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Mechanobiologic Mechanisms of Osteocyte Regulation of Bone-Cartilage Crosstalk

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Mechanobiologic Mechanisms of Osteocyte Regulation of Bone-Cartilage Crosstalk

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Contributions

I would like to acknowledge all of my co-authors on previously published manuscripts and book chapters that are contained in this dissertation. Chapter 3 is based on work in the peer-reviewed article titled “Mechanosensitive Control of Articular Cartilage and Subchondral Bone Homeostasis Requires Osteocytic TGFβ Signaling” by Karsyn N. Bailey, Jeffrey Nguyen, Cristal S. Yee, Neha S. Dole, Alexis Dang, and Tamara Alliston published in *Arthritis and Rheumatology*. Components of chapter 5 are based on the published peer-reviewed book chapter titled “Joint Cross-talk Among Bone, Synovium, and Articular Cartilage” by Courtney M. Mazur, Karsyn N. Bailey, and Tamara Alliston in *Orthopaedic Basic Science: Foundations of Clinical Practice*.

Abstract

Mechanobiologic Mechanisms of Osteocyte Regulation of Bone-Cartilage Crosstalk

by

Karsyn N. Bailey

Osteoarthritis (OA) is a degenerative joint disease characterized by disruption of multiple joint tissues, including articular cartilage and subchondral bone, resulting in chronic joint pain. The precise contributions of articular cartilage and subchondral bone in the etiology of OA are yet to be established, but growing evidence suggests that crosstalk between these two tissues is critical for joint homeostasis. Among the factors important for skeletal health is transforming growth factor beta (TGFβ) signaling, which is known to play an important role in each of the tissues of the synovial joint. The careful regulation of TGFβ signaling in the joint is dose-dependent and relies upon coordinated downstream signaling, mechanical cues, and tissue-specific function.

This work identifies osteocytic TGFβ signaling as a key component of bone-cartilage crosstalk and the mechanosensitive response to joint injury. In mice, increased joint loading due to injury represses osteocytic TGFβ signaling at the subchondral bone plate. Furthermore, the subchondral bone in transgenic mice with an osteocyte-intrinsic loss of TGFβ signaling is unable to respond to mechanical changes with injury, identifying a bidirectional role for osteocytic TGFβ signaling in joint injury. Without osteocytic TGFβ signaling, articular cartilage degeneration is worsened due to disrupted mechanosensation. Additionally, this work investigates the transient receptor potential vanilloid 4 (TRPV4) calcium ion channel as a possible calibrator of osteocytic TGFβ signaling. Findings from this work reveal osteocytes, and specifically osteocytic TGFβ signaling, as major contributors to joint homeostasis and OA progression.

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List of Abbreviations

IF	Immunofluorescence
MLI	Meniscal-ligamentous injury
MMP	Matrix metalloproteinase
OA	Osteoarthritis
PLR	Perilacunar/canalicular remodeling
SBP	Subchondral bone plate
TβRII	TGFβ type II receptor
TGFβ	Transforming growth factor beta
TRPV4	Transient receptor potential vanilloid 4

Chapter 1

The Sophisticated Function of TGFβ in the Synovial Joint

Introduction

The synovial joint consists of multiple tissues, including articular cartilage, subchondral bone, meniscus, and synovium, that engage in crosstalk to maintain joint health (1). During joint degeneration, the disruption of one tissue can lead to overall deterioration by shifting biomechanical loads, increasing inflammation, altering paracrine factors, or inducing aberrant cell-intrinsic signaling. The transforming growth factor beta (TGFβ) signaling pathway plays an intricate role in maintaining joint crosstalk by carefully regulating homeostasis of each tissue. The delicate balance of TGFβ signaling throughout the joint is non-linear and relies upon control of effector selection, mechanical cues, and spatial localization. Here we discuss the apparent sophistication of the function of TGFβ signaling in joint homeostasis and disease.

Molecular Function of the TGFβ Signaling Pathway

The TGFβ signaling pathway controls cell behavior through hierarchical, context-dependent, coordinated regulation (2-4). Latent TGFβ ligand remains locally sequestered and inactive within the extracellular matrix (ECM) and can be activated through a variety of mechanisms, including mechanical and chemical cues (2). This regulation of ligand is especially relevant in tissues with abundant ECM, such as hyaline cartilage and bone, and ensures that the effect of TGFβ is local and highly controlled. There are three differentially regulated TGFβ ligand isoforms, TGFβ1, β2, and β3, that are secreted as inactive ligand, consisting of the latency-associated peptide and the mature peptide. This complex is then secured to the ECM through latent TGFβ binding proteins (LTBP) and other proteins, such as biglycan, fibrillin, and thrombospondin,

many of which have also been implicated in joint homeostasis or disease (5). Not only do these mechanisms function to sequester TGFβ in the ECM, but they also add multiple layers of regulation to TGFβ activation, to be discussed later.

Once latent TGFβ ligand becomes activated, it binds to two type II serine/threonine kinase receptors (TβRII), which then recruit and phosphorylate two type I serine/threonine kinase receptors (TβRI) to form a heterotetrameric transmembrane receptor complex (6). The type I receptors are also termed activin receptor-like kinases (ALKs) and calibrate ligand binding affinity and effector selection. Upon ligand binding, the receptors are phosphorylated, which permits effectors to be recruited and activated, including the canonical Smad2/3 protein and multiple non-canonical effectors, such as Smad1/5/8, Erk, JNK, Akt, and p38 (5). Smad2/3-dependent TGFβ signaling is mediated by the ALK5 type I receptor, and Smad1/5/8-dependent TGFβ signaling is mediated by the ALK1 type I receptor, illustrating one mechanism of effector selection in TGFβ signaling (5). Once activated, phosphorylated Smad3 (pSmad3) forms a complex with the co-Smad Smad4 and localizes to the nucleus where it binds directly to Smad-binding elements in the promotor, or indirectly to other transcription factors, to alter gene expression (6).

TGFβ signaling exhibits exquisite internal control through negative feedback by a variety of mechanisms. Inhibitory Smads Smad6 and Smad7 are induced by TGFβ, activin, and BMP signaling and regulate signaling by competitively interfering with the receptor-Smad complex to prevent Smad activation (7). Additionally, Smad6 and Smad7 also inhibit TGFβ signaling by recruiting Smurf E3 ubiquitin ligases, Smurf1 and Smurf2, which ubiquitylate the TGFβ receptors and Smads, tagging them for degradation (7). Multiple other mechanisms of transcriptional and post-transcriptional control, including targeting components of the TGFβ pathway through microRNAs (miRNAs), sumoylation, and regulated localization, also tune the functional activity

of this pathway (5, 7). Another level of complexity in effector regulation is that in certain contexts, non-canonical effectors can themselves regulate the activity of Smad2/3, thus impacting downstream signaling and gene expression (2).

Here we focus on the function of TGFβ in skeletal cells and tissues in joint health and disease. Spatial and temporal control of TGFβ signaling is exerted at multiple levels of the pathway and is context dependent. Disrupting this balance can lead to joint degeneration.

Overview of Joint Health and Disease

While there are a variety of joint diseases, this review will focus primarily on the impact of TGFβ signaling in osteoarthritis (OA). OA is a chronic and debilitating joint disease that diminishes mobility and causes severe pain. OA affects 30 million Americans (8) and is a leading cause of disability globally (9). There are no currently available pharmacologic agents that can delay or prevent the development of OA, and end-stage OA often deteriorates to the point that a joint replacement is necessary. Given the dearth of treatment options for those suffering from OA, there is a pressing need to understand the cellular mechanisms that contribute to joint homeostasis. As mentioned previously, hierarchical regulation of the TGFβ signaling pathway indicates that differential function in various levels of the pathway could result in dysregulated TGFβ signaling. Importantly, either diminished or excessive TGFβ signaling can perturb joint health.

One genetic syndrome vividly demonstrates the importance of the TGFβ pathway on joint health. Aneurysms osteoarthritis syndrome (AOS) is a familial syndromic form of aortic aneurysm and early-onset OA that is the result of loss-of-function mutations of *SMAD3*, such as a truncating mutation removing the MH2 domain in one family or missense mutations resulting in conformational changes of the SMAD3 protein disrupting protein trimerization in other families

(10, 11). Other musculoskeletal anomalies can also be present in patients with AOS, including intervertebral disc degeneration, osteochondritis dissecans, and meniscal anomalies (11). Most mutations that lead to AOS are located in the MH2 domain, which is important for Smad association with TGFβ receptors, SMAD3/SMAD4 oligomerization, and Smad-dependent transcriptional control (11). Despite the loss-of-function mutation in *SMAD3*, patients with AOS paradoxically demonstrate increased total levels of SMAD3 protein, excessive activation and nuclear localization of SMAD2 and SMAD3, and higher levels of TGFβ1 in the aortic wall (10). Taken together, these observations suggest a role for enhanced TGFβ signaling in AOS. While the level of TGFβ and downstream effectors was not assessed in musculoskeletal tissues, it is possible that early-onset OA in these patients coincides with excessive TGFβ signaling in the joint.

Dysregulated TGFβ signaling has been found in various synovial joint tissues in OA. Bone from patients with OA has exhibited increased expression of TGFβ ligands and *SMAD3*, even at sites distant to joint degeneration (12-14), in addition to changes in collagen I proportion, *RUNX2* expression, osteocalcin, and WNT signaling targets, each of which plays a role in osteoblast differentiation (12, 14, 15). In human cartilage, *SMAD3* mRNA expression was higher in OA cartilage than in controls (16), providing evidence that in some cases, the TGFβ/SMAD3 pathway may be overactive in OA cartilage leading to degeneration. In synovium and synovial fluid from patients with OA, the amount of TGFβ ligand is a strong predictor of OA progression (17).

Genome-Wide Association Studies (GWAS) provide compelling evidence of the multi-level genetic role of TGFβ signaling in joint disease. Single nucleotide polymorphisms (SNPs) in introns or exons of the *SMAD3* gene have been associated with increased risk of OA (18-21). More specifically, a missense mutation of an exon of the *SMAD3* gene correlates with increased serum levels of MMP2 and MMP9, enzymes that play a role in articular cartilage degeneration (18).

Genetic variation in intronic SNPs of *SMAD3* also increases risk of hip OA and knee OA, although the specific function of the SNPs is unclear given their intronic location in the *SMAD3* gene (19-21). In concert with the increased risk of OA with *SMAD3* mutations, *SMAD3* may have a pleiotropic effect on bone mineral density (20). At the level of the ligand, a SNP within the *LTBP3* gene, a member of the latent TGFβ binding protein that would regulate the availability and activity of TGFβ ligand, was associated with the risk and clinical severity of hip OA (22). Noncoding mutations in *GDF5*, a member of the TGFβ super family, or p38 MAP kinase-mediated signaling, a non-canonical TGFβ signaling pathway, are causal variants in OA development (23). A single variant within the *TGFB1* gene can likewise be postulated as causal for OA development, which coincided with significant genome-wide enrichment for TGFβ-signaling-pathway genes, such as *LTBP1*, *LTBP3*, *SMAD3*, and *RUNX2* (24). Evidence of a genetic basis for TGFβ mutations in OA development motivates the mechanistic study of TGFβ in joint health in order to identify therapeutic targets.

Mechanical Regulation of TGFβ Signaling

At the cellular-level, changes in the mechanical microenvironment can alter the effect of TGFβ signaling at different hierarchies of the signaling pathway in each joint tissue (25). Cellular forces resulting from changes in ECM stiffness or cytoskeletal tension can directly activate TGFβ, rendering it available for downstream signaling (26), and colocalization of TGFβ receptors TβRI and TβRII for downstream signaling in chondrocytes is sensitive to cytoskeletal tension (27). A discrete cell substrate stiffness that is consistent with the physiologic stiffness of healthy articular cartilage stimulates maximal TGFβ-dependent Smad3 phosphorylation, nuclear localization, and

transactivation of chondrogenic genes; substrates that are more or less stiff reduce the chondrogenic influence of TGFβ signaling (28).

Joint loading presents a tissue-level mechanical cue that regulates the function of TGFβ ligand to modulate metabolic activity of chondrocytes. Both the superficial zone and the synovial fluid secrete latent TGFβ, which becomes activated with joint motion, and accumulates in the superficial zone of articular cartilage (29). Mechanical shearing within the joint activates latent TGFβ1 in the synovial fluid, rendering it available to stimulate TGFβ signaling in chondrocytes (30). As active TGFβ builds in the superficial zone, it is unable to penetrate deeper into middle and deep zones of cartilage (29). This proposes a mechanism by which chondrocytes in the superficial zone receive adequate active TGFβ ligand in order to maintain homeostatic TGFβ signaling. In fact, physiologic dynamic compression of devitalized cartilage explants does not directly activate ECM-bound latent TGFβ in the deep zone of articular cartilage (31). Therefore, a chondrocyte-intrinsic activation of TGFβ, such as through enzymatic activation, is required to liberate TGFβ and stimulate TGFβ signaling in the deep zone. Taken together, articular cartilage relies upon both mechanical loading and chemical activation to obtain active TGFβ in different articular cartilage zones.

Both physiologic and excessive loads of articular cartilage result in rapid induction of the canonical TGFβ signaling pathway (32). In response to dynamic compression of either physiologic or excessive force, articular cartilage explants increase the mRNA levels for established TGFβ-inducible genes *Serpine1* and *Smad7*, and production of TGFβ1 mRNA through an ALK5-dependent mechanism, demonstrating that TGFβ signaling and TGFβ production rely upon cartilage loading (32). Loss of compressive loading of cartilage explants results in a rapid loss of TGFβ signaling, as shown by the reduction of pSmad2 in chondrocytes and decreased expression

of TGFβ-responsive genes *Alk5*, *Smad7*, and *Serpine1*, that is restored upon re-loading (33). As a consequence of the unloading-induced loss of TGFβ signaling, *Col10a1* mRNA expression increased, a marker of chondrocyte hypertrophy, linking the function of joint loading and cartilage homeostasis through a TGFβ-dependent mechanism (33).

In response to load in either cartilage or bone, relative ALK5 expression increases, but the consequences of this change for joint homeostasis depend upon the cell compartment. In cartilage, loading reduced ALK1 expression and increased ALK5 expression to support canonical TGFβ signaling, unveiling one mechanism by which the balance between canonical and non-canonical TGFβ signaling in cartilage is preserved (32, 33). In aging cartilage, the relative ratio of ALK1/ALK5 is increased, thus favoring a non-canonical TGFβ signaling pathway (34, 35). In response to either physiologic or excessive mechanical load, aged cartilage has a reduced ability to induce canonical TGFβ signaling, both in reduced overall level of phosphorylated Smad2 as well as diminished nuclear localization of phosphorylated Smad2 (36). This demonstrates that loading as a mechanism to improve cartilage health may lose its efficacy as cartilage ages, and thus other avenues may become relevant as canonical TGFβ signaling is less favored. Interestingly, perturbed joint loading due to transection of the anterior cruciate ligament (ACL) increased ALK5 in subchondral bone mesenchymal stem cells (MSCs) without increasing ALK1, supporting Smad2/3 signaling (37), uncovering a tissue-specific response to altered joint loading.

Subchondral bone, likewise, is dynamically loaded in joint motion and provides mechanical support to the overlying cartilage. The load-induced response of bone is largely mediated by osteocytes, the primary mechanosensors of bone (38). Osteocytic TGFβ signaling is required for bone mechanosensation, as well as the control of bone quality (39, 40). Increased loading on the articular cartilage and underlying subchondral bone as a result of meniscal injury

in mice represses osteocytic TβRII in the subchondral bone plate (41). Likewise, mice with a transgenic ablation of TβRII in osteocytes demonstrate an inability of the subchondral bone to respond to injury-induced load, illustrating that TGFβ signaling is both regulated by and required for osteocytic mechanosensitivity (41). In the same model of osteocytic TGFβ deficiency, articular cartilage deteriorates, suggesting that the mechanosensitive function of the subchondral bone plate requires osteocytic TGFβ signaling in order to maintain the health of cartilage (41).

Tissue-specific Function of TGFβ Signaling in Joint Disease

TGFβ plays a tissue-specific function in joint homeostasis and joint disease, contributing to the sophisticated function TGFβ in the joint. For example, inhibiting TGFβ within the joint can prevent osteophyte formation, but simultaneously exacerbate cartilage degeneration (42). Tissue-level evidence in clinical specimens offers insight into the role of TGFβ in joint disease, and transgenic models of joint disease with a tissue-specific ablation of key receptors or effectors within the TGFβ signaling pathway provide a powerful tool for isolating the tissue- or cell-type-specific function of TGFβ in joint disease.

Bone-specific role of TGFβ in joint health and disease

The association of altered bone mass and mineralization with OA progression (1), coupled with differences in expression of genes critical for TGFβ signaling in OA bone (12-14), has led to an interest in understanding the role of TGFβ in bone during joint degeneration. Among the possible mechanisms by which bone may contribute to cartilage degeneration in joint disease, disrupted TGFβ signaling may participate by altering joint mechanics, disrupting nutrient and vasculature exposure through the osteocytic lacunocanalicular network, or changing levels of

paracrine factors that disrupt cartilage health. All three bone-cell types – osteoblasts, osteoclasts, and osteocytes – are distinctly regulated by TGFβ signaling in health and disease.

Studies of TGFβ signaling in whole-bone samples from OA human joints have found an increase in TGFβ ligand production and SMAD3 gene expression, relative to non-arthritic joints, even in bone specimens taken from sites distant from the osteoarthritic joint (12-14). Microarray gene expression of bone from OA patients demonstrates significant increases in expression of SMAD3 and of the osteogenic transcription factor and known TGFβ target gene, RUNX2 (12). These disruptions in TGFβ signaling occur in the context of altered WNT/β-catenin signaling (12, 14). Taken together, the differential gene expression in OA bone is consistent with perturbed osteoblast differentiation, altered bone formation, and decreased mineralization. Given the mixture of bone-cell types within whole bone, further delineating the specific contributions of each cell type and TGFβ signaling is necessary.

There is substantial evidence for an osteoblast-specific role of TGFβ in human OA using *in vitro* methods for primary osteoblast culture, the study of which allows for mechanistic insight into the function of TGFβ in osteoarthritic osteoblasts. Similar to whole bone extracts, primary osteoblasts obtained from osteoarthritic bone expressed increased levels of TGFβ and SMAD3 (43-47), which is responsible for aberrant matrix mineralization. OA osteoblasts exhibit a TGFβ-dependent abnormal ratio of COL1A1 to COL1A2, thus blunting the ability of osteoblasts to mineralize bone matrix (15, 45). When TGFβ1 is inhibited, OA osteoblasts are able correct the abnormal COL1A1 to COL1A2 ratio and mineralize normally (45). Increased SMAD3 in OA osteoblasts was associated with the calcium: phosphorous ratio in bone mineral, indicating SMAD3 may also be related to the dysregulated mineral deposition (43).

TGFβ signaling in OA osteoblasts exhibits an epistatic relationship with the Wnt signaling pathway, an important regulatory pathway in bone formation (43, 46, 47). TGFβ antagonizes the Wnt signaling pathway by increasing expression of Dickkopf-1 (DKK2), which diminishes osteoblastic mineralization in response to BMP-2 (46). Increased hepatocyte growth factor (HGF) expression in OA osteoblasts is responsible for increased TGFβ1 expression, which subsequently inhibits the Wnt signaling pathway and blunts BMP-2-dependent mineralization (47). Loss of HGF, therefore, favors BMP-2 signaling through Smad1/5/8 and stimulates the canonical Wnt signaling pathway (47).

Camurati-Engelmann disease (CED) is a disease of poor bone quality due to excessive levels of TGFβ1. A model of CED, in which the Col1A1 promotor drives expression of TGFβ1 with the point mutation H222D, results in higher levels of active TGFβ1 secreted by osteoblasts (37). Coupled with the disrupted subchondral bone, these mice demonstrate thinning articular cartilage with relative hypocellularity, thickening calcified cartilage, and excessive subchondral bone vascularity (37). The subchondral bone in these mice exhibits increased levels of nestin-positive MSCs and osterix-positive osteoprogenitors, suggesting that excessive TGFβ signaling alters the joint environment to recruit MSCs which increases the number of available osteoprogenitors (37). This MSC-dependent mechanism illustrates how excessive TGFβ secreted by subchondral bone osteoblasts can exacerbate OA.

On the other hand, inadequate subchondral bone TGFβ signaling also has negative consequences for joint homeostasis. Osteocytic TGFβ signaling is essential for osteocyte function, regulating the ability of osteocytes to maintain the lacunocanalicular network through osteocytic perilacunar/canalicular remodeling (PLR) (39, 40). In addition to their role in maintaining bone quality, osteocytes have recently emerged as cellular contributors to OA (41, 48). More

specifically, osteocytic TGFβ signaling is required for the response of subchondral bone to joint injury (41). Without this key signaling pathway, subchondral bone homeostasis is disturbed, resulting in cartilage degeneration and OA (41).

As mediators of bone homeostasis, osteoclasts are inevitably involved in joint health. The altered mass and mineralization in OA subchondral bone suggests an imbalance in bone remodeling by osteoblasts and osteoclasts, for which TGFβ is a critical coupling factor (49). Osteoclasts can play a direct or indirect role on the effect of TGFβ within the joint. Directly, osteoclasts require TGFβ for osteoclastogenesis and locally control the level of active TGFβ by liberating and activating latent TGFβ from the bone matrix (50-52). Aberrant TGFβ signaling within the joint can have broader effects on osteoclasts indirectly through osteoblast-osteoclast coupling in bone remodeling and by regulating RANKL production from osteocytes (39, 53). Therefore, targeting osteoclasts in animal models can have broader impacts on the level of active TGFβ signaling throughout the joint. For instance, an ACLT model of OA with alendronate reduced local TGFβ release, indirectly disrupting other joint tissues (54).

Cartilage-specific role of TGFβ in joint health and disease

Human osteoarthritic cartilage shows a complex disruption of homeostatic TGFβ signaling at the level of ligand, receptor, effector, and negative regulators, underscoring the importance of studying the hierarchy of altered TGFβ signaling in OA. There are at least three ways that altered TGFβ signaling in cartilage is implicated in OA. First, excessive activity of the pathway; in human OA cartilage, mRNA gene expression of TGFβ1 and β2 ligands was positively correlated with cartilage degeneration (55). Second, shifting from canonical to non-canonical signaling; on the receptor level, expression of ALK1 was correlated with chondrocytic MMP13 expression, whereas

ALK5 was correlated with aggrecan and type II collagen, suggesting a role for TGFβ signaling effector selection in expression of genes that are protective or deleterious for cartilage (34). Likewise, SMAD3 mRNA expression was significantly higher in OA cartilage, unexplained by DNA methylation in the promoter region (16). Third, aberrant negative regulation of the signaling pathway; human OA cartilage expressed higher levels of Smurf2, a multi-level negative regulator of TGFβ signaling (56). Smurf2 ubiquitylates Smads and the TβRI receptor to promote degradation and reduce TGFβ signaling. Therefore, high expression of Smurf2 indicates a general reduction in TGFβ signaling. In addition to direct regulation of the signaling pathway, TGFβ signaling can regulate and be regulated by microRNAs (miRNAs) to affect downstream signaling and OA gene expression. Expression of miR-140, for instance, is repressed by TGFβ signaling and significantly reduced in human OA cartilage (57-59), whereas miR-455, which promotes TGFβ/Smad3 signaling, is also repressed in OA cartilage (60). Overall, these observations demonstrate that aberrant TGFβ signaling in human OA cartilage, whether by suppressed or excessive activity, a shift from canonical to non-canonical signaling, or aberrant negative regulation can be associated with degeneration. Thus, TGFβ in cartilage plays a sophisticated and complex function and motivates the study of the multi-level regulation of TGFβ signaling in chondrocytes.

The need for an optimal level and type of TGFβ signaling is reinforced by findings in animal models. Increased TGFβ in the synovial fluid, whether by intra-articular administration of TGFβ or overexpression of TGFβ by the synovium, induces cartilage degeneration and osteophyte formation and results in increased mRNA of TGFβ1, β2, and β3 in the joint capsule substantially higher than controls (61-63). Conversely, inhibition of endogenous TGFβ by systemic injection of scavenging soluble TβRII prevents osteophyte formation but, likewise, induces proteoglycan degeneration in cartilage (42). At the chondrocyte-level, although the expression of both receptors

declines with age, the number of cells expressing ALK5 in OA cartilage reduce more rapidly than those expressing ALK1, consequently increasing the ALK1/ALK5 ratio and favoring Smad/1/5/8 signaling and increasing MMP13 expression (34). Together, excessive or inhibited TGFβ or a shift in the balance of canonical and non-canonical TGFβ signaling can, in certain contexts, exacerbate cartilage degeneration, highlighting the importance of optimal TGFβ signaling.

Transgenic mouse models with multi-system disrupted TGFβ signaling at the level of the receptor or effector have likewise demonstrated the requirement of TGFβ signaling in cartilage health. Mice with a dominant-negative mutation of TβRII in the articular cartilage, synovium, periosteum, and perichondrium exhibit a severe OA phenotype with substantial cartilage loss and osteophyte formation (64). This mutation suppresses the responsiveness to TGFβ ligand, thus promoting terminal differentiation of chondrocytes, reducing proteoglycan synthesis, and increasing type X collagen (64). Mice with a global loss of canonical TGFβ effector Smad3 similarly develop severe OA (65, 66). Taken together, these two mouse models illustrate the chondroprotective role of TGFβ in synovial joint tissues, motivating mechanistic study of the tissue-specific effect of TGFβ in articular cartilage.

Because OA involves the coordinated disruption of multiple joint tissues, mouse models employing chondrocyte-intrinsic mutations in the TGFβ signaling pathway can diminish the apparently confounding effects of TGFβ signaling in joint crosstalk. Toggling this pathway by targeting the receptors, effectors, and negative regulators in chondrocyte-intrinsic models, for example driving transgenic expression of Cre recombinase or other genes using a Col2a1 promoter, isolates the relative chondroprotective nature of TGFβ. As mentioned previously, Smurf2 is overexpressed in human OA cartilage, disrupting homeostatic TGFβ signaling (56). Mice with a chondrocyte-intrinsic overexpression of Smurf2 developed spontaneous OA with

decreased articular cartilage, increased subchondral bone sclerosis, and increased expression of type X collagen and MMP13 in the articular cartilage (56), demonstrating the chondrocyte-intrinsic requirement of balanced TGFβ signaling. Likewise, mice with a chondrocyte-intrinsic loss of *Smad3* develop OA and have decreased aggrecan and collagen II expression and increased MMP13 expression in the articular cartilage, thus indicating that chondrocyte-intrinsic *Smad3* is essential for expression of collagen II and aggrecan and the suppression of MMP13 (67). At the receptor level, ablating either chondrocyte-specific TβRII (68) or ALK5 (69) induces joint degeneration by increasing genes destructive for cartilage. More specifically, deletion of TβRII in chondrocytes exacerbates OA and induces Runx2, MMP13, and Adamts5 expression in articular cartilage (68). Interestingly, the deleterious effects of ablation of chondrocytic TβRII were mediated by MMP13 or Adamts5, and deletion of MMP13 or Adamts5 or treatment of MMP13 inhibitor ameliorated the OA phenotype (68). In a separate study, cartilage degeneration was rescued in mice with a chondrocyte-intrinsic ablation of TβRII by upregulating pSmad2 and downregulating MMP13, introducing a possible treatment targeting this pathway (70). Alternatively, deletion of ALK5 from chondrocytes resulted in a spontaneous OA phenotype with articular cartilage degradation, synovial hyperplasia, osteophyte formation, and subchondral bone sclerosis by reducing Smad3 activity and increasing *Mmp13*, *Adamts5*, and *Col10* expression (69).

TGFβ plays a key function in chondrocyte differentiation. Through Smad2/3-dependent signaling, TGFβ inhibits chondrocyte maturation (71, 72) and represses *ColX* expression (73), a marker of chondrocyte terminal differentiation. During chondrogenic differentiation of MSCs, Smad3 increases transcriptional activity of SOX9 and expression of *COL2A1* (72). Prior to chondrogenesis, MSCs require both ALK5-dependent Smad2/3 activity and ALK1-dependent Smad1/5/8 activity, in concert with Smad4, to induce collagen II expression (74-76). However,

the function of Smad1/5/8 shifts during chondrogenesis to promote terminal differentiation such that it inhibits collagen II production and increases MMP13, ColX, and alkaline phosphatase (74). TGFβ is able to induce its own signaling by increasing the expression of TGFβ receptors and Smad3 (77). However, long-term TGFβ exposure activates negative feedback loops that can reduce mRNA stability of TGFβ receptors, decrease Smad3 expression, and increase Smad7 expression to shift expression from chondroprotective genes such as Sox9 and COL2A1 to COL10A1 and induce chondrocyte maturation (77). In addition to the availability of TGFβ ligand and the relative balance of Smad2/3 signaling, the chondrogenic response to TGFβ depends on mechanical cues from the ECM (28), as mentioned previously.

Genes that are beneficial or deleterious for cartilage homeostasis are precisely controlled by TGFβ signaling, depending upon the presence or dominance of TGFβ receptor and the relative favoring of canonical and non-canonical signaling. At the TβRI level, the relative balance of ALK1/ALK5 regulates the chondroprotective function of TGFβ signaling. Whereas ALK5-dependent TGFβ signaling results in increased Collagen II and aggrecan, ALK1-dependent TGFβ signaling increases MMP13 expression in cartilage (34). Additionally, TGFβ/ALK5 signaling is required for proteoglycan 4 (PRG4) expression through regulating protein kinase A (PKA)-CREB signaling (69). Repression of cartilage destructive enzyme MMP13 by TGFβ requires TβRII, Smad3, and Runx2 (67, 68). TGFβ treatment of chondrocytes downregulated MMP13 and COLX expression and inhibition of TβRII using siRNA upregulated MMP13 and COLX expression (68). Co-transfection that inhibited both TβRII and Runx2 prevented this increase, indicating that Runx2 mediates the increase in MMP13 expression with impaired TGFβ signaling (68). Furthermore, chondrocytic Smad3 is required to repress Runx2-inducible MMP13 expression (67). Chondrocytes exhibit a time-dependent response to exogenous TGFβ, with an early repression of

MMP13 requiring Smad3 and a later induction of MMP13 requiring p38 (67). Importantly, in the absence of Smad3, non-canonical TGFβ signaling through p38 was favored, inducing the cartilage destructive-enzyme MMP13 (67). TGFβ also induces expression of ECM degradation inhibitor tissue inhibitor of metalloproteinases-3 (TIMP-3) through Smad2/3-dependent and ERK1/2-dependent mechanisms (78, 79).

In addition to its role in regulating expression of MMP13, chondrocyte-intrinsic Smad3 tempers the relative favoring of BMP signaling in chondrocytes. Primary chondrocytes from mice lacking Smad3 show enhanced BMP-related gene expression, resulting in increased phosphorylated Smad1/5/8 and collagen X expression, accelerating chondrocyte maturation (80). Interestingly, these chondrocytes increased *Smad1*, *Smad5*, *BMP2*, and *BMP6* mRNA expression, indicating a shift toward BMP signaling without Smad3 (80). This aberrant signaling could be blocked by overexpressing Smad2 and Smad3 or inhibiting BMP signaling (80).

TGFβ and synovial inflammation

As in cartilage, a balanced level of TGFβ signaling in the synovium and synovial fluid is required for healthy joint homeostasis, with insufficient or excessive TGFβ levels compromising joint health. TGFβ can induce a context-dependent suppressive or inflammatory immune response within the synovium (81). The synovium is a source of TGFβ ligand within the joint (30, 82), and the level of TGFβ in synovial fluid correlates positively with OA severity (17). TGFβ is also a key mediator of synovial hyperplasia in rheumatoid arthritis (83). Either excessive or suppressed TGFβ in the synovial joint can result in synovial hyperplasia. For instance, mice with reduced TGFβ signaling throughout the joint due to a dominant-negative mutation of TβRII in multiple joint tissues exhibit synovial hyperplasia (64). Excessive levels of TGFβ within the joint as a result of

intra-articular injection of exogenous TGFβ likewise induce synovial hyperplasia (61). On the other hand, synovial inflammation with joint degeneration involves production of inflammatory cytokines, such as IL-1. TGFβ antagonizes the degenerative effects of IL-1 on cartilage (84, 85). Together, TGFβ and the synovium can participate in bidirectional feedback, where synovium production of TGFβ can have broader effects on other joint tissues, and TGFβ signaling in other joint tissues can affect the synovium.

Mesenchymal stem cells and TGFβ in joint health and disease

In addition to the role of subchondral bone osteoblasts, osteoclasts, and osteocytes in synovial joint homeostasis, there is evidence of an influx of bone marrow MSCs into the subchondral bone during OA progression, which induce angiogenesis and differentiate into osteoblasts to further perturb the subchondral bone environment (37). Joint injury as a result of ACL transection increases the level of nestin-positive MSCs in the subchondral bone marrow and osteoprogenitor clusters in the bone marrow (37, 86). Systemic administration of a TβRI inhibitor can reduce the number of MSCs and osteoprogenitors, normalize the subchondral bone, and attenuate OA after injury (37). The benefits of the TβRI inhibitor on joint health are dose-dependent; higher concentrations of the TβRI inhibitor improve subchondral bone structure, but also induce proteoglycan loss, underscoring the tight control of TGFβ that is necessary for joint health (37). The relative benefits of targeting TGFβ signaling within the subchondral bone are mediated by nestin-positive MSCs; a nestin-positive MSC-specific ablation of TβRII recapitulates these findings by improving the subchondral bone microarchitecture and attenuating proteoglycan loss after ACL transection (37).

With increased levels of TGFβ in circulation after injury, targeting the TGFβ signaling pathway at the level of the ligand, rather than receptor, could likewise normalize the aberrant environment after injury. Local treatment to the subchondral bone or systemic treatment with an inhibitor of TGFβ1, β2, and β3 reduces the number and mobilization of nestin-positive MSCs in the subchondral bone and diminishes angiogenesis after ACL transection (37, 86). This effect was also dose-dependent, such that lower and higher concentrations led to proteoglycan loss (86). As discussed below, when considering the benefits of inhibiting excess TGFβ signaling post-injury, care must be taken not to compromise the essential role of this growth factor in other joint cell types where it also supports joint homeostasis.

TGFβ in Aging and OA

Aging can have broad effects on TGFβ signaling in multiple joint tissues. Because the prevalence of OA increases with age, considering the shift in function of TGFβ with age could identify a distinct age-related pathophysiology of cartilage degeneration. Furthermore, studying spontaneous age-related OA allows for the evaluation of joint degeneration in the absence of the mechanical and inflammatory changes that occur with joint injury in post-traumatic OA models.

Among the many roles of TGFβ in the joint is its interaction with IL-1, a pro-inflammatory cartilage destructive cytokine that is upregulated in age and in injury (87). TGFβ and IL-1 interact, such that TGFβ can protect against the deleterious effects of IL-1 on proteoglycan synthesis (84, 85). With age, however, IL-1-induced cartilage degeneration outpaces the protective effects of TGFβ (84, 85, 88), mediated in part by nitric oxide production (85). While TGFβ blocks IL-1-induced nitric oxide production in young mice, TGFβ is unable to induce the same in response old mice, likely due to decreased expression of TGFβ receptors (85). Interestingly, while TGFβ

showed protective effects on cartilage synthesis in the presence of IL-1, it exacerbated the inflammatory response, generating a severe synovitis, underscoring the importance of delineating the tissue-specific effects of TGFβ (84). A similar role for IL-1 and TGFβ crosstalk has been shown in equine cartilage, where TGFβ-induced proteoglycan synthesis was diminished by the addition of IL-1 (89).

Aging cartilage exhibits downregulation of TGFβ1, β2, and β3 expression, diminished TGFβ-induced proteoglycan synthesis, and decreased expression of both TβRI and TβRII (82, 88). This age-related suppression of TGFβ ligands and receptors results in decreased phosphorylated Smad2 without a reduction in overall Smad2 expression, suggesting reduced active canonical TGFβ signaling (88). Furthermore, a mouse model of spontaneous OA demonstrated lower levels of TGFβ3 ligand and phosphorylated Smad2 over the course of OA progression with a complete loss by one year of age, coinciding with an increase in BMP-2 expression with age (82).

Across species, articular cartilage exhibits a shift from canonical to non-canonical TGFβ signaling during aging (34, 35, 90). In murine cartilage, both ALK1 and ALK5 are expressed, thus phosphorylating both Smad1/5/8 and Smad2/3 in response to stimulation with TGFβ (34). Although expression of TGFβ type I and type II receptors decreases with age and with cartilage degeneration, the number of cells expressing ALK5 declines more markedly than those expressing ALK1, resulting in an increased ALK1/ALK5 ratio in cartilage from aged mice, thus favoring TGFβ-induced Smad1/5/8 signaling and MMP13 expression (34). A similar increase in ALK1/ALK5 ratio is observed in aged bovine cartilage (35), and aged chondrocytes from guinea pigs demonstrate a progressive shift of Smad2/3 signaling to Smad1/5/8 signaling (90), illustrating the age-related shift in TGFβ function across multiple species. Aged bovine cartilage demonstrates a reduced activation of Smad2/3 signaling and nuclear localization in response to either stimulation

with TGFβ or mechanical activation (36). These changes with age precede gross degeneration of cartilage, and therefore may be early signs of OA (36). Together, these findings emphasize the function of the relative balance of canonical and non-canonical TGFβ signaling in cartilage health.

Interactions of TGFβ and Other Tissues

Aging can also impact the TGFβ-induced collagen production in ligamental fibroblasts. With aging, the baseline levels of collagen and the ability of medial collateral ligament (MCL)-derived fibroblasts to synthesize collagen in response to TGFβ were diminished (91). However, while overall collagen synthesis was less at all doses in MCL-derived fibroblasts from older rabbits, the sensitization to TGFβ was higher in aged animals, such that relative to controls lacking TGFβ, collagen synthesis increased with increasing doses of TGFβ (91). Therefore, these age-dependent changes in ligaments could change the mechanical environment of the joint.

One area of interest in the intersection of TGFβ and joint homeostasis is the extent of crosstalk with the nervous system. TGFβ and nerve growth factor (NGF) demonstrate similarities in structure (5) and NGF is a key driver of the musculoskeletal pain response. Anti-NGF therapies in OA have reduced knee OA pain (92), but increased incidence of rapidly progressive OA (93), therefore understanding the extent to which TGFβ is involved in this could provide further context for this complex nature of targeting NGF.

Targeting TGFβ for Treatment of Joint Disease

The role of TGFβ in joint health and disease relies upon exquisite control of multiple factors, including optimal dose, tissue-specific effects, control of downstream TGFβ signaling, and local mechanical cues, thus illustrating the careful consideration that must be taken when

developing TGFβ-targeting treatments for OA. In homeostasis, TGFβ signaling exhibits multi-level regulation, resulting in precise control of the pathway and a context-dependent role in joint health. For instance, injury can induce excessive levels of TGFβ ligand within the joint (37), and therefore returning TGFβ to homeostatic levels may encourage joint health. However, injury also represses osteocytic TβRII in the subchondral bone, and reduced levels of TGFβ ligand may further reduce downstream osteocytic TGFβ signaling (41). Furthermore, in aging cartilage, the relative balance of canonical and non-canonical TGFβ signaling is disrupted (34, 35, 90), complicating the efforts of targeting this pathway by enhancing or inhibiting TGFβ.

Therapeutics have been developed that target different levels of the TGFβ signaling pathway in the clinical setting, primarily in the context of cancer or fibrotic disease (94, 95). Recently, human chondrocytes virally transduced with a gene containing TGFβ1 have been employed in the setting of patients with OA in a phase II clinical trial, which improved pain with potential reduction of progression of cartilage degeneration (96, 97). Further work is needed to fully uncover the relative benefit of delivery of TGFβ1-producing chondrocytes. Overall, the complex nature of the TGFβ signaling pathway suggests an etiology-dependent regulation of TGFβ may be better suited as a successful therapeutic of OA.

In conclusion, TGFβ plays a sophisticated function in the joint that is non-linear and depends upon effector selection, physical and mechanical cues, and tissue-specific function. Regulation of the TGFβ signaling pathway can occur at different levels and disrupting the homeostatic TGFβ signaling at any level of the pathway can have broad effects across multiple joint tissues as a result of joint crosstalk.

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Chapter 2

Materials and Methods

Mice

To assess the role of osteocytic TGFβ signaling in joint homeostasis, mice with an osteocyte-specific ablation of TGFβ type II receptor (TβRII) were generated by breeding homozygous TβRII$^{fl/fl}$ mice that possess *loxP* sites flanking exon 4 of the TβRII gene (Jackson Laboratories #012603) with hemizygous DMP1-Cre$^{+/-}$ mice (9.6-kb promoter), which express Cre primarily in osteocytes (Jackson Laboratories #023047) (1, 2). Mice were bred to generate TβRII$^{ocy-/-}$ mice (DMP1-Cre^{+}; TβRII$^{fl/fl}$) and littermate control mice (DMP1-Cre^{-}; TβRII$^{fl/fl}$). Animals were housed in groups in a pathogen-free facility at 22°C with a 12-hour light/dark cycle and supplied with standard irradiated mouse chow and water ad libitum. All animal procedures were conducted with approval from the Institutional Animal Care and Use Committee (IACUC) at the University of California San Francisco.

To analyze the basal joint phenotype, hindlimbs were collected from control and TβRII$^{ocy-/-}$ male and female mice at 16 weeks of age (n=7-11 mice per group). To analyze the effect of joint injury, male control and TβRII$^{ocy-/-}$ mice were aged to 8 weeks of age and subjected to bilateral sham surgery or meniscal-ligamentous injury (MLI) surgery, as described (n=9-11 mice per group) (3). Briefly, under sterile conditions, mice were anesthetized with general isoflurane, and incisions were made through the skin and joint capsule medial to the patella of both hind limbs. Once exposed, the medial collateral ligament (MCL) was transected, and the medial meniscus was removed. Sham-injured animals received bilateral incisions without MCL transection or meniscal removal. Incisions were closed with sutures, and animals received an injection of a sustained release buprenorphine analgesic (1 mg/kg). Importantly, bupivacaine was not used as a local

anesthetic because it is chondrotoxic and interferes with chondrocyte mechanotransduction (4-6). Hindlimbs were collected from euthanized mice 8 weeks post-surgery at 16 weeks of age. The effect of aging on the joint phenotype in TβRII$^{ocy-/-}$ mice was evaluated in hindlimbs collected from male control and TβRII$^{ocy-/-}$ mice at 6 months and 12 months of age (n = 8-11 mice per group).

The role of TRPV4 on cortical and subchondral bone osteocytes was assessed using mice with a systemic loss of TRPV4 (TRPV4$^{-/-}$) and wildtype C57BL/6 (TRPV4$^{+/+}$) littermates. Cortical bone was obtained from ulnas from 12-month-old male and female TRPV4$^{-/-}$ mice (7) (Gift from Dr. Wolfgang Liedtke) and compared with cortical femur from 12-month-old male C57BL/6 mice that, due to sample availability, were not littermates (n = 3-5 mice per group). Subchondral bone was obtained from coronally sectioned knees from 32-week-old male TRPV4$^{-/-}$ and littermate TRPV4$^{+/+}$ mice fed a control diet (n = 3 per genotype) (10% kcal; #12540B, Research Diets, Inc.) (8) (Gift from Dr. Farshid Guilak).

Table 2.1: Mouse lines

Name	Background	Source	Reference
DMP1-Cre$^{+/-}$	C57BL/6NJ	Jackson Labs, 023047	(1)
TβRII$^{fl/fl}$	C57BL/6J	Jackson Labs, 012603	(2)
TRPV4$^{-/-}$	C57BL/6J	Gift from Dr. Wolfgang Liedtke and Dr. Farshid Guilak	(7)
TRPV4$^{fl/fl}$	C57BL/6	Gift from Dr. Swapnil Sonkusare	(9)

MicroCT Analysis

A 4-mm region of the left hindlimb, centered on the joint, was scanned using a Scanco μCT50 specimen scanner with an X-ray potential of 55 kVp, current of 109 μA, voxel size of 10 μm, and integration time of 500 milliseconds. Thresholding and quantification of two regions of interest were performed as described (10-12).

First, the tibial plateau subchondral bone region of interest was designated 200 μm from the proximal surface of the tibia to exclude the subchondral bone plate and extended 250 μm

distally. Contours were delineated by a blinded reviewer at the periosteal surface with a linear boundary bisecting the medial and lateral halves, and bone volume fraction (BV/TV) was reported.

Second, the femoral subchondral bone plate (SBP) region of interest was contoured in a blinded manner in sagittal microCT images of the femoral condyles. Femoral SBP thickness, rather than tibial SBP thickness, was analyzed because tibial SBPs were too thin and too close to the growth plate for meaningful analysis (13). A grayscale 3-D map indicating SBP thickness was used for quantification. Using ImageJ, circles of diameter 750 μm were consistently placed on grayscale images, centered on the lateral anterior, medial anterior, lateral posterior, and medial posterior regions, as described (13). Mean pixel intensity in a defined area was quantified and then converted to SBP thickness in μm (n=9-10 mice per group). In representative samples, grayscale images were converted to pseudocolor for illustration in figures.

Qualitative differences in shape between control and TβRII$^{ocy-/-}$ mice were identified on 2D sagittal images of the knee, using cross-section of the patella as a landmark. Differences were consistently observed by a blinded grader (n=7-11 mice per group). Three-dimensional models of the femoral condyle SBP from one female control mouse and one female TβRII$^{ocy-/-}$ mouse were generated, and a series of homologous landmarks were applied in the software Landmark Editor to visualize differences in shape. A video transitioning between both models was created by warping the two individual objects using a thin-plate spline, which estimates changes in the objects using the minimum bending energy to transform one object configuration into another.

Histology

Tissues of interest were placed in 10% neutral buffered formalin for 48 hours and decalcified in 0.5M EDTA for two weeks. Samples were then dehydrated in ethanol and infiltrated

with CitriSolv. For knee analysis, right hind limbs were paraffin-embedded at 90 degrees of flexion in the coronal orientation. For cortical bone, ulnas or femurs were paraffin-embedded in the axial orientation. All samples were sectioned at 6 μm thickness and adhered to positively charged uncoated glass slides. Brightfield images were acquired on a Nikon Eclipse E800 microscope and fluorescent images were acquired on a Leica DMi8 confocal microscope.

Immunofluorescence

Immunofluorescence (IF) was used to identify cells expressing TβRII or sclerostin in the subchondral bone and articular cartilage of the knee. Sections (6 μm) were deparaffinized and hydrated prior to antigen retrieval using incubation in Innovex Uni-Trieve mild temperature retrieval solution (NB325) in a 65°C water bath for 30 minutes for detection of TβRII, or Trypsin/EDTA at 37°C for 25 minutes for detection of sclerostin. Samples were blocked with Background Buster (Innovex) for 10 minutes at room temperature, incubated in PBS/0.1% Tween for 5 minutes, and incubated overnight at room temperature with rabbit anti-TGF beta Receptor II antibody (1:500 for male mice, 1:250 for female mice, Abcam ab186838), goat anti-SOST/Sclerostin (1:200, R&D Systems AF1589), or species-specific non-immune IgG negative controls. After three 20-minute washes in PBS and one five-minute wash in PBS with 0.1% Tween-20, secondary antibody conjugated to Alexa Fluor 594 was applied for 60 minutes at room temperature (for TβRII: goat anti-rabbit 1:1000, Invitrogen AB_2534079; for sclerostin: donkey anti-goat 1:1000, Invitrogen AB_2534105). Following secondary antibody incubation, slides were again washed in PBS for three 20-minute washes and one five-minute wash in PBS with 0.1% Tween-20, and background was reduced using a 10-minute incubation in copper sulfate. Slides were washed again in two 10-minute PBS washes, and slides were then mounted with Prolong

gold antifade reagent with DAPI. Positively-stained cells were counted by a blinded grader in 20x visual fields from each joint quadrant using ImageJ and reported as % positive cells in each respective tissue (n=4-8 mice per group).

Histological Stains

The osteocytic lacunocanalicular network of subchondral bone was visualized using the Ploton silver stain with a Cresyl Violet counterstain, as described (10, 12, 14). Briefly, following deparaffinization and rehydration, slides were incubated in a silver solution containing 10% silver nitrate in water and a gelatin-formic acid solution for one hour. Slides were then rinsed in water for three five-minute washes, incubated in a 5% sodium thiosulfate solution for 10 minutes, and rinsed again in water for three five-minute washes. For the Cresyl Violet counterstain, samples were incubated for 10 minutes in a 0.1% Cresyl Violet working solution in acetic acid and sodium acetate. Slides were then dehydrated and mounted using CoverSafe Mounting Medium. Articular cartilage was visualized using the Safranin-O/Fast Green stain, in which sections (6 μm) were selected from the mid-joint region using the identification of the ACL and PCL as landmarks and stained, as described (12, 15).

To determine mean canalicular length, four images per joint quadrant were acquired within the subchondral bone at 100x visual field, and length was quantified in a blinded manner using ImageJ by tracing 10 canaliculi per osteocyte and three osteocytes per image (48 osteocytes per animal, n=5 mice per group). Canalicular lengths were averaged to obtain a mean canalicular length for each sample, and mean lengths were averaged and reported for each group.

For OA score, each quadrant of the knee (medial tibia, lateral tibia, medial femur, lateral femur) was imaged and graded by three blinded graders using modified Mankin (16) and OARSI

(17) grading schemes (n=9-11 per group). Scores from all graders were averaged to obtain a mean score, and mean scores were then averaged within each group. Total score represents the sum of all four quadrants.

Fluorescent Canalicular Analysis

Distal femurs for fluorescent canalicular network analysis were cleaned of soft tissue and fixed in 10% neutral buffered formalin for 48 hours at 4°C. Samples were demineralized in 0.5M EDTA (pH 7.5) for two weeks and cryoprotected in a sucrose gradient of 15% sucrose in PBS for 30 minutes and then 30% sucrose in PBS for 30 minutes. Femurs were embedded in Tissue-Tek OCT in the sagittal orientation, with the lateral side facing down. Femurs were cryosectioned until sections were approximately mid-way through the lateral femoral condyle and adhered to slides using the Leica CryoJane Tape-Transfer System. Cryosections were stained to visualize osteocytic cell membrane surfaces, F-actin cytoskeleton, and cell nuclei, as previously described (18-20). Briefly, cryosections were incubated with the hydrophobic lipophilic dye DiI (ThermoFisher) at 100 μM in 50% DMSO in PBS for six days at 4°C for the cell membrane surfaces, then Alexa Fluor 488-Phalloidin (ThermoFisher) at 165 nM overnight at 4°C for F-actin cytoskeleton, and then DAPI at 4 μM in PBS at room temperature for 30 minutes for cell nuclei. Samples were optically cleared in sequential incubations with 10%, 25%, 50% 2,2-thiodiethanol (TDE) dilutions in PBS for two hours each at room temperature, concluding with 95% TDE dilution overnight at 4°C. Sections were covered with 97% TDE dilution and a coverslip and sealed with a toluene free nail polish. Slides were imaged on a Leica DMi8 confocal microscope and presented as merged composite 3D images.

Cell Culture

In vitro studies were performed using OCY454 osteocyte-like cells (21). OCY454 cells were grown on tissue-culture treated plastic dishes coated with collagen type I in alpha-MEM with nucleosides (12571, Gibco) with 10% FBS and 1% antibiotic-antimycotic, maintained at 33°C and passaged every 2-3 days. For experiments noted as using differentiated OCY454 cells, cells were cultured to full confluence and then transferred to 37°C for 14 days for differentiation, with media changed every 2-3 days, prior to treatment.

The dose-dependent effect of pharmacologic TRPV4 activation and inhibition on downstream TGFβ signaling was assessed by pre-treating cells with TRPV4 agonist GSK1016790A (GSK101, Sigma-Aldrich) or TRPV4 antagonist (HC06747, Tocris Bioscience) for 30 minutes, and then stimulating with TGFβ-1 (5 ng/ml, Peprotech) for one hour. All groups within an experiment received the same concentration of DMSO, the vehicle in which HC06747 and GSK101 were delivered.

Western Blotting

Downstream effects of TRPV4 activity on TGFβ signaling were evaluated using Western analysis with commercially available antibodies of phosphorylated TGFβ effectors: anti-phospho-Smad3 (ab52903, Abcam), anti-phospho-AKT (4060, Cell Signaling), and anti-phospho-p38 (9211, Cell Signaling). Anti-beta actin antibody (ab8226, Abcam) was used to normalize for total protein content.

Cells were lysed with RIPA buffer (50mM Tris pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EDTA, supplemented with phosphatase (Pierce) and protease (cOmplete Mini, Roche) inhibitor tablets, and 1mM PMSF) and sonicated on ice using a cuphorn

sonicator (5x 15 s pulse, 45 s between pulses). Lysates were cleared by centrifugation at 10000 g for 10 minutes at 4°C and frozen at -20°C.

Western analysis was carried out using an SDS/PAGE protocol on 10% polyacrylamide gels and 5% stacking gels prepared in the lab. Protein lysates were thawed on ice and protein samples were prepared with 5x Laemmli buffer and boiled at 90°C for 10 minutes. Equal volumes of protein were loaded in each well. For 10-well gels, 50 µl was loaded into each well, containing 10 µl 5x Laemmli buffer diluted with 40 µl of lysate. For 15-well gels, 25 µl was loaded into each well, containing 5 µl 5x Laemmli buffer diluted with 20 µl of lysate. The gel was run at 60V until the protein passed through the stacking gel, and then at 150V until the 37 kDa band of the ladder was near the bottom of the gel. Protein was transferred from the gel to a nitrocellulose membrane at 100V for one hour. Blots were blocked in 5% milk in TBS for one hour and then washed three times in TBST for five minutes each. Blots were probed with primary antibody for one hour at room temperature or overnight at 4°C. Primary antibodies were prepared in 1% milk in TBST for β-actin or 5% bovine serum albumin for antibodies for phosphorylated proteins. Following primary antibody incubation, blots were washed three times in TBST for 10 minutes each, and then probed with anti-mouse and anti-rabbit secondary antibodies conjugated to 800CW IRDye fluorophores for one hour at room temperature. Blots were washed five times in TBST for five minutes each and then replaced with TBS until imaging. Protein was detected using an Odyssey infrared imaging system (LI-COR Biosciences), and images were quantified using Image Studio Lite (LI-COR Biosciences). Band intensity for proteins of interest was normalized to the band intensity of β-actin.

Table 2.2: Antibodies Used for Immunostaining and Western Analysis

Target	Product Number	Concentration
Immunostaining		
TβRII	Abcam ab186838	1:500 for male mice, 1:250 for female mice
Sclerostin	R&D Systems AF1589	1:200
Alexa Fluor 594 Goat anti-rabbit	Invitrogen AB_2534079	1:1000
Alexa Fluor 594 Donkey anti-goat	Invitrogen AB_2534105	1:1000
Western Blotting		
pSmad3	Abcam ab52903	1:2000
β-actin	Abcam ab8226	1:2500
pAkt	Cell Signaling 4060	1:2000
p-p38	Cell Signaling 9211	1:1000
800CW Goat anti-rabbit	LI-COR AB_621843	1:15,000
800CW Donkey anti-mouse	LI-COR AB_621847	1:15,000

RNA Sequencing

To identify changes in osteocytic gene expression with age, RNA enriched for osteocytic populations was isolated from humeri from C57BL/6 mice across four ages: 2 months, 12 months, 24 months, and 30 months. Humeri were dissected to remove the muscle and periosteum, the epiphyseal ends were trimmed, and marrow was removed by centrifugation (22). Bones were flash frozen in liquid nitrogen, immersed in QIAzol, and homogenized on ice with a Polytron tissue homogenizer. RNA extraction was completed using the miRNeasy Mini Kit (Qiagen), according to manufacturer's instructions.

For RNA-seq, samples were sequenced on the Illumina HiSeq 4000 at the UCSF Functional Genomics Core. Single-end 50bp RNA-seq reads were aligned to the Ensembl mouse GRCm38.87 reference genome using STAR 2.5.2a aligner. We obtained 745 million total reads with an average of 78.7% of these reads aligning uniquely to the mouse genome. Read counts were normalized to the those from 2-month-old control mice and reported as such.

Statistical Analysis

Comparisons between genotypes, for each male mice and female mice, were tested using an unpaired two-tailed Student's t-test. Comparisons among genotype and injury for male control and male TβRII$^{ocy-/-}$ mice and comparisons among TGFβ and TRPV4 treatments for *in* vitro experiments employed two-way ANOVA followed by Tukey post-hoc tests. Statistical analyses were performed in GraphPad Prism 8 (GraphPad Software, Inc.). As appropriate for each outcome, values are expressed as mean ± SD for outcomes reporting a mean of individual measurements or ± SEM for outcomes reporting a mean of mean measurements, and p-values less than 0.05 were considered statistically significant, with sample size "n" specified in figure legends.

Additional Considerations

The MLI model was employed to reliably induce posttraumatic OA. In studies detailed herein, MLI surgeries were bilateral, and the right hindlimb was used for histological analysis and the left hindlimb was used for microCT analysis, which limited the conclusions that could be drawn from our findings. Given the distinct differences in SBP thickness and condylar shape in TβRII$^{ocy-/-}$ mice, future studies should utilize the same limb for microCT and histological analysis, which would provide more spatial context for the genotype-dependent differences. Logistically, samples can be fixed and scanned for microCT prior to decalcification and paraffin embedding. Conducting these analyses on the same limb would also allow for additional assays to be completed on the contralateral limb, such as 3D fluorescent canalicular analysis, which could give insight into the changes of the canalicular network at the osteochondral junction.

In the studies that investigated the role of osteocytic TGFβ signaling in age-related OA, there were originally three age groups: 6 months, 12 months, and 18 months. Difficulties in aging

TβRII$^{ocy-/-}$ mice increased the fatality rate and shortened their lifespan, resulting in premature euthanasia of TβRII$^{ocy-/-}$ at 15 months. Unfortunately, several control mice were aged to 17 months, so this aged group did not have appropriately age-matched controls. The OA phenotype, as well as the bone phenotype, in this aged group suggest a confounding effect with the older control mice. Further work will utilize age-matched 15-month-old control mice to complete this group and further dissect the role of osteocytic TGFβ on age-related OA. For all Mankin scores reported here, the same three blinded graders evaluated the OA phenotype in these mice: CM, JS, and CY. As further work collects appropriate age-matched control mice, it will be critical to calibrate scores to the original graders in order to compare to the 6-month- and 12-month-old groups.

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Chapter 3

Mechanosensitive Control of Articular Cartilage and Subchondral Bone Homeostasis Requires Osteocytic TGFβ Signaling

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Abstract

Objective: TGFβ signaling plays a complex tissue-specific and non-linear role in osteoarthritis (OA). We sought to identify the osteocytic contributions of TGFβ signaling to OA.

Methods: To uncover the role of osteocytic TGFβ signaling in joint homeostasis, we used 16-week-old male and female mice with an osteocyte-intrinsic ablation of TGFβ type II receptor ($T\beta RII^{ocy-/-}$) and assessed defects in cartilage degeneration, subchondral bone plate (SBP) thickness, and SBP sclerostin expression. To further investigate these mechanisms in 16-week-old male mice, we perturbed joint homeostasis using the medial meniscal/ligamentous injury (MLI) model at 8 weeks of age, which preferentially disrupts the mechanical environment of the medial joint to induce OA.

Results: In all contexts, independent of sex, genotype, or medial or lateral joint compartment, increased SBP thickness and SBP sclerostin expression were spatially associated with cartilage degeneration. Male $T\beta RII^{ocy-/-}$ mice, but not female $T\beta RII^{ocy-/-}$ mice, have increased cartilage degeneration, increased SBP thickness, and higher levels of SBP sclerostin, demonstrating that the role of osteocytic TGFβ signaling on joint homeostasis is sexually dimorphic. With changes in joint mechanics following injury, control mice increase SBP thickness, subchondral bone volume, and SBP sclerostin expression. $T\beta RII^{ocy-/-}$ mice, however, are insensitive to subchondral bone changes with injury, suggesting that SBP mechanosensation requires osteocytic TGFβ signaling.

Conclusion: Our results provide new evidence that osteocytic TGFβ signaling is required for a mechanosensitive response to injury, and that osteocytes control SBP homeostasis to maintain cartilage health, uncovering osteocytic TGFβ signaling as a novel therapeutic target for OA.

Introduction

Osteoarthritis (OA), considered a "whole joint" disease, is characterized by the irreversible disruption of multiple joint tissues including articular cartilage and subchondral bone (1). While the relationship between cartilage degeneration and subchondral bone changes is an established hallmark of OA, the causal mechanisms driving the coordinated loss of homeostasis in these two tissues remains unclear. The subchondral bone plate (SBP), for example, undergoes dynamic remodeling in OA, with thinning and increased remodeling in early joint disease and thickening in spatial association with cartilage degeneration in late-stage OA (1-7). SBP changes can impact both the mechanical and biological environment of the joint. Given its proximity to the articular cartilage, the SBP can impact the stress concentration at the articular cartilage during joint loading (2), which is one possible explanation for the spatial association of SBP thickening and cartilage degeneration. Additionally, the SBP acts as a physical boundary, regulating cartilage exposure to vasculature, oxygen, nutrients, and cytokines (1). During OA progression, the SBP permeabilizes with increased vascular invasion, disrupting the homeostatic biological environment and aggravating cartilage degeneration (1). While SBP thickening is considered an indisputable sign of late-stage OA, the underlying mechanisms of the thickening are still largely unknown, and whether it contributes to or is the consequence of cartilage degeneration remains elusive.

The transforming growth factor beta (TGFβ) signaling pathway plays an important role in both bone and cartilage homeostasis, and imbalanced TGFβ signaling results in disease in each tissue, such as osteogenesis imperfecta in bone (8) and OA in cartilage (9-11). TGFβ signaling is critical for maintaining bone mass and quality (12-15) and balanced TGFβ signaling in each tissue compartment is integral for joint health. In some contexts, TGFβ signaling protects against OA (9-11, 16-19), and in other contexts TGFβ signaling exacerbates OA (20, 21). Two key concepts

clarify the sophisticated function of TGFβ in joint homeostasis. First, TGFβ action in the joint is tissue compartment- and cell type-specific. Second, the effect of TGFβ on the joint is non-linear, such that an optimal level of signaling is required for joint homeostasis, and highly active, suppressed, or an imbalance of canonical and non-canonical TGFβ signaling drives joint degeneration. More specifically, loss-of-function mutations in the canonical TGFβ effector SMAD3 increase the incidence of OA in humans (16, 18) and in mice (9-11). In the absence of chondrocyte-intrinsic Smad3, TGFβ signaling activates non-canonical pathways that prevent cartilage anabolism and stimulate MMP13-dependent cartilage catabolism (9-11). Conversely, systemic inhibition of TGFβ signaling in mice has been shown to exert dose-dependent benefits on joint health, in part, by suppressing TGFβ signaling within mesenchymal stem cells (MSCs) (20, 21). Taken together, this further emphasizes the need to uncover the precise role of TGFβ signaling in each joint tissue to maintain homeostasis.

Alterations in systemic, chondrocytic, and MSC-intrinsic TGFβ signaling have profound effects on joint homeostasis. The role in OA of TGFβ signaling in bone cell types is less well-defined. Recent evidence demonstrates a new role for osteocytes in human and mouse OA (22); however, the role of osteocyte-intrinsic TGFβ signaling in joint homeostasis is unknown. In addition to coordinating the flow of nutrients through the canalicular network and regulating the mechanosensitivity of bone, osteocytes may also impact cartilage through a process known as perilacunar/canalicular remodeling (PLR) (23). During PLR, osteocytes secrete acid and proteases to dynamically resorb and then replace their surrounding bone matrix to maintain the mineral homeostasis, the canalicular network, and bone quality (24-27). Osteocyte-intrinsic inhibition of TGFβ signaling causes PLR suppression, including disrupted canalicular networks, dysregulation of PLR enzyme expression, increased trabecular bone volume, and inferior bone quality in male,

but not in female, mice (23, 28). Thus, osteocytic TGFβ signaling functions to maintain both biological and structural properties of bone, each of which could impact cartilage integrity. Combined with the known role of TGFβ signaling in skeletal mechanobiology (29-32), osteocytic TGFβ signaling may contribute to joint homeostasis through a number of mechanisms.

To dissect the osteocyte-specific contributions of TGFβ signaling on joint homeostasis, we tested the hypothesis that loss of osteocytic TGFβ signaling exacerbates OA through its control of subchondral bone. We used a DMP1-Cre transgenic mouse model (33) to ablate the TGFβ type II receptor (TβRII) (34) to study the osteocyte-intrinsic role of TGFβ signaling on subchondral bone and OA development in male and female mice. Our findings reveal that osteocytic TGFβ signaling is critical for cartilage health and SBP homeostasis in male, but not female, mice. Additionally, in the setting of disrupted joint mechanics due to injury, we find that osteocytic TGFβ signaling is required for mechanosensitive changes in SBP thickness and sclerostin expression. This work advances our understanding of the cellular mechanisms involved in bone-cartilage crosstalk and implicates osteocytes as drivers of OA through their control of the SBP.

Results

Requirement of osteocytic TβRII for subchondral bone canalicular homeostasis

To determine the specificity of the DMP1-Cre promotor in the joint, we evaluated the expression of TβRII in control (DMP1-Cre^{-};TβRII$^{fl/fl}$) and TβRII$^{ocy-/-}$ (DMP1-Cre^{+};TβRII$^{fl/fl}$) knees. Relative to a control non-immune IgG, immunofluorescence revealed TβRII expression throughout the meniscus, cartilage, subchondral bone, and bone marrow of control mice (**Figure 3.1A, 3.1C**). In male TβRII$^{ocy-/-}$ mice, TβRII expression was apparent in all of these tissues and reduced in subchondral bone osteocytes. A blinded semiquantitative analysis of TβRII expression

revealed a significant 38% decrease in percentage of TβRII-positive osteocytes within the subchondral bone between control and TβRII$^{ocy-/-}$ male mice, with no change in percentage of TβRII-positive articular chondrocytes (**Figure 3.1B**) or in osteocyte density. This selective disruption of TβRII in subchondral bone allows us to evaluate the role of osteocytic TGFβ signaling in joint homeostasis.

We have previously reported that male TβRII$^{ocy-/-}$ mice demonstrate hallmarks of suppressed osteocyte perilacunar/canalicular remodeling (PLR) within the cortical femur, with reduced expression of several essential PLR enzymes and a corresponding reduction in canalicular length (23, 28). We evaluated the effect of osteocyte-intrinsic TβRII-deficiency on canalicular length in subchondral bone. TβRII$^{ocy-/-}$ mice exhibit a significant reduction in canalicular length within the subchondral bone of the tibial plateau and femoral condyles compared with control mice at 16 weeks of age (**Figure 3.1D, 3.1E**). In our model of an osteocyte-specific loss of TGFβ signaling, we find evidence of impaired PLR in the subchondral bone of male TβRII$^{ocy-/-}$ mice.

Requirement of osteocytic TβRII for cartilage homeostasis is sexually dimorphic.

Given the evidence for osteocyte dysfunction in human OA (22) and the cell-type specific role of TGFβ in the pathogenesis of OA, we evaluated the cartilage phenotype of TβRII$^{ocy-/-}$ mice to elucidate the role of osteocytic TGFβ signaling in joint homeostasis. When compared with control mice, male TβRII$^{ocy-/-}$ mice had significantly increased Mankin scores in the whole joint and in the medial and lateral compartments, with a disruption of articular cartilage structure, loss of proteoglycan staining, increased hypertrophic chondrocytes, and increased subchondral bone thickness (**Figure 3.1F-3.1I**). TβRII$^{ocy-/-}$ mice did not have a statistically significant increase in OARSI score (data not shown).

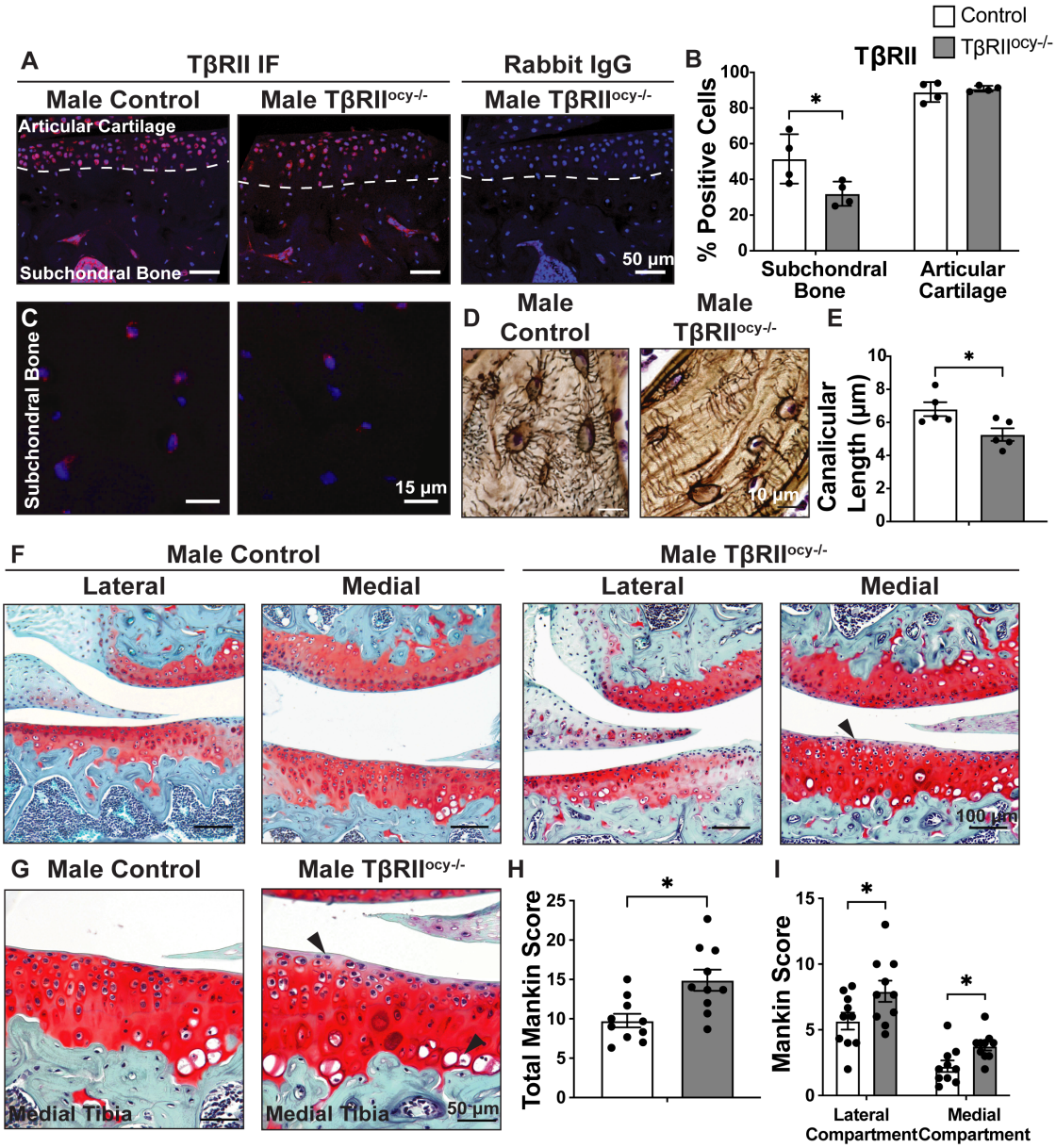


Figure 3.1: Osteocytic TGFβ signaling is required for canalicular network and articular cartilage homeostasis in 16-week-old male mice. Immunofluorescence (IF) of TβRII shows a significant repression in subchondral bone osteocytes with no difference in expression in articular chondrocytes in male TβRII$^{ocy-/-}$ mice compared with control mice (A, scale bar = 50 μm, C, scale bar = 15 μm), demonstrating an osteocyte-specific loss of TβRII in TβRII$^{ocy-/-}$ mice (B, n = 4 mice per genotype). Lacunocanalicular networks of the subchondral bone are disrupted in male TβRII$^{ocy-/-}$ mice compared with control mice (Ploton Silver stain, D, scale bar = 10 μm), resulting in significantly decreased canalicular length (E, n = 5 mice per genotype). 16-week-old male TβRII$^{ocy-/-}$ mice have significantly increased Mankin scores in the whole joint (H, n = 10 mice per genotype) and the medial and lateral joint compartments (I, n = 10 mice per genotype) when compared with control mice (Safranin-O/Fast Green stain, F, scale bar = 100 μm, G, scale bar = 50 μm). Arrows denote proteoglycan loss and hypertrophic chondrocytes in TβRII$^{ocy-/-}$ medial tibia. Bar graphs represent mean ± SD (B) and mean ± SEM (E, H, I). *p<0.05 between genotypes by unpaired Students t test.

Although female TβRII$^{ocy-/-}$ mice also had reduced osteocytic expression of TβRII in the subchondral bone by 45% (**Figure 3.2A, 3.2B**), for canalicular length and Mankin score, female TβRII$^{ocy-/-}$ mice were indistinguishable from female controls (**Figure 3.2C-3.2G**). Therefore, phenotypic differences in male and female TβRII$^{ocy-/-}$ mice are not the result of differences in reduction of osteocytic TβRII, and the requirement of osteocytic TβRII for subchondral bone lacunocanalicular network maintenance and articular cartilage homeostasis is sexually dimorphic.

Increased subchondral bone plate thickness in male, but not female, TβRII$^{ocy-/-}$ joints

The mechanisms by which osteocytic defects in TGFβ signaling drive cartilage degeneration in male mice are unknown but could be the result of disrupting the mechanical or biological environment of the subchondral bone in the joint. Changes in joint mechanics and subchondral bone plate (SBP) thickness are closely linked to OA development, suggesting that disruption of subchondral bone mechanics exacerbates cartilage degeneration (2, 35). To examine the relationship between osteocytic TβRII, subchondral bone mechanics, and cartilage degeneration, we quantified SBP thickness in male and female mice, focusing on the load-bearing posterior regions of control and TβRII$^{ocy-/-}$ distal femora (7). We found that the SBP of male TβRII$^{ocy-/-}$ mice is significantly thicker than in control mice on both the lateral and medial condyles (**Figure 3.3A**, **3.3B**). The load-bearing femoral SBP thickness of female TβRII$^{ocy-/-}$ mice, however, does not significantly differ from female controls (**Figure 3.3F**, **3.3G)**. Coupled with the sex-specific effect of osteocytic TGFβ signaling on the canalicular network and SBP thickness in the bone, the sexual dimorphism in OA raises the possibility that altered mechanics at the joint in male

TβRII$^{ocy-/-}$ mice leads to joint degeneration, while female TβRII$^{ocy-/-}$ mice have relatively normal joint mechanics and therefore healthy cartilage homeostasis.

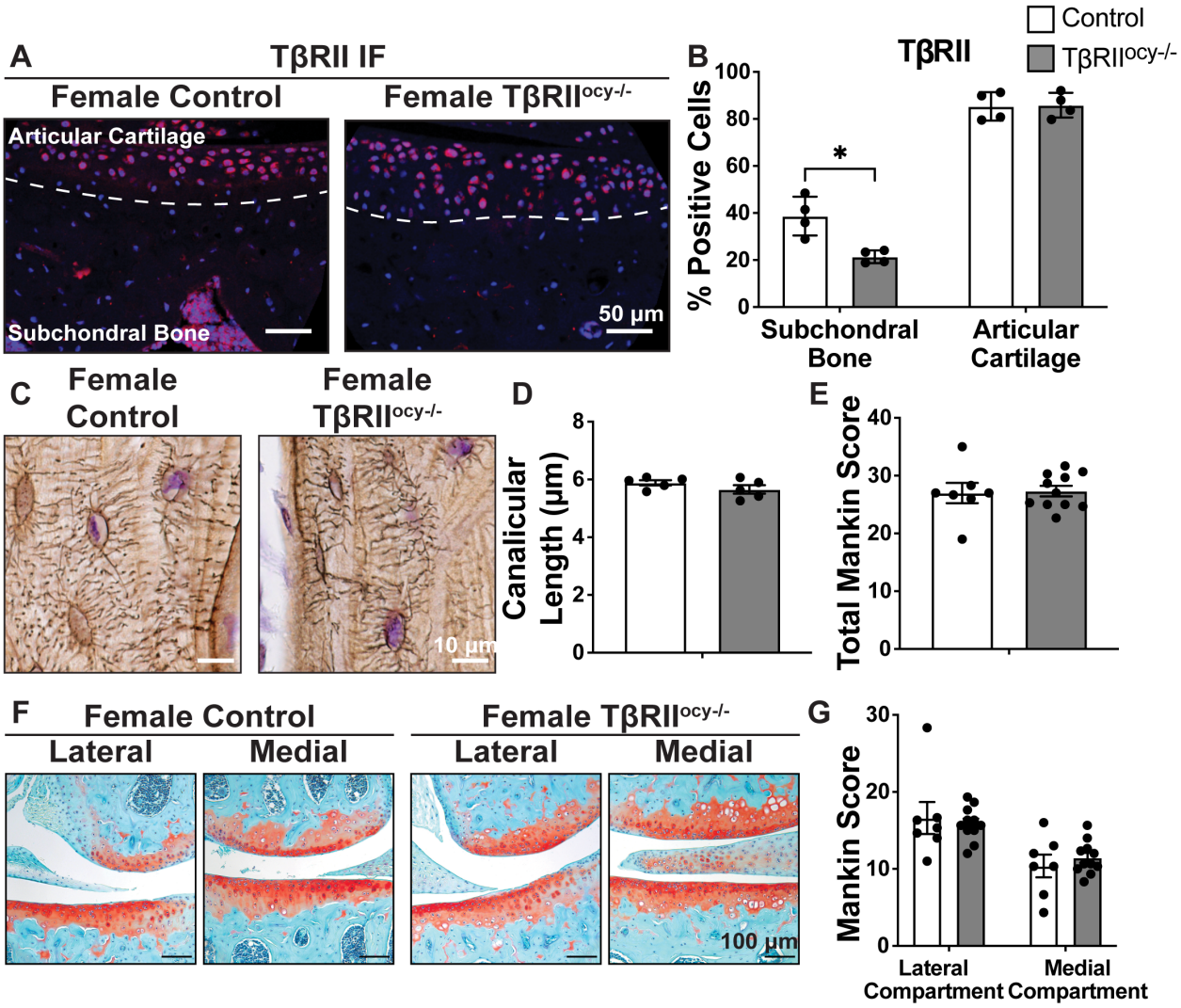


Figure 3.2: The requirement of osteocytic TβRII for canalicular network and articular cartilage homeostasis is sexually dimorphic. Compared with female control mice, female TβRII$^{ocy-/-}$ mice demonstrate a repression of TβRII-positive osteocytes in the subchondral bone with no difference in TβRII expression in articular chondrocytes using immunofluorescence (IF) of TβRII (A, B, scale bar = 50 μm, n = 4 mice per genotype). Lacunocanalicular networks between female TβRII$^{ocy-/-}$ mice and female control mice are indistinguishable (Ploton Silver stain, C, scale bar = 10 μm), resulting in no difference in canalicular length (D, n = 5 mice per genotype). Female TβRII$^{ocy-/-}$ mice at 16 weeks of age show no differences in cartilage degeneration when compared with control mice (Safranin-O/Fast Green stain, F, scale bar = 100 μm), resulting in no difference in Mankin scores in the whole joint (E, n = 7-11 mice per genotype) or in the medial and lateral joint compartments (G, n = 7-11 mice per genotype). Bar graphs represent mean ± SD (B) and mean ± SEM (D, E, G). *$p<0.05$ between genotypes by unpaired Students t test.

Coupled deregulation of sclerostin expression, SBP thickness, and cartilage homeostasis

Sclerostin is a negative regulator of bone formation, and TGFβ regulates load-dependent bone formation in a sclerostin-dependent manner (32, 36). We sought to determine if SBP sclerostin expression is sensitive to osteocytic TGFβ signaling, and furthermore, if this regulation is sexually dimorphic. Therefore, we examined sclerostin expression in male and female TβRII$^{ocy-/-}$ SBP. Sclerostin was not detected in chondrocytes or articular cartilage in any condition (**Figure 3.3C, 3.3H, 3.4B**). We find a significant increase in sclerostin expression in male TβRII$^{ocy-/-}$ SBP when compared with male controls (**Figure 3.3C-3.3E**), corresponding to the increase in SBP thickness observed in TβRII$^{ocy-/-}$ mice. We previously observed persistently elevated levels of sclerostin in unloaded and loaded bone from male mice expressing a dominant negative form of TβRII under the control of the osteocalcin promoter (32), and we find the same to be true in male mice with an osteocytic ablation of TβRII. However, the female SBP showed no genotype-dependent difference in sclerostin expression (**Figure 3.3H-3.3J**), indicating that sclerostin expression at the SBP in TβRII$^{ocy-/-}$ mice is also sexually dimorphic. Thus, we observe a close relationship among cartilage degeneration, SBP thickness, and deregulated sclerostin expression; in male TβRII$^{ocy-/-}$ mice, we observe a genotype-dependent increase in cartilage degeneration in concert with increased SBP thickness and increased sclerostin expression, whereas in female TβRII$^{ocy-/-}$ mice, none of these outcomes differ from female controls. The extent to which these structural or biological changes in subchondral bone drive OA is unclear.

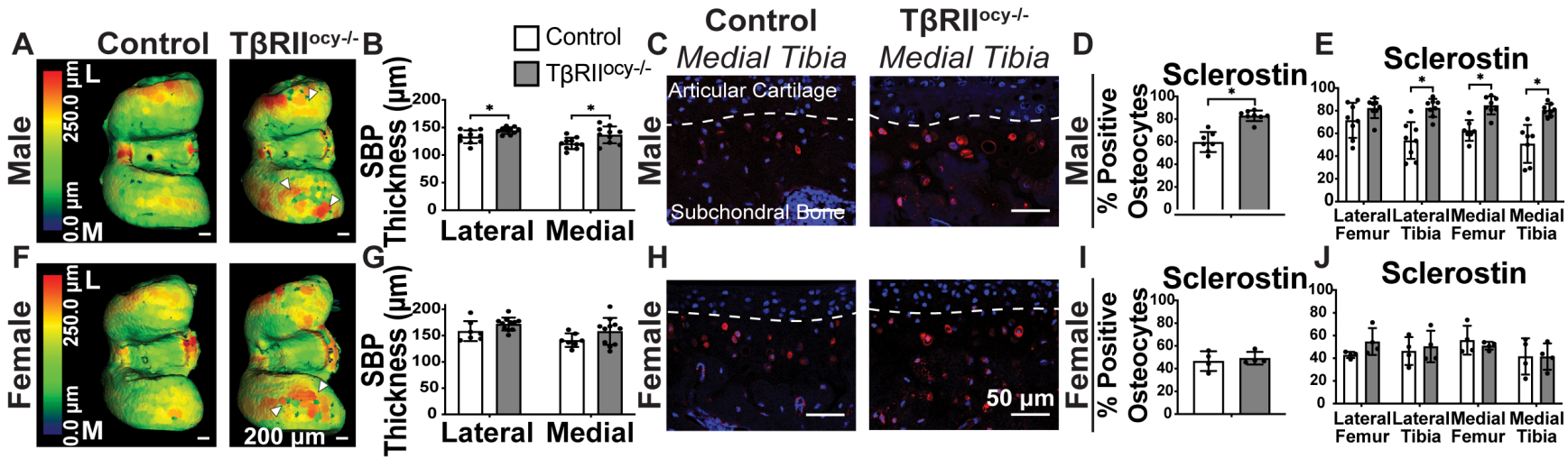


Figure 3.3: Subchondral bone plate thickness and sclerostin expression are increased in male, but not in female, TβRII$^{ocy-/-}$ mice. Male TβRII$^{ocy-/-}$ femoral condyles have spatial differences, denoted by white arrows, in subchondral bone plate (SBP) thickness compared with male control mice (microCT thickness maps, A, scale bar = 200 µm), resulting in significantly higher SBP thickness in the medial and lateral condyles (B, n = 10 mice per genotype). SBP thickness in female TβRII$^{ocy-/-}$ mice is not significantly different from female control mice (F, G, scale bar = 200 µm, n = 7-11 mice per genotype). Immunofluorescence (IF) of sclerostin at the SBP is significantly increased in male TβRII$^{ocy-/-}$ mice compared with male control mice overall and in the lateral tibia, medial femur, and medial tibia joint quadrants specifically (C, D, E, scale bar = 50 µm, n = 8 mice per genotype). IF of sclerostin in female TβRII$^{ocy-/-}$ mice is indistinguishable from female control mice (H, scale bar = 50 µm), resulting in no significant differences in sclerostin expression overall or in each independent joint quadrant (I, J n = 4 mice per genotype). Bar graphs represent mean ± SD. *p<0.05 between genotypes by unpaired Students t test.

Osteocytic TGFβ signaling is required for injury-induced changes in subchondral bone and sclerostin expression

Given the need to clarify the relationship between OA progression and the changes in SBP thickness and sclerostin expression in TβRII$^{ocy-/-}$ mice, and the extent to which these may be mechanically regulated, we implemented an OA model in which a meniscal ligamentous injury (MLI) preferentially disrupts the mechanical environment on the medial side of the joint. MLI of control mice significantly increased SBP thickness, subchondral bone volume, and SBP sclerostin expression on the medial side of the joint but elicited only a more modest increase in SBP thickness on the lateral side (**Figure 3.4**). Importantly, MLI did not affect any of these parameters on either the 'loaded' medial side or the lateral side of the TβRII$^{ocy-/-}$ subchondral bone, indicating that injury-induced changes in subchondral bone require osteocytic TGFβ signaling (**Figure 3.4E, 3.4H, 3.4K**). Furthermore, on the medial side, apparent genotype-dependent differences in subchondral bone volume and sclerostin expression in sham mice disappeared following MLI (**Figure 3.4F, 3.4I)**. Together with prior evidence implicating TGFβ in the anabolic response of bone to mechanical load (32), these data reveal that osteocytic TGFβ signaling is required for mechanosensitive changes in subchondral bone formation and sclerostin expression.

Increased cartilage degeneration in injured male TβRII$^{ocy-/-}$ mice

Compartmental evaluation of OA severity in this medial injury model allows dissection of mechanisms of joint crosstalk. We find that injury further exacerbates joint degeneration in TβRII$^{ocy-/-}$ mice compared with injured control mice, resulting in a significantly increased total Mankin score due to more severe disruption of the articular cartilage structure, loss of proteoglycans, and increased hypertrophic chondrocytes (**Figure 3.5A**, **3.5B**). Compartmental

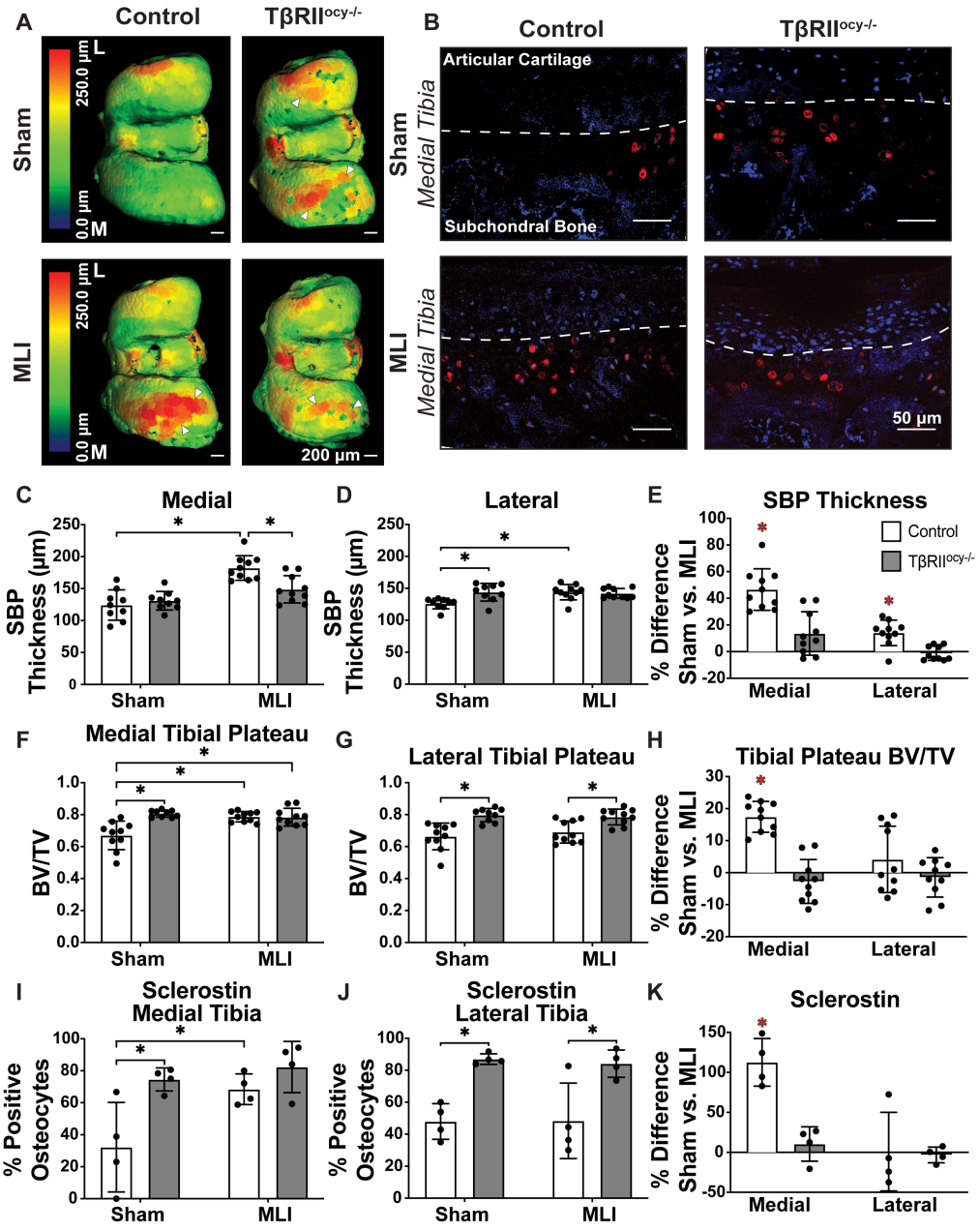


Figure 3.4: Osteocytic TβRII is critical for injury-induced changes in subchondral bone and sclerostin expression. MicroCT thickness maps show spatial differences, denoted by white arrows, in subchondral bone plate (SBP) thickness of the femoral condyles (A, scale bar = 200 µm). Injury in control mice resulted in a significant increase in SBP thickness on the medial and lateral femoral condyles (C, D, n = 9-10 mice per group). Subchondral bone volume fraction (F, G, n = 9-10 mice per group) and immunofluorescence (IF) of sclerostin in the SBP (B, I, J, scale bar = 50 µm, n = 4 mice per group) reveal a genotype-dependent increase in sham mice in the medial and lateral tibia, and an injury-dependent increase in the medial tibia of control mice. TβRII$^{ocy-/-}$ mice were insensitive to injury-induced changes in SBP thickness (E, n = 9-10 mice per group), subchondral bone volume fraction (H, n = 9-10 mice per group), and sclerostin expression (K, n = 4 mice per group). Bar graphs represent mean ± SD. *p<0.05 between groups by two-way ANOVA and Tukey post-hoc test.

analysis reveals that the increased Mankin score in TβRII$^{ocy-/-}$ joints following MLI results from increased cartilage degeneration on the uninjured lateral side of the TβRII$^{ocy-/-}$ joints relative to controls (**Figure 3.5D**). Importantly, on the injured medial side, the effect of MLI on cartilage in the control and TβRII$^{ocy-/-}$ mice is indistinguishable (**Figure 3.5C**). Thus, on the injured medial side of the joint, cartilage degeneration correlates with increased subchondral bone and sclerostin levels, independently of genotype. On the uninjured lateral side, where the mechanical environment is less affected, we continue to observe worsened cartilage degeneration in TβRII$^{ocy-/-}$ mice, similar to that observed in the basal cartilage phenotype in TβRII$^{ocy-/-}$ mice (**Figure 3.1**). Overall, independent of genotype, sex, or injury, we find that increased SBP thickness corresponds spatially to cartilage degeneration. These findings suggest a critical role for osteocytic TGFβ signaling in controlling the SBP response to changes in the mechanical environment with injury.

Interestingly, our data suggest that osteocytic TGFβ signaling plays a bidirectional role in response to injury. Injury diminishes the genotype-dependent repression of osteocytic TβRII expression in the subchondral bone, resulting in a decrease ($p=0.051$) in osteocytic TβRII expression in injured control mice (**Figure 3.6A, 3.6B**), further implicating osteocytic TGFβ signaling as a key player in the mechanosensitive response to injury. Additional research will be needed to investigate mechanisms responsible for the more severe cartilage degeneration in male TβRII$^{ocy-/-}$ joints following MLI and the role that osteocytes play in cartilage homeostasis and disrupted joint mechanics with injury. Several factors may contribute. First, our findings suggest that the worsened cartilage phenotype on the lateral side in TβRII$^{ocy-/-}$ mice with MLI **(Figure 3.5D)** is due, at least in part, to the underlying increased SBP thickness, subchondral bone volume, and SBP sclerostin levels prior to injury **(Figure 3.4D, 3.4G, 3.4J)**. Second, changes in SBP thickness are insufficient to explain why injured TβRII$^{ocy-/-}$ mice exhibit more cartilage

degeneration than sham TβRII$^{ocy-/-}$ mice **(Figure 3.5B)**. Indeed, TβRII$^{ocy-/-}$ mice were insensitive to injury-induced changes in SBP thickness, subchondral bone volume, or sclerostin levels **(Figure 3.4C-3.4K)**. This indicates that other factors with injury independent of changes in bone, such as inflammation or increased direct loading on the cartilage with meniscectomy, worsen cartilage degeneration in injured TβRII$^{ocy-/-}$ mice. We additionally observe a number of genotype-dependent differences that may participate. As expected, sham TβRII$^{ocy-/-}$ mice demonstrated a significant

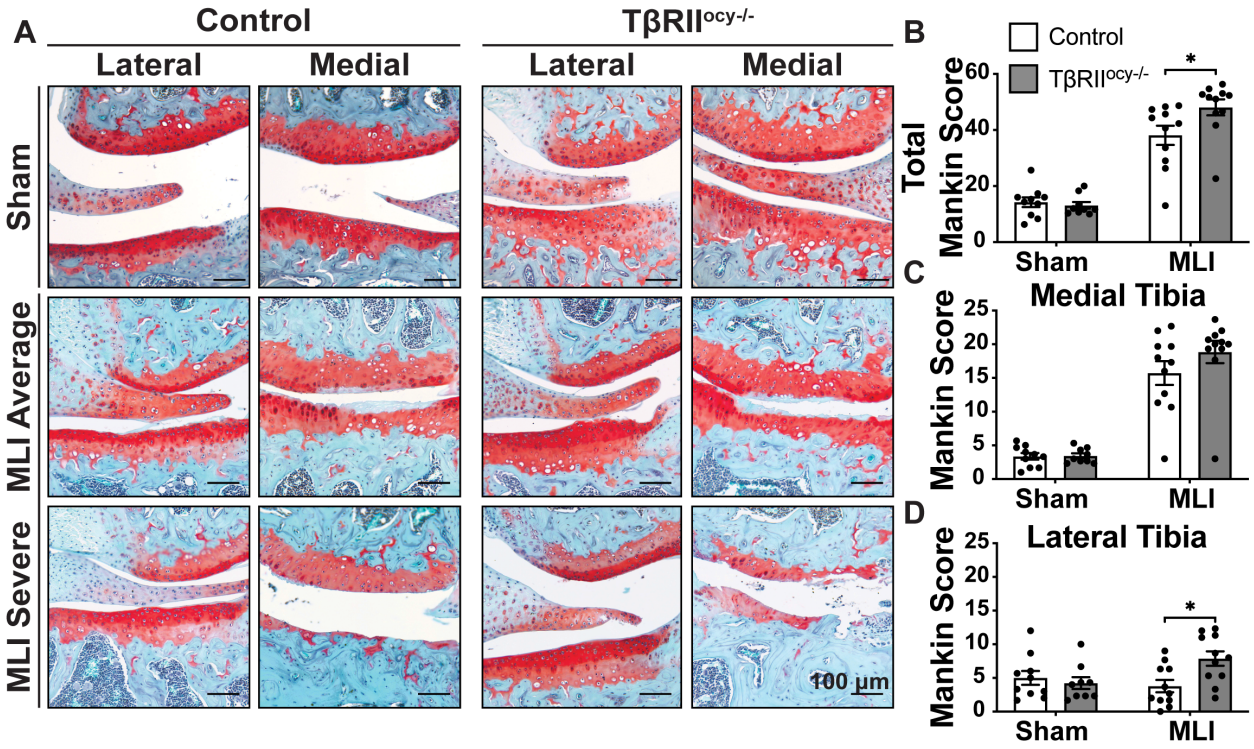


Figure 3.5: Loss of osteocytic TGFβ signaling exacerbates joint degeneration with injury. Cartilage degeneration is worsened on the medial side with injury in TβRII$^{ocy-/-}$ and control mice, and injured TβRII$^{ocy-/-}$ mice have worsened OA phenotype compared with injured control mice (Safranin-O/Fast Green stain, A, scale bar = 100 μm), resulting in significantly higher whole joint Mankin score in injured TβRII$^{ocy-/-}$ mice compared with injured control mice. Medial injury increased total joint Mankin score (B, n = 9-11 mice per group) and medial tibia Mankin score (C, n = 9-11 mice per group) in control and TβRII$^{ocy-/-}$ mice when compared with respective sham controls. There was no difference between injured TβRII$^{ocy-/-}$ mice and injured control mice in the medial tibia (C, n = 9-11 mice per group), but significantly higher Mankin score in the lateral tibia of injured TβRII$^{ocy-/-}$ mice compared with injured control mice (D, n = 9-11 mice per group). Bar graphs represent mean ± SEM. *$p<0.05$ between groups by two-way ANOVA and Tukey post-hoc test.

decrease in canalicular length when compared with sham control mice (**Figure 3.6C, 3.6D**). In the context of MLI, this TβRII-dependent reduction in canalicular length was lost (**Figure 3.6D**). Finally, we detect qualitative differences in shape of the femoral condyles in TβRII$^{ocy-/-}$ mice relative to control mice (**Supplemental Figure 3.1C, 3.1F**). Since these qualitative differences in joint shape were apparent in both male and female TβRII$^{ocy-/-}$ mice, the extent to which they play a causal role in cartilage degeneration in this model remains to be determined**.**

Discussion

In this study, we show that loss of osteocytic TGFβ signaling can play a causal role in articular cartilage degeneration through its spatial control of subchondral bone. Using an established mouse model of osteocyte-specific ablation of TβRII (23), we observed a close relationship among cartilage degeneration, thickening of the SBP, and increased osteocytic sclerostin expression in male TβRII$^{ocy-/-}$ mice and injured control mice, which additionally showed an injury-dependent decrease in osteocytic TβRII. On the contrary, female TβRII$^{ocy-/-}$ mice do not show these differences, indicating a sex-specific role for osteocytic TGFβ signaling in cartilage homeostasis. With changes in joint mechanics due to injury, control mice increase SBP thickness and sclerostin expression in spatial association with cartilage degeneration and repress osteocytic TβRII within the subchondral bone. Without osteocytic TGFβ signaling, however, injured TβRII$^{ocy-/-}$ mice do not increase SBP thickness and sclerostin expression, suggesting that mechanosensation at the SBP requires osteocytic TGFβ signaling. Overall, we find that increased SBP thickness and increased SBP sclerostin expression, whether by loss of osteocytic TGFβ signaling or by medial injury, drives cartilage degeneration. Together, this work provides new

insight into the factors driving changes in SBP thickness during joint degeneration, identifying osteocytes as potential targets for OA therapeutics.

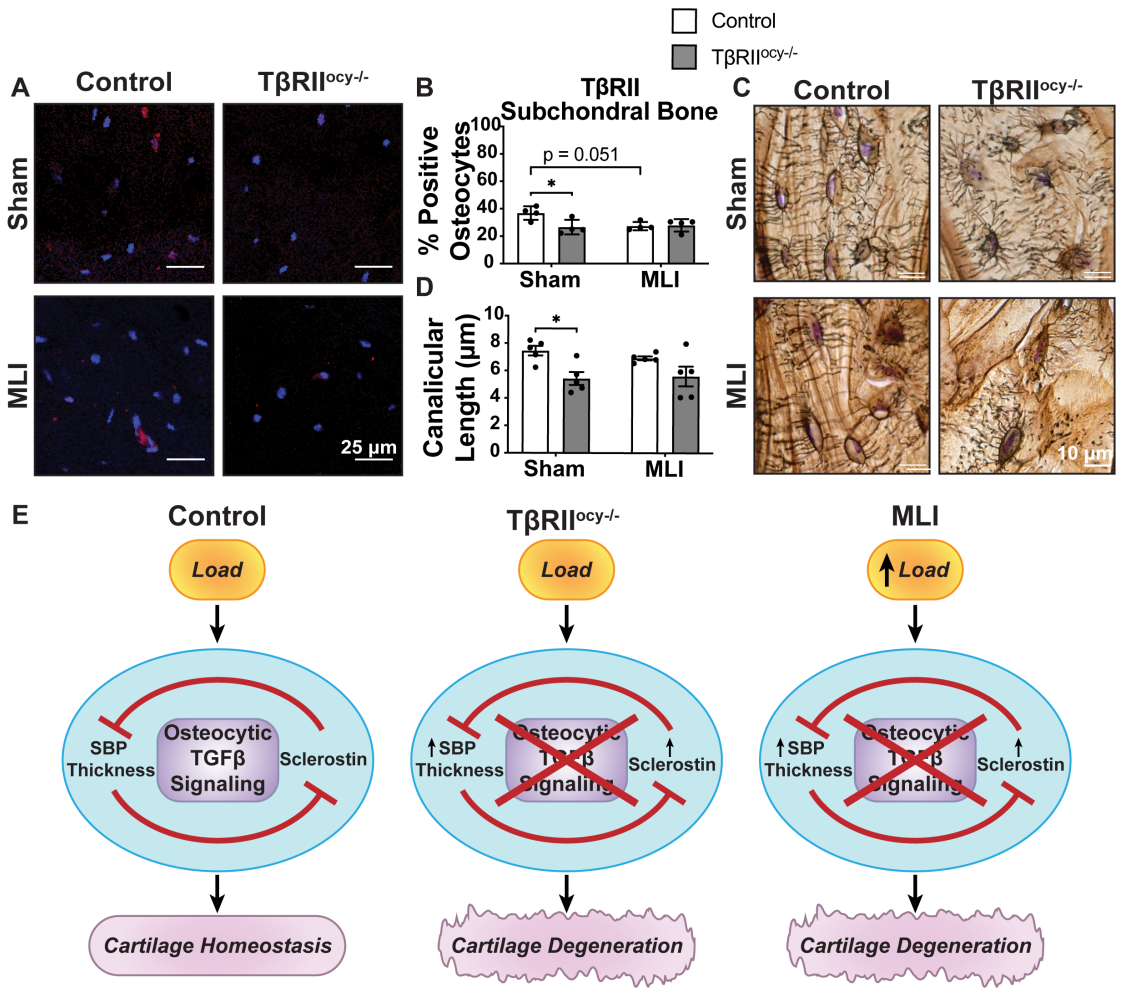
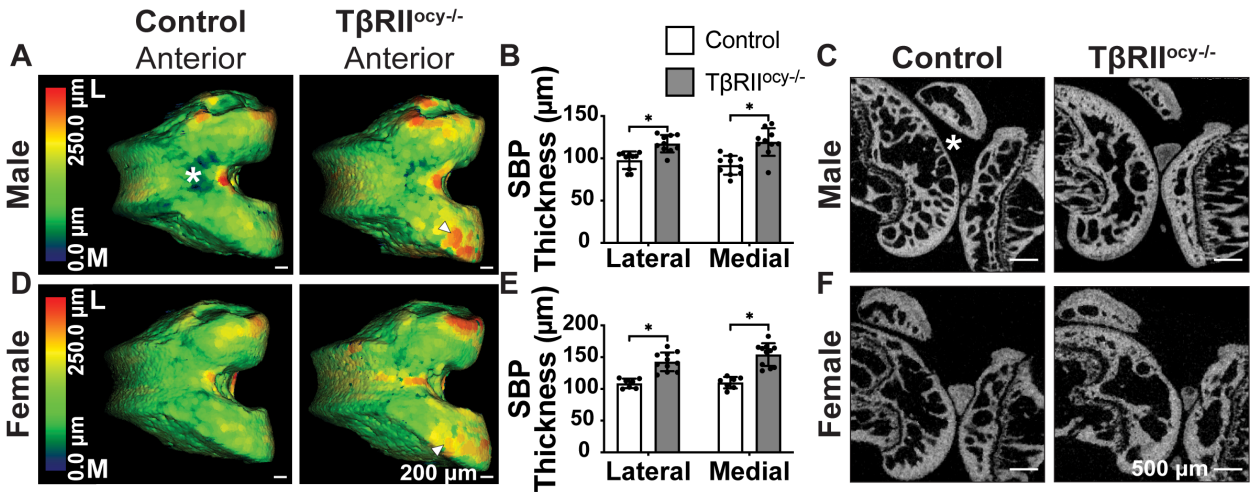


Figure 3.6: Injury represses osteocytic TGFβ signaling and diminishes genotype-dependent differences in canalicular length. Compared with sham-operated control mice, sham-operated TβRII$^{ocy-/-}$ mice possess fewer TβRII-positive osteocytes in the subchondral bone using immunofluorescence (IF) of TβRII (A, B, scale bar = 25 μm, n = 4 mice per genotype). Injury eliminates this genotype-dependent difference, resulting in a decrease in TβRII-positive osteocytes in injured control mice when compared with sham-operated control mice (p=0.051). Lacunocanalicular networks in the subchondral bone are disrupted between sham-operated control and TβRII$^{ocy-/-}$ mice, but not between injured control and TβRII$^{ocy-/-}$ mice (Ploton Silver stain, C, scale bar = 10 μm), resulting in a significant decrease of canalicular length between sham groups, but not between injured groups (D, n = 5 mice per group). Bar graphs represent mean ± SD (B)_and mean ± SEM (D). *p<0.05 between groups by two-way ANOVA and Tukey post-hoc test. Mechanosensitive function of bone requires osteocytic TGFβ to regulate subchondral bone thickness (SBP) and sclerostin in a concerted manner to maintain joint homeostasis (E).

Bone quality (37, 38) and development of OA (39, 40) are known to have sex-specific differences in humans and in mice. The prevalence of OA is higher in women than in men, particularly after menopause, demonstrating a need to better understand the sex-specific pathogenesis of OA (40). We have recently shown that osteocytic TGFβ signaling induces PLR in a sex-specific manner (28). While male mice with an osteocytic inhibition of TGFβ signaling demonstrate hallmarks of suppressed PLR in the cortical bone, including blunted canalicular networks, reduced expression of PLR enzymes, and defective bone quality, PLR and bone quality in female mice with an osteocytic inhibition of TGFβ signaling remain intact (28). In this study, male TβRII$^{ocy-/-}$ mice showed a disruption of the canalicular network within the subchondral bone, increased SBP thickness, and signs of worsened joint degeneration at 16 weeks of age. Female TβRII$^{ocy-/-}$ mice, however, showed no differences in the canalicular network, SBP thickness, or joint degeneration, demonstrating that the role of osteocytic TGFβ signaling on subchondral bone PLR and joint homeostasis is sexually dimorphic. These findings are consistent with the idea that altered mechanics at the SBP lead to cartilage degeneration. Interestingly, female TβRII$^{ocy-/-}$ joints were not entirely normal. Both male and female TβRII$^{ocy-/-}$ joints show qualitative shape differences of the femoral condyle, including a marked thickening of the SBP in the anterior region and an apparent rounding of the condyle, as well as increased SBP thickness in the relatively unloaded anterior region of the femoral condyle (**Supplemental Figure 3.1**). These differences in female TβRII$^{ocy-/-}$ joints, however, appear insufficient to affect joint degeneration at this time point, and further work is needed to understand the extent to which osteocytes respond to changes in the mechanical environment due to injury in female TβRII$^{ocy-/-}$ mice. Osteocytes emerge as important regulators of SBP thickness and joint shape, and female TβRII$^{ocy-/-}$ mice are thus able to maintain joint health in the absence of osteocytic TGFβ signaling.

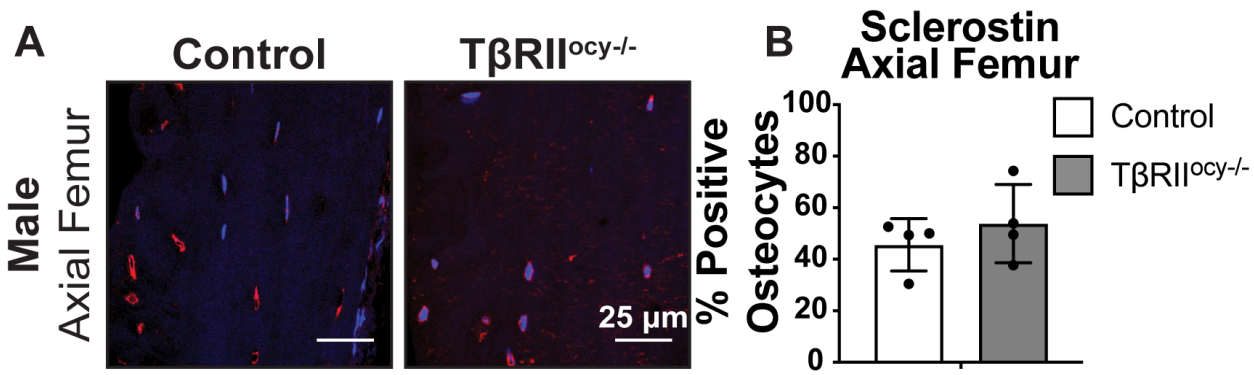


Supplemental Figure 3.1: TβRII$^{ocy-/-}$ mice have increased SBP thickness in the anterior region and altered joint shape of the femoral condyle. Male TβRII$^{ocy-/-}$ mice (microCT thickness maps, A, scale bar = 200 µm) and female TβRII$^{ocy-/-}$ mice (D) have spatial differences, denoted by white arrows, in subchondral bone plate (SBP) thickness of the anterior region of the femoral condyles compared with respective control mice, resulting in significantly higher SBP thickness in the relatively unloaded anterior regions of the medial and lateral condyles (B, E, n = 7-11 mice per genotype). Male TβRII$^{ocy-/-}$ mice (2D sagittal microCT image, C) and female TβRII$^{ocy-/-}$ mice (F) show qualitative differences in shape of the femoral condyles, including a marked thickening of the SBP in the anterior region and an apparent rounding of the condyle, when compared with respective control mice. White asterisk on A and C used as landmark for orientation. Bar graphs represent mean ± SD. *$p<0.05$ between genotypes by unpaired Students t test.

SBP thickness changes over the course of OA progression, and the spatial association of thickening of the SBP with cartilage degeneration is a known hallmark of late-stage OA (1-7). Additionally, TGFβ signaling participates in mechanoregulation of the skeleton (29-32), which, in the context of osteocytic TGFβ signaling, could impact the response of the SBP to changes in joint mechanics. Using TβRII$^{ocy-/-}$ mice, we find evidence that the mechanoresponse to injury at the SBP requires osteocytic TGFβ signaling. Unlike injured control mice, injured TβRII$^{ocy-/-}$ mice are insensitive to injury-induced changes in SBP, subchondral bone volume, and SBP sclerostin expression. Notably, injured TβRII$^{ocy-/-}$ mice still have worsened cartilage degeneration, which was primarily driven by an increase in OA score on the uninjured side in the lateral tibia (**Figure 3.5D**). This suggests that the underlying increase in SBP thickness on the 'uninjured' lateral side of TβRII$^{ocy-/-}$ mice is sufficient to disrupt the normal mechanical environment of the joint and drives

cartilage degeneration. Notably, although we have previously observed an injury-dependent disruption of the lacunocanalicular network in the subchondral bone in a different mouse model of PLR suppression (22), we do not observe an injury-dependent decrease in canalicular length in this study, possibly due to differences in genetic perturbations in these mouse models or selected regions of interest. Together, these findings indicate that osteocytic TGFβ signaling is critical for the spatial association between cartilage degeneration and SBP thickness in late-stage OA and the mechanosensitive response to changes in biomechanical load with injury.

In contrast to previous studies, we observed an increase in sclerostin expression in the setting of increased SBP thickness and cartilage degeneration. Other studies have shown an inverse relationship between the expression of sclerostin and OA severity in humans (41) and a repression of sclerostin associated with increased SBP thickness in late-stage OA in mice (7). Because sclerostin expression is induced by TGFβ signaling (32, 36), repressed in response to load (42), and stimulates PLR (27), we expected to observe a decrease in sclerostin expression in TβRII$^{ocy-/-}$ mice and in injured control mice. Instead, we surprisingly observed a significant increase in sclerostin-positive osteocytes in all cases with increased SBP thickness, which was spatially confined to the SBP and not the axial femur (**Supplemental Figure 3.2**). One possible explanation of these findings is that with increased SBP thickness, osteocyte sensitivity to equivalent loads may be reduced, with a corresponding inability to suppress sclerostin expression. Additionally, an upregulation of sclerostin levels may indicate a compensation for defective PLR due to loss of osteocytic TGFβ signaling (27) or a change in the dynamics of osteocytic differentiation in TβRII$^{ocy-/-}$ subchondral bone. Taken together, our studies and others (7) demonstrate that coordinated regulation of sclerostin and the SBP is critical in joint homeostasis, which is likely dynamic over the course of OA progression. While sclerostin has been studied in the context of



Supplemental Figure 3.2: Osteocytic sclerostin expression in the cortical femur is insensitive to osteocytic TGFβ signaling. Immunofluorescence (IF) of sclerostin at the axial cortical femur in male TβRII$^{ocy-/-}$ mice is indistinguishable from male control mice (A, B, scale bar = 25 μm, n = 4 mice per genotype). Bar graphs represent mean ± SD.

OA in mice and rats, either through sclerostin inhibition or treating with recombinant Sost protein (43, 44), the extent to which sclerostin exacerbates the joint phenotype remains unclear. Given the current clinical use of romosozumab, an anti-sclerostin antibody, for the treatment of human osteoporosis (45, 46), it is important that further work resolve the mechanistic relationship between sclerostin expression throughout the joint and OA over the course of disease progression.

Limitations of this study include the use of a mouse model in which osteocytic TβRII is constitutively ablated, which confounds the observed TβRII$^{ocy-/-}$ joint phenotype with the effects of TβRII in joint development. Additionally, while this work describes a correlation among osteocytic TGFβ signaling, SBP thickness, and SBP sclerostin expression, further work is needed to establish a causal role for SBP sclerostin in the cartilage phenotype. While uninjured TβRII$^{ocy-/-}$ mice have an increased Mankin score when compared with uninjured control mice, sham-operated TβRII$^{ocy-/-}$ mice do not have a genotype-dependent increase in cartilage degeneration, suggesting that other factors may be impacting the health of the joint, such as an inflammatory effect following incision in the sham-operated groups. Finally, additional work is needed to elucidate the mechanisms by which joint injury represses osteocytic TβRII expression in subchondral bone.

In summary, our findings demonstrate that mechanoregulation of bone requires osteocytic TGFβ signaling to regulate sclerostin and SBP thickness and maintain cartilage homeostasis (**Figure 3.6E**). In settings with increased SBP thickness, such as male TβRII$^{ocy-/-}$ mice or on the medial side of injured control mice, we observed increased sclerostin expression at the SBP and exacerbated joint degeneration. In contrast, we did not observe increased SBP thickness, sclerostin expression, or OA development in female TβRII$^{ocy-/-}$ mice. These findings suggest that osteocyte-intrinsic defects in TGFβ signaling can play a contributory role in OA progression, further implicating osteocytes as being a key participant of healthy joint homeostasis through bone-cartilage crosstalk.

Acknowledgements

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Chapter 4

TRPV4 is Essential for Perilacunar/Canalicular Remodeling and Osteocytic TGFβ Signaling

Introduction

Identifying mechanisms of OA progression is central for developing new therapeutics. The precise contributions of articular cartilage and subchondral bone in the etiology of OA are yet to be established, but growing evidence suggests that crosstalk between these two tissues is critical to maintain joint health (1-4). Cartilage relies upon the subchondral bone for mechanical and vascular support. Osteocytes have become a cell type of interest in bone-cartilage crosstalk because of their role in bone quality and ability to facilitate cellular communication, mechanosensation, and nourishment through the osteocytic lacunocanalicular network. Through a process known as perilacunar/canalicular remodeling (PLR), osteocytes dynamically maintain the lacunocanalicular network by acidifying their microenvironment and secreting matrix metalloproteinases (MMPs), cathepsin K, and other enzymes (5, 6). Osteocytes have been shown to play a role in human and mouse OA. Subchondral bone in human OA exhibits hallmarks of suppressed PLR when compared to healthy cadaveric controls, including blunted canalicular networks, disrupted collagen alignment, and decreased expression of MMP13, an enzyme essential for healthy PLR (4). Further, loss of osteocytic TGFβ signaling (7, 8) or osteocytic MMP13 (4) result in impaired PLR and exacerbated OA (4, 9). Results from our lab, among others (10), provide evidence for suppressed PLR in aging, which may be a major contributor to age-related joint degeneration as a result of disrupted joint crosstalk.

TGFβ signaling, a key pathway involved in bone-cartilage crosstalk, exhibits dynamic tissue-specific effects in the maintenance of bone and cartilage (11). The sophisticated function of

TGFβ in the joint requires an optimal level of signaling, such that highly active, suppressed, or imbalanced canonical and non-canonical TGFβ signaling causes joint degeneration. In healthy murine cartilage, for instance, TGFβ signaling represses expression of the enzyme MMP13 (12), but during aging, TGFβ signaling increases expression of MMP13 and drives cartilage destruction (13). In bone, however, osteocytic MMP13 induced by TGFβ signaling is required for osteocyte-mediated PLR (4, 7, 8). Loss of osteocytic TGFβ signaling exacerbates OA through its mechanosensitive control of articular cartilage and subchondral bone (9). Though both cartilage and bone rely upon TGFβ signaling, the function of TGFβ within each compartment is deregulated in joint disease (9, 12, 13).

Though the factors that contribute to these compartment-specific effects of TGFβ signaling are unclear, identifying molecular regulators of TGFβ signaling in skeletal cells could lay the groundwork for identifying therapeutic targets to restore cellular homeostasis in the joint. The transient receptor potential vanilloid 4 (TRPV4) channel, a mechanosensitive Ca^{2+}-permeable ion channel (14), is a channel of interest due to its ability to calibrate TGFβ signaling in non-skeletal cells (15-18). iPSCs derived from patients with a gain of function TRPV4 mutation show reduced chondrogenic response to TGFβ, suggesting that TGFβ-mediated chondrogenic potential is TRPV4-dependent (19). Similarly, dermal, lung, and cardiac fibroblasts have each demonstrated reduced TGFβ-inducible differentiation when TRPV4 activity is inhibited (15-18, 20). Others have shown that TGFβ signaling in fibroblasts results in increased expression and activation of TRPV4 (15-18, 20). While these experiments demonstrate a bidirectional relationship between TRPV4 and TGFβ in other cell types, the relationship between TRPV4 and TGFβ in osteocytes is unknown. In osteocytes, TRPV4 signaling is critical for mechanotransduction and mechanosensitive repression of sclerostin (21, 22), but the extent to which this is TGFβ-dependent remains to be determined.

Similar to TGFβ, TRPV4 also demonstrates a tissue-specific effect in joint disease (23-25). Whereas mice with a systemic ablation of the TRPV4 channel exhibit an age-related OA phenotype of severe cartilage degeneration and increased subchondral bone mass (23), mice with a cartilage-specific loss of TRPV4 surprisingly show attenuated age-related OA (25). These compartment-specific effects suggest that TRPV4 in tissues other than cartilage plays a contributory role in age-related OA. Though the exact function of TRPV4 in bone is yet to be established, mice with a systemic ablation of TRPV4 exhibit increased bone mass and reduced bone material strength with a decreased resistance to stress and decreased elastic modulus (26). Given that osteocytes regulate bone mass and bone quality (4, 7, 8), and also play a role in OA (4, 9), these data support the premise of an osteocyte-intrinsic role for TRPV4 in maintaining PLR and healthy joint crosstalk.

To test this hypothesis, we evaluated the role of TRPV4 in osteocytic PLR and osteocyte-intrinsic TGFβ signaling. We find evidence that systemic loss of TRPV4 disrupts the osteocytic lacunocanalicular network and suppresses PLR in cortical and subchondral bone. In concert with PLR gene expression with age, TRPV4 expression changes with age, mimicking key age-related PLR changes. Finally, we see an osteocyte-intrinsic effect of pharmaceutical activation or inhibition of TRPV4 activity on TGFβ signaling. These findings motivate further work on the osteocyte-intrinsic role of TRPV4 in TGFβ signaling and PLR.

Results

Systemic loss of TRPV4 impairs osteocyte connection in cortical bone

Evidence of alterations in bone mass and bone quality in TRPV4$^{-/-}$ mice (23, 26) led us to hypothesize that TRPV4$^{-/-}$ mice show hallmarks of disrupted osteocytic PLR. We evaluated the

lacunocanalicular network in cortical bone from 12-month-old male and female TRPV4$^{-/-}$ mice (Gift from Dr. Wolfgang Liedtke). Relative to male control mice, both male TRPV4$^{-/-}$ mice (p=0.09) and female TRPV4$^{-/-}$ mice (p=0.07) had decreased canalicular length within the cortical bone (**Figure 4.1**). The availability and quality of samples limits the conclusions that can be drawn from these findings. First, comparisons were not able to be made with control littermates, nor were they from the same bone; femoral cortical bone was evaluated in male control mice and ulnar cortical bone was evaluated in TRPV4$^{-/-}$ mice. Additionally, we and others (10) have shown that the canalicular network in wildtype mice degenerates with age, resulting in a significant reduction in canalicular length by 12 months of age. Therefore, genotype-dependent differences in the canalicular network between control and TRPV4$^{-/-}$ mice may be more pronounced in bone from younger mice.

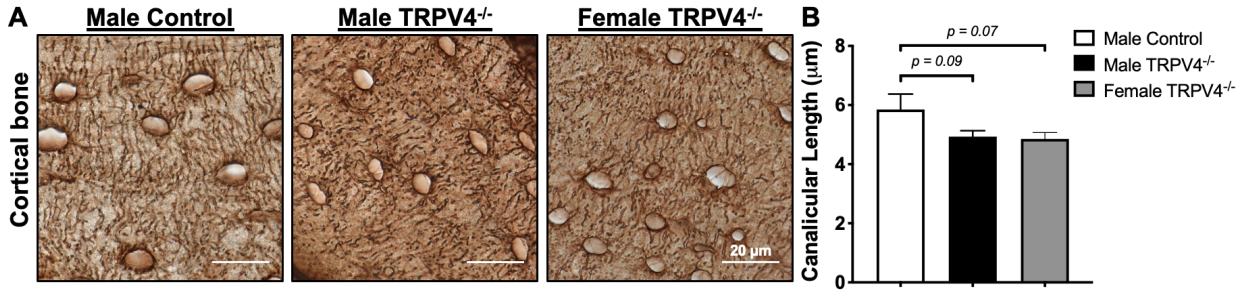

Figure 4.1: Systemic loss of TRPV4 reduces canalicular length in cortical bone at 12 months of age. Cortical bone from 12-month-old male and female TRPV4$^{-/-}$ mice shows disrupted canalicular network (Ploton Silver stain, A, scale bar = 20 µm) and reduced canalicular length (B, n = 3-5 mice per group) when compared with 12-month-old male controls. Bar graph represents mean ± SEM. P-value vs. male control.

TRPV4 is critical for subchondral bone osteocyte lacunocanalicular network maintenance

Given the experimental limitations of the canalicular findings in cortical bone, we obtained histological specimens from the hindlimb of 32-week-old male TRPV4$^{-/-}$ mice and their littermate controls (Gift from Dr. Farshid Guilak) to evaluate the canalicular network in TRPV4$^{-/-}$ subchondral bone. In other models of PLR suppression, we have found reduced canalicular length

within the subchondral bone (4, 9, 27). In tibial plateau subchondral bone from male TRPV4$^{-/-}$ mice, we observe a significant 25% decrease in canalicular length overall and in both the medial and lateral tibia when compared with control littermates (**Figure 4.2**). There was no joint compartment-specific effect within each genotype. These findings demonstrate a severe disruption of the lacunocanalicular network in the absence of systemic TRPV4, suggesting that TRPV4 is critical for osteocytic perilacunar/canalicular remodeling (PLR).

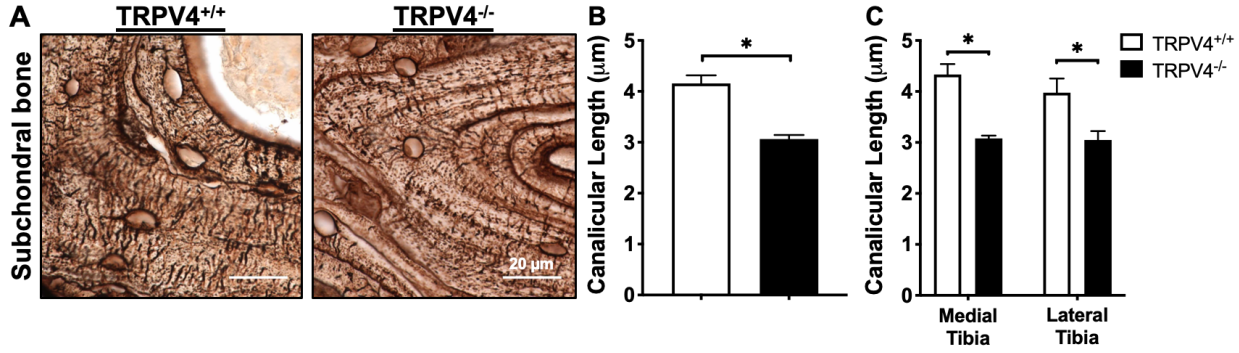

Figure 4.2: TRPV4$^{-/-}$ subchondral bone shows hallmarks of defective PLR. TRPV4$^{-/-}$ subchondral bone (Ploton Silver stain, A, scale bar = 20 μm) demonstrates disorganized canalicular networks and significantly reduced canalicular length overall (B, n = 3 mice per genotype) and in the medial and lateral tibia specifically (C, n = 3 mice per genotype) compared with control littermates. Bar graphs represent mean ± SEM. *p<0.05 between genotypes by unpaired Students t test.

Age-related changes in TRPV4 expression in osteocytes mimic changes in PLR-critical genes

To identify differential gene expression with aging, we performed RNA-seq on mRNA isolated from C67BL/6 bone across several ages (2 months, 12 months, 24 months, and 30 months), which was enriched for osteocytic populations. We observe an age-dependent change in expression of genes critical for PLR, such as MMP2, MMP13, and MMP14 (**Figure 4.3**). Specifically, by 12 months of age, these genes are significantly reduced in C57BL/6 bone. This reduction lasts through 24 and 30 months for MMP2 and MMP14, but interestingly, expression of MMP13 rebounds by 24 months of age. Notably, the change in TRPV4 gene expression with age

resembles the age-related changes in expression of MMPs, specifically MMP13. Because these PLR genes are induced by TGFβ (7, 8), this finding is consistent with the hypothesis that TRPV4 works in concert with TGFβ signaling in bone to modulate PLR.

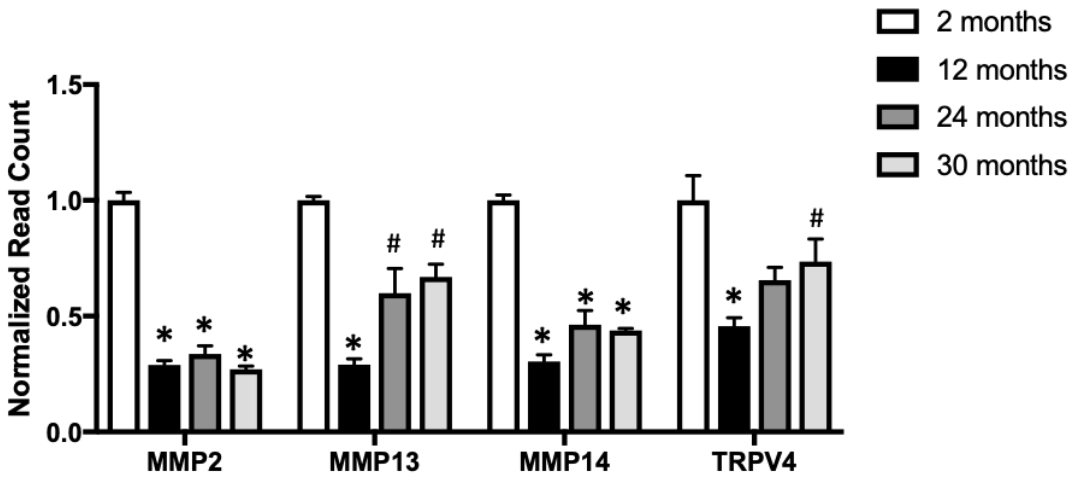

Figure 4.3: Age-dependent change in PLR gene expression. RNA-Seq from C57BL/6 bone enriched for osteocytic populations shows an age-dependent change in expression of PLR genes: MMP2, MMP13, and MMP14. Expression of TRPV4 with age resembles that of essential PLR genes, consistent with the hypothesis that TRPV4 works in concert with TGFβ to control PLR. Data presented as mean ± SD of n=4, *$p \leq 0.05$ vs. 2 months, $^{\#}p \leq 0.05$ vs. 12 months.

TRPV4 activity modulates osteocyte-intrinsic TGFβ signaling

TRPV4 activity has been shown to shift the function of TGFβ signaling in non-skeletal cells (15-18). To understand the relationship between TRPV4 and TGFβ signaling in osteocytes, we used the OCY454 osteocyte-like cell line, a robust murine cell line that abundantly expresses TRPV4 (28) and SOST/Sclerostin (29). Using the TRPV4 antagonist HC06747, we evaluated the effect of TRPV4 inhibition on the activity of downstream TGFβ effectors to identify the mechanisms by which TRPV4 calibrates TGFβ signaling in osteocytes. In OCY454 cells, we find that the TGFβ-dependent induction of Smad3 phosphorylation shows a dose-dependent repression when TRPV4 activity is inhibited (**Figure 4.4**). This remained true in both undifferentiated OCY454 cells and OCY454 cells that had been differentiated for 14 days.

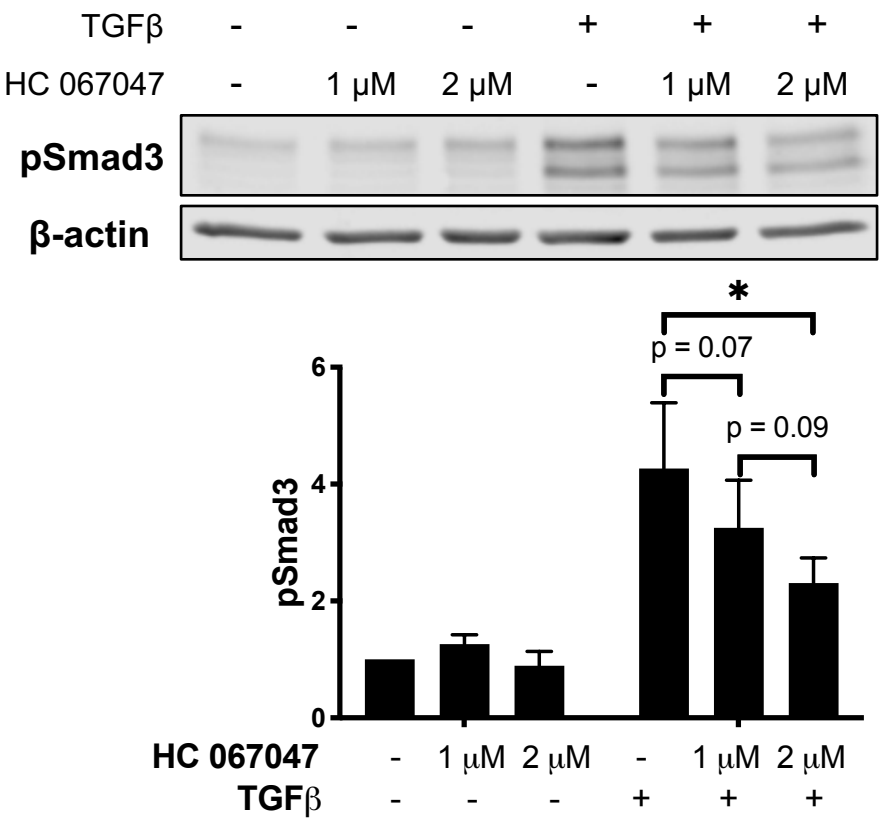

Figure 4.4: TRPV4 antagonism modulates canonical TGFβ signaling in a dose-dependent manner. With increasing dosage of TRPV4 antagonist HC06747, pretreated for 30 minutes, OCY454 cells show reduced phosphorylation of TGFβ effector Smad3 one hour after TGFβ treatment. Data presented as mean ± SD of n = 4 biological replicates, *p≤0.05.

Conversely, we assessed the dose-dependent effect of pharmacologic TRPV4 activation on TGFβ signaling using the TRPV4 agonist GSK1016790A (GSK101). Surprisingly, similar to the effect of TRPV4 antagonism using HC06747, pharmacologically inducing TRPV4 activity using GSK101 also resulted in a dose-dependent repression of the canonical TGFβ effector, Smad3 phosphorylation (**Figure 4.5**). In the absence of TGFβ, GSK101 does not affect the levels of pSmad3, demonstrating that the role of TRPV4 activation on Smad3 phosphorylation is TGFβ-dependent. Taken together, both TRPV4 inhibition using HC06747 and TRPV4 activation using GSK101 repress downstream canonical TGFβ signaling, disrupting homeostatic TGFβ signaling.

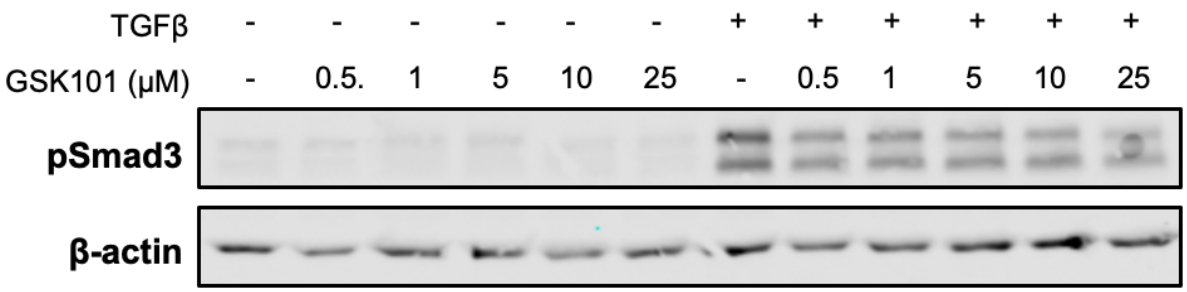

Figure 4.5: TRPV4 agonism exhibits a dose-dependent repression of canonical TGFβ signaling in osteocytes. One hour of TGFβ treatment in OCY454 cells results in induction of canonical TGFβ effector pSmad3. Thirty-minute pretreatment with TRPV4 agonist GSK101 results in a dose-dependent decrease in Smad3 activity. In the absence of TGFβ, GSK101 does not induce pSmad3. N = 1 biological replicate.

Discussion

This work provides evidence for a TRPV4-dependent role in osteocytic PLR *in vivo* and in osteocyte-intrinsic TGFβ signaling *in vitro*. In cortical and subchondral bone from mice with a systemic loss of TRPV4, we observe a reduction in canalicular length, a hallmark of suppressed osteocytic PLR, suggesting that TRPV4 is critical for preserving the lacunocanalicular network. The lacunocanalicular network degenerates with age (10), and we find evidence that expression of TRPV4 in C57BL/6 mice changes with age in a pattern resembling the changes in PLR gene expression with age. In OCY454 osteocyte-like cells, we identify TRPV4 activity as a regulator of canonical TGFβ signaling, such that either activation or inhibition of TRPV4 reduces activity of the canonical effector Smad3. Together, these findings serve as a framework of the role of TRPV4 in TGFβ-mediated PLR and motivates future studies investigating the osteocyte-intrinsic role of TRPV4 in PLR. Uncovering this relationship has implications for understanding bone fragility and OA in aging.

TRPV4 loss of function mutations in humans have been linked to several bone pathologies including increased risk of fragility fractures without a loss of bone mineral density (26), genetic

bilateral avascular necrosis of the femoral head (30, 31), and familial digital arthropathy-brachydactyly (FDAB), a severe inherited OA of the hands and feet (32). Human mutations in TGFβ signaling result in similar phenotypes, including osteogenesis imperfecta in bone (33) or a syndromic form of early-onset OA (34, 35). Given that TRPV4 calibrates TGFβ signaling in non-skeletal cells (15-18), and our data show that TRPV4 activity in osteocytes can similarly regulate canonical TGFβ signaling, the analogous clinical phenotypes in human mutations of TRPV4 and TGFβ may be related mechanistically.

Male TRPV4$^{-/-}$ mice have increased cortical and trabecular bone mass and decreased elastic modulus (23, 26). While these mice have reduced osteoclastic activity (26, 36-38), the role of osteocytes in this phenotype is unknown. Here, we uncover a role for TRPV4 in maintaining the lacunocanalicular network in cortical and subchondral bone, demonstrating that loss of TRPV4 has an osteocytic effect. Osteocytes are able to control osteoclast activity through production of RANKL (39), which is diminished in mice deficient in osteocytic TGFβ signaling, which also show elevated trabecular bone volume (7). Therefore, an osteocyte-intrinsic role for TRPV4 could be responsible, at least in part, for the reduced osteoclastogenesis and increased trabecular bone volume observed in TRPV4$^{-/-}$ mice. Further exploration of other hallmarks of PLR, such as PLR enzyme expression, in these mice is needed, and the extent to which this is osteocyte-intrinsic remains to be determined.

Loss of TRPV4 has a sexually dimorphic effect on bone and on the joint. Male TRPV4$^{-/-}$ mice have a severe disruption of bone homeostasis, including 20% increase in cortical and trabecular bone mass, increased intracortical porosity, and hypomineralized bone matrix (26). Likewise, male TRPV4$^{-/-}$ mice have a severe OA phenotype that develops by 9 months of age (23). Female TRPV4$^{-/-}$ mice, however, do not demonstrate a genotype-dependent change in any of these

outcomes. We have recently shown a similar sexual dimorphism in mice with an osteocyte-intrinsic inhibition of TGFβ signaling: male, but not female, mice show hallmarks of PLR suppression, reduced bone quality, and OA development (8, 9). Our observations of the lacunocanalicular network in cortical bone from TRPV4$^{-/-}$ mice suggests that both male and female TRPV4$^{-/-}$ mice have reduced canalicular length relative to male controls, and the canalicular network does not differ between male and female TRPV4$^{-/-}$ mice at 12 months of age. This does not rule out the possibility that loss of TRPV4 has a sex-specific effect on the osteocytic lacunocanalicular network. First, we were limited in availability of controls, precluding a comparison to female controls. Second, an aging-related reduction in canalicular length may be confounding sex-specific differences. Additional comprehensive analysis would be necessary to overcome the experimental limitations and understand whether the effect of TRPV4 on the lacunocanalicular network is sexually dimorphic.

While these experiments in OCY454 cells demonstrate that pharmacological TRPV4 activation or inhibition calibrates canonical TGFβ signaling in osteocytes, further work is needed to better understand the effect on activity of non-canonical effectors of TGFβ signaling, such as Akt and p38, and downstream gene expression of PAI-1, a TGFβ-responsive gene, as well as PLR enzyme expression. Preliminary experiments investigating the effect of TRPV4 activity on the phosphorylation of Akt and p38 remain inconclusive due to variable effects of TGFβ treatment on downstream effector phosphorylation. Additionally, utilizing tools to genetically ablate TRPV4 *in vitro* would provide further context to these findings.

Male mice systemically lacking TRPV4 exhibit severe OA, but mice with a chondrocyte-intrinsic ablation of TRPV4 surprisingly have attenuated OA, suggesting that cell-types other than chondrocytes are responsible for the development of OA in TRPV4$^{-/-}$ mice. We have recently

shown that osteocytes can play a causal role in OA (4, 9). More specifically, without osteocytic TGFβ signaling, subchondral bone is unable to respond to disrupted joint mechanics with injury resulting in worsened cartilage degeneration. Osteocytes are considered the primary mechanosensors of bone (5, 40), and TRPV4 is an important mechanosensitive component of the osteocyte (28, 41). The extent to which the OA phenotype in TRPV4$^{-/-}$ mice is due to deregulated mechanosensation is motivation for future study.

Overall, this work investigates a previously unexplored relationship between TRPV4 and TGFβ-dependent PLR in osteocytes, yet further exploration is necessary to fully untangle the epistatic relationship between TRPV4 activity and TGFβ signaling in osteocytes and the extent to which this plays a role in osteocytic PLR. Future experiments should use an osteocyte-specific mouse model to ablate the TRPV4 channel to understand to what extent the bone and OA phenotype in TRPV4$^{-/-}$ mice is the consequence of defective TRPV4 activity in osteocytes. Identifying a therapeutic target that restores the balance of TGFβ activity may overcome the challenges and side effects associated with treatments that target the TGFβ pathway specifically.

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Chapter 5

Conclusions and Future Directions

Synovial joints facilitate smooth motion and load transfer through integrated function of multiple tissues, including articular cartilage, bone, ligaments, tendons, synovium, and menisci (1, 2). Under normal conditions, the biological and mechanical activities of these tissues are exquisitely coordinated. They develop together, often share a blood supply, exchange signaling molecules, and mechanically load each other almost constantly throughout life (1-3). While crosstalk among these tissues in healthy joints supports homeostasis, it can alternatively drive a positive-feedback loop of joint destruction if any of the joint components become perturbed by injury or diseases, such as osteoarthritis (OA) (1-3). OA is considered a "whole joint" disease, in recognition of the coordinated degradation and interaction of multiple tissues within the joint.

The TGFβ signaling pathway is closely involved in maintaining healthy joint crosstalk because of its dose-dependent, context-dependent, and tissue-specific function in synovial tissues that carefully interact to coordinate joint health. Additionally, osteocyte-intrinsic TGFβ signaling is required for healthy bone quality and osteocytic perilacunar/canalicular remodeling (PLR), the process by which osteocytes maintain their lacunocanalicular network (4, 5). While the tissue-specific function of TGFβ signaling in chondrocytes, osteoblasts, and MSCs has been studied in the context of OA (6-10), the osteocyte-intrinsic role of TGFβ in OA was unknown. This work uncovered the osteocytic contributions of TGFβ signaling to joint homeostasis, and these findings presented a novel understanding of osteocytic TGFβ in the mechanosensitive response to injury (11). Overall, osteocytic TGFβ signaling is identified as a key component in bone-cartilage crosstalk. Still, questions remain regarding the role of subchondral bone osteocytes in the joint, a few of which are detailed in this chapter.

Future Directions

The role of osteocytic TGFβ signaling in age-related OA

Male mice with an osteocyte-intrinsic ablation of TGFβ type II receptor (TβRII), driven by the DMP1-Cre promoter and referred to as TβRII$^{ocy-/-}$ mice (DMP1-Cre^{+};TβRII$^{fl/fl}$), demonstrate signs of worsened cartilage degeneration at 16 weeks of age (11). To understand if TβRII$^{ocy-/-}$ mice exhibit a similar genotype-dependent increase in incidence of age-related OA, we evaluated the OA phenotype in 6-month-old and 12-month-old male TβRII$^{ocy-/-}$ and control mice. TβRII$^{ocy-/-}$ mice had worsened cartilage degeneration and a significantly increased Mankin score in the lateral tibia at both 6 month and 12 months of age (**Figure 5.1**). Interestingly, the increased total Mankin score in injured TβRII$^{ocy-/-}$ mice was likewise driven by a significant increase in Mankin score in the lateral tibia (**Figure 3.5D**), illustrating a joint compartment-specific effect in TβRII$^{ocy-/-}$ mice. Unlike 16-week-old TβRII$^{ocy-/-}$ mice, neither age group demonstrated a genotype-dependent increase in total joint Mankin score. Increased cartilage degeneration in TβRII$^{ocy-/-}$ mice is associated with increased thickness of the subchondral bone plate (SBP) (11), so evaluating SBP thickness in these aged mice could help elucidate the compartment-specific contributions to OA. Furthermore, with age, TGFβ signaling in chondrocytes shifts from canonical to non-canonical signaling (12-14). Whether there are similar changes in osteocytic TGFβ signaling with age are not yet known, but this could impact osteocytic PLR, bone quality, and joint homeostasis.

Importantly, this study initially included three age groups to assess the osteocyte-intrinsic role of TGFβ with age: 6 months, 12 months, and 18 months of age. Due to sample availability and mouse fatality rate, TβRII$^{ocy-/-}$ mice in the 18-month-old group were only aged to 15 months, while control mice were 17 months at the time of collection. Mice in this age group did not demonstrate a genotype-dependent increase in Mankin score in any joint compartment, but

whether this is due to the difference in ages between the control and TβRII$^{ocy-/-}$ mice or an age-related loss of genotype-dependent effects requires further investigation with appropriate age-matched control mice.

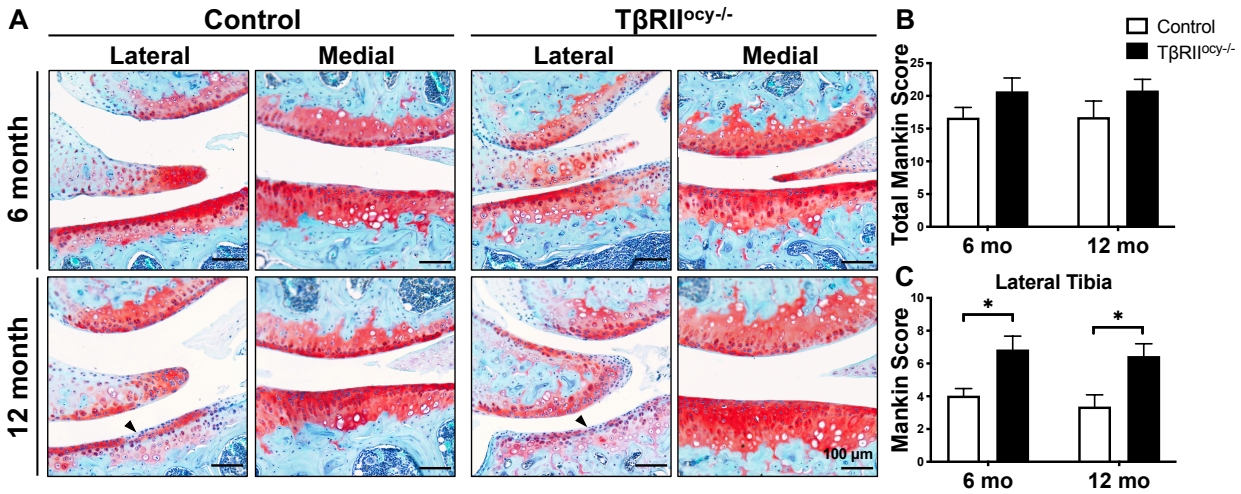

Figure 5.1: Loss of osteocytic TGFβ signaling has a compartment-specific effect on cartilage degeneration with age. At 6 and 12 months of age, male TβRII$^{ocy-/-}$ mice do not have significantly different total joint Mankin scores (B, n = 8-11 per group) when compared with control mice (Safranin-O/Fast Green stain, A, scale bar = 100 μm), but do demonstrate genotype-dependent increases in Mankin score specifically in the lateral tibia (C, n = 8-11 mice per group). Arrows denote proteoglycan loss in the lateral tibia. Bar graphs represent mean ± SEM. *p<0.05 between groups by two-way ANOVA and Tukey post-hoc test.

Osteocytic contributions to joint shape

As illustrated in **Supplemental Figure 3.1**, both male and female TβRII$^{ocy-/-}$ mice exhibit qualitative differences in joint shape of the distal femoral condyle on 2D sagittal images of microCT scans, demonstrating that osteocytes play a role in regulating joint shape. Preliminary data of three-dimensional comparisons of joint shape created in collaboration with Dr. Nathan Young better illustrate the genotype-dependent change in condylar shape (**Figure 5.2**), displaying the transition from the femoral condyle of a female control mouse to that of a female TβRII$^{ocy-/-}$ mouse. More comprehensive analysis is necessary to determine whether these qualitatively apparent differences correspond to significant differences in quantitative parameters.

Changes in bone area and shape are associated with pain and radiographic progression of human OA (15), and three-dimensional shape analysis could provide insight into the factors disrupting joint mechanics and leading to joint degeneration in male TβRII$^{ocy-/-}$ mice (11). It could also be used to give insight into the sexual dimorphism of the OA phenotype in TβRII$^{ocy-/-}$ mice. Although both male and female TβRII$^{ocy-/-}$ mice demonstrate shape changes, only male TβRII$^{ocy-/-}$ mice exhibit an OA phenotype, which may be related to altered mechanics that could be further studied with these tools (11). Additionally, future investigations could employ this method of analysis to more fully understand the extent to which this genotype-dependent shape difference is impacted by development and age. TβRII$^{ocy-/-}$ mice have a constitutive ablation of osteocytic TβRII, and thus, shape analysis in younger mice could elucidate whether this difference in shape is present at birth or develops over the first 16 weeks of life.

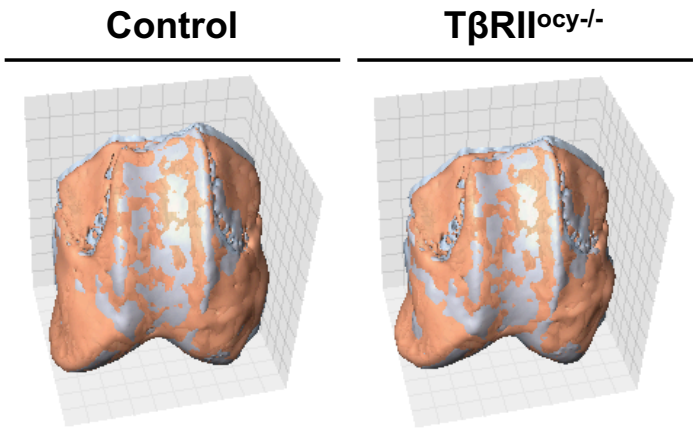

Figure 5.2: Disrupted osteocytic TGFβ signaling alters femoral condyle joint shape. Three dimensional models of the distal femoral condyle of female control and TβRII$^{ocy-/-}$ mice show changes in shape, including an apparent shrinking and squaring of the condyle. Red - control, blue - TβRII$^{ocy-/-}$.

Exploration of Mechanisms of Joint Crosstalk

Osteocytes play a role in cartilage degeneration in humans and in mice (11, 16). Disrupted mechanosensation at the subchondral bone plate is one mechanism, presented in Chapter 3, by which osteocytes in subchondral bone can contribute to cartilage degeneration (11). Another possible mechanism by which osteocytes can support cartilage homeostasis is by impacting nutrient and vascular exposure through the canalicular network. Osteocytic TGFβ is critical for the maintenance of the lacunocanalicular network (4, 5), and 3D fluorescent analysis of the canalicular network allows for imaging at the subchondral bone plate and osteochondral junction. In collaboration with Charles Schurman, this imaging technique was employed to elucidate this intimate relationship between the canalicular network and the articular cartilage (**Figure 5.3**), which lays the groundwork for a more rigorous understanding of how models of perilacunar/canalicular remodeling (PLR) suppression can disrupt communication with the articular cartilage.

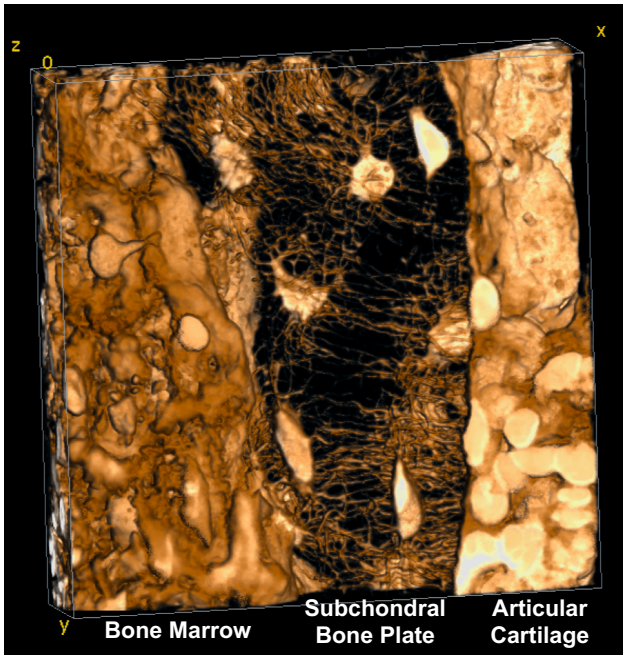

Figure 5.3: Fluorescent canalicular analysis for visualization of the osteocytic network at the osteochondral junction. The distal femoral condyle of a 6-month-old control mouse exhibits close canalicular interaction with the articular cartilage at the subchondral bone plate (n = 1 mouse).

Harnessing Joint Crosstalk for Therapeutic Targets

Taken together, the work presented in this dissertation provides further evidence of bone-cartilage crosstalk in the pathogenesis of OA and implicates osteocytes as critical components of joint homeostasis. Given the current dearth of disease-modifying osteoarthritis drugs (DMOADs), the search for new therapies has increasingly shifted from cartilage to other affected joint tissues, including subchondral bone and synovium. Additionally, radiographic evidence of OA, such as joint space narrowing, poorly correlates with pain (17), suggesting that clinical improvements in some widely-used clinical outcome measures of OA may not be sufficient to achieve a therapeutic benefit to patients.

Treating joint disease by targeting subchondral bone

In light of the evidence of subchondral bone changes preceding cartilage degeneration in OA, particularly an increase in bone remodeling in the early stages of the disease, clinical trials have targeted bone resorption to mitigate OA severity. Many randomized control trials (RCTs) have been conducted using bisphosphonates, antiresorptive drugs that inhibit osteoclast-mediated bone resorption. We discuss these results in detail because bisphosphonates are already in clinical use for treatment of osteoporosis. Therefore, success in RCTs could allow bisphosphonates to quickly be employed to treat OA.

Once-daily oral risedronate has been shown to improve pain, function, and stiffness in patients with knee OA, with a trend toward attenuation of joint-space narrowing (18). These patients also demonstrated significantly reduced markers of cartilage degradation and bone resorption, suggesting an arrest of pathologic joint remodeling. A separate study, however, similarly tested the effect of oral risedronate on knee OA but found that risedronate did not

significantly reduce radiographic progression of OA, and while it did reduce pain by 20%, this was also true of patients treated with placebo (19). While this study also observed a dose-dependent reduction in urinary cartilage degradation markers, it was in the absence of any other observed improvement in OA, demonstrating potential diagnostic limitations of urinary cartilage degradation markers.

The effect of another bisphosphonate, zoledronic acid, has been tested in the setting of a single intravenous infusion in patients with knee OA (20). This study reports temporal effects of bisphosphonates on pain and bone marrow lesion (BML) size. Pain scores were significantly reduced in patients receiving zoledronic acid compared with placebo after 6 months, but not after 3 months or 12 months. Additionally, reduction in total BML area was greater in patients who received zoledronic acid compared with placebo after 6 months, with a trend after 12 months. BMLs and OA progression are closely linked, so a clinically meaningful reduction in BML area may be a good surrogate outcome for OA progression and indicate potential efficacy for treatment. Interestingly, the findings from this study were specific to one of two pain scales, highlighting the relative subjectivity of these scales and emphasizing the importance of the chosen pain scale in evaluating drug efficacy.

While these studies reinforce the importance of joint crosstalk and show clinical promise on their own, a meta-analysis of RCTs showed that bisphosphonate treatment of OA provides no significant benefit over placebo in pain relief, functional improvement, or prevention of radiographic progression (21). It did find, however, that bisphosphonates displayed no significant differences in adverse outcomes relative to placebo groups. These mixed results might reflect the need to identify the subset of OA patients with a disease course that may benefit from targeting bone resorption. If used earlier in OA progression in patients with a specific etiology,

bisphosphonates may show more success and could be a good DMOAD option given their safety profile.

Two Phase III clinical trials of symptomatic knee OA testing the efficacy of treating OA with oral calcitonin also had mixed results (22). Calcitonin, also an anti-resorptive, inhibits bone resorption by binding to and activating the calcitonin receptor on osteoclasts. Each of these clinical trials demonstrated a comparable reduction in pain, function, and stiffness scores in the treatment and placebo groups, with no effect on joint space narrowing. The modest effects of calcitonin on markers of cartilage degradation and bone resorption did not correspond with changes in other OA outcomes.

Strontium ranelate, also used as an osteoporosis drug, inhibits bone resorption and induces bone formation. In an RCT, patients with knee OA received daily strontium ranelate for three years to observe its effect on OA progression. Strontium ranelate limited joint space narrowing over the course of the study and improved pain outcomes (23). Further stratification of patients from this study identified a relationship between BMLs, a pathologic feature that predicts OA progression to total knee arthroplasty, and efficacy of strontium ranelate (24). Since strontium ranelate reduced BML size, this treatment may limit joint deterioration enough to protect or delay some OA patients from requiring a total knee arthroplasty.

Studies employing antiresorptives to treat OA in clinical trials have provided mixed results at best. With our current understanding of OA progression, antiresorptives would be most effective in early disease, a time at which patients may be asymptomatic. This poses a challenge of identifying patients to treat and suggests that some patients in these clinical trials may have OA that is too advanced to benefit from antiresorptive therapy. Longitudinal data on patients undergoing antiresorptive treatment is needed to answer these questions.

Treating joint disease by targeting synovium

Due to the association of synovitis with OA development, as well as the relative success in targeting local and systemic inflammation in the treatment of rheumatoid arthritis, clinical trials have tested the effect of inflammation-suppressing drugs, such as corticosteroids, on OA. In spite of the hypothesis that reducing synovitis may attenuate OA, an RCT that administered intra-articular triamcinolone every 12 weeks for 2 years found that relative to placebo, this corticosteroid failed to reduce knee pain and exacerbated cartilage loss (25). Although intra-articular corticosteroid injection is commonly used clinically for short term pain relief, treating OA with sustained intra-articular corticosteroids is not effective and could instead worsen disease.

An RCT studying the effect of chondroitin sulfate, a glycosaminoglycan with both anti-inflammatory effects and direct effects on chondrocytes *in vitro*, found that patients who received chondroitin sulfate had significantly reduced BML area and cartilage volume loss than those who received placebo (26). Interestingly, between treated and control groups, no differences were detected in synovial thickness or pain, as assessed by two different pain scores. These findings reinforce the discordance between radiographic outcomes, such as BMLs and cartilage volume, and the experience of pain. Successful therapeutics will need to consider each of these outcomes to demonstrate efficacy in treating OA. Better understanding the role of the synovium in OA could advance the development of new therapies that more effectively prevent cartilage degeneration while improving joint pain.

Treating joint disease by targeting pain

The complex relationship between cartilage degeneration and joint pain poses a significant problem for the development of DMOADs (17). Some clinical trials have, instead of targeting

cartilage, bone, or synovium, specifically treated OA pain using non-steroidal anti-inflammatory drugs (NSAIDs) or inhibitors of nerve growth factor. Meta-analysis of NSAIDs treatment for OA, specifically considering the COX-2 prostaglandin synthase inhibitor celecoxib, demonstrates a small improvement in pain (27). This benefit was no longer significant when the meta-analysis considered only high-quality clinical trials. Other pain-specific treatments have targeted nerve growth factor, which is produced in the synovium in OA and is stimulated by IL-1β and TNF-α to further induce proliferation of the synovium (28). Expression of nerve growth factor by osteoclasts in osteochondral channels is also strongly associated with pain in OA (29). Tanezumab, a humanized monoclonal antibody that binds to and inhibits nerve growth factor, has been tested as an analgesic for knee OA. Patients who received tanezumab had a reduction in joint pain and improvement in function when compared with placebo, with mild and moderate adverse events (30). Radiographic OA progression was not assessed.

While some treatments effectively reduce pain, none meaningfully prevent or delay OA progression. In fact, the combined use of NSAIDS with inhibitory nerve growth factor antibodies increased the prevalence of rapidly progressive OA (31). These findings reinforce the idea that pain is an important physiological process, and specifically reducing pain in the absence of other disease-modifying benefits may paradoxically worsen OA. Analysis of serological biomarkers is underway in patients from the tanezumab study, in an effort to predict which patients will benefit and which may be predisposed to rapidly progressive OA (31).

Treating joint disease by increasing bone-cartilage crosstalk

Microfracture, a technique in which small holes are drilled through a cartilage defect and into the subchondral plate, is used in some cases of OA and osteochondral defects to surgically

induce physical crosstalk within the joint (32). These holes permit the exchange of marrow contents into the joint and synovial fluid into the marrow space, in a procedure that stimulates formation of a 'super clot' within the cartilage defect. This migration of marrow stromal cells and growth factors yields a largely fibrocartilaginous repair tissue within the cartilage defect. However, microfracture has been shown to increase bone tissue overgrowth, subchondral bone cyst formation, and subchondral plate fragility. While patients can experience short-term functional improvements, microfracture has not demonstrated long-term efficacy and patients still often require total joint arthroplasty. Biological control of growth factors in the marrow contents may improve outcomes of microfracture. In an osteochondral defect rabbit model, microfracture with oral losartan, a TGFβ1 blocker, improves cartilage repair by reducing fibrocartilage and increasing hyaline-like cartilage when compared with microfracture alone (33). Therefore, local modulation of TGFβ signaling may be one approach to enhance joint crosstalk to restore joint health.

Improving therapeutic approaches

Many of the above-mentioned treatments have attenuated the phenotype of OA in animals, but none have successfully delayed or prevented OA in humans. These paradoxical findings highlight the need for further research, especially to elucidate the mechanisms of crosstalk among tissues in the healthy joint and in OA. The lack of success in developing DMOADs brings to light a few key areas for improvement.

Even though OA is considered one disease, it can arise from several originating causes, including joint injury, obesity, and age, among others. Joint crosstalk is one of the processes that may result in a common disease outcome, since deregulation of any individual tissue can trigger pathological behaviors in others. Given the multiple OA phenotypes, certain therapeutic

approaches may be more efficacious in some etiologies than in others. Additional research is needed to better understand these OA phenotypes, diagnose them in humans, and stratify treatments accordingly.

While it is becoming increasingly important to elucidate the longitudinal disease progression in order to determine the appropriate time of intervention, current radiographic standards are unable to detect the relative stage of OA. Additionally, patients often present at the clinic with prolonged pain, at which point OA may have progressed past the opportunity for successful interventional treatment. Longitudinal MRI tracking of joint outcomes, such as BMLs and synovitis, may improve detection of early OA in time for patients to benefit from certain therapeutics.

The limitations of observing disease progression in humans underscore the importance of animal studies to test the causal role of joint crosstalk mechanisms. However, current outcomes of OA in animals may not be representative of disease in humans. For instance, histologic evidence of cartilage degeneration tracks with OA severity in animal models, but not necessarily in humans, where a reduction in cartilage degradation has little to no relationship to pain (19). Development of OA animal models that better recapitulate human disease is needed.

Finally, pain is a fundamental symptom of joint disease that plays a complex, and incompletely understood, role in OA. Consequently, current outcome measures in clinical trials, such as pain assessment scores, radiographic joint space narrowing, or cartilage volume, may not be appropriate to determine if a given therapeutic is disease-modifying. Radiologic assessment of OA and pain are poorly correlated (17), and placebo can result in a marked amelioration of pain. Furthermore, RCTs have employed distinct pain scores to evaluate pain improvement, which can complicate drawing conclusions about the effect of a specific treatment on pain, especially in meta-

analyses. Additional consideration of pain in OA, its causes, and treatments in the design of basic and clinical research is needed.

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