MODULATING HEDGEHOG SIGNALING CAN ATTENUATE
THE SEVERITY OF OSTEOARTHRITIS

by

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for the degree of Doctor of Philosophy
Graduate Department of the Institute of Medical Science
University of Toronto

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ABSTRACT

Osteoarthritis is associated with the irreversible degeneration of articular cartilage. Notably, in this condition, articular cartilage chondrocytes undergo phenotypic and gene expression changes that are reminiscent of their end-stage differentiation in the growth plate during skeletal development. Hedgehog (Hh) signaling regulates normal chondrocyte growth and differentiation; however, the role of Hh signaling in chondrocytes in osteoarthritis is unknown. Here I examined human osteoarthritic samples and mice in which osteoarthritis was surgically induced and find that Hh signaling is activated in osteoarthritis. Using several genetically modified mice, I found that higher levels of Hh signaling in chondrocytes cause a more severe osteoarthritic phenotype. Furthermore, I show in mice and in human cartilage explants that pharmacological or genetic inhibition of Hh signaling reduces the severity of osteoarthritis and that runt-related transcription factor-2 (Runx2) potentially mediates this process by regulating a disintegrin and metalloproteinase with thrombospondin type 1 motif-5 (Adamts5) expression. Together, these findings raise the possibility that Hh blockade can be used as a therapeutic approach to inhibit articular cartilage degeneration.

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Table of Abbreviations

ADAMTS5	a disintegrin-like and metallopeptidase with thrombospondin type 1 motif, 5
BMP	bone morphogenic protein
BV/TV	bone volume (%)
CASP3	caspase 3
CATNB	beta-catenin
COL1A1	collagen, type I, α 1
COL2A1	collagen, type II, α 1
COL10A1	collagen, type X, α 1
COMP	cartilage oligomeric matrix protein
ECM	extra-cellular matrix
FGF	fibroblast growth factor
FZ	Frizzled
FRZB	frizzled-related protein
GDF	growth differentiation factor
Hh	Hedgehog
Ihh	Indian hedgehog

IL	interleukin
L, l	litre
MMP	matrix metalloproteinase
mm	millimetre
μm	micrometer
NO	nitric oxide
OA	osteoarthritis
PGE2	prostaglandin E2
PTCH1	Patched1
PTHrP	parathyroid hormone related protein
GLI1	glioma-associated oncogene homolog-1
GLI2	glioma-associated oncogene homolog-2
GLI3	glioma-associated oncogene homolog-3
RT-PCR	reverse transcription polymerase chain reaction
RUNX2	runt-related transcription factor 2
SFRP-1	secreted frizzled-related peptide 1
SMO	Smoothened
WT	wild-type
LRP5/6	low density lipoprotein receptor-related protein 5/6

SNP	single nucleotide polymorphism
TCF/LEF	T-cell factor/lymphoid enhancer factor
Tb.N.	trabecular number (/mm)
Tb.Sp	trabecular spacing (μm)
Tb.Th	trabecular thickness (μm)
TGF	transforming growth factor
TNF	tumour necrosis factor
VEGF	vascular endothelial growth factor
WNT	wingless

CHAPTER ONE

INTRODUCTION

BACKGROUND

Osteoarthritis (OA) is the most common degenerative disease of the joint, with over 200 million people afflicted worldwide¹. In synovial joints, articular cartilage lines the ends of bones, providing a cushion for gliding and movement. In OA, the articular cartilage degrades and has a limited capacity to regenerate. Over time, the underlying subchondral bone thickens, and the formation of osteophytes or “bone spurs” ensues (**Figure 1**). The result is decreased joint function, pain with physical activity, joint stiffness with inactivity, tenderness, and swelling. Currently, with OA, there is no cure². Inevitably, over time, when symptoms can no longer be managed, the joint needs to be replaced³.

Over the past 20 years, the disease burden of OA has been significant. In Canada during the early 1990s, an estimated 5 billion dollars were spent annually for arthritis-related treatments. In 2008, conservative estimates of arthritis management, the majority of which is OA, increased to 23 billion dollars⁴. Over 4 million or 1 in 10 Canadians suffer from OA, and those affected are over the age of 50 years. As the “baby-boomer” generation ages, these costs will continue to increase, and new treatments will need to move beyond symptom management.

Articular cartilage

In a healthy joint, hyaline articular cartilage consists of specialized cells called chondrocytes that are sparsely populated in a well-hydrated, extracellular matrix (ECM), 60% to 80% in water by total weight⁵. The remaining 20% to 40% by weight consists mainly of two structural protein components: type II collagen, a long triple-helical peptide that provides a fibrillar meshwork, and aggrecan, a larger proteoglycan made up of chondroitin and keratan sulfates. Aggrecan further organizes into larger aggregates, bound together by hyaluronic acid and link protein. Other large proteoglycans (e.g. cartilage oligomeric matrix protein or COMP, versican, perlecan), glycoproteins, and minor component of collagens IX, XI, III, V, VI, X, XII, and XIV all help to confer the unique biomechanical properties of articular cartilage: a cushion for compression forces and a gliding surface for shearing forces in the joint⁵.

Histological and microscopic examination reveals the organization of articular cartilage into four stratified zones. The tangential (superficial) zone is made up of flattened chondrocytes and collagen fibrils that lie tangential to the articulating surface, in opposition to the shearing forces during movement. The transitional (intermediate) zone contains chondrocytes that are smaller and rounder, and consists mostly of proteoglycans and collagen fibrils that run obliquely. This

arrangement of the extra-cellular matrix (ECM) helps in the transition of shearing forces at the surface layer to the compression forces deep in the cartilage. In the radial (deep) zone, collagen fibres are organized perpendicular to the subchondral bone that, along with the highest proteoglycan content of the four layers, help distribute compression forces. The chondrocytes are larger and round, and are metabolically the most synthetically active. The calcified cartilage zone is characterized by the tide mark that delineates uncalcified and calcified cartilage, and by its interdigitated-anchoring into the subchondral bone. Here the chondrocytes are enlarged and predominantly express type X collagen (COL10A1)⁶.

Etiology and pathophysiology of OA

The most common cause of OA is from a secondary event (e.g. joint trauma, childhood joint deformity, underlying metabolic disorder). OA can also develop from a primary genetic mutation (e.g. type II collagen) or from an idiopathic etiology. In any case, OA arises when an irreversible imbalance occurs between the synthesis and degradation of the ECM, resulting in a number of molecular changes in chondrocyte phenotype and gene expression⁶⁻⁸(**Figure 2**).

As observed in both murine and human osteoarthritic cartilage samples, chondrocytes (even those cells that reside in uncalcified cartilage) undergo clonal proliferation, hypertrophy with the upregulation of type X collagen⁹, and apoptosis marked by caspase 3 (CASP3) overexpression¹⁰. It is believed this increase in CASP3 is induced, in part, by the excess production of nitric oxide (NO) in osteoarthritic cartilage samples¹¹⁻¹³. There is also upregulated gene and protein expression of matrix degradation enzymes MMP13¹⁴, a matrix metalloproteinase that cleaves type II collagen, and a disintegrin and metalloproteinase with thrombospondin type 1 motif-5 (ADAMTS5)¹⁵, the most common of the aggrecanases that cleave aggrecan. The result is the production of ECM degradation products, the so-called neoepitopes, of type II collagen^{16,17} and aggrecan^{18,19}.

Based on expression data from human samples of osteoarthritic cartilage and synovium, and from pharmacological blockade experiments in animal models of OA, cytokines interleukin (IL)-1 β ²⁰ and tumour necrosis factor (TNF)- α ²¹ are overexpressed in OA chondrocytes and synoviocytes, resulting in the induction of MMPs, other cytokines such as IL-8, IL-6, as well as prostaglandin E2 (PGE2)²². Furthermore, the enzymatic degradation products of the type II collagen and aggrecan are released into the synovial fluid, upregulating inflammatory cytokines

in chondrocytes and synoviocytes, which, in turn, further induce matrix degradation and chondrocyte hypertrophy^{17,23}.

There is also greater mineralization of the cartilage ECM in OA, remodeling of the underlying subchondral bone, with the upregulated levels of type I collagen and bone morphogenic proteins (Bmps)^{24,25}. At the level of calcified cartilage, bone replaces cartilage through a process of endochondral ossification, with upregulated expression of vascular endothelial growth factor (VEGF) receptors in osteoarthritic cartilage and osteophytes which are thought to promote vascular invasion²⁶.

Developmental signaling pathways in the pathophysiology of OA

Although OA is thought of as a disease primarily of the old, a number of signaling pathways crucial to normal embryonic development are dysregulated in OA. These pathways include Wingless (WNT) signaling, transforming growth factor (TGF) signaling, and fibroblastic growth factor (FGF) signaling²⁷⁻³⁰.

In canonical Wnt signaling, Wnt ligand binds to its Frizzled (FZD) and Low-Density Lipoprotein (LDL) receptor related proteins (LRP)5/6 coreceptors, and beta-catenin translocates to the nucleus where it binds to the T-cell factor (TCF)/lymphoid enhancer-binding factor (Lef) family of transcription factors,

activating transcription (**Figure 3**). In the absence of the Wnt ligand, beta-catenin is phosphorylated in the cytoplasm, targeting it for ubiquitin-mediated degradation. When the ligand binds to its FZD and LRP5/6 coreceptors, the multimeric protein complex is inhibited. Wnt ligands that activate beta-catenin mediated signaling such as Wnt7b are overexpressed during OA³¹. Wnt ligands can also activate the non-canonical pathway, however, their involvement in OA based on gene expression data from mice is inconclusive³².

In human and murine OA, increased protein levels of beta-catenin have been found in degenerated cartilage and osteophyte samples³³. As shown in mice, the bone-resorption pattern of rheumatoid arthritis was reversed to the bone-forming pattern of osteoarthritis by inhibiting Dickkopf-1 (Dkk-1), a negative regulatory molecule of the canonical Wnt pathway³⁴. There is also the suggestion of Wnt signaling and a genetic susceptibility to OA, with single nucleotide polymorphisms (SNPs) in Wnt receptors LRP5 and FRZB associated with hip OA in population studies^{35,36}. Furthermore, expression studies involving Wnt-induced signaling protein (WISP)-1³² show an upregulation in human OA synovial samples, and its overexpression in mice delivered by adenovirus injection into the knee joint results in the development of OA with cartilage damage by MMP-13 and IL-1³⁷.

During OA, increasing levels of beta-catenin have also been suggested to, in turn, promote osteophyte formation by upregulating levels of Bmp-2 in mice and human samples³⁸⁻⁴⁰. Bmp-2 is a member of the Transforming Growth Factor super family of signaling proteins, which also contains growth differentiation factors (Gdfs), TGF-betas. In addition to its role in osteophyte bone formation, increased Bmp-2 levels are found in both human and murine OA cartilage where they help to promote chondrocyte differentiation and hypertrophy in comparison to areas of least OA involvement⁴¹. In contrast, TGF-beta levels are observed initially to increase during the onset of OA, and then decrease during later stages, based on expression studies in human osteoarthritic cartilage samples²⁴. TGF-beta is thought to act as an anabolic factor by suppressing type II collagen degradation²⁴. Genome wide scans have reveal the susceptibility of TGF signaling in mainly bone related diseases, which are thought, in part, to influence and promote articular cartilage degeneration and OA. However, SNP in GDF5 has recently been reported to be associated with OA susceptibility⁴².

In articular cartilage, FGF-2 is thought to serve as a chondroprotective agent⁴³ by suppressing IL-1– or TNF-alpha– stimulated aggrecanase activity. The joints in mice deficient of FGF-2 are indistinguishable from wild-type littermates until 12

months of ages or with surgical induction of OA, at which point cartilage degradation and increased expression of Adamts5 take place⁴³. FGF is normally bound to the heparan sulfate chains of the proteoglycan perlecan in the pericellular matrix, where it acts as a mechanotransducer by activating intracellular others pathways including ERK, one of the MAPKs.

Expression data from both human and murine OA cartilage samples also reveal an increased level of parathyroid hormone receptor related protein (PTHrP), decreased levels of Sox-9, and decreased levels of insulin growth factor (IGF)-1, all of which are expressed and influence the normal maintenance and development of chondrocytes in the growth plate⁶.

Joint development and articular chondrocytes

Notably, the same developmental signaling pathways that are dysregulated in OA are crucial to normal growth and maturation of cartilage⁶. Thus, understanding how chondrocytes behave in diseases such as osteoarthritis may lie in understanding how chondrocytes originate as early as joint development⁴⁴. During joint formation, mesenchymal progenitor cells of the limb bud rapidly divide, expressing type II collagen, and areas of greater proliferation (often referred to as

condensation) form the interzone, the presumptive joint⁴⁵⁻⁴⁹. Cells in the interzone then express Wnt9a (formerly Wnt14)⁵⁰, which then induces the expression of the joint specific marker Gdf5 (a member of the TGF family of proteins)⁵¹. The cells of the interzone subsequently lose their expression of type II collagen and Sox9 whilst increasing type I and III collagen expression. The interzone then adopts a three-layered structure: a dense intermediate layer whose cells will eventually form the ligaments, menisci, and joint capsule; and two outer layers whose cells express type II collagen and will become the hyaline articular cartilage of adjacent long bones⁵².

The importance of Gdf5 in joint development was first identified from the examination of point mutations in mice with brachypodism, a condition characterized by shortened limbs and abnormal joint development while sparing the axial skeleton⁵³. Along with Gdf5, Gdf6 and Gdf7 are also expressed in the interzone, and null-mutants of Gdf6 also exhibit specific joint fusions in the hands and wrist⁵⁴. Mice deficient in Gdf7, however, exhibit seminal vesicle formation defects with no skeletal abnormalities⁵⁴. To test the redundancy of Gdfs in the interzone, a double null mutant of Gdf5:Gdf6 showed that the severity of skeletal defects increase compared to Gdf5 null mutants, with the failure of joint formation and abnormalities observed in both the appendicular and axial skeleton⁵⁴. Notably,

although movement in embryonic joints is crucial for its normal development, Gdf5 expression is not affected by immobilization; in contrast, Fgf2, which is normally expressed alongside the joint cavity, is diminished without joint movement⁴³.

Other members of the TGF super-family of proteins expressed at sites of future joints include Bmp2, and Bmp4⁵⁵. Whereas Gdf5 binds to its Bmpr1b receptor, Bmp2 and 4 bind to the Bmpr1a. To determine the role of Bmp2 and 4 in the developing joint, conditional mice with a floxed Bmpr1a allele were crossed with mice expressing cre-recombinase under Gdf5 promoter regulatory elements⁵⁶. The conditional cre-loxP Bmpr1a mutants have joints that are indistinguishable from their wild-type littermates; however, a number of skeletal defects develop post-natally, including premature osteoarthritis in these mice deficient in Bmpr1a early in joint formation.

Along with Wnt 9a⁵⁰, Wnt4 and Wnt16 are expressed in the interzone²⁸, all of which activate beta-catenin mediated signaling in the canonical pathway. To examine the role of beta-catenin in joint development, a variety of mice deficient in beta-catenin have been generated and their joint phenotype examined. In conditional mice deficient in beta-catenin in cells expressing type II collagen or Gdf5, joint formation is impaired, with joint fusion occurring throughout the

skeleton, but not completely ablated^{57,58}. Examination of joints of mice deficient in beta-catenin in cells where Gdf5 is expressed also revealed defects in the superficial layer of cells within the articular cartilage. Furthermore, ectopic expression of Wnt9a or a constitutively activated form of beta-catenin in chondrocytes using similar conditional Gdf5 and Col2a1 cre-loxP mice or by cre virus induction resulted in ectopic joint formation and impaired endochondral ossification^{57,59}. Taken together, the data suggest that a persistent level of beta-catenin signaling is beneficial to joint formation and that it can influence chondrocyte lineage differentiation and maintenance.

In addition to its induction of Gdf5 expression and its role upstream of beta-catenin, Wnt9a is thought to also activate the expression of Indian hedgehog (Ihh), accounting for the expression of Gli1 and Gli3 (transcription factors of the hedgehog signaling pathway) at the interzone during joint development^{28,49}. In mice deficient in both Wnt9a and beta-catenin, Ihh gene expression was downregulated at the forming joint, and *in vivo* chromatin immunoprecipitation demonstrated the interaction of beta-catenin/Lef1 complex with the *Ihh* promoter⁶⁰. This interaction corroborates with other studies on the role of Ihh in joint development. In Ihh null mutant mice or conditional mice whereby Ihh is deficient in cells expressing Gdf5,

joints induction occurs but are misformed and/or fused⁴⁹. Given that both Wnt signaling and Hedgehog (Hh) signaling can also negatively-regulate each other, this interaction of Wnt9a induction of *Ihh* expression through beta-catenin mediated transcription is recognized to be both temporally and spatially confined.

Growth plate chondrocyte development

Adjacent to the forming interzone, cells of the cartilaginous anlage continue to proliferate, expressing type II collagen, and differentiate into larger cells that undergo hypertrophy. These hypertrophic chondrocytes express type X collagen and become the master coordinator of events in long bone development: vascularization, mineralization, and bone collar formation. Cavitation then ensues and the chondroprogenitors flanking this region proliferate and organize themselves to what will become the epiphyseal growth plate^{27,61}.

Closer examination of the growth plate also reveals the arrangement of chondrocytes into zones or stages of lineage differentiation: smaller sized cells near the secondary ossification centre, the proliferating or pre-columnar chondrocytes, appear flattened and tightly stacked. Moving towards the diaphysis, cells differentiate into columnar chondrocytes which are round, slightly larger, and

become the predominant cell type of the growth plate up until its closure; cells continue to increase in size, becoming prehypertrophic chondrocytes; finally, cells fully enlarge to become hypertrophic chondrocytes, at the junction between growth plate and new bone formation.

An important pathway in regulating the differentiation of chondrocytes of the growth plate is the Hh signaling pathway²⁷ (**Figure 3**). In mammals, there are three Hh ligands (Shh, Dhh, and Ihh)⁶² and Ihh is the major Hh ligand produced in chondrocytes. Ihh binds to the Patched-one (PTCH1) receptor, which releases its inhibition on Smoothened (SMO). SMO can then signal to process the GLI family of transcription factors, upregulating downstream target genes, which include the Hh signaling pathway members *GLI1* and *PTCH1*, and *HHIP*⁶³. In chondrocytes and limb development, Gli1 and Gli2 are thought to be activators of the pathway while the cleavage product of Gli3 represses Hh signaling^{64,65}.

As first described in the chick and later in mice, Ihh is expressed in prehypertrophic and hypertrophic chondrocytes, and then signals to its receptor Ptch in the perichondrial region near the articular cartilage, whereby it induces PTHrP expression. PTHrP then binds to its receptor Pthr1 in periarticular and articular chondrocytes, and in a negative feedback loop, regulates the rate of

chondrocyte differentiation, whereby chondrocytes are maintained in their proliferative state and are downregulated for *Ihh* expression^{27,66-68}. Using conditional mice deficient in PTHrP and *Smo* (or *Ptch*) in chondrocytes, Hh signaling was shown to regulate chondrocyte hypertrophy independent of PTHrP⁶⁹. However, during embryonic development, the effects of *Ihh*-PTHrP appear to dominate and thus mask this effect⁶⁹. Hh signaling has also been shown to promote chondrocyte proliferation, since mice deficient of *Ihh* show a significant reduction in the rate of chondrocyte proliferation in the growth plate⁷⁰. Moreover, *Ihh* can also act independently of PTHrP to stimulate the differentiation of periarticular to columnar chondrocytes^{68,71}. Growth plates in mice deficient in the PTH/PTHrP receptor exhibited an upregulation of PTHrP, accelerating periarticular chondrocyte differentiation, and elongation of the columnar region. These roles of Hh signaling in growth plate chondrocytes have been further corroborated at the transcriptional level in mice deficient in *Gli2*⁷² and/or *Gli3*⁷³, where *Gli3* repressor is thought to negative regulate the differentiation periarticular to columnar chondrocytes and to regulate the transition of prehypertrophic to hypertrophic chondrocytes in a PTHrP-dependent and independent manner. Thus, in the mouse, Hh signaling influences the chondrocyte phenotype in the growth plate during

embryonic development by promoting chondrocyte proliferation and controlling the rate of chondrocyte hypertrophy.

Wnt signaling also plays a crucial role in controlling the chondrocyte phenotype in the growth plate⁵⁹. First expressed in the developing limb bud, Wnt5a is later expressed in a region between proliferating and pre-hypertrophic chondrocytes, overlapping with Ihh expression in prehypertrophic chondrocytes⁴⁸. Mice deficient in Wnt5a are delayed in Ihh and Pthrp expression in prehypertrophic chondrocytes, and in Col10a1 expression in hypertrophic chondrocytes^{48,74}. Overexpression of Wnt5a in non-hypertrophic chondrocytes generates a similar phenotype that cannot be rescued with Ihh overexpression. Together, these data suggest that Wnt5a inhibits the transition of periarticular chondrocytes to proliferating chondrocytes but promotes the differentiation of proliferating chondrocytes to prehypertrophic chondrocytes. In contrast, Wnt5b is expressed in a region between prehypertrophic chondrocytes and hypertrophic chondrocytes. In the same study, data from mice deficient in Wnt5a and with overexpression of Wnt5b in the non-hypertrophic chondrocytes, suggested that Wnt5b promotes differentiation of periarticular chondrocytes to proliferating chondrocytes, but negatively regulates the transition from prehypertrophic to hypertrophic

chondrocytes. These effects of Wnt5a and Wnt5b are thought to be independent of *Ihh*.

Wnt 5a is thought to negatively regulate beta-catenin, and as such, Wnt5a deficient mice exhibit similar growth plates to mice with stabilized beta-catenin in non-hypertrophic chondrocytes. Beta-catenin has also been studied in context with the characteristic transcription factors expressed during all stages of chondrocyte development, namely Sox9. Sox9 is expressed as early as mesenchymal precursors to chondrocytes and is required throughout chondrocyte differentiation but is absent in hypertrophic chondrocytes. Sox9 has also been shown to bind to the promoter region of *Col2a1* gene, and mice deficient in Sox9 do not undergo chondrocyte differentiation and formation^{75,76}. In one study, beta-catenin is suggested to regulate chondrocyte differentiation by interacting directly with Sox9⁷⁷, and in another study, *in vitro* data suggest that Sox9 may regulate beta-catenin by promoting phosphorylation, thereby targeting beta-catenin for degradation⁷⁸.

Given their overlapping roles, the interactions between Wnt, Hh and PTHrP signaling pathways have also been studied in the growth plate chondrocyte development²⁸. In mice deficient in Wnt9 and in both Wnt9a and *Catnb*, *Ihh*

expression was downregulated; and with the finding of putative Tcf/Lef1 binding sites on the *Ihh* promoter, *Ihh* expression is believed to be regulated, in part, by beta-catenin-mediated transcription⁶⁰. In another study using mice deficient in beta-catenin and/or *Ptch* from chondrocytes, beta-catenin is believed to be upstream of *Ihh* signaling in regulating chondrocyte survival, inhibiting apoptosis⁷⁹. Using mice deficient in *Pthrp* and beta-catenin, it has suggested that beta-catenin regulates chondrocyte hypertrophy independently of PTHrP⁸⁰, similar to previous findings on the *Ihh*-*Pthrp* axis.

Similar to Wnt signaling, FGF signaling is thought to play multiple roles in the growth plate. Mutations in the FGF receptor (FGFR)3 gene results in its activation, causing achondroplasia, which is the most common form of dwarfism in humans. In mice, *Fgfr3* is normally expressed in perichondrium, the domain that encompasses proliferating and prehypertrophic chondrocytes. Mice deficient in *Fgfr3* experience prolonged endochondral bone growth, which is thought to arise from an increased rate of chondrocyte proliferation and differentiation⁸¹, which is also consistent with data generated in mice deficient in *Fgf18* (*Fgf18* signals through *Fgfr3* to promote chondrogenesis)^{38,82,83}. Furthermore, conditional mice activated for *Fgfr3* signaling (achieved by overexpression of *Fgf9* in cells expressing under type II collagen

regulatory elements) develop achondroplasia, exhibiting a significant reduction in proliferating and hypertrophic zones in the growth plate and decreased *Ihh* and *Bmp4* expression⁸⁴.

TGF-beta signaling is also thought to regulate growth plate chondrocytes (for a review see reference 55), since *Smad2* and *Smad3* are expressed in proliferating chondrocytes and hypertrophic chondrocytes respectively and have been shown *in vitro* to promote chondrocyte maturation with upregulated *Col10a1* expression. BMPs are thought to promote proliferation of chondrocytes in the growth plate as *Noggin*-null mice exhibit overgrown skeletal elements (*Noggin* is an antagonist to *Bmp*). Both *Bmp2* and *Bmp6* are expressed in hypertrophic chondrocytes, however loss of function double mutants of *Bmp2*^{+/-};*Bmp6*^{-/-} reveal a reduction of bone formation, indicating a paracrine function for BMPs on the osteoblast rather than an autocrine role in the hypertrophic chondrocyte⁸⁵. Mice deficient in *Bmpr1a* and *Bmpr1b* receptors exhibit delayed differentiation, with the presence of a hypertrophic zone, albeit significantly reduced^{41,86,87}. Thus, similar to Wnts and Fgfs, Tgf signaling appears to have differential roles in influencing the chondrocyte phenotype in the growth plate.

Developmental signaling pathways in post-natal chondrocytes

The use of mice with cell-specific and inducible expression of cre-recombinase has permitted loss- and gain-of-function studies otherwise associated with embryonic lethality. For example, mice conditionally deficient in beta-catenin die before birth⁸⁸. However, transgenic mice expressing the inhibitor of beta-catenin and T cell factor (ICAT) driven by Col2a1 regulatory elements permit the post-natal investigation of beta-catenin downregulation in chondrocytes^{89,90}. In these mice, the articular and growth plate chondrocytes show a delay of chondrocyte differentiation with smaller proliferating and hypertrophic zones in the growth plate, decreased chondrocyte proliferation, increased apoptosis, and decreased levels of MMP-13 (which is typically upregulated in OA).

Mice stabilized for beta-catenin in chondrocytes are also embryonically lethal. To circumvent this issue, mice with the same tamoxifen-induced expression of cre-recombinase in chondrocytes were crossed with conditional beta-catenin floxed mice, resulting in stabilization of beta-catenin⁹¹. With tamoxifen administration at 3 and 6 months and examination of joints at 2 months thereafter, these mice exhibited accelerated chondrocyte maturation and hypertrophy, increased apoptosis, and increased MMP-13 destruction of the articular cartilage. Thus, taken together, post-

natal dysregulation of beta-catenin in joint chondrocytes alters chondrocyte proliferation and hypertrophy, thereby altering cartilage integrity⁹².

The post-natal role of Hh signaling in joint chondrocytes has also been investigated. Using mice with tamoxifen-inducible expression of cre-recombinase in chondrocytes, conditional ablation of *Ihh* at time points of 4 days to 8 weeks after birth showed growth plate cartilage defects of increased hypertrophic zones, increased PTHrP expression, and decreased mineralization and trabecular bone density in long bones^{69,93,94}. These data correlate with another study examining limbs at earlier time points, whereby mice deficient in *Smo* in chondrocytes exhibit dysregulation of PTHrP expression by *Ihh* signaling and trabecular bone defects⁹⁵. Moreover, *Gli2* deficient mice exhibit bone defects of impaired endochondral bone development and osteopenia, and pharmacological blockade of Hh signaling 7 days after birth results in the premature closure of the growth plate in mice⁷². Also, activation of Hh signaling in chondrocytes, as seen in mice with *Gli2* overexpression under *Col2a1* regulatory elements, results in the development of enchondromas in the growth plate^{96,97}. Taken together, this data reinforces the importance of Hh signaling in regulating the post-natal regulation and maintenance of chondrocytes of hyaline cartilage.

Disruption of TGF-beta signaling through its family-member receptor Bmpr1a in mice during joint formation results in joint changes later in aged mice⁵⁶. Cre-loxP mice deficient in Bmpr1a when Gdf5 is expressed exhibit distal joint defects in ankles and phalanges, which include synovial membrane hyperplasia, articular cartilage surface deterioration, and accelerated chondrocyte maturation as early as 1 week of age⁵⁶. At 7 weeks of age, continued deficiency of Bmpr1a results in complete loss of proteoglycan staining in the growth plate in Safranin-O sections, and at 9 months, the articular layer is completely absent.

Pharmacological modulators of developmental signaling pathways

High throughput screening and drug design has identified a number of pharmacological agents that modulate ligand-dependent signaling pathways, such as Wnt, Hh, TGF, and FGF². However, for cartilage and bone, some agents that remain in use were discovered with less conventional methods and act downstream of ligand receptors.

For example, lithium has been used safely and effectively for over half a century in the treatment of bipolar illness. Lithium stimulates the upregulation of beta-catenin levels in the cytoplasm by inhibiting GSK-beta. In the absence of Wnt

ligand, beta-catenin is phosphorylated in the cytoplasm by a protein of the multimeric protein complex that consists minimally of GSK3-beta, Axin and APC. This phosphorylation targets beta-catenin for ubiquitin-mediated degradation. When the ligand binds to its Frizzled and LRP5/6 coreceptors, the multimeric protein complex is inhibited. As a result, beta-catenin accumulates in the cytoplasm and then translocates to the nucleus where it binds to the family of Tcf/Lef transcription factors activating transcription.

Lithium treatment enhanced bone formation and improved bone mass in mice via activation of the canonical Wnt pathway²⁸. Moreover, in mice with surgically-induced fractures, upregulation of beta-catenin by lithium treatment enhanced fracture repair when administered after the fracture occurs and the cartilage template is formed, while treatment with lithium before and during the early stages of fracture repair and cartilage formation proved deleterious⁹⁸.

Initially noted for its teratogenic effects⁹⁹, cyclopamine is a steroidal alkaloid that inhibits the Hh signaling pathway by binding downstream of the Patched receptor onto Smoothened¹⁰⁰. Its use as an antitumour agent was first described in the treatment of medulloblastomas as seen in mice harboring a heterozygous mutation in Ptch¹⁰¹. Since then, a number of neoplasias involving activation of Hh

signaling have been investigated for their treatment by pharmacological modulators of Hh signaling, including a number of bone and cartilage tumours and pathologies^{64,102-105}. For example, Hh signaling blockade is effective in mice in the treatment of synovial chondromatosis¹⁰⁶ and human chondrosarcoma xenografts¹⁰⁷. Similar to the dose and efficacy of cyclopamine, the small molecule N-[(3S,5S)-1-(2H-benzo[3,4-d]1,3-dioxolan-5-ylmethyl)-5-(piperazinylcarbonyl)pyrrolidin-3-yl]-N-(3-methoxyphenyl)methyl]-3,3-dimethylbutanamide (C₃₁H₄₂N₄O₅) has also been characterized as an effective pharmacological agent in Hh signaling blockade¹⁰⁸. Currently, some of these Hh blockade agents are in Phase II clinical trials.

One of the downstream effects of Pthrp signaling is the activation of PKA. Thus, a commonly utilized pharmacological agent to modulate and study Pthrp signaling is H89 which inhibits PKA. Examination of the growth plates of mice deficient in Gli2 and Gli3 treated with PTHrP and H89 suggests that PTHrP regulates chondrocyte proliferation through Gli3 in a ligand-independent, PKA-dependent manner¹⁰⁹.

Animal models of OA

Both primary and secondary OA have been reproduced in animal models, the former through the advent of transgenic and gene-targeted modifications in mice and the latter largely with microsurgical revisions of the synovial joint¹¹⁰.

For OA arising from a primary genetic etiology, a number of mice have been characterized and generated. The *Col2a1^{sedc}* mouse harbours a homozygous missense mutation in *Col2a1* resulting in a number of defects including osteoarthritis^{111,112}. A transgenic mouse with an arginine-to-cysteine substitution at position 519 of the human COL2A1 gene has also been generated resulting in generalized OA with mild chondrodysplasia¹¹³. The Del1 transgenic mouse contains 6 copies of a deletion mutation in the *Col2a1* gene, resulting in generalized OA lesions¹¹⁴. The STR/ort mouse strain, discovered from the inbreeding of the STR/1N strain, develops naturally occurring OA at a high rate in the medial tibial plateau of the knee¹¹⁵. Also, mice deficient in *Mig-6* develop the early onset of osteoarthritis at 1.5 months of age^{31,116}. A more exhaustive list of genetic animal models of OA is provided in **Table 1**¹¹⁷.

For post-traumatic induced OA, animal models studies have involved synovial joints of dogs, sheep, rabbits, horses, mice, cows, pigs, rats, and mice¹¹⁸⁻¹²¹.

Traditionally, the most studied form of surgically induced OA is by arthrotomy and ACL transection, especially for larger animals. However, with sequencing of the human and murine genome and the generation of genetically modified mice, the last 5 years has seen the emergence of the more difficult surgical induction of OA in the rat¹²² and recently in the mouse knee joint by arthrotomy and medial meniscus removal and/or ligament resection¹²³. However, given the miniature scale of murine joints, cartilage and bone regeneration studies *in vivo* continue in the larger sheep and bovine joints.

Current therapies in the management of OA

The majority of medical therapies for OA focus on the relief of symptoms with the use of analgesics and anti-inflammatory drugs. Bisphosphonates and nutraceuticals, hyaluronans, and inhibitors of MMPs, IL-1, iNOS, PPAR, and ICE offer little or no improvement of symptoms². For larger weight bearing joints, a number of different surgical techniques have been explored to restore articular cartilage defects³. These procedures include microfracture of the subchondral bone, osteochondral and cell transplantation, and autologous and/or tissue-engineered grafts. Unfortunately, all have been unable to restore the cartilage surface and

prevent further cartilage degradation and subchondral bone changes. Moreover, these therapies involve surgical interventions that, in some cases, require total joint arthrotomy and even the harvesting of healthy cartilage in the joint, resulting in further damage¹²⁴. Thus far, there are no universally successful treatments to restore normal articular cartilage and chondrocyte function.

Therefore, a novel biological approach to address articular cartilage deterioration in OA would be to modulate signaling pathways to improve the joint phenotype. One such pathway that is important in the joint, both embryonically and post-natally, is the Hh signaling pathway. Whether Hh signaling is dysregulated in OA and whether modulating Hh signaling in OA can alter the joint phenotype is unknown.

HYPOTHESIS

Hh signaling is dysregulated in OA, and modulating Hh signaling can alter the joint phenotype and gene expression in the joint.

RATIONALE

During OA, articular chondrocytes undergo phenotypic changes that are reminiscent of terminal growth plate chondrocyte development (**Figure 4**)⁶. Since Hh signaling plays an important role in regulating the chondrocyte phenotype of the growth plate and in the post-natal maintenance of articular cartilage and trabecular bone, Hh signaling dysregulation may, in part, result in the changes in phenotype and gene expression that occur to articular chondrocytes during OA. Moreover, it is reasonable to consider this pathway and its influence on the joint phenotype, since aberrant Hh signaling is associated with a number of bone and joint pathologies (e.g. chondrosarcomas, osteosarcomas, and synovial chondromatosis). Finally, using human OA cartilage samples and given the availability of genetically modified mice and pharmacological agents characterized for Hh signaling blockade, I will be able to test whether Hh signaling modulation can alter the OA phenotype and gene expression changes in mice and human cartilage samples.

OBJECTIVES

To test my hypothesis, I will answer the following questions:

1. Is Hh signaling dysregulated in human and murine OA?
2. Does activated Hh signaling predispose to OA?
3. Can Hh signaling modulation attenuate the OA phenotype?
4. How does Hh signaling influence the genes expressed in OA?

Figure 1. Schematic representation of a normal synovial joint (left) and one with osteoarthritis (right). Osteoarthritis is characterized by the irreversible degeneration of articular cartilage, subchondral bone changes with the formation of osteophytes (bone spurs), and thickening of the synovium (adapted from Aigner et al, 2007).

Figure 2. Schematic representation of the pathological events associated with OA. During OA, chondrocytes undergo changes to their phenotype, which include hypertrophy, and gene expression changes, which include the upregulation of MMP-13, type X collagen, and ADAMTS-5 (adapted from Krasnokutsky et al., 2008).

Figure 3. Schematic representations of the canonical Wnt signaling (left, A) and Hh signaling (right, B) pathways. In the absence of the Wnt ligand, beta-catenin is targeted for ubiquitin-mediated protein degradation. In the presence of the ligand, beta-catenin does not become phosphorylated, and thus accumulates in the cytoplasm and translocates to the nucleus where it binds to the Tcf/Lef1 family of transcription factors, activating transcription. In Hh signaling, Ptch1 receptor represses Smo receptor, resulting in pathway inactivation. With binding of the Hh ligand, Ptch depresses its inhibition on Smo, activating the Gli family of transcription factors and transcription of downstream targets of Hh signaling, GLI1, PTCH1, and HHIP which are also component of the pathway (adapted from Day et al., 2008).

Osteoarthritis	Developmental model: endochondral ossification	
Pathomechanisms	Development steps	
Differentiation	Chondrogenesis
Anabolism	Matrix synthesis	
Proliferation	Proliferation	
Catabolism	Matrix degradation	
Hypertrophy	Hypertrophy	
Calcification	Calcification	
Cell death/apoptosis	Cell death/apoptosis	

Figure 4. Schematic representation of the growth plate (right) showing the different stages of chondrocyte growth and differentiation. Notably, the steps involved in the developmental model of endochondral ossification are recapitulated in articular chondrocytes during OA (adapted from Aigner et al., 2007).

Gene	Gene defect or modification	Features
<i>Extracellular matrix</i>		
<i>Col2a1</i>	Heterozygous knockout or mutation	OA-like changes during aging
<i>Col9a1</i>	Transgenic truncation	Mild chondrodysplasia; OA
<i>Col9a1</i>	Knockout	Early onset OA
<i>Col11a1</i>	<i>Cho/+</i> ; spontaneous deletion	OA-like changes during aging
Aggrecan	<i>Cmd/+</i> ; spontaneous deletion	OA-like changes during aging
Fibromodulin	Knockout	Tendon mineralization; OA
Fibromodulin/biglycan	Double knockout	Ectopic mineralization; early-onset OA
Matrilin-3	Knockout	OA-like changes with aging
<i>Proteinases</i>		
Mmp9	Knockout	Exacerbation of surgically induced OA
Mmp13	Postnatal transgenic	Increased susceptibility to OA
Adamts5	Knockout	Decreased susceptibility to OA
<i>Cytokines and related molecules</i>		
IL-1 β	Knockout	Exacerbates OA in STR/ORT mice; protects against surgically induced OA
IL-6	Knockout	Severe spontaneous OA with subchondral bone sclerosis in aging males but not in females
ICE	Knockout	Exacerbates OA in STR/ORT mice
MK2	Knockout	More severe in surgically induced OA
NOS2	Knockout	Reduced proteoglycan depletion and restoration of IGF-1 responsiveness
<i>Growth factor signaling</i>		
BmpR1a	Postnatal conditional knockout	OA-like cartilage degeneration
Tgfb β RII	Transgenic dominant-negative truncation	Enhanced chondrocyte hypertrophy and progressive skeletal degeneration with OA
Smad3	Targeted disruption	Enhanced chondrocyte hypertrophy and OA-like cartilage erosion
Ank	<i>ank/ank</i> ; homozygous truncation mutation	Early-onset OA associated with crystal deposition
Npp1	Knockout	OA associated with crystal deposition
α 1-integrin	Knockout	Accelerated cartilage degradation

Table 1. Genetic mouse models of and susceptibility to OA. The severity of the OA due to surgical instability induced by destabilization of the medial meniscus depends on the wild-type strain, the 129/SvEv strain developing the most severe OA, followed by C57B10.RIII, C57BL/6, FVB/N, and DBA. MMP-3, Cox-1, Cox-2, Jak3, MMP-12, cPLA2a, and ADAMTS-4 KO mice display no exacerbation of or protection against OA pathology (adapted from Bronner et al, 2007).

CHAPTER TWO

Hh signaling is activated in osteoarthritis

Modulating Hh signaling can attenuate the severity of osteoarthritis

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* Performed all procedures except for sectioning and immunostaining

SUMMARY

During osteoarthritis, articular chondrocytes undergo changes in phenotype and gene expression that resemble normal growth plate chondrocyte development. Hedgehog signaling is important in regulating growth plate chondrocytes and the post-natal maintenance of articular cartilage and trabecular bone. However, whether Hh signaling is associated with osteoarthritis is unknown. Here I ask whether Hh signaling is dysregulated in human and murine OA cartilage. We found at least a 2.5-fold increase of *PTCH*, *GLI1*, and *HHIP* in the most involved areas of human OA cartilage compared to the least involved areas. The severity of OA was determined histologically using the ICRS grading scale. Accompanying this increase in expression of Hh signaling targets was enhanced immunostaining for PTCH and HHIP, and at least a 4-fold increase in the expression of *ADAMTS5*, *COL10A1*, and *MMP13*, known genes upregulated during OA. Similarly, in murine OA cartilage samples from mice with surgically-induced OA, there was at least a 4-fold increase in *Ptch1* and *Hhip*, enhanced immunostaining for Ptch1 and Hhip, and at least a 10-fold increase in *Adamts5*, *Col10a1*, and *Mmp13* compared to sham-operated samples. We also found typical changes to subchondral bone histomorphometric parameters, with increases in BV/TV, Tb.Th and decreases in Tb.N. and Tb.Sp, and mild

synovitis. Taken together, here we show that Hh signaling upregulation is associated with human and murine OA cartilage.

INTRODUCTION

A number of developmental signaling pathways are important to the normal embryonic development and differentiation of chondrocytes of hyaline cartilage. One such example is the Hedgehog signaling pathway. In the growth plate, Ihh ligand, produced by prehypertrophic chondrocytes, signals to regulate chondrocyte proliferation and rate of chondrocyte hypertrophy^{27,66,67,69}. Ihh ligand binds to its receptor Patched, which then depresses its inhibition on Smoothened, resulting in the activation of Gli mediated transcription of downstream targets, *PTCH*, *GLI1*, and *HHIP*⁶⁴.

Interestingly, during OA, articular chondrocytes undergo phenotypic and gene expression changes that recapitulate normal growth plate chondrocyte development⁶. Moreover, Hh signaling is important in the post-natal maintenance of articular cartilage and trabecular bone^{69,93,95}. I hypothesize that, during OA, Hh signaling is dysregulated in articular cartilage.

For this investigation, human OA cartilage samples and murine knee joints from mice in which OA was surgically-induced were used. The severity of OA was assessed by histology and graded using the International Cartilage and Repair Society (ICRS)¹²⁵ scoring method. Also, the real-time PCR expression of known

genes upregulated in OA, namely *ADAMTS5*, *COL10A1*, and *MMP13*, were examined. To determine the level of Hh signaling, downstream targets PTCH and HHIP were examined by real-time RT-PCR, and to evaluate protein expression, immunostained sections for Ptch, Hhip, type X collagen, the aggrecan NITEGE neoepitope¹²⁶, and the collagen C1,2C (Col2 $\frac{3}{4}$ C_{short}) neoepitope¹⁶ were performed. The subchondral bone also undergoes remodeling during OA, and these changes were assessed by radiography and bone histomorphometric parameters¹²⁷ of BV/TV and Tb.Th (which typically increase in OA) and Tb.N and Tb.Sp (which typically decrease in OA). Synovitis, which is atypical in OA, were characterized using the *Krenn* scoring scale^{128,129}.

RESULTS

Characterization of human osteoarthritis samples

In order to determine whether Hh signaling was dysregulated in human OA cartilage, I characterized the severity of osteoarthritis across different areas in the joint. To this end, human articular cartilage samples were obtained from ten

patients undergoing total knee replacement. The mean age of the patients was 64.4 years. The cartilage was harvested across both tibial plateaus and femoral condyles, and for each area, the sample was portioned for real-time PCR expression studies, for histology and immunostaining, and for explant culture (see Chapter 4 for explant studies). To assess the level of OA involvement in these areas, the cartilage was prepared for histology (Safranin-O and H&E staining) and was graded in accordance to the International Cartilage and Repair Society (ICRS) histological grading scale. The ICRS scores were graded by blinded observers Justyna Bartoszko, Michael Khoury, Brian Seeto, and myself. To further characterize the OA cartilage, levels of expression of known genes expressed in OA cartilage, namely *ADAMTS5*, *COL10A1*, *RUNX2*, and *MMP-13*^{15,17,122}, were examined by real-time PCR. I carried out these experiments along with the assistance of Brian Seeto.

As shown in **Figure 5** and **Table 2**, the most involved areas of OA were associated with worse ICRS scoring (summary score 8.0) in comparison to in the least involved areas compared (summary score 15.2). Moreover, there was substantial loss of articular cartilage above the tide mark, loss of proteoglycan content, increase clonal proliferation, hypertrophy of chondrocytes, and subchondral bone remodeling. All six ICRS scoring parameters showed significant

differences between most and least involved areas, with the greatest difference was observed in the surface (summary scores of 2.5 – vs – 0.5) and cell distribution (summary scores of 1.3 – vs – 0.5) parameters. The most involved area turned out to be adjacent to lesions in the medial femoral condyle and medial tibial plateau, and the least involved area was within the lateral femoral condyle and lateral tibial condyle. A Mann-Whitney *U*-test was used to determine significance in ICRS scores.

There was also a substantial associated increase ($p < 0.05$) in genes known to be upregulated in OA in the most involved areas compared to the least (**Figure 6**). Namely, at least a 4-fold increase in *ADAMTS5*, at least a 5-fold increase type X collagen expression, and at least a 8-fold increase in *MMP13* expression was observed. The significance in real-time PCR expression levels was determined using a *t*-test at a 95% confidence interval.

Hh signaling is activated in human OA cartilage

To assess whether Hh signaling is dysregulated in human OA, the same areas described above were also used. To determine the level of Hh signaling in these samples, expression studies on Hh target genes *GLI1*, *PTCH* and *HHIP* were performed by myself and Brian Seeto, and immunostaining for PTCH and HHIP were

performed by Heather Whetstone, with sample preparation and sectioning done by myself and Lily Morikawa respectively.

We found a significant ($p=0.005$) increase in expression of downstream Hh signaling targets, with at least a 2.5-fold increase in *PTCH1*, at least a 4-fold increase in *GLI1*, and at least a 2.5-fold increase in *HHIP* expression in the most involved areas of OA cartilage samples compared to the least (**Figure 6**). Also, immunostaining for PTCH and HHIP were significantly enhanced in the most involved areas compared to the least (**Figure 7**), with immunostaining scores of 3.8 – vs – 0.8, $P=0.006$ for Ptch and 3.6 – vs – 0.8 for Hhip, $P=0.006$ respectively. The significance between immunostaining samples was determined using a *U*-test. The protein expression of Hh signaling targets appears throughout all levels of the articular cartilage, both above and below the tide-mark. The samples with the highest levels of Hh signaling activation turned out to be samples from the medial femoral condyle and medial tibial plateau, and the samples where the level of Hh signaling upregulation was the lowest were from the lateral femoral condyle and lateral tibial condyle.

Characterization of murine osteoarthritic samples

To investigate whether Hh signaling may also be activated in murine OA cartilage, a model of surgically-induced post-traumatic OA was chosen. OA in knees of mice was induced by arthrotomy and excision of the medial meniscus (meniscectomy). For sham-operated knees, only arthrotomy was performed (the joint capsule was breached and then closed). I performed all operations.

At least six knees in each group (meniscectomy, sham-operated) were characterized for their OA phenotype using radiography (radiographs not shown), ICRS histological grading, synovitis scoring, immunostaining scoring, and subchondral bone histomorphometry. For gene expression studies, another six knees for each group were harvested for their articular cartilage post-mortem by careful microdissection from the underlying bone. These samples were examined for the expression of known genes upregulated in OA, *Adamts5*, *Col10a1*, and *Mmp13*. To corroborate these expression studies, immunostaining of type X collagen (a marker of chondrocyte hypertrophy), the aggrecan NITEGE neoepitope (a cleavage, enzymatic degradation product of aggrecanase) and the C1,2C (Col2 ³/₄ C_{short}) neoepitope (a cleavage, enzymatic degradation product of collagenases for type I and type II collagen) were performed. I prepared all samples for histology

and immunostaining, Lily Morikawa and Heather Whetstone performed sectioning, and immunostaining was carried out primarily by Heather Whetstone, with assistance from Justyna Bartoszko, Michael Khoury, and myself. To grade these immunostained sections, a semi-quantitative immunostaining score was used. For subchondral bone histomorphometry, four parameters were quantified: the bone volume to total volume ratio (BV/TV, %), trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, /mm), and trabecular spacing (Tb.Sp, μm), all according to Parfitt's formulae and as previously described^{117,130-132}. All grading was carried out blinded by Brian Seeto, Justyna Bartoszko, Michael Khoury, and myself.

As shown in **Figure 8**, histological examination of joints 16-weeks post-operative revealed a substantial loss of articular cartilage above the tide mark, loss of proteoglycan content, increase clonal proliferation, and hypertrophy of chondrocytes. As shown in **Table 2**, there was a significant difference across all ICRS graded parameters, with worse ICRS summary scores for knees with meniscectomy versus without (4.6 – vs – 17.4, $p=0.0026$). The significance was determined by a Mann-Whitney *U*-test. The most notable differences observed were in the parameters of the cartilage surface (3.0 with meniscectomy – vs – 0.6 without), chondrocyte distribution (3.0 with meniscectomy – vs – 0.8 without),

subchondral bone (3.0 with meniscectomy – vs – 0.4 without), and cartilage mineralization (3.0 with meniscectomy – vs – 0.0 without). The ICRS changes in the subchondral bone and cartilage mineralization were consistent with differences determined by bone histomorphometry. In comparison of knees with meniscectomy to sham-operated controls, increases in BV/TV (48.83 – vs – 35.78) and Tb.Th (92.31 – vs – 59.64) and decreases in Tb.N (6.73 – vs – 1.68) and Tb.Sp. (229.14 – vs – 150.36) were noted (**Table 3**).

As shown by real-time PCR, there is also at least a 50-fold increase in *Col10a1*, at least a 35-fold increase in *Adamts5*, and at least a 10-fold increase in *Mmp13* expression, known genes upregulated in OA (**Figure 9**). Moreover, there is enhanced immunostaining of ECM proteins associated with OA in joints with meniscus removal compared to sham-operated knees, with significant differences observed in the semi-quantitative scores in knees with meniscectomy for type X collagen (2.8 – vs – 7.4, $P=0.026$), aggrecan NITEGE neoepitope (0.6 – vs – 5.2, $P=0.06$), and collagenase neoepitope C1,2C ($\text{Col2 } \frac{3}{4} \text{ C}_{\text{short}}$) (0.6 – vs – 4.4, $P=0.026$) (**Figure 10**). As expected in non-inflammatory arthritis, there is also little or low-grade synovitis scored in all murine knee joints samples, with the regular finding of increased hypertrophy of the synovium (**Table 4**).

Hh signaling is activated in murine OA

Using the same murine knees and cartilage samples that were characterized for OA above, the level of Hh signaling was also investigated by real-time PCR of *Ptch1* and *Hhip* and immunostaining for Ptch1 and Hhip. At 4, 8, and 16 weeks post-operatively, articular cartilage samples from mice that underwent meniscectomy or a sham-operation were harvested for expression studies. At 16 weeks post-operatively, immunostained sections of Ptch and Hhip were prepared and semi-quantitatively scored. I prepared all samples for histology and immunostaining, Lily Morikawa and Heather Whetstone performed sectioning, and staining was carried out with the assistance of Heather Whetstone, Justyna Bartoszko, Michael Khoury, and myself. To grade these immunostained sections, a semi-quantitative immunostaining score was employed. All grading was carried out blinded by Brian Seeto, Justyna Bartoszko, Michael Khoury, and myself.

As shown in **Figure 9**, there was more than a 4-fold increase in *Ptch1* and *Hhip* expression in murine OA cartilage in which OA was surgically induced at the 16-week time point ($p < 0.05$ for the 16 week time point), with enhanced immunostaining of Ptch1 and Hhip throughout all levels of the articular cartilage in comparison to sham-operated controls (**Figure 11**). The significance in expression

was determined using a t-test. Scoring of the immunostained sections shown significantly enhanced expression in knees with meniscectomy for both for *Ptch* (1.4 – vs – 3.8, $p=0.006$) and *Hhip* (1.2 – vs – 4.2, $p=0.0026$) in comparison to sections prepared from sham-operated knees.

DISCUSSION

Here we show that Hh signaling is upregulated in human and murine OA cartilage. In human osteoarthritic knees, articular cartilage from the medial femoral condyles and medial tibial plateau were associated with the greatest upregulation of Hh signaling targets *PTCH1*, *GLI1*, and *HHIP*, worst histological ICRS scores, and greatest upregulation of OA markers *ADAMTS5*, *COL10A1*, and *MMP13*. These areas also showed significantly enhanced immunostaining for PTCH, HHIP, type X collagen, NITEGE neoepitope, and collagenase neoepitope C1,2C (Col2 $\frac{3}{4}$ C_{short}). In mice knees with surgically-induced OA, there was a significant upregulation of *Ptch1* and *Hhip* expression, worst ICRS histological scores, and significant increase in *Adamts5*, *Col10a1* and *Mmp13* expression. This data corroborated with

significantly enhanced immunostaining of Ptch1, Hhip, type X collagen, NITEGE neoepitope, and collagenase neoepitope C1,2C (Col2 $\frac{3}{4}$ C_{short}). Moreover, substantial changes to subchondral bone were observed, with increases to the histomorphometric parameters of BV/TV and Tb.Th. and decreases to Tb.N. and Tb.Sp. While bone histomorphometry is commonly performed on plastic embedded samples, our characterization of OA required samples to be embedded in paraffin for histology and immunostaining. Thus, in arthritis studies, the use of paraffin embedded sections for bone histomorphometry has been performed, as previously described^{117,130,131}. Of note, in our analysis, a relative comparison of parameters was considered, and as such, our findings should not be compared or considered as absolute values.

Our choice of using a mouse model of OA by way of medial meniscectomy was deliberate and our reasons two-fold. First, an animal model with post-traumatic induction of osteoarthritis is representative of the human condition, whereby OA develops as a result of joint instability from a secondary event¹¹⁰. Second, in humans, OA typically progresses gradually over a number of years. Thus, medial meniscectomy allowed us to study the disease in mice up to 26 weeks of age, which is long-term relative to the maturity and lifespan of mice and is consistent

with previous findings of surgically induced OA. Other, more surgically-invasive murine OA models (e.g. total meniscectomy, ligament resection) would have shortened the onset of and hastened the progression towards a severe phenotype¹²³.

The upregulation of Hh signaling in OA may be explained by both external and genetic factors. *Ihh* has previously been reported as a mechanoresponsive gene in chondrocytes^{133,134}. As such, in post-traumatic injury or in cases of abnormal joint morphology, Hh signaling could be upregulated in chondrocytes during OA due to abnormal forces on the joint. During chondrocyte development, beta-catenin has been identified upstream of Hh signaling⁶⁰. Thus, given that beta-catenin is known to be upregulated in OA and since there are a number of genetic polymorphisms of Wnt pathway components implicated in the genetic susceptibility to OA³³, another explanation that would account for Hh signaling activation could be due to the upstream activation of canonical Wnt signaling in chondrocytes during OA.

Alongside changes to the articular cartilage, upregulated Hh signaling in OA correlated with changes to the underlying subchondral bone, as evidenced by ICRS and bone histomorphometry. Normally, whereas growth plate cartilage disappears in adulthood arresting long bone growth, articular cartilage persists throughout a lifetime. However, during OA, upregulated Hh signaling in chondrocytes may also

influence the rate of subchondral bone formation and turnover, which may be similar to endochondral ossification of the growth plate.

There are etiologies alternative to joint injury from which OA can arise; for example, a primary genetic mutation in *COL2A1* results in articular cartilage deterioration and subchondral bone remodeling. Such is also the case in the *Col2a1^{sedc}* mice which harbour a homozygous null-mutation in *Col2a1*¹¹¹. In transgenic mice carrying an internally deleted human type II collagen gene (*COL2A1*), there is increased bone resorption¹³⁵, opposite to what occurs in post-traumatic OA in both mice and humans¹³⁰. Specifically, although cartilage deterioration occurs in the *Col2a1^{sedc}* mouse, the subchondral bone in the OA joints of mutant *Col2a1* mice show decreased bone volume, decrease trabecular thickness, and increased trabecular spacing¹³⁵. As such, mice with primary OA would likely differ in their dysregulation of Hh signaling compared to mice with secondary OA. Therefore, articular cartilage deterioration does not necessarily explain the increases in cartilage mineralization and subchondral bone remodeling observed in secondary OA. Instead, upregulated Hh signaling in chondrocytes may account for the changes in subchondral bone remodeling and osteophyte formation during secondary OA.

MATERIALS AND METHODS

Human OA cartilage samples

Human articular cartilage samples were obtained from 10 osteoarthritic patients undergoing total knee replacement. The mean age of the patients was 64.4 years. Cartilage was harvested from the femoral and tibial condyles and plateaus, placed in 1X PBS solution (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH of 7.4). Each area of cartilage was portioned for histology, RNA expression studies, and for explant culture (see Chapter 4). Samples were histologically graded by blinded observers using the ICRS scale¹²⁵. A summary score was calculated and statistical significance determined using a Mann-Whitney *U*-test. Immunostaining of PTCH1, HHIP, type X collagen, and neoepitopes were also performed. (See RNA, Histology, Immunohistochemistry methods below). All samples were obtained under the appropriate ethics approval.

Surgery to induce osteoarthritis in mice

Osteoarthritis was surgically induced in mice via removal of the medial meniscus in the knee joint as previously described^{15,123}. Briefly, mice were anesthetized, and betadine was used to disinfect and surgically prepare the knee.

Under sterilize conditions, an incision was made along the medial border of the patella tendon, the skin retracted, another incision was made through the joint capsule, and the medial meniscus was located and excised. The joint capsule was closed, typically with one simple-interrupted suture, followed by the subcutaneous and skin tissues, with another one or two sutures. Antibiotic ointment was applied to the wound and pain-killer medications were administered subcutaneously. The mouse was then monitored, and pain medications administered twice daily for 3 days post-operatively.

For sham-operated controls, all surgical procedures were the same except that meniscectomy was not performed and the joint capsule was only breached and closed. All mice were monitored daily for 1 week post-operatively for any visible signs of distress or pain. All procedures were performed under the appropriate approval of animal protocols.

Radiography, histology, and immunohistochemistry

After 4, 8, and 16 weeks post-operatively, mice with meniscectomy and sham-operated controls were sacrificed by cervical dislocation and their knee joints were radiographed using the Faxitron MX20 X-ray system. Both lateral and

anterior-posterior views were taken at several different fields of view. Samples for histology were prepared by fixation in 4% paraformaldehyde solution (10% formalin), decalcification in 19% ethylenediaminetetraacetic acid (EDTA) at pH 7.4, dehydrated under serial concentrations of ethanol (70%, 80%, 85% 95% 100%), and embedded into paraffin. Serial sections of 5 μ m were obtained, with representative sections taken at 50 μ m intervals across the entire joint. For ICRS histological scoring, synovitis scoring, and bone histomorphometry, both Safranin O and H&E staining sections were prepared.

For immunostaining, serial sections of 5 μ m were deparaffinized (xylene washes), rehydrated through an alcohol gradient to water (100%, 95%, 80%, 70%) and digested with 0.4 units/L chondroitinase ABC in 1 M Tris pH8.0 (Sigma, St. Louis, MO) for 30 min @ 37°C. Endogenous peroxide activity was blocked with 3%(v/v) peroxide in methanol for 15 minutes at room temperature, and nonspecific binding was blocked with 2% (v/v) normal horse serum (Vectorlabs) in 1X PBS for 30 minutes. Primary antibody was incubated overnight to either Patched (sc-6147) (Santa Cruz Biotechnology), Hhip (sc-25465) (Santa Cruz Biotechnology), the aggrecanase NITEGE neoepitope, the C1,2C (Col2 $\frac{3}{4}$ C_{short}) neoepitope (IBEX Pharmaceuticals Inc. Montreal, QC), or type X collagen (Quartett, Germany).

Biotinylated secondary antibody and avidin-linked peroxidase (Vectastain Universal Elite ABC kit, Vectorlabs, Burlingame, CA) were used to detect binding of the antibody. Normal rabbit serum was used in control sections.

ICRS scoring, synovitis scoring, and immunostaining

For each sample, a minimum of 12 levels were graded using the ICRS method of scoring¹²⁵, a synovitis scoring scale¹²⁸, and a semi-quantitative scoring system. These analyses were carried out by 3 observers under blinded conditions.

The ICRS scale grades cartilage by evaluating 6 features: 1) surface, 2) matrix, 3) cell distribution 4) cell population 5) subchondral bone and 6) cartilage mineralization (calcified cartilage) (Table 1). Each parameter carries a maximum score of 3, and thus, a maximum ICRS summary score is 18.

The severity of synovitis was scored using the *Krenn* scale, which grades histological sections based on three criteria: 1) the synovial cell lining cell layer, 2) density of resident cells (e.g. cellularity, multinucleated giant cells, pannus formation and rheumatoid granulomas), and 3) inflammatory infiltrate might occur (e.g. lymphocytes or plasma cells). All defined histopathological qualities are graded from absent (0), slight (1) and moderate (2) to strong (3), with summaries ranging

from 0 to 9. Values of 0 to 1 corresponds to no synovitis (inflammatory grade = 0); 2 to 3 to a slight synovitis (inflammatory grade 1); 4 to 6 to a moderate synovitis (inflammatory grade 2), and 7 to 9 to a strong synovitis (inflammatory grade 3).

The immunostaining semi-quantitative score consists of grading each sample twice, once for the percentage of labeled cells, and the second for the intensity of the immunostaining. For percentage of labeled cells, the value of 0 corresponds to an absence of labeling over the cells; 1 is to 30% of cells are labeled; 2 is to 30–60%; 3 is to 60%). For intensity of the immunostaining, the value of 0 corresponds to no staining; 1 indicates weak staining; 2 indicates mild staining; and 3 indicates strong staining). Both parameters multiplied to give a final quotation score between 0 (absence of labeling and no staining) to 9 (>60% and strong staining).

Bone histomorphometry

The same number of histological sections graded under the ICRS and synovitis scale were analyzed and quantified using ImageJ v.1.41 software (NIH), and the bone parameters of bone volume (BV/TV, %), trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, /mm), and trabecular spacing (Tb.Sp, μm) were scored by blinded observers as previously described¹²⁷. Briefly, according to

Parfitt's formula, to calculate BV/TV, 2D measurements were made of the trabecular bone area (B.Ar in mm²) and total cancellous tissue area (T.Ar), whereby

$$BV/TV = 100 \text{ B.Ar}/T.Ar.$$

Tb.Th was derived from the trabecular perimeter (B.Pm) and bone area (B.Ar),

$$Tb.Th = (B.Ar/B.Pm)(\pi/2)$$

From these parameters, Tb.N and Tb.Sp were then derived,

$$Tb.N = (B.Pm/T.Ar) * 10.$$

$$Tb.Sp = (1000 * T.Ar - B.Ar)/B.Pm.$$

For each parameter, all errors were calculated from the average of the mean at a 95% confidence interval. For each sample, a minimum of 12 levels were examined, and for each level or section, a minimum of three representative fields were identified. For each section, the calcified cartilage/subchondral bone junction was used as the upper limit of each field, which was readily determined using both Safranin-O and H&E stained serial sections. The depth of measurement was limited to the upper limit down to 500 μ m, consistent to methods previously described^{117,130-132}. All statistical differences between parameters of samples with meniscectomy and with sham-operation were compared using a student's t-test at $p < 0.05$.

RNA extraction, reverse transcription, and real-time PCR

Human and mice articular cartilage samples were snap frozen in liquid nitrogen, total RNA was extracted using Trizol (Invitrogen), and reverse transcription was carried out using Superscript II (Invitrogen). TaqMan assays were performed on an ABI7900HT in accordance to the manufacturer's instructions, with relative expression normalized to an endogenous reference control ($2^{-\Delta Ct}$). In human samples, downstream Hh signaling targets *PTCH1*, *GLI1*, *HHIP* were examined against housekeeping reference genes *GAPDH* and *ACTB*. Similarly for mouse samples, *Ptch1*, *Gli1*, and *Hhip* were examined against *Gapdh* and *Actb*. For known upregulated targets of OA, *ADAMTS5*, *COL10A1*, and *MMP13* were examined against genes *GAPDH* and *ACTB*. For mouse samples, *Adamts5*, *Col10a1*, and *Mmp13* were examined against *Gapdh* and *Actb*. Taqman primers were obtained from Applied Biosystems. For human expression studies, the gene expression normalized to the endogenous control was arbitrarily set to "1" for the samples with the least OA involvement. As such, by evaluating $2^{-\Delta\Delta Ct}$ between the least involved and most involved samples, the fold-change in gene expression was determined. Similarly, for murine expression studies, the gene expression normalized to the endogenous control was arbitrarily set to "1" for sham operated samples. As such, by evaluating

$2^{-\Delta\Delta C_t}$ between the sham-operated and samples from knees with meniscectomy, the fold-change in gene expression was determined. All samples were performed in triplicate, and all values were reported as the mean and errors as 95% confidence intervals. Statistical significance was calculated using a student's t-test at $p < 0.05$.

Figure 5. Representative histological Safranin O sections, comparing the least involved (left) and most involved (right) segment of human osteoarthritic cartilage. Proteoglycan stains red in the sections. Scale bar, 400 μm .

Figure 6. Hedgehog signaling targets are expressed in osteoarthritis (OA). Real-time RT-PCR expression of Hh signaling targets (*PTCH1*, *GLI1* and *HHIP*) and known markers of osteoarthritis (*ADAMTS5*, *COL10A1* and *MMP13*) in human in osteoarthritic cartilage samples obtained from patients undergoing total knee replacements (mean age = 64.4 years). The level of expression was arbitrarily defined as '1' in the least involved segment of the knee (black), and data from the most involved segment are given as the mean (grey). The error bars are 95% confidence intervals (n = 10; *P < 0.05).

Figure 7. Protein expression of Hh downstream targets in human osteoarthritic samples. Enhanced immunostaining of PTCH and HHIP in the most involved samples (right) compared to the least (left) are shown.

Figure 8. Representative histological Safranin O sections showing typical changes in mouse knees with medial meniscectomy (right) compared to knees with arthrotomy only (left). Data from 16-week post-operative samples are shown. Proteoglycan stains red in the sections. Scale bar, 200 μm .

Figure 9. Real time RT-PCR data for the expression of Hh signaling targets and markers of osteoarthritis in mouse articular cartilage samples microdissected from knees with surgical removal of their medial meniscus (grey) and knees without (black). Data from 16-week post-operative samples are shown. The expression level for the knee with arthrotomy only was arbitrarily defined as '1' (black), and data from knees with meniscectomy are given as the mean (grey) *Ptch1* and *Hhip* are Hh target genes, while *Adamts5*, *Col10a1*, and *Mmp-13* are genes known to be upregulated in OA (n = 6, *P < 0.05).

Figure 10. Protein expression for type X collagen (top), aggrecan NITEGE epitope (middle) and type I and II collagen C1,2C (Col2 ^{3/4} C_{short}) neoepitopes in murine osteoarthritic samples. Data from 16-week post-operative samples are shown. Immunostaining is enhanced in samples with surgical induction of OA in the knee by arthrotomy and meniscetomy (representative section on the right) compared to sham operation (representative section on the left).

Figure 11. Protein expression of Hh downstream targets in murine osteoarthritic samples. Data from 16-week post-operative samples are shown. Immunostaining of Ptch and Hhip is enhanced in samples with surgical induction of OA in the knee by arthrotomy and meniscetomy (representative section on the right) compared to sham operation (representative section on the left).

ICRS CARTILAGE SCORING SCALE	Mouse		Human	
	Sham	Surgery	Least Involved	Most Involved
1) Surface	3.0 ± 0.0	0.6 ± 0.1	2.5 ± 0.2	0.5 ± 0.1
2) Matrix	2.7 ± 0.2	1.8 ± 0.1	2.7 ± 0.2	2.0 ± 0.1
3) Cell Distribution	3.0 ± 0.0	0.8 ± 0.2	1.3 ± 0.1	0.5 ± 0.2
4) Cell Population Viability	2.7 ± 0.2	1.0 ± 0.1	3.0 ± 0.0	2.3 ± 0.1
5) Suchondral Bone	3.0 ± 0.0	0.4 ± 0.1	2.7 ± 0.2	1.2 ± 0.2
6) Cartilage Minerlization	3.0 ± 0.0	0.0 ± 0.0	3.0 ± 0.0	1.5 ± 0.2
ICRS SUMMARY SCORE	17.4 ± 0.2	4.6 ± 0.2	15.2 ± 0.2	8.0 ± 0.2
P-value (U-test)	P=0.0026		P=0.0066	

Table 2. ICRS Histological Scores for OA Cartilage.

Human osteoarthritic samples obtained from patients undergoing total knee replacement were scored, comparing the most and least involved samples for eight patients (mean age of 64.4 years). The knee joints of mice were also scored, comparing mice that underwent surgical removal of their medial meniscus to a sham operation (n=6). Data from 16-week post-operative samples are shown.

BONE HISTOMORPHOMETRY	Mouse	
	Sham	Surgery
BV/TV (%)	35.78 ± 3.75	48.83 ± 2.38
Tb.Th (μm)	59.64 ± 2.82	92.31 ± 3.92
Tb.N (mm ⁻¹)	6.73 ± 0.52	1.68 ± 0.71
Tb.Sp (μm)	229.14 ± 8.88	150.36 ± 9.64

Table 3. Bone Histomorphometry in Mice. Subchondral bone analysis in the knee joint of mice with surgically-induced OA by arthrotomy and meniscectomy exhibit increases in bone volume (BV/TV) and trabecular thickness (Tb.Th), and decreases in trabecular number (Tb.N) and trabecular spacing (Tb.Sp) in comparison to mice with a sham operation. Data from 16-week post-operative samples are shown.

SYNOVITIS SCORING SCALE	Mouse	
	Sham	Surgery
1) Synovial lining cell layer	0.8 ± 0.1	1.6 ± 0.1
2) Density of resident cells	0.2 ± 0.1	0.8 ± 0.1
3) Inflammatory infiltrate	0.2 ± 0.1	0.6 ± 0.1
SYNOVITIS SCORE	1.2 ± 0.2	3.0 ± 0.2

Table 4. Synovitis Grading. Low-grade synovitis is present in mice with surgical removal of their medial meniscus, which is typical in non-inflammatory arthritis such as trauma-induced osteoarthritis. There is a slight increase in the number of layers of synovial cells in mice with meniscectomy versus sham operated knees. Data from 16-week post-operative samples are shown.

CHAPTER THREE

Hh signaling activation predisposes to the osteoarthritis

Modulating Hh signaling can attenuate the severity of osteoarthritis

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* Performed all procedures except for sectioning and immunostaining

SUMMARY

In Chapter Two, we found that Hh signaling is upregulated in human and murine OA cartilage. There are a number of cartilage and bone pathologies in humans and mice associated with activated Hh signaling; however, whether Hh signaling activation also predisposes to OA is unknown. Using several genetically modified mice varied in their level of Hh signaling activation, we compared *Ptch1*^{+/-}, *Col2a1-Gli2*, and *COL2-rtTA-Cre;Gt(ROSA)26Sor^{tm1}(Smo/YFP)Amc* mutants, and found at least a 2-fold, 4-fold, and 15-fold increase in the expression of *Ptch1* and *Hhip* and enhanced expression of *Ptch1* and *Hhip*. We then characterized the knee joints of these *Ptch1*, *Gli2*, and *Smo* mutants and found at least a 2.5-, 4-, and 22-fold increased in the expression of *Adamts5*, *Col10a1*, and *Mmp13*, accompanied by enhanced immunostaining of type X collagen, aggrecan and collagen neoepitopes. Also, with greater levels of Hh signaling activation, there was a significant decline in ICRS scores and increase subchondral bone changes revealed by radiography and bone histomorphometry. Taken together, we show that the level of Hh signaling activation in mice predisposes to and positively correlates with the severity of the OA joint phenotype and expression of OA markers.

INTRODUCTION

During embryonic development in mice, Hh signaling has been shown to regulate chondrocyte differentiation, and post-natally, Hh signaling is required in the maintenance of chondrocytes in articular cartilage and the growth plate. In Chapter 2, upregulation of Hh signaling was shown to be associated with human and murine osteoarthritic cartilage samples. Hh signaling activation predisposes to a number of pathological conditions in cartilage and bone. For example, mice deficient in the Hh transcriptional repressor Gli3 are predisposed to synovial chondromatosis¹⁰⁶, which involves the ectopic formation of cartilaginous nodules in the synovial membrane. In the *Col2a1-Gli2* mouse, overexpression of the transcriptional activator Gli2 under type II collagen promoter regulatory elements results in the development of enchondromas of the growth plate^{96,97}. Aberrant Hh signaling activation also predisposes to the development of chondrosarcomas¹⁰⁷ and osteosarcomas¹³⁶. Here I ask whether the level of Hh signaling activation predisposes to the severity of the joint phenotype in OA.

For this investigation, several genetically modified mice activated for Hh signaling will be examined for their joint phenotype and expression of known genes upregulated in OA. The *Ptch1*^{+/-} mouse harbors a heterozygous null mutation of

Ptch1, which results in the upregulation of Hh target genes¹³⁷. With a heterozygous deficiency in *Ptch1*, there is decreased inhibition of *Smo* thereby activating downstream the Gli family of transcription factors. The *Col2A1-Gli2* transgenic mouse overexpresses the full-length form of the Hh-activated transcriptional factor Gli2 in chondrocytes under Col2A1 regulatory elements⁹⁷. In chondrocytes when Ihh ligand binds to Patched, the full length Gli2 protein is cleaved downstream, acting as a transcriptional activator, which results in the upregulation of downstream targets of Hh signaling. The *Gt(ROSA)26Sor^{tm1}(Smo/YFP)Amc* mouse, which expresses the constitutively active W539L point mutation of the mouse smoothened homolog (*Drosophila*) gene (*SmoM2*) upon cre recombination, was crossed with *COL2-rtTA-Cre* mice so that *SmoM2* was expressed in chondrocytes when doxycycline was administered^{138,139}

To investigate the level of Hh signaling activation, the real-time PCR expression of downstream targets of Hh signaling, namely *Ptch1* and *Hhip*, was examined, alongside semiquantitative scoring of immunostained samples of *Ptch1* and *Hhip*. To characterize the knee joint, radiography; ICRS histological grading; real-time PCR expression of known genes upregulated in OA *Adamts5*, *Col10a1*, and

Mmp13; bone histomorphometric measurements of BV/TV, Tb.Th., Tb.N., and Tb.Sp.; and semi-quantitative scoring of synovitis was performed.

RESULTS

Hh signaling activation in mice produces an OA-like phenotype and upregulated gene expression of known OA markers

To investigate whether Hh signaling activation predisposes to OA in mice, several genetically modified mice were examined. The *Ptch1*^{+/-} and *Col2a1-Gli2* mutants were compared against wild-type littermate controls, and the *COL2-rtTA-Cre; Gt(ROSA)26Sor^{tm1(Smo/YFP)Amc}* mouse was compared against mice not expressing *COL2-rtTA-Cre* but still treated with doxycycline. Doxycycline was administered to these mice at ten weeks of age. At least eight mutant mice and littermate controls were used for real-time PCR expressions studies and the same number used for radiography histological grading, and immunostaining scoring. For each of the three groups of mutant and littermate control mice, the joint was examined at eighteen weeks of age. In mice where Hh signaling was activated in chondrocytes

(*Col2a1-Gli2* and *COL2-rtTA-Cre;Gt(ROSA)26Sor^{tm1(Smo/YFP)Amc}* mice) there was no premature closure of the growth plate, however, some exhibited the formation of enchondromas similar to observed elsewhere^{96,97}. I bred and genotyped all mice colonies using a previously reported breeding scheme¹⁴⁰.

To determine the level of Hh signaling activation, real-time PCR experiments were performed on articular cartilage samples carefully microdissected from the underlying bone. For each group, articular cartilage samples were obtained from 8 mutant mice and 8 littermate controls. To corroborate these expression studies, sections immunostained for *Ptch1* and *Hhip* were also prepared. I prepared all knee samples, Lily Morikawa and Heather Whetstone performed sectioning of samples, and Heather Whetstone performed immunostaining. Brian Seeto assisted me in the PCR experiments.

As shown in **Figure 12**, all mutant mice exhibited upregulated expression of downstream targets of Hh signaling. In ascending order, articular cartilage samples from the knee joints of *Ptch1*^{+/-}, *Col2a1-Gli2*, and *COL2-rtTA-Cre;Gt(ROSA)26Sor^{tm1(Smo/YFP)Amc}* mice showed at least a 2-fold, 4-fold, and 15-fold increase in real-time PCR expression of downstream targets *Ptch1* and *Hhip*. Alongside these changes, there was enhanced protein expression of *Ptch* and *Hhip*

as evidenced by their immunostaining (**Figure 13**). Moreover, the semi-quantitative scores for Ptch immunostaining in *Ptch1*^{+/-} mutants compared to wild-type littermate controls were 0.6 – vs – 3.6, P=0.0026, for *Col2a1-Gli2* mutants compared to littermate controls were 0.6 – vs – 4.4, p=0.006, and for *COL2-rtTA-Cre;Gt(ROSA)26Sor^{tm1(Smo/YFP)Amc}* mice and littermate *Gt(ROSA)26Sor^{tm1(Smo/YFP)Amc}* mice were 0.4 and 6.0, P=0.008. In all cases, immunostaining of downstream Hh signaling targets was observed in all chondrocytes both above and below the tide-mark.

To characterize the joint phenotype, a number of methods were used. Murine knee joints were examined by radiography and histological sections were scored using the ICRS histological grading scale and a standardized synovitis scoring scale, and were examined for bone histomorphometric parameters. To determine the level of gene expression, real-time PCR of known markers of OA, namely *Adamts5*, *Col10a1*, and *Mmp13* were also examined. Sectioned samples were also immunostained for type X collagen, and the aggrecan neoepitope NITEGE and collagen neoepitope C1,2C (Col2 ³/₄ C_{short}) and scored using a semi-quantitative method previously described. I performed all experiments, Brian Seeto also performed real-time PCR experiments, ICRS scoring was also performed blinded by

Justyna Bartoszko, Michael Khoury, Brian Seeto, I prepared all samples for histology and immunostaining, Lily Morikawa and Heather Whetstone performed sectioning, and Heather Whetstone performed immunostaining.

In the same order for Hh signaling activation, radiographs of knee joints of *Ptch1*^{+/-}, *Col2a1-Gli2*, and *COL2-rtTA-Cre;Gt(ROSA)26Sor^{tm1(Smo/YFP)}Amc* mice show greater joint space narrowing and increased bone changes, with osteophyte formation in the knees of *COL2-rtTA-Cre;Gt(ROSA)26Sor^{tm1(Smo/YFP)}Amc* mice (**Figure 14**). Histology of these knees (**Figure 15**) and ICRS scoring shows substantial changes, with the most significant decreases in the parameters of cell distribution, subchondral bone, and cartilage mineralization (**Table 5**). There is also a decrease in the amount of articular cartilage above the tide mark, a greater loss in proteoglycan content, an increase in clonal proliferation of chondrocytes, and increased subchondral bone remodeling in mice with higher levels of Hh signaling. Interestingly, in *Ptch1*^{+/-} mutants, over 90% of all chondrocytes above the tide-mark were hypertrophic in comparison to wild-type littermate yielding less than 20%, and there was a substantial loss of proteoglycan staining in the articular cartilage compared to the growth plate (internal control) and to wild-type littermate controls. The ICRS summary scores for wild-type littermate controls compared to

Ptch1^{+/-} mice was 17.5 –vs- 14.8 (p=0.00475), for controls compared to *Col2A1-Gli2* transgenic mice was 17.1 –vs- 7.0 (p=0.0026), and *COL2-rtTA-Cre;Gt(ROSA)26Sor^{tm1(Smo/YFP)}Amc* mice in which the constitutively active Smoothed is not expressed compared to mice in which it was activated post-natally was 17.2 –vs- 0.6 (p=0.0026).

The ICRS scores and radiographs also corroborated with the real-time expression of known upregulated genes in OA (**Figure 12**). Compared to littermate controls, *Ptch1*^{+/-}, *Col2A1-Gli2* and *COL2-rtTA-Cre;Gt(ROSA)26Sor^{tm1(Smo/YFP)}Amc* mice showed at least an 8-, 13-, and 28-fold increased expression of *Adamts5*, at least a 2.5-, 4-, and 32-fold increase in *Col10a1*, and a 3-, 12-, and at least a 22-fold increase in *Mmp13* expression. These changes in gene expressed also were corroborated with enhanced immunostaining of type X collagen, and NITEGE and C1,2C (Col2 ^{3/4} C_{short}) neoepitopes (**Figure 16**). For type X collagen, the semi-quantitative immunostaining scores for wild-type littermate controls compared to *Ptch1*^{+/-} mice were 1.2 – vs – 4.4, P=0.0026), for controls compared to *Col2A1-Gli2* transgenic mice were 0.6 – vs – 5.8 (P=0.006), and for *Gt(ROSA)26Sor^{tm1(Smo/YFP)}Amc* mice in which the constitutively active Smoothed is not expressed compared to *COL2-*

rtTA-Cre;Gt(ROSA)26Sor^{tm1(Smo/YFP)Amc} mice in which it was activated post-natally were 0.6 – vs – 7.6 (P=0.0026).

Overall, amongst all Hh signaling mutant variants, bone histomorphometric parameters BV/TV and Tb.Th values decreased with increased Hh signaling activation in comparison to littermate controls. However, with ubiquitous Hh signaling activation as seen in the *Ptch1*^{+/-} mice, the bone histomorphometric parameters BV/TV and Tb.Th both showed a significant decrease in mutants in comparison to wild-type littermates. As shown in **Table 6**, for BV/TV, mutants scored 27.94% – vs – 53.34% for wild-type littermates, and for Tb.Th, mutants scored 49.82 μ m – vs – 56.76 μ m compared to wild-type littermates. In contrast, both BV/TV and Tb.Th values were increased in the *Col2A1-Gli2* and *Gt(ROSA)26Sor^{tm1(Smo/YFP)Amc}* mutants in comparison to controls. For BV/TV, *Col2A1-Gli2* mutants scored values of 84.58% versus 61.22% for wild-type littermates; and *COL2-rtTA-Cre;Gt(ROSA)26Sor^{tm1(Smo/YFP)Amc}* mutants scored values of 120.60% versus 67.27% for *Gt(ROSA)26Sor^{tm1(Smo/YFP)Amc}* mice. As expected, there was a decrease in Tb.N. in all mutants compared to controls, with no significant difference between mutants. For Tb.Sp., a significant decrease was observed in all mice with

activated Hh signaling in comparison to controls, with a values of Tb.Sp. decreasing with increasing Hh signaling activation.

All mice examined exhibited low-grade synovitis (**Table 7**), which suggests that arthritis (if any) displayed in these mutant mice is not inflammatory by nature. Taken together, the level of Hh signaling activation in mice predisposes to a deterioration of the articular cartilage and overall joint phenotype.

DISCUSSION

Our examination of the knee joints from *Ptch1*^{+/-}, *Col2A1-Gli2*, and *COL2-rtTA-Cre;Gt(ROSA)26Sor^{tm1(Smo/YFP)Amc}* mice shows the expression level of downstream Hh signaling targets *Ptch1*, *Gli1*, and *Hhip* is associated and positively correlates with the severity of the OA phenotype and with the expression of known genes upregulated in OA *Adamts5*, *Col10a1*, and *Mmp13*.

For *Ptch1*^{+/-} mutants, ubiquitous Hh signaling activation resulted in changes that were subtle on x-ray, but substantial under joint histology, immunostaining, and real-time PCR expression studies. For mutants at 18 and 26 weeks of age,

articular chondrocytes underwent greater hypertrophy, there was a substantial decrease in proteoglycan staining of the extracellular matrix, and little or no synovitis was observed. All of these histological changes are characteristic of articular cartilage in post-traumatic secondary osteoarthritis, although joint trauma or injury did not precipitate these findings. Our examination of the subchondral bone revealed a decrease in BV/TV and Tb.Th in mutants compared to wild-type littermates. In contrast to our findings on the articular cartilage, these changes in the joint by bone histomorphometric parameters are opposite to what is typically observed in secondary osteoarthritis, and are also opposite to what is observed in younger *Ptch1*^{+/-} mice mutants 8 weeks of age where an increase in BV/TV and Tb.Th is reported¹⁴¹.

Why ubiquitous Hh signaling activation in our aged *Ptch1*^{+/-} mice might result in decreased bone volume and trabecular thickness may be explained through two previous studies on mice with *Ptch1* insufficiency^{94,141}. One report examined the same *Ptch1*^{+/-} mice mutants used in our experiments, and the other made use of cre-loxP technology to generate mice with a homozygous null mutation for *Ptch1* in mature osteoblasts and osteocytes. In both studies, increased Hh signaling was found to promote osteoblastogenesis and osteoclastogenesis, and bone formation

and bone resorption. For *Ptch1*^{+/} mice at 8 weeks, the balance favoured bone formation over resorption as evidenced in increased values of BV/TV and Tb.Th. However, as observed in the other study with homozygous mutants, increased Hh signaling by *Ptch1* ablation in mature osteoblasts was found to promote osteoclast formation by upregulating PTHrP and RANKL⁹⁴. The result was cre-loxP mice with severe osteopenia. Thus, it is likely then that the shift we observed towards an osteopenic phenotype in our aged *Ptch1*^{+/-} mutants of 18 and 26 weeks of age resulted from increased Hh signaling in mature osteoblasts that promoted osteoclast formation and subsequent bone resorption.

Whereas the radiographic changes associated with OA that were subtle with *Ptch1* mutants, there is visible narrowing of the joint space and osteophyte formation in the *Col2A1-Gli2* and *COL2-rtTA-Cre;Gt(ROSA)26Sor^{tm1(Smo/YFP)Amc}* mutant mice. For these mutants, Hh signaling is activated specifically in chondrocytes. In mice with *Gli2* overexpression, histological findings also revealed chondrocyte hypertrophy and decrease proteoglycan staining, both above and below the tide-mark; however, unlike the *Ptch1* mutants, the articular cartilage of these mice was observed, at times, to be defibrillated at the surface, which is characteristic of changes observed in secondary osteoarthritis induced by trauma or injury.

Complete articular cartilage defibrillation was observed in the *SmoM2* mutants, with the most substantial articular cartilage deterioration as evidenced by the worst ICRS scores.

In the case of all of the mice with activated Hh signaling, the arthritis that was characterized was not post-traumatic in origin, the subchondral bone changes showed a significant increase in BV/TV and Tb.Tm alongside decreases in Tb.N and Tb.Sp compared to littermate controls. Moreover, with constitutive activation of Hh signaling as seen in the *COL2-rtTA-Cre;Gt(ROSA)26Sor^{tm1(Smo/YFP)}Amc* transgenic mice, the radiographic changes are substantial with the formation of osteophytes and decreased joint space. It is clear from our characterization of the OA phenotype in these mice that changes to the chondrocytes and their metabolism results in the changes to the underlying subchondral bone. In contrast, in mutant mice defective for type II collagen, changes to the subchondral bone histomorphometric parameters are opposite¹³⁵, with decreases in BV/TV and Tb.Tm and an increase in Tb.Sp. for trabecular spacing. Therefore, it is likely that with greater Hh signaling activation in chondrocytes, there is a greater rate of cartilage turnover and replacement with bone.

Notably, the increases in real-time PCR expression of *Adamts5*, *Col10a1*, and *Mmp13* were not necessarily emulated in the corresponding immunostained sections for type X collagen, aggrecan neoepitope NITEGE, and collagen neoepitope C1,2C (*Col2*^{3/4} *C_{short}*). This difference between gene and protein expression suggests regulatory mechanisms are present at the protein level (e.g. translation, phosphorylation, ubiquitin-mediated degradation, enzyme inhibitors) to help offset the substantial increases that were observed in gene expression.

OA can develop from subtle changes in joint shape, causing abnormal mechanical stresses as in the case of post-traumatic arthritis. Therefore, activation of Hh signaling throughout development in the *Ptch1*^{+/-} and *Col2A1-Gli2* transgenic mice exhibit Hh signaling activation throughout development could result in subtle changes in joint architecture and in the development of OA. However, our data on the *COL2-rtTA-Cre;Gt(ROSA)26Sor*^{tm1(Smo/YFP)Amc} transgenic mouse shows that activation of Hh signaling in chondrocytes after mice are a few months old also predisposes to a deleterious joint phenotype, with changes in cartilage and bone that are typical in post-traumatic arthritis, which suggests that a change in the joint shape alone is not responsible for the development of OA.

The development of OA can also differ amongst mice of different genetic background. For example, for mice induced with OA by medial meniscus destabilization, the characteristic histological changes are greatest amongst 129/SvEv mice, followed by C57BL6 and 129/SvInJ; and FVB/n and 129/SvJ strain are relatively protected from the development of spontaneous OA up to 1 year in age¹⁴². All mice characterized in our studies are of a C57BL/6 background and, for transgenic mice, there is the additional 129/SvJ strain introduced at the stage of construct injection into embryonic stem cells. Both C57BL/6 and 129/SvJ strain of mice have been reported to share similar histological findings and gene expression changes with induction of OA via medial meniscus destabilization. Moreover, for each group of mutant and littermate controls, our histological characterization of OA was corroborated with expression studies of Hh signaling targets and known upregulated genes in OA. Thus, although the differences in background strain can account for differences in OA, it is more likely that the changes we observed at the joint amongst our mutant mice were due to the substantial differences in the level of Hh signaling.

Doxycycline is a known inhibitor of matrix metalloproteinases, and as such, has been suggested to influence the joint phenotype in mice under investigation for

arthritis. However, the protective effect of doxycycline, if any, is offset by the effects of Hh signaling activation in all mice examined, significantly increased in *MMP13* expression and collagen neoepitope immunostaining, with the *COL2-rtTA-Cre;Gt(ROSA)26Sor^{tm1(Smo/YFP)Amc}* transgenic mouse exhibiting the most severe OA phenotype.

Finally, in the Hh signaling pathway, Ptch is upstream of Smo, and Smo is upstream of Gli2. Based on our joint analyses, the severity of OA is more dramatic with activation pathway in chondrocytes (as seen in the *Col2A1-Gli2* and *COL2-rtTA-Cre;Gt(ROSA)26Sor^{tm1(Smo/YFP)Amc}* mice). Furthermore, in chondrocytes, activation of the pathway upstream at the level of receptor Smo produces a more severe OA phenotype than with activation of the pathway downstream at the level of transcription factor Gli2. As such, the converse may be true, where Hh signaling blockade upstream may improve the OA phenotype greater than if the blockade were to occur downstream of the pathway.

METHODS AND MATERIALS

Genetically modified mice

The knee joints of *Ptch1*^{+/-}, *Col2A1-Gli2*, and *COL2-rtTA-Cre*; *Gt(ROSA)26Sor^{tm1(Smo/YFP)}Amc* mice were examined for their joint phenotype and gene expression at 18 weeks of age. The *Col2A1-Gli2* transgenic mouse overexpresses the full length form of the Hh-activated transcriptional factor Gli2 in chondrocytes under *Col2A1* regulatory elements⁹⁷. In the case of *Ptch1*^{+/-} and *Col2A1-Gli2* mice, wild-type littermate mice were used as controls using a previously reported breeding strategy⁹⁷. The *Gt(ROSA)26Sor^{tm1(Smo/YFP)}Amc* mouse, which expresses the constitutively active W539L point mutation of the mouse smoothened homolog (*Drosophila*) gene (*SmoM2*) upon cre recombination, was crossed with *COL2-rtTA-Cre* mice so that *SmoM2* was expressed in chondrocytes when doxycycline is administered^{138,139}. Doxycycline was administered daily at a concentration of 2 mg/ml in the drinking water containing 5% sucrose, changing the solution every other day as previously described¹³⁸. Control mice consisted of mice not expressing *COL2-rtTA-Cre* but still were treated with doxycycline.

The *Smo*^{tm2Amc} transgenic mouse, which expresses a null allele of *Smo* upon the exposure to cre-recombinase, was crossed with the *COL2-rtTA-Cre* mouse to generate mice in which a null allele of *Smo* is expressed upon the administration of doxycycline. Doxycycline was administered in the drinking water¹³⁸ resulting in downregulated Hh signaling blockade in chondrocytes^{138,143}. As a control, littermates that do not express *COL2-rtTA-Cre* but were still treated with doxycycline were used. Cre-mediated recombination was confirmed by fluorescence and PCR.

For each genotype, at least 8 mice were examined for their joint phenotype (radiology, histology and at least 8 mice were used for gene expression studies with microdissected articular cartilage).

For genotyping of *Ptch1*^{+/-} mice, DNA was prepared from tail biopsies, using primers 5'-CACGGGTAGCCAACGCTATGTC and 5'-GCCCTGAATGAACTGCAGGACG for the mutant sequences and 5'-CTGCGGCAAGTTTTTGGTTG and 5'-AGGGCTTCTCGTTGGCTACAAG for the wild-type sequences. PCR was carried out for using a mixture of all 4 sequences for 35 cycles of amplification under the following conditions: denaturation, 94°C/30s; annealing, 69°C/60s; extension, 72°C/45s. The

PCR product has a size of 479 bp for homozygous mutant, 200 bp and 479 bp for heterozygous mutant, and 200 bp for the wild-type.

For genotyping of *Col2a1-Gli2* mice, DNA was prepared from tail biopsies, using primers 5' CTCTGCTAACCATGTTTCATG and 5'CAGAGGACAGGCCTTTTTC for the forward and reverse sequences respectively. PCR was carried out for 35 cycles of amplification under the following conditions: denaturation, 95°C/30s; annealing, 58°C/30s; extension, 72°C/45s. The PCR product has a size of 244 bp.

For genotyping *COL2-rtTA-Cre* mice, DNA was prepared from tail biopsies, using primers 5'-CTCTCTTGTCAGCACCGTTCT and 5'-CTCTGCACCTTGGTGATCAA for the COL2A1 promoter and the rtTA sequences respectively. PCR was carried out for 40 cycles of amplification under the following conditions: denaturation, 95°C/30s; annealing, 58°C/30s; extension, 72°C/45s. The PCR product has a size of 663 bp.

For genotyping *Gt(ROSA)26Sor^{tm1(Smo/YFP)Amc}* mice, DNA was prepared from tail biopsies, using primers 5'-AAGTTCATCTGCACCACCG and 5'-TCCTTGAAGAAGATGGTGCG for the mutant sequences and 5'-CGTGATCTGCAACTCCAGTC and 5'-GGAGCGGGAGAAATG GATATG for the wild-type

sequences. For the mutant, PCR was carried out for 35 cycles of amplification under the following conditions: denaturation, 95°C/30s; annealing, 60°C/60s; extension, 72°C/60s. For the wild-type, PCR was carried out for 35 cycles of amplification under the following conditions: denaturation, 94°C/30s; annealing, 66.8°C/60s; extension, 72°C/60s. The PCR product has a size of 173 bp for homozygous mutant, 173 bp and 410 bp for heterozygous mutant, and 410 bp for the wild-type.

Joint phenotype analysis

As outlined in greater detail in Chapter Two, Safranin-O and H&E histological sections were prepared and graded by radiography, by the ICRS scoring scale, by the *Krenn* scale for synovitis, the *Klein* semi-quantitative scale for immunostaining, and bone histomorphometry. For radiography, images were obtained from both the lateral and anterior-posterior planes of view. For histological grading, a minimum of 12 sections were graded for each sample, and grading was performed blinded. All values were reported as a mean with standard errors calculated at a confidence interval of $p < 0.05$. For statistical differences between values, a Mann-Whitney *U*-test was performed.

RNA expression studies

Articular cartilage was microdissected from the knee joints of mice at 18 and 26 weeks of age, to correspond to the post-operative ages of mice with surgical induction of OA by medial meniscectomy. As also outlined in Chapter Two, mice articular cartilage samples were snap frozen in liquid nitrogen, total RNA was extracted using Trizol (Invitrogen), and reverse transcription performed using Superscript II (Invitrogen). For downstream Hh signaling target, *Ptch1*, *Gli1*, and *Hhip* were examined against *Gapdh* and *Actb*. For known upregulated targets of OA, *ADAMTS5*, *COL10A1*, and *MMP13* were examined against housekeeping genes *GAPDH* and *ACTB*. For mouse samples, *Adamts5*, *Col10a1*, and *Mmp13* were examined against *Gapdh* and *Actb*. The gene expression normalized to the endogenous control was arbitrarily set to “1” for sham operated samples. As such, by evaluating $2^{-\Delta\Delta C_t}$ between littermate controls and mutant knee cartilage samples, the fold-change in gene expression was determined. All samples were performed in triplicate, and all values were reported as the mean and errors as 95% confidence intervals. All samples were performed in triplicate. Taqman primers were obtained from Applied Biosystems. Statistical significance was calculated using a t-test at $p < 0.05$.

Figure 12. Real time RT-PCR data showing relative expression of Hh signaling targets and genes expressed in osteoarthritis from mouse articular cartilage samples microdissected from knees of the genetically modified mice with activated Hh signaling compared to wild-type littermates (or, in the case of *COL2-rtTA-Cre;Gt(ROSA)26Sortm1(Smo/YFP)Amc*-transgenic mice, mice that do not express *COL2-rtTA* were used for comparison). As the level of *Ptch* and *Hhip* (downstream targets of Hh signaling) increases, the level of known genes normally upregulated in OA (*Adamts5*, *Col10a1*, *Mmp13*) increases. The level for expression from control knees was arbitrarily defined as '1', and data for the transgenic mice are given as the mean. The error bars are 95% confidence intervals (n = 6, *P < 0.05). Data from mice at 18 weeks of age are shown.

Figure 13. Protein expression of Hh downstream targets in murine osteoarthritic samples. There is increased enhancement of Ptch and Hhip immunostaining as the level of Hh signaling activation increases (representative sections shown). Data from mice at 18 weeks of age are shown.

Figure 14. Representative radiographs (lateral view) of murine knee joints with activated Hh signaling compared to control (WT, left), showing progressively worsening radiographic features as the level of Hh signaling activation increases (left to right). Scale bar, 1mm. Data mice at 18 weeks of age are shown.

Figure 15. Representative Safranin O histological sections from knees of wild-type mice and mice with activated Hh signaling (*Ptch1*^{+/-}, *Col2a1*-Gli2-transgenic and COL2-rtTA-Cre; *Gt(ROSA)26Sortm1(Smo/YFP)Amc*-transgenic mice), showing progressively worse histological findings associated with osteoarthritis (left to right). Data from mice at 18 weeks of age are shown. Scale bar, 200 μ m.

Figure 16. Protein expression of type X collagen, aggrecan NITEGE neoepitope, and the type I and type II collagen C1,2C neoepitopes in murine osteoarthritic samples. Immunostaining becomes more enhanced as the level of Hh signaling activation increases. Data from mice at 18 weeks of age are shown.

ICRS CARTILAGE SCORING SCALE	<i>Ptch1</i> ^{+/-}		<i>Col2A1</i> -Gli2		<i>COL2-rTA-Cre</i> ; <i>Gt(ROSA)26Sor</i> ^{<i>tm1(Smo^{YFP})Amc</i>}	
	Wt	Mutant	Wt	Mutant	Wt	Mutant
1) Surface	3.0 ± 0.0	2.8 ± 0.1	3.0 ± 0.0	1.8 ± 0.2	2.8 ± 0.1	0.0 ± 0.0
2) Matrix	2.7 ± 0.2	2.0 ± 0.2	2.7 ± 0.1	1.8 ± 0.2	3.0 ± 0.0	0.2 ± 0.2
3) Cell Distribution	3.0 ± 0.0	2.0 ± 0.2	3.0 ± 0.0	1.2 ± 0.1	2.7 ± 0.1	0.0 ± 0.0
4) Cell Population Viability	2.8 ± 0.1	2.2 ± 0.1	2.7 ± 0.2	1.4 ± 0.2	3.0 ± 0.0	0.4 ± 0.2
5) Suchondral Bone	3.0 ± 0.0	2.8 ± 0.1	3.0 ± 0.0	0.8 ± 0.1	2.7 ± 0.2	0.0 ± 0.0
6) Cartilage Mineralization	3.0 ± 0.0	3.0 ± 0.0	2.7 ± 0.2	0.0 ± 0.0	3.0 ± 0.0	0.0 ± 0.0
ICRS SUMMARY SCORE	17.5 ± 0.0	14.8 ± 0.0	17.1 ± 0.2	7.0 ± 0.2	17.2 ± 0.2	0.6 ± 0.2
P-value (U-test)	P=0.0475		P=0.0026		P=0.0026	

Table 5. ICRS Histological Grading. The knee joints from mice upregulated for Hh signaling were scored, examining *Ptc* +/- mice, *Col2a1*-Gli2 mice, and *COL2-rTA-Cre*; *Gt(ROSA)26Sortm1(Smo/YFP)Amc* mice versus their wild-type littermate controls. With increasing levels of Hh signaling activation in mice, there is worse ICRS-scored OA. Data from mice at 18 weeks of age are shown.

BONE HISTOMORPHOMETRY	<i>Ptch1</i>		<i>Col2a1-Gli2</i>		<i>COL2-rtTA-Cre; Gt(ROSA)26Sor^{tm1(Smo/YFP)Amc}</i>	
	Wt	Mutant	Wt	Mutant	Wt	Mutant
BV/TV (%)	39.17 ± 2.89	27.94 ± 1.32	36.39 ± 2.75	53.34 ± 1.35	34.23 ± 1.61	72.02 ± 1.82
Tb.Th (µm)	56.76 ± 1.90	49.82 ± 1.64	61.22 ± 2.13	84.58 ± 1.80	67.27 ± 1.69	120.60 ± 2.48
Tb.N (mm ⁻¹)	4.74 ± 0.66	1.80 ± 0.89	5.69 ± 0.54	1.80 ± 0.72	5.60 ± 0.43	1.98 ± 0.58
Tb.Sp (µm)	206.13 ± 12.05	149.62 ± 5.84	205.48 ± 10.65	138.93 ± 5.43	181.16 ± 11.01	126.46 ± 3.65

Table 6. Bone Histomorphometry in mice. Compared to their littermate controls, subchondral bone analysis in the knee joints of Col2a1-Gli2–transgenic and COL2-rtTA-Cre; Gt(ROSA)26Sortm1(Smo/YFP)Amc–transgenic mice with activated Hh signaling exhibit increases in bone volume (BV/TV) and trabecular thickness (Tb.Th), and decreases in trabecular number (Tb.N) and trabecular spacing (Tb.Sp). Notably, these changes are typical with secondary OA that is trauma induced. The *Ptch1*+/- mouse, notably, showed decreases in all four parameters measured.

SYNOVITIS SCORING SCALE	<i>Ptch1</i>		<i>Col2a1-Gli2</i>		<i>COL2-rTA-Cre; Gt(ROSA)26Sor^{tm1(Smo/YFP)Amc}</i>	
	Wt	Mutant	Wt	Mutant	Wt	Mutant
1) Synovial lining cell layer	0.6 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	1.8 ± 0.1
2) Density of resident cells	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.8 ± 0.1
3) Inflammatory infiltrate	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.8 ± 0.1
SYNOVITIS SCORE	1.0 ± 0.2	1.2 ± 0.2	1.0 ± 0.2	1.6 ± 0.2	1.2 ± 0.2	3.4 ± 0.2

Table 7. Synovitis Grading. Low-grade synovitis is present in the *Ptc +/-* mice and *Col2a1-Gli2* mice, which is typical in non-inflammatory OA. The *COL2-rTA-Cre; Gt(ROSA)26Sortm1(Smo/YFP)Amc* mutants, however, were slightly increased in their low-grade/mild-grade synovitis, and these mutants were associated with the worst ICRS-scored OA and highest level of Hh signaling activation and upregulation of known genes in OA. Data from mice at 18 weeks of age are shown.

CHAPTER FOUR

Hh signaling modulation can alter osteoarthritis

Modulating Hh signaling can attenuate the severity of osteoarthritis

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* Performed all procedures except for sectioning and immunostaining

SUMMARY

In Chapters Two and Three, we found that Hh signaling is upregulated in human and murine osteoarthritic cartilage samples, and that the level of Hh signaling activation predisposes to the severity of OA in mice. Whether Hh signaling modulation can alter the OA phenotype and the expression of known markers in OA are unknown. Here we show using human osteoarthritic cartilage explant samples that the expression level of known OA markers *ADAMTS5*, *COL10A1*, and *MMP13* can be up- or downregulated with Hh ligand treatment or pharmacological blockade respectively. Furthermore, using mice that are surgically-induced with OA by medial meniscectomy, we show that Hh signaling blockade pharmacologically or genetically in the *COL2-rtTA-Cre; Smo^{tm2Amc}* mouse can attenuate the OA cartilage phenotype and the expression of *Adamts5*, *Col10a1*, and *Mmp13* in cartilage. *Adamts5* is not regulated directly by Hh signaling through the Gli family of transcription factors. Using siRNA, here we show that Hh signaling modulates OA by regulating *Adamts5* expression through Runx2.

INTRODUCTION

Aberrant Hh signaling activation has been reported in a number of pathological conditions: medulloblastoma, basal cell carcinoma, prostate cancer, pancreatic cancer, gastrointestinal malignancies, and ovarian cancer are a few examples^{102,104}. With respect to bone and cartilage pathologies, Hh signaling upregulation has also been shown in chondrosarcomas, osteosarcomas, synovial chondromatosis^{32,97,105-107,136}, and as shown in Chapters Two and Three, osteoarthritis. Hh signaling blockade by pharmacological agents has been effective in attenuating a number of these pathologies *in vivo* in mice, including *Ptch1*^{+/-} mutants with medulloblastoma¹⁰¹, *Gli3*^{+/-} mutants with synovial chondromatosis¹⁰⁶, and in human chondrosarcoma xenografts in mice¹⁰⁷. Whether Hh signaling modulation can alter the OA phenotype and known genes upregulated in OA are unknown. I speculate that genetic and pharmacological blockade of Hh signaling can attenuate cartilage deterioration in OA, and as such, also downregulates known genes that are normally upregulated in OA.

Pharmacological inhibitors of Hh signaling work by binding to Smoothened, inactivating its ability to act downstream of processing the Gli family of transcription factors^{100,103,104,144-151}. These derivatives include cyclopamine, which

was first discovered for its teratogenic effects of holoprosencephaly, and other chemically-related derivatives such as $C_{31}H_{42}N_4O_5$.

Hh signaling could alter the OA phenotype by regulating gene expression through Gli-mediated transcription or through other pathways. *ADAMTS5*, *COL10A1*, and *MMP13* do not contain in their promoter regions any Gli-consensus binding sequences, and as such, are unlikely to be regulated directly by Hh signaling. It has been shown that the promoter region of *Adamts5* contains Runx2-consensus binding sequences¹⁵², and that Hh signaling regulates the expression of *Runx2* in chondrocytes. Therefore, using siRNA, whether Hh signaling can regulate *Adamts5* gene expression through Runx2 was investigated.

RESULTS

Genetic and pharmacological blockade of Hh signaling in mice with OA

To determine whether Hh signaling blockade attenuates the severity of OA, OA was surgically-induced in mice by arthrotomy and excision of the medial meniscus in the knee. Sham-operated controls consisted of arthrotomy only. Two

groups of mice were investigated: mice with pharmacological blockade and mice with genetic blockade of Hh signaling.

For pharmacological blockade experiments, OA was surgically induced in wild-type mice were surgically induced OA at 10 weeks of age and treated 1 week post-operative with a daily IP injection of the small molecule inhibitor agent C₃₁H₄₂N₄O₅. Mice were examined at 4, 8, and 16 weeks of treatment. As controls, mice with meniscectomy were administered with a daily IP injection of a carrier only; mice with a sham operation of arthrotomy only were administered a carrier only; and mice with a sham operation were administered the small molecule inhibitor. Eight mice were examined for each treatment or control group. I performed all surgeries and treatments.

For mice with genetic blockade of Hh signaling, OA was also surgically-induced in the knee by medial meniscectomy in cre-loxP mice at 10 weeks of age. Specifically, the *Smo*^{tm2Amc} transgenic mouse, which expresses a null allele of *Smo* upon the exposure to cre-recombinase, was crossed with the *COL2-rtTA-Cre* mouse to generate mice in which a null allele of *Smo* is expressed upon the administration of doxycycline in their drinking water. As controls, littermates that do not express *COL2-rtTA-Cre* were used, and amongst these mice, meniscectomy was performed

and doxycycline was administered; meniscectomy was performed and drinking water without doxycycline was administered; and sham operation was performed and doxycycline was administered. Mice were examined monthly and the longest time-point was 16 weeks of Hh blockade. Each treatment and control group consisted of eight mice. I bred and genotyped all mice, and performed all surgeries and treatments.

To assess the effectiveness of Hh signaling blockade, articular cartilage was microdissected from mice at 4, 8, and 16 weeks post-operatively and real-time PCR of downstream targets *Ptch1* and *Hhip* were performed. For both pharmacological and genetic blockade, sixteen mice were examined at each time point in these expression studies, (8 with meniscectomy and Hh signaling blockade, and 8 with meniscectomy only and carrier). Another sixteen mice were examined at each time point for each group (8 with meniscectomy and Hh signaling blockade and 8 with meniscectomy only and carrier).

To characterize the OA phenotype, joints were examined at 8 and 16 weeks post-operative by radiography, ICRS scoring, bone histomorphometry, synovitis scoring, and immunostaining of type X collagen and neoepitope degradation products for aggrecanase and collagenase. The same mice characterized for *Ptch1*

and *Hhip* immunostaining were used for these assessments (8 with meniscectomy and Hh signaling blockade and 8 with meniscectomy only). Also, real-time PCR experiments of known genes upregulated in OA, *Adamts5*, *Col10a1*, and *Mmp13*, were performed. Similarly, for these assessments, the same articular cartilage samples used to determine real-time PCR expression of Hh signaling targets were used for each group (8 with meniscectomy and Hh signaling blockade and 8 with meniscectomy only and carrier).

With pharmacological Hh blockade, we found a significant decrease in expression of downstream Hh signaling targets *Ptch1* and *Hhip* in mice with meniscectomy and Hh signaling blockade in comparison to mice with meniscectomy and carrier treatment across all time points (**Figures 17 and 18**). At 16 weeks of treatment, the significant decline in *Ptch1* and *Hhip* expression was 0.6 and 0.3 respectively.

Moreover, significant improvement of the joint phenotype was observed in mice with Hh signaling blockade pharmacologically and genetically. At sixteen weeks of treatment in mice with meniscectomy, histological examination (**Figure 19**) and ICRS scores increased substantially with Hh blockade (summary score 4.2 in mice with meniscectomy but without pharmacologic blockade -vs- 13.2 in mice

with meniscectomy and with pharmacologic blockade, $p=0.0154$, and 4.4 –vs– 12.2, for genetic blockade, $p=0.0125$) (**Tables 8 and 9**). Moreover, there was also a significant decline in gene expression of *Adamts5* and *Col10a1* across all timepoints, both of which are otherwise known to be upregulated in OA (**Figure 17 and 18**). At sixteen weeks of Hh signaling blockade, *Adamts5* and *Col10a1* levels decreased to 0.6 and 0.2 fold respectively with pharmacological treatment and 0.5 and 0.3 respectively with genetic blockade. However, the level of *Mmp13* expression did not appear to change with Hh signaling blockade across all time points. The downregulation of OA markers with pharmacological treatment was also corroborated with a decrease in immunostaining for type X collagen and neopeptides for NITEGE, and the C1,2C ($\text{Col2}^{3/4} \text{C}_{\text{short}}$) (**Figure 20**).

The individual ICRS parameters for bone remodeling showed significant improvements with Hh signaling blockade. Namely, for the subchondral bone and cartilage mineralization, the ICRS scores in mice with meniscectomy and pharmacological blockade versus meniscectomy and carrier treatment were 2.8 –vs– 0.6 and 3.0 –vs– 0.0 respectively; the scores in mice with meniscectomy and genetic blockade versus meniscectomy and carrier treatment were 1.2 –vs– 0.5, and 2.4 –vs– 0.2 (**Table 8**). However, our bone histomorphometric analysis showed only

an improvement in Tb.Th. in mice with meniscectomy and pharmacological treatment versus mice with meniscectomy and carrier treatment (**Table 10**).

Overall, all mice exhibited low-grade synovitis at all time points, which is typical in secondary osteoarthritis (**Table 11**).

Hh signaling alters known OA markers in human osteoarthritic cartilage

To determine whether Hh signaling modulation could alter the gene expression of known OA markers, human articular cartilage explants cultures were established from six patients undergoing total knee replacement. Prior to culture, human articular cartilage samples were first characterized by ICRS scoring, immunostaining for neoepitopes of aggrecan and collagen, and by real-time PCR of downstream Hh signaling targets and known markers of OA (Chapter One). Explant cultures were then treated with either Hh ligand or carrier as a control to upregulate Hh signaling, or with the pharmacological hedgehog blocking agent or carrier for 24 hours as previously reported¹⁰⁸. The pharmacological Hh blocking agent C₃₁H₄₂N₄O₅ was used. The effectiveness of Hh modulation was verified by determining the expressing of *PTCH1*, *GLI1*, and *HHIP* target genes, and markers of osteoarthritis *ADAMTS5*, *RUNX2*, *COL10A1*, and *MMP13* were also examined.

In our human cartilage explants treated with Hh ligand, we found a significant increase in downstream Hh signaling targets *PTCH1*, *GLI1*, and *HHIP*, and in correlation with a significant increase of osteoarthritic markers *ADAMTS5*, *RUNX2*, and *COL10A1*, with at least a 5-, 2.5-, and 11-fold increase respectively (**Figure 21**); however, with pharmacological Hh blockade, the same markers are decreased by at least 0.5, with *COL10A1* expression decreased by 0.2 (**Figure 21**).

Hh signaling regulates *Adamts5* expression through Runx2

We then asked how Hh signaling might regulate the OA phenotype by investigating the regulation of known genes upregulated in OA. For *ADAMTS5*, *COL10A1*, and *MMP13*, promoter analyses did not reveal at GLI consensus binding sites, and as such, Hh signaling likely does not regulate their expression. *ADAMTS5* is known to be regulated at the transcriptional level by *RUNX2*¹⁵², a gene also known to be expressed in osteoarthritis¹⁵³ and that is transcriptionally regulated by Hh signaling^{154,155}. As such, it is possible that Hh signaling regulates the expression of *ADAMTS5* by regulating the expression of *RUNX2*. To test for this possibility, primary cell cultures from articular chondrocyte samples were established from murine knee joints at P5. Chondrocytes cultures were grown to 70% confluence,

and for 24 hours, they were transfected with either an siRNA construct for Runx2 or a scrambled control, and treated with Hh ligand, the hedgehog blocking agent, or carrier as a control. With Hh ligand stimulation, there is at least a 2.5-fold increase in expression of *ADAMTS5* in chondrocytes compared to treatment with a carrier. With pharmacological blockade of Hh signaling, there is downregulated expression of *ADAMTS5* at least 0.5-fold compared to treatment with a carrier. However, with either Hh ligand stimulation or pharmacological blockade of Hh signaling, there is no difference in expression of *ADAMTS5* in cells expressing a scrambled siRNA sequence compared to cells in which *RUNX2* is knocked down by siRNA (**Figure 22**).

DISCUSSION

Here we show that pharmacological or genetic blockade of Hh signaling in mice can downregulate the expression of *Adamts5*, *Col10a1*, and *Mmp13* in articular cartilage normally upregulated in OA, and substantially improves the joint phenotype as shown by radiography, increased ICRS scores, decreased immunostaining of neoepitopes, and improved bone histomorphometric

parameters. Furthermore, we show that Hh signaling upregulation in human OA articular cartilage explants increases the expression of known OA markers, and downregulation of Hh signaling also decreases their expression. Using siRNA for Runx2, we suggest that Hh signaling influences the OA phenotype, in part, by regulating the expression of Adamts5 through Runx2.

In previous studies in mice deficient post-natally of Smoothed in chondrocytes, 14-month old mutants appear to have a thicker articular cartilage in comparison to littermate controls⁶⁹. Moreover, when Hh signaling is activated during development and post-natally in genetically modified mice, the rate of chondrocyte hypertrophy is accelerated. With these findings in mind, it is not surprising that Hh signaling activation in OA influences the articular chondrocyte phenotype, and that Hh signaling blockade would oppose the rate of chondrocyte hypertrophy. Our data on the pharmacological or genetic blockade of Hh signaling further supports this finding, as the fold decreased of *COL10A1* expression and decrease in immunostaining for type X collagen were substantial.

Previous studies with transient Hh signaling blockade using a pharmacological inhibitor resulted in premature closure of the growth plate in young mice 10 to 14 days old¹⁵⁶, but with no apparent effects in adult mice. In

comparison to another long-term treatment study in mice¹⁵⁷, our treatments with mice with daily IP injection with Hh signaling blockade for 16 weeks is the longest of such studies to date. Although no apparent growth plate defects could be determined, there were notable differences in the bone histomorphometric parameters in mice with 16 weeks of Hh signaling blockade. Typically, in post-traumatic OA, there are increases to the bone histomorphometric parameters of BV/TV and Tb.Th. and decreases to Tb.N. and Tb.Sp. We observed no statistical difference in Tb.Th with genetic or pharmacological blockade of Hh signaling determined by *U*-test, while changes to the other parameters hold to the same trends. Therefore, although the overall joint phenotype is significantly improved with attenuation of articular cartilage deterioration, Hh signaling blockade does not improve the subchondral phenotype to the same degree. Mice that are Gli2 deficient are osteopenic and demonstrate delayed endochondral ossification⁷²; moreover, Ihh and Gli2 promote osteoblastogenesis through Runx2¹⁵⁴. Thus, while long-term Hh signaling blockade in mice may significantly improve the cartilage phenotype in mice with secondary post-traumatic osteoarthritis, the deficiency of Hh signaling in bone metabolism may need to also be considered.

We found that Hh signaling blockade in human articular cartilage explants results in the downregulation of genes *ADAMTS5*, *COL10A1*, and *MMP13*, and in mice with pharmacological or genetic blockade of Hh signaling, there is also significant decrease in expression of *Adamts5* and *Col10a1*. However, *Mmp13* expression in these mice did not change with Hh signaling blockade. One possible explanation relates to the how *Mmp13* expression may be activated in these mice. *Mmp13* has been shown *in vitro* to be upregulated in chondrocytes subjected to cyclic mechanical strain^{158,159}. As aforementioned, the subchondral bone in these mice is compromised with Hh signaling blockade. Thus, even if Hh signaling blockade could inhibit *Mmp13* expression, the forces experienced by articular chondrocytes as a result of subchondral bone changes and joint movement may offset any effects of Hh signaling blockade *in vivo*.

The downregulation of *COL10A1* and *MMP13* is consistent with previous findings that Hh signaling regulates the rate of chondrocyte hypertrophy in the growth plate, and since type X collagen and collagenase 3 are markers of chondrocyte hypertrophy, Hh signaling blockade would result in decreased *COL10A1* expression.

Mice deficient in *Adamts5* are protected from articular cartilage deterioration in a similar model of surgically induced OA. Our data corroborates previous work that Hh signaling regulates *Adamts5* expression through *Runx2*. The regulation of other genes involved in osteoarthritis, namely *Col10a1* and *Mmp13* have also been shown in promoter studies to be under the transcriptional regulation of *Runx2*. Therefore, it is likely that Hh signaling regulates the expression of *Col10a1* and *Mmp13* through *Runx2*.

Taken together, our data shows that inhibition of Hh signaling attenuates the severity of OA in mice, and downregulates the expression of known OA-associated genes in human OA cartilage explants. By inhibiting terminal differentiation of chondrocytes, cartilage regeneration may be enhanced. Since there are a number of Hh-blocking agents poised for trials in humans¹⁰⁵, this raises the intriguing possibility that OA might be prevented in part using such agents. In cartilage regeneration strategies, there the new chondrocytes often develop a hypertrophic phenotype, and as such, inhibition of Hh signaling may also improve outcomes in cartilage repair and/or regeneration engineered therapies. However, transient inhibition of Hh signaling in mice as early as 4 days of age results in premature closure of the growth plate¹⁵³. Moreover, as aforementioned, mice deficient in *Gli2*

develop osteopenia⁷² and Hh signaling is important in regulating cell cycle regulators in stem cells to control hematopoietic regeneration¹⁶⁰. Therefore, Hh signaling blockade in humans must circumvent these potential side-effects in order to emerge as a bonafide therapy.

MATERIALS AND METHODS

Hh signaling blockade in mice with surgically-induced OA

As outlined in greater detail in Chapter Two, mice were surgically-induced with OA by medial meniscectomy at 10 weeks of age. For sham-operated controls, only arthrotomy was performed. For pharmacological blockade of Hh signaling, mice were administered with daily IP injections with N-[(3S,5S)-1-(2H-benzo[3,4-d]1,3-dioxolan-5-ylmethyl)-5-(piperazinylcarbonyl)pyrrolidin-3-yl]-N-(3-methoxyphenyl)methyl]-3,3-dimethylbutanamide (C₃₁H₄₂N₄O₅) at a dose of 50 mg/kg each day¹⁰⁸, or carrier as a control. The daily IP injections started one week after the surgery. For genetic blockade of Hh signaling, *COL2-rtTA-Cre* mice were crossed with *Smo^{tm2Amc}* mice using a previously reporting breeding scheme. The resulting *COL2-rtTA-Cre* X *Smo^{tm2Amc}* mouse expresses the null allele of *Smo* in chondrocytes upon the administration of doxycycline. As a control, littermates that do not express *COL2-rtTA-Cre* but were still treated with doxycycline were used. Cre-mediated recombination was confirmed by fluorescence and PCR. Knee joints were examined at 4, 8, and 16 weeks post-operative for their phenotype and for

real-time PCR expression studies. All studies were conducted under the appropriate animal protocols.

Joint phenotype analysis

As outlined in greater detail in Chapters Two and Three, Safranin-O and H&E histological sections were prepared and graded by radiography, by the ICRS scoring scale, by the *Krenn* scale for synovitis, the *Klein* semi-quantitative scale for immunostaining, and bone histomorphometry. All grading was performed blinded. All values were reported as a mean with standard errors calculated at a confidence interval of $p < 0.05$. For statistical differences between values, a Mann-Whitney *U*-test was performed.

RNA expression studies

As outlined in greater detail in Chapters Two and Three, articular cartilage was microdissected from the knee joints of mice at 18 and 26 weeks of age, correspond to 8 and 16 post-operative time-points. RNA was extracted, reverse transcription performed, and real-time PCR experiments were carried out, with relative expression normalized to an endogenous reference control ($2^{-\Delta Ct}$). In human

samples, downstream Hh signaling targets *PTCH1*, *GLI1*, *HHIP* were examined against housekeeping reference genes *GAPDH* and *ACTB*. Similarly for mouse samples, *Ptch1*, *Gli1*, and *Hhip* were examined against *Gapdh* and *Actb*. For known upregulated targets of OA, *ADAMTS5*, *COL10A1*, and *MMP13* were examined against genes *GAPDH* and *ACTB*. For mouse samples, *Adamts5*, *Col10a1*, and *Mmp13* were examined against *Gapdh* and *Actb*. Taqman primers were obtained from Applied Biosystems. For human expression studies, the gene expression normalized to the endogenous control was arbitrarily set to “1” for the samples with the least OA involvement. As such, by evaluating $2^{-\Delta\Delta C_t}$ between control and treated samples, the fold-change in gene expression was determined. Similarly, for murine expression studies, the gene expression normalized to the endogenous control was arbitrarily set to “1” for sham operated samples. As such, by evaluating $2^{-\Delta\Delta C_t}$ between the control and samples from knees with Hh signaling blockade, the fold-change in gene expression was determined. All samples were performed in triplicate, and all values were reported as the mean and errors as 95% confidence intervals. A t-test was used to determined statistical differences between values.

Human explant cultures

Human articular cartilage samples were cut into 2mm cubes and placed into DMEM culture media with 0.1% bovine serum albumin, 50 µg/ml ascorbic acid, and 1X antibiotic antimycotic. For Hh signaling upregulation, samples were treated for 24 hours with Shh-N at 5 µg/mL (R&D Biosystems) or tomatidine (R&D Biosystems) at 5 µg/mL as a control. For Hh signaling blockade, samples were treated for 24 hours with the pharmacological blocking agent $C_{31}H_{42}N_4O_5$ at 1 µM or carrier as a control.

siRNA experiments

Chondrocyte cell cultures were established from mice knee joints as previously described. Briefly, articular cartilage was microdissected from joints of newborn mice 5 to 7 days old. The samples were incubated at 37°C in collagenase D at 3mg/mL in DMEM for 45 minutes, fragmented by pipeting, followed by additional incubation at 37°C in collagenase D at 0.5mg/mL in DMEM overnight. The smaller remaining samples were further fragmented by pipeting and the entire suspension is left to stand for 2 minutes to allow any larger fragments to settle. The cell suspension was carefully withdrawn, passed by gravity through a 48 µm nylon

mesh, and centrifuged for 10 minutes at 200*g*. The supernatant was removed, the cell pellet was resuspended, and the cells were plated at a density of 8×10^3 cell/cm². At 70% confluence, siRNA experiments were performed.

A mouse pGIPZ lentiviral Runx2 shRNA_{mir} set (RMM4532, Open Biosystems/Thermo Scientific) was used for targeted inhibition of Runx2 mRNA. A FITC conjugated siRNA-A (sc-37007, Santa Cruz Biotechnology) consisting of a scrambled sequence that will not lead to degradation of any known cellular mRNA was used as a negative control according to the manufacturer instructions. After transfection, cells were treated with hedgehog ligand at 5 µg/mL, the hedgehog blocking agent at 1 µM, or carrier as a control. Transfected cells expressing GFP were sorted and knockdown of gene expression was confirmed using qRT-PCR.

Figure 17. Real-time RT-PCR data of articular cartilage from mice treated with the Hh blockade agent or carrier control. Data from samples obtained 16 weeks after surgery are shown. The level of expression in mice treated with the carrier is arbitrarily defined as '1', and the level of expression from mice treated with the blocking agent is given as the mean. The error bars are 95% confidence intervals (n = 6, *P < 0.05).

Figure 18. Real-time RT-PCR expression of Hh target genes and known genes expressed in OA in mice with surgically-induced OA subjected to Hh pharmacological blockade. Data from articular cartilage samples obtained from mice treated with a pharmacological Hh signaling blocking agent for 4, 8 and 16 weeks post-operative (grey, left to right). The level of expression in mice treated with the a carrier is arbitrarily defined as '1', and the level of expression from mice treated with the blocking agent is given as the mean. The error bars are 95% confidence intervals (n = 6, *P < 0.05).

Figure 19. Hh signaling blockade attenuates the severity of surgically-induced osteoarthritis in mice. (**a–c**) Representative histological sections stained with Safranin O of knees from mice in which osteoarthritis was surgically-induced by removal of the medial meniscus 4 months after surgery. (**a**) The knee from a representative control mouse without Hh signaling blockade, showing features of osteoarthritis such as a loss of

proteoglycan staining. The growth plate (red color) at the bottom of the section acts as an internal control for Safranin O staining intensity. **(b)** A section from a mouse treated with pharmacological Hh signaling blockade, showing improved histological characteristics. **(c)** A section from a COL2-rtTA-Cre; Smotm2Amc-transgenic mouse in which Hh signaling was genetically inhibited, showing similar improvements in histological characteristics (representative sections shown). Scale bar, 400 μm .

Figure 20. Mice with surgical removal of their medial meniscus in the knee develop OA and show enhanced immunostaining of type X collagen, and neoepitopes for aggrecan NITEGE and type I and II collagen C1,2C (Col2 ^{3/4} Cshort) (representative sections, left). However, upon Hh signaling blockade achieved either genetically (representative section, right) or with the use of a pharmacological agent (representative section, middle), protein expression is decreased. Data from mice 4 months after surgery are shown.

Figure 21. Hh modulation alters the expression of genes implicated in osteoarthritis in human samples. **(a)** Real-time RT-PCR expression data from human osteoarthritic articular cartilage explants treated with Hh ligand stimulation, showing increased expression of downstream targets of Hh signaling and genes expressed in osteoarthritis. Samples treated with the carrier are arbitrarily defined as '1', and the level of expression from Hh ligand treated samples is given as a mean. The error bars are 95% confidence intervals ($n = 6$, $*P < 0.05$). **(b)** Real-time RT-PCR expression from human cartilage explants treated with Hh blockade. Samples treated with the carrier are arbitrarily defined as '1', and data from samples treated with Hh blockade are given as means. The error bars are 95% confidence intervals ($n = 6$, $*P < 0.05$).

Figure 22. The regulation of *Adamts5* expression by Hh signaling is mediated by Runx2. With Hh ligand stimulation (left), there is upregulated expression of *Adamts5* in chondrocytes (grey) compared to treatment with a carrier (black). With pharmacological blockade of Hh signaling (second from the right), there is downregulated expression of *Adamts5* (grey) compared to treatment with a carrier (black). However, with either Hh ligand stimulation (second from the left) or pharmacological blockade of Hh signaling (right), there is no difference in expression of *ADAMTS5* in cells expressing a scrambled siRNA sequence (black) compared to cells in which Runx2 is knocked down by siRNA (grey). Samples treated with the carrier or scrambled sequence are arbitrarily defined as '1', and data from samples treated with Hh ligand or pharmacological blockade are given as a mean. The error bars are 95% confidence intervals (n = 4, *P < 0.05).

ICRS CARTILAGE SCORING SCALE	COL2-rtTA-Cre; <i>Smo</i> ^{tm2Amc}		Mouse Hh pharmacological blockade	
	Wt	Mutant	No Surgery	Surgery
1) Surface	2.7 ± 0.2	2.4 ± 0.2	3.0 ± 0.0	1.8 ± 0.1
2) Matrix	2.7 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	2.8 ± 0.1
3) Cell Distribution	3.0 ± 0.0	0.8 ± 0.2	2.7 ± 0.2	0.6 ± 0.2
4) Cell Population Viability	3.0 ± 0.0	2.6 ± 0.1	3.0 ± 0.0	2.2 ± 0.2
5) Suchondral Bone	2.7 ± 0.1	1.2 ± 0.2	2.8 ± 0.1	2.8 ± 0.2
6) Cartilage Minerlization	3.0 ± 0.0	2.4 ± 0.2	3.0 ± 0.0	3.0 ± 0.0
ICRS SUMMARY SCORE	17.1 ± 0.2	12.2 ± 0.2	17.3 ± 0.2	13.2 ± 0.2
P-value (U-test)	P=0.0102		P=0.0475	

Table 8. ICRS Histological Scores for OA Cartilage. Mice with Hh signaling blockade were examined and their knee joints scored, comparing surgically-induced OA versus a sham operation. Hh signaling blockade was achieved *in vivo* genetically using the COL2-rtTA-Cre; *Smo*^{tm2Amc} mouse and pharmacologically using a small-molecule agent. With Hh signaling blockade, there is an associated improvement of ICRS-scored OA. All errors are reported at a 95% confidence interval. A Mann-Whitney *U*-test was used to determine statistical differences (n=6). Data from mice 4 months after surgery are shown.

ICRS summary score	Weeks Post-Operative Treatment	COL11-rtTA-Cre; <i>Smo</i> ^{tm2Amc}		Mouse	
		Wt - Sham	Mutant - Surgery	Hh pharmacological blockade	Surgery
				Sham	
	4 weeks	18.0 ± 0.0	15.9 ± 0.2	17.8 ± 0.1	16.3 ± 0.2
	8 weeks	17.6 ± 0.2	13.8 ± 0.2	17.5 ± 0.2	14.7 ± 0.2
	16 weeks	17.1 ± 0.1	12.2 ± 0.2	17.3 ± 0.2	13.2 ± 0.1

Table 9. ICRS summary scores for mice surgically-induced with OA by medial meniscectomy with genetic or pharmacological blockade of Hh signaling. Mice were examined at 4, 8, and 16 weeks post-operative, comparing mice with medial meniscectomy versus mice with a sham operation.

BONE HISTOMORPHOMETRY	<i>COL2-rtTA-Cre;</i> <i>Smo^{tm2Amc}</i>		Mouse	
	Wt	Mutant	Hh pharmacological blockade	
			No Surgery	Surgery
BV/TV (%)	29.84 ± 3.82	39.29 ± 4.84	35.35 ± 4.92	56.64 ± 1.38
Tb.Th (μm)	65.90 ± 5.30	73.30 ± 2.18	75.50 ± 2.53	66.85 ± 2.88
Tb.N (mm ⁻¹)	5.80 ± 0.45	1.03 ± 0.21	5.91 ± 0.42	1.07 ± 0.49
Tb.Sp (μm)	174.27 ± 14.01	136.83 ± 7.08	176.45 ± 8.85	95.79 ± 10.44

Table 10. Bone Histomorphometry in Mice. With Hh signaling blockade, subchondral bone analysis in the knee joint of mice with surgically-induced OA by meniscectomy exhibit an increase in bone volume (BV/TV) and decreases in trabecular number (Tb.N) and trabecular spacing (Tb.Sp) in comparison to mice with a sham operation. However, with either pharmacological or genetic Hh signaling blockade, an improvement in trabecular thickness (Tb.Th) is observed. All errors are reported at a 95% confidence interval. Data from mice 4 months after surgery are shown.

SYNOVITIS SCORING SCALE	<i>COL2-rtTA-Cre;</i> <i>Smo^{tm2Amc}</i>		Mouse	
	Wt	Mutant	Hh pharmacological blockade No Surgery	Surgery
1) Synovial lining cell layer	0.6 ± 0.1	1.6 ± 0.1	0.6 ± 0.1	1.4 ± 0.1
2) Density of resident cells	0.2 ± 0.1	0.8 ± 0.1	0.2 ± 0.1	0.8 ± 0.1
3) Inflammatory infiltrate	0.2 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.4 ± 0.1
SYNOVITIS SCORE	1.0 ± 0.2	2.8 ± 0.2	1.0 ± 0.2	2.6 ± 0.2

Table 11. Synovitis Grading. Low-grade synovitis is present in mice with surgical removal of their medial meniscus, which is typical in non-inflammatory arthritis such as trauma-induced osteoarthritis. There is a slight increase in the number of layers of synovial cells in mice with meniscectomy versus sham operated knees. Genetic or pharmacological blockade of Hh signaling did not reduce the low-grade synovitis present in mice that underwent arthrotomy and medial meniscectomy. All errors are reported at a 95% confidence interval. Data from mice 4 months after surgery are shown.

CHAPTER FIVE

Conclusions and future directions

In this work, I asked whether a fundamental embryonic signaling pathway – the Hh signaling pathway, which is normal to the development and maturation of chondrocytes of hyaline cartilage – is dysregulated in a disease that affects primarily the old – the degenerative joint diseases of osteoarthritis. I found that Hh signaling is upregulated in human and murine OA cartilage, that activating the pathway predisposes to OA in mice, and that blocking the pathway *in vivo* in mice and *in vitro* in human OA cartilage improves the joint phenotype and markers of the disease. The purpose of this Chapter then is to integrate and propose further research into these findings.

1) Genes expressed during OA recapitulate gene expression in normal chondrocyte development of hyaline cartilage

I have shown the expression of downstream Hh signaling targets *PTCH1*, *GLI1*, and *HHIP* are upregulated in osteoarthritic articular cartilage samples. Other genes that are also developmentally expressed and/or influence normal growth plate chondrocyte development may regulate Hh signaling in OA. Such examples that are likely involved and could also be investigated in OA include the PTHrP,

beta-catenin mediated signaling, and Fgf-18 mediated signaling. The same experiments outlined in this work could be used in these investigations.

2) Aberrant Hh signaling activation upregulates genes associated with OA

I have shown using genetically modified mice activated for Hh signaling and human cartilage samples treated with Hh ligand that known genes associated with OA also become upregulated. Using siRNA, I suggest that for one of these genes, *Adamts5*, expression may be regulated indirectly by Hh signaling. Other similar experiments could be performed for *Col10a1*, and *Mmp13*. Moreover, other genes may also be OA regulated by Hh signaling. Thus, microarray experiments on human/murine OA cartilage explants or human/murine chondrocyte cultures with Hh signaling modulation (e.g. by pharmacological blockade, siRNA for Hh signaling components, Hh ligand stimulation) would lead to the determination of novel expression targets that are dysregulated in normal and/or osteoarthritic articular chondrocytes¹⁶¹.

3) Hh signaling blockade attenuates genes associated with OA

Using human OA articular cartilage, I have shown that a small-molecule inhibitor $C_{31}H_{42}N_4O_5$ can be used to downregulate known genes of OA, *ADAMTS5*, *COL10A1*, and *MMP13*. The use of pharmacological Hh blockade agents that bind and inactivate Smoothened are currently in clinical trials, and thus future commercially-benefiting considerations could involve the synthesis of small-molecule derivatives that are analogs and chemically-related to these agents. Moreover, there are other mechanisms to which Hh signaling downregulation can be achieved downstream of Smoothened. For example, the processed form of Gli3 acts a repressor of Hh signaling and SUFU is thought to act as a negative regulator of Hh signaling. Targeting these pathway components in OA could also lead into novel genes that are regulated by Hh signaling during OA, and translate into novel therapies.

4) Understanding articular chondrocyte development can lead to novel therapeutic targets in OA

Knowledge of Hh signaling in normal chondrocyte development contributed to our understanding of the pathological condition of OA. Thus, developing a

further understanding of chondrocyte development and how Hh signaling might interact with other developmental signaling pathways could translate into a greater understanding of OA and into future treatments.

For example, previous work examining the fate of progenitor cells in the limb suggest that cells that will become articular chondrocytes differ from those that will become cells of the growth plate. Therefore, identifying which genes are involved in the regulating these population of cells could provide insight into how to regulate the cell fate of articular chondrocytes in OA. These novel gene targets would then, in turn, be examined for their regulation by and/or of Hh signaling in OA. In addition to the experimental procedures outlined in this body of work, murine limb explant cultures, mouse embryonic fibroblast cultures, micromass cultures, microarray, and chromatin immunoprecipitation analysis would also be used to address this fundamental question in chondrocyte development.

As evidenced in both loss- and gain-of function studies post-natally in chondrocytes, modulating beta-catenin mediated signaling results in an OA-like phenotype in the joint. Since the level of beta-catenin is important in maintaining healthy articular chondrocytes, investigating how Hh signaling might interact with

beta-catenin could also provide insight into how to attenuate articular cartilage deterioration in OA. In previously published work, the *SFRP-1* gene has been shown to be a downstream target of Hh signaling, with Gli-consensus binding sequences identified in the *SFRP-1* promoter region. As such, Hh signaling could negatively regulate beta-catenin mediated signaling in chondrocytes through SFRP-1. Along with the experiments outlined above, the use of genetically modified mice altered for pathway components (*Sfrp-1*^{-/-} mouse, *Ctnnb1*^{tm2Kem} mouse, *Catnb*^{lox(ex3)} mouse), mice with expressing cre-recombinase under specific-promoter regulatory elements (e.g. *Col2*-*COL2-rtTA-Cre*), and pharmacological agents such as lithium that upregulate beta-catenin levels in future experiments could identify genes involved in this interaction, which could translate into novel therapies in OA.

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