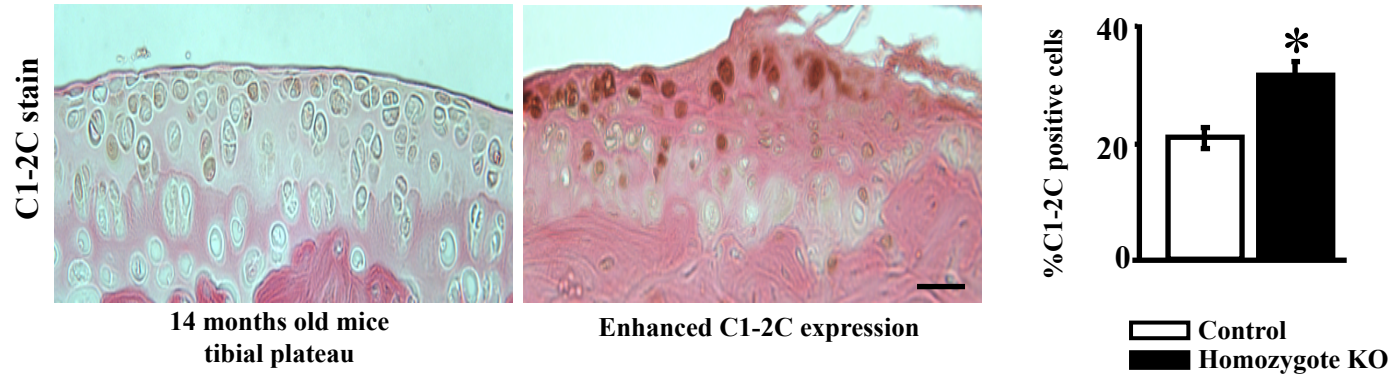
9A


Control
Homozygote KO
MMP-13 stain
%MMP-13 positive cells
75
50
25
0
*
Enhanced MMP-13 expression

9B


VDIPEN stain
%VDIPEN positive cells
50
25
0
*
Enhanced VDIPEN expression

9C


C1-2C stain
%C1-2C positive cells
40
20
0
*
14 months old mice
tibial plateau
Enhanced C1-2C expression
Control
Homozygote KO

10

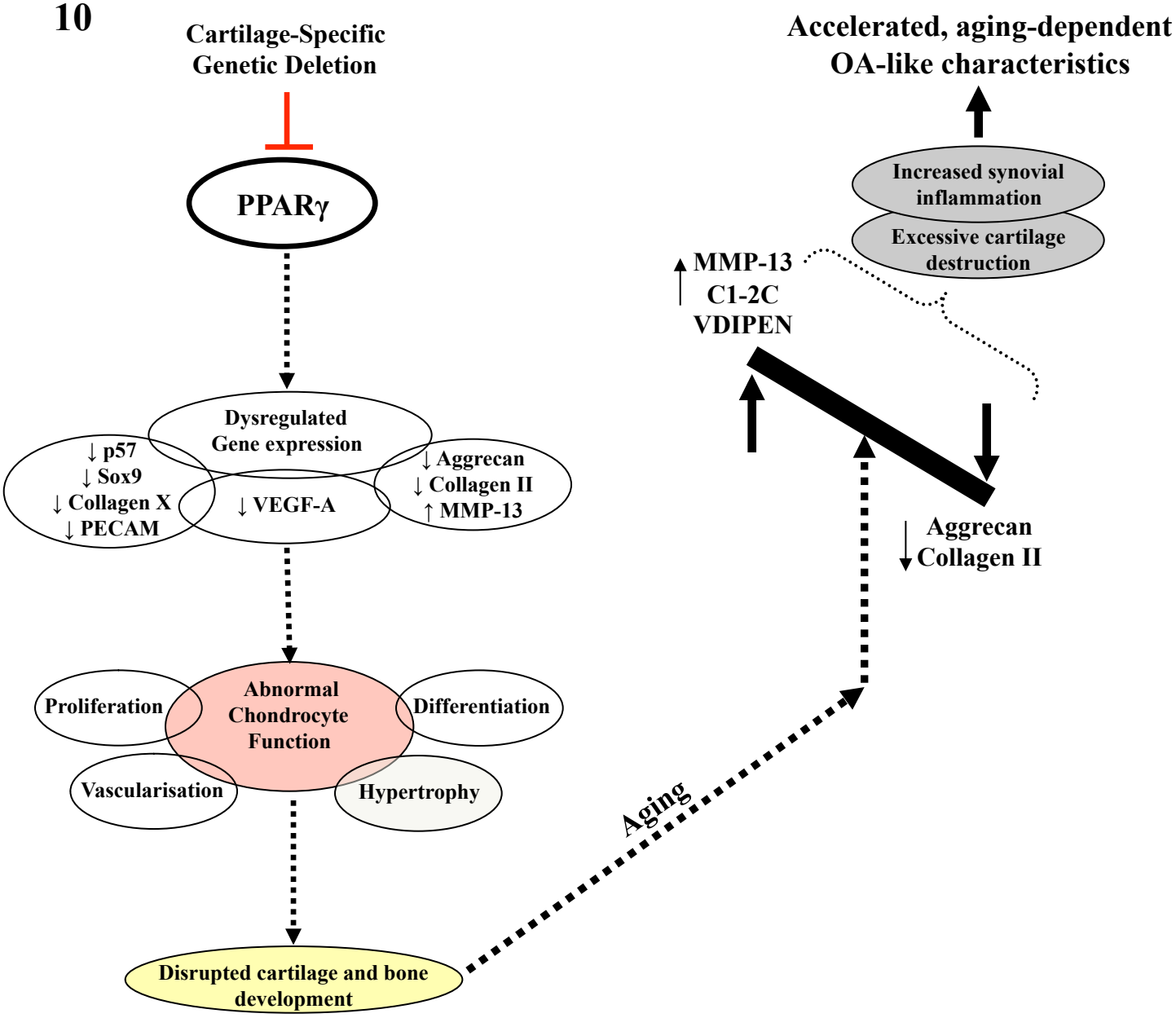

Cartilage-Specific
Genetic Deletion
PPARγ
Dysregulated
Gene expression
↓ p57
↓ Sox9
↓ Collagen X
↓ PECAM
↓ VEGF-A
↓ Aggrecan
↓ Collagen II
↑ MMP-13
Proliferation
Abnormal
Chondrocyte
Function
Differentiation
Vascularisation
Hypertrophy
Disrupted cartilage and bone
development
Aging
Accelerated, aging-dependent
OA-like characteristics
Increased synovial
inflammation
Excessive cartilage
destruction
↑ MMP-13
C1-2C
VDIPEN
↓ Aggrecan
↓ Collagen II

Table I: Histopathological scoring of cartilage degradation and synovial inflammation in PPARγ heterozygous and homozygous KO mice versus control mice during aging mice.

	Control	Heterozygous KO	Homozygous KO
OARSI Scores (0-6)	1.8 ± 0.200	2.6 ± 0.245*	3.8 ± 0.374 *,+
Inflammation score (0-3)	0.5 ± 0.297	1.5 ± 0.288 *	2.75 ± 0.25 *,+
Cellularity (per plane)	118.33 ± 7.05	101.66 ± 3.17 *	71.38 ± 4.05 *, +

Values are expressed at mean ± SEM from *n*=5-6 representative mice in each group. '*' indicates significance between heterozygous and homozygous KO versus control mice. '+' indicates significance among all three means. *,+, $P < 0.05$

GENERAL DISCUSSION

The research study presented in this thesis was conducted at two stages of development, during embryonic and early postnatal stages, and aging stages. Therefore, one can draw a principal conclusion from each developmental stage. Firstly, it can be deduced that PPARγ regulates normal cartilage and endochondral bone growth and development during early stages. Secondly, PPARγ-deficiency results in accelerated and spontaneous OA during aging. Overall, we were able to demonstrate that PPARγ plays a pivotal role in coordinating diverse aspects of skeletal morphogenesis.

Although determining the specific *in vivo* function and the signaling pathways affecting PPARγ expression and activity in cartilage growth and development is complex, by beginning to gain an understanding of the role of PPARγ, this study has created advances in the field, thus allowing us to be one step closer in achieving therapeutic efficacy for OA with potentially less side effects.

Our studies have revealed that PPARγ is a critical regulator of cartilage health and physiology during aging and the lack of PPARγ leads to accelerated and spontaneous OA; however, some matters remain unresolved. It is not known whether the observed accelerated OA is secondary to the observed early growth and developmental defects or whether it is caused by the independent roles of PPARγ in articular cartilage. Therefore, looking forward, it is important to answer this question, which is possible through the cartilage-specific inactivation of PPARγ in adult mice using an inducible system. This system is currently being employed by our research team and will allow for the bypass of early growth and developmental defects caused by PPARγ inactivation. This will clarify whether age-related defects in PPARγ KO mice

are as a result of early growth and developmental defects or occur independently during aging.

Another important aspect that is essential to examine in the future is the downstream signaling pathway responsible for PPARγ actions in cartilage destruction. This will further clarify the role of PPARγ in cartilage growth and development. Recent *in vitro* studies have shown that PPARγ can regulate the transcription of phosphatase and tensin homolog (PTEN), a known tumour suppressor that is primarily described in cancer cells. Activation of PPARγ through agonists, especially rosiglitazone, has been shown to increase functional PTEN protein levels. PTEN is a critical negative regulator of the cell survival signaling pathway initiated by PI3K [309, 310]. Loss of PTEN function leads to an accumulation of PIP3 and an activation of kinases of the Akt family. To our knowledge, no studies have described the relationship between PPARγ signaling and the PTEN/Akt pathway in chondrocyte biology and OA. Therefore, it is important to determine whether the PTEN/Akt pathway is the downstream signaling pathway with which PPARγ regulates the expression of catabolic and inflammatory markers. Loss of PPARγ and subsequent downstream alterations in the PTEN/Akt pathway may in part contribute towards increased expression of OA catabolic and inflammatory markers, thus enabling the articular cartilage of PPARγ-deficient mice to be more susceptible to degradation during aging. With safety issues such as myocardial infarction and cardiovascular mortality arisen with PPARγ synthetic agonists, targeting the PTEN/Akt pathway downstream of PPARγ could provide alternative therapeutics against OA and related diseases.

CONCLUSION

In summary, the studies I have undertaken have allowed me to draw two principal conclusions about the specific *in vivo* role of PPARγ in cartilage growth and development. Firstly, cartilage-specific PPARγ-deficiency in embryonic and early postnatal growth stages results in abnormal cartilage growth and development. Secondly, absence of PPARγ in cartilage during aging results in accelerated and spontaneous OA. Overall, for the first time, our results demonstrate an important *in vivo* role of PPARγ in endochondral ossification, cartilage growth and development, and age-dependent OA through the use of cartilage-specific PPARγ KO mice. From this study, I have gained important insight into the role of PPARγ in cartilage growth and development and learned that it is a critical regulator of cartilage health and physiology in early cartilage growth and development, and aging.

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