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TGF- β /Smad3 Regulation of Bone, Cartilage, and Tendon

Matrix Composition and Material Properties

by

Carol G. Chen

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Abstract

The unique material properties of skeletal matrices are critical for musculoskeletal tissue function. For example, bone matrix material properties (BMMPs) – including elastic modulus, hardness, and fracture toughness – reflect the ability of bone matrix to resist deformation and catastrophic failure. However, the mechanisms by which these properties are established remain unclear. Many reports support a role for TGF- β and Smad3 in regulating the skeletal matrix. Thus, we hypothesized that Smad3 is essential for maintaining the extracellular matrix in bone, cartilage, and tendon.

Previously, our lab determined that TGF- β regulates BMMPs in development by signaling through its intracellular effector, Smad3. Here, we showed that BMMPs could also be regulated by pharmacologically disrupting TGF- β signaling. To further dissect the mechanism of BMMP regulation, we identified the downstream targets of TGF- β signaling. In vitro studies showed that TGF- β inhibits osteoblast differentiation by Smad3 repression of Runx2 function. Using nanoindentation, we found that TGF- β regulation of BMMPs through Smad3 is also Runx2-dependent. We further show that osteopontin, a matrix protein regulated by both TGF- β and Runx2, is essential for normal bone matrix material properties.

Our results establish a mechanism through which TGF- β regulates BMMPs to maintain healthy bone. This suggests a paradigm in which matrix material properties are established by growth factors through their regulation of lineage-specific transcription factors. Indeed, our further studies in cartilage and tendon indicate an essential role for TGF- β /Smad3 signaling in regulating cell lineage-specific transcription factor expression and activity as well as matrix structure and composition. We anticipate that our research will lead to the identification of novel diagnostic strategies and therapeutics for the prevention or treatment of skeletal diseases, as well as aid in the development of improved biomaterials for tissue regeneration.

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Chapter 1

Introduction

I. Overview

A major function of the musculoskeletal system – mainly comprised of muscle, bone, cartilage, tendons, and ligaments – is to provide structure, support, stability, and movement to the body. As such, it has an essential mechanical function, which is compromised in injury and diseases such as osteoarthritis and osteoporosis (in which cartilage and bone tissue are degraded, respectively). For example, in osteoarthritis, a progressive degeneration of articular cartilage leads to pain, stiffness, and loss of mobility. The articular cartilage matrix loses its integrity; as a result, joints become functionally impaired and unable to support normal loads.

Orthopaedic problems such as these are already a major cause of disability, and are growing due to rises in the active, aging, and obese populations. Osteoarthritis afflicts more than 20.7 million Americans and results in a cost of approximately \$65 billion per year in healthcare expenditures and productivity loss (AF 2008). More than 40 million Americans either already have osteoporosis or are at high risk due to low bone mass. In 2002, between \$12.2 and \$17.9 billion was spent on nursing homes and hospitals for people with osteoporosis-related fractures. The indirect costs of osteoporosis due to loss of productivity and lost wages is unknown (NIH 2011). Consequently, more research in the past couple of decades has focused on understanding the etiology of – and discovering therapies for – orthopaedic disorders, especially in the fields of molecular biology, tissue engineering, biomechanics, and biomaterials. Due to the challenges inherent in these complex biomechanical tissues, treatments for these injuries and disorders is often sub-optimal, either accompanied by undesirable side effects or treating symptoms without

addressing the underlying causes of disease. Furthermore, some of these musculoskeletal tissues, cartilage and tendon in particular, intrinsically have a limited capacity for repair, and successful treatment will need to address both slowing and stopping the progression of degradation as well as regeneration of native tissue.

Each musculoskeletal tissue is composed of cells within a unique matrix, which undergoes changes in disease. Though there is much research identifying the factors and some of the mechanisms that regulate bone and cartilage homeostasis (Goldring and Goldring 2007; Lombardi, Di Somma et al. 2010; Unnanuntana, Rebolledo et al. 2010; Donatti, Koch et al. 2011; Lewiecki 2011), the specific mechanisms through which the matrix is regulated are not yet well understood. Moreover, during healing, the regenerated tissue is frequently structurally different than native tissue and is functionally inferior. These structure and composition changes lead to changes in the matrix material properties, which are critical for both mechanical function and tissue maintenance. Therefore, my thesis focuses on exploring the biological factors and mechanisms that specify the musculoskeletal tissue matrix and regulate its material properties. Specifically, I investigated the role and mechanism of transforming growth factor beta (TGF- β)/Smad3 signaling in bone, cartilage, and tendon in regulating matrix constituents, structure, function, and material properties. I hypothesized that TGF- β signaling through Smad3 regulates transcriptional factors in bone, cartilage, and tendon to specify the tissue matrix.

II. Musculoskeletal Tissues: Form and Function

The tissues that I investigate in this thesis – bone, cartilage, and tendon – have analogous basic structures and compositions. In its simplest description, all these tissues are a combination of cells, fibers, and ground substance. The fibers in each of these tissues is collagen, which provides toughness and tensile properties. The ground substance in bone is hydroxyapatite, or mineral; in cartilage and tendon, it comprises glycosaminoglycans and water. These constituents provide the tissue

with specific resistance to compressive forces. The following sections will describe the form and function of each tissue in more detail.

A. Bone

The function of bones is to move, support, and protect various organs of the body. They are composed of multiple tissues including mineralized osseous tissue, marrow, nerves, and blood vessels. Aside from their mechanical function, bones also synthesize red blood cells and participate in many metabolic activities, such as calcium phosphate, glucose, and fertility regulation (Lee, Sowa et al. 2007; Oury, Sumara et al. 2011). For the remainder of this thesis, the focus will be on the biology and mechanical function of the weight-bearing long bones. Therefore, this section will focus on the form and function of cortical bone.

Cortical bone is the hard layer of bone surrounding the marrow cavity in long bones such as tibiae and femurs. The cortical bone matrix consists of both inorganic and organic constituents. The inorganic part of bone is composed of carbonated hydroxyapatite while the organic part is composed mainly of type I collagen (Col1) fibrils, with small amounts of growth factors and noncollagenous proteins such as osteocalcin, osteonectin, osteopontin, bone sialoprotein, biglycan, decorin, and other glycosaminoglycans. The collagen fibrils align to form lamellae, which are organized into osteons through which run vessels and nerves (1.1). Mature bone cells, osteocytes, are located within lacunae.

The main cells that regulate matrix production and resorption are osteoblasts and osteoclasts, respectively. In diseases such as osteoporosis, the tightly controlled balance of matrix anabolism and catabolism is disrupted in favor of catabolism, resulting in bone with decreased bone mass and mechanical integrity.

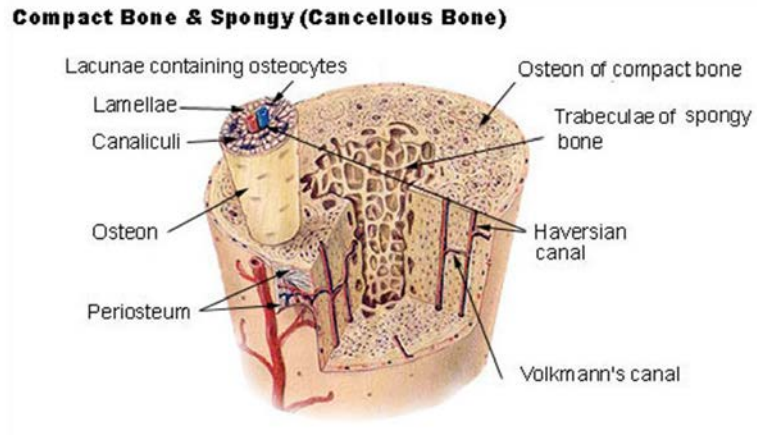


Figure 1.1. Schematic of compact (cortical) bone structure. Image from the U.S. National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) Program, <http://training.seer.cancer.gov/anatomy/skeletal/tissue.html>.

B. Cartilage

Articular cartilage is a flexible connective tissue that covers the end of bones in synovial joints (1.2). Its main function is to cushion and lubricate the joints. The unique composition of articular cartilage gives it the ability to withstand 1-2 million walking cycles a year and sustain loads of up to 10 times the body weight (Schmalzried, Szuszczewicz et al. 1998; Silva, Shepherd et al. 2002; Mow and Huiskes 2005). Because it lacks both a blood supply and nerves, it has a limited repair capacity, though the few chondrocytes in the tissue have the ability to deposit new matrix.

Articular cartilage consists primarily of water (60-85%) and an organized matrix consisting of mostly type II collagen (Col2, 15-22%) and glycosaminoglycans (4-7%) (Poole, Kojima et al. 2001). Articular cartilage can be divided into distinct zones, which are easily distinguished by changes in collagen organization. In the superficial zone (outermost layer), the collagen fibers lie in an orientation parallel to the joint surface. In the middle zone, the fibers are randomly organized, and in the deep zone, the fibers are perpendicular to the joint surface (1.3). The glycosaminoglycans exist mostly as aggregating

proteoglycans, called aggrecan (Agn, 50-85% of total proteoglycan weight). The only cells found in articular cartilage are chondrocytes, and they occupy 3-5% of the tissue by volume.

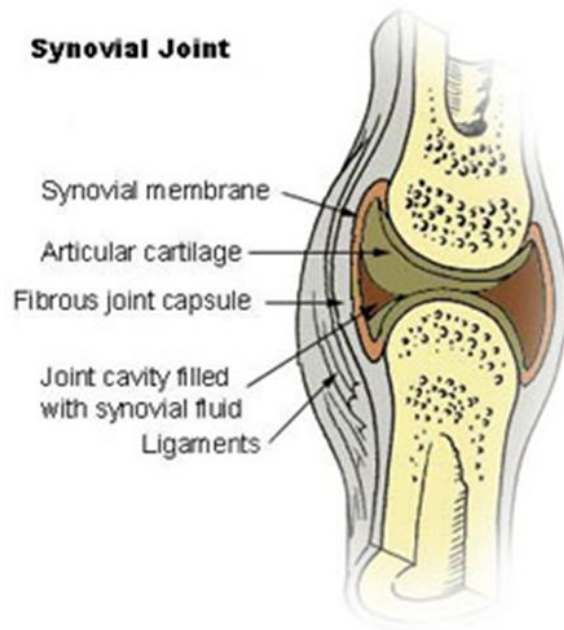


Figure 1.2. Schematic of a joint indicating articular cartilage location. Image from the U.S. National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) Program, <http://training.seer.cancer.gov/anatomy/skeletal/articulations.html>.

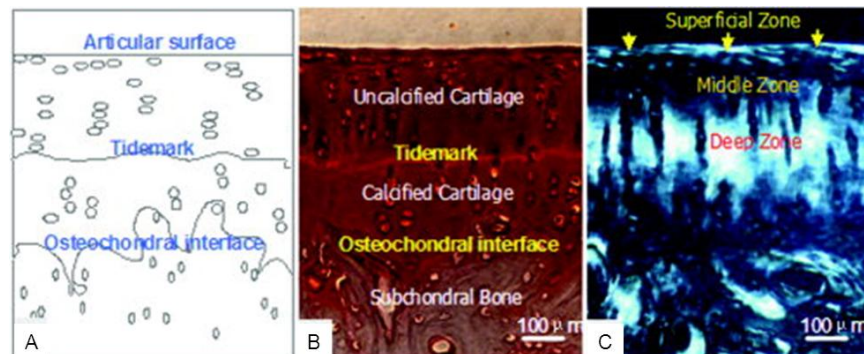


Figure 1.3. Illustration of cartilage zones in a rabbit metacarpophalangeal joint. (A) Schematic of a cross section. (B) Safranin-O-stained section. (C) Section observed under polarized light. Image from (Li, Pruitt et al. 2006).

C. Tendon

Tendon is a fibrous connective tissue that normally connects muscle to bone (1.4 A). It functions to transmit forces as well as modulate forces by acting as a spring. Because the tissue is elastic, it can store and recover energy efficiently. Furthermore, because tendons are flexible, muscles can generate greater forces since they are then able to function with little to no change in length.

Tendons are composed of parallel bundles of collagen fibers bound tightly together (1.4 B). They are about 60-80% water, and the remaining dry mass is composed of 86% collagen – mostly Col1, 2% elastin, and 1-5% proteoglycans (Lin, Cardenas et al. 2004). Like cartilage, tendon has a limited capacity for repair and relatively few cells reside in the tendon matrix.

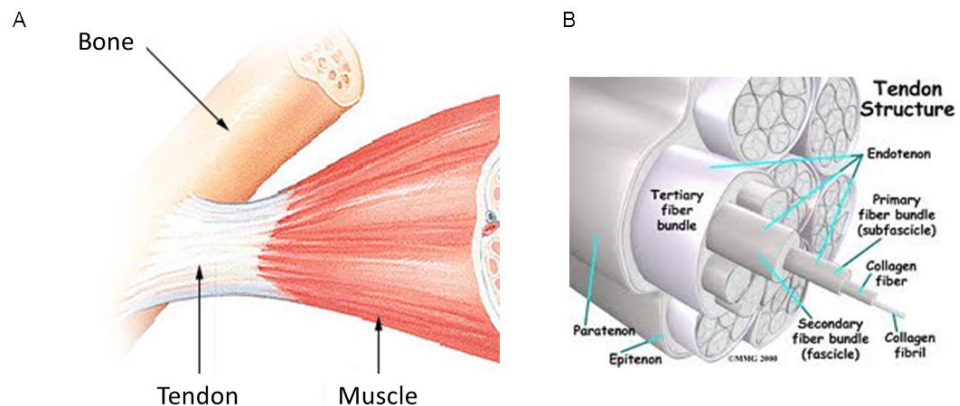


Figure 1.4. Schematics of tendon structure. (A) Tendon connects bone to muscle. Image from the U.S. National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) Program, <http://training.seer.cancer.gov/anatomy/muscular/structure.html>. (B) Organization of collagen fibers in tendon. Image from <http://www.eorthopod.com/content/achilles-tendon-problems>.

III. The Importance of Matrix Material Properties

The matrix material properties of musculoskeletal tissues are critical for normal function. Measurements such as fracture toughness, elastic modulus, hardness, and

yield strength have become useful in determining changes in the mechanical behavior of musculoskeletal tissues and thus their propensity for deformation and catastrophic failure.

For example, bone mineral density (BMD), or the areal density of bone, measured in g/cm^2 , has been the most common diagnostic to assess fracture risk (Fogelman and Blake 2000; Lochmuller, Lill et al. 2002). However, less than half of non-vertebral fractures can be explained by BMD alone (Schuit, van der Klift et al. 2004). Thus, other aspects of bone are important in assessing fracture risk, which are grouped under the term *bone quality* (Hernandez and Keaveny 2006). These characteristics include geometry, architecture, mineral concentration, and matrix material properties. In cases such as osteogenesis imperfecta, aging, diabetes and chronic corticosteroid use, increased incidence of fracture is not accompanied by significant changes in BMD (Wang, Shen et al. 2002; Van Staa, Laan et al. 2003; Kozloff, Carden et al. 2004; Nalla, Kruzic et al. 2004; Schwartz and Sellmeyer 2007; Saito and Marumo 2010). Localized changes in the elastic modulus of the bone matrix have been found in mice treated with glucocorticoids, indicating a possible correlation between the matrix properties and fracture risk (Lane, Yao et al. 2006).

In cartilage, it is well-documented that diseases such as osteoarthritis (OA) result in changes in the material properties and mechanical behavior of cartilage matrix. As OA progresses, articular cartilage loses its tissue-level mechanical integrity, becoming up to 20% less stiff in bulk mechanical properties than healthy cartilage (Knecht, Vanwanseele et al. 2006). Several studies have also investigated the matrix-level changes in elastic modulus as a result of cartilage degradation in animal models as well as human OA (Stolz, Raiteri et al. 2004; Gottardi, Cardinali et al. 2007; Stolz, Gottardi et al. 2009).

Besides their potential diagnostic utility, matrix stiffness has emerged as a potent regulator of cell behavior. For example, abnormally stiff matrices promote breast tumorigenesis and liver fibrosis (Yeh, Li et al. 2002; Paszek, Zahir et al. 2005). In

addition, matrix stiffness has been implicated in the control of mesenchymal stem cell differentiation (Engler, Sen et al. 2006). The stiffness of polyacrylamide matrices was shown to be a stronger agonist of mesenchymal stem cell differentiation into osteoblasts than traditional soluble agonists ascorbic acid and β -glycero-phosphate. Conversely, culture of the same cells in a matrix with lower stiffness potentiated neurogenic differentiation. Our lab is currently exploring the interactions between biological and physical cues. Consistent with previous reports, the data indicate that not only are physical cues as strong as biological cues in chondrogenic differentiation, but optimum levels of chondrocyte-specific genes are expressed on polyacrylamide gels of a specific stiffness, comparable to normal cartilage tissue (Allen and Alliston, unpublished observations). Thus, the matrix environment, particularly the matrix stiffness, exerts a strong effect on cell behavior and differentiation and is a key property of a variety of tissues and in a variety of diseases.

In tendon, the stiffness of tendon, measured by tension testing of whole tissue, is a critical factor in determining the damage to muscle fibers during lengthening contractions. A study demonstrated that the force deficit after a contraction-induced injury is directly related to the strain on the muscle fibers during the lengthening contraction (Brooks, Zerba et al. 1995). In myostatin-deficient mice, the force deficit after a two-stretch lengthening contraction protocol was 15% greater than in control mice (Mendias, Marcin et al. 2006). These mice also had significantly increased tendon stiffness (Mendias, Bakhurin et al. 2008), suggesting that the increased strain felt by the muscle fibers may have been due to changes in material properties of tendon.

Overall, it is clear that the matrix material properties are not only important for the mechanical function of the musculoskeletal system, but also for normal cell behavior.

IV. TGF- β /Smad3 Regulation of Musculoskeletal Matrix Material Properties

In each of these musculoskeletal tissues, the specific matrix produced by the resident cells is uniquely suited to its function. The deposition as well as the turnover rate of the matrix is tightly regulated, and diseases such as osteoarthritis and osteoporosis can be traced back to disruptions in this control (Goldring and Marcu 2009; Syed, Iqbal et al. 2010). Though some of the factors involved in matrix regulation have been identified, the mechanisms that regulate the development and maintenance of the matrix through specification of matrix proteins and organization are still largely unknown.

Much evidence supports a role for TGF- β /Smad3 signaling in regulating the matrix composition and material properties of bone, cartilage, and tendon. First, our lab previously demonstrated that the elastic modulus and hardness of cortical bone was reduced in D4 and D5 mice with overexpression of TGF- β while the converse was true in mice with reduced TGF- β signaling either due to the expression of a dominant negative TGF- β receptor (DNT β RII) or systemic deletion of Smad3 (Balooch, Balooch et al. 2005) (1.5 A). Further, in vitro studies in osteoblasts show that TGF- β signaling through Smad3 represses Runx2, a key osteoblastic transcription factor, through recruitment of a histone deacetylase (Alliston, Choy et al. 2001; Kang, Alliston et al. 2005) (1.5 B). Whether this pathway is relevant in vivo, as well as in the TGF- β -mediated regulation of bone extracellular matrix material properties, are some of the questions I address in this thesis; in addition, I investigate whether the matrix constituent osteopontin (OPN), shown to be regulated by both TGF- β and Runx2 (Wai, Mi et al. 2006; Duvall, Taylor et al. 2007), is a downstream target of TGF- β signaling in the regulation of bone matrix material properties.

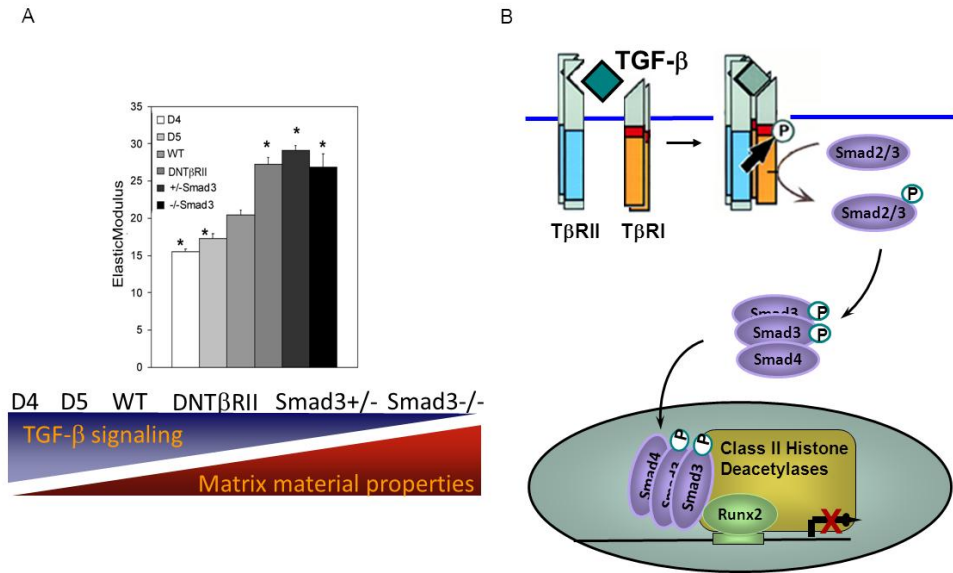


Figure 1.5. TGF- β signaling in bone. (A) The regulation of elastic modulus by TGF- β (Balooch, Balooch et al. 2005). (B) TGF- β signaling pathway in osteoblast differentiation; may be involved in defining matrix material properties (Alliston, Choy et al. 2001; Kang, Alliston et al. 2005).

The existence of a parallel mechanism has yet to be elucidated in chondrocytes and cartilage. However, evidence suggests that TGF- β and Smad3 inhibition of Runx2 play a role in the progression of chondrocyte differentiation (Ferguson, Schwarz et al. 2000). Smad3 $-/-$ mice, as well as mice that overexpress a dominant negative TGF- β type II receptor or Runx2, both exhibit premature chondrocyte hypertrophy (Zheng, Zhou et al. 2003). Also, both type X collagen (ColX) and matrix metalloproteinase (MMP-13), which are cartilage extracellular matrix proteins induced by Runx2 during terminal chondrocyte differentiation, are also regulated by TGF- β (Zheng, Zhou et al. 2003; Dong, Soung do et al. 2006; Selvamurugan, Jefcoat et al. 2006). ColX and MMP-13 participate in cartilage matrix structure and remodeling and are misregulated in OA. ColX associates with type II collagen fibrils, interacts with matrix components such as proteoglycans, and is expressed prior to mineral deposition by cultured chondrocytes (Schmid and Linsenmayer 1990; Kirsch, Swoboda et al. 1992). MMP-13 degrades collagen II, aggrecan, and other cartilage

extracellular matrix proteins (Stickens, Behonick et al. 2004). As chondrocytes progress from the resting state to hypertrophy, ColX and MMP-13 expression increases, altering the matrix composition and presumably altering its material properties. Thus, I investigated the changes in matrix composition and matrix material properties that result from disruption of the TGF- β signaling pathway in Smad3-deficient mouse cartilage.

Lastly, though less well-studied, TGF- β has also been implicated in tendon development and maintenance. Disruption of TGF- β signaling by either ablating TGF- β 2 and TGF- β 3 signaling, or inactivating the TGF- β type II receptor, both resulted in the development of embryos missing most of their tendons (Pryce, Watson et al. 2009). Furthermore, myostatin, a TGF- β superfamily cytokine which activates Smad3, like TGF- β itself, was shown to regulate the mechanical properties and gene expression of tendons (Mendias, Bakhurin et al. 2008). Mice lacking myostatin had stiff, brittle tendons; they also had decreased expression of scleraxis (Scx), a tendon lineage-specific transcription factor, tenomodulin (Tnmd), a tendon-selective gene, and Col1 (Mendias, Bakhurin et al. 2008). Therefore, I investigated the role of TGF- β /Smad3 signaling in the regulation of the tendon matrix in Smad3-deficient mice and developed an in vitro system for further studies on the mechanisms through which this regulation might occur.

V. Clinical Relevance

In the long term, this research has the potential to make significant contributions to clinical problems. First, understanding the regulation of matrix material properties may provide new targets for therapeutic strategies to treat or prevent osteoporosis, OA, and other degenerative diseases. Furthermore, changes in material properties due to aging and OA are detectable by indentation well before detection by radiographic and arthroscopic evaluation, the current diagnostic tools in use today (Stolz, Gottardi et al. 2009). This highlights a possible use for clinical indenters as a

diagnostic tool, since the matrix material properties may reflect changes that can be used as a sign of disease (Hansma, Yu et al. 2009). Lastly, understanding the molecular regulation of the development and maintenance of a healthy musculoskeletal matrix will improve the ability to regenerate and heal these tissues when damaged due to disease or trauma. I anticipate that this research will contribute to the identification of novel diagnostic strategies and therapeutics for the prevention or treatment of skeletal diseases, as well as aid in the development of improved biomaterials for tissue regeneration.

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Chapter 2

Materials and Methods

Mice

The mice used in these experiments are described in table 2.1.

Mouse	Description
D4	16-fold overexpression of TGF- β 2 driven by the osteocalcin promoter ¹
D5	2.5-fold overexpression of TGF- β 2 driven by the osteocalcin promoter ¹
DNT β RII	Expresses a cytoplasmically truncated type II TGF- β receptor driven by the osteocalcin promoter ²
Smad3 ^{-/-}	Systemic inactivation of Smad3 to produce Smad3-null mice ³
Runx2 ^{+/-}	Systemic heterozygous inactivation of Runx2 ⁴
Smad3 ^{ex8/ex8}	Systemic inactivation of Smad3 by deletion of exon 8 ⁵
OPN ^{-/-}	Systemic inactivation of osteopontin by deletion of exons 4-7 ⁶
Smad3 ^{fl/fl}	Two loxP sites flanking Smad3 exons 2 and 3 ⁷
Col1-Cre;Smad3 ^{fl/fl}	Conditional inactivation of Smad3 in Col1-expressing tissues (predominantly bone) ⁸
Col2-Cre;Smad3 ^{fl/fl}	Conditional inactivation of Smad3 in Col2-expressing tissues (predominantly cartilage) ⁹

Table 2.1. Description of mouse genotypes. References: 1. (Erlebacher and Derynck 1996), 2. (Filvaroff, Erlebacher et al. 1999), 3. (Datto, Frederick et al. 1999), 4. (Otto, Thornell et al. 1997), 5. (Yang, Letterio et al. 1999), 6. (Liaw, Birk et al. 1998), 7. (Li, Pangas et al. 2008), 8. (Liu, Woitge et al. 2004), 9. (Ovchinnikov, Deng et al. 2000)

Real-time (Quantitative) Reverse Transcriptase-PCR (RT-PCR)

For analysis of gene expression in mouse tissue, calvaria, femur, costal cartilage or tail, flexor digitalis longus, and tibialis anterior tendons were isolated from 2-month old mice. For calvaria, the periosteum was removed with forceps carefully. For femurs, the ends were removed and the marrow was flushed several times with PBS using a 25 gauge syringe so that the remaining tissue used consisted only of cortical bone. For costal cartilage, each individual rib was carefully cleaned of the membranous connective tissue between each rib. For tendon, the epitenon was carefully removed with a #11 scalpel and the dissection was performed with the aid of loupes. All tissues were snap frozen in liquid N₂ and stored in liquid N₂.

Calvaria, femur and costal cartilage were homogenized using the same method, in a ceramic mortar and pestle partially submerged in liquid N₂. The tissues were ground into a fine dust and immediately put into Trizol (Invitrogen). Tendon was placed directly into Trizol and homogenized with a glass tube mortar and pestle. After homogenization, RNA was extracted using the Purelink RNA Mini Kit (Invitrogen). cDNA was reverse transcribed from the RNA using the iScript cDNA synthesis kit (BioRad). Transcripts were amplified using the primer sets shown in table 2.2. Results were detected based on amplicon binding of Sybr Green (BioRad) using the CFX96 Real-Time PCR Detection System (BioRad, Hercules, CA).

Gene	Forward	Reverse
OCN	5'-CAAGTCCCACACAGCAGC TT- 3'	5'-AAAGCCGAGCTGCCAGAGTT-3'
OPN	5'-AGAGAGCGAGGATTCTGTGG-3'	5'-CCTCAGTCCA TAAGCCAAGC-3'
Smad3	5'-CACAGCCACCATGAATTACG-3'	5'-TGGAGGTAGAACTGGCGTCT-3'
Runx2	5'-CCCAGCCACCTTTACCTACA-3'	5'-CAGCGTCAACACCATCATTC-3'
SOST	5'-TCCTCCTGAGAACAACCAGAC-3'	5'-TGTCAGGAAGCGGGTGTAGTG-3'
MMP-13	5'-CGGGAATCCTGAAGAAGTCTACA-3'	5'-CTAAGCCAAAGAAAGATTGCATTTC-3'
Sox9	5'-TCGCCTTCCCCGGGTTTAGAGC-3'	5'-GGCGGCGGGCACTTAGCAGA-3'
Col2	5'-ACGAAGCGGCTGGCAACCTCA-3'	5'-CCCTCGGCCCTCA TCTCTACA TCA-3'
ColX	5'-TGCCCGTGTCTGCTTTTACTGTCA-3'	5'-TCAAA TGGGATGGGGGCACCTACT-3'
Scx	5'-CCTTCTGCCTCAGCAACCAG-3'	5'-GGTCCAAAGTGGGGCTCTCCGTGAC-3'
Tnmd	5'-TGTA CTGGATCAATCCCACTCT-3'	5'-GCTCATTCTGGTCAATCCCCT-3'
TenC	5'-AGGCGATCCCAGCCAGTCAGT-3'	5'-ATGGACGGGGCACCTCCTGTC-3'

Table 2.2. Real-time RT-PCR primer sets

For real-time RT-PCR analysis of Saos-2 cells treated with TGF- β (Peprotech) and tendon fibroblasts, β -mercaptoethanol was used to lyse the cells and the RNeasy Mini Kit (Qiagen) was used to purify the RNA. Subsequent steps were performed the same as with RNA from tissues, described above.

Nanoindentation

Nanoindentation was used to determine the elastic modulus and hardness of various mouse bones. For details, see Chapter 3, Nanoindentation. Briefly, dissected cochlea, malleus, calvariae, tibiae, and femurs were stored in Hank's Balanced Salt Solution (HBSS) with 0.05% sodium azide at 4° C for a maximum of one week. They were then embedded in Stycast 1266 epoxy (Emerson and Cuming) and polished with abrasive paper and diamond suspensions down to 0.25 mm. Indentation was performed with a Berkovitch tip (Triboindenter, Hysitron) using a

trapezoidal loading function. A peak load of 600 μN was held for 10s before unloading and calculation of the elastic modulus and hardness.

Indentation-type Atomic Force Microscopy (IT-AFM) of Polyacrylamide Gels

IT-AFM was used to evaluate the elastic moduli of polyacrylamide gels. For details, see Chapter 4, Indentation Testing for Biological Soft Tissues: Nanoindentation and AFM. Briefly, AFM indentation was performed as described on polyacrylamide gels with 1, 2, and 3% piperazine diacrylamide cross-linker (Hansma, Yu et al. 2009).

Transmission Electron Microscopy (TEM)

TEM was used to qualitatively assess the mineral in mouse tibiae. To prepare the bones, mouse tibiae were fixed in anhydrous ethylene glycol for 24 h, dehydrated by rinsing in 100% ethanol three times for 5 min in acetonitrile, and then infiltrated with resin (Agar Scientific) over several days. Mounting resin was prepared with 12 g Quetol, 6.5 g methyl nadic anhydride (MNA), 15.5 g nonenylsuccinic anhydride (NSA), and 0.7 g benzlydimethylamine (BDMA). The samples were agitated at room temperature in 1:1 solutions of acetonitrile and resin for 1 day, then 1:2 acetonitrile resin for 1 day, and finally 100% resin for 3 days under vacuum; the resin was changed every 24 h. Samples were then cured in fresh resin for 24 h at 60 °C. Silver to gold sections (70–90 nm) were cut onto distilled water with an ultramicrotome using a 35° diamond knife. Samples were collected immediately on lacy carbon 300 mesh copper grids. For TEM analysis, bright field imaging was performed on a JEOL 2000 FX TEM using operating voltages of 120 and 200 kV.

Auditory Function

Evoked auditory brainstem response thresholds were measured in 2-month-old male mice (Akil, Chang et al. 2006).

Fracture Toughness

Dissected mouse femurs were tested in bending to measure the fracture toughness, as previously described (Ritchie, Koester et al. 2008; Turner, Chen et al. 2010). Sharp notches were made using 1 μm diamond polishing solution to cut the bone midshaft through the posterior wall of the femur. The resulting micro-notches were maintained at $\sim 1/3$ of the bone diameter in

length with a reproducible notch root radius of $\sim 10\ \mu\text{m}$. Femurs were tested in a three-point bending configuration with a custom-made rig for the ELF 3200 mechanical testing machine (ELF3200, EnduraTEC, Minnetonka, MN). Testing was conducted in displacement control at a cross-head displacement rate of 0.001 mm/s. Half-crack angle at point of instability was determined by scanning electron microscopy. Fracture toughness, K_{IC} , was calculated using a stress-intensity solution for a circumferential through-wall flaw in cylinders.

X-ray Tomography

XTM studies were used to assess the degree of mineralization of the bone; procedures were based on the work of Kinney et al. (Kinney, Marshall et al. 1994). Whole mouse femurs were scanned to determine the degree of bone mineralization. Imaging was performed at the Advanced Light Source (ALS) on Beamline (8-3-2) at the Lawrence Berkeley National Laboratory by obtaining two-dimensional radiographs as the specimens were rotated through 180° in 0.5° increments. The radiographs were reconstructed into 2,500 slices by Fourier-filtered back projection with a 4.5 mm resolution. The attenuation coefficient (μm^{-1}) of each pixel relates directly to bone mineral concentration. The degree of bone mineralization (DBM) was obtained from the following equation:

DXA

Analysis of bone mineral density (BMD) was performed on dissected mouse femurs using a PIXImus mouse densitometer (GE Lunar II, Faxitron Corporation, Wheeling, IL).

Micro-Computed Tomography (μ -CT)

μ -CT was used to assess the volumetric bone mineral density and geometric and architectural parameters of bone. Mouse tibiae were scanned using the Scanco Viva-CT 40 scanner (Scanco Medical AG, Bassersdorf, Switzerland). The μ -CT scanner was operated at the peak energy of 70 kVp, current of 114 μ A, integration time of 381 ms, and a 10 μ m voxel resolution. A semi-automatic adaptive slice-wise contouring method was used for determining the boundaries for both cortical bone and trabecular bone, and the contours were manually verified for consistency and continuity.

Histology

To analyze the structure of mouse bones, tibiae were isolated from 2-month old mice and fixed in 4% paraformaldehyde at 4° C overnight and decalcified in 19% EDTA. The decalcified tissues were then dehydrated and embedded in paraffin. Sections were cut longitudinally and stained with Masson's Trichrome.

To analyze the proteoglycan content in articular cartilage and the growth plate, knee joints were isolated from 2-month old mice and prepared as described above. Sagittal sections were made, stained with Safranin-O, and counterstained with Fast Green.

To analyze the structure of tendons, FDLs were isolated from 2-month old mice and fixed as described above. After dehydration and embedding in paraffin, the tendons were sectioned longitudinally and stained with hematoxylin and eosin.

After staining, all sections were rinsed, dehydrated, and mounted using standard procedures.

Polarized Light Microscopy

Masson's Trichrome-stained tibiae were examined under polarized light using Nikon Microphot-FX microscope with polarizing filters (Nikon Microphot-FX, Nikon, Melville, KY) to assess collagen fiber organization.

Dimethylmethylene Blue (DMMB) Assay

To quantify the glycosaminoglycan (GAG) content in mouse articular cartilage, the DMMB assay was performed on mouse femoral head cartilage. The left and right femoral head cartilage was gently dislocated from the bone by squeezing with forceps. The two pieces of tissue were then digested with 20 units/ml papain (Sigma) with 2mM L-cysteine hydrochloride (Sigma) and 2mM disodium EDTA in a total volume of 250 ml PBS at 60° C overnight. The samples were then treated with DNase I (Roche) and diluted 1:10. The samples were compared to a standard curve made with chondroitin sulfate isolated from bovine trachea (Sigma). Absorption was measured at 525 nm using a spectrophotometer. The GAG content was normalized to DNA measured using Quant-iT PicroGreen (Invitrogen).

Modified Mankin Scoring

To assess the degree of cartilage degeneration, the Modified Mankin scoring system was used to evaluate the Safranin-O stained knee joints. Eight sections per group were scored by a blinded orthopaedic surgery resident. Mouse articular cartilages were evaluated based upon pericellular and background Safranin-O staining, chondrocyte arrangement, and structural condition of the articular cartilage. The score for normal mouse articular cartilage is 0 and the maximum score for degenerative articular cartilage is 10, see table 7.1.

Immunohistochemistry

To visualize the location of type II collagen and aggrecan in mouse articular cartilage, immunohistochemistry was carried out on paraffin embedded sections after antigen retrieval with 2.5% hyaluronidase (Sigma) and as previously described (Shibata, Fukada et al. 2001), respectively. The following antibodies were used: mouse aggrecan 12/21/1-C-6 (Developmental Studies Hybridoma Bank) and rabbit collagen II (AbCam). The Mach4 universal detection system (Biocare Medical) was used to detect the primary antibodies and the DAB Substrate Kit for Peroxidase (Vector Laboratories) was used to stain the tissues.

Tendon Fibroblast Cultures

To develop an in vitro model to study the mechanisms that regulate the function and differentiation of tendon cells, we used primary tendon fibroblasts from mice. See Appendix D for

a detailed protocol. Briefly, FDL tendons were isolated from P10 and 6-8 week old mice and the cells were isolated and cultured. For studies with TGF- β , cells were treated with 5 ng/ml TGF- β 1 (Peprotech) when they reached confluency for 24 hours.

Adenoviral Infection of Tendon Fibroblasts

To delete Smad3 from cultured tendon fibroblasts, spinfection was performed on cells isolated from Smad3^{fl/fl} mice (Suter, Gouthro et al. 2006). To prepare for the infection, 1 ml of 4 mg/ml polybrene was added to each ml of Optimem media. For a 6 well plate, you need 1 ml/well. After 10 minutes, virus was added at 100 pfu/cell. Replication-deficient adenoviruses expressing Cre recombinase and GFP were used. After 10 more minutes, 1 ml of the media with polybrene and virus was added to each well. The plates were then spun at 1520 RPM for 45 minutes at 37° C. At the end of the spin, 1 ml of tendon cell culture media (see Appendix D) was added to each well. The plates were then incubated overnight and the media with virus was removed the next day and new media was added. Cells were then left to grow to confluency before harvesting for RNA isolation and real-time RT-PCR.

Statistics

Statistical analyses of gene expression, nanoindentation, fracture toughness, XTM, unconfined compression, DXA, μ CT and DMMB of groups were compared using the two-tailed Student's T-test with a significance threshold of $p = 0.05$. The p value for multiple regression analysis comparing elastic modulus with ABR thresholds was also determined using the two-tailed Student's T-test.

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Chapter 3

Nanoindentation

Introduction

Essential functions of skeletal tissues such as bone, cartilage and tendon are mechanical and structural. Many diseases such as osteoporosis and osteoarthritis are marked by a deterioration in both mechanical and material properties. Osteoporosis often results in an increase in bone fragility and many studies have shown that osteoarthritis is correlated to a reduction in the stiffness of articular cartilage (Setton, Mow et al. 1994; Hyttinen, Toyras et al. 2001; Knecht, Vanwanseele et al. 2006). Thus, characterizing the material properties and mechanical behavior of skeletal tissues, as well as their relationships to disease progression, is of critical clinical significance.

Early studies utilized tools that test bulk material and mechanical properties at the macroscale. In the majority of these studies, pieces of or whole tissue with sizes on the order of centimeters or larger are placed in a mechanical tester and undergo compression or tension. Although useful, these data are of insufficient resolution to understand many biological processes due to methodological limitations. Bone and other biological tissues are heterogeneous and often exhibit localized differences in material properties. Multiple studies suggest that the material properties of the microstructural elements of bone, such as individual trabeculae or osteons, is distinct from macroscale measurements. The observation that laminate osteons have a Young's modulus, defined as the ratio of stress to strain and a measure of stiffness, that differs from that of bulk bone corroborates this conclusion (Rho, Tsui et al. 1997). Therefore, to investigate intrinsic material properties on a more locally precise scale, indentation procedures, which were originally used to test stiffness and strength in ceramics, metals and polymers, have been adapted for use on biological tissues.

In particular, nanoindentation has emerged as a useful and widely employed technique. Previous studies have used this technique to characterize such diverse biological tissues as bone, dentin, enamel, cartilage, and arteries (Ebenstein and Pruitt 2004; Balooch, Balooch et al. 2005; Li, Pruitt et al. 2006; Saeki, Hilton et al. 2007). In nanoindentation, a load is applied to a hard tip, or probe, of known material properties such that it induces deformation of the test sample surface. Information about the applied load and tip displacement is recorded and the material properties of the test sample may be calculated using equations based on elastic contact theory. Probe diameters may be in the range of tens of nanometers to ~100 microns, which facilitate the measurement of material properties with increased spatial resolution. Besides microscale capabilities, these probes are ideal for testing tissues from small animal models, such as mice, which are often too small and difficult to evaluate using traditional tools. Thus, nanoindentation, coupled with the powerful utility of genetic mouse models, has made it possible to explore the relationship of biological signaling pathways to the specification of material properties. In this thesis, nanoindentation will be used to measure bone matrix material properties in genetic mouse models.

Instrumentation

The instrument used in these studies to measure the material properties of bone is the Triboindenter (Hysitron, Inc., Minneapolis, MN), a high-throughput, automated platform for nanoindentation, imaging, and other nanomechanical characterization techniques. The system consists of a motorized XYZ staging system, high resolution top-down optics, and the TriboScanner, which includes a three-axis piezo positioner, the transducer, and the indenter tip. The optics and the TriboScanner are mounted on a granite bridge which is on a granite stage, all within an acoustic enclosure. With the active vibration control stage, the enclosure ensures that ambient noise, vibration, and thermal drift effects are minimal. The instrumentation located within the acoustic enclosure is shown (3.1).

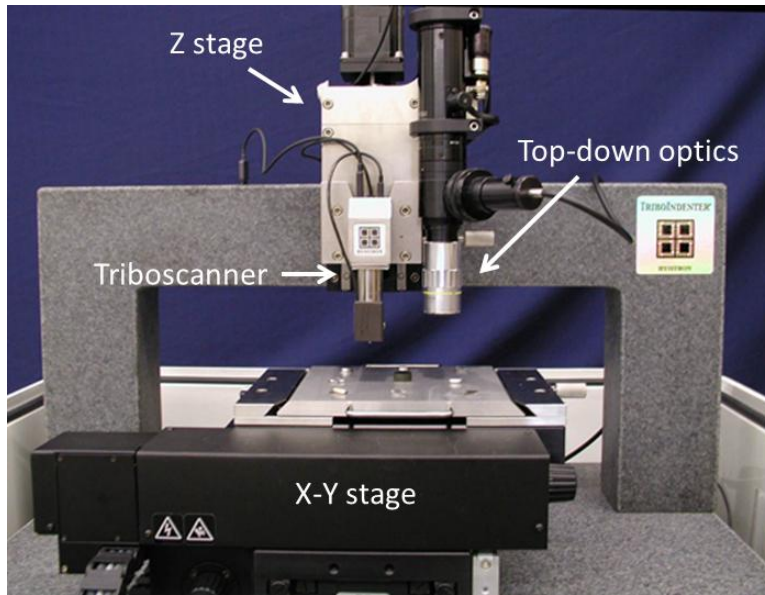


Figure 3.1. Photograph of the Triboindenter system with the top of the enclosure removed. Image courtesy of Hysitron, Inc.

Tip geometry may be regarded as the most important experimental selection (Oyen Cook 2009). Sharper tips are more suitable for stiffer, harder materials or the characterization of finer features while more obtuse or blunt tips are generally used for softer materials to prevent excessive plastic deformation, fracture, or penetration. Another consideration when choosing tip geometry is the deformation mode of interest. The major deformation modes in instrumented indentation are elastic, brittle, elastic-plastic, viscous-elastic, and viscous-elastic-plastic. Elastic deformation is reversible. When a load is applied to a purely elastic material, the strain is instantaneous and once the load is removed, the material returns to its original shape just as quickly. Materials with brittle behavior typically exhibit very little plastic deformation before fracture. An elastic-plastic material exhibits both elastic behavior and permanent (plastic) deformation, a viscous-elastic material exhibits elastic behavior and a non-linear strain rate, and viscous-elastic-plastic materials exhibit all three modes of deformation. Typical load-displacement curves for each mode are shown (3.2 A-E) (Oyen and Cook 2009). Consistent with previous studies, these studies use the standard Berkovitch (three-sided pyramidal) tip for

measuring the material properties of dry bone, which predominantly exhibits elastic-plastic deformation (3.3 A-C) (Balooch, Balooch et al. 2005; Lane, Yao et al. 2006) (Appendix A).

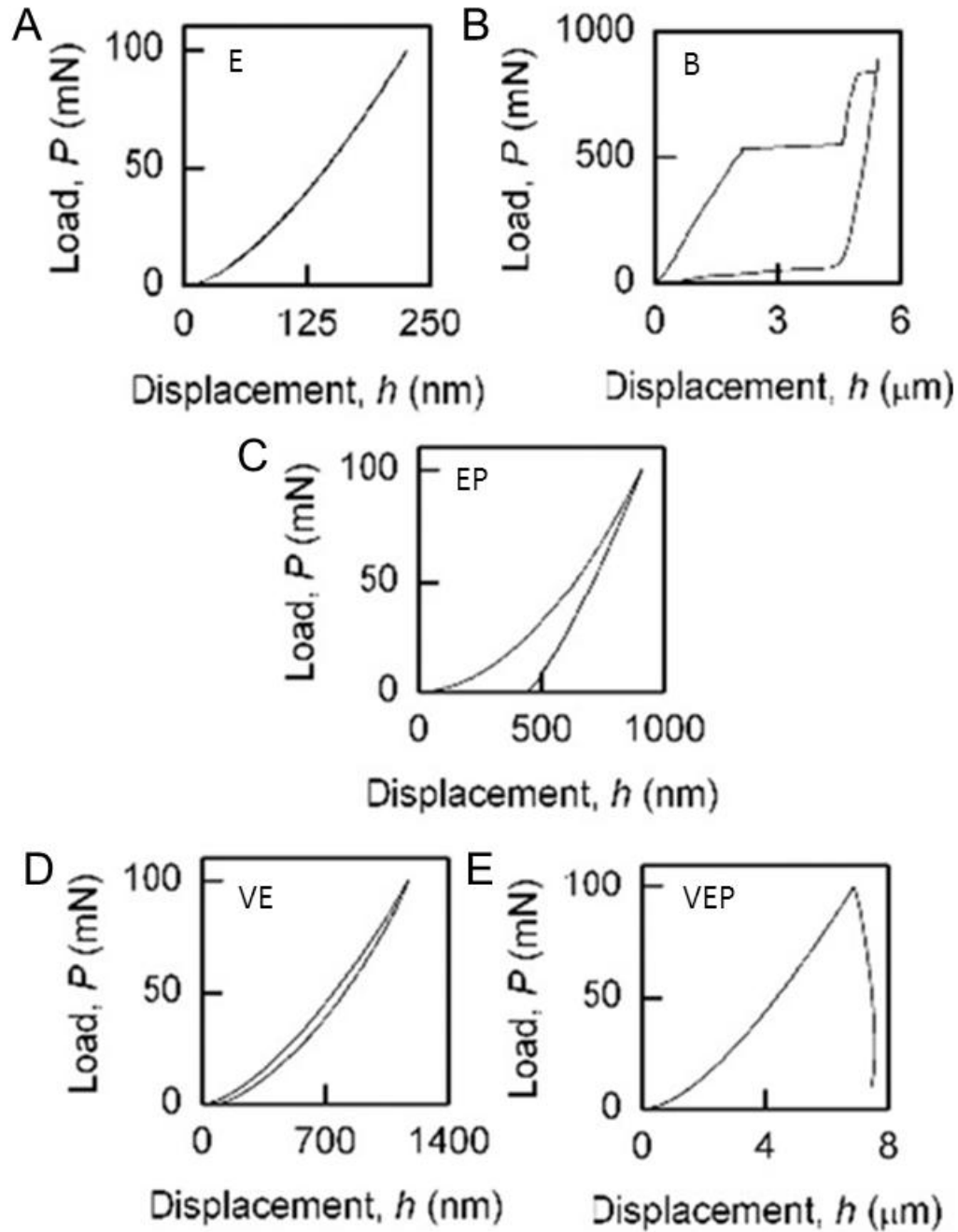


Figure 3.2. Typical load-displacement curves for major modes of deformation. Load-displacement curves for elastic (A), brittle (B), elastic-plastic (C), viscous-elastic (D), and viscous-elastic-plastic (E) behavior.

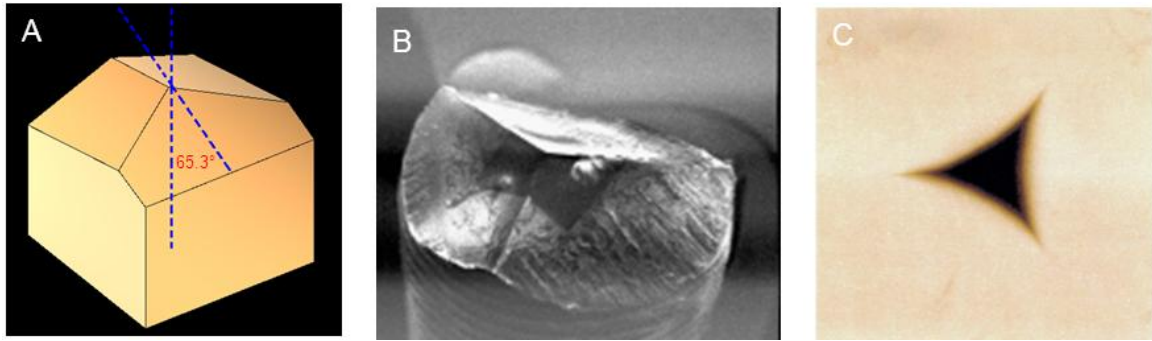


Figure 3.3. The Berkovitch Tip. (A) Schematic of Berkovitch tip geometry. (B) Photograph of Berkovitch tip. (C) AFM image of indent made by Berkovitch tip. 3.0 μm scan of 10,000 μN indent on fused quartz.

Nanoindentation Procedure (Please refer to Appendix B for a detailed protocol.)

I. Sample Preparation

Sample preparation is of paramount importance in nanoindentation. Critical factors include minimizing compliance in sample mounting, creating a smooth, even, level surface for probing, maintaining tissue integrity, and fixation of the sample on the testing stage. For these studies, mouse bones were embedded in Stycast 1266 and parallel cross-sectional cuts were made to expose mid-tibial region with a low speed saw to ensure a flat surface. The section of embedded bone was then glued onto metal specimen discs with cyanoacrylate and polished with diamond polish down to 0.25 μm . The metal specimen discs are necessary for the specimen to be firmly held onto the magnetic stage of the TriboIndenter.

II. Calibrating the TriboIndenter

Several steps must be taken to calibrate the instrument. First, the Advanced Z-Axis Calibration must be performed to correct for the amount of electrostatic force necessary to deform the spring in the transducer. Then the tip-optics offset calibration must be performed so that indent locations correctly correspond to the optical focal point. Once this is done, if there have been any tip or stage modifications, the machine compliance must also be calibrated. In indentation instruments, the measured displacement is the sum of the indentation depth and the

displacement associated with the system, termed the load-frame compliance. Since material properties are calculated from load-displacement curves, it is essential to ascertain the compliance associated with the instrument. The final calibration necessary is the tip area function. This process results in an equation relating the projected contact area to the indent depth, which is necessary for subsequent analysis to determine material properties. This will be discussed in more detail in section IV, Data Analysis.

III. Making an Indent (Data Acquisition)

The first step in making an indent is to specify the testing mode. For the Triboindenter, there are three modes available: open loop, load control, and displacement control. In open loop control, there is no feedback for the real-time force and displacement. The force is calculated based upon the voltage applied to displace the tip. However, this force is not the true force applied to the sample because changes in the transducer configuration are not taken into account. The force generated by the electrostatic voltage is the sum of the force applied to the sample plus the force used to deform the internal spring in the transducer.

For more precise control of the nanoindenter tip, load or displacement control should be used. In these modes, the piezo controller provides feedback to specify the electrostatic force (voltage) in real-time, allowing precise control of the nanoindenter tip in the z-axis. In displacement control, the force is adjusted in response to continuous monitoring of displacement. In our studies, we used load control to increase precision and accuracy of measurements.

After choosing the testing mode, the user must specify the load function. The simplest load function consists of two segments, a loading and unloading sequence. This is commonly performed on materials that exhibit little or no time-dependent behavior, such as aluminum. Bone, though mostly exhibiting an elastic-plastic mode of deformation, displays viscous behavior that cannot be considered negligible. Thus, for these studies, a trapezoidal load function is used (3.4). The specific load function used on bone in these studies consists of a loading rate of 100 $\mu\text{N/s}$ to a maximum load of 600 μN , followed by a hold period of 10 seconds followed by

unloading at the same rate as the load sequence. A 10 second hold was ascertained to be long enough for time-dependent behavior of the material to equilibrate.

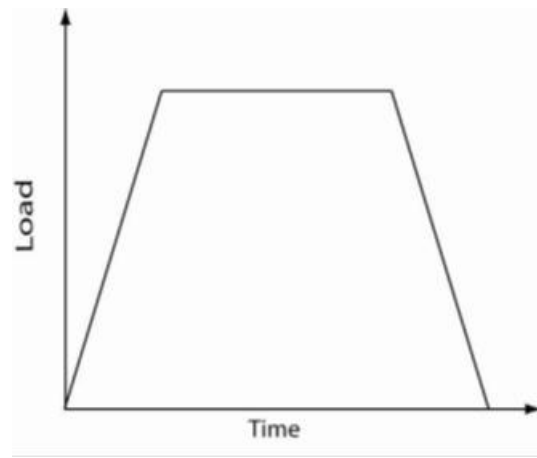


Figure 3.4. Schematic of a trapezoidal loading scheme.

Indents were performed after in situ imaging provided topographical information so that asperities, lacunae and any voids could be avoided. At least 5-10 μm spacing between indents was used to ensure that the stress field from one indent did not affect the next. Multiple indents were performed on each sample such that the effect of surface roughness was minimized (Gerberich, Yu et al. 1998; Klapperich, Pruitt et al. 2001)

IV. Data Analysis

Figure 3.5 shows a typical load-displacement (L-D) curve and a schematic of the resultant indent. This data, with the area function, may be used to calculate the material properties of elastic modulus (E) and hardness (H). Elastic modulus reflects the stiffness, or resistance to elastic deformation, of a material while hardness indicates the resistance to plastic deformation. The most widely used and accepted method of analyzing nanoindentation data is known as the Oliver Pharr method, based on elastic contact theory (Oliver and Pharr 1992; Nix 1997). The key assumption of the Oliver Pharr method is that the initial unloading portion of the

load-displacement curve is dominated by the elastic response and thus may be used to calculate the elastic properties of a material.

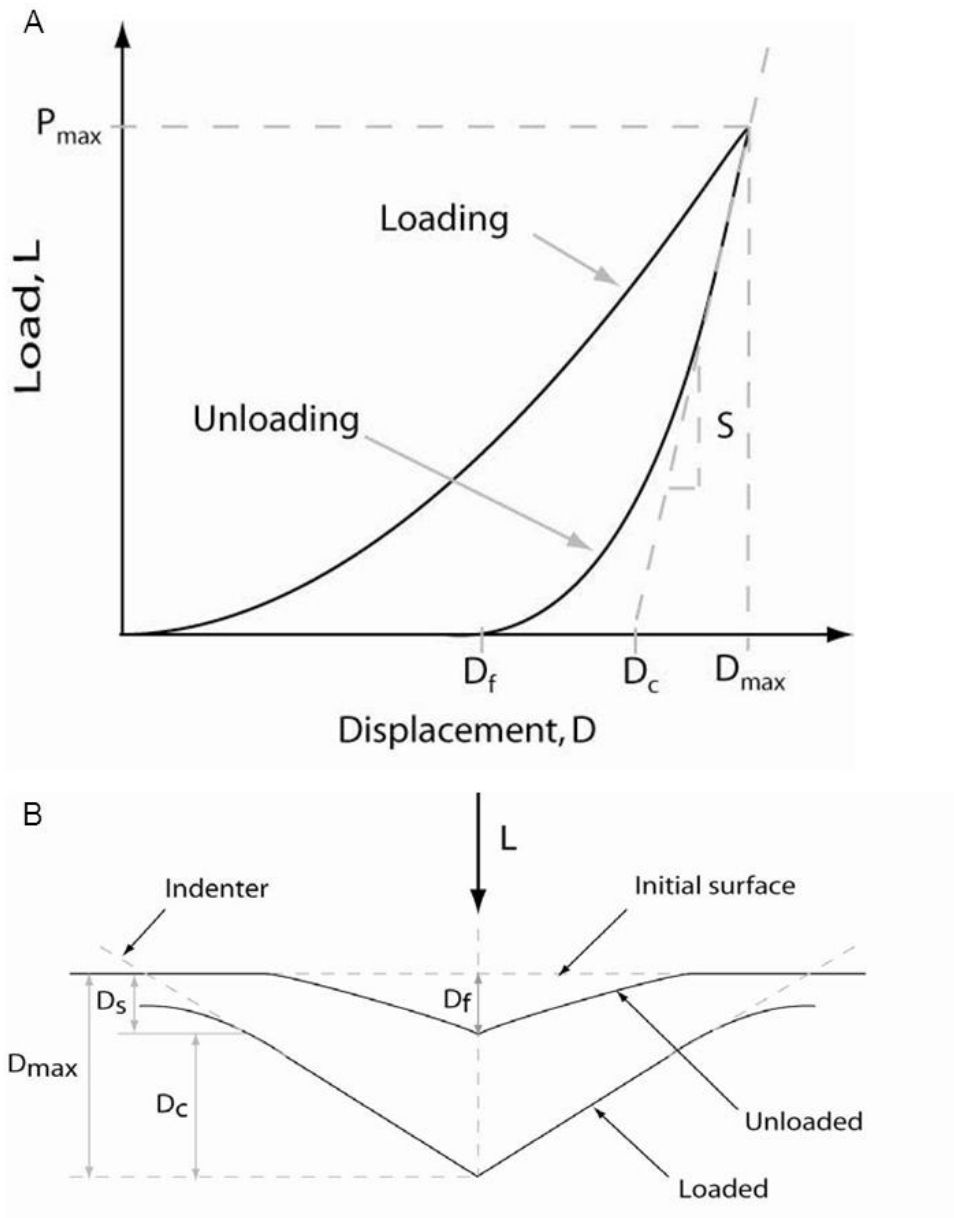
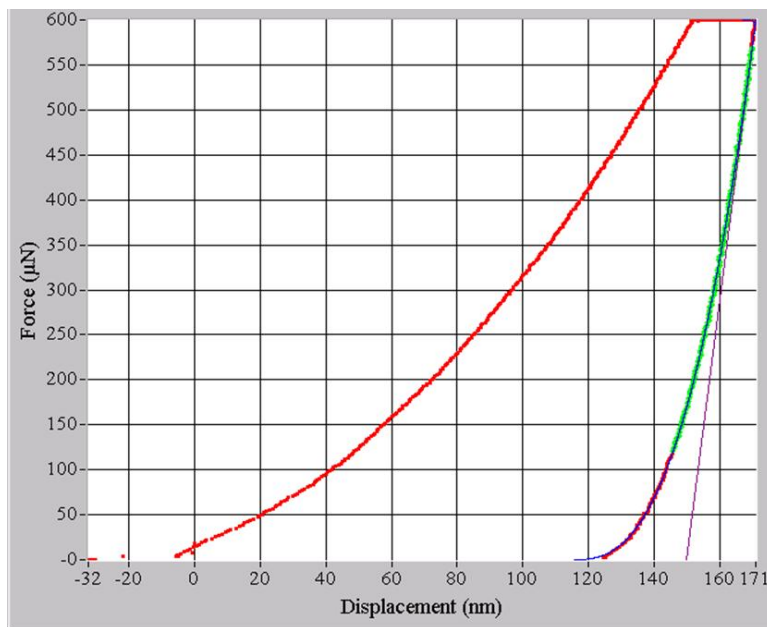


Figure 3.5. Typical nanoindentation load-displacement curve and indent schematic. (A) Typical nanoindentation load-displacement curve; S is the stiffness, P_{max} is the maximum load measured during indentation, D_f is the final depth (due to material plasticity), D_c is the contact depth at peak load, and D_{max} is the maximum depth. (B) Schematic of a typical indent; D_s is the amount of sink-in.

According to the Oliver-Pharr method, hardness and reduced elastic modulus may be defined as:

where α and m are fitting constants (Oliver and Pharr 1992). Furthermore, they derived an equation to determine the contact depth, D_c , in terms of maximum load and unloading stiffness:



cortical bone with significantly lower elastic modulus than any measured in WT bone (3.7 C).

Further analysis by other investigators showed that MMP-13 bones displayed mineral heterogeneity not seen in WT bones (data not shown).

Conclusion: Nanoindentation shows localized changes in MMP-13 bone. Studies are ongoing to investigate whether this is a downstream target of TGF- β signaling and to evaluate other changes in the bone matrix due to loss of MMP-13.

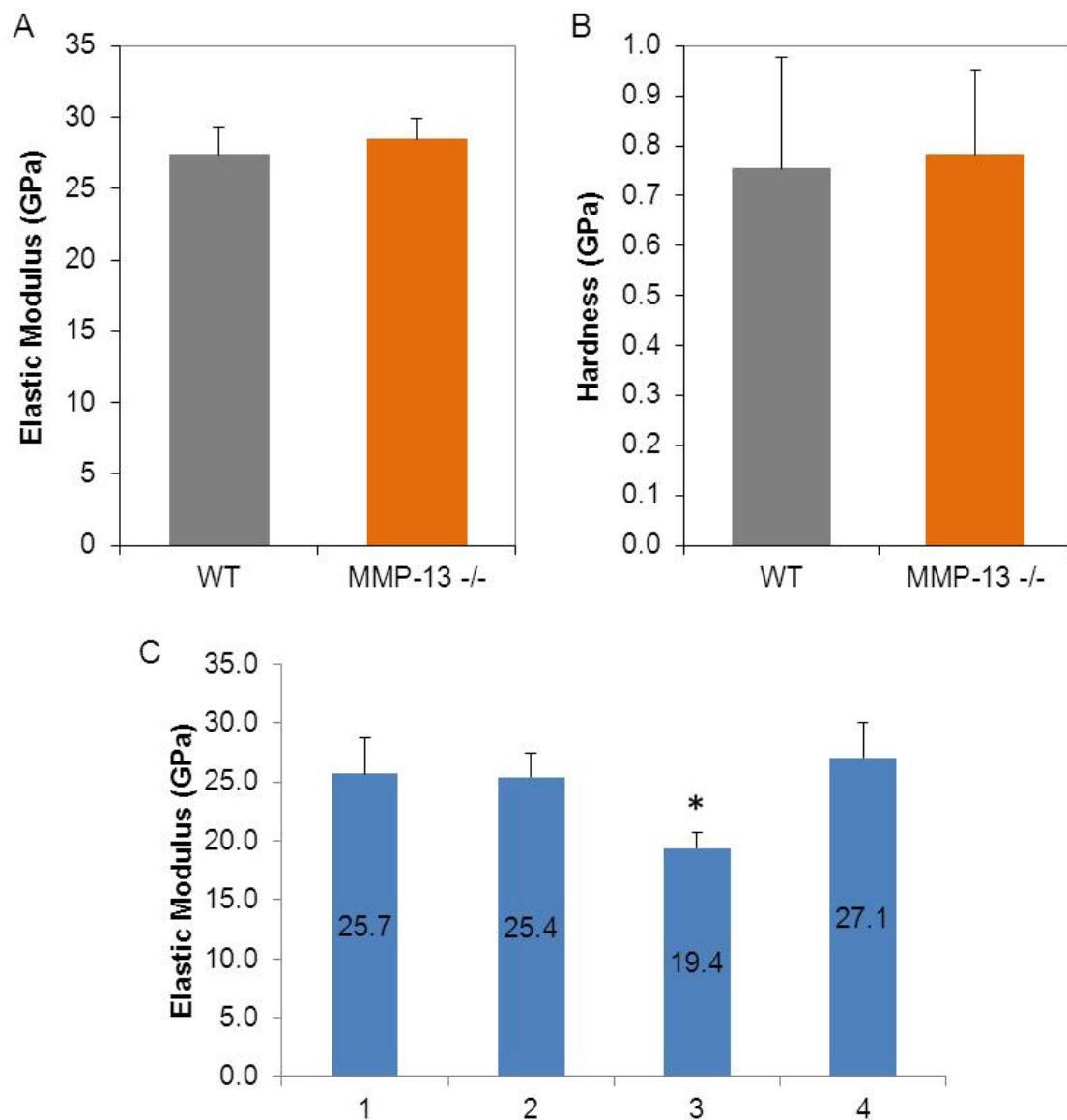


Figure 3.7. Elastic modulus of MMP-13 bone. No significant differences were found in

average elastic modulus (A) and hardness (B) (n = 5 for MMP-13^{-/-} and n = 4 for WT). However, one MMP-13 specimen displayed a region of significantly lower modulus (C). Ten indents were made in four distinct regions around the mid-cortical bone. * p value < 0.0001.

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Chapter 4

Indentation Testing for Soft Biological Tissues: Nanoindentation and AFM

Introduction

A natural extension of nanoindentation is to apply the measurement and analysis techniques beyond bone to other biological tissues, especially soft tissues. In the past decade, groups have begun to use indentation techniques to assess soft tissues such as cartilage (Ebenstein, Kuo et al. 2004; Cao, Youn et al. 2006), mammary glands (Levental, Yu et al. 2009), and vascular tissue (Ebenstein and Pruitt 2004). This chapter is an introduction to the application of nanoindentation and the use of the atomic force microscope (AFM) for measuring the material properties of soft materials such as cartilage or polyacrylamide gels.

There are several challenges in measuring the material properties of cartilage due to its anisotropy, heterogeneity, viscoelasticity, and hydration. The majority of experiments have been on the macroscale, which offers limited information since OA-related changes are localized and relatively healthy and degraded tissue may coexist in a given specimen (Tang, Souza et al. 2011). Nevertheless, previous studies show that the bulk compressive modulus is decreased in human as well as canine and lapine models of OA (Setton, Mow et al. 1994; Sah, Yang et al. 1997; Silver, Bradica et al. 2001). To investigate these material property changes on a more locally precise scale, indentation procedures have been adapted for use on soft biological tissues. Additionally, nanoindentation allows the evaluation of very small specimens, such as those from mice, which opens the possibility of investigating biological regulation of material properties.

Our studies initially focused on using nanoindentation to assess mouse femoral head cartilage. To date, very few studies have used nanoindentation to measure the material properties of cartilage, and even fewer have investigated mouse cartilage. Ebenstein pioneered

initial studies, evaluating the mechanical properties of repair cartilage formed after microfracture treatment of chondral defects in rabbit knees (Ebenstein, Kuo et al. 2004). Subsequently, nanoindentation was able to show that the elastic modulus of C57BL/6 mouse tibial articular cartilage was similar to that measured in both humans and rats (Cao, Youn et al. 2006). The indenter geometry used in this study was a 110 μm flat punch, while collagen fibers have diameters in the 100 nm range. Thus, even this setup has limited sensitivity in resolving local variations in cartilage properties. Indeed, a clinical indenter was not able to demonstrate statistically significant differences between healthy and arthritic human cartilage (Tkaczuk 1986). Furthermore, a search through current literature reveals that forays into investigating the molecular mechanisms that regulate cartilage matrix material properties has not resulted in publishable data. Based on conversations with other investigators and published studies, nanoindentation is able to differentiate severely degraded tissues, but does not have high enough resolution to measure the subtle changes that occur due to abnormalities in molecular signaling early in disease before blatant histological and morphological changes (Stolz, Raiteri et al. 2004; Stolz, Gottardi et al. 2009).

With the advent of IT-AFM (indentation-type AFM), in which probe diameters can be as small as tens of nanometers, it has now become possible to measure material properties with increased spatial resolution. Stolz et al. showed that IT AFM could be used to probe the changes in material properties due to cartilage degradation by enzymes (Stolz, Raiteri et al. 2004). Furthermore, they showed that although a 2.5 μm spherical tip was unable to resolve differences in digested porcine cartilage, a tip with a nominal radius of 20 nm did have the needed sensitivity and resolution. Importantly, they have also used the same technique to show increasing stiffness in femoral head cartilage of 19-month old mice when compared to 1-month old mice (Loparic, Wirz et al. 2010).

Instrumentation

Nanoindentation

The instrument used to perform nanoindentation on cartilage was the same as that used for the bone studies, the Hysitron Triboindenter (see chapter 3, figure 3.1). The one adjustment made to the setup was to change the Berkovitch tip to a 100 μm spherical tip. As discussed previously, tip geometry is of paramount importance and spherical tips are preferred for soft tissues. A spherical tip is better at achieving uniform contact on irregular surfaces and does not introduce the complication of stress concentrations at the edges of flat punch and Berkovitch tips (Ebenstein and Pruitt 2004). The spherical indenter is frequently employed for measuring the material properties of cartilage (Ebenstein, Kuo et al. 2004; Stolz, Raiteri et al. 2004; Li, Pruitt et al. 2006).

IT-AFM

The AFM we used is the MFP-3D-BIO (Asylum Research, Santa Barbara, CA), which can be used for both imaging and force measurements (Darling, Wilusz et al. 2010). How AFM works is illustrated by a schematic shown in figure 4.1 A. Basically, a cantilever with a sharp tip, usually composed of silicon or silicon nitride, is used to scan or probe a sample surface. As the tip contacts the surface, the cantilever deflects. This deflection is measured using a laser reflected from the top of the cantilever, which is detected by photodiodes. Thus, the topography of a sample surface may be measured. Indentation measurements use Hooke's Law to calculate the force as a function of cantilever deflection.

The instrument consists of an AFM head on an inverted optical platform for viewing of transparent samples and top-down optics for viewing opaque samples and the laser positioning on the cantilever. This is mounted on a vibration isolation table, all of which are situated within an acoustic enclosure. Figure 4.1 B shows the AFM head, inverted optical platform, and top-down optics. The cantilever is mounted on the AFM head, which also includes the photodiode laser detectors, a piezo actuator and a nanopositioning system (NPS) sensor which provides precise measurements of the cantilever Z position (4.1 C, D). Our system also has an XY scanner for precise X-Y positioning.

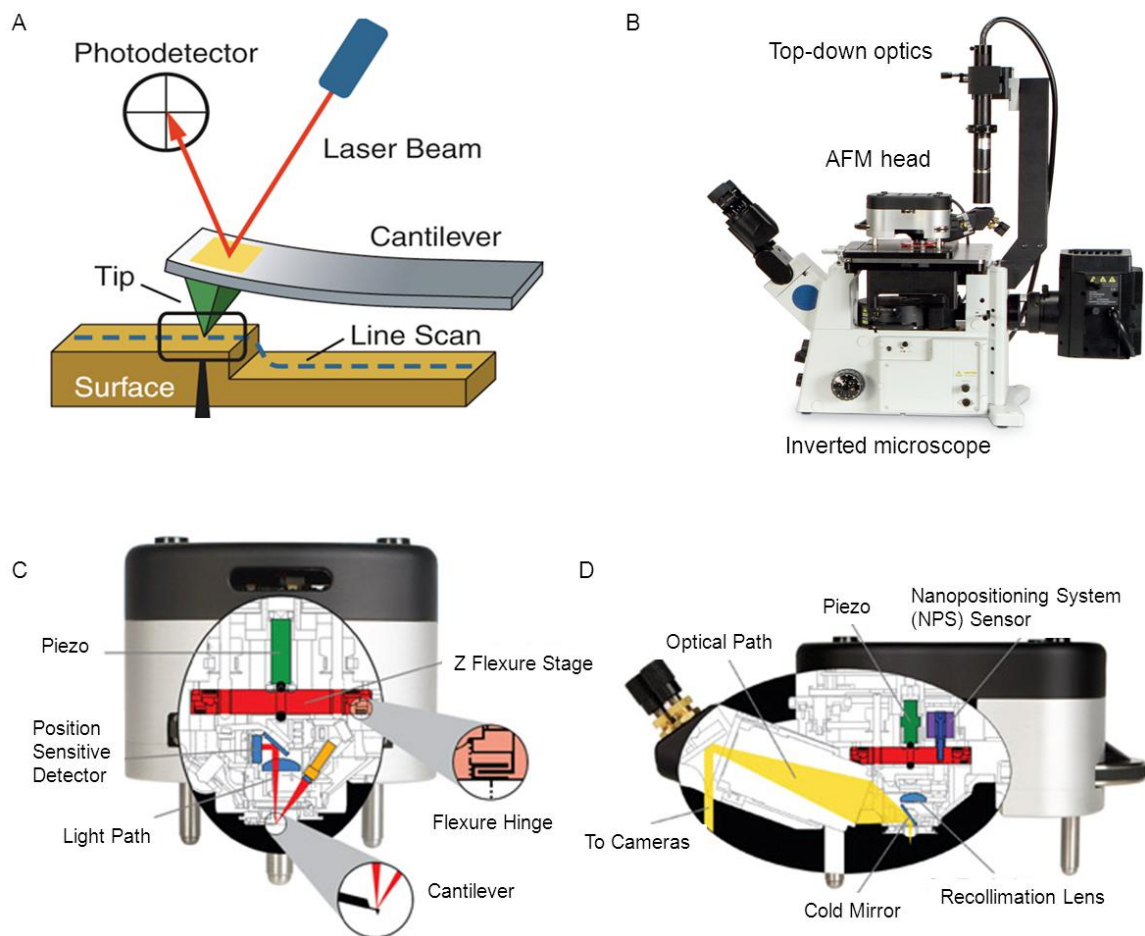


Figure 4.1. The MFP-3D-BIO AFM. (A) Schematic of how AFM works. (B) Photo of the instrument. Acoustic enclosure and vibration table not shown. Image courtesy of Asylum Research. Photo and schematic of the AFM head and its inner components, front view (C) and side view (D). Images courtesy of Agilent Technologies (A) and Asylum Research (B-D).

As in nanoindentation, the tip geometry is of critical importance. As discussed, spherical tips are most suited for cartilage. For AFM, the spring constant of the cantilever must also be specified. AFM cantilevers come in a variety of stiffnesses, from as small as 0.006 N/m to hundreds of N/m. The choice of spring constant is as important as the choice of tip geometry. If a cantilever is much stiffer than the sample, then contact will not cause the cantilever to deflect. If however, the cantilever is much less stiff than the sample, then contact with the sample surface will cause large deflections, resulting in the sample seeming much stiffer than it actually is. Consistent with previous studies and with nanoindentation, the cantilevers used in our studies were silicon nitride with a nominal spring constant of 0.06 N/m and a borosilicate glass sphere tip (Stolz, Raiteri et al. 2004; Loparic, Wirz et al. 2010).

Nanoindentation Procedure (Please refer to Appendix C for a detailed protocol)

I. Sample Preparation

The main challenges in cartilage nanoindentation are the rapid degradation of the tissue and the hydration level. Cartilage rapidly degrades, so all of our studies were performed on the same day as tissue isolation. Furthermore, about 85% of cartilage matrix is water, which is an essential component in defining the material properties. Therefore, the tissues were kept hydrated by submersion in Hank's balanced salt solution (HBSS) to best mimic a physiological environment (Habelitz, Marshall et al. 2002). For these studies, mouse femoral heads were isolated with a portion of the femur still attached for embedding purposes. Immediately after drying the remaining femoral shaft with a Kimwipe, the femur was embedded in a pool of 5-minute epoxy in a plastic tissue culture dish so that the femoral head cartilage surface did not contact the epoxy and was the highest point. After the epoxy had set for 5 minutes, the tissue was re-submerged in HBSS.

II. Calibrating the Triboindenter

The calibration of the Triboindenter for cartilage measurements is the same as for bone, with a few minor adjustments. First, the advanced Z-axis calibration must be done in the same environment as the sample, in this case, in HBSS, to account for the additional interactions

between the tip and the fluid. Second, the tip-optics offset, usually done on single crystal aluminum, must be done on agarose gel, since the large radius spherical tip cannot make a clear plastic imprint on aluminum (Ebenstein and Pruitt 2004). Lastly, the fused silica used to calculate tip area function for Berkovitch tip indentation into bone is inappropriate to find the area function for spherical indenter indents into cartilage. The displacement of cartilage is much larger than that into hard tissues and calibration on fused silica cannot capture the depth range of indentation into cartilage. Thus, an ideal spherical area function was used to evaluate the nanoindentation data in these studies.

III. Making an Indent (Data Acquisition)

Adjustments must be made in the loading scheme as well. Displacement control was used in these studies because it enables precise capture of the specimen surface as well as control of tip penetration, of critical importance in layered materials like cartilage. A trapezoidal loading function, similar to the one used for bone, was employed in these studies, though the dwell period for cartilage is much longer, up to 120 seconds, to allow for dissipation of viscoelastic effects and for the sample to approach quasi-equilibrium. Loading and unloading rates of 200 nm/s, a hold period of 60 seconds, and a maximum indent depth of 2 μm were used for these studies.

IV. Data Analysis

The Oliver Pharr method was used to calculate the elastic modulus (see Chapter 3: Nanoindentation). An ideal spherical area function was used to calculate the contact area:

Cartilage samples were prepared in the same way as for nanoindentation. Due to the inferior top-down optics of the system, testing on cartilage was abandoned after several extensive attempts to overcome this limitation. Instead, polyacrylamide gels were used to validate IT-AFM for measuring the material properties of soft, hydrated materials. Polyacrylamide gels were prepared as described previously (Hansma, Yu et al. 2009).

II. Calibrating the AFM

The two calibrations that are needed before beginning measurement are the spring constant of the cantilever and the inverse optical lever sensitivity (InvOLS). The spring constant may be derived by measuring the thermal fluctuations of the cantilever in air (Hutter and Bechhoefer 1993). The InvOLS is a constant that scales the changes in laser position, recorded as voltage, to changes in cantilever deflection. This is done by performing a force measurement on a hard surface (such as fused silica) such that the distance that the piezo moves is the same as the cantilever deflection, since there is no deformation. The InvOLS may be calculated as the inverse of the linear slope of the resulting voltage versus distance curve.

III. Making an Indent (Data Acquisition)

The first step in making an indent is to engage the tip so that contact occurs within the Z-axis travel of the cantilever. Next, the trigger point and the approach and retract rates must be specified. This is equivalent to specifying the load function in nanoindentation. For our studies on polyacrylamide gels, we used a trigger point of 10nN, which means that as soon as the force measured reads 10nN, the cantilever tip will retract. The approach and retraction rates were both 1 $\mu\text{m/s}$. At least 20 indents were performed on each gel, resulting in at least 20 load-displacement curves per sample.

IV. Data Analysis

For the gel studies, we analyzed the load-displacement curves by fitting the approach data to the Hertz model, consistent with other studies (Darling, Zauscher et al. 2006; Darling, Wilusz et al. 2010). The approach was used for analysis rather than the retract portion of the

load-displacement curve so that adhesion could be neglected. The Hertz equation which describes the indentation assumes an infinitely hard sphere indenting a flat, deformable substrate:

Conclusion: In agreement with previous reports, nanoindentation was unable to detect differences in cartilage though it could resolve differences in agarose. Agarose, when imaged with both nano- and micrometer scale tips, appear to be amorphous. However, when cartilage is imaged with nanometer scale tips, the molecular structure of the collagen can be resolved. Thus, it was hypothesized that nanoindentation could detect changes on the micrometer scale, but does not have the resolution to probe the molecular changes we expected to see in our mouse model.

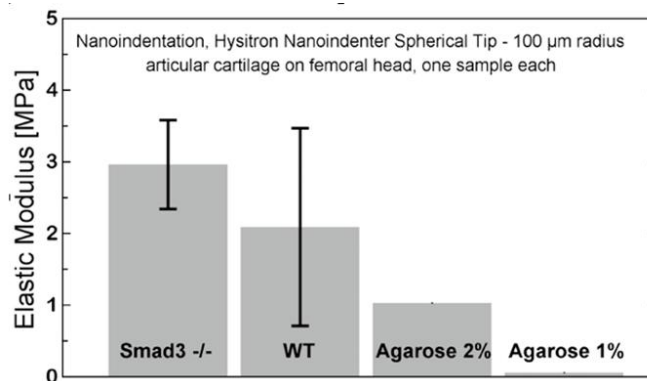


Figure 4.2. Nanoindentation of mouse cartilage and agarose gels. No significant differences were detected in mouse femoral head cartilage though differences were detected in 1% and 2% agarose gels.

B. IT-AFM Case Study (For details, see published paper in Appendix A.)

Background: Since we could not detect differences with nanoindentation, we looked to IT-AFM to measure material properties. Unfortunately, the optics were not optimal to reproducibly identify and measure comparable regions of mouse articular cartilage. It was extremely difficult to locate areas of interest. However, we were able to validate the IT-AFM by measuring the elastic modulus of more homogeneous polyacrylamide gels with elastic moduli in the range previously reported for cartilage.

Methods: AFM indentation was performed as described above on polyacrylamide gels with 1, 2, and 3% piperazine diacrylamide cross-linker. Elastic modulus data acquired from IT-

AFM testing were compared to measurements made by nanoindentation, Tissue Diagnostic Instrument (TDI) indentation, and unconfined compression.

Results: The elastic modulus values we measured by IT-AFM were comparable to the values measured by other testing modalities (4.3).

Conclusion: The MFP-3D-BIO has the potential to be a powerful tool in measuring the material properties of soft, hydrated materials like cartilage. We are currently exploring new methods to measure mouse cartilage, such as by sectioning whole tissue with a microtome so that the inverted optical microscope may be used to place indents on the area of interest. This has previously been done successfully on mouse knee joint cartilage (Darling, Wilusz et al. 2010).

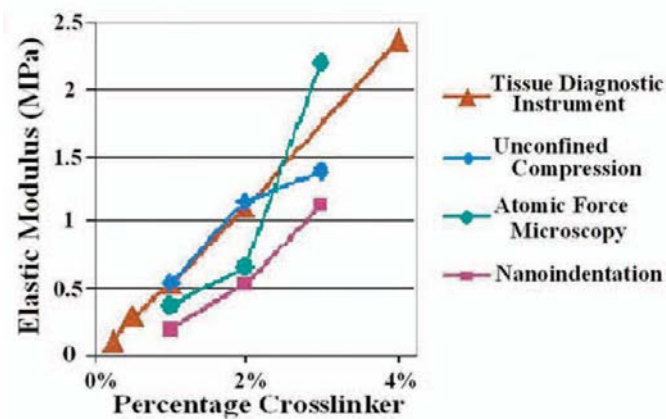


Figure 4.3. Elastic moduli of polyacrylamide gels. IT-AFM, nanoindentation, the TDI and unconfined compression were used to measure the elastic modulus of polyacrylamide gels.

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Chapter 5

TGF- β and Runx2 regulation of bone matrix material properties

Introduction

The unique material properties of bone matrix are critical for tissue function. Though bone mineral density (BMD) is the most common diagnostic for fracture risk (Fogelman and Blake 2000; Lochmuller, Lill et al. 2002), less than half of non-vertebral fractures can be explained by BMD alone (Schuit, van der Klift et al. 2004). Furthermore, there are cases in which patients exhibit normal bone mass but an increased risk of fracture, such as in osteogenesis imperfecta, aging, diabetes, and chronic corticosteroid use. This suggests that other aspects of bone such as microarchitecture, geometry, and matrix material properties, altogether described by the term “bone quality” (Hernandez and Keaveny 2006), may play an important role in predicting fracture risk. Bone matrix material properties (BMMPs), including elastic modulus, hardness, and fracture toughness, reflect the ability of bone matrix to resist deformation and catastrophic failure. However, the mechanisms that establish and maintain these properties and link them to tissue-specific function remain unclear.

Currey provided early evidence that material properties may be biologically regulated (Currey 1999). Following this, our lab demonstrated that TGF- β regulates BMMPs by signaling through its intracellular effector, Smad3, during development (Balooch, Balooch et al. 2005). Nanoindentation was used to show significant differences in elastic modulus and hardness of bone matrix from transgenic mice with disruptions in TGF- β signaling. While D4 and D5 mice, which express 16 and 2.5-fold increased levels of TGF- β in bone (Erlebacher and Derynck 1996), exhibit decreased elastic modulus and hardness, Smad3^{-/-} mice, in which a major effector of TGF- β signaling is ablated, exhibit increased elastic modulus and hardness. This was the first

description that bone matrix material properties are not only anatomically distinct but that they are also regulated through a classical biochemical signaling pathway. Subsequently, another study showed that glucocorticoid treatment in mice induced localized changes in the elastic modulus and hardness of trabecular bone (Lane, Yao et al. 2006). These studies indicate that bone matrix material properties are biologically regulated, though the cellular and molecular changes that occur downstream of TGF- β or glucocorticoid signaling were largely unexplored. Because understanding the biological mechanisms that define bone matrix material properties are so critical for the ability of bone to resist fracture, we sought to investigate these mechanisms further by seeking to identify possible targets of TGF- β /Smad3 signaling in the regulation of bone matrix material properties.

In vitro studies showed that TGF- β inhibits osteoblast differentiation and osteocalcin expression by Smad3 repression of Runx2 expression and function (Alliston, Choy et al. 2001). Whether this pathway is conserved in vivo is unknown, though some data suggests that TGF- β also represses Runx2 in vivo. For example, mice that overexpress TGF- β 2 in osteoblasts and Runx2 $\pm$ mice display overlapping bone phenotypes, both possessing dysplastic clavicles and open cranial sutures (Erlebacher and Derynck 1996; Otto, Thornell et al. 1997). For these reasons, we decided to investigate the role of Runx2 as an effector of TGF- β in the regulation of bone matrix material properties.

The specification of bone matrix material properties is likely achieved through regulation of bone matrix protein expression. Bone matrix is a composite of organic and inorganic constituents. The inorganic, or mineral, part is composed of carbonated hydroxyapatite crystals. The organic part is predominantly type I collagen, but also contains non-collagenous proteins such as osteocalcin, osteopontin (OPN), osteonectin, bone sialoprotein and others that participate in bone metabolism and structure. Though the roles of collagen and mineral in bone quality have been studied extensively, relatively little is known about the influence of non-collagenous proteins on bone matrix material properties (Zioupou, Currey et al. 1999; Silva, Brodt et al. 2006; Tang, Zeenath et al. 2007; Roschger, Paschalis et al. 2008; Busse, Hahn et al. 2009).

Osteopontin emerged as a candidate downstream of TGF- β /Smad3 in the regulation of bone matrix material properties. First, OPN transcription is regulated by TGF- β and Runx2 (Wai, Mi et al. 2006; Duvall, Taylor et al. 2007). Second, OPN has been implicated in the control of bone matrix mineralization due to its localization in the bone extracellular matrix and ability to bind calcium (Chen, Bal et al. 1992; McKee and Nanci 1996). Third, in vitro studies show that OPN controls mineral crystal growth (Qiu, Wierzbicki et al. 2004). Finally, OPN deficient mice have increased mineral crystal maturity (Boskey, Spevak et al. 2002). Therefore, we hypothesized that TGF- β establishes and maintains bone matrix material properties, in part, through regulation of the osteogenic lineage-specific transcription factor Runx2 and Runx2-target genes such as osteopontin.

Though it is clear that TGF- β signaling plays an important role in establishing bone matrix material properties during development, the role of TGF- β in the maintenance of post-natal bone quality is not known. The healthy post-natal skeleton responds to changes in the internal or external environment by coordinately changing bone mass and parameters of bone quality to increase fracture resistance. Mechanical loading increases bone mass, whereas in osteogenesis imperfecta, changes in geometry, composition, and matrix material properties improve fracture resistance independently of changes in bone mass (McBride, Shapiro et al. 1998; Kozloff, Carden et al. 2004). The process by which post-natal bone responds to the environment to maintain overall bone mass and bone quality is called adaptation. For example, several studies have reported on how bone adapts to mechanical load, or stress and strain, by changing its size, shape, and density (Turner, Anne et al. 1997; Turner 2006). Loss of this adaptive response to load, disease, or hormonal status is typical of metabolic skeletal diseases such as osteoporosis. Recently molecular regulators such as SOST have been implicated in the adaptive deposition of new bone mass in response to mechanical load. However, the molecular mechanisms that coordinate adaptive skeletal response to maintain bone quality remain unclear. To address this question, we used a pharmacological TGF- β type I receptor (T β RI) inhibitor, SD-208 (Uhl, Aulwurm et al. 2004), to systemically inhibit TGF- β function in post-natal mice. We hypothesized

that, as in development, post-natal inhibition of TGF- β function can increase mineralization and bone matrix material properties.

Results

TGF- β regulates Runx2 in vivo

To evaluate whether TGF- β repression of Runx2 is conserved in vivo, we first confirmed the skeletal phenotype in the mice we used for our experiments. Consistent with previous studies, both D4 mice, in which TGF- β is overexpressed in osteoblasts, as well as Runx2 $^{+/-}$ mice displayed absent clavicles, as validated by x-ray (5.1 A) (Erlebacher and Derynck 1996; Otto, Thornell et al. 1997). The similarity of the skeletal phenotype suggests that TGF- β and Runx2 act via the same pathway. We next checked the mRNA expression of Runx2 in D4 mice that overexpress an activated form of human TGF- β type 2 under control of the osteocalcin promoter, as well as mice with a disruption in the TGF- β signaling pathway. In D4 mice, which express a 16-fold increase in TGF- β in bone, the expression of Runx2-regulated genes Runx2 and osteocalcin in calvaria was reduced 50% when compared to WT (5.1 B) Conversely, DNT β RII mice, which express a dominant negative form of the TGF- β type II receptor, also under control of the osteocalcin promoter, (Filvaroff, Erlebacher et al. 1999), exhibited a more than two-fold increase in both Runx2 and osteocalcin mRNA expression levels (5.1 C). Altogether, these results indicate that TGF- β represses Runx2 expression and transactivation of its target genes in vivo as it does in vitro.

TGF- β regulation of BMMPs is Runx2-dependent

Though Runx2 is repressed by TGF- β in vivo, it remained unclear whether TGF- β regulates bone matrix material properties in a Runx2-dependent manner. Previously, we determined that increased TGF- β signaling resulted in reduced bone matrix elastic modulus while the converse resulted in increased elastic modulus. Consistent with Runx2 having a role in TGF- β regulation of BMMPs, Runx2 $^{+/-}$ mouse cochlea exhibited a significant reduction in elastic

modulus when compared to WT mice (5.2 A). To examine whether Runx2 lies downstream of TGF- β in the control of BMMPs, we crossed Runx2^{+/-} mice with DNT β RII mice. We hypothesized that inhibition of TGF- β signaling would be sufficient to rescue the Runx2 phenotype if Runx2 indeed regulates BMMPs downstream of TGF- β . Though clavicles were still absent in DNT β RII;Runx2^{+/-} mice, elastic modulus, hardness, and mineralization defects were partially rescued in these mice (5.2 B and C). Crossing Runx2^{+/-} mice with DNT β RII mice did not fully restore elastic modulus, possibly because of residual TGF- β signaling in the DNT β RII mice. Thus, one way in which TGF- β regulates matrix material properties is through repression of Runx2. Further study to evaluate the mRNA expression levels of Runx2 in these mice will elucidate the relative role of TGF- β repression of Runx2 in regulating BMMPs.

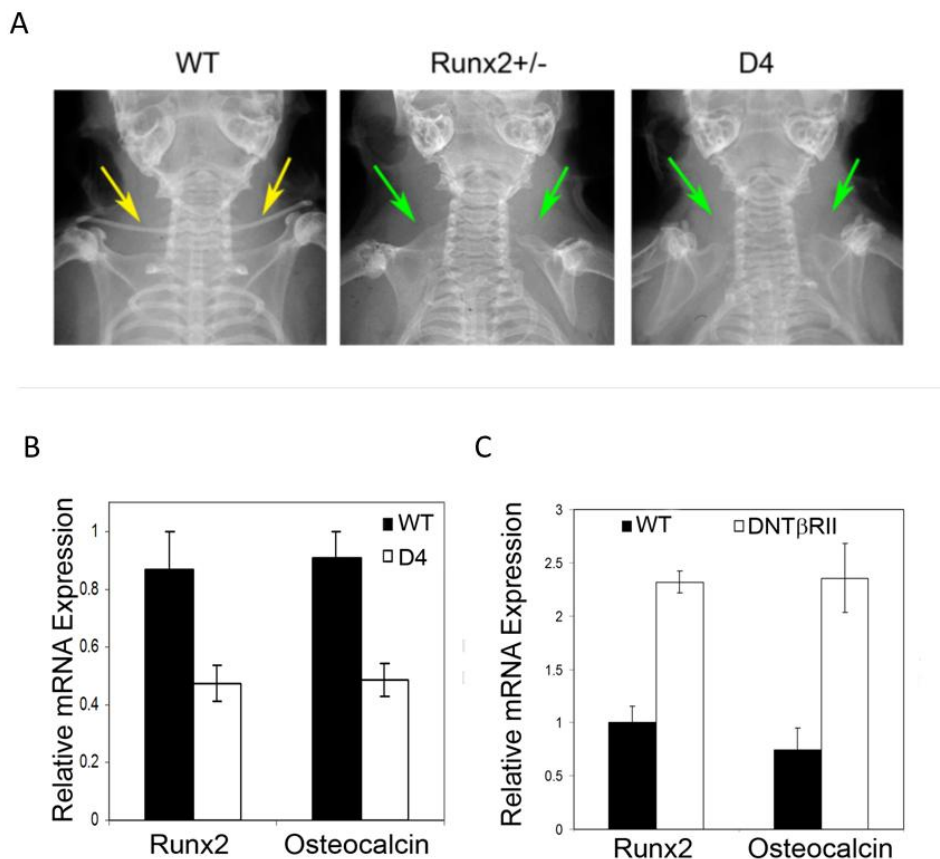


Figure 5.1. TGF- β regulates Runx2 in vivo. X-ray confirmed absent clavicles (green arrows) in Runx2^{+/-} and D4 mice with elevated TGF- β (A). Calvaria from D4 mice exhibit reduced

expression of Runx2 and osteocalcin, a Runx2-target gene (B), while those from DNT β RII mice with reduced TGF- β exhibit increased Runx2 and osteocalcin expression (C).

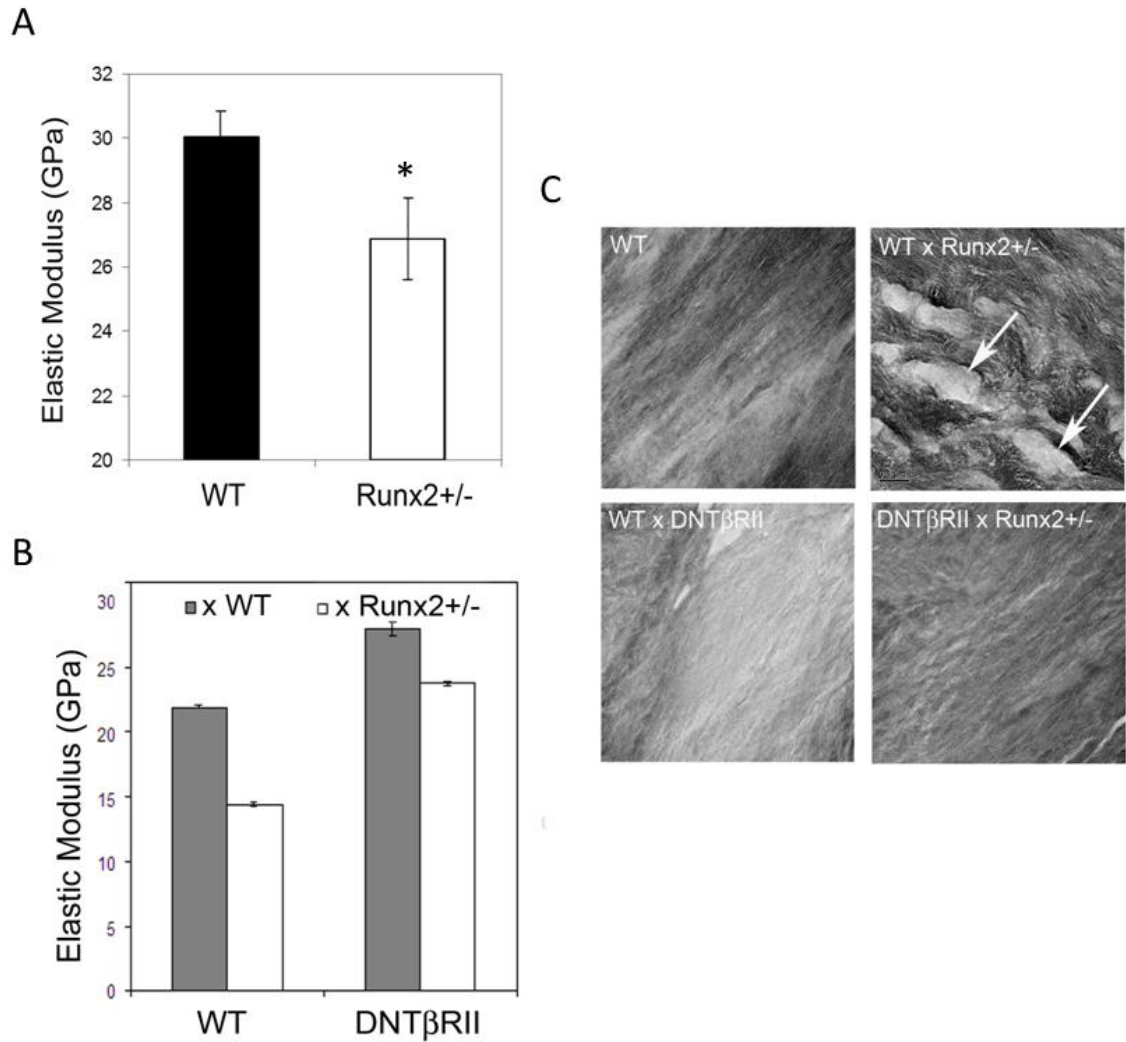


Figure 5.2. TGF- β regulation of BMPs is Runx2-dependent. The elastic modulus of cochlear capsule bone matrix from Runx2^{+/-} mice is reduced relative to WT (*p value < 0.01) (A), which can be rescued by inhibition of TGF- β signaling (B). TGF- β inhibition also rescues defective mineralization (C).

BMMPs are anatomically distinct and required for normal bone function

The material properties of specific bones are unique, as evidenced by Currey's studies in various species. In these studies, age-, species-, and anatomical location-dependence of BMMPs were observed (Currey 1999). This raises the question: is there a specific function for these specialized matrix material properties, aside from fracture resistance? Confirming Currey's reports, we observed significant differences in the matrix elastic modulus of several mouse bones (5.3 A). Strikingly, the cochlea and the malleus, two ear bones, were the stiffest, with an elastic modulus of 30 GPa. This unique property piqued our interest since many bone diseases present with associated hearing loss, such as Paget's disease, osteogenesis imperfecta, and cleidocranial dysplasia (Visosky, Johnson et al. 2003; Hartikka, Kuurila et al. 2004; Monsell 2004). Therefore, we tested the hearing of WT, Runx2, and D4 mice using the auditory brainstem response (ABR). Interestingly, there was a significant correlation between the elastic modulus and the auditory threshold (5.3 B), indicating that the matrix defects could account for hearing loss. For more details, please refer to our previously published paper in Appendix A.

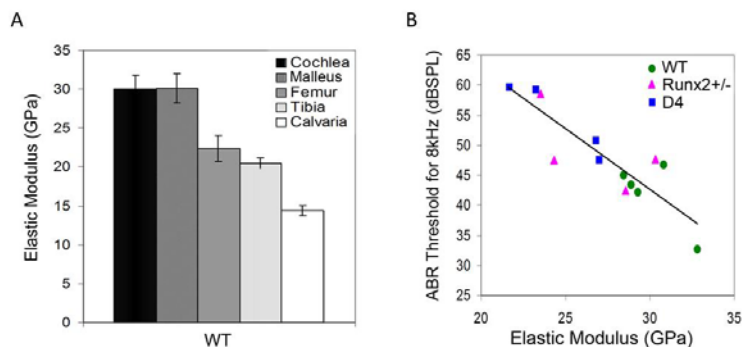


Figure 5.3. BMMPs are anatomically distinct and functionally essential. Elastic moduli of mouse bones show bone-specific differences (A). These differences are required for normal bone function, as illustrated by a multiple linear regression analysis comparing elastic modulus to hearing thresholds for each ear. Therefore, the uniquely hard elastic modulus of ear bones is a critical determinant of auditory function (p -value < 0.01, $R^2=0.77$) (B).

OPN is required for normal BMMPs but is not a downstream target of TGF- β in their regulation

Though we found that TGF- β /Smad3 regulation of BMMPs occurs through repression of Runx2, none of these genes are physical constituents of bone matrix. Thus, we next explored the question of how this signaling pathway could regulate bone matrix proteins. We chose to investigate OPN because it has been shown to be regulated by both TGF- β and Runx2 (Wai, Mi et al. 2006; Duvall, Taylor et al. 2007). Though reductions in maximum load and torque, as well as work-to-failure, were found in OPN-deficient mice, the underlying changes in bone matrix material properties were unclear. Previous studies support a role for OPN in regulating bone matrix properties. First, OPN has been shown to facilitate osteoclast attachment and guides bone mineralization by influencing mineral crystal shape and size (Hunter, Hauschka et al. 1996; Giachelli and Steitz 2000; Qiu, Wierzbicki et al. 2004). Furthermore, OPN is present on fracture surfaces with other phosphorylated matrix proteins. Mediated by Ca²⁺ ions, these proteins can form networks that repeatedly dissipate large amounts of energy (Fantner, Adams et al. 2007; Zappone, Thurner et al. 2008). Consistent with previous reports, the elastic modulus was decreased by 15% in OPN^{-/-} mouse tibiae compared to WT (Kavukcuoglu, Denhardt et al. 2007) (5.4 A). Notably, we found that the fracture resistance of femurs from these mice, as measured by fracture toughness, was also significantly decreased, by 30% (5.4 B).

We next evaluated the TGF- β regulation of OPN in vivo and in vitro. Previous studies reported both an induction and a repression of OPN by TGF- β depending on the cell type, dose, and time points evaluated. However, the regulation of OPN expression by TGF- β in vivo has not been examined. Consistent with Noda et al., we observed that TGF- β induced OPN mRNA expression in Saos-2 osteosarcoma cells within 4 hours (Noda, Yoon et al. 1988) (5.4 C). However, significant differences in OPN expression were not detected between calvarial bone from DNT β R11 mice and WT littermates in vivo (5.4. D). Therefore, though OPN is involved in the specification of BMMPs, our data indicates that it is unlikely to be the target of TGF- β /Smad3/Runx2 in their regulation of bone matrix elastic modulus and hardness. For further information on our analysis of the structure, composition, and material properties of OPN-deficient mice, please refer to our previously published paper in Appendix A.

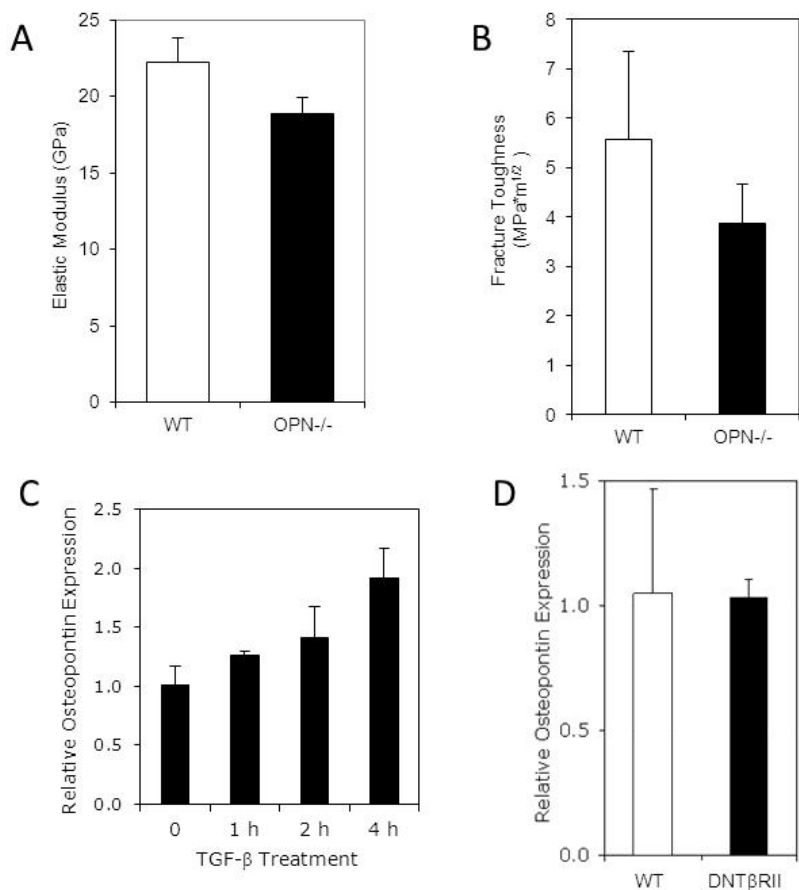


Figure 5.4. OPN is required for normal BMMPs but is not a downstream target of TGF-β in their regulation. Elastic modulus in OPN^{-/-} bone matrix was lowered to (20.3 ± 1.0) GPa compared to (23.9 ± 1.7) GPa for WT bone (p value ≤ 0.02) (A). Fracture toughness, a measure of the macro-mechanical behavior of bone, was reduced to (3.87 ± 0.79) MPa·m^{1/2} in OPN^{-/-} bone compared to WT bone (5.55 ± 1.78) MPa·m^{1/2} (p value ≤ 0.03) (B). Therefore, bone quality is reduced in OPN-deficient mice. Though we hypothesized that OPN was a downstream target of TGF-β and Runx2 in BMMP regulation, as suggested by TGF-β induction of OPN gene expression in vitro, (C), no differences in OPN gene expression were detected in bone from mice with altered TGF-β signaling (D).

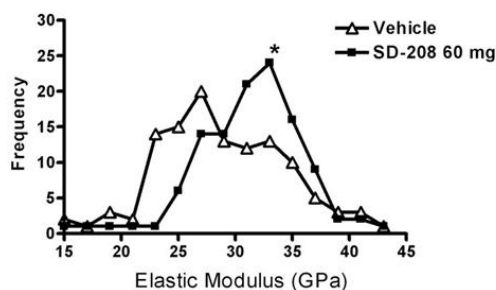
Interestingly, recently studies have shown that TGF-β and Smads regulate the expression and maturation of microRNAs, which participate in regulating the stability or translational efficiency of mRNA (Davis, Hilyard et al. 2008; Sun, Zhang et al. 2008). This opens the possibility that TGF-β could have non-genomic effects on post-transcriptional or post-translational regulation of OPN. Future work should address whether TGF-β/Smad3 might

participate in miRNA regulation of matrix constituents involved in specifying BMMPs. A first step would be to use microarray analysis of bone tissue in *Smad3*^{-/-} mice to identify miRNAs regulated by TGF- β in bone.

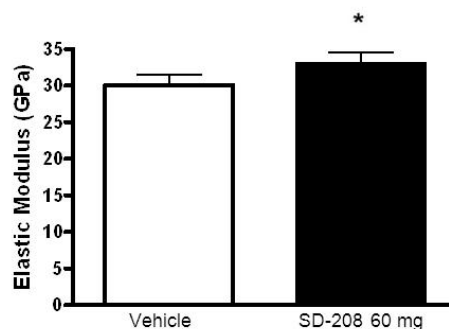
Pharmacologic inhibition of T β RI increases BMMPs

Thus far, we have shown the effects of TGF- β signaling on the BMMPs of genetically modified mice. Though our data indicates that inhibition of TGF- β signaling in development causes enhancement of BMMPs, it was still unknown whether an adaptive response could be achieved by post-natal disruption of TGF- β signaling. To examine the role of TGF- β in the maintenance of the post-natal skeleton, we used a pharmacological TGF- β type I receptor (T β RI) inhibitor, SD-208, to systemically inhibit TGF- β function in post-natal mice and then measured the elastic modulus of tibial cortical bone using nanoindentation. As in development, disruption of TGF- β signaling post-natally increases the frequency of sites with an elevated local elastic modulus (5.5 A) as well as the mean elastic modulus of bone matrix (5.5 B). This is consistent with increases seen in bone mineral concentration by XTM (5.5 C). Ultimately, compression testing of vertebrae from male mice treated with vehicle or SD-208 (60 mg/kg) showed an increased peak load-to-failure following T β RI inhibition (5.5 D).

A



B



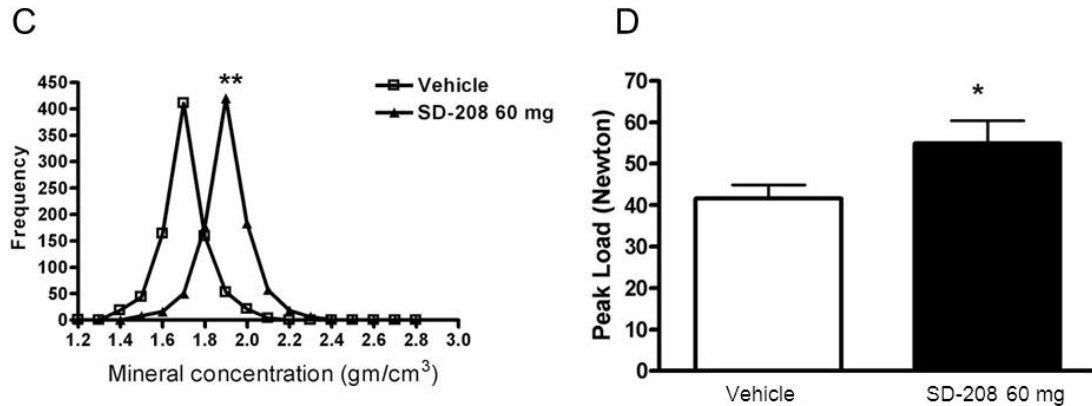


Figure 5.5. T β RI inhibitors increase bone matrix material properties and mineralization. Nanoindentation of tibial cortical bone shows that SD-208 (60 mg/kg) increases the frequency of sites with an elevated local elastic modulus (A). SD-208 also increases the mean elastic modulus (B) of bone matrix (* $p < 0.05$). Consistent with this, femoral bone mineral concentration as measured by XTM is also increased in treated mice (C). Unconfined compression testing of vertebrae from male mice treated with vehicle or SD-208 (60 mg/kg) shows an increased peak load-to-failure following T β RI inhibition (D). (* p value < 0.05 , ** p value < 0.01)

Discussion

Our studies explored the underlying mechanisms and functional role of BMMP regulation by TGF- β signaling. It had been previously shown that TGF- β represses Runx2 through a Smad3-dependent mechanism in osteoblasts and that TGF- β could regulate bone matrix material properties, though the mechanism was unknown (Alliston, Choy et al. 2001; Balooch, Balooch et al. 2005). Here, we found that TGF- β regulation of BMMPs through Smad3 is partially Runx2-dependent. Not only did Runx2 expression levels change in correlation to level of TGF- β signaling, Runx2 $\pm$ mouse bones furthermore exhibited reduced elastic modulus and mineralization defects, which were rescued by crossing with mice with reduced TGF- β signaling. Therefore, TGF- β regulates BMMPs through the same Runx2-dependent mechanism used to regulate osteoblast differentiation, by repressing the activity of Runx2. This is the first report that implicates a transcription factor in the regulation of extracellular matrix material properties. Additionally, we found that deregulation of this pathway compromises the distinctly hard cochlear bone matrix, which is significantly correlated with hearing loss. This demonstrates the essential

nature of these properties for healthy tissue function and highlights a heretofore undiscovered function for these tissue-specific extracellular matrix material properties.

We also studied the role of osteopontin (OPN), a non-collagenous bone matrix protein that is regulated by both TGF- β and Runx2 in vitro. We found that OPN-deficient mouse bones had decreased elastic modulus and fracture toughness when compared to WT littermates. Recent work has indicated that non-collagenous proteins may have overlapping roles, which may be related to the relatively mild phenotype we saw in OPN-deficient mice. Vashishth's group at Rensselaer Polytechnic Institute has recently found that osteocalcin/osteopontin double knockout mouse has a more severe bone phenotype than either alone, suggesting that the relationship between phenotype and TGF- β regulation in OPN-deficient mice may be masked by compensation (personal communication).

Although tangential, it is interesting to note the emerging body of work elucidating the role of non-collagenous proteins in endocrine signaling. Osteocalcin has been shown to regulate glucose metabolism by regulating insulin secretion, and this is hypothesized to occur through stimulation of osteoclast activity which regulates the bioavailability of osteocalcin (Clemens and Karsenty 2010). Subsequently, osteocalcin was shown to also regulate male fertility by inducing testosterone production (Oury, Sumara et al. 2011). Since osteocalcin-deficient mice show no overt abnormalities in ECM mineralization, these new studies suggest that the osteocalcin may play a more critical role in endocrine signaling than in bone-intrinsic regulation. OPN, too, has been shown to participate in bone-extrinsic activity, notably in cancer and tumor metastasis (Wai and Kuo 2008; Shevde, Das et al. 2010).

Our studies demonstrate that apart from its role in endocrine signaling, OPN plays an important role in establishing BMMPs. Further studies are ongoing to identify potential targets of TGF- β /Smad3 repression of Runx2 in the regulation of BMMPs.

Lastly, we examined the role of TGF- β in the post-natal regulation of BMMPs. We administered SD-208, a TGF- β type I receptor kinase inhibitor (T β RI), to mice and evaluated its

effects on material properties. The administration of T β RI inhibitors was shown to increase bone elastic modulus. This is consistent with the developmental bone phenotypes of mice with inhibition of TGF- β /Smad3 signaling, suggesting that an adaptive response can be achieved by post-natal inhibition of TGF- β signaling. This shows that TGF- β has a role in both establishing and maintaining BMMPs, highlighting a possible therapeutic use for reduction of TGF- β signaling in the treatment of bone diseases characterized by low bone mass and bone fragility. Further studies evaluating potential side effects are required, as TGF- β has a critical role in normal physiological processes and disease, such as development of immune cells, inflammation, and cancer metastasis (Sanjabi, Zenewicz et al. 2009; Bieri and Moses 2010).

Our results establish a mechanism through which TGF- β regulates BMMPs to maintain healthy bone (5.6). Interestingly, the material properties of dentin are also regulated by TGF- β (Saeki, Hilton et al. 2007). Furthermore, that TGF- β can also regulate skin extracellular matrix material properties indicates that there may be a generalized mechanism for TGF- β regulation of extracellular matrix material properties. This suggests a paradigm in which matrix material properties are established by growth factors through their regulation of lineage-specific transcription factors. Reports of TGF- β controlling the expression and activity of many lineage-specific transcription factors, particularly Runx2 in bone, Sox9 in cartilage, scleraxis in tendon, and myogenin in muscle further support for this idea (Alliston, Choy et al. 2001; Zhu, Goldschmidt-Clermont et al. 2004; Furumatsu, Tsuda et al. 2005; Lorda-Diez, Montero et al. 2009). Therefore, TGF- β regulation of matrix material properties may not only function in bone, but also in other musculoskeletal tissues and beyond.

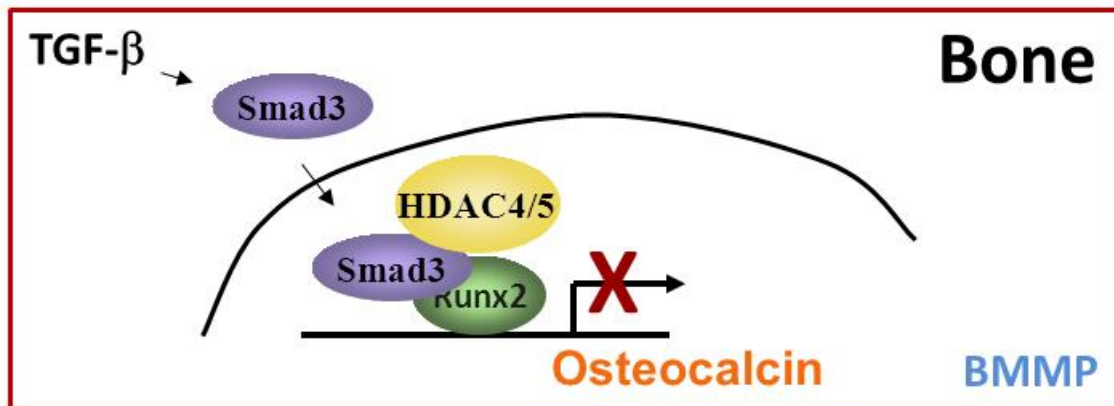


Figure 5.6. TGF-β regulation of bone matrix material properties.

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Chapter 6

Role of osteoblast-intrinsic TGF- β /Smad3 signaling in the bone matrix

Introduction

Several studies highlight the complex roles that TGF- β and Smad3 play in controlling the expression of bone matrix constituents and tissue level mechanical properties. Previously, we found that heterozygous loss of Smad3 (Smad3^{+/-} mice) increased the fracture toughness of bone. Smad3^{-/-} mice, however, showed the opposite trend (Balooch, Balooch et al. 2005). It was hypothesized that since Smad3^{-/-} mice experience immune, gastrointestinal, and endocrine defects, systemic effects may have played a part in specifying bone matrix mineral concentration. In addition, these mice exhibit other skeletal abnormalities, including decreased bone densities in areas other than joints, kyphoscoliosis, and distorted forelimbs, possibly due to a patterning defect (Yang, Chen et al. 2001). Therefore, the extent to which bone-intrinsic TGF- β /Smad3 signaling regulates matrix constituents and material properties is unclear.

In order to uncouple both the systemic effects and effects due to abnormal skeletal development from osteoblast signaling events in our studies, we used the Cre/Lox system to specifically delete Smad3 in osteoblasts. Floxed-Smad3 (Smad3^{fl/fl}) mice, developed by Dr. Jon Graff, were bred with Col1-Cre mice, with Cre expression targeted to osteoblasts by a 2.3 kb fragment of the Col1 promoter, to generate Col1-Cre;Smad3^{fl/fl} mice. For the Smad3^{fl/fl} mice, the targeting vector was constructed to contain two loxP sites flanking Smad3 exons 2 and 3, which were deleted in previously generated Smad3-null mice (Zhu, Richardson et al. 1998; Li, Pangas et al. 2008). This strategy should truncate the gene such that the carboxyl-terminal active domain would not be produced. Furthermore, point mutations in exon 2 have been shown to create loss of function Smad3 proteins. Lastly, sequence analysis indicated that alternative splicing around

exon 2 would shift the open reading frame (Zhu, Richardson et al. 1998). Bone-specific expression of Cre recombinase has been previously verified (Liu, Woitge et al. 2004).

We hypothesized that since TGF- β signaling through Smad3 represses Runx2 in osteoblasts, the bone-specific deletion of Smad3 would result in increased mineral concentration and bone matrix material properties. To test our hypothesis, we evaluated material properties, bone mineral density, and gene expression in bones from Col1-Cre;Smad3^{fl/fl} mice. Though our results are inconclusive thus far due to incomplete Cre-mediated recombination of the floxed Smad3 allele in our mice, our results coupled with further study will help elucidate the specific role of TGF- β /Smad3 signaling in bone.

Results

Insufficient deletion of Smad3 in bone and no significant changes in gene expression

To determine whether bone-intrinsic loss of Smad3 results in changes in gene expression, we harvested femurs to check for reduction of Smad3 and concomitant changes in the lineage-specific transcription factor Runx2 and bone matrix proteins osteocalcin (OCN) and type I collagen (Col1), signaling mediator sclerostin (SOST), and matrix degrading enzyme matrix metalloproteinase-13 (MMP13), all of which have the potential to impact matrix material properties. Surprisingly, we were not able to measure significant decreases in Smad3 expression in the cortical bone of femurs (6.1 A). According to reports detailing the generation of Col1-Cre mice, the expression of Cre recombinase is higher in calvaria (Liu, Woitge et al. 2004). Consistent with this, calvaria from Col1-Cre;Smad3^{fl/fl} mice exhibited a 50% reduction in Smad3 expression despite the fact that femurs showed no reduction in Smad3 mRNA expression (6.1 B). It is possible that insufficient recombination in femurs results in negligible inhibition of Smad3 mRNA expression levels. A significant difference in mRNA expression was only detected in OCN in femurs, which exhibited a modest two-fold increase (6.1 A), which is consistent with our expectations. Though not significant, a similar trend was observed in calvariae. Only one other

gene demonstrated a consistent trend in both tissues, Col1, which was decreased, though not significantly so, in both tissues (6.1 A and B).

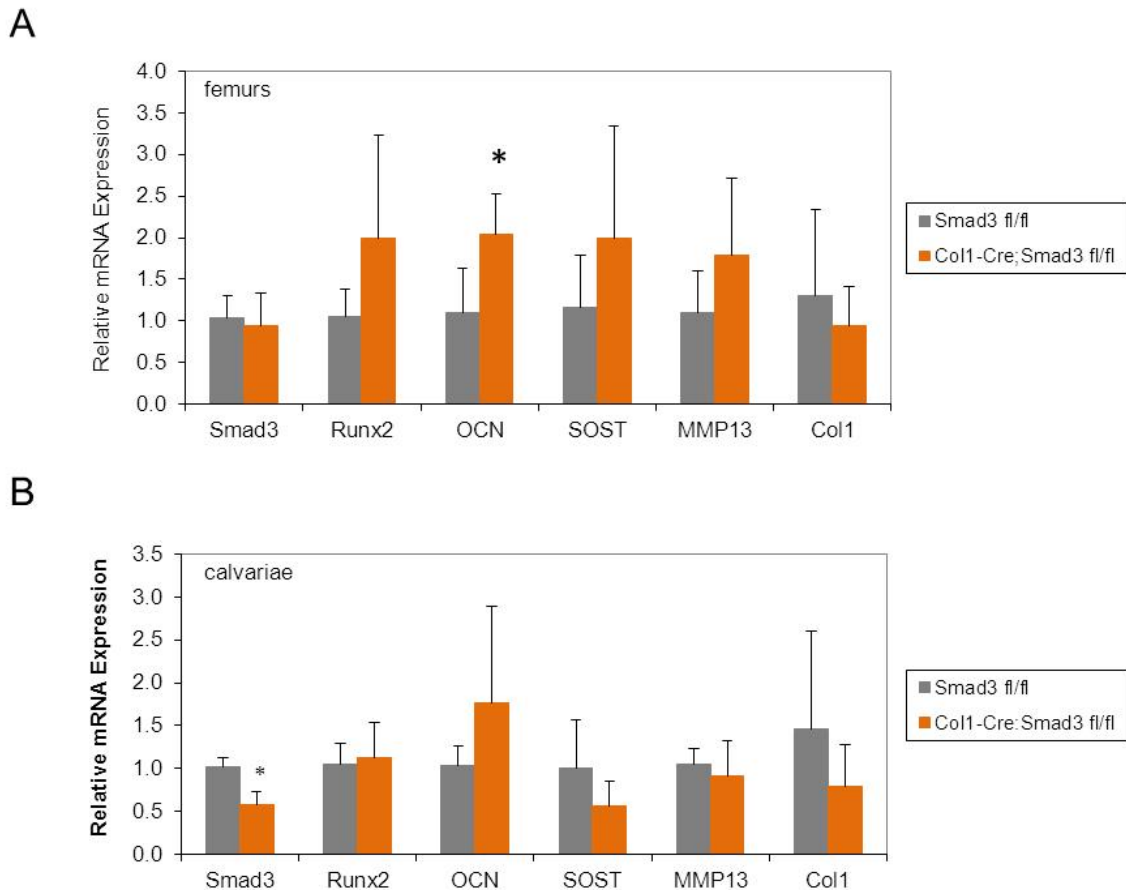


Figure 6.1. Insufficient deletion of Smad3 in femurs and largely no differences in osteogenic gene expression. (A) Smad3 mRNA expression in Col1-Cre;Smad3^{fl/fl} mouse femurs show no significant difference when compared to smad3^{fl/fl} controls. Osteogenic genes also exhibited no significant differences except for OCN (n = 6). (B) In calvariae, a partial decrease in Smad3 mRNA expression did not result in differences in osteogenic gene expression (n = 6). * p value < 0.02.

Reduced material properties in Col1-Cre;Smad3^{fl/fl} mouse femurs

Though no significant changes in gene expression were uncovered, the confirmation of partial Smad3 deficiency led us to evaluate the bone phenotype in these mice. One of the essential functions of bone is to provide support and structure for the body, and as such, a critical property of bone is its fragility or resistance to fracture. Bone fracture resistance is significantly

derived from its ability to impede the propagation of cracks once a fracture is initiated. Fracture toughness testing measures a material property of bone, specifically the ability of a bone to resist crack propagation independently of its ability to resist crack initiation. To evaluate whether Smad3 has a role in the ability to resist crack propagation, femurs were harvested from Col1-Cre;Smad3^{fl/fl} and Smad3^{fl/fl} mice and assessed for fracture toughness. Femurs were notched with razors at the mid-diaphysis and loaded to failure in a three-point bending configuration (6.2 A). Surprisingly, the fracture toughness of Smad3-deficient bone ($Kc = 3.52 \text{ MPa}\cdot\text{m}^{1/2}$) was 27% lower than control, Smad3^{fl/fl} mice ($Kc = 4.81 \text{ MPa}\cdot\text{m}^{1/2}$) (6.2 B).

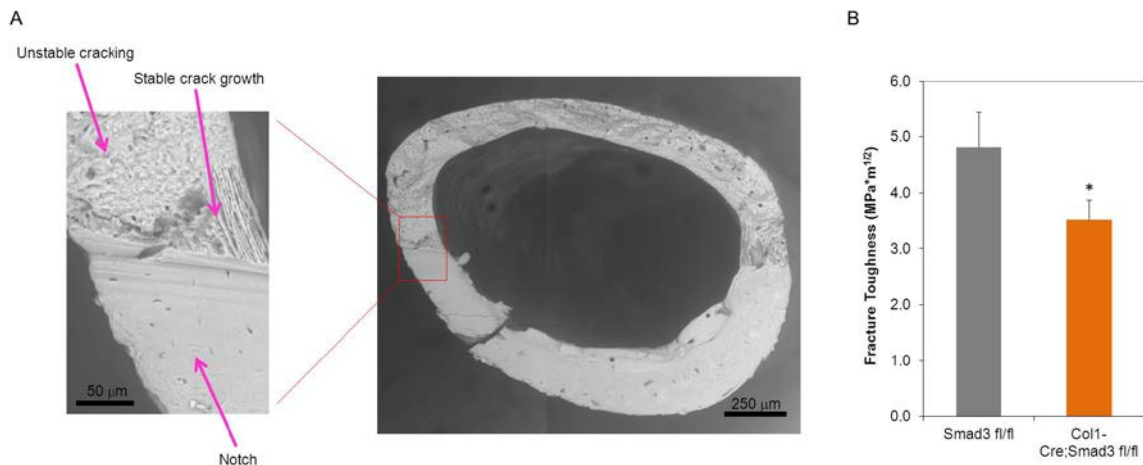


Figure 6.2. Decreased fracture toughness in Col1-Cre;Smad3^{fl/fl} mouse femurs. (A) SEM image showing the cross section of a femur after loading to failure. Visible are a smooth notched region from which extends a stable crack growth region followed by unstable cracking. (B) Col1-Cre;Smad3^{fl/fl} femurs exhibit a significant decrease in fracture toughness ($n = 6$ for Smad3^{fl/fl}, $n = 5$ for Col1-Cre;Smad3^{fl/fl}). * p value < 0.003 .

Many toughening mechanisms occur at smaller length scales. Thus, nanoindentation was used to evaluate the local material properties of the tibial cortical bone matrix. This high resolution approach allows the analysis of material properties independently of size and geometry. We measured elastic modulus and hardness, which reflect the ability of the matrix to resist plastic and elastic deformation, respectively. Similar to the reduction in fracture toughness, both were reduced, elastic modulus by 16% and hardness by 20% (6.3 A and B).

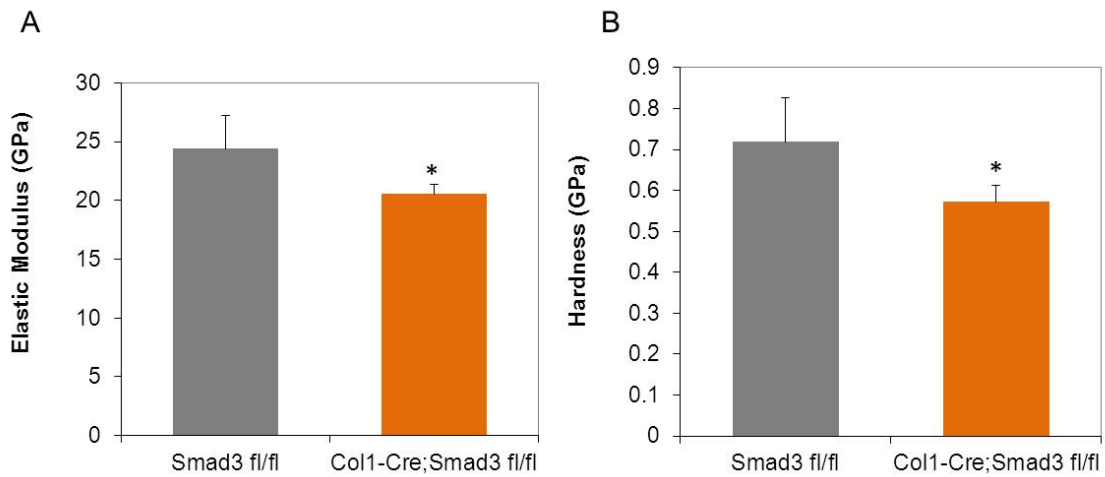


Figure 6.3. Decreased matrix material properties in Col1-Cre;Smad3^{fl/fl} mouse tibiae. Cross-sections of polished tibial cortical bone matrix were assessed by nanoindentation to determine elastic modulus (A) and hardness (B) (n = 5). * p value < 0.05.

Absence of changes in bone mass, architecture, mineral distribution and morphology

Decreases in fracture toughness, elastic modulus, and hardness may be a result of either a decrease in bone mineral density or changes in the geometry or architecture of bone. Thus, we used DXA and micro-computed tomography (μ CT) to evaluate several bone parameters in Col1-Cre;Smad3^{fl/fl} mice. There were no significant differences in femur BMD as measured by DXA (6.4). μ CT of tibiae was consistent, showing no significant differences in either trabecular or cortical BMD (Table 6.1). Indeed, there were no differences in trabecular volume, spacing, number, thickness, or any other architectural parameter (Table 6.1). Hence, neither BMD nor architectural changes could account for the Smad3-deficiency-related changes in fracture toughness and matrix material properties.

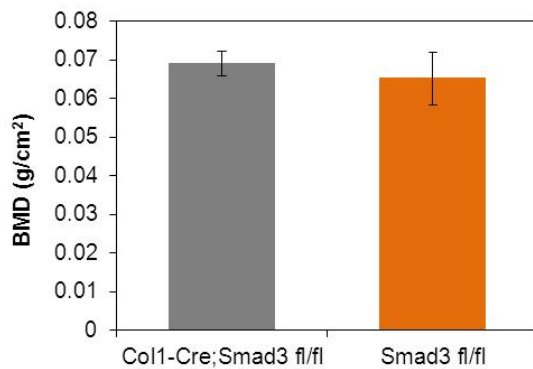


Figure 6.4. No significant differences in femoral bone mineral density in Col1-Cre;Smad3^{fl/fl} mice. DXA analysis of dissected femurs showed no changes in areal bone mineral density.

Table 6.1. Bone structural parameters and volumetric BMD are not affected by Smad3 deficiency

	Col1-Cre; Smad3 fl/fl	Smad3 fl/fl
BV/TV	0.149 ± 0.028	0.166 ± 0.025
Conn. Density (mm ⁻³)	259.984 ± 71.984	313.652 ± 76.818
Tb.Sp (mm)	0.148 ± 0.010	0.138 ± 0.013
Tb.N (mm ⁻¹)	6.642 ± 0.376	7.038 ± 0.513
Tr.Th (mm)	0.038 ± 0.003	0.0394 ± 0.002
Trabecular vBMD (mg/cm ³)	299.366 ± 39.475	320.094 ± 33.008
Cortical Thickness	0.321 ± 0.018	0.322 ± 0.024
Cortical vBMD (mg/cm ³)	1045.471 ± 38.083	1026.986 ± 49.579

Micro-computed tomography was used to assess several quantitative parameters of bone.

Previous work in our lab and beyond has consistently led to the idea that increased heterogeneity in mineral distribution can affect the mechanical competence of bone (Busse, Hahn et al. 2009; Thurner, Chen et al. 2010). Thus, we used the data from μ CT to analyze the mineral variability in both the proximal tibia and the tibiofibular junction. Neither location exhibited differences in mineral distribution (6.5 A and B).

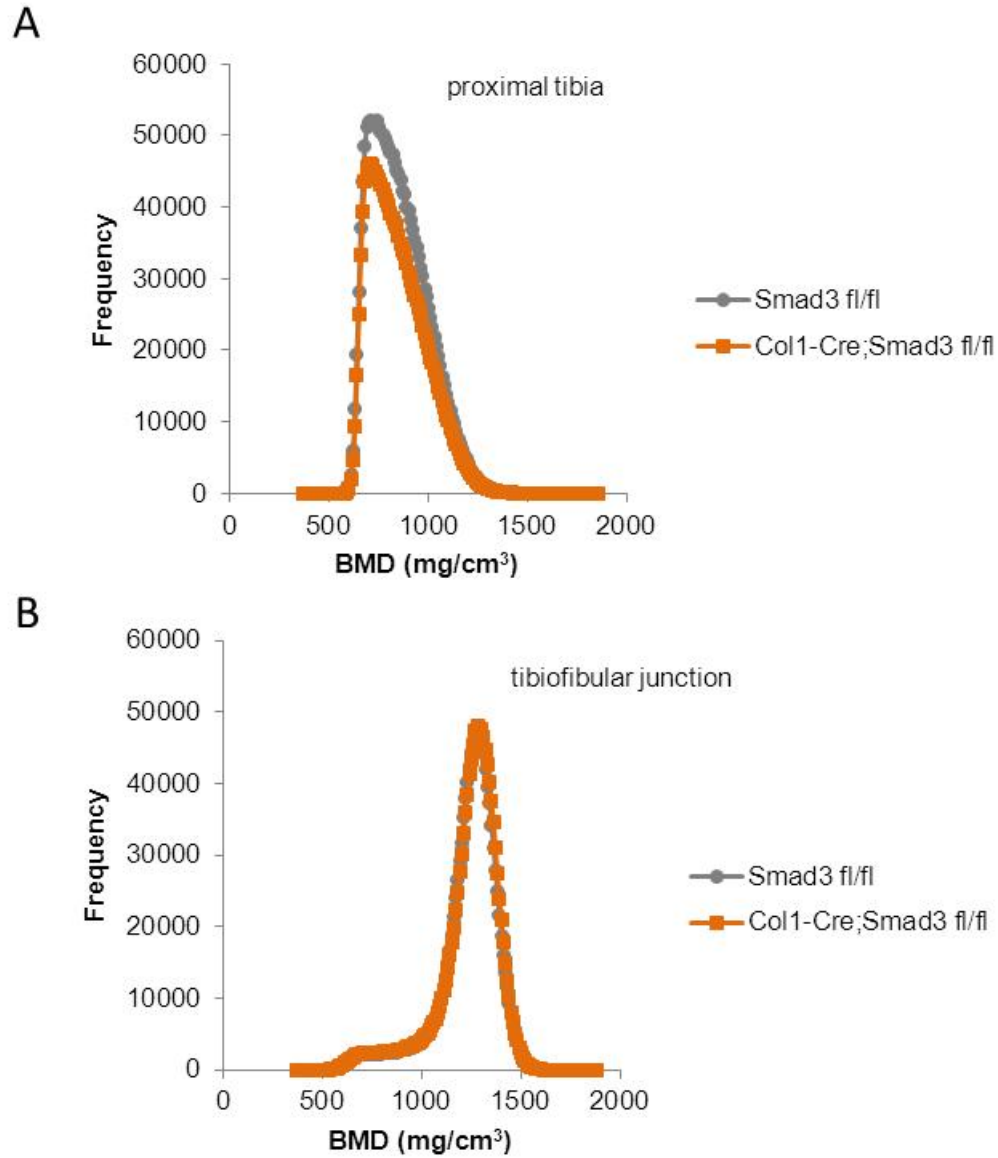


Figure 6.5. Mineral distribution unaffected in *Col1-Cre;Smad3*^{fl/fl} mice. No significant differences were found in the mineral distribution of *Col1-Cre;Smad3*^{fl/fl} mice compared to *Smad3*^{fl/fl} mice in the proximal tibia (A) and the tibiofibular junction (B).

Since we did not uncover any differences in mineral or microarchitecture that could account for the reduction in the material properties of *Col1-Cre;Smad3*^{fl/fl} bone, we sectioned demineralized tibiae and stained with trichrome to evaluate gross histological changes (6.6 A-H).

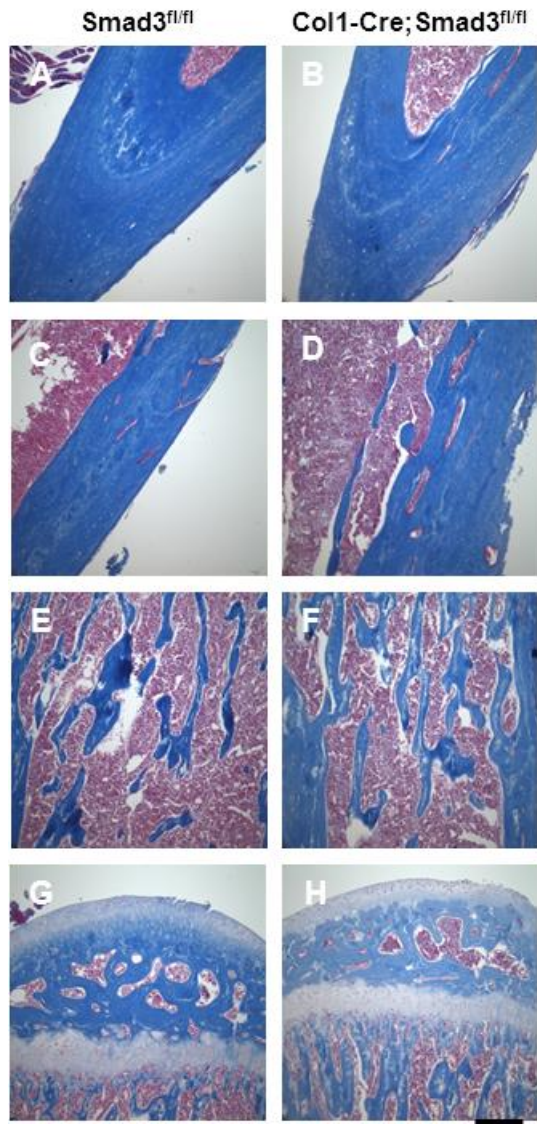


Figure 6.6. Histology of Col1-Cre;Smad3^{fl/fl} and Smad3^{fl/fl} mice. Tibiae of mice were sectioned longitudinally and stained with Masson's Trichrome. No differences were seen in Col1-Cre;Smad3^{fl/fl} mice (B, D, F, H) when compared to Smad3^{fl/fl} mice (A, C, E, G). Bar: 250 μ m.

Though histologic analysis did not show in any striking differences, we are continuing our investigation to elucidate the underlying mechanism through which bone-intrinsic Smad3-deficiency can cause reductions in fracture toughness, elastic modulus, and hardness. Because we did not find abnormalities in the mineral, future studies will probe the possible effects that loss of Smad3 may have on collagen organization and the organic components of bone. Preliminary

analysis with polarized light microscopy indicates that the collagen matrix may be disorganized in these bone-intrinsic Smad3-deficient mice (6.7 A and B).

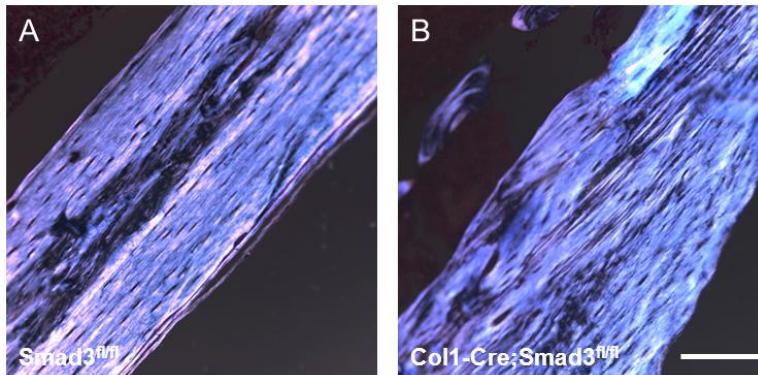


Figure 6.7. Polarized light microscopy of Smad3^{fl/fl} and Col1-Cre;Smad3^{fl/fl} mouse tibia. Polarized light microscopy shows an increase in matrix disorganization in Col1-Cre;Smad3^{fl/fl} mouse tibia (B) when compared to Smad3^{fl/fl} mouse tibia controls (A). Bar: 250 μ m.

Discussion

In this study, we evaluated gene expression and the bone phenotype in Col1-Cre;Smad3^{fl/fl} mice. There were largely no differences in gene expression in Col1-Cre;Smad3^{fl/fl} bone when compared to Smad3^{fl/fl} controls, likely partially due to incomplete deletion of Smad3 expression. No changes in bone mineral density or heterogeneity, or microarchitecture, were measured either. Despite this, we did observe significant decreases in elastic modulus, hardness, and fracture toughness.

Two possible explanations may account for these confounding observations. First, though we had Smad3^{fl/fl} controls, we have not yet evaluated Col1-Cre mice. Thus, it has not been established whether the expression of Cre itself could cause the differences in matrix material properties that we found. Even if there was no appreciable reduction in Smad3 expression, the presence of Cre in the bones of Col1-Cre;Smad3^{fl/fl} mice could potentially have effects. Future studies should measure the elastic modulus, hardness, and fracture toughness of bones from Col1-Cre mice to control for the effects of Cre. Second, it is also possible that the reduction in gene expression was not able to be detected by real-time RT-PCR. The inability to

detect a reduction in Smad3 mRNA expression was baffling because we not only checked for the presence of both Cre and floxed Smad3 by PCR, but additionally checked for recombination, ensuring that the portion of Smad3 flanked by loxP sites was indeed deleted. However, real-time RT-PCR checks the mRNA expression in a whole piece of homogenized tissue, and does not have the resolution to identify local excision of Smad3. Future studies should address this issue by evaluating local Smad3 expression using in situ hybridization. Local changes in Smad3 could potentially underlie the changes in material properties and matrix organization that we observed.

Assuming that Smad3 deletion was successful, these data contribute to our understanding of the bone-intrinsic role of Smad3. Previously, we found that systemic loss of Smad3 resulted in a decrease in BMD and fracture toughness in bone, though the elastic modulus was increased. Contrary to this, Col1-Cre;Smad3^{fl/fl} mice display no changes in BMD and decreases in both fracture toughness and elastic modulus. This suggests mineralization is more greatly influenced by bone-extrinsic Smad3 signaling, and the fracture toughness phenotype in Smad3^{-/-} mice likely results from the decrease in bone mineralization. In Col1-Cre-Smad3^{fl/fl} mice, however, the decrease in fracture toughness was not accompanied by a decrease in BMD, indicating that some other mechanism is behind the changes in matrix material properties. Interestingly, in Smad3^{-/-} mice, the elastic modulus is increased despite a decrease in BMD, suggesting that the organic constituents of the matrix also significantly contribute to the matrix material properties. Our data showing increased collagen disorganization in Col1-Cre;Smad3^{fl/fl} bone matrix support this idea and is a potential explanation for the decreased bone matrix material properties we observed. Thus, our data indicates that bone-intrinsic Smad3 signaling plays an essential role in specifying the organic portion of bone matrix.

Future work will focus on elucidating the mechanisms underlying the Smad3 regulation of the collagen matrix in bone. Collagen cross-linking, a post-translational modification of collagen, is one aspect of organic matrix organization that is both biologically regulated and also plays a key role in the mechanical competency of bone. The mechanisms of cross-linking may be divided into two groups: non-enzymatic and enzymatic. Non-enzymatic glycation-induced cross-

links have previously been implicated in bone fragility (Tang, Zeenath et al. 2007; Saito and Marumo 2010). Future studies will investigate whether this mechanism is involved in the decrease in Col1-Cre;Smad3^{fl/fl} fracture toughness. Another promising avenue that we will explore is lysyl oxidase-mediated collagen cross-linking. Previous reports have demonstrated that lysyl oxidase cross-links in bone may contribute to bone quality and that TGF- β regulates both the mRNA expression level and activity of lysyl oxidase (Feres-Filho, Choi et al. 1995; Saito and Marumo 2010). Whether these specific mechanisms underlie the Smad3-dependent changes in the bone matrix will be interesting to explore.

Finally, Smad3 has recently been shown to participate in the miRNA processing complex by interaction with Drosha (Davis, Hilyard et al. 2010). This represents another way in which Smad3 might regulate gene expression – through miRNA-mediated translational repression or mRNA degradation. It is possible that the differences between the Smad3^{+/-} and Smad3^{-/-} phenotype are not only a result of Smad3-mediated signaling, but also a result of Smad3-dependent miRNA regulation of gene expression. Future studies should investigate this hitherto unexplored mechanism in bone.

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Chapter 7

Role of chondrocyte-intrinsic TGF- β /Smad3 signaling in the cartilage matrix

Introduction

Like bone, articular cartilage has a unique matrix composition fitting its essential mechanical function. A viscoelastic tissue, it is able to withstand 1-2 million walking cycles a year and sustain loads of up to 10 times the body weight (Silva, Shepherd et al. 2002). In disease, such as osteoarthritis (OA), articular cartilage loses its mechanical integrity, becoming up to 20% less stiff in bulk mechanical properties relative to healthy cartilage (Mow and Huijskes 2005). This degradation is associated with loss of proteoglycans and disruption of the collagen extracellular matrix (ECM) (Knecht, Vanwanseele et al. 2006).

While chondrocytes in articular cartilage and growth plate cartilage share some similarities, they are distinct cell populations. During skeletal development and in the growth plate, chondrocytes form a temporary cartilaginous template that eventually undergoes endochondral ossification and is replaced by bone (Hall 2005). They progress through stages of resting, proliferation, and maturation, and finally reach terminal differentiation and become hypertrophic chondrocytes, producing a unique set of matrix proteins and signaling factors at each step (Hall and Newman 1991; Stevens and Williams 1999). The chondrocytes in permanent cartilage at joints, however, do not undergo endochondral ossification and arrest at the stage of resting chondrocytes. Loss of this stable resting chondrocyte phenotype is associated with cartilage degeneration in OA (Yang, Chen et al. 2001). Although an increasing number of studies address the control of chondrocyte differentiation and stability in articular cartilage, the molecular mechanisms for alterations in cell differentiation and matrix composition, as seen in osteoarthritis, have not been fully explored.

TGF- β is a key factor that has been implicated in the control of normal matrix gene expression and matrix material properties. In development, TGF- β helps to maintain the prehypertrophic chondrocyte phenotype and regulates the production of cartilage matrix proteins (Furumatsu, Tsuda et al. 2005). It also appears to be important in the maintenance of the resting chondrocyte phenotype in articular cartilage. Blocking TGF- β signaling by expressing a dominant negative TGF- β receptor (DNT β RII mice) in murine skeletal tissues resulted in the accelerated progression of articular chondrocyte maturation and an osteoarthritic phenotype (Serra, Johnson et al. 1997). Furthermore, the application of TGF- β to developing chick limb buds showed down-regulation of type X collagen and inhibition of chondrocyte maturation to the hypertrophic phenotype (Ferguson, Schwarz et al. 2004). Type X collagen is frequently used as a marker of progression towards chondrocyte maturation.

Studies of Smad3, one of the key effectors of TGF- β signaling, provide additional evidence of the involvement of TGF- β in the control of chondrocyte differentiation. Deficiency of Smad3 leads to OA-like joint disease and acceleration of chondrocyte differentiation. Notably, it was found that the inhibition of chondrocyte differentiation by TGF- β was diminished in Smad3 $^{-/-}$ sternal chondrocytes when compared to WT controls (Li, Darowish et al. 2006). In addition, the joints of Smad3 $^{-/-}$ mice become progressively osteoarthritic, as demonstrated by increased hypertrophic chondrocytes and ColX staining, the appearance of osteophytes, and decreased proteoglycans (Yang, Chen et al. 2001).

Despite the profusion of evidence that TGF- β /Smad3 signals promote matrix deposition and maintenance, the mechanism remains inconclusive. Though injections of TGF- β directly into mouse knees initially stimulated proteoglycan synthesis, examination at longer time points indicate the appearance of proteoglycan depletion, formation of osteophytes, and lesions, all signs of OA and matrix degradation (van Beuningen, Glansbeek et al. 2000). However, in these experiments, the effects of TGF- β injection are not limited to chondrocytes and cartilage. Indeed, in all the aforementioned reports, the role of TGF- β in cartilage has been studied using mouse

models with systemic disruptions in TGF- β /Smad3 signaling. TGF- β has many effects in multiple tissues and cell types. Skeletal, immune, gastrointestinal, and endocrine defects as a result of Smad3-deficiency could potentially affect the metabolism of cartilage. Supporting this, our lab has previously shown that some aspects of the Smad3-deficient bone phenotype are indirect results of Smad3 ablation in other tissues on bone (Balooch, Balooch et al. 2005). The extent to which Smad3 deficiency in other tissues contributes to the cartilage phenotype, as opposed to chondrocyte-intrinsic effects of Smad3 deficiency, remains unknown. Thus, in our studies, a tissue-specific approach to explore disruption of TGF- β signaling through Smad3 was used.

Much of the previous work investigating the mechanisms of cartilage matrix degradation has focused on examining the growth plate and applying the findings to articular cartilage. In Smad3^{-/-} and DNT β R11 mice, which express the transgene driven by the metallothionein promoter, growth plates exhibit accelerated terminal chondrocyte differentiation marked by increased hypertrophic chondrocytes and ColX expression (Serra, Johnson et al. 1997; Yang, Chen et al. 2001). Because many of the signs of OA-related cartilage degradation recapitulate the process of endochondral ossification, reports often extend the molecular mechanisms that occur in growth plate chondrocytes to articular chondrocytes undergoing abnormal terminal differentiation, as in OA. However, this is inexact. For example, it was found that TGF- β inhibition of chondrocyte hypertrophy is PTHrP (parathyroid hormone-related peptide)-dependent. Terminal differentiation in PTHrP-null embryos was not inhibited by TGF- β in metatarsal rudiments (Serra, Karaplis et al. 1999). In addition, expression of Ihh (indian hedgehog) has also been found to inhibit chondrocyte hypertrophy, and Ihh and PTHrP were proposed to participate in a negative feedback loop in which Ihh expression in prehypertrophic chondrocytes stimulated expression of PTHrP in the perichondrium which then inhibits chondrocyte hypertrophy (Lanske, Karaplis et al. 1996; Vortkamp, Lee et al. 1996). Subsequently, studies reported that Ihh inhibition of chondrocyte hypertrophy is dependent on TGF- β expression in the perichondrium, since Ihh was found to induce TGF- β expression in the perichondrium and removal of the perichondrium prevented Ihh from inhibiting terminal differentiation (Alvarez, Sohn et al. 2002).

These data taken together suggest that in normal growth plate chondrocyte maturation, *Ihh* stimulates TGF- β in the perichondrium, which then induces PTHrP expression to inhibit terminal differentiation. However, this pathway only applies to endochondral ossification, since articular cartilage lacks a perichondrium.

Little is known of the articular chondrocyte-specific mechanisms that govern matrix metabolism and there are very few reports on gene expression changes accompanying matrix degradation in cartilage. The studies cataloging the cartilage phenotypes of the *Smad3*^{-/-} and *DNT β RII* mice showed a decrease in ColX expression in articular cartilage matrix at six months, but did not report any earlier gene expression changes (Serra, Johnson et al. 1997; Yang, Chen et al. 2001). At six months, the articular surface already exhibits severe erosion. Visible changes in articular cartilage were detectable at P30, indicating that changes in gene expression may occur earlier, likely even preceding histologic changes. Studies of the intervertebral disc, a tissue similar to articular cartilage, in *Smad3*^{-/-} mice reported significant changes in Col2, Agn, and ColX as early as P10 (Li, Liang et al. 2009). Therefore, we became interested in examining the changes in gene expression and the cartilage matrix as a result of chondrocyte-specific *Smad3*-deficiency in articular cartilage.

Although many studies show that TGF- β induced *Smad3* signaling is required for maintaining the resting chondrocyte state in both articular and growth plate cartilage, it is unclear what the downstream effectors are. In osteoblasts, *Smad3* mediates TGF- β inhibition of *Runx2*, as well as the resulting repression of osteoblast differentiation genes such as osteocalcin, through the recruitment of HDAC4 (histone deacetylase 4) (Alliston, Choy et al. 2001). The existence of a parallel mechanism has yet to be elucidated in chondrocytes. However, evidence suggests that TGF- β and *Smad3* inhibition of *Runx2* play a role in the progression of chondrocyte differentiation (Ferguson, Schwarz et al. 2000), and HDAC4-null mice display premature chondrocyte hypertrophy (Vega, Matsuda et al. 2004). This is consistent with the observation that mice that overexpress *Runx2* exhibit premature chondrocyte hypertrophy (Zheng, Zhou et al. 2003). Also, both ColX and MMP-13, which are cartilage extracellular matrix proteins induced by

Runx2 during terminal chondrocyte differentiation, are also regulated by TGF- β . (Zheng, Zhou et al. 2003; Dong, Soung do et al. 2006; Selvamurugan, Jefcoat et al. 2006) Moreover, TGF- β also regulates the expression of Col2, another key cartilage extracellular matrix protein, through the control of Smad3-dependent Sox9 activity via a histone deacetylase, CREB-binding protein (CBP) (Furumatsu, Tsuda et al. 2005). Thus, we hypothesize that Smad3 acts in chondrocytes to define cartilage matrix composition through regulation of Runx2 and Sox9 expression and function.

Results

Col2-Cre;Smad3^{fl/fl} mice display degeneration in articular cartilage

To evaluate the chondrocyte-intrinsic role of Smad3 in cartilage development and maintenance, we first assessed the skeletal phenotype of mice with a conditional deletion of Smad3 in chondrocytes. To generate these, mice with floxed Smad3 alleles (Li, Pangas et al. 2008) were crossed with Col2-Cre transgenic mice (Ovchinnikov, Deng et al. 2000). Knee joints harvested at P60 were analyzed histologically. Mutant mice expressing Cre were compared with control mice confirmed to be Cre-negative by PCR. Previous studies showed that articular cartilage from mice with a global disruption of Smad3 (Smad3^{ex8/ex8}) exhibits a progressively degenerative phenotype starting at approximately 3-4 weeks of age which becomes more obvious at 6-8 weeks of age (Yang, Chen et al. 2001). Consistent with the global Smad3 knockout phenotype, Col2-Cre;Smad3^{fl/fl} knee joints showed deterioration of the smooth articular cartilage surface (7.1 B) while control joints remained smooth and intact (7.1 A). In the most severe cases, the commencement of fibrillation can be detected accompanied by a loss of resting chondrocytes, especially in the superficial layer, along with an increase in hypertrophic chondrocytes (7.1, B, D, and E).

Although previous studies observed no differences before 3-4 weeks of age in Smad3^{ex8/ex8} mice, we sought to determine whether the deterioration of cartilage was accelerated in Col2-Cre;Smad3^{fl/fl} cartilage. Therefore, we evaluated the knee joints of P14 mice. Consistent

with the findings in global Smad3 knockout mice, articular cartilage in both mutant and control are indistinguishable at P14 (7.1, F and G). Cell density and morphology are identical and the joint surfaces appear smooth and intact. Together, these observations demonstrate that chondrocyte-intrinsic Smad3 plays an important role in articular cartilage maintenance and matrix structure and function.

In principle, a cartilage degenerative phenotype could result from skeletal malformations occurring during development. Smad3^{ex8/ex8} mice are frequently kyphotic and move with difficulty (Yang, Chen et al. 2001). In contrast, no such skeletal defects were observed in Col2-Cre;Smad3^{fl/fl} mice at P60 as they were indistinguishable from their Smad3^{fl/fl} littermates in mobility and gross morphology (data not shown). These observations suggest that the progressive cartilage degeneration seen in Smad3-deficient mice is due to the intrinsic loss of Smad3 in chondrocytes and not a secondary effect of skeletal structural deformities.

Heterogeneity and decreased proteoglycan content in Col2-Cre;Smad3^{fl/fl} mice

Because an osteoarthritic phenotype and abnormal chondrogenic differentiation are often associated with changes in proteoglycan content, we evaluated Safranin-O staining in mutant and control knee joints. Consistent with the global Smad3^{ex8/ex8} phenotype, no qualitative differences in Safranin-O staining were detected in tibial articular cartilage (7.1, F and G) of P14 mice. Interestingly, although overall we found decreases in Safranin-O staining in mutant articular cartilage compared to control articular cartilage in P60 mice (7.1, A-E), there were also cases of increased staining (data not shown). This finding is corroborated by previous studies, which have reported that the progression of osteoarthritis is marked by an increase in both the synthesis and the degradation of aggrecan, one of the major proteoglycans found in articular cartilage (Poole, Rizkalla et al. 1993; Aigner, Zien et al. 2001). The joints exhibiting the most severe degradation of the articular cartilage surface had the least staining, especially in regions of overt fibrillation. These changes in proteoglycan coupled with our histologic observations indicate that the progressive degeneration of the articular surface is a focal event, as evidenced by the heterogeneity of the matrix.

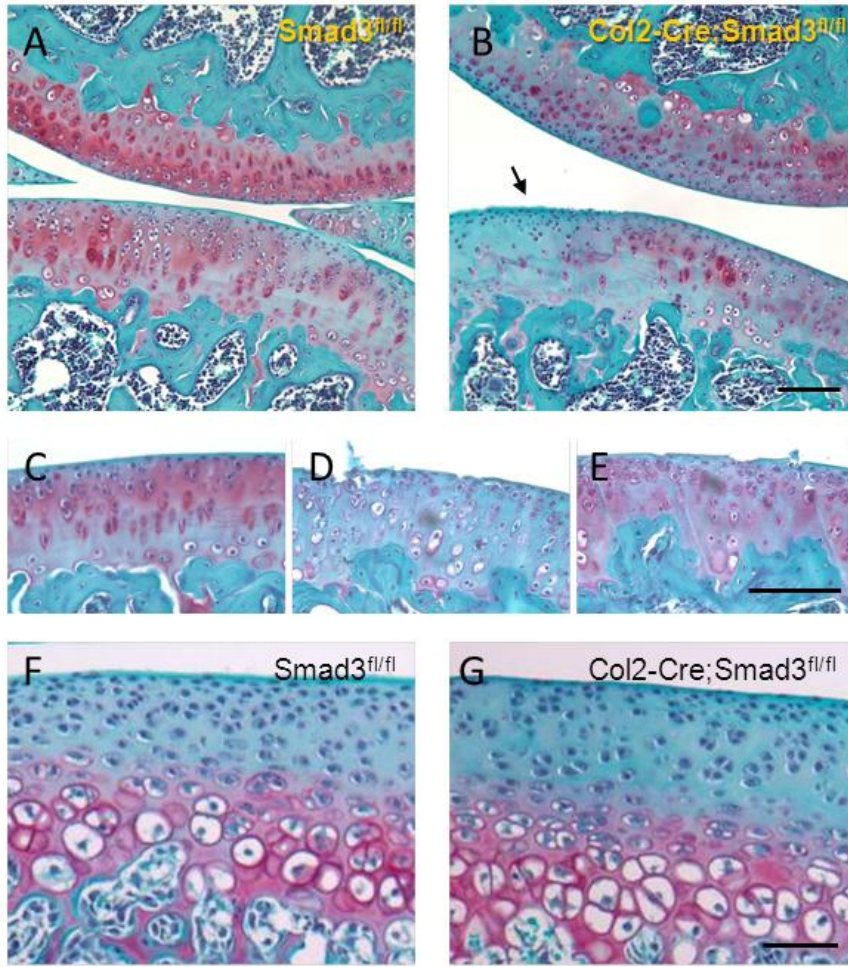


Figure 7.1. Histology and proteoglycan expression of articular cartilage in Col2-Cre;Smad3^{fl/fl} mice. (A and B) Safranin-O staining of knee joints of P60 Smad3^{fl/fl} (A) and Col2-Cre;Smad3^{fl/fl} (B) mice. Arrow points to surface roughening. (C-E) Magnification of articular surface of P60 Smad3^{fl/fl} (C) and Col2-Cre;Smad3^{fl/fl} (D and E) mice show fibrillation and decreased staining intensity. (F and G) Safranin-O staining of knee joints of P14 Smad3^{fl/fl} (F) and Col2-Cre;Smad3^{fl/fl} (G) mice. Bar: (A-E) 100μm; (F and G) 250μm

No differences found in quantification of bulk proteoglycan content by DMMB

To further quantify the changes in proteoglycan production in articular cartilage, we performed the DMMB assay on femoral head cartilage. The DMMB assay involves the specific binding of dimethylmethylene blue (DMMB) to glycosaminoglycans (GAGs), which are linked to proteins to form proteoglycans. This interaction results in the formation of a complex, the

absorbance of which can be measured and then correlated to a standard to quantify GAG content. Analysis revealed no significant differences in overall proteoglycan content in P14 and P60 animals (data not shown, 7.2). Though Safranin-O staining showed qualitative differences in proteoglycan amount, there were regions of more and less intense staining such that it is possible that the overall amount of proteoglycan changes very little. Furthermore, the DMMB assay may not have the resolution to detect the apparent cartilage matrix heterogeneity since it measures proteoglycan content from digest of a whole piece of tissue.

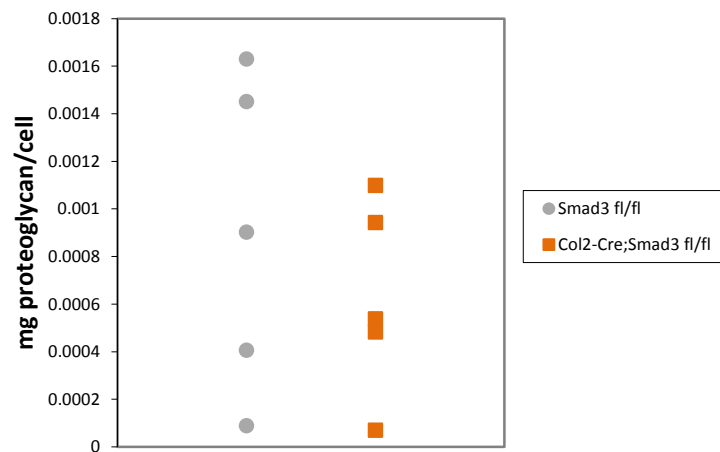


Figure 7.2. No significant differences in proteoglycan content in Col2-Cre;Smad3^{fl/fl} articular cartilage. Femoral heads were digested and the DMMB assay was used to analyze proteoglycan content (n=5). DNA amount calculated from the PicoGreen assay was used to normalize proteoglycan data.

Col2-Cre;Smad3^{fl/fl} mice exhibit disorganized growth plates

We next examined epiphyseal growth plates to ascertain whether loss of Smad3-related defects were present. Normal growth plates contain four different cell types arranged in columns: resting, proliferating, maturing and hypertrophic chondrocytes, each with its own distinct morphology (7.3, A and B). As with the articular cartilage phenotype and consistent with Smad3^{ex8/ex8} mice, no differences were observed in Col2-Cre;Smad3^{fl/fl} when compared to controls at P14 (7.3, A and B). However, P60 mutant growth plates exhibited disorganization in the arrangement of chondrocyte columns, reproducing the phenotype seen in global Smad3

knockout mice (7.3, C and D). Both P14 and P60 growth plates exhibited strong Safranin-O staining, compared to articular cartilage, though no significant differences were observed between mutant and control mice (7.3, A-D). The defects we observed were not evident in younger mice, indicating that the formation of articular cartilage and the growth plate are not affected and that Smad3 is involved in maintenance rather than development of articular cartilage and the epiphyseal growth plate.

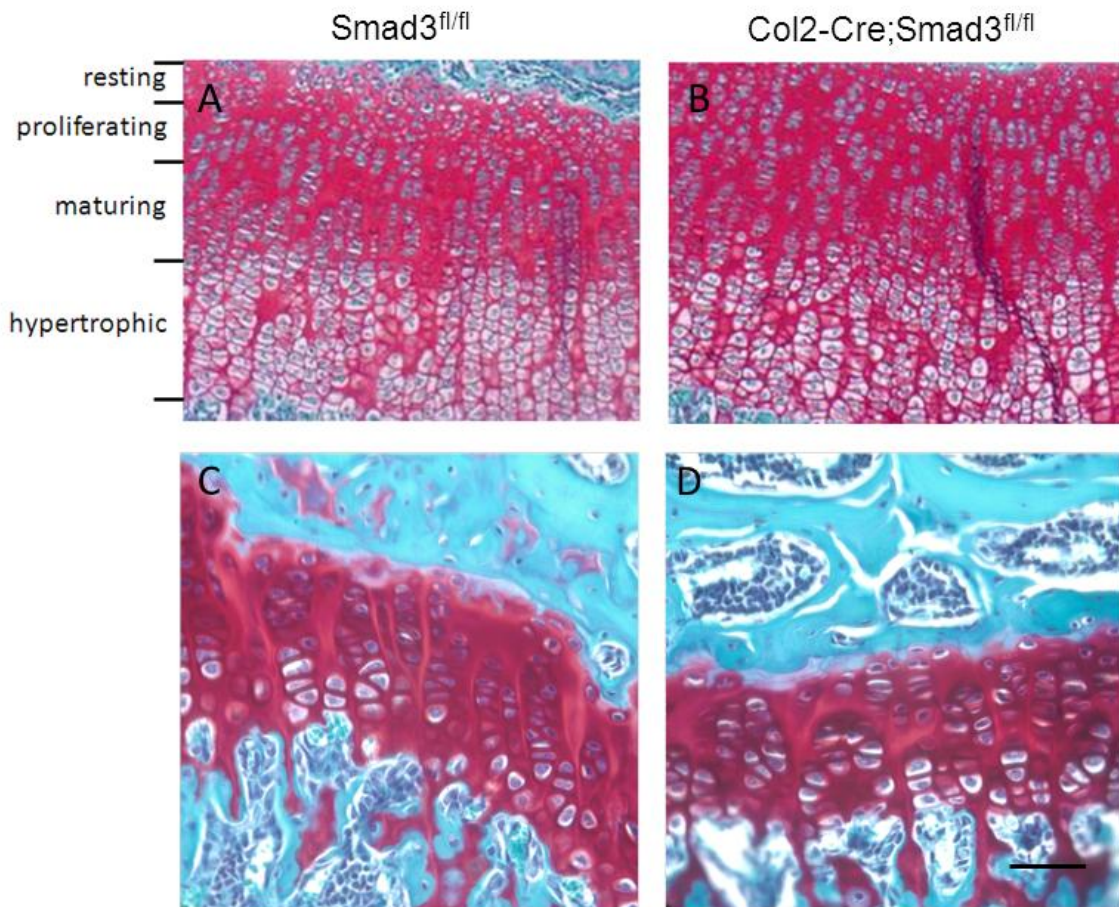


Figure 7.3. Histology and proteoglycan expression of epiphyseal growth plate in Col2-Cre;Smad3^{fl/fl} mice. (A and B) Safranin-O staining of growth plates from P14 Smad3^{fl/fl} (A) and Col2-Cre;Smad3^{fl/fl} (B) mice. (C and D) Safranin-O staining of growth plates from P60 Smad3^{fl/fl} (C) and Col2-Cre;Smad3^{fl/fl} (D) mice. Bar: 100 μ m.

Cartilage from mice with impaired TGF- β /Smad3 signaling display an OA phenotype

To further evaluate the overall form and structure of the articular cartilage, we applied the Modified Mankin scoring system previously used to quantify the condition of mouse articular cartilage (Xu, Polur et al. 2009). Eight Safranin-O stained sections of anatomically matched locations representing four P60 mice from each group were analyzed and given a score between 0 and 10, with 0 indicative of normal cartilage and 10 indicative of the maximum score for degenerative articular cartilage or most severe osteoarthritis (Table 7.1). Intensely enhanced pericellular staining (7.1 B), a decrease in background staining (7.1 B, D, and E), and fibrillation beyond the superficial layer (7.1 D and E) were observed in mutant joints at P60. The median scores for control and mutant animals were 2.5 and 4.5, respectively. A statistical comparison using the Mann-Whitney test indicated a significant difference between the two groups, with the p value less than 0.002 (Table 7.2). Thus, the chondrocyte-specific deficiency of Smad3 results in morphological changes that parallel the progression of osteoarthritis.

Table 7.1: Modified Mankin scoring system used to evaluate articular cartilage in mice

Feature	Grade
(1) Pericellular Safranin-O staining	
a. Normal	0
b. Slightly enhanced	1
c. Intensely enhanced	2
(2) Background Safranin-O staining	
a. Normal	0
b. Slight increase or decrease	1
c. Severe increase or decrease	2
d. No staining	3
(3) Arrangement of chondrocytes	
a. Normal	0
b. Appearance of clustering	1
c. Hypocellularity	2
(4) Cartilage structure	
a. Normal	0
b. Fibrillation in superficial layer	1
c. Fibrillation beyond superficial layer	2
d. Missing articular cartilage	3

Table 7.2: Modified Mankin scores of mouse knee joints

Genotype	n	Median	Range
Smad3 ^{fl/fl}	8	2.5	1-3
Col2-Cre;Smad3 ^{fl/fl}	8	4.5	3-6*

*p value < 0.002 vs. control group (Smad3^{fl/fl})

Loss of Smad3 results in reduced Col2 mRNA expression

Because we saw differences in cartilage matrix structure and composition, we studied the relationship of gene expression to these changes. To explore the mechanism by which loss of Smad3 could result in these changes, we evaluated the expression of Sox9 and Runx2 mRNAs in Smad3^{fl/fl} and Col2-Cre;Smad3^{fl/fl} mouse costal cartilage. Though costal cartilage is not considered articular cartilage, it is similarly composed and is also classified as hyaline cartilage. Furthermore, costal cartilage articulates with the sternum, can undergo calcification with age, and osteoarthritis can affect the sternocostal and sternoclavicular joints. Sox9 is a key chondrogenic transcription factor while Runx2 is a key osteogenic transcription factor. TGF- β has previously been shown to regulate the expression and function of both transcription factors, a regulation that is required for at least some of its effects on chondrocyte and osteoblast differentiation (Alliston, Choy et al. 2001; Furumatsu, Tsuda et al. 2005; Kang, Alliston et al. 2005). Though there was a partial reduction in Smad3 expression in Col2-Cre;Smad3^{fl/fl} mice (~60%), Sox9 and Runx2 expression in these mice were not significantly different than in control mice at either P14 (data not shown) or P60 (7.4 A).

We next evaluated the effect of chondrocyte-specific Smad3 ablation on the expression levels of key cartilage matrix constituents. No significant differences were detected in the mRNA levels for the matrix proteoglycan aggrecan (Agn) or collagen types II and X (Col2, ColX) at P14 (data not shown) and Agn at P60 (7.4 B), which is consistent with the DMMB results. However, type II collagen (Col2) and type X collagen (ColX) mRNA expression was significantly reduced in P60 mutant costal cartilage, ~50% and ~30% lower than control expression levels, respectively

(7.4 B). Contrary to our results, previous studies showed that Smad3-deficient sternal chondrocytes in monolayer exhibited increased expression of both Col2 and ColX (Li, Darowish et al. 2006). It was unclear, however, whether this effect would be replicated in vivo. Cellular signaling is often altered when cultured in monolayer (Albrecht, Underhill et al. 2006) and chondrocytes in particular dedifferentiate and lose their characteristic phenotype when cultured in 2D (Benya and Shaffer 1982). Earlier reports show that in vitro, Smad3 directly induces Col2 expression via a transcriptional complex with Sox9 and CBP/p300 (Furumatsu, Tsuda et al. 2005). Our data provides the first evidence that this pathway has a key role in regulating cartilage maintenance and chondrocyte differentiation in vivo. Thus, even though changes in Sox9 expression were not detected, the loss of Smad3 would prevent the activation of Sox9-dependent transcription of Col2 and so the effect of Smad3 deletion was most apparent on this gene. Furthermore, this suggests that the level of Col2 expression is carefully regulated by Smad3 to maintain a normal cartilage matrix, and further, that reduction of Col2 expression may be an early indicator of degeneration. This is consistent with evidence that mutations of the Col2 gene can cause osteoarthritis (Ala-Kokko, Baldwin et al. 1990; Eyre, Weis et al. 1991; Vikkula, Palotie et al. 1993). It is possible that other events in cartilage degeneration are secondary to a loss of Col2 expression in some cases of osteoarthritis.

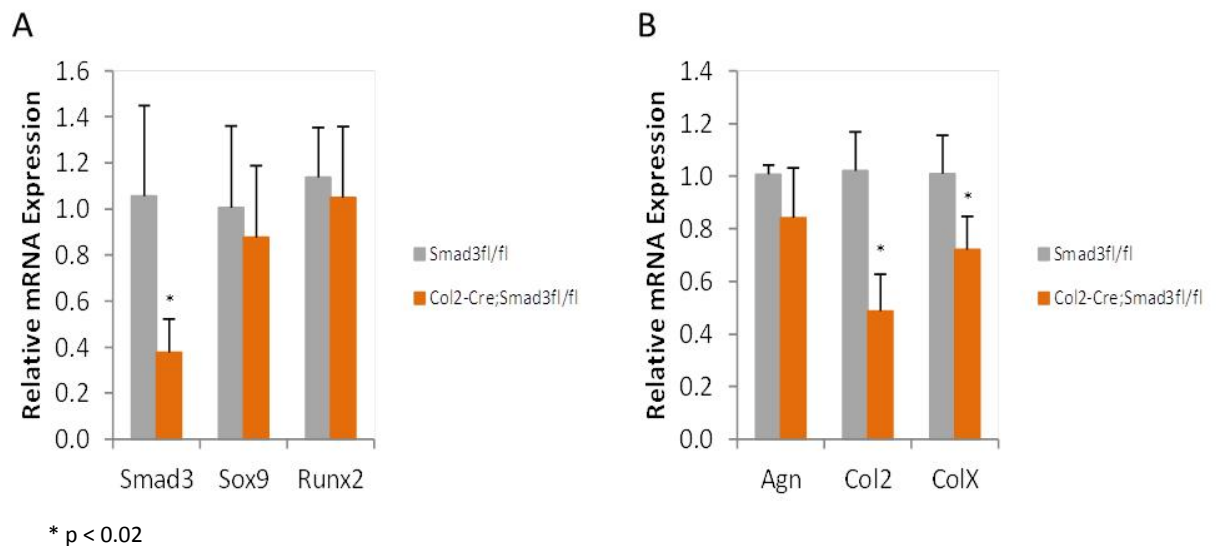


Figure 7.4. Gene expression changes in cartilage resulting from Smad3-deficiency in chondrocytes. (A and B) Real-time RT-PCR analysis of costal cartilage harvested from P60

Smad3^{fl/fl} and Col2-Cre;Smad3^{fl/fl} mice (n = 5). Values are normalized to RPL19 expression and are shown relative to Smad3^{fl/fl} costal cartilage. * p value < 0.02 vs. control group (Smad3^{fl/fl})

Decreased expression of Agn and Col2 protein in Col2-Cre;Smad3^{fl/fl} articular cartilage

Differences in the cartilage matrix can result from changes in composition or changes in the organization of the constituents. To further explore the heterogeneous matrix organization apparent in the Safranin-O stained articular cartilage, we sought to examine the expression level and localization of key cartilage matrix constituents Col2 and Agn by immunohistochemistry. Consistent with a decreased level of mRNA expression, less intense Col2 staining was observed in Col2-Cre;Smad3^{fl/fl} articular cartilage (7.5, A and B). This is also consistent with an osteoarthritic phenotype, as one of the major hallmarks of the disease is the degradation of collagen type II, the main constituent of articular cartilage. Though we did not observe a decrease in Agn mRNA expression, immunohistochemical analysis shows that there is a marked decrease in Agn staining in Col2-Cre;Smad3^{fl/fl} articular cartilage (7.5, C and D). This suggests that though the Agn transcription might not be affected by loss of Smad3, the synthesis and degradation of Agn protein may be regulated by a Smad3-dependent signaling pathway. Another possibility is that there are differences in Agn expression at different anatomical sites. Real-time RT-PCR measurement of mRNA expression was analyzed using costal cartilage while immunohistochemistry was analyzed on articular cartilage. It is possible that analysis of articular cartilage could reveal a reduction in Agn mRNA expression that is not present in costal cartilage. Future work will address this by using real-time RT-PCR to evaluate Agn expression in articular cartilage from Smad3^{fl/fl} and Col2-Cre;Smad3^{fl/fl} mice.

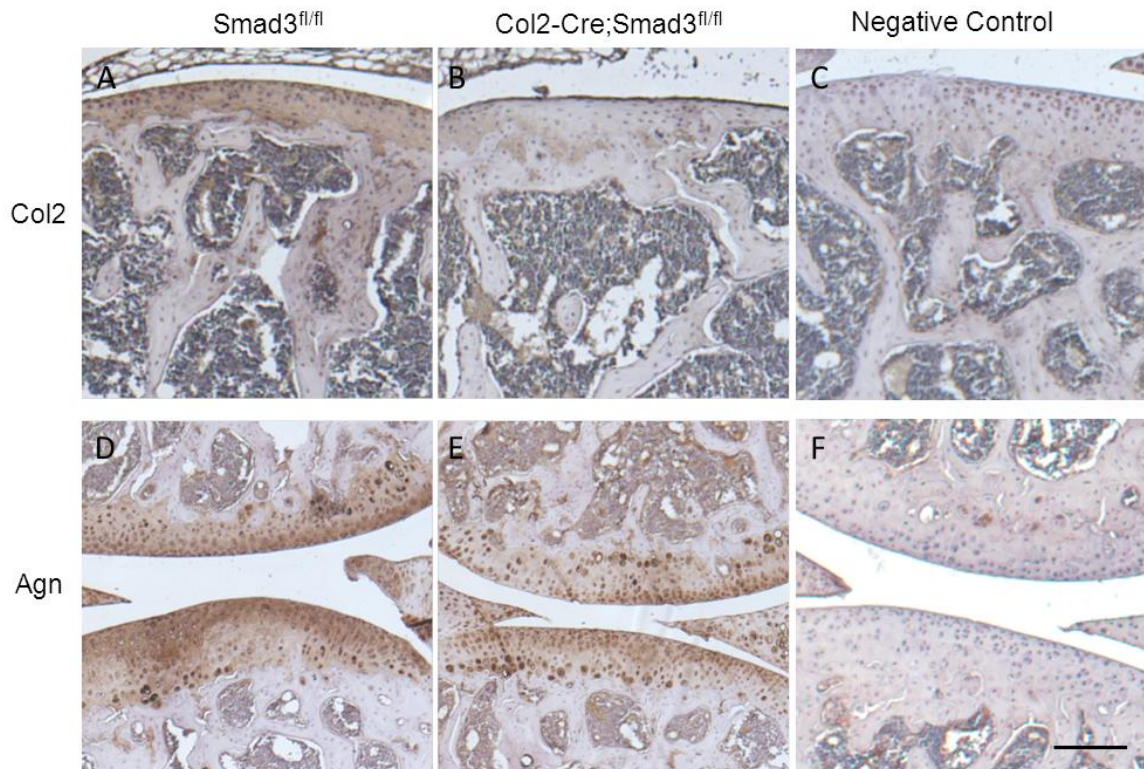


Figure 7.5. Reduction of Col2 and Agn expression in the articular cartilage of P60 Col2-Cre;Smad3^{fl/fl} mice compared to Smad3^{fl/fl} mice. Immunohistochemical detection of Col2 (A and B) and Agn (D and E) in Smad3^{fl/fl} (A and D) and Col2-Cre;Smad3^{fl/fl} (B and E) knee joints. (C and F) Negative controls (IgG) show no non-specific staining. Bar: 250 μ m.

Discussion

Osteoarthritis, the most common disease of the joints, results from the degeneration of articular cartilage. Evidence suggests that deregulation of TGF- β signaling may have a key role in the pathogenesis of the disease (Serra, Johnson et al. 1997; Yang, Chen et al. 2001; Blaney Davidson, van der Kraan et al. 2007), though specific signaling mechanisms remain largely undefined. Here, we investigated the chondrocyte-intrinsic role of Smad3 in the development and maintenance of articular cartilage and explored the mechanisms through which the TGF- β /Smad3 signaling pathway specifies the cartilage matrix. We found that chondrocyte-specific loss of Smad3 in mice results in a progressively degenerative cartilage phenotype that mimics

osteoarthritis. These characteristics include loss of a smooth articular cartilage surface, localized reduction in proteoglycan, and, in the most extreme cases, fibrillation, loss of cartilage, and accelerated chondrocyte differentiation. Furthermore, the growth plates of these mice exhibited disorganization. This is consistent with previous work documenting that mice with systemic deletion of Smad3 results in cartilage degeneration associated with the release of TGF- β /Smad3 repression of chondrocyte differentiation (Yang, Chen et al. 2001). To provide further evidence of the presence of abnormal hypertrophy and accelerated differentiation, future work will focus on the expression of maturation and differentiation markers such as ColX and osteopontin.

It is notable that though Col2-Cre;Smad3^{fl/fl} have a similar phenotype to mice with systemic deletion of Smad3, it is less severe. This may be due to incomplete deletion of Smad3, as evidenced by the Smad3 expression level of Col2-Cre;Smad3^{fl/fl} mice, ~40% of the normal expression level. Additionally, systemic Smad3 loss results in an abnormal bone phenotype, functional defects in the immune system, and changes in metabolism (Datto, Frederick et al. 1999; Yang, Chen et al. 2001; Balooch, Balooch et al. 2005). Lastly, in our mouse model, Cre is only expressed after chondrocytes are differentiated, that is, after Col2 is already expressed, so Smad3 may also be important in the commitment of the chondrocytic lineage. Prx1-Cre mice, which express Cre in the developing limb bud mesenchyme, may be useful in exploring the role of Smad3 in chondrocyte lineage commitment (Logan, Martin et al. 2002). Our work attempts to clarify and highlight the importance of the chondrocyte-specific role of Smad3 in maintaining normal articular cartilage.

We have further elucidated the means by which TGF- β regulates cartilage matrix composition in vivo. Previous work has focused on the role of TGF- β /Smad3 signaling in repressing chondrocyte hypertrophic differentiation. In osteoblasts, Smad3 mediates TGF- β inhibition of Runx2, as well as the resulting repression of osteoblast differentiation genes such as osteocalcin (Alliston, Choy et al. 2001). Furthermore, evidence suggests that TGF- β and Smad3 inhibition of Runx2 may also play a role in the progression of chondrocyte differentiation (Ferguson, Schwarz et al. 2000; Dong, Soung do et al. 2006). Both ColX and MMP-13, which are

cartilage extracellular matrix proteins induced by Runx2 during terminal chondrocyte differentiation, have been shown to be regulated by TGF- β (Zheng, Zhou et al. 2003; Selvamurugan, Kwok et al. 2004; Dong, Soung do et al. 2006; Selvamurugan, Jefcoat et al. 2006). Our data now show that Smad3 induction of Col2 expression may be essential for maintaining articular cartilage matrix integrity and may occur earlier than changes in chondrocyte differentiation. This highlights the differential roles that TGF- β has in chondrocyte differentiation – promoting synthetic activity early on as well as repressing later events, such as hypertrophy, in these cells.

Col2 is the most abundant protein in articular cartilage and is a major structural component. There are numerous studies detailing the degradation of this protein as a key event in the progression of osteoarthritis (Tchetina, Squires et al. 2005; Nemirovskiy, Dufield et al. 2007; Settle, Vickery et al. 2010). TGF- β and Smad3 regulate the expression of Col2 during the initiation of chondrogenesis by forming a transcriptional complex with Sox9 (Furumatsu, Tsuda et al. 2005). Thus, even though we did not see changes in Sox9 expression, loss of Smad3 resulted in a reduction in Col2 mRNA expression levels, likely due to the inability to recruit the Smad3-CBP complex to coactivate Sox9 transcriptional function. Though previous studies have shown the activity of this pathway in cell culture experiments, our data now suggests that the pathway is active in vivo. Furthermore, not only is this pathway critical in cartilage development, it is essential for cartilage maintenance.

Interestingly, we did not see an increase in ColX mRNA expression, which has frequently been found to accompany cartilage degradation and is associated with osteoarthritis (Aigner, Zien et al. 2001; Yang, Chen et al. 2001; Tchetina, Squires et al. 2005). Moreover, osteoarthritis has been described as the loss of mechanisms that arrest chondrocyte differentiation and ColX is a marker of terminal chondrocyte differentiation (Drissi, Zuscik et al. 2005). Smad3^{ex8/ex8} mice were shown to have increased ColX in both the articular cartilage and in the growth plate (Yang, Chen et al. 2001). Our results, however, show that at P60, there is a decrease in ColX mRNA in costal cartilage. Although it is possible that chondrocytes in costal cartilage behave differently than the

ones in knee joint articular cartilage, another explanation for the discrepancy could be that the increase in ColX expression occurs at a later time point than the loss of Col2 expression in the progression of cartilage degeneration. Smad3^{ex8/ex8} mice were shown to be indistinguishable from wild-type mice until 3-4 weeks. Reports show that they have more intense ColX staining at 6-months of age; it is unknown when changes in the expression level of ColX initiate. Additionally, while previous studies have shown that MMP-13, another marker of terminal differentiation that has been shown to be induced by Runx2 in osteoarthritis (Wang, Manner et al. 2004), it has also been shown to have suppressed promoter activity mediated by a Runx2 binding site (Varghese, Rydziel et al. 2005). This is interesting in that it opens the possibility that another signaling pathway that is not yet well understood could suppress ColX expression levels similarly. It will be interesting to investigate whether Smad3 repression of Runx2 or other unexplored signaling pathways could play a role in the progression of osteoarthritis. Currently, however, our results indicate that this is not a significant factor in the initiation and early development of the osteoarthritic phenotype.

Our results also seemingly contradict previous studies that show a gradual increase in Col2 gene expression in Smad3-deficient chondrocytes over time, when compared with wild-type chondrocytes (Li, Darowish et al. 2006). However, these studies were of sternal chondrocytes in monolayer and do not reflect a physiologically relevant environment. Moreover, gene expression studies of the intervertebral disc show that P10 Smad3^{-/-} mice display an increase in Col2 mRNA expression followed by a gradual decrease when compared to control animals (Li, Liang et al. 2009). P30 mice had significantly decreased levels of Col2 mRNA. This is consistent with reports of an initial upregulation of matrix synthesis in osteoarthritis followed by a decrease in synthesis and degradation.

In line with our gene expression data, we further demonstrate that Col2 protein levels are decreased in Col2-Cre;Smad3^{fl/fl} mouse articular cartilage as shown by immunohistochemistry. Interestingly, we did not see changes in Agn gene expression, though there was a marked decrease in Agn protein. This suggests that Smad3 may have a role in regulating the activity or

expression of aggrecanases, such as ADAMTS4/5. TGF- β has previously been shown to stimulate ADAMTS4 expression in chondrocyte cultures (Moulharat, Lesur et al. 2004) and ADAMTS4 is elevated in human osteoarthritic cartilage (Murphy and Nagase 2008). However, the mechanism is unknown and it is unclear whether this effect would be replicated in vivo. It will be interesting to further investigate the physiological mechanisms of TGF- β signaling in regulating both the synthesis and degradation of cartilage matrix proteins.

Studies in Smad3-deficient chondrocytes also suggest disruption of TGF- β signaling can lead to activation of other pathways, specifically indicating a shift from TGF- β towards BMP signaling (Li, Darowish et al. 2006). Furthermore, TGF- β itself can signal through non-Smad3 pathways, including TAK-1, MAPK, and even ALK1, previously thought to be activated only by BMP (Goumans and Mummery 2000; Qiao, Padilla et al. 2005). Our data is consistent with the idea that TGF- β has possible switch-like activities. It can activate both Smad3 and Smad1/5/8 pathways, which are pro-chondrogenic and pro-osteogenic, respectively. The Smad1/5/8 pathway is known to induce chondrocyte terminal differentiation (Ito, Akiyama et al. 1999), and our data shows that a loss of Smad3 is associated with increased chondrocyte hypertrophy. Further study is necessary to elucidate the activation of the BMP pathways.

Overall, our results demonstrate that TGF- β both promotes the expression of normal cartilage constituents (Col2 and Agn) and prevents abnormal terminal differentiation. Consequently, disruption of TGF- β signaling through the loss of Smad3 results in cartilage degradation and mimics osteoarthritis. Furthermore, we present evidence that TGF- β /Smad3 signals are essential in the regulation of the composition of cartilage matrix, as in bone, though studies are needed to evaluate their role in controlling matrix material properties. Though these mechanisms are not fully uncovered, our studies suggest that one pathway may be through Smad3 regulation of Sox9 function. Interestingly, opens the possibility that there may be a conserved role for Smad3 in regulating skeletal matrix material properties by regulating the activity of lineage-specific transcription factors such as Runx2 and Sox9 (7.6). Understanding the TGF- β /Smad3 signaling pathways in cartilage has become important as a recent study has

reported the association of Smad3 genetic variations and the risk of developing osteoarthritis in humans (Valdes, Spector et al. 2010). Future studies should focus on elucidating the crosstalk between pathways and tracking the molecular changes that occur in these models of osteoarthritis to identify potential therapeutic targets.

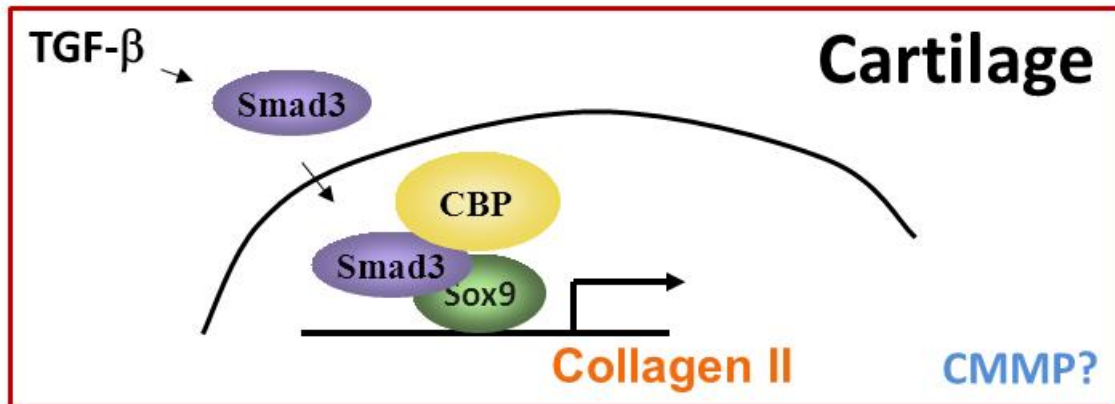


Figure 7.6. TGF- β regulation of cartilage matrix material properties.

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Chapter 8

Role of TGF- β /Smad3 signaling in the tendon matrix

Introduction

Tendons are an integral component of the musculoskeletal system. As the connectors between bone and skeletal muscles, their function is to transmit forces between skeletal muscles and bones to enable motion across joints. Like bone and cartilage, tendon possesses a unique extracellular matrix that defines its mechanical and material properties, which are essential for its physiological function. Though studies have reported the effects of exercise, immobilization and aging on the structure and function of tendons, little is known of the molecular framework that guides tendon development and tenocyte differentiation.

The elucidation of key molecular pathways and factors is only beginning to emerge, made possible by the relatively recent identification of a tenocyte lineage specification transcription factor, scleraxis (Scx) (Schweitzer, Chyung et al. 2001). Mice deficient in Scx displayed severe tendon defects and impaired locomotion. The tendon extracellular matrix in these mice have decreased expression and organization of Col1, demonstrated by in situ hybridization (Murchison, Price et al. 2007). Consistent with this, Scx has been shown to regulate Col1 in tendon fibroblasts by cooperating with NFATc4 to activate Col1 expression (Lejard, Brideau et al. 2007). Though prior studies address the important role of these factors, the mechanisms that guide expression and activity of these factors remain largely unexplored.

TGF- β , considered a key regulator of the differentiation and growth of bone and cartilage, has also been shown to be essential for normal tendon development. Ablation of TGF- β 2 and TGF- β 3 in mice results in the complete loss of tendons, as does limb-specific deletion of the TGF- β type II receptor (Pryce, Watson et al. 2009). Moreover, it was demonstrated that TGF- β

induces both Scx and Col1 expression (Chan, Fu et al. 2008; Lorda-Diez, Montero et al. 2009; Pryce, Watson et al. 2009).

Evidence implicates Smad3 in the TGF- β regulation of tendon-specific genes. First, Smad3 is a key effector of TGF- β signaling in diverse musculoskeletal tissues, including bone, cartilage, and muscle. TGF- β /Smad3 signaling regulates the lineage-specific transcription factors Runx2, Sox9, and MyoD to control osteogenic, chondrogenic, and myogenic differentiation (Alliston, Choy et al. 2001; Liu, Black et al. 2001; Furumatsu, Tsuda et al. 2005). Whether the TGF- β regulation of Scx, a tendon-specific transcription is Smad3-dependent is unknown. Second, myostatin, a member of the TGF- β superfamily of cytokines, has been shown to phosphorylate Smad3 in tendon fibroblasts and upregulates Scx and the tendon-selective gene tenomodulin (Tnmd) (Mendias, Bakhurin et al. 2008). Therefore, we hypothesize that Smad3 is essential for normal tendon development and gene expression.

Results

Tendons in Smad3-deficient mice display abnormal morphology

Although the bone and cartilage defects in Smad3-deficient mice have been described (Yang, Chen et al. 2001), the role of TGF- β /Smad3 in tendon structure and organization remains to be evaluated. Very recent work has focused on how Smad3 affects the healing process in tendons (Katzel, Wolenski et al. 2010). Interestingly, in these studies, Smad3 flexor digitorum longus (FDL) tendons exhibited a significant 39% decrease in maximum tensile strength. However, the changes in the tendon extracellular matrix that may underlie this decrease in breaking force were not investigated. To examine the appearance of Smad3^{-/-} tendons, we investigated the histologic structure of Smad3^{-/-} mouse tendons. To that end, we harvested flexor digitorum longus tendons from P60 Smad3^{-/-} mice, sectioned and stained them, and compared them to WT littermates. Strikingly, while the cells in the WT tendons were arranged in parallel lines of as many as ten cells in a row, the Smad3^{-/-} tendons had markedly crooked lines

of cells that were much more disorganized (8.1, A and B). Furthermore, the Smad3^{-/-} matrix itself appeared crimped while the WT controls were smooth and flat. In some areas, there was evidence of gaps within the tissue (8.1 C) and an increase in fraying (8.1 D). Overall, there is a visible reduction in the structural integrity of Smad3^{-/-} mouse tendon, which could contribute to the decreased tensile strength observed.

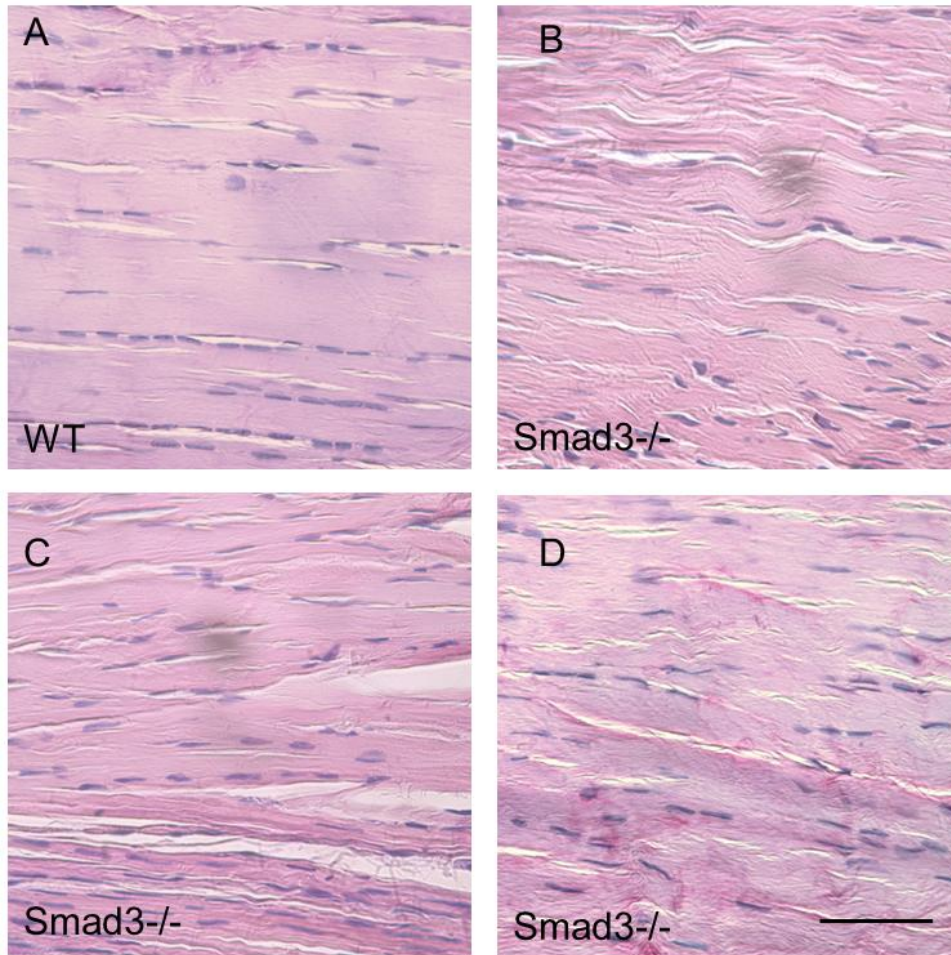


Figure 8.1. Tendons from Smad3^{-/-} mice are abnormal. H & E staining of FDL tendons from Smad3^{-/-} mice show crimped fibers (B), large gaps (C), and cell disorganization.

Decreased expression of tendon marker genes and matrix constituents in Smad3^{-/-} mice

The changes apparent in the tendon matrix of Smad3^{-/-} mice led us to examine whether these changes were correlated with differences in gene expression. To explore the mechanisms

by which loss of Smad3 could alter the tendon matrix, we used real time RT-PCR to evaluate scleraxis (Scx), a transcription factor which marks tendon cell fate (Cserjesi, Brown et al. 1995; Schweitzer, Chyung et al. 2001), tenomodulin (Tnmd), a tendon-specific marker which has a role in tenocyte proliferation and collagen fibril morphology (Docheva, Hunziker et al. 2005), collagen type I (Col1), the major matrix constituent of tendon, and Tenascin C, another tendon matrix component. In tail tendons from P60 mice, loss of Smad3 results in an average ~43% decrease in Scx mRNA levels, ~50% decrease in TenC, and almost no expression of both Tnmd and Col1 (> 99% decrease) (8.2).

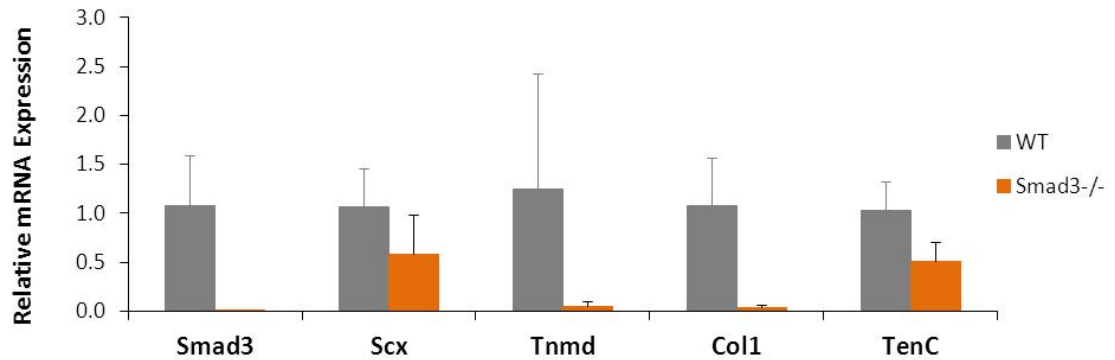


Figure 8.2. Tendon specific gene expression decreased in Smad3^{-/-} mouse tendons. Smad3-deficiency results in decreases in Scx, Tnmd, Col1, and TenC mRNA expression levels.

Generating an in vitro model for studying primary tendon fibroblasts from mice

To better understand the mechanisms that regulate the function and differentiation of tendon cells, we next sought to develop an in vitro culture system for Smad3-deficient tendon fibroblasts. Though previous studies have reported the use of primary tendon fibroblasts isolated from murine tendons (Bi, Ehrichtiou et al. 2007; Mendias, Bakhurin et al. 2008), there are very few such studies to date and the cells themselves have not been well characterized. We started by isolating cells from FDLs of 6 week old mice with the epitenon carefully removed, as previously described (Bi, Ehrichtiou et al. 2007). These tendons were then digested with collagenase and

disperse to release cells from the tendon matrix and subsequently cultured on collagen type I-coated plates in α MEM supplemented with 20% FBS and 1% penicillin streptomycin. Initially, we used four mice and there were too few cells to count on the day of harvest. Consistent with previous reports (Bi, Ehrlichou et al. 2007), only a small population of cells formed adherent cell colonies and the cells remained quiescent for 2-5 days before dividing to form colonies in our first trials (8.3, A and B). After 13 days in culture, the RNA was isolated and tendon marker gene expression was evaluated by real time RT-PCR and compared to expression levels in isolated murine tendon tissue. Consistent with previous results found in human and rat primary tendon cells, *Tnmd* expression is significantly reduced in cultured murine tendon fibroblasts (> 99.9%), indicating that cells grown in two-dimensional cell cultures progressively de-differentiate (8.3 C) (Jelinsky, Archambault et al. 2010). Interestingly, *Scx* expression was 80% higher in cultured tendon fibroblasts than tendon tissue, though this is only from one trial (8.3 C). Though the cultured cells seem to de-differentiate, as evidenced by the loss of *Tnmd* expression, the presence of *Scx* expression indicates that the cells may retain an earlier state of tendon differentiation or a tendon progenitor state. Moreover, *Tnmd* generally represents a later stage of tendon differentiation (Shukunami, Takimoto et al. 2006).

TGF- β regulates Tendon Gene Expression in vitro

Our observations that the expression of key tendon factors was altered in *Smad3*-deficient tendons support an important role for TGF- β signaling in tendon. Consequently, we isolated tendon fibroblasts from C56BL/6 mice and treated them with TGF- β to further examine the effect of TGF- β activation on the gene expression of *Scx*, *Tnmd*, *TenC* and *Col1* (8.4). No change was observed in *Scx* expression as a result of the addition of 5 ng/ml of TGF- β . However, consistent with previous studies, TGF- β treatment resulted in an upregulation of key tendon matrix genes, a 50-fold increase in *Tnmd*, a 30% increase in *Col1*, and a 2.5-fold increase in *TenC* (Lorda-Diez, Montero et al. 2009; Pryce, Watson et al. 2009).

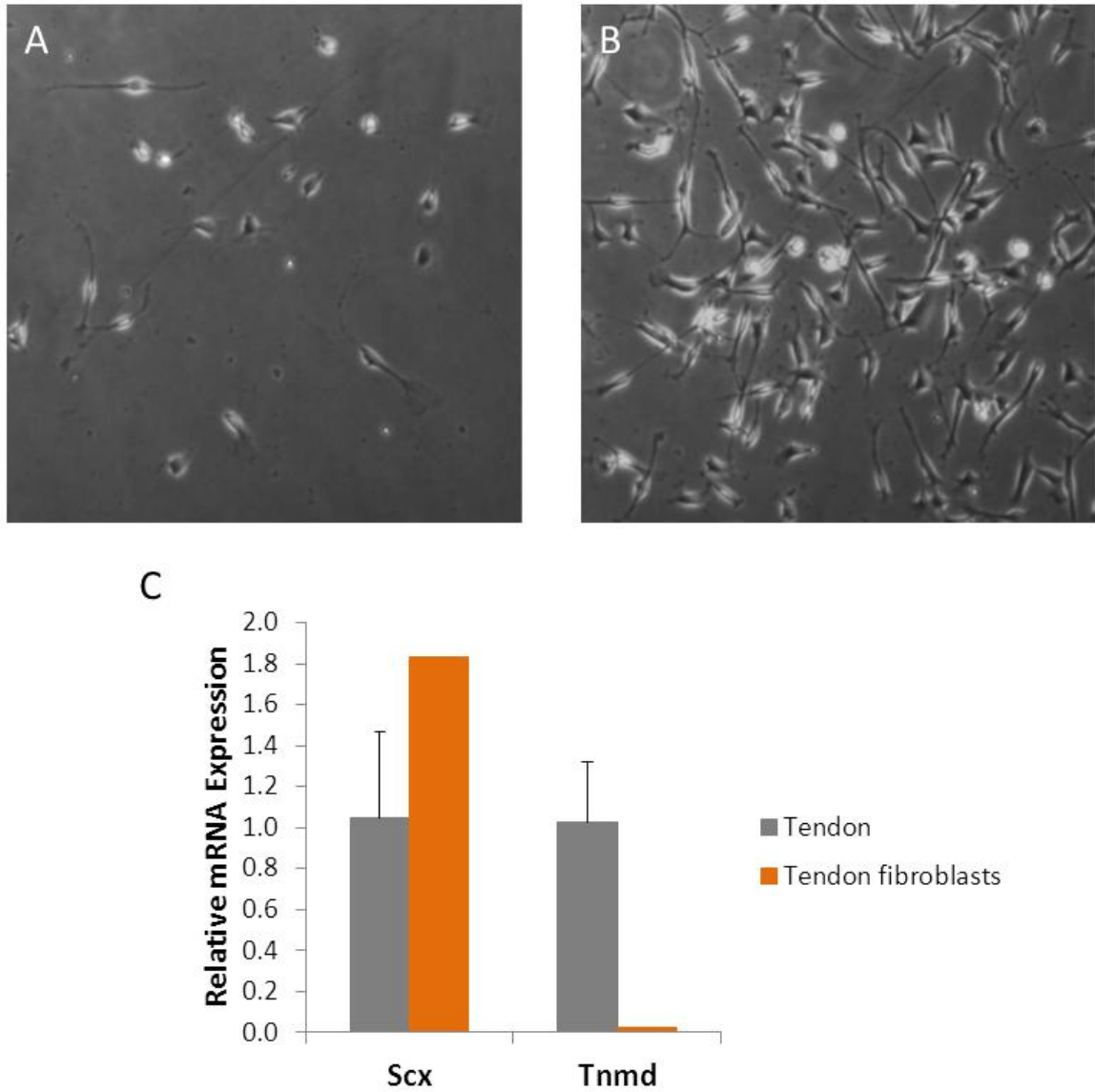


Figure 8.3. Characterization of tendon fibroblasts isolated from murine FDLs. (A) Primary tendon fibroblasts on day two of culture. (B) Primary tendon fibroblasts on day 4 of culture. (C) Tendon-specific gene expression in tail tendons and primary tendon fibroblasts after 13 days of culture. Values are normalized to RPL19 expression.

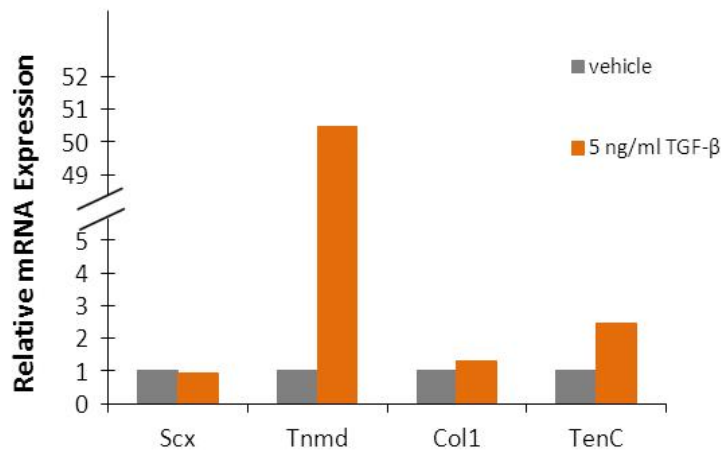


Figure 8.4. TGF-β regulation of tendon-specific gene expression. Treatment of mouse tendon fibroblasts with 5 ng/ml TGF-β for 24 hours resulted in induction of Tnmd, Col1, and TenC.

Generating an in vitro model of Smad3-deficiency in tendon fibroblasts

The next step was to delete Smad3 in primary tendon fibroblasts. Using Smad3^{fl/fl} mice, we harvested FDLs from four 8-week old animals and passaged them two times when they reached ~70-80% confluency. After two weeks, we infected them overnight with Cre-recombinant adenovirus (Ad-Cre) and GFP adenovirus (Ad-GFP) as a control. The next day, the cells clearly expressed GFP, indicating that the infection was successful (8.5, A and B). Furthermore, the Smad3 mRNA expression level in Ad-Cre-infected cells was negligible when compared to Ad-GFP controls (8.5 C).

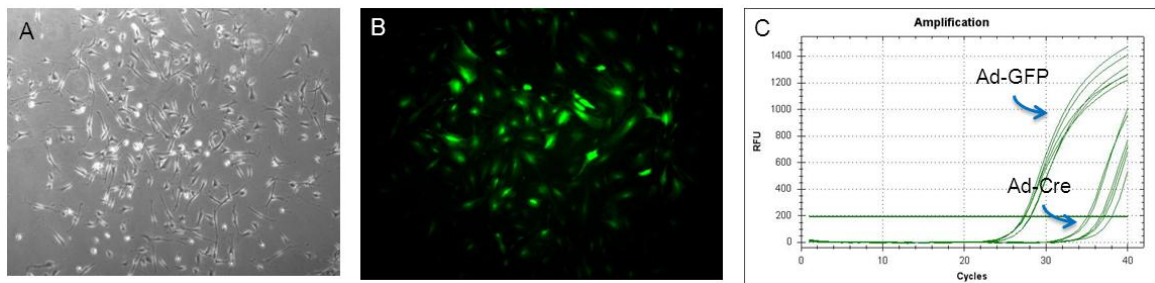


Figure 8.5. Ad-Cre deletion of Smad3 from primary tendon fibroblasts. (A) Primary tendon fibroblasts from Smad3^{fl/fl} mice before Ad-Cre infection. (B) Control cells show successful infection with Ad-GFP. (C) Amplification plots show the reduction of Smad3 in Ad-Cre-infected tendon fibroblasts.

The tendon fibroblasts harvested from 6-8 week old mice contained so few cells that it took at least 2-3 weeks and two passages to grow enough cells for an experiment. Our results indicate that in this time, the cells de-differentiate to a degree and behave differently than they do in vivo. Thus, we decided to use FDL tendons from much younger, P10 mice, which are more highly cellular. Initiating experiments with more cells would both decrease the opportunity to de-differentiate and the number of cell divisions, thereby enhancing the ability of the cells to preserve their differentiated phenotype. However, one potential drawback is that tendons from younger mice appeared less fibrous, were more mucinoid, and did not possess the same structural integrity as those from adult mice. This implies that the tissue matrix and the cells are not yet mature and may further differentiate to a mature tenocyte phenotype.

Tendon fibroblasts were harvested from four P10 Smad3^{fl/fl} mice. Many more cells were visible from the onset of culture, unlike in the cultures from 6 week old mice. On day one, cells were already 30-40% confluent and were actively dividing (8.6 A). These cells were infected with Ad-Cre and Ad-GFP after eight days in culture, RNA was isolated after 10 days in culture, and real time RT-PCR was used to evaluate gene expression. Similar to our in vivo studies, the tenocytes from young mice displayed decreasing Tnmd and Col1 mRNA expression as a result of Smad3-deficiency, though to a much lesser extent than in vivo (8.6 B). Tnmd and Col1 were reduced by 27% and 32% in vitro, compared to > 99% in vivo (Compare to 8.2). Scx and TenC, which displayed much smaller reductions in vivo (8.2), showed no changes in vitro (data not shown). The relatively modest reductions we observed in vitro when compared to in vivo experiments indicate that the changes we see in vivo are not cell autonomous – the extracellular environment in these two-dimensional cultures is different from the three-dimensional physiological environment in vivo. Though preliminary, these studies suggest that this cell model may be suitable for some in vitro analysis of the role of Smad3 in tenocyte differentiation and gene expression.

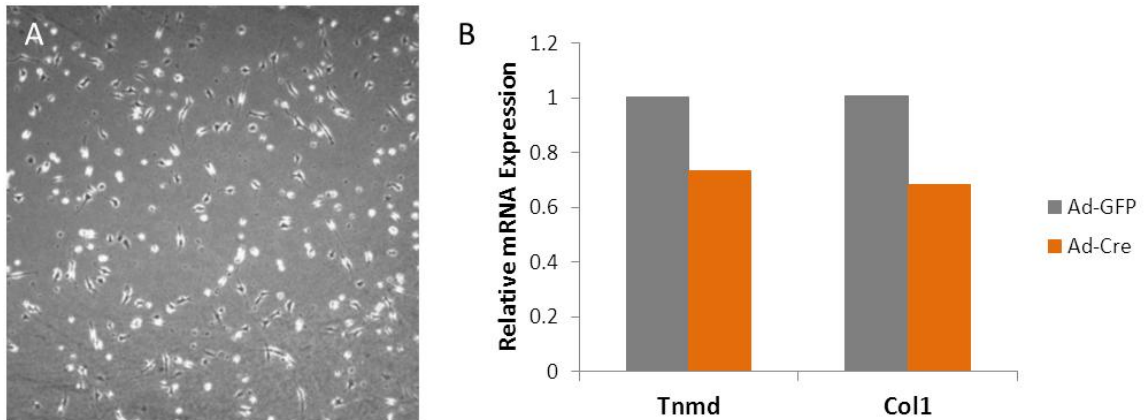


Figure 8.6. Smad3-deficiency in tendon fibroblasts results in decreases in tendon specific gene expression. (A) P10 tendon fibroblasts from mouse FDLs on day one of culture. (B) Real-time RT-PCR analysis of Ad-Cre-infected mouse primary tendon fibroblasts compared to Ad-GFP infected controls. Values are normalized to RPL19 expression. All gene expression experiments using cultured tendon fibroblasts represent one biological replicate per condition.

Discussion

TGF- β /Smad3 signaling has been shown to be key in both cell differentiation and matrix specification in bone, cartilage and muscle. To determine whether this pathway also has a role in regulating tenocyte gene expression and tendon matrix, we examined the tendons of Smad3^{-/-} mice. Histologic analyses showed that the FDLs from Smad3^{-/-} mice were disorganized compared to tendons isolated from WT littermates. There were visible gaps in the matrix, crimping, and the cells were scattered and arranged more haphazardly. Gene expression analysis using real-time QPCR showed a decrease in Scx. This suggests that the TGF- β regulation of Scx in vivo is Smad3-dependent. Furthermore, expression levels of genes previously found to be induced by Scx, Col1 (Lejard, Brideau et al. 2007; Murchison, Price et al. 2007) and Tnmd (Shukunami, Takimoto et al. 2006), were found to be subject to even greater repression.

To further evaluate the mechanism through which Smad3 regulates tendon gene expression, and thus matrix composition, we developed an in vitro system to study primary

tendon fibroblasts. Though still in the stages of optimization, we have shown that the cells have Scx expression approximately equivalent to tendon tissue itself, thereby demonstrating that the cells retain some degree of tendon gene expression. Further, these cells respond to TGF- β treatment in a manner consistent with previous studies, resulting in the upregulation of Tnmd, Col1 and TenC. Finally, we successfully deleted Smad3 expression in these cells using Ad-Cre and show that the tendon-selective gene, Tnmd, and the tendon matrix protein, Col1, are decreased in these cells, consistent with the in vivo findings from tendons of Smad3^{-/-} mice.

While some understanding of the processes in tendons is gained from these in vitro experiments, further work must be done to ensure a model fitting physiologic processes. Ongoing work will focus on the characterizing the tendon-specific gene expression at multiple time points to track the changes that occur as a result of two-dimensional culture. Following this, we will investigate whether Smad3 is required for tendon gene expression in our model system and compare it to the in vivo data. It is possible that many processes that are seen in vivo are not cell autonomous -- matrix-cell interactions, cell-cell interactions, mechanical stimulation are likely all involved, and the results from these experiments will help elucidate these processes.

Based upon the work we have accomplished already, future work will evaluate whether Smad3 is directly involved in regulating tendon-specific gene expression by determining the physical interactions of Smad3 binding with promoter regions of tendon-specific genes and whether this binding occurs as a result of TGF- β signaling. We will test the hypothesis that, as in bone and cartilage, Smad3 has a conserved role in regulating tendon matrix material properties by regulating the function of the lineage-specific transcription factor Scx (8.7).

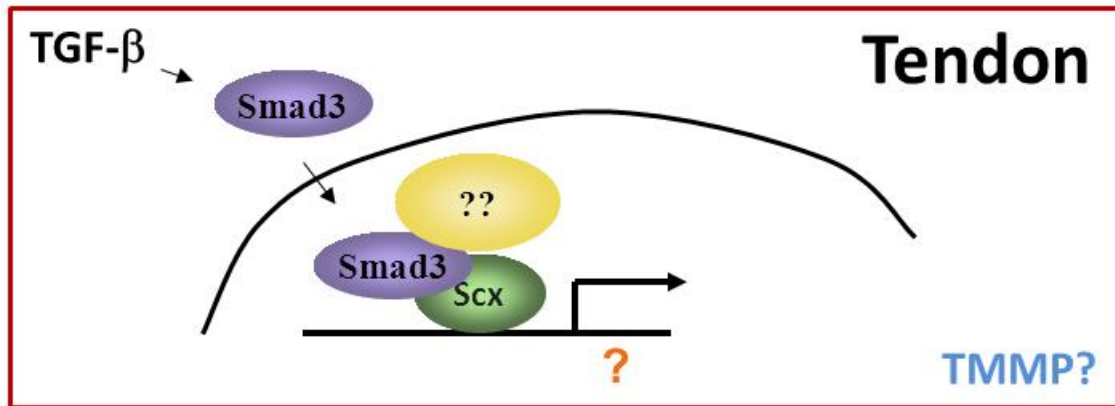


Figure 8.7. Proposed schematic of TGF-β regulation of tendon matrix material properties.

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Chapter 9

Summary and Future Directions

In summary, our studies explored the mechanisms and functional role of TGF- β /Smad3 regulation of the bone, cartilage, and tendon matrices. In bone, we demonstrated that TGF- β /Smad3 regulates matrix material properties via a Runx2-dependent pathway. Runx2^{+/-} mouse bones exhibited reduced elastic modulus, which was rescued by crossing with mice with reduced TGF- β signaling (5.2 A and B). Therefore, one mechanism through which TGF- β regulates BMMPs is by the same Runx2-dependent mechanism used to regulate osteoblast differentiation. Additionally, we found that deregulation of this pathway compromises the distinctly hard cochlear bone matrix which is correlated with hearing loss, demonstrating the essential nature of these properties for healthy tissue function (5.3 A and B). The TGF- β /Smad3 signaling pathway was shown to be equally important in the maintenance of the structure and composition of cartilage and tendon matrices. Our results led us to generate a model by which tissue-specific extracellular matrix material properties are established by virtue of lineage-specific transcriptional factors which also direct cell differentiation.

Interestingly, our data indicate that disruption of the TGF- β /Smad3 signaling pathway affects both the collagen and ground substance in musculoskeletal tissue matrix. In bone, disruption of TGF- β signaling results in changes in mineral concentration as well as collagen organization (5.5C, 6.7 A and B); in cartilage and tendon, the mRNA expression levels of the major collagen constituents, Col2 and Col1, respectively, were significantly decreased (7.4 B, 8.2). Additionally, blocking TGF- β signaling resulted in notable decreases in proteoglycan in the articular cartilage matrix (7.1 A-E, 7.5 D and E). These changes in matrix constituents likely result in alterations in matrix material properties, as we show in bone, though more work is necessary to elucidate how the matrix material properties in cartilage and tendon will be affected.

Initially, one of the main goals of my research was to measure the cartilage matrix material properties in Smad3-deficient mice to explore the relationship of signaling pathways to the specification of material properties. Our underlying hypothesis was that the material properties of cartilage, like those of bone, are carefully regulated and act as cues that direct cell function. Accordingly, loss of either biological or physical homeostasis destroys cues that are required to retain normal cell behavior and tissue matrix. This hypothesis is consistent with observations that OA can be triggered by either mechanical injury or by genetic factors (Davis, Ettinger et al. 1989; Wilder, Hall et al. 2002; Kizawa, Kou et al. 2005; Goldring and Goldring 2007; Lohmander, Englund et al. 2007).

Furthermore, the specialized material properties of articular cartilage are essential for normal joint function, but are lost in OA (Setton, Mow et al. 1994; Sah, Yang et al. 1997; Silver, Bradica et al. 2001). A recent indentation study of load displacement behavior in human cartilage detected up to a ~15-fold reduction in cartilage matrix stiffness that correlated with OA severity (Stolz, Gottardi et al. 2009). Loss of matrix material properties may be a factor that contributes to OA progression. If the material properties of cartilage matrix are cues that retain chondrocytes in a stable articular phenotype, small changes in these properties could have significant effects on chondrocyte differentiation. Interestingly, studies of mice deficient in a specific proteolytic enzyme (MMP-13) concluded that remodeling of extracellular matrix is required for terminal chondrocyte differentiation. In the absence of this enzyme, terminal chondrocyte differentiation is inhibited by unknown mechanisms (Stickens, Behonick et al. 2004). Among the possibilities is that MMP-13-dependent changes in matrix stiffness are required for terminal chondrocyte differentiation. In support of this, our lab has seen evidence that the elastic modulus of the substrate on which chondrocytes are grown affects chondrogenic gene expression (Allen and Alliston, unpublished observations). Thus, a long term goal is to develop a framework for understanding the biological mechanisms that specify matrix material properties, as well as how these material properties can direct cell behavior and how these pathways intersect (9.1). Our studies begin to fill in this framework by investigating the mechanism through which TGF-

β /Smad3 signaling can regulate musculoskeletal extracellular matrices as well as the identifying matrix factors that are involved (Table 9.1).

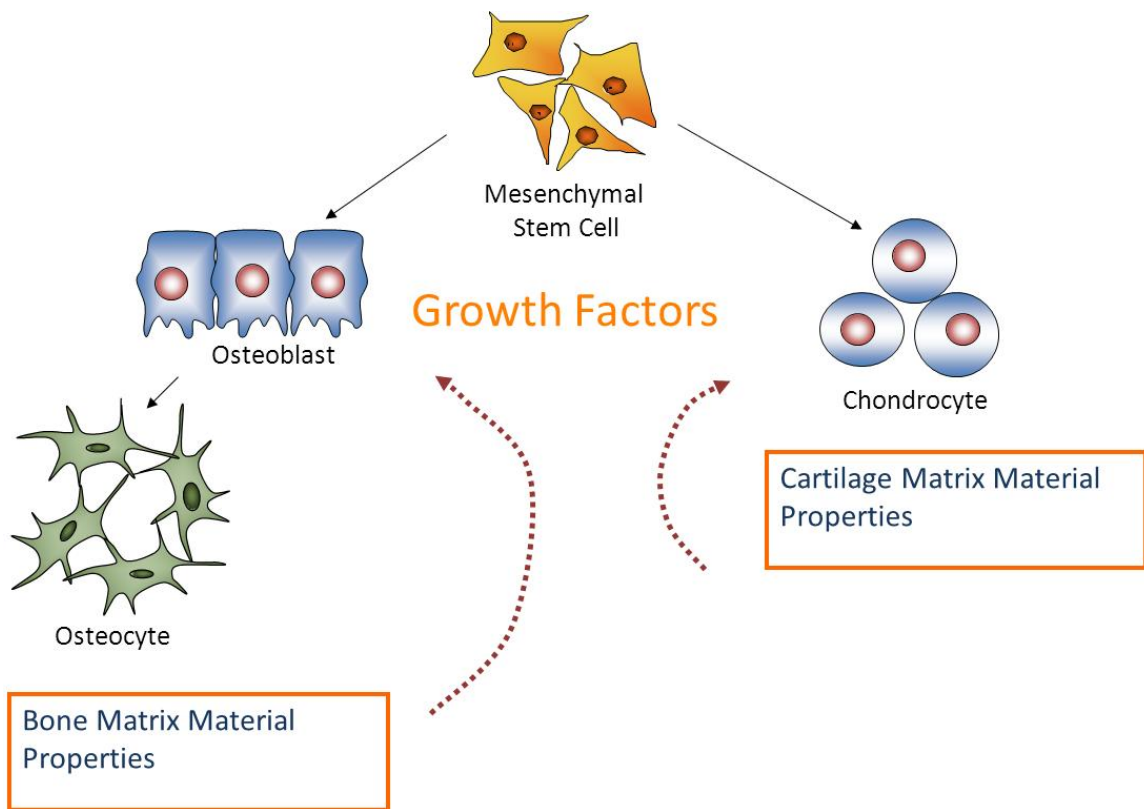


Figure 9.1: Convergence of biological and mechanical pathways

	Bone	Cartilage	Tendon
Mechanical Quality (macro/material)	Reduced macromechanical properties, Increased BMMP ³	(Likely reduced macromechanical properties, CMMP unknown)	(Likely reduced macromechanical properties, TMMP unknown)
Maintenance (histology)	Osteopenia ^{1,2}	Osteoarthritis ^{1,7}	Severe Tissue Disruption ⁷
Development (histology)	Effects on bone geometry ^{1,2}	Growth plate defects ^{1,7}	Unknown
In Vivo Transcription Factor Expression (tissue qRTPCR)	No significant change in Runx2 in calvaria or femur of Col I CRE:Smad3 fl/fl ⁷	No significant change in Sox9 in Col II CRE:Smad3 fl/fl ⁷	Reduced Scx expression in Smad3-/- ⁷
In Vivo Differentiation (tissue qRTPCR)	Increased osteocalcin in Col I CRE:Smad3 fl/fl femur ⁷	Decreased Col II in Col II CRE:Smad3 fl/fl ⁷	Decreased Col I and tenomodulin in Smad3-/- ⁷
In Vitro Transcription Factor Expression	Increased Runx2 expression and function ⁴	Reduced Sox9 expression and function ⁵	Under investigation ⁷
In Vitro Differentiation	Enhanced Differentiation ⁴	Enhanced terminal chondrocyte differentiation ⁶	Under investigation ⁷
Summary	Smad3 represses Runx2 to inhibit osteoblast differentiation	Smad3 promotes chondrocyte differentiation	Smad3 is required for tendon development and cell differentiation

Table 9.1: Effect of Smad3-deficiency on skeletal tissues and skeletal cell differentiation. References: 1. (Yang, Chen et al. 2001), 2. (Borton, Frederick et al. 2001), 3. (Balooch, Balooch et al. 2005), 4. (Alliston, Choy et al. 2001), 5. (Furumatsu, Tsuda et al. 2005), 6. (Li, Darowish et al. 2006), 7. This dissertation.

Advances in techniques reported in the past two years coupled with my initial forays in into nanoindentation and IT-AFM have now made it feasible for our lab to measure the cartilage matrix material properties of the mouse models in this thesis. Using AFM-indentation with both micro- and nano-sized probes, it was found that Col9a1^{-/-} knockout mice, which display a progressively osteoarthritic phenotype, exhibit decreasing microstiffness after six months of age. Most promisingly, nanoscale measurements of stiffness were able to show differences in mice as young as one month old (Stolz, Gottardi et al. 2009). This technique is also useful for high resolution imaging, such that information about both material properties and structure can be correlated. Interestingly, subsequent work has used this technique to probe the pericellular matrix in chondrocytes in situ, and has found that the elastic modulus of the pericellular matrix was significantly decreased when compared to the interterritorial matrix (Darling, Wilusz et al.

2010). AFM-indentation is such a high resolution technique that it is now possible to detect material property changes that occur as a result of molecular changes. Indeed, a study on porcine cartilage found that though the microstiffness was unchanged after digestion of the proteoglycan moiety, the nanostiffness progressively increased with time (Stolz, Raiteri et al. 2004). Based on these studies, we are continuing our investigation of the material properties of Smad3-deficient mouse cartilage. Interestingly, our Agn immunohistochemistry in mouse articular cartilage shows a gradient of expression, with highest expression occurring at the cartilage surface (7.5 D). This gradient is lost in the Smad3-deficient cartilage (7.5 E), and future studies will address how this loss of proteoglycan affects the material properties of articular cartilage matrix, with the goal of correlating this molecular constituent to physical properties.

If our hypothesis regarding the relationship between biological and physical cues is correct, then there is a need to address both the material property and biological changes that occur in degenerative diseases such as OA. Once a disease state is initiated, regardless of the cause, it progresses and may even accelerate due to the proposed interaction and feedback between physical and biochemical cues. Therefore, it is critical to identify the cues and understand the crosstalk between these cues in order to develop therapies that treat or halt the progression of diseases.

Our work furthers the case that TGF- β may be involved in linking these biochemical and mechanical factors. TGF- β is essential for maintaining a normal extracellular matrix in bone, cartilage, and tendon, regulating both the structure and composition. Disruption of this pathway compromises matrix material properties in bone, and likely as well in both cartilage and tendon. Furthermore, chondrocytes are known to respond to exogenously applied load and changes in the matrix environment affect how this load is perceived by embedded chondrocytes. Independent of the addition of biochemical factors, elastic modulus of the matrix regulates the expression level of TGF- β -regulated genes such as Agn and ColX. Remarkably, the physical cue of elastic modulus was as strong as the biochemical cue of TGF- β in inducing Agn expression, and combining these cues led to a synergistic effect (Allen and Alliston, unpublished

observations). To further study whether TGF- β plays a role in the crosstalk between biological and mechanical factors, future studies may investigate the effect of elastic modulus on gene expression levels in cells with Smad3-deficiency, with and without the addition of TGF- β . Another interesting question to pursue: do changes in TGF- β signaling occur as a result of mechanically induced osteoarthritis? There are many reports of surgically-induced arthritis in mice, and studying them could provide information on how physical and cellular/molecular cues interact. Recent data in our lab using mice that express a synthetic Smad2/3 promoter-Luciferase reporter construct suggest this could be the case. We detect significant increases in the level of Smad3 transactivation in bones that experience mechanical load, relative to unloaded bones. While our studies implicate TGF- β signaling as a required participant in load-induced bone formation, load-sensitive TGF- β signaling may also participate in the progression of OA.

The prevention and treatment of OA is a major unmet medical need. Newer therapies transplant a patient's own chondrocytes to an early-stage cartilage defect, but these do not restore the unique biological and mechanical properties of cartilage. With the promise of stem cells and novel biomaterials, minimally invasive cell-based therapies may be on the horizon. An ideal treatment would regenerate cartilage with native biological and mechanical behavior. Similar to cartilage, tendon and ligament have limited repair capacities and injuries to these tissues are difficult to treat; successful regeneration could address these issues. Development of these novel therapies will require interdisciplinary research that considers both the biological and mechanical features of musculoskeletal tissue.

This dissertation adds to our understanding of the mechanisms through which the musculoskeletal tissue matrix is maintained. Identification of factors important for matrix integrity may lead to the discovery of new molecular therapeutic targets. For example, SD-208, a TGF- β receptor I inhibitor, was originally investigated for cancer therapy. However, we have found that it may also be useful in treating skeletal fragility. Additionally, understanding the development and maintenance of normal tissue matrix will aid in the creation of regenerative tissues by identifying

biochemical and mechanical factors that can be controlled to produce tissues and cells that behave like healthy bone, cartilage, and tendon.

Altogether, our results suggest a new paradigm in which matrix material properties are established by growth factors through their regulation of lineage-specific transcription factors. Therefore, TGF- β regulation of matrix material properties may not only function in bone, but also in cartilage, where TGF- β regulates the chondrocyte lineage-specific transcription factor Sox9 (Furumatsu, Tsuda et al. 2005), and in tendon, where TGF- β regulates the tenocyte-specific transcription factor Scx (9.2). It will be interesting to see whether other growth factors, such as FGF, BMP, and Wnt, all known to regulate many cellular functions in skeletal tissues, also function through this mechanism. Innovations in the treatment of musculoskeletal diseases can best be accomplished by increasing our understanding of the mechanisms through which molecular pathways specify the tissue matrix.

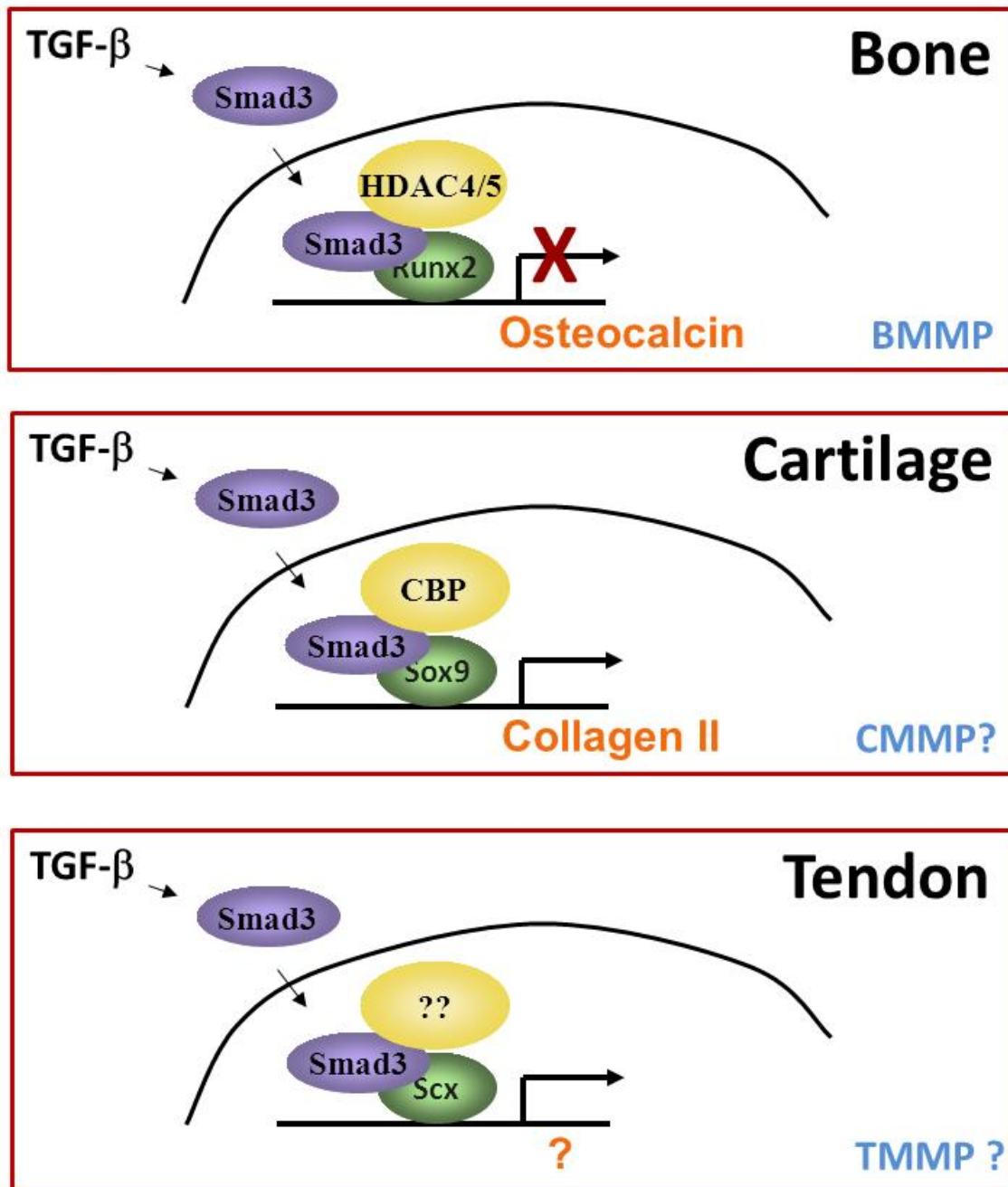


Figure 9.2. Our proposed mechanisms by which extracellular matrix material properties are regulated by TGF-β.

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Pharmacologic Inhibition of the TGF- β Type I Receptor Kinase Has Anabolic and Anti-Catabolic Effects on Bone

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Abstract

During development, growth factors and hormones cooperate to establish the unique sizes, shapes and material properties of individual bones. Among these, TGF- β has been shown to developmentally regulate bone mass and bone matrix properties. However, the mechanisms that control postnatal skeletal integrity in a dynamic biological and mechanical environment are distinct from those that regulate bone development. In addition, despite advances in understanding the roles of TGF- β signaling in osteoblasts and osteoclasts, the net effects of altered postnatal TGF- β signaling on bone remain unclear. To examine the role of TGF- β in the maintenance of the postnatal skeleton, we evaluated the effects of pharmacological inhibition of the TGF- β type I receptor (T β RI) kinase on bone mass, architecture and material properties. Inhibition of T β RI function increased bone mass and multiple aspects of bone quality, including trabecular bone architecture and macro-mechanical behavior of vertebral bone. T β RI inhibitors achieved these effects by increasing osteoblast differentiation and bone formation, while reducing osteoclast differentiation and bone resorption. Furthermore, they induced the expression of Runx2 and EphB4, which promote osteoblast differentiation, and ephrinB2, which antagonizes osteoclast differentiation. Through these anabolic and anti-catabolic effects, T β RI inhibitors coordinate changes in multiple bone parameters, including bone mass, architecture, matrix mineral concentration and material properties, that collectively increase bone fracture resistance. Therefore, T β RI inhibitors may be effective in treating conditions of skeletal fragility.

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Introduction

In skeletal development, each bone is formed with a distinctive size, geometry, architecture, and material properties. Among the many growth factors and hormones involved in this process [1–3], transforming growth factor- β (TGF- β) is sequestered at high levels in bone matrix and is a critical regulator of osteogenesis [4]. Bone mass is dramatically affected by developmental manipulation of TGF- β signaling in genetically modified mouse models [5–9]. In addition to bone mass, TGF- β regulates bone matrix material properties, which impact the ability of bone to resist fracture [10]. However, little is known about the role of TGF- β in the post-natal skeleton, which responds to changes in bone or the environment to retain or improve bone quality, fundamentally defined as the ability to resist bone fracture [11].

The effects of postnatal manipulation of TGF- β signaling on bone mass and quality are difficult to predict based on developmental studies. For example, osteoporosis and bone fragility are observed in mice with increased TGF- β production [6], as well as in those that are deficient in Smad3 [8,9], a key TGF- β effector. Conversely, other mouse models with reduced TGF- β signaling have increased bone mass and quality [7,10]. In addition, the roles of TGF- β on the proliferation, differentiation, and apoptosis of cells in both the osteoblast and osteoclast lineages have been extensively studied [4,12–14]. In spite of this wealth of information, the net effect of postnatal TGF- β signaling on bone remains unknown.

The recent development of specific inhibitors of the TGF- β type I receptor (T β RI) kinase that block most if not all TGF- β signaling events [15–17] now enables an investigation of this fundamental

question. ATP-competitive inhibitors of the TβRI kinase, such as SD-208, can effectively limit TGF-β-mediated lung fibrosis and tumorigenesis *in vivo* at doses that are too low to exert non-specific effects on other kinases [17–20]. Since such inhibitors are in clinical trials for cancer and other disorders, it is crucial to define the effects of TGF-β blockade on the skeleton.

Maintenance of the postnatal skeleton depends on the functional coordination between bone-depositing osteoblasts and bone-resorbing osteoclasts [21]. Both cell populations express and respond to TGF-β, and TGF-β has been suggested to couple osteoblast and osteoclast activity [4]. TGF-β promotes osteoprogenitor proliferation and inhibits terminal osteoblast differentiation, in part by repressing the function of osteogenic transcription factor Runx2 [22]. TGF-β also regulates osteoblast expression of osteoclast regulatory factors m-CSF, RANKL, and OPG [23–25], whereas resorbing osteoclasts release and activate matrix-bound latent TGF-β, which feeds back to modulate osteoblast and osteoclast function [26–28]. Because the effects of TGF-β on osteoblast and osteoclast function are dynamic, dose-dependent, and specific for each cell type and stage of differentiation [4,12–14], prior studies do not indicate how the cell types present in mature bone will respond to a systemic alteration in TGF-β signaling.

In the current study, we found that the TβRI kinase inhibitor, SD-208, affects osteoblast and osteoclast function to coordinately regulate several bone parameters, resulting in increased bone mass and trabecular bone volume, as well as increased mineral concentration and elastic modulus of bone matrix. This was associated with an increased resistance to vertebral fracture. These results suggest that pharmacologic inhibition of TGF-β signaling may have therapeutic utility in a variety of bone diseases characterized by poor bone quality, low bone mass and a propensity to fracture.

Results

Pharmacologic inhibition of the TβRI kinase increases bone mineral density

To determine the effects of pharmacologic inhibition of TGF-β signaling on bone, mice were treated for 6 weeks with either of two doses of SD-208, a small molecule that blocks ATP binding to the type I TGF-β receptor to specifically inhibit its kinase activity [17]. The 20 mg/kg SD-208 dose was chosen to achieve specific inhibition of the TβRI kinase, whereas the 60 mg/kg dose was chosen to achieve a maximal response with minimal inhibition of other pathways [19]. Using mice that express luciferase under the control of a TGF-β-responsive Smad binding element (SBE-Luc mice) [29], we confirmed the ability of SD-208 to inhibit endogenous and exogenously applied TGF-β function in bone *in vivo* and *ex vivo* (Fig. 1a, 1b). As expected, the well-established TGF-β-inducible expression of PAI-1 [30] was inhibited by SD-208 in calvarial explants, whereas the expression of reported targets of TGF-β repression, Runx2 and osteocalcin [22], was induced by SD-208 (Fig. 1c).

Longitudinal examination of the bone mineral density (BMD) by dual energy X-ray absorptiometry (DXA) showed the normal increase in BMD between 1 and 2.5 months of age. Accordingly, vehicle-treated male and female mice showed an increase of 21.8% and 29.6%, respectively, in whole body BMD after 6 weeks (Fig. 2a, 2b). Although low dose SD-208 did not affect whole body BMD, both male and female mice treated with high dose SD-208, showed significantly greater bone accrual over the same time period, with an additional 4.12% increase in male ($p<0.001$) and 5.2% increase in female ($p<0.001$) whole body BMD. The SD-

208-induced increase in whole body BMD was comparable to that observed following an 8-week treatment with bisphosphonates, which can increase whole body BMD by 5% [31].

More pronounced effects were apparent in the tibia and femur, where the BMD was already significantly increased within 3 weeks of SD-208 treatment relative to vehicle-treated controls (Fig. 2c–2f). After 6 weeks, SD-208 significantly increased the BMD in male mice by 20% in the tibia ($p<0.001$), 14.8% in the femur ($p<0.001$) and 8.9% in the lumbar spine ($p<0.01$) relative to vehicle-treated mice. SD-208 increased the tibial, femoral and lumbar spine BMD in female mice by 16.3% ($p<0.001$), 11.4% ($p<0.01$) and 17.9% ($p<0.001$), respectively. Dose-dependent increases in BMD were most apparent in the femur (Fig. 2e, 2f). Thus, systemic pharmacologic inhibition of TGF-β signaling increases the BMD.

Inhibition of the TβRI kinase increases trabecular bone

To determine if the increased BMD resulted from changes in cortical or trabecular bone, dissected femora and tibiae were analyzed using micro-computed tomography (micro-CT). Reconstructed images of trabecular bone in the distal femur showed a dose-dependent increase in trabecular bone volume following 6 weeks of SD-208 treatment in both male and female mice (Fig. 3a). This increase in trabecular bone was noted in the secondary spongiosa and did not extend to the diaphysis (Figure S1). At the high dose, SD-208 increased the femoral trabecular bone volume of male and female mice by 57.6% and 264%, respectively (Fig. 3b, Table 1). Remarkably, high-dose SD-208 increased the trabecular density of male and female femora by 192% and 581%, respectively (Fig. 3c). Increases in trabecular number and thickness were associated with a corresponding decrease in trabecular separation following treatment with SD-208 (Fig. 3d, 3e, Table 1). As shown by these and other parameters, SD-208 greatly improved trabecular bone microarchitecture in male and female femora and tibiae (Table 1). In contrast, SD-208 caused no significant differences in measured cortical bone parameters (Table 2). Therefore, the effect of 6 weeks of pharmacologic inhibition of TβRI function on BMD appears to be specific to the trabecular bone.

Inhibition of TβRI affects both osteoblasts and osteoclasts

Increased BMD may be due to increased osteoblast activity, reduced osteoclast activity or both. Quantitative histomorphometry confirmed the SD-208 dose-dependent increase in trabecular bone that was observed by micro-CT (Fig. 4a). The significantly increased bone volume (Fig. 4b) was accompanied by a TβRI inhibitor dose-dependent increase in osteoblast number (Fig. 4c). Importantly, even the most specific low dose of SD-208 (20 mg/kg) caused significant increases in male and female bone volume and osteoblast numbers ($p<0.05$). In addition, the osteoclast numbers were reduced in the femora of SD-208 treated mice (Fig. 4d). Bones from male mice treated with the highest dose of SD-208 had twice as many osteoblasts and half as many osteoclasts as the vehicle-treated controls (Fig. 4).

These data suggest that inhibition of TGF-β signaling increases bone mass by enhancing bone formation and inhibiting bone resorption. Dynamic histomorphometry revealed that SD-208 stimulates a dose-dependent increase in the mineral apposition rate and bone formation rate in male mice (Fig. 4e, 4f). Female mice showed the same trend. Collectively, these analyses demonstrate that TβRI inhibitors increase bone mass in mature mice via anabolic and anti-catabolic mechanisms.

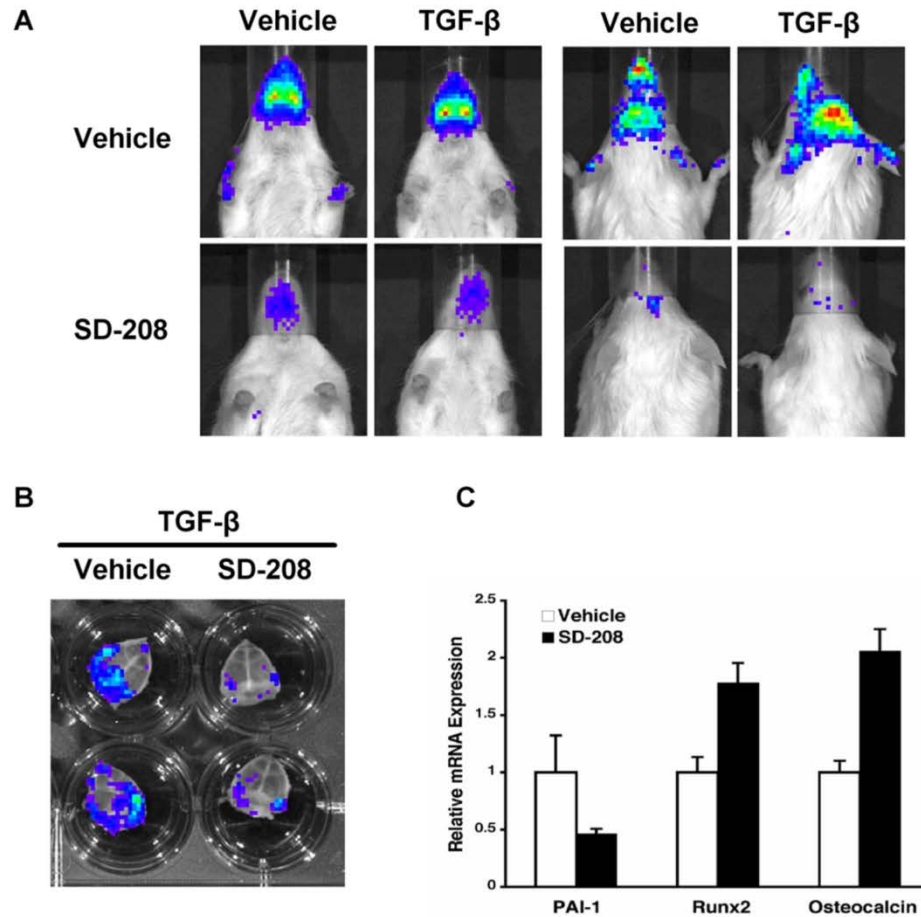


Figure 1. SD-208 inhibition of TGF- β function in vivo. Five hours after TGF- β administration, SBE-Luc mice showed increased bioluminescence on the dorsal and ventral surfaces of the head where relatively little superficial tissue covers skeletal elements (calvarial bone and jaws) (a). Mice pretreated with SD-208 showed less basal and TGF- β -inducible luminescence than vehicle-treated controls (a, lower panels). SD-208 also inhibited reporter activity in SBE-Luc mouse calvarial explants cultured overnight with TGF- β (b). SD-208 treatment of calvarial explants inhibits expression of the TGF- β -inducible gene, PAI-1 [30], but induces expression of Runx2 and osteocalcin, osteoblast marker genes that are targets of TGF- β repression [22].
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T β RI inhibition promotes osteoblast differentiation and inhibits osteoclast differentiation

To determine if the changes in osteoblast and osteoclast numbers and activity resulted from changes in cell differentiation, bone marrow stromal cells that were isolated from vehicle- and SD-208-treated mice were examined ex vivo in osteoblast or osteoclast differentiation assays (Fig. 5a–5c). In vivo exposure to SD-208 enhanced the osteoblast differentiation (CFU-Ob, Fig. 5a) with no detectable effect on osteoprogenitor recruitment (CFU-F,

Fig. 5b). Conversely, marrow stromal cells from mice treated with SD-208 formed fewer multinucleated cells that express the functional osteoclast marker TRAP (Fig. 5c). Thus, in vivo inhibition of T β RI with SD-208 promotes osteoblast differentiation and inhibits osteoclast differentiation.

To investigate the effect of T β RI inhibitors on the expression of osteoblast and osteoclast regulatory factors, we utilized primary calvarial osteoblasts, which retain the capacity to differentiate into mineralizing osteoblasts, and have an intact autocrine TGF- β

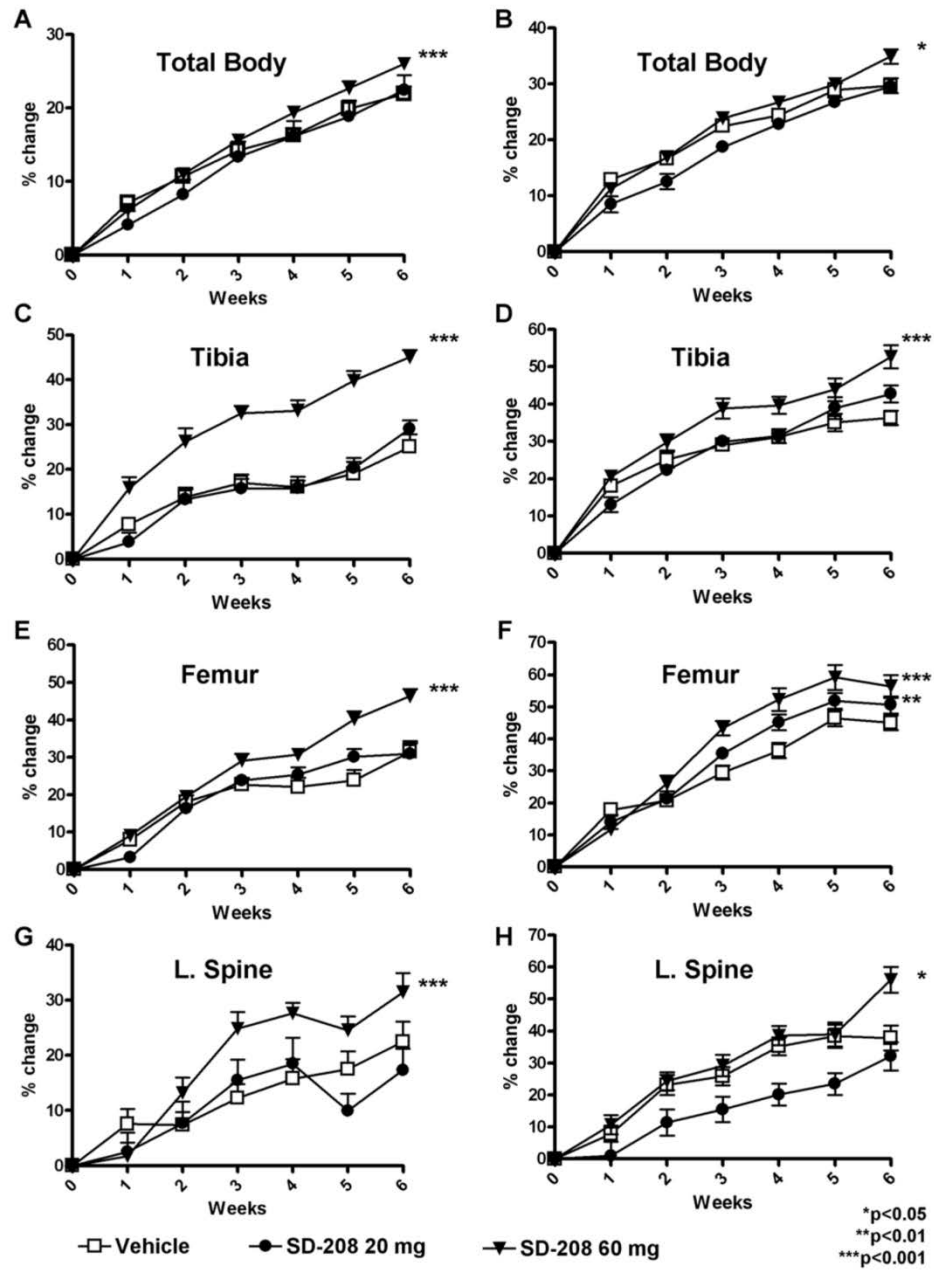


Figure 2. Pharmacologic TβRI inhibition increases BMD. DXA was used to measure BMD longitudinally for male (a, c, e, g) and female mice (b, d, f, h) treated with or without the TβRI inhibitor SD-208 at 20 mg/kg or 60 mg/kg. SD-208 treatment at the 60 mg/kg dose caused an increase in total body (a, b) tibia (c, d), femur (e, f), and lumbar spine (g, h) BMD. SD-208 at the 20 mg/kg dose increased femoral BMD in female mice (f). Data represent mean ± SEM ($p < 0.05$, as determined by two-way analysis of variance (ANOVA)).
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regulatory pathway. As in calvarial explants treated with SD-208 (Fig. 1c), another ATP-competitive TβRI kinase inhibitor, SB431542, inhibits the expression of the TGF-β-inducible gene, PAI-1 [30], in primary calvarial osteoblasts (Fig. 5d). As shown previously [22], Runx2 expression was reduced after 48 h of treatment with added TGF-β (Fig. 5d). In contrast, TβRI inhibitors induce Runx2 expression, consistent with the increased osteoblast numbers, bone formation rate, and osteoblast differentiation potential observed in SD-208-treated mice (Fig. 4c, 4f, 5a, 5d).

RANK ligand (RANKL) promotes osteoclast differentiation, function and survival [21]. After 48 h of treatment, TβRI inhibitors reduced the expression of RANKL mRNA by approximately 50% compared with the mRNA levels observed in vehicle-treated primary calvarial osteoblasts (Fig. 5e). The reduced expression of this osteoclastogenic factor is consistent with the decreased osteoclast numbers and differentiation capacity observed in SD-208-treated mice (Figs. 4d and 5c). However, RANKL function is antagonized by osteoprotegerin, the expression of which is also reduced by TβRI-I treatment. Similar results were observed after 2 h of TβRI-I treatment (data not shown). Though the inhibition of TGF-β signaling impacts both of these critical regulators of osteoclast differentiation and function, the relative RANKL/OPG ratio is unchanged. Therefore, the effect of inhibition TβRI function on other factors which regulate osteoblast and osteoclast function was investigated.

Recently, ephrin B2 and EphB4, a transmembrane ligand and receptor respectively, have been implicated as factors that couple osteoblast and osteoclast activities in bone metabolism [32]. Bidirectional signaling between ephrin B2, expressed by osteoblasts and osteoclasts, and EphB4 on osteoblasts increases osteoblast differentiation and inhibits osteoclast differentiation [32]. However, the ability of TGF-β to control ephrin signaling in bone metabolism has not been reported. Inhibition of TβRI function significantly increased the expression of both ephrin B2, the ephrin that inhibits osteoclast differentiation (Fig. 5f), and EphB4, the Eph receptor that induces osteoblast differentiation. TGF-β signaling crosstalk with the ephrin pathway may contribute to the anabolic and anti-catabolic effects of SD-208 on bone, though additional experiments are needed to establish a functional link. By affecting osteoblast and osteoclast differentiation, numbers and activity (Figs. 4c–f, 5a, 5c), the TβRI inhibitor dramatically shifts bone toward a state of metabolic anabolism.

TβRI inhibitors increase bone matrix mineral concentration, material properties and fracture resistance

The net effect of TβRI inhibitors on bone is increased BMD, which reflects both bone mass and mineral concentration (Fig. 2). With the monochromatic light from synchrotron radiation, X-ray tomographic microscopy (XTM) permits direct quantification of the mineral concentration of bone matrix with an 8 μm resolution [33]. Analyses of femoral bone showed that SD-208 treatment resulted in a higher degree of mineralization of bone matrix (Fig. 6a). The SD-208-dependent increase in mineral concentration was evident in both the diaphysis and epiphysis (data not shown), suggesting that the mineralization of both cortical and trabecular bone were affected.

Mineral concentration is a major determinant of bone matrix material properties [34]. Using nanoindentation, material properties such as the elastic modulus and hardness of bone matrix can be determined independently of changes in bone mass or structure [35,36]. We have previously used this approach to demonstrate that TGF-β signaling in osteoblasts regulates the elastic modulus and hardness of bone matrix in genetically-modified mice [10]. Treatment of mice with SD-208 increased the elastic modulus of cortical bone relative to vehicle-treated controls. Although the measured modulus values in each group overlapped, more than half of the measurements in vehicle-treated bone were below 28 GPa, whereas less than a quarter of the values measured in SD-208-treated mice were in the same range (Fig. 6b).

Treatment with the TβRI inhibitor SD-208 affects bone on several levels, including bone mass (Figs. 2–4), bone mineral concentration and bone matrix material properties (Figs. 6a, 6b). These observations led us to perform macro-mechanical testing to evaluate the ability of SD-208-treated bones to resist fracture. Compression testing of vertebrae showed that inhibition of the TβRI kinase increased the load-to-failure relative to vehicle-treated controls (Fig. 6c, Table 3). When the femora were tested using notched or unnotched three-point bending, SD-208-dependent differences in peak load, stiffness, or fracture toughness were not observed (Table 4). The increased load-to-failure of SD-208-treated vertebral bone, but not femoral bone, is entirely consistent with the increase in trabecular but not cortical bone volume after 6 weeks of SD-208 treatment. Together these data demonstrate that TGF-β inhibitors drive functionally significant and coordinated increases in trabecular bone mass, mineral concentration and bone matrix material properties.

Discussion

Here we explored the role of TGF-β signaling in postnatal bone by systemic administration of a TβRI inhibitor to mature mice. Pharmacologic inhibition of TGF-β signaling resulted in dose-dependent increases in BMD, trabecular microarchitecture, bone matrix elastic modulus and mineral concentration. These coordinated changes in bone mass and parameters of bone quality improved the ability of vertebral bone to resist fracture. By targeting key regulatory pathways in osteoblasts and osteoclasts, TβRI inhibitors increased the number of osteoblasts and the bone formation rate, while reducing osteoclast numbers. Therefore, TβRI inhibition elicits both anabolic and anti-catabolic activities to improve bone quality.

The TβRI inhibitor-dependent increase in tibial BMD exceeded the physiologic increase in BMD over this time period or those induced by comparable regimens utilizing clinically available bisphosphonates or PTH [31,37]. TβRI inhibitors may have more profound effects since they both stimulate bone formation and inhibit bone resorption, rather than the uncoupled effects of PTH to stimulate osteoblast activity or bisphosphonates to inhibit osteoclast activity. The effects of the TβRI inhibitor on adult bone are consistent with the developmental bone phenotypes of mice with partial inhibition of TGF-β/Smad signaling, as observed in Smad3+/- mice or DNTβRII mice that express a dominant negative TGF-β type II receptor in osteoblasts [7,10]. In contrast, more complete inhibition of TGF-β signaling in

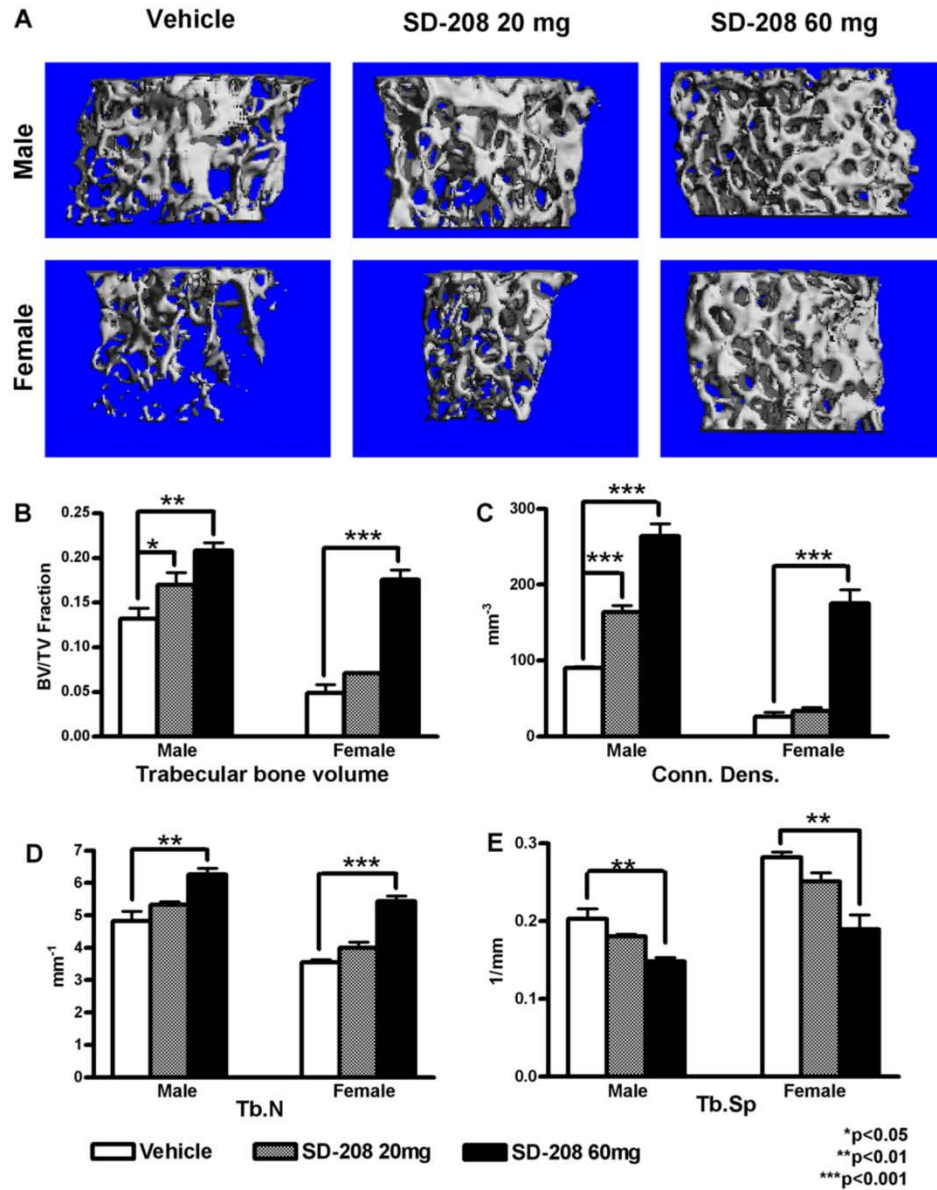


Figure 3. Pharmacologic T β RI inhibition increases trabecular bone volume. Micro-CT images show increased femoral trabecular bone volume following SD-208 treatment in male and female mice, relative to vehicle-treated controls (a). Quantitative analyses show that SD-208 increased trabecular bone volume (BV/TV, fraction) (b), connectivity density (c), and trabecular number (d), but decreased trabecular spacing (e) in male and female femora. Data represent mean $\pm$ SEM ($p < 0.05$, as determined by one-way ANOVA Newman-Keuls multiple comparison test). doi:10.1371/journal.pone.0005275.g003

Table 1. Trabecular bone structural parameters are affected by TβRI inhibition.

	Male				Female			
	Tibia		Femur		Tibia		Femur	
	Vehicle	20 mg SD-208	60 mg SD-208	Vehicle	Vehicle	20 mg SD-208	60 mg SD-208	Vehicle
TVB	0.11±0.006	0.182±0.018**	0.20±0.009**	0.132±0.017	0.09±0.009	0.113±0.007	0.161±0.014**	0.048±0.009
DT.Tb.Th	0.048±0.0006	0.049±0.002	0.047±0.0004	0.049±0.0012	0.045±0.0003	0.048±0.0009	0.046±0.0005	0.045±0.001*
DT.Tb.N	4.44±0.19	5.53±0.10**	6.19±0.15**	4.82±0.29	3.34±0.15	3.69±0.15	5.22±0.39**	3.54±0.08
DT.Tb.Sp	0.20±0.01	0.16±0.004	0.15±0.003*	0.20±0.01	0.14±0.004**	0.26±0.012	0.11±0.016**	0.28±0.006
Conn.Dens.	60.52±4.13	129.6±12.06**	197.3±15.16**	90.32±1.61	47.13±5.21	50.55±6.01	138.2±25.01**	25.7±6.13
TRI SMI	2.36±0.06	1.98±0.13	1.97±0.082	2.39±0.11	2.33±0.07	2.15±0.12	2.34±0.044	3.21±0.048
TRI DA	2.13±0.06	2.11±0.07	1.92±0.06	1.33±0.009	2.34±0.066	2.6±0.023**	2.01±0.089**	1.4±0.106

Micro-computed tomography was used to assess several quantitative parameters of trabecular bone structure. The mean values and standard deviations are presented here. Significant differences between vehicle and SD-208 treated groups are indicated (*p<0.05, **p<0.01, ***p<0.001). doi:10.1371/journal.pone.0005275.t001

Smad3^{-/-} and TGF-β1^{-/-} mice is associated with low bone mass and poor bone quality, which may result, in part, from the significant systemic effects of Smad3 and TGF-β1 deletion [5,8,9].

Some effects of TβRI inhibition on bone resulted from the reduction in osteoclast numbers and differentiation potential in SD-208-treated mice (Figs. 4d, 5c). This in vivo response is striking because TGF-β has been shown to inhibit and promote osteoclast differentiation in vitro, depending on the timing, dose and experimental cell population [12]. TGF-β can act by binding directly to its receptors on osteoclasts and their progenitors, or by acting on osteoblasts to regulate the expression of osteoclast regulatory factors, such as RANKL and OPG [6,7,23–25,38–40]. Though the current study does not explore the extent to which SD-208 affects osteoclasts directly or indirectly through osteoblast-dependent mechanisms, SD-208 can directly inhibit osteoclast function in a purified osteoclast precursor population (Guise, personal communication). In addition, TβRI inhibitors regulated osteoblast expression of osteoclast regulatory factors such as RANKL, OPG, ephrin B2 and EphB4 (Fig. 5). Likely, a combination of direct and indirect mechanisms is responsible for the anti-catabolic and anabolic effects of TβRI inhibitors in vivo.

Treatment of mice with TβRI inhibitors resulted in increased osteoblast numbers and differentiation, and increased bone formation. Consistent with these data, reduced TGF-β signaling in Smad3^{+/+} mice or DNTβRII mice also relieves the suppression of osteoblast differentiation by TGF-β, which is exerted by Smad3 and histone deacetylases [22,41], thereby contributing to increased BMD [7,10]. The increased osteogenic differentiation in response to TβRI inhibitors may also reflect a decrease in repression of BMP signaling by the inhibitory Smad6 [42]. Although changes in Smad6 expression were not observed in our experimental conditions, the BMP antagonist, Noggin, reversed some effects of TβRI inhibitors on gene expression (data not shown), affirming the previous observation that increased BMP signaling contributes to the osteogenic activity of TβRI inhibitors [42]. Therefore, despite the ability of TGF-β to promote or inhibit specific stages of osteoblast differentiation [13], the net effect of TβRI inhibitors on osteoblasts in vivo is to increase bone formation.

Ultimately, the ability of bone to resist fracture is the most clinically desirable outcome [11]. TβRI inhibition increased the peak load that vertebral bone can sustain prior to fracture, in part due to the potent anabolic effect of TβRI inhibitors on trabecular bone. Although a 6-week treatment with TβRI inhibitors was insufficient to increase cortical bone mass or geometry, it significantly increased the mineralization and material properties of cortical bone matrix, when measured using high-resolution XTM and nanoindentation. These data suggest that optimization of the dose or duration of therapy may result in detectable changes in cortical bone mass and macromechanical behavior. Furthermore, our data indicate that TGF-β signaling helps define bone matrix material properties postnatally as it does in development [10], although the effect was more modest than that observed in genetically modified mice. Although the elastic modulus of bone matrix often correlates with mineral content [1], Smad3 also regulates material properties independently of mineralization, as has recently been shown in skin [43]. The mechanisms by which TGF-β regulates the material properties of extracellular matrices remain unknown.

In conclusion, pharmacologic inhibition of TGF-β signaling in postnatal bone increases bone quality. Coupling of osteoblast and osteoclast activity may be critical for the ability of TGF-β to coordinately control bone mass, architecture, and the material properties of bone. Therefore, therapies that produce a reliable

Table 2. Cortical bone structural parameters are not affected by TβRI inhibition.

	Male			Female		
	Vehicle	SD-208 20 mg	SD-208 60 mg	Vehicle	SD-208 20 mg	SD-208 60 mg
Cort. CSA	0.183±0.019	0.182±0.016	0.172±0.007	0.155±0.007	0.156±0.006	0.152±0.003
Cort. Th.	0.201±0.005	0.178±0.006	0.186±0.005	0.186±0.011	0.195±0.005	0.188±0.004
Total CSA	0.329±0.038	0.346±0.035	0.405±0.025	0.281±0.017	0.285±0.015	0.281±0.007
Perios. Perim.	1.057±0.098	1.202±0.081	0.986±0.057	0.964±0.047	0.977±0.045	0.958±0.017
Diam. Mid Shaft	0.592±0.017	0.546±0.017	0.577±0.004	0.554±0.012	0.555±0.005	0.560±0.010
Med. Area	0.182±0.040	0.218±0.098	0.214±0.019	0.105±0.009	0.109±0.008	0.110±0.005
Endosteal Perim	0.761±0.078	0.891±0.233	0.729±0.055	0.571±0.049	0.596±0.038	0.614±0.020
Mid Diam	0.447±0.054	0.427±0.077	0.551±0.013	0.352±0.008	0.351±0.003	0.343±0.011

Micro-computed tomography was used to assess several quantitative parameters of cortical bone structure. The mean values and standard deviations are presented here.

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reduction in TGF-β signaling may have significant clinical benefit in the treatment of diseases characterized by low bone mass and bone fragility. However, TβRI-inhibition may be counter-indicated for the treatment of existing bone fractures, where TGF-β plays a role in fracture repair. Additional studies evaluating the efficacy and potential sex-specificity of the mature skeletal response to TβRI inhibitors, particularly in ovariectomized animals, would be needed to determine their potential therapeutic value for post-menopausal osteoporosis. Careful consideration of safety is essential, given the critical role of TGF-β in normal physiological processes including the control of cell proliferation, differentiation, and apoptosis in many tissues.

Materials and Methods

Ethics Statement

In all studies, mice were handled and euthanized in accordance with approved institutional, national and international guidelines.

TβRI inhibitor treatment

Four-week old male and female C57BL/6 mice were treated for 6 weeks with vehicle (1% methylcellulose) or SD-208 (20 mg/kg once daily or 60 mg/kg twice daily) by gavage. As described, SD-208 is a specific inhibitor of the TGF-β type I receptor, developed by Scios, Inc. [17]. Based on the mouse monitoring parameters of our treatment protocol, no adverse effects of SD-208 on mouse health were detected during the study. At 10 and 3 days prior to euthanasia, an intraperitoneal injection of calcein (Sigma C-0875, 0.02 mg/g) was administered to all mice. Forelimbs, hindlimbs, and spines were collected. For studies using SBE-luciferase mice [29], mice were treated with vehicle or 60 mg/kg SD-208 as above for 3 days, prior to an intraperitoneal injection of TGF-β1 (10 μg/kg). Five hours later, mice were administered luciferin (150 mg/kg) intraperitoneally, anaesthetized with isoflourane, and imaged 10 minutes later using a bioluminescence imaging system (Xenogen).

Bone mineral density (BMD) measurement

BMD was measured using a PIXImus mouse densitometer (GE Lunar II, Faxitron Corp., Wheeling, IL) (N = 15/group). Total body measurement was performed excluding the calvarium, mandible and teeth. Regions of interest were defined as the distal

femur and proximal tibia just beneath the growth plate (12×12 pixels) and the lower lumbar spine (20×50 pixels). Values were expressed as percentage change in BMD over the pretreatment scan.

Histomorphometry

For demineralized bone histomorphometry, tissues were fixed for 48 h in 10% formalin, demineralized in 10% EDTA for 2 weeks, and embedded in paraffin to generate 3.5 μm longitudinal sections. Trabecular bone volume of the secondary spongiosa (BV/TV%) and osteoblast number (N.Ob/high power field) were measured on hematoxylin and eosin stained sections of the distal femur, proximal tibia, and lumbar vertebrae (N≥12 mice/group). Tartrate resistant acid phosphatase (TRAP) stained sections were used to quantify osteoclast number (N.Oc/BS/mm). Dynamic bone histomorphometry was performed on 7 μm thick sections of mineralized lumbar vertebrae embedded in methylmethacrylate using standard procedures. The mineral apposition rate (MAR, μm/day) and bone formation rate (BFR/BS, μm³/μm²/day) were measured on vertebral trabecular bone using fluorescence microscopy to visualize calcein labels as described [44].

Micro-computed tomography (micro-CT)

Formalin fixed tibiae and femora were imaged with micro-CT using a microCT-40 (Scanco Medical AG, Bassersdorf, Switzerland) using a voxel size of 12 μm in all dimensions (N≥12 mice/group). The region of interest comprised 240 transverse CT slices representing the entire medullary volume with a border lying approximately 100 μm from the cortex [45]. Morphometric variables were computed using direct, three-dimensional techniques that do not rely on assumptions about the underlying structure. Fractional bone volume (BV/TV, Fraction) and architectural properties of trabecular reconstructions, apparent trabecular thickness (Tb.Th., μm), trabecular number (Tb.N., mm⁻¹), trabecular spacing (Tb.Sp., 1/mm), and connectivity density (Conn.D., mm⁻³) were calculated as described [46].

Cortical bone assessment by micro-CT

The CT images of the mid-diaphysis of the tibia were segmented into bone and marrow regions by applying a visually chosen, fixed threshold for all samples, after smoothing the image with a three-dimensional Gaussian low-pass filter. The outer

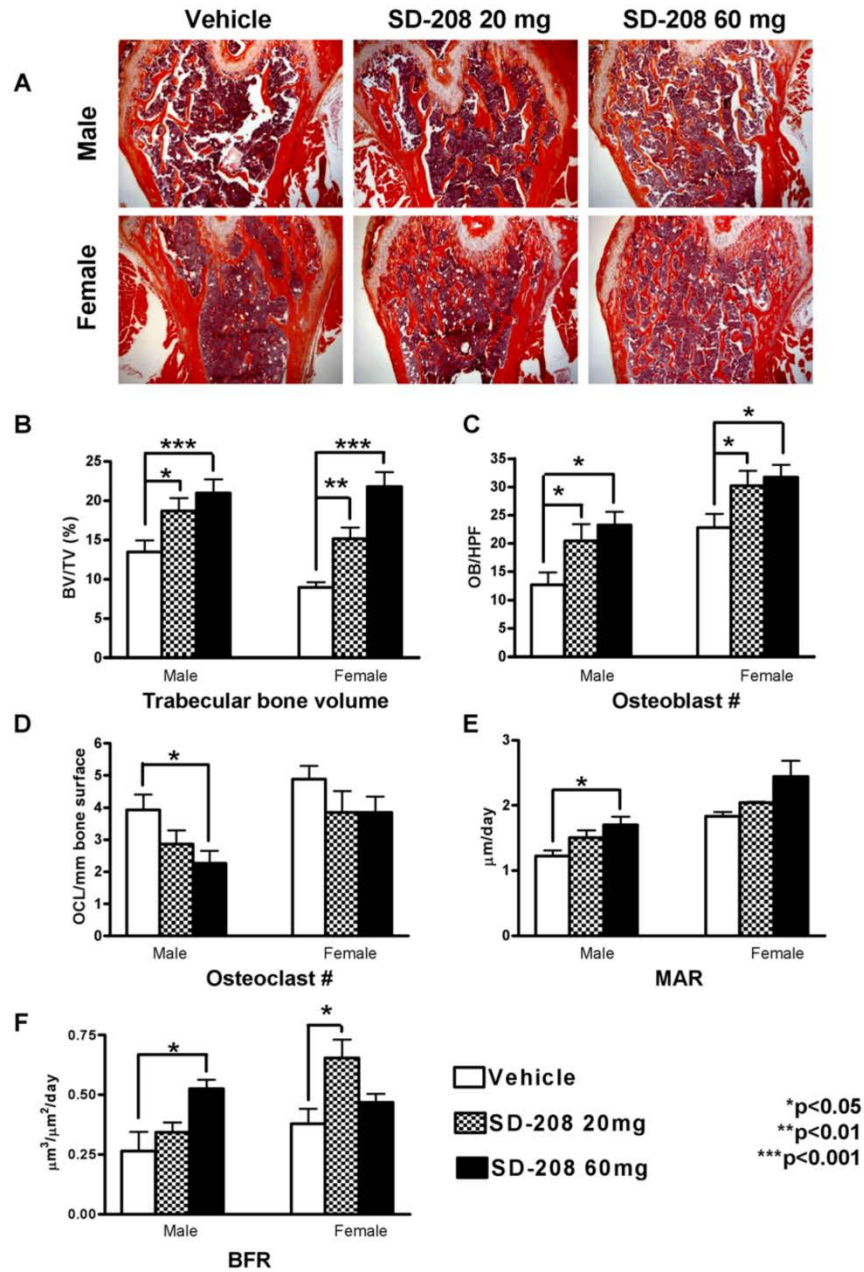


Figure 4. Pharmacologic T β RI inhibition increases osteoblast numbers but reduces osteoclast numbers. Representative H&E stained sections of femoral bone show the SD-208-dependent increase in trabecular bone in male and female mice (a). Histomorphometry shows that SD-208 increases trabecular bone volume in the femur (b) and tibia (data not shown), as well as osteoblast number (c) in a dose-dependent manner for male and female mice. Osteoclast numbers are reduced by SD-208 (60 mg/kg) in male mice (d). Dynamic histomorphometry of male mouse lumbar vertebrae shows that SD-208 treatment (60 mg/kg) increased mineral apposition rate (MAR) (e) and bone formation rate (BFR) (f). Data represent mean $\pm$ SEM (* p <0.05, ** p <0.01, *** p <0.001, as determined by one-way ANOVA Newman-Keuls multiple comparison test). doi:10.1371/journal.pone.0005275.g004

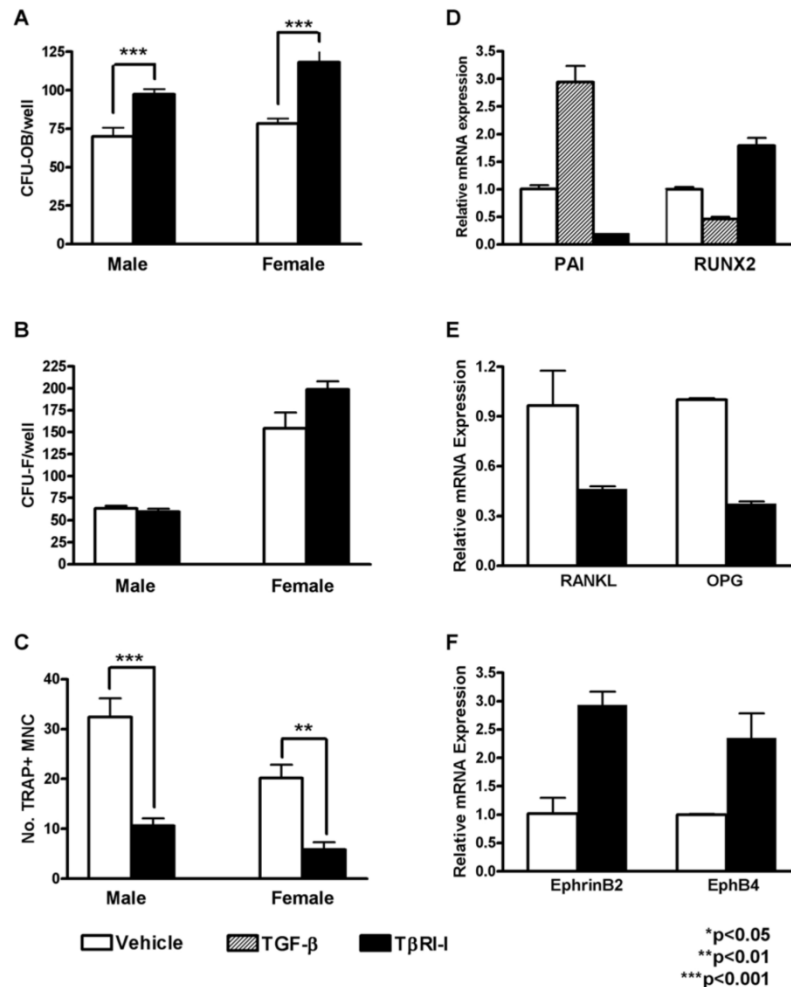


Figure 5. T β RI inhibition promotes osteoblast differentiation and bone deposition but inhibits osteoclast differentiation. Bone marrow isolated from male and female mice treated with SD-208 (60 mg/kg) has increased numbers of osteoblast colony forming units (CFU-OB) (a) with no change in the number of colony forming units (CFU-F) (b). The number of TRAP-positive multinucleated cells (TRAP+ MNC) is lower in cultures from SD-208 treated mice than from vehicle-treated controls (c). Primary calvarial osteoblasts treated with T β RI-inhibitor SB431542 (10 μ M) or vehicle for 48 h show altered mRNA expression of PAI-1 (d) and several osteoblast and osteoclast regulatory factors including Runx2 (d), RANKL and OPG (e), and ephrinB2 and EphB4 (f). Data represent mean $\pm$ SEM (* p <0.05, ** p <0.01, *** p <0.001, as determined by unpaired t-test). doi:10.1371/journal.pone.0005275.g005

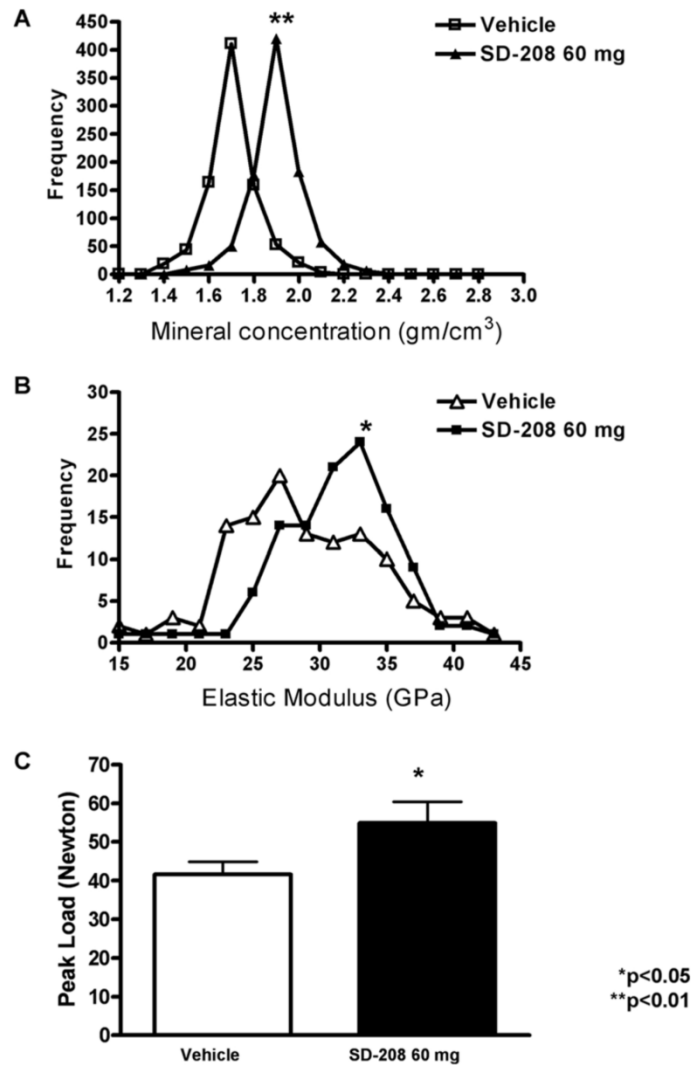


Figure 6. T β RI inhibitors increase bone mechanical and material properties. Analysis of each pixel from XTM scans of femora show that SD-208 (60 mg/kg) increases bone matrix mineral concentration with a mean of $1.90 \text{ g/cm}^3 \pm 0.066$, relative to a mean mineral concentration of $1.54 \text{ g/cm}^3 \pm 0.069$ for vehicle-treated controls ($p < 0.05$, as determined by unpaired t-test) (a). Analysis of elastic modulus values from nanoindenters applied to tibial cortical bone showed a similar shift ($p < 0.05$) (b). Unconfined compression testing of vertebrae from male mice treated with vehicle or SD-208 (60 mg/kg) shows an increased peak load-to-failure following T β RI inhibition ($p < 0.05$) (c). doi:10.1371/journal.pone.0005275.g006

contour of the bone was found automatically with the built-in Scanco iterative contouring tool. Total area (TA) was calculated by counting all voxels within the contoured bone area, (BA) by counting all voxels that were segmented as bone, and marrow area

(MA) was calculated as TA-BA. This calculation was performed on all 30 slices (1 slice = $12.5 \mu\text{m}$), using the average for the final calculation. The outer and inner perimeter of the cortical midshaft was determined by a three-dimensional triangulation of the bone

Table 3. Macromechanical testing of vertebrae.

	Male Vertebrae	
	Vehicle	SD-208 60 mg
Peak Load	41.55±3.34	54.95±5.45*
Stiffness	98.61±11.52	111.1±18.70

Mean values±SEM for macromechanical tests of vertebral peak load and stiffness are shown. The significance of differences between vehicle and SD-208 (60 mg/kg) treated groups is indicated with p values (*p<0.05).
doi:10.1371/journal.pone.0005275.t003

Table 4. Macromechanical testing of femora.

	Male Femora	
	Vehicle	SD-208 60 mg
Peak Load	16.09±0.90	15.55±0.52
Stiffness	40.32±5.59	47.10±1.40
Fracture Toughness	4.367±0.783	4.755±0.509

Mean values±SEM for macromechanical tests of femoral peak load, stiffness and fracture toughness are shown.
doi:10.1371/journal.pone.0005275.t004

surface (BS) of the 30 slices, and cortical parameters were calculated as described [47].

Marrow stromal cell differentiation assays

Bone marrow stromal cells were flushed from 6 femora and tibiae per treatment group, collected by centrifugation (1500 rpm, 10 minutes), resuspended (αMEM, 10% FCS), and incubated for 2 h at 37°C. For osteoblast assays, cells were cultured in αMEM, 15% FBS, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate. The number of alkaline phosphatase-positive osteoblast progenitor forming colonies (CFU-F) and Alizarin Red-positive osteoblast forming colonies (CFU-OB) was quantified microscopically after 9 or 28 days of culture, respectively, as described [48,49]. For osteoclast progenitor assays, non-adherent cells were cultured for 6 days in 10% αMEM, 1% FBS and 10⁻⁸ M 1α,25(OH)₂ vitamin D₃. Cultures were fixed and stained for microscopic quantification of multinucleated (MNC) TRAP+ cells.

Tissue culture, RNA isolation, and quantitative reverse transcription PCR

Calvarial explants were isolated from 10 day old SBE-Luc mice and cultured overnight in DMEM supplemented with 10% fetal bovine serum and 5 ng/ml TGF-β1 in the presence of either 150 nM SD-208 or an equivalent volume of vehicle (1% methylcellulose). Following culture, explants were moved to media containing luciferin (150 mg/ml) for immediate visualization of luciferase reporter activity with a bioluminescent imaging system (Xenogen). Explants were then crushed in liquid nitrogen using a mortar and pestle prior to additional tissue disruption in Trizol with a Omni-GLH homogenizer (Omni Scientific). Following Trizol extraction, RNA was further purified using RNeasy columns (Qiagen).

Primary calvarial osteoblasts were isolated from 3 to 5-day old mice and cultured in osteogenic conditions as described [22]. Cells were treated with a commercially available TGF-β receptor type I

inhibitory compound suspended in DMSO (SB431542, Sigma) for 48 h. All other cells received an equivalent quantity of DMSO in the presence or absence of TGF-β (5 ng/ml). Total RNA was purified using RNeasy columns (Qiagen) and reverse transcribed for the analysis of gene expression. Transcripts were amplified using primers sets for PAI-1 5'-AACCAATTACTGAAAA-CTGCACAA-3' (forward) and 5'-TCCGGTGGAGACATAA-CAGATG-3' (reverse), Runx2 5'-CCCAGCCACCTTTACC-TACA-3' (forward) and 5'-CAGCGTCAACACCATCATT-3' (reverse), OPG 5'-AGAGCAACCTTCCAGCTGC-3' (forward) and 5'-CTGCTCTGTGGTGAAGTTCCG-3' (reverse), RANKL 5'-CACCATCAGCTGAAGATAGT-3' (forward) and 5'-CCAA-GATCTCTAACATGACG-3' (reverse), EphrinB2 5'-TCGAA-CTCCAAATTTCTACCC-3' (forward) and 5'-TGCTTGGTCT-TTATCAACCA-3' (reverse), EphB4 5'-CAAAGTATGCAGA-GCCTGTG-3' (forward) and 5'-CCGGTAATACCCAAATTC-GAC-3' (reverse). Results were detected based on amplicon binding of Sybr Green using quantitative RT-PCR and are representative of at least three independent experiments.

X-ray tomography (XTM)

XTM studies were used to assess the degree of mineralization of the bone; procedures were based on the work of Kinney *et al.* [33]. Whole male mouse femora were scanned to determine the degree of bone mineralization (N = 3/group). Imaging was performed at the Advanced Light Source (ALS) on Beamline (8-3-2) at the Lawrence Berkeley National Laboratory by obtaining two-dimensional radiographs as the specimens were rotated through 180° in 0.5° increments. The radiographs were reconstructed into 2,500 slices by Fourier-filtered back projection with a 4.5 μm resolution. The attenuation coefficient (mm⁻¹) of each pixel relates directly to bone mineral concentration. The degree of bone mineralization (DBM) was obtained from Eq. (1):

$$DBM = \frac{\mu_i - \mu_o}{\mu_m - \mu_o} * C \quad (1)$$

where μ_i is measured attenuation coefficient at pixel i , μ_o is the attenuation coefficient of organic, μ_m is attenuation coefficient of mineral, and C represents the density of hydroxyapatite.

Nanoindentation

Dissected male mouse tibiae were embedded in a two-component epoxy resin (Stycast 1266) prior to sectioning with a precision low-speed saw to generate mid-tibial cortical bone surfaces for nanoindentation. A nanoindenter (Triboindenter, Hysitron, Minneapolis, MN) with a Berkovich tip was used to evaluate polished samples (0.25 μm) under dry conditions as described [10]. Indents were applied using a trapezoidal loading profile with a loading rate of 200 μN/second, peak load of 600 μN, and a hold period of 10 seconds. From the resulting load-deformation curves, local elastic modulus and hardness were calculated as described [50]. Three sets of 20 nanoindentation points were performed in a line with a 5 μm separation. Statistical analyses show the mean and standard error of the median elastic modulus values for each of 3 individual animals per group.

Macroscopic mechanical testing

Whole bone strength and load to failure were determined by mechanical testing of vertebrae and intact tibiae for at least 12 mice per treatment group as previously described [45]. Thawed bones were hydrated in saline for 1 h before testing at room temperature using a MTS 858 Bionex Test Systems load frame (MTS Systems Corp, Eden Prairie, MN). Vertebral bodies (L4)

were prepared with flat and parallel cranial and caudal ends by removing the soft cartilage to expose the bone, prior to compression testing at a rate of 3 mm/minute. Tibiae were tested in a three-point bending configuration with their anterior side down on two horizontal supports spaced 7 mm apart; the central loading point was displaced downward at 0.1 mm/second on the posterior surface of the diaphysis at the midpoint of the bone length. For all tests, load-displacement data were recorded at 100 Hz (TestWorks 4.0, MTS). Curves were analyzed to determine measures of whole-bone strength, primarily peak load and stiffness [47]. Load-to-failure was recorded as the load after a 2% drop from peak load.

Fracture toughness testing was performed on at least 10 isolated femora per condition. Thawed samples were notched using a razor blade followed by a micronotching technique. Notches were evaluated to ensure that they were through-wall but notched less than 1/3 of the bone diameter. Samples were tested in 37°C HBSS in a three-point bending configuration with a custom-made rig for the ELF 3200 mechanical testing machine (ELF3200, Bose, EnduraTEC, Minnetonka, MN), in general accordance with ASTM Standard E-399 and E-1820 [51,52] and as previously described [53]. Scanning electron microscopy was used to image fracture surfaces to measure the crack area and point of failure. The fracture toughness, K_{IC} , was calculated using a stress-intensity solution for circumferential through-wall flaw in cylinders [43,54]. Macro-mechanical testing was performed on male and female femora, with no SD-208-dependent differences observed in either group.

Supporting Information

Figure S1 The diaphysis is not filled by trabecular bone following SD-208 treatment. Although increased trabecular bone

in femora from SD-208-treated mice (60 mg/kg) is evident in reconstructed micro-CT images, the trabecular bone does not extend past the distal third of the femur. The scale bar is 1 mm. The diaphysis is not filled by trabecular bone following SD-208 treatment. Although increased trabecular bone in femora from SD-208-treated mice (60 mg/kg) is evident in reconstructed micro-CT images, the trabecular bone does not extend past the distal third of the femur. The scale bar is 1 mm.

Found at: doi:10.1371/journal.pone.0005275.s001 (1.00 MB TIF)

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Author Contributions

Conceived and designed the experiments: KM RD. Performed the experiments: KM SIM. Analyzed the data: KM. Contributed reagents/materials/analysis tools: KM. Wrote the paper: KM. Wrote the manuscript with critical input from all authors: TA. Planned and performed most of the experiments with assistance from CRM, HD, MN, XHP: KSM. Planned and performed nanoindentation analyses: CC. Performed XTM: GB. Participated in study design: ES. Administered drugs and did DXA measurements: HD RM. Performed CFU-F & CFU-OB: MN. Did the histology: XHP. Planned and performed gene expression studies: DN TA. Performed microCT: WH JB. Participated in study design: DW. Planned fracture toughness tests and X-ray tomography, which were performed by SSI-M and GB respectively: RR. Planned micro-tomography and macromechanical tests, which were performed by JWB and WRH: LS. Designed and coordinated the study and supervised all experiments: TAG TA.

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The tissue diagnostic instrument

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Tissue mechanical properties reflect extracellular matrix composition and organization, and as such, their changes can be a signature of disease. Examples of such diseases include intervertebral disk degeneration, cancer, atherosclerosis, osteoarthritis, osteoporosis, and tooth decay. Here we introduce the tissue diagnostic instrument (TDI), a device designed to probe the mechanical properties of normal and diseased soft and hard tissues not only in the laboratory but also in patients. The TDI can distinguish between the nucleus and the annulus of spinal disks, between young and degenerated cartilage, and between normal and cancerous mammary glands. It can quantify the elastic modulus and hardness of the wet dentin left in a cavity after excavation. It can perform an indentation test of bone tissue, quantifying the indentation depth increase and other mechanical parameters. With local anesthesia and disposable, sterile, probe assemblies, there has been neither pain nor complications in tests on patients. We anticipate that this unique device will facilitate research on many tissue systems in living organisms, including plants, leading to new insights into disease mechanisms and methods for their early detection. © 2009 American Institute of Physics. [DOI: 10.1063/1.3127602]

I. INTRODUCTION

The tissue diagnostic instrument (TDI) was redesigned from the bone diagnostic instrument^{1,2} so as to measure tissue mechanical properties subcutaneously and *in vivo* with additional probe assemblies and an adjustable compliance (Fig. 1). It consists of a thin probe assembly that can penetrate skin and soft tissue to reach deep tissues. The disposable, sterilizable probe assembly consists of an outer reference probe made from a 23 gauge hypodermic needle and an inner test probe made from stainless steel wire ranging from 175 to 300 μm in diameter and from 2 to 90 mm in length. Since friction between the test probe and the reference probe increases with length, it is desirable to use only the length needed to access the desired tissue location. The test probe is held in a nickel tube that couples to a magnet, which in turn is linked to a force generator. During operation the force generator oscillates the probe within the tissue of interest and

concurrently measures the force and displacement. The maximum values for force and displacement are 12 N and 600 μm . The probe is typically operated at a frequency of 4 Hz because this is rapid enough to allow hand holding yet sufficiently slow to allow easy decoupling of the elastic and viscous response of the tissue (see supplementary material³ for more details including force and displacement ranges.)

II. MEASUREMENTS

We first illustrate TDI use in human spinal disks that are composed of a thick outer ligament (*annulus fibrosus*) and a central swelling hydrogel (*nucleus pulposus*). Spinal disk degeneration can be the underlying cause of back pain leading to significant morbidity and societal expense. Intervertebral disks are one of the most highly loaded tissues in the body, and consequently material property insufficiency can lead to damage accumulation, inflammation, and pain. Disk degen-

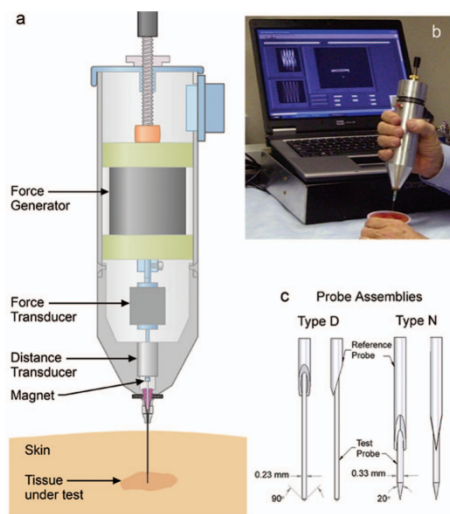


FIG. 1. (Color) The TDI. (a) The TDI can measure mechanical properties of tissues under test even if they are covered with skin and other soft tissues because it has a probe assembly that can be inserted subcutaneously into the tissue under test. (b) It can be handheld and is connected to a computer for data generation, acquisition, and processing. In this photo it is being used to measure differences in the mechanical properties of fruit and gel in a snack food. (c) A probe assembly for the TDI consists of a test probe, which moves displacements of the order of $200\ \mu\text{m}$ relative to the reference probe. The reference probe serves to shield the test probe from the influence of the skin and soft tissue that must be penetrated to reach the tissue under test. Type D probes are good for very soft tissue, such as the murine breast tissue in Fig. 3. Type N probes are good for stiffer tissue, such as the spinal disk tissue in Fig. 2. The screw at the top of the TDI (a) can adjust the compliance of the TDI, as discussed in the supplementary material.

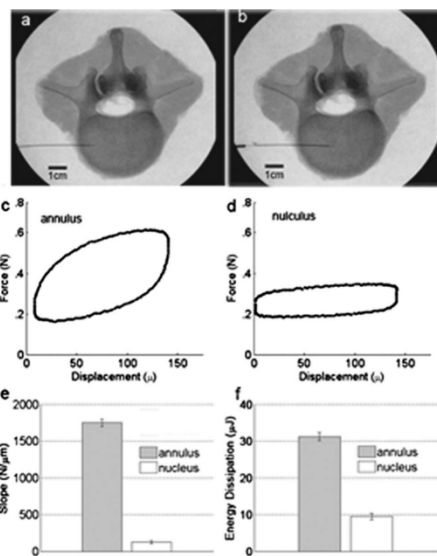


FIG. 2. Demonstration of the ability of the TDI to distinguish between the annulus and nucleus of a human intervertebral disk. (a) X-ray image of transverse view of a cadaver lumbar motion segment L12 with test probe located in annulus. (b) Similar view with probe centered in the nucleus. (c) Force vs displacement curve measured by the TDI during a cyclic load cycle (4 Hz) in the annulus. (d) Force vs displacement curve measured in the nucleus. Note that the annulus is much stiffer (higher slope) and dissipates more energy (higher area enclosed by the curve). (e) Histogram comparing the average least-squares slope for ten cycles in the annulus vs in the nucleus. (f) Histogram comparing the average energy dissipation for ten measurements in the annulus vs in the nucleus ($31.8 \pm 1.1\ \mu\text{J}$ vs $9.7 \pm 0.6\ \mu\text{J}$; $p < 0.01$). The error bars indicate standard deviation for the ten measurements within the annulus and within the nucleus in the disk.

eration is currently diagnosed using imaging techniques, such as magnetic resonance.⁴ Unfortunately, these methods can only indirectly suggest disk mechanical properties, which currently cannot be measured *in vivo*.

Using image-guided, percutaneous placement [Figs. 2(a) and 2(b)], disk material properties can now be measured safely *in vivo* using a type N probe assembly [Fig. 1(c)]. The novel sharpening of the reference probe for type N probe assembly decreases the problem of tissue being caught between the test probe and the reference probe during insertion and thus decreases the friction between the test probe and the reference probe. The friction between the test and reference probes is typically $\sim 0.02\ \text{N}$. The specific value is recorded by the software before testing a sample and is removed from the samples' force versus distance plot before analysis. The force versus displacement data are plotted in real time and recorded digitally [Figs. 2(c) and 2(d)]. The slope of the force versus displacement curve provides a measure of disk elasticity: in the case of a simple spring, the slope would be the spring constant. The energy dissipation in the force versus displacement curve is the area inside the curve and is a measure of the viscous behavior. Viscosity is absent from a simple spring yet is large for a purely viscous material such

as petroleum jelly, which has an elasticity near zero.

There are significant differences (p values of < 0.01) in slope (N/m) and energy dissipation (μJ) between the *annulus fibrosus* and the *nucleus pulposus* (Fig. 2). The *annulus fibrosus* has both higher slope and energy dissipation. These results are representative of our measurements on 11 disks: the slope and energy dissipation are always greater in the annulus than the nucleus. This observation of higher slope or stiffness in the annulus is consistent with previous experiments measuring the compressive properties of both annulus and nucleus.^{5,6} However, a precise comparison with established mechanical data is not readily available because mechanical testing in this manner at high spatial resolution has not been possible previously. Because these properties are known to change with age and degeneration, an eventual goal would be to determine whether *in vivo* measures of annulus and nucleus material properties provide novel data that improve back pain diagnosis and treatment. We note that the 23 gauge needle is consistent with the recent recommendation⁷ that a spinal needle smaller than or equal to 22 gauge should be used to prevent postsurgery leakage.

An epithelial tissue such as the mammary gland is an

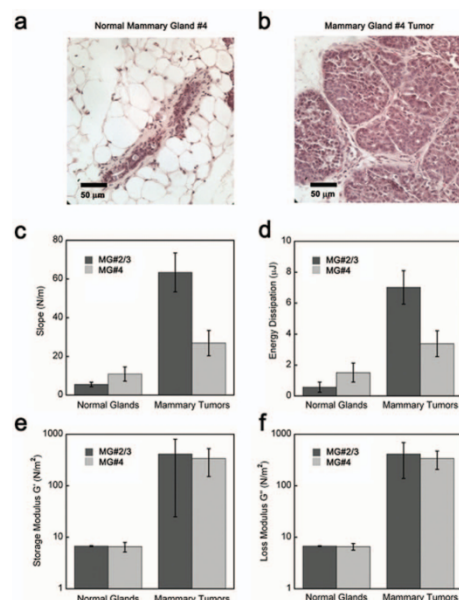


FIG. 3. (Color) Demonstration of the ability of the TDI to distinguish between normal mammary glands and tumors. Hematoxylin and eosin (H&E) staining of (a) the representative normal FVB murine mammary gland and (b) the matched malignant MMTV-PyMT+/- murine mammary gland that were tested in this experiment. (c) The mammary tumors have significantly ($P < 0.001$) higher slopes, a measure of elasticity, for both the thoracic # 2/3 and the inguinal #4 tissues. (d) The mammary tumors have significantly higher energy dissipation for the thoracic # 2/3 tissue ($P < 0.001$), but the difference for the inguinal #4 tumor was not significant. Histogram comparing (e) the elastic modulus and (f) the loss modulus, as measured by rheology, for the normal mammary glands and mammary tumors after the TDI measurements (two subregions for each mammary gland, ten measurements for each region). Note that the results for elasticity and loss modulus for the two techniques reproduce the same general trends. The error bars in the measurements indicate standard deviation for all the measurements.

example of the softest tissue that can be probed with the current TDI. Figure 3 shows a paired-comparison of the mammary glands #2/3 and #4 from normal Friend Virus B₆ mice and tumors arising in the matched mammary glands of their MMTV-PyMT+/- (Mouse Mammary Tumor Virus-Polyoma Middle T Antigen) littermates. These data are representative of the data in an ongoing study of various tumors. The results of that study are beyond the scope of this paper, but we can report that all 30 tumors are stiffer (have higher slope) than all 15 normal mammary glands in tissue site-matched and age-matched mice. Normal murine mammary glands have elastic modulus below 1 kPa as measured with a conventional rheometer and with the TDI device. This value is comparable with our calibration curves on polyacrylamide (PA) gels (see supplementary material) that demonstrate TDI sensitivity below 1 kPa. Normal and transformed human breast tissue is considerably stiffer than mouse tissue⁸ and is therefore well within the range of the TDI. Tissue stiffness

increases in many breast cancers. To quantify stiffness and improve breast tumor detection, imaging modalities such as sonoelastography and Magnetic Resonance elastography have been used.^{9,10} The TDI offers a tractable and economical approach to measure breast stiffness *in situ* with millimeter resolution. Our preliminary trials on human breast tissue from cadavers showed detectable variations in mechanical properties between different locations in the same specimen with a spatial resolution of 2 mm (data not shown). Based on these observations an eventual goal would be to use the TDI for localization of human breast cancer *in situ*. A foreseeable clinical application includes using the device to define margins of affected tissue and sites for biopsy. The TDI measurement could easily be combined with biopsy; the test probe could be withdrawn into the reference probe to collect a biopsy sample after mechanical testing. We are currently investigating the molecular mechanism of how the mechanical properties contribute to breast cancer; the TDI can be applied to clarify the molecular link between matrix materials properties of tissues and tumor risk (i.e., breast cancer in women with mammographically dense breasts^{11,12}).

Prior to clinically apparent symptoms of osteoarthritis, the material quality and mechanical function of cartilage matrix are compromised.¹³ The ability to noninvasively probe the material quality of this stratified tissue will complement and extend current diagnostic capabilities.¹³ Furthermore, detection of cartilage degeneration early in osteoarthritis may increase the success of therapeutic intervention. To that end, the ability of the TDI to distinguish the elastic modulus of synthetic materials with moduli comparable to cartilage was validated against well-established methods including atomic force microscopy, nanoindentation, and bulk stress relaxation (Fig. 4). In addition to accurately measuring the elastic modulus of PA gels with a range of moduli from 0.2 to 1 MPa, the TDI could measure the elastic modulus of a stiff gel that was inferior to a compliant gel, demonstrating its ability to noninvasively evaluate a stratified material [Fig. 4(d)]. When applied to cartilage, the TDI readily discriminated between a young healthy cadaveric specimen and an old degenerated surgical specimen that were probed *in situ* [Fig. 4(f)].

Human dentin is an example of the hard tissue that can be probed with our current device (Fig. 5). One unsolved problem for practicing dentists is deciding when a sufficient depth has been reached when drilling to remove carious dentin from a cavity. One proposed solution has been the development of a new experimental polymer bur (from SS White Burs, Inc., Lakewood, NJ), which is designed to remove soft decaying dentin but blunt on harder healthy dentin and thus self-limit the tissue amount removed. Here we show the properties of the remaining dentin after a first excavation by such a bur and then after a second excavation with a second polymer bur of the same type (Fig. 5). Next was a third excavation with a #4 round carbide bur and finally a cavity preparation into presumably sound dentin using a #330 carbide bur. Note that even after the cavity preparation, the dentin did not have the full elastic modulus of the healthy dentin. Our primary focus was on relative values as excavation proceeded. The elastic modulus, as calculated from force

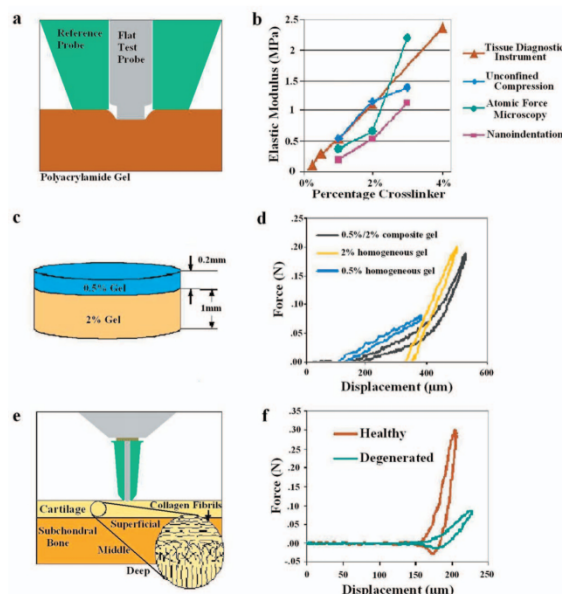


FIG. 4. (Color) Demonstration of the ability of the TDI to distinguish differences in moduli of stratified materials such as cartilage. (a) The elastic modulus can be determined with the type V probe assembly that indents soft materials rather than penetrating them, as above. (b) PA gels with elastic moduli in the range previously reported for cartilage (0.2–1 MPa) were used to validate the TDI relative to other established methods, including atomic force microscopy, nanoindentation, and bulk stress relaxation. PA gel moduli increased dose-dependently with cross-linker concentration ($p=0.012$). (c) To construct a stratified elastic modulus gel, a 0.2 mm thick layer of “compliant” PA gel was poured over a prepolymerized 1 mm thick “stiff” PA gel. (d) The force vs displacement curve produced by the TDI revealed two distinct slopes on the loading curve for the stratified gels. Each slope of the composite gel matches the corresponding slopes for homogeneous 0.5% and 2% PA gel, demonstrating the capability to analyze stratified materials, such as cartilage. (e) A schematic of the indentation tests performed on cartilage, which were performed in hydrated conditions with phosphate buffered saline. (f) Using similar test conditions, the TDI easily distinguished between young cadaveric cartilage and aged degenerated cartilage measured *in situ*.

versus displacement curves generated by the TDI and analyzed using a modified Oliver and Pharr¹⁴ method, for the dentin left in the cavity by the polymer bur was below that for healthy dentin far from the cavity [Fig. 5(c)]. Please see the appendix for the details of the modified method. The hardness, as calculated from force versus displacement curves generated by the TDI and analyzed using a modified Oliver and Pharr¹⁴ method, for the dentin left in the cavity by the polymer bur was below that for healthy dentin far from the cavity [Fig. 5(c)]. One-way analysis of variance gives values of $P<0.0001$ for the elastic modulus and $P=0.0008$ for the hardness, indicating that the variation among means is significantly greater than expected by chance. Thus the TDI has the potential to quantify the properties of dentin left in a cavity and could be used to study the outcome of various treatment strategies for how much degenerated dentin is removed before filling the cavity.

The absolute value for the elastic modulus of our “healthy dentin” is well within the range of existing measurements but below the value of 20–25 GPa recommended in a recent critical reevaluation of the literature.¹⁵ The reason is probably the storage of the teeth in water for weeks before measurement.¹⁶ To our knowledge, the TDI is the first instru-

ment that can measure elastic modulus and hardness inside irregularly shaped, fully hydrated dentin cavities. It could be used for research projects without further modification. For individual clinical use, a smaller, less expensive version with an angled probe would be desirable. The experiments on dentin reported here build on a rich history of measuring mechanical properties with indentation methods.^{14,17,18} Of special interest is recent work modeling size effects with finite element analysis¹⁹ because extensions of work such as this may lead to a more quantitative understanding of TDI measurements on soft tissue as well as hard tissue.

The hard tissue, bone, is of particular interest medically because of the growing incidence of debilitating bone fracture as our population ages.²⁰ Changes in bone material properties are believed to play a role in fracture risk.^{21–23} With the top screw backed off, as discussed in the supplementary materials,³ the TDI functions as a Bone Diagnostic Instrument,^{1,2} which may, after clinical tests, prove useful in quantifying the component of bone fragility due to degraded material properties. Figure 6 shows tests of the TDI, working as a bone diagnostic instrument, on a living patient to determine if the procedure is painful or results in complications. Neither this patient nor the others tested to date experienced

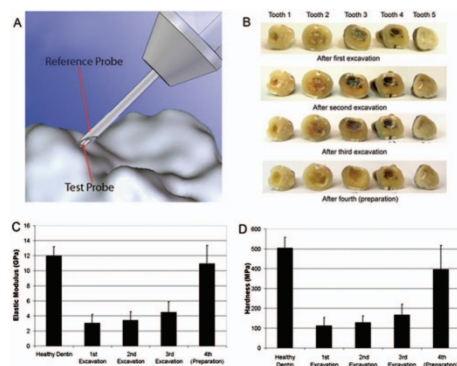


FIG. 5. (Color) Demonstration of the ability of the TDI to measure the elastic modulus and hardness of human dentin to quantify the properties of the dentin left in a tooth cavity after each of multiple excavations and finally preparation. (a) The probe assembly for these measurements was designed to indent the hard tissue. The test probe was sharpened into a 90° cone with a 30 μm radius at the end (drawing by Haykaz Mkrtchyan). (b) The teeth after the various excavations and finally preparation. At each successive stage of excavation and preparation more dentin was removed from the cavity. [(c) and (d)] The elastic modulus and hardness of the dentin remaining in the cavity was significantly ($p=.01$) less than that of healthy dentin. The error bars indicate standard deviation of the ten measurements that were taken on each of the five teeth (a total of 50 measurements). Note that the elastic modulus of the healthy dentin is over 10 GPa, over seven orders of magnitude greater than the normal mammary glands (Fig. 3), demonstrating the range of the TDI.

any pain beyond the initial “stick” when the local anesthesia was injected. There have been no complications.

III. DISCUSSION OF NEW POSSIBILITIES

It might, in the future, be possible for the TDI to measure the interaction forces between antibody coated test probes and tissues. This would allow measurements of single molecule interactions as is currently achieved with an atomic force microscope.²⁴ Rupture forces in the range of 20–140 pN have been measured for many receptor-ligand interactions with single-cell force spectroscopy.²⁵ With these interaction forces, we can make order of magnitude estimates of forces we might find when trying to rotate or translate a test probe that had bound to a tissue with many molecular bonds in parallel. Assuming a molecular density of 1 molecule/10 nm^2 , an interaction force per molecule of 50 pN, a coated region of area of $4 \times 10^{-6} \text{ m}^2$ (the exposed area of the type D probe), and a fractional binding of 1%, we would get a force of $50 \text{ pN/molecule} \times 4 \times 10^{-6} \text{ m}^2 \times 1 \text{ molecule}/10 \text{ nm}^2 \times .01 = 200 \text{ mN}$. The present lower limits of sensitivity of the TDI for forces come from the friction between the test probe and the reference probe, of the order of 10 mN, and from the force noise in our force transducer, of the order of 5 mN in a 1 kHz bandwidth. Thus forces of the magnitude that could be expected from molecular interactions with coated tips should be measurable. A big problem could be nonspecific interaction masking specific interactions. A proof of concept experimental approach to

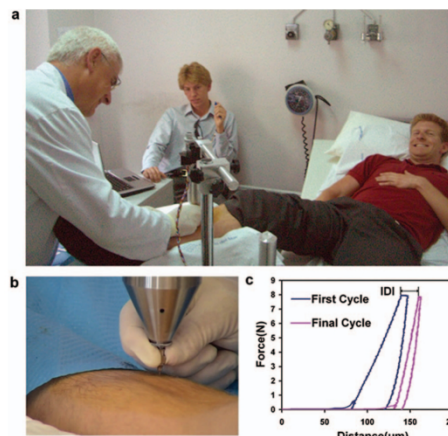


FIG. 6. (Color) Demonstration of the ability of the TDI to do measurements on a living patient. (a) The probe assembly of the TDI is lowered by a physician (A.D.P.) to penetrate the skin and soft tissue covering the tibia of the patient (D.B.) after the test site has been sterilized and locally anesthetized. (b) Close up of the physician's hand on the probe assembly as he lowers it to the bone surface. (c) Representative force vs distance curves measured on the bone of the patient. This patient and the other patients tested to date experienced neither pain nor complications with the procedure. The most important parameter is the indentation distance increase (IDI) defined in the image as the increase in indentation distance from the first cycle to the last. In model systems the IDI is greater for more easily fractured bone. Other parameters such as the creep at nearly constant force (the plateau on the top of the curves), the elastic modulus, the energy dissipation, and the hardness can also be determined from analysis of the force vs distance curves.

overcoming this masking effect would be to use a test probe coated on just one side that was exposed to the tissue under test through a window in the wall of a closed-end reference probe. The difference in the forces between the test probe and the tissue under test for the coated versus uncoated side could be measured. This could naturally be extended with multiple coatings on multiple strips on the test probe, each exposed one by one through a slit in the wall of the closed-end reference probe. We emphasize, however, that proof of concept experiments will be necessary to evaluate this potential application of the TDI.

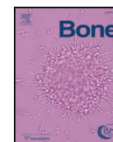
It is important to note that though the present instrument is able to make basic measurements in a wide range of tissues (almost all tissues in the human body from very soft breast tissue to hard, mineralized tissues), it is in a very early stage of development. More versatile instruments with more measurement modalities, such as mentioned above, and more user convenience features, such as wireless operation, are possible. Specialized instruments for specific measurements in specific tissues could be developed at a small fraction of the cost of the fully versatile instrument. The device could also be modified to assess materials properties of various bioengineered artificial three dimensional tissues.^{26,27}

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Osteopontin deficiency increases bone fragility but preserves bone mass

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ABSTRACT

The ability of bone to resist catastrophic failure is critically dependent upon the material properties of bone matrix, a composite of hydroxyapatite, collagen type I, and noncollagenous proteins. These properties include elastic modulus, hardness, and fracture toughness. Like other aspects of bone quality, matrix material properties are biologically-defined and can be disrupted in skeletal disease. While mineral and collagen have been investigated in greater detail, the contribution of noncollagenous proteins such as osteopontin to bone matrix material properties remains unclear. Several roles have been ascribed to osteopontin in bone, many of which have the potential to impact material properties. To elucidate the role of osteopontin in bone quality, we evaluated the structure, composition, and material properties of bone from osteopontin-deficient mice and wild-type littermates at several length scales. Most importantly, the results show that osteopontin deficiency causes a 30% decrease in fracture toughness, suggesting an important role for OPN in preventing crack propagation. This significant decline in fracture toughness is independent of changes in whole bone mass, structure, or matrix porosity. Using nanoindentation and quantitative backscattered electron imaging to evaluate osteopontin-deficient bone matrix at the micrometer level, we observed a significant reduction in elastic modulus and increased variability in calcium concentration. Matrix heterogeneity was also apparent at the ultrastructural level. In conclusion, we find that osteopontin is essential for the fracture toughness of bone, and reduced toughness in osteopontin-deficient bone may be related to the increased matrix heterogeneity observed at the micro-scale. By exploring the effects of osteopontin deficiency on bone matrix material properties, composition and organization, this study suggests that reduced fracture toughness is one mechanism by which loss of noncollagenous proteins contribute to bone fragility.

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Introduction

Currently, bone mineral density (BMD) is the most common diagnostic used to assess fracture risk [1,2], yet less than half of non-vertebral fractures can be explained by BMD alone [3]. The limitations of using solely BMD for the prediction of fracture risk can even be shown *in vitro*. The mechanical competence of healthy human bone is

compromised when exposed to chemical treatments that affect the organic matrix but not BMD [4–7]. These findings are not surprising given that the inorganic mineral is only one of several constituents that make up the complex hierarchical composite that is bone. To better reflect these complexities, the scientific and medical communities have adopted the term “bone quality” to summarize the aspects of bone that contribute to fracture risk but are not encompassed by BMD measurements [8]. Bone quality, the subject of growing interest and research efforts, comprises a number of parameters such as the microarchitecture of trabecular bone, prevalence of microcracks, bone geometry and, importantly, bone matrix material properties.

Bone matrix material properties, including elastic modulus, hardness, and fracture toughness, reflect the ability of bone to resist deformation and catastrophic failure. Even without changes in bone mass, average mineralization or bone shape, the alteration of bone matrix material properties can dramatically impact the mechanical

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competence of bone. For example, it is known clinically that defects in bone matrix material properties due to increased collagen cross-linking likely contribute to bone fragility in diabetes [9,10], whereas collagen point mutations contribute to bone fragility in osteogenesis imperfecta [11]. Even in healthy tissue, bone matrix material properties are biologically regulated [12,13] and anatomically distinct [14]. The unique material properties of a specific bone are present across multiple species, suggesting that their regulation is functionally advantageous and evolutionarily conserved. However, the mechanisms that specify the material properties of bone matrix remain largely unknown.

Bone matrix is a composite of osteoblast-derived collagen and noncollagenous proteins that undergoes mineralization. Both the mineral and organic components of bone matrix contribute to its characteristic hardness and toughness, and defects in the composition or organization of either can cause bone fragility. While the roles of mineral and of collagen type I in bone quality have been investigated in some detail [15–20], we know little about the influence of the noncollagenous proteins on bone matrix material properties. Noncollagenous proteins such as osteocalcin, osteopontin (OPN) and others comprise a relatively small percentage of the bone matrix volume or weight, but they may contribute to bone matrix quality in a number of ways. Noncollagenous proteins control hydroxyapatite crystal nucleation, growth, shape and size as well as facilitate attachment between the major organic (collagen) and inorganic (hydroxyapatite) phases [21–26]. In addition to their effects on mineral, it has more recently been suggested that the intrinsic properties of certain noncollagenous proteins could also be important for the material properties of bone matrix [27]. Networks of highly phosphorylated proteins such as OPN exhibit a molecular self-healing character allowing them to repeatedly dissipate large amounts of energy when loaded in tension [6]. Such protein networks were also found to have an energy storage mechanism and, importantly, exhibit large cohesion and toughness [28]. Hence, the role of noncollagenous proteins, particularly OPN, as determinants of bone matrix material properties and fracture resistance warrants further study.

Although OPN deficiency has previously been shown to impair the macro-mechanical properties of whole bones, the mechanism for this impairment remains unclear. Duvall et al. [29] found a decreased maximum load and torque, as well as work to failure in OPN-deficient mice, but these tests do not explore the underlying changes in bone matrix material properties and ultrastructure. Therefore, the origin of the effect of OPN on bone quality is unclear, which is in part due to the multiple roles attributed to it. OPN facilitates osteoclast attachment and guides mineral deposition by influencing crystal shape and size [23,25,30]. OPN, as well as other noncollagenous proteins, are enriched in cement lines, lamellar interfaces, and interfibrillar spaces of mineralized collagen fibrils [31–34]. High-resolution imagery shows that bone ultimately fails through delamination of mineralized collagen fibrils [6,35–37]. Therefore, perturbation of either the mineral or organic components at these interfaces due to genotype, such as OPN deficiency, or disease, such as osteoporosis, could dramatically impact fracture resistance. Therefore, we hypothesized that OPN, because of its effects on bone mineral and its localization in interfibrillar spaces, is critical for the material properties of bone matrix.

In order to investigate this hypothesis, bones of male OPN-deficient (OPN^{−/−}) mice and wild-type littermates (WT) were evaluated to determine the role of OPN in bone structure, composition, and mechanical and material behavior. Our most important result was a significant decrease in fracture toughness due to OPN deficiency. Fracture toughness is influenced by a multitude of factors including uncracked-ligament bridging, microcracking, crack deflection, and porosity [38–40]. As these factors arise at multiple length scales, our analysis extended from the whole bone level down to the composition and organization of individual matrix constituents.

Materials and methods

Mice

Osteopontin-deficient mice (OPN^{−/−}) on a C57BL/6 background were purchased from the Jackson Laboratory [41]. Protocols were performed as approved by the Institutional Animal Care and Use Committee. Male mice were sacrificed at 8 weeks of age. Harvested hind limbs and spines were cleaned of soft tissue. Left tibiae were stored in Hanks' balanced salt solution (HBSS) containing 0.05% NaN₃ at 4 °C for a maximum of 5 days, whereas all other bones were wrapped in HBSS soaked gauze and stored in sealed plastic pouches at −80 °C.

Fracture toughness

Dissected femora from 9 mice of each genotype were tested in bending to measure the fracture toughness. For such measurements, ASTM standards [42] require that fracture is initiated from a sharp precrack. Generally, this is achieved by fatigue precracking, but this is not really feasible for small mouse bones. Because of this, machined notches have often been used, but more often than not, these are not sharp enough to obtain an accurate toughness measurement. Accordingly, we use a micro-notching technique here, where machined notches are sharpened by “polishing” with a razor blade using 1 μm diamond polishing solution to cut the bone midshaft through the posterior wall of the femur. The resulting micro-notches were maintained at ~1/3 of the bone diameter in length with a reproducible notch root radius of ~10 μm [43]. In this study, femora were tested in 37 °C HBSS in a three-point bending configuration with a custom-made rig for the ELF 3200 mechanical testing machine (ELF3200, EnduraTEC, Minnetonka, MN), in general accordance with ASTM Standard E-399 [42] and E-1820 [44] and as discussed in previously developed methods for small animal bone testing [43]. Testing was conducted in displacement control at a cross-head displacement rate of 0.001 mm/s. Half-crack angle at point of instability was determined by scanning electron microscopy. Fracture toughness, K_{IC} , was calculated using a stress-intensity solution for a circumferential through-wall flaw in cylinders [45]. This methodology and its motivations are discussed in more detail by Ritchie et al. [43].

Areal bone mineral density (aBMD) measurement

aBMD of dissected spines ($N=9$ WT, 11 OPN^{−/−}) and femurs ($N=6$ WT, 7 OPN^{−/−}) was measured using a PIXImus mouse densitometer (GE Lunar II, Faxitron Corp., Wheeling, IL). Importantly, it should be noted that this areal BMD measurement is a combined measure of tissue mineral content as well as the structural organization of bone.

Micro-computed tomography

Dissected femora from 6 mice of each genotype were subjected to micro-computed tomography to determine cortical thickness. Femora were scanned fully hydrated in a benchtop CT 160Xi tomography system (XTEK, Tring, UK) using an acceleration voltage of 75 kV and a current of 60 mA. 1920 projections were recorded for each bone. Bone was segmented from reconstructed volumes using an adaptive threshold algorithm [46], from which the region of interest (ROI) containing only the midshaft of each bone was chosen for quantitative analysis. Segmentation and processing of ROIs was done using algorithms programmed in IDL (ITT, Boulder, CO). As each slice was roughly perpendicular to the long axis of the bone, a 2D component labeling algorithm allowed detection of the main bone compartment as well as the medullary cavity and outside void space. A thickness map of the cortical bone was obtained by filling the outside void space and performing a 3D distance transformation. The individual

thickness values were obtained from the outermost voxels of the cortical bone surface using morphological erosion, which allowed determination of average cortical bone thickness.

Synchrotron radiation micro-computed tomography

Femora from 2 mice of each genotype were tomographically imaged using synchrotron radiation. These experiments were performed at Beamline ID19 of the European Synchrotron Radiation Facility (ESRF). A 2 mm long region of the midshaft of each femur was scanned, as described in [47], at a photon energy of 20 keV and a voxel size of 1.4 μm . Histograms of reconstructed volumes were bimodal distributions with peaks representing either bone or void space. For each sample, the peak representing bone was fitted with a Lorentzian function and analyzed in terms of peak position and full width at half maximum (FWHM), which represent average calcium concentration and calcium concentration variability, respectively. In addition, the volumes were processed for bone porosity using both IDL and ImageJ. In brief, bone was segmented using an adaptive threshold algorithm [46]. Subsequently the volumes were subjected slice by slice to a 2D component labeling algorithm in order to suppress noise arising from soft tissue remnants on the outside surface of the bone as well as in the medullary cavity. Using morphological operators, all internal voids were filled. The resulting closed bone space was used as a mask on the original volumes containing only the main bone component. The resulting volume contained only the void spaces, which were analyzed using a component labeling algorithm that was programmed to return voids larger than 274 μm^3 . Detected particles were ordered by size and a particle size distribution curve was computed. From these data we computed BV/TV values for the cortical bone as well as the ratios of lacuna pore space (PS_{LAC}) and blood vessel pore space (PS_{VES}) to the total pore space (PS). Based on the particle distribution curves, the size threshold for blood vessels was set at 1098 μm^3 .

Nanoindentation

Dissected tibiae from 4 mice of each genotype were encased in a 2-component epoxy (Stycast 1266, Emerson & Cuming, Henkel, Dusseldorf Germany) without infiltration. Bones were sectioned with a diamond wafering blade at the midshaft perpendicular to the long axis to generate cortical bone surfaces for nanoindentation. Retrieved sections were polished with sandpaper and diamond suspensions down to a particle size of 0.25 μm . Nanoindentation was performed in dry conditions with a Triboindenter (Hysitron, Minneapolis, MN) equipped with a Berkovich tip as described in [12]. Indents were applied using a trapezoidal loading profile with a loading rate of 100 $\mu\text{N/s}$, peak load of 600 μN , and a hold period of 10 s. From the resulting load-deformation curves, local elastic modulus was calculated as described in [48]. Three sets of 10 nanoindentation points were performed in a line with a 5 μm separation. Mean values for each mouse were averaged to determine the average and standard errors for elastic modulus and hardness for each genotype.

Quantitative back scattering imaging (qBEI)

Sections of tibial cortical bone adjacent to those used for nanoindentation were carbon-coated in preparation for qBEI. QBEI images for each of 5 bones for each genotype were acquired using a Hitachi S-4300SE/N environmental scanning electron microscope (ESEM) (Hitachi America, Pleasanton, CA) at 20 keV electron beam energy and 1000 $\times$ magnification under high-resolution image mode as described in [49]. For each bone, three images of mid-cortical bone matrix were taken in regions that generally corresponded to those tested by nanoindentation. As described in detail by Roschger et al. [49], grayscale values from QBEI images were calibrated using hydroxyapatite and carbon standards (MAC Consultants, St. Ives,

UK) and beam current was controlled using a Faraday cup in the center of the standard. Fitting the bone mineral density distribution with a Gaussian curve allowed determination of the calcium concentration (weight %) and variability. Graphs indicate the mean and standard error of values for 5 mice, each of which reflects the mean of the three qBEI measurements.

Small angle X-ray scattering (SAXS)

Tibiae from 4 wild-type and 3 OPN $^{-/-}$ mice were thawed, air-dried and embedded in a 2-component epoxy (Stycast 1266). Embedded tibiae were sectioned parallel to their long axis using a diamond wafering blade. Cut sections were thinned to approximately 300 μm in thickness using a polishing wheel. SAXS patterns for several sections of the cortical bone were acquired using a benchtop system with Cu K α radiation (Nanostar, Bruker, Madison, WI) as well as a dedicated experiment at beamline 8.3.3 at the Advanced Light Source (ALS) at the Lawrence Berkeley National Laboratory using an X-ray energy of 10 keV. Acquired patterns were absorption-corrected and azimuthally integrated to calculate mean crystal thickness [50]. Values using these two approaches were pooled.

Transmission electron microscopy (TEM)

Two methods of sectioning were chosen: ultramicrotomy (UM) and focused ion beam milling (FIB). 1. Ultramicrotomy: additional tibiae from 2 mice of each genotype were fixed in anhydrous ethylene glycol for 24 h, dehydrated by rinsing in 100% ethanol three times for 5 min in acetonitrile, and then infiltrated with resin (Agar Scientific) over several days. Mounting resin was prepared with 12 g Quetol, 6.5 g methyl nadic anhydride (MNA), 15.5 g nonenylsuccinic anhydride (NSA), and 0.7 g benzyltrimethylamine (BDMA). The samples were agitated at room temperature in 1:1 solutions of acetonitrile and resin for 1 day, then 1:2 acetonitrile resin for 1 day, and finally 100% resin for 3 days under vacuum; the resin was changed every 24 h. Samples were then cured in fresh resin for 24 h at 60 $^{\circ}\text{C}$. Silver to gold sections (70–90 nm) were cut onto distilled water with an ultramicrotome using a 35 $^{\circ}$ diamond knife. Samples were collected immediately on lacy carbon 300 mesh copper grids. 2. Focused ion beam milling: for each of 2 tibiae per genotype, focused ion beam milled sections of approximately 80 nm thickness were prepared using FIB 200 (FEI Company, Hillsboro, OR) which is a single focused gallium ion beam (FIB) instrument. The samples were first coated with approximately 40–50 nm of gold to minimize specimen charging during FIB-milling. Before milling, a platinum layer, 20 $\mu\text{m} \times 2.5 \mu\text{m}$ and 1 μm thick, was deposited over the area of interest on each of the two coated samples. Thin sections were prepared parallel to the long axis of the samples. The FIB ion beam was operated at 30 kV with beam currents that ranged from 3 nA to 100 pA with the lower current used for final polishing of the electron-transparent portion of the sample.

For TEM analysis, bright field imaging was performed on a JEOL 2000 FX TEM using operating voltages of 120 and 200 kV. Multiple longitudinal and transverse sections were investigated for each bone to assess nanoscale tissue organization. Quantitative analyses included measurement of hydroxyapatite dimensions, collagen fibril diameter and D-banding. To study the collagen fibril diameter, selected sections were decalcified in 5% ethylene-di-aminetetraacetic acid (EDTA) disodium salt (Sigma, St. Louis, MO) in 0.1 M Pipes buffer (pH 7.4) for 15 min. The decalcified sections were subsequently stained with uranyl-acetate and lead citrate for 5 min in each. The collagen fibril banding pattern was also evident in non-stained TEM sections prepared by FIB.

The thickness of the crystals was measured from TEM images using methods described in [51]. Crystals were observed both on edge and *en face* within the mineralized collagen fibrils. Crystals oriented *en*

face were plate-like in shape with no well defined profile, whereas crystals on edge were needle-shaped with a well defined, oblong profile. The plate-like dimension of the crystals could not be determined accurately due the scarcity of clearly visible plate-like crystals. In comparison, the needle-like crystals were generally uniform and much easier to isolate and measure. The thickness of these needle-like crystals were measured for both UM and FIB prepared OPN^{−/−} and WT bone sections using $N=50$ for apatite thickness.

In order to confirm the 3D morphology of the crystals and the supposition that differences in contrast observed from the crystals observed on edge and *en face* arises from differences in their orientation, tilting experiments were performed. Nine images were taken with a variation of tilt angles ranging from -20° to $+20^\circ$ with 5° increments. Ultramicrotomy prepared WT and OPN^{−/−} were imaged to determine a difference in 3D morphology of crystal apatite. These tilting experiments confirmed that the needle-like crystals were actually plate-like crystallites viewed on edge.

Demineralized bone testing

Dissected femora from 5 mice of each genotype were thawed and cut at both ends to permit flushing with HBSS to remove bone marrow. The samples were demineralized in a 19% EDTA solution containing 0.05% NaN₃, 2 $\mu\text{g}/\text{ml}$ leupeptin and 2 $\mu\text{g}/\text{ml}$ aprotinin for 3 days. Subsequently, the samples were soaked in 110 mM NaCl, 40 mM CaCl₂, and 10 mM HEPES overnight, after which they were prepared for tensile testing according to Silva et al. [19]. Tensile testing was performed in 20 °C HBSS using custom grips and a small scale mechanical tester (ELF3200, Bose) at a displacement rate of 0.1 mm/s. Digital video recordings of the specimens, marked with Verhoeff's stain at two points, allowed determination of strain in the gauge region of the samples using a custom algorithm (Labview 8.6, National Instruments, Austin, TX), from which tensile strength and elastic modulus were calculated. Sample width was determined from digital images. Thickness was based on measurements using a pair of calipers and micro-CT. In brief, we compared cortical thickness of a set of mineralized femora determined using micro-CT and image processing, including a distance transform, with cortical thickness of a set of demineralized femora determined using a pair of calipers and found good agreement of average thicknesses without any significant differences between the two methods. Therefore, calipers were used measure cortical thickness of the remaining demineralized samples used for tension testing in order to minimize protein degradation due to ambient exposure.

Statistics

Statistical comparisons of data for OPN^{−/−} and WT mouse bones were compared using two tailed Student's *T*-test using a significance threshold of $p=0.05$.

Results

Reduced fracture toughness in OPN-deficient bone

Bone's fracture resistance is significantly derived from its ability to impede the propagation of cracks once a fracture is initiated [52]. Fracture toughness testing measures a material property of bone, specifically the ability of a bone to resist crack propagation independently of its ability to resist crack initiation. To determine if OPN is important for the ability of bone to resist crack propagation, dissected femora from wild-type and OPN^{−/−} mice were prepared for fracture toughness testing. Femora were notched at the mid-diaphysis and loaded to failure in a three-point bending configuration (Fig. 1A,B). The fracture toughness of wild-type femora ($K_{\text{IC}}=5.55 \pm 1.78 \text{ MPa m}^{1/2}$)

was consistent with previously reported values [12,43]. However, the fracture toughness of OPN^{−/−} femora ($3.87 \pm 0.79 \text{ MPa m}^{1/2}$) was 30% lower than wild-type values ($p<0.05$, Fig. 1C). This result is significantly above the coefficient of variation (18%) for analysis of fracture toughness in mouse bone. Therefore, the reduced fracture toughness indicates that OPN deficiency compromises the ability of bone to resist crack propagation.

Though the reduced fracture toughness of the OPN-deficient bone likely contributes to the bone fragility reported by Duvall et al. [29], the mechanisms by which OPN contributes to fracture toughness remain unclear, in part due to the multiplicity of roles for this protein. For example, OPN has been previously been shown to influence bone remodeling [53] and mineral crystallization [54], both of which may impact the ability of bone to resist fracture. In an effort to better understand the contribution of the OPN protein to the mechanical competence of bone, the structural and material properties of bone from OPN-deficient mice were investigated at multiple length scales.

Loss of fracture toughness is not accompanied by loss of bone mass or cortical thickness

At the largest length scale, bone mass and structure influence the ability of bone to resist fracture. Accordingly, DEXA and micro-CT analysis were used to evaluate bone mass and structure in wild-type and OPN-deficient mice. DEXA analysis showed a significant increase in the aBMD of the OPN-deficient mouse spine, which is composed primarily of trabecular bone (Fig. 2A). Since OPN-deficient mice have previously been reported to possess increased trabecular bone volume [55], this observation validated the OPN-deficient phenotype of the mice used in this study. However, OPN-dependent differences in areal aBMD were not apparent in femora (Fig. 2B). The structural features of cortical bone were examined more carefully using micro-CT since this is the region that was assayed in fracture toughness tests. The cortical thickness of OPN-deficient femora ($318 \pm 36 \mu\text{m}$) did not differ significantly from that of wild-type bones ($303 \pm 28 \mu\text{m}$) (Figs. 2C, D), a finding that is consistent with Koyama et al. [56]. Hence, there are no macroscopic changes in cortical bone mass or geometry which could be responsible for the OPN-dependent reduction in fracture toughness.

Absence of changes in porosity in OPN-deficient mice

Microstructural features such as microcracks or changes in bone porosity can impact the fracture toughness of bone. To assess the effect of OPN deficiency on cortical bone porosity, bones were examined by synchrotron radiation micro-computed tomography (SRμCT). Figs. 3A and B show reconstructed slices of cortical bone from wild-type and OPN-deficient femora, respectively. Quantitative analyses revealed no significant differences in overall cortical bone volume or porosity ($\text{BV}/\text{TV}_{\text{WT}} 0.9386 \pm 0.0011$, $\text{BV}/\text{TV}_{\text{OPN}^{-/−}} 0.9317 \pm 0.0042$) (Fig. 3C). Reconstructed images, such as the example shown in Fig. 3D, were used to evaluate the distribution of pore size. A threshold set based on pore size was used to distinguish between osteocyte lacunae which represent approximately 47–48% of the pore volume (Fig. 3F), and blood vessels which comprise the remaining 52–53% (Fig. 3G). No differences in the distribution of pores for osteocyte lacunae or vasculature were observed between wild-type and OPN-deficient mice (Fig. 3H). In addition, pore organization also appeared to be very similar with no evidence of the qualitatively striking differences in blood vessel or lacunae organization that were reported by Schneider et al. [47]. Therefore, the loss of fracture toughness in OPN-deficient bones is also not a result of microstructural defects in porosity.

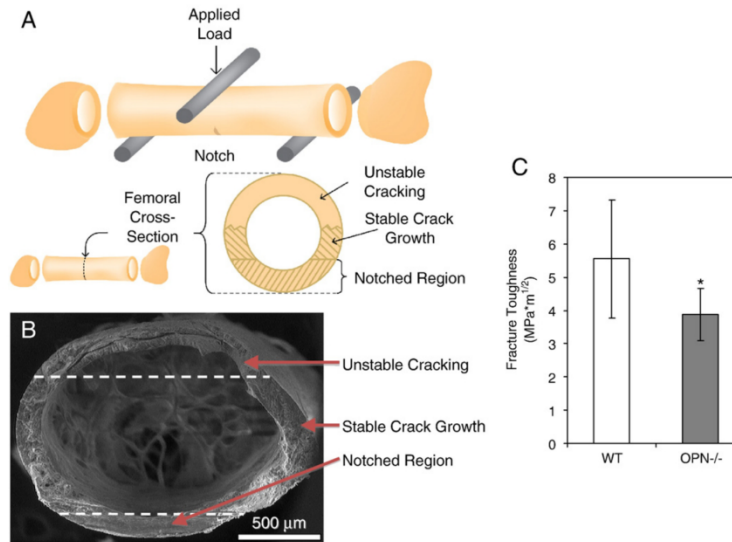


Fig. 1. OPN-deficient bone has reduced fracture toughness. The test configuration is shown schematically (A). A notched femur is tested in three-point bending, with the polished notch on the posterior of the femur, opposite the applied load. An examination of the broken cross section (bottom of schematic A and SEM image (B)) after the test reveals a smooth notched region, from which extends a stable crack growth region, followed by unstable cracking for the remaining circumference of the sample (approximate borders between these regions are indicated with dashed lines in the SEM image B). Further details on this fracture toughness testing procedure are described in [43]. Asterisks (*) indicate statistically significant differences in fracture toughness ($p < 0.05$) in WT and OPN-deficient bone (C).

OPN deficiency results in decreased indentation modulus

These data suggests that the factors responsible for the reduced fracture toughness were independent of bone size and shape. Several proposed mechanisms for bone toughening occur at smaller length scales. Therefore, we analyzed bones from WT and OPN-deficient mice using several higher resolution approaches. Nanoindentation was used to evaluate the local material properties of tibial cortical bone matrix. The hardness reflects the ability to resist plastic deformation whereas the indentation modulus reflects the ability of the mineral/protein composite to resist elastic deformation. Although bone matrix hardness

did not differ between wild-type and OPN-deficient mice, the indentation modulus for OPN-deficient mice was reduced to 20.3 ± 1.0 GPa compared to 23.9 ± 1.7 GPa ($p < 0.05$, Fig. 4A). The 15% decrease in indentation modulus in OPN-deficient mice is consistent with the recent observations of Kavukcuoglu et al. [57].

Increased calcium variability in osteopontin-deficient bone matrix

Defects in either the mineral or the organic matrix can alter the indentation modulus. To relate the changes in local indentation modulus to changes in local mineral content, cross-sections of wild-

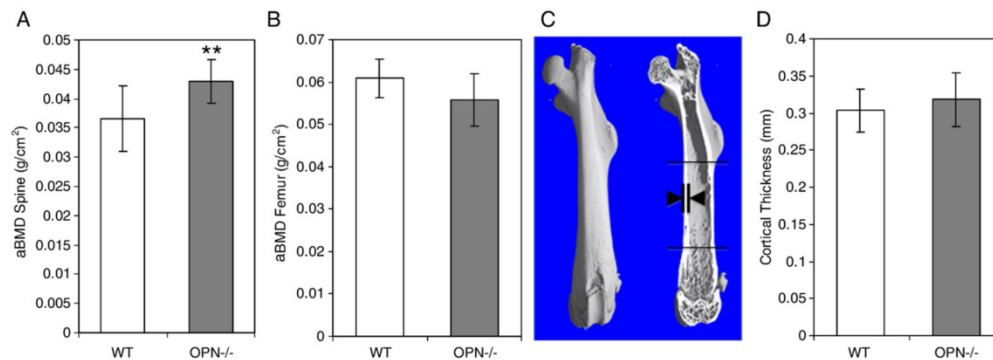


Fig. 2. Femoral bone mineral density and cortical thickness are not affected by OPN deficiency. DEXA analysis of dissected spines (A), but not femora (B), showed OPN-dependent differences in areal bone mineral density (aBMD) (** $p < 0.01$). As shown by the arrows in panel C, micro-computed tomography data of dissected femora from OPN^{-/-} mice and their wild-type littermates were used to determine cortical thickness (D).

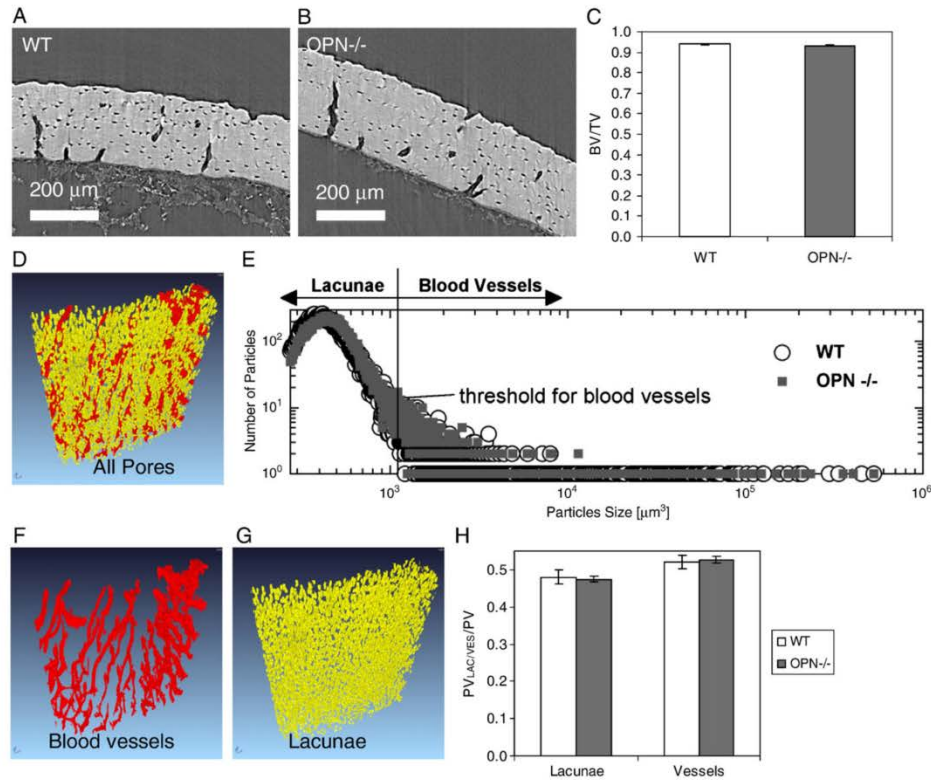


Fig. 3. Cortical bone porosity is not affected by OPN deficiency. Synchrotron radiation micro-computed tomographic (SRμCT) analysis of cortical femoral bone from OPN-/- mice (B) and wild-type littermates (A) showed no differences in bone volume (BV/TV) (C). The size distribution of cortical bone pores was assessed in wild-type (open circles) and OPN-/- mice (gray squares) using reconstructed images (D, E). Based on particle size, pores were segmented as blood vessels (F) or osteocyte lacunae (G). Quantitative analysis of lacunar pore volume (PV_{lac}/PV) and blood vessel volume (PV_{ves}/PV) showed no OPN-dependent differences (H).

type and OPN-deficient tibial cortical bone were evaluated by quantitative backscattered electron imaging (qBEI). This approach was chosen because it queries bone matrix at the same length scale as

nanindentation; nanindentation probes matrix material properties in the superficial 4 μm, whereas qBEI, at 20 keV electron energy, quantifies the mineral concentration of the superficial 2 μm of the section [58]. Figs. 5A and B show representative qBEI images from wild-type and OPN-deficient bone matrix, each of which approximately corresponds to the sites of nanindentation. Interestingly, significant differences in average matrix calcium concentration were not apparent (Fig. 5C). However, the average full width at half maximum (FWHM) of the bone mineral density distribution curves was increased by 5% to 2.89 ± 0.06 wt.% Ca in OPN-deficient bone compared to 2.76 ± 0.05 wt.% Ca in the WT bone, demonstrating an increase in calcium variability (Fig. 5D). Therefore, OPN-deficient bone matrix has a significantly higher variability of calcium concentration. Though not significantly different, a similar trend was observed by SRμCT (Fig. 5E).

A combination of *in vitro* and *in vivo* studies has implicated OPN in the control of mineral crystal maturity and size [23,53,54,59]. Therefore, we hypothesized that OPN deficiency might have more significant effects on crystal organization and size than it did on mineral concentration. Small angle X-ray scattering (SAXS) was performed to evaluate the effect of OPN deficiency on bone matrix mineral organization. In SAXS, changes in the symmetry of the diffraction pattern indicate altered organization of the matrix,

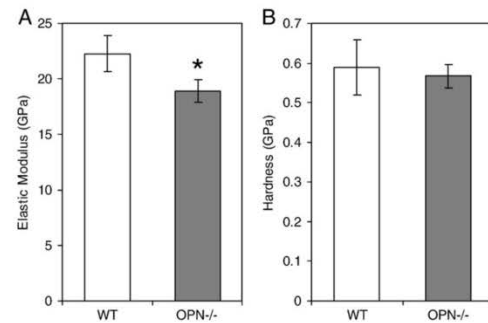


Fig. 4. OPN deficiency impairs the elastic modulus of cortical bone matrix. Cross-sections of polished mid-tibial cortical bone matrix were assessed by nanoindentation to determine elastic modulus (A) and hardness (B) (* $p < 0.05$).

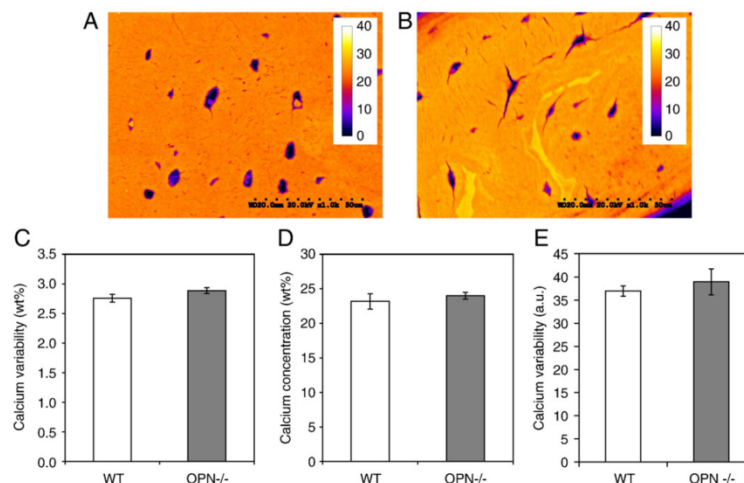


Fig. 5. OPN deficiency increases mineral heterogeneity. Bone specimens equivalent to those in Fig. 4 were assessed by quantitative backscattered electron imaging to evaluate local variation in matrix mineralization in wild-type (A) and OPN^{-/-} (B) mice. Mineral concentration in bones was calibrated to standards to produce a pseudo-color scale, with yellow regions indicating the highest mineral concentration (given in wt%). Though no differences in mineral concentration were detected (C), OPN^{-/-} bone matrix had significantly increased mineral heterogeneity (D) (**p* < 0.05). A similar, however, insignificant trend in mineral heterogeneity was also detected in the samples imaged with synchrotron radiation micro-computed tomography (SRμCT) (E).

whereas the *t*-parameter can be used to determine crystal thickness. Osteopontin-dependent differences were not apparent either in the distinct diffraction features originating from the collagen D-banding or in the ellipsoid pattern originating from the alignment of the mineral crystals along the long axis of the bone (Fig. 6A). Fig. 6B shows the quantitative analysis of mean crystal thickness in OPN-deficient bone relative to WT matrix.

To examine the effect of OPN deficiency on bone matrix organization, transmission electron microscopy studies were performed. However, these studies did not provide conclusive evidence with regards to differences in collagen organization (Fig. 6) in OPN-deficient bone or changes in apatite length, collagen fibril width, or collagen fibril banding (Table 1). So while bone matrix did show increased heterogeneity in qBEL images (Fig. 5) a similar increase in heterogeneity could not be detected at the ultrastructural level.

Tension testing of demineralized osteopontin-deficient bone matrix

The subtle differences in matrix mineralization in OPN-deficient mice suggested that the OPN protein itself might contribute to the mechanical properties of bone. *In vitro* studies have shown that OPN can form self-healing networks with very high toughness that might be important for the material properties of bone, independently of the role of OPN in regulating mineral deposition [28,60]. To examine the role of OPN in bone matrix material properties in the absence of mineral, test specimens were cut in a standard geometry from demineralized femora of wild-type and OPN-deficient mice [19]. Specimens evaluated in tension tests showed no differences in ultimate peak tensile strength or elastic modulus (Fig. 7). Although an intrinsic contribution of the OPN protein to the mechanical behavior of bone matrix was not detectable at the macro-scale, studies on smaller scale specimens or in different geometries may be more informative.

Discussion

By combining approaches that assess the material properties and composition at different levels of bone hierarchy, the present results reveal that OPN participates in establishing bone matrix material properties. Most notably, OPN deficiency results in 30% decreased fracture toughness, suggesting an important role for OPN in impeding crack propagation. This marked decline in fracture toughness was independent of changes in whole bone mass, structure, or matrix porosity. The search for mechanisms potentially responsible for the loss of fracture toughness revealed that OPN-deficient cortical bone has a significantly reduced indentation modulus. Though the reduction in modulus did not correspond to local changes in mineral concentration, the mineralization of OPN-deficient bone matrix was more heterogeneous. Some of these features resemble the compositional and mechanical properties of osteoporotic bone matrix, which also exhibits a significant loss of toughness [18]. In conclusion, we find that OPN is essential for the fracture toughness of bone. By exploring the effects of OPN deficiency on bone matrix material properties, composition and organization, this study narrows the search for mechanisms by which OPN toughens bone, and how toughness is lost in skeletal disease such as osteoporosis.

The results of this study extend and confirm previous observations that OPN plays a critical role in the mechanical behavior of bone. The reduced fracture toughness in the current study likely contributes to the decreased work to failure observed by Duvall et al. [29]. Work to failure indicates the ability of bone to resist both crack initiation and crack propagation, whereas our fracture toughness measurements evaluate resistance to crack propagation. Since microcracks invariably pre-exist in bone, the latter measurements represent a more appropriate measure of resistance to fracture. Therefore, at least part of the impaired work to failure is due to the role of OPN in impeding crack propagation. Similar to Kavukcuoglu et al. [57], our nanoindentation studies showed a decrease in elastic modulus. Although Duvall et al., reported an increase in elastic

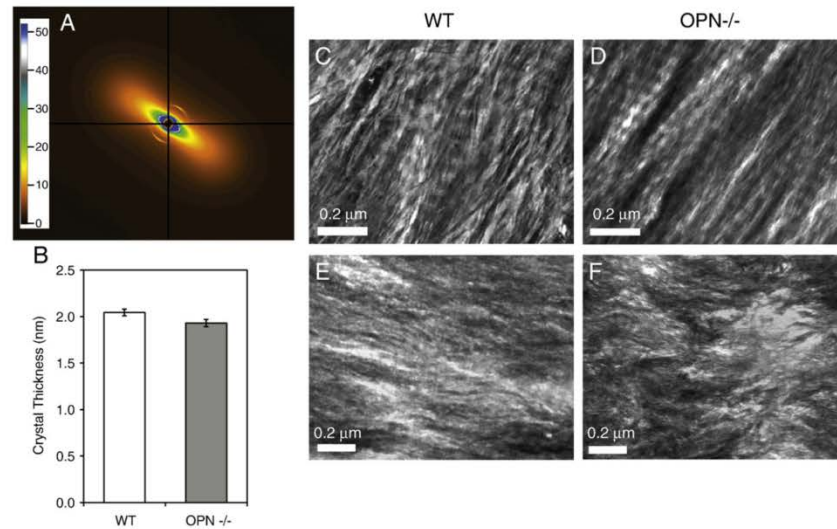


Fig. 6. Analysis of mineral and collagen size and organization. Panel A shows a representative diffraction pattern from small angle X-ray scattering analysis, which showed trends ($p = 0.09$) towards lower crystal thickness in OPN-deficient (1.930 ± 0.039 nm) vs. WT (2.044 ± 0.036 nm) tibial cortical bone (B). Representative images of samples prepared by focused ion beam (FIB) milling (C, D) showed no differences in collagen organization; but those prepared by ultramicrotomy showed an increase in matrix disorganization in OPN-/- femoral cortical bone matrix (F) relative to wild-type (E).

modulus in OPN-deficient bone matrix, this value was ascertained in whole bone three-point bending studies. Differences between elastic modulus values measured by nanoindentation and those acquired by three-point bending have previously been reported [61]. Such differences can be explained by the anisotropic properties of bone and the contribution of hierarchical structural elements that influence mechanical behavior when going from the nano- or micrometer level to the whole bone. While elastic modulus and fracture toughness are related, the dependence of elastic modulus on fracture toughness is not linear, which suggests that the smaller decrease in elastic modulus in OPN-deficient bone alone cannot explain the detected decrease in fracture toughness.

The reduced fracture toughness of OPN-deficient bone is also not explained by structural changes. Fracture toughness tests evaluate the material properties of cortical bone, which showed no difference in cortical thickness or bone volume. The fact that we did not detect such changes in cortical bone suggests that growth and development in OPN-deficient mice is normal, which is in accordance with previous communications [62]. In contrast, spine aBMD, was increased. aBMD is a combined measure of both structure and tissue mineral content and represents, in the case of the spine, primarily trabecular bone, due to its high volume fraction in vertebrae. Trabecular bone is most sensitive to changes in osteoclast activity, which is significantly affected by OPN deficiency [55]. Another important structural

parameter to consider in cortical bone is the porosity and pore size distribution. Pores detected by SRμCT may result from lacunae, vasculature or microdamage [47,63]. Marked changes in pore size distribution or organization could critically influence the ability of cortical bone to resist crack propagation. As our data show, however, no such changes are present in OPN-deficient femora suggesting that the effect of OPN on fracture toughness is not due to changes in the size distribution or organization of pores in cortical bone.

Progressing to the micrometer length scale, qBEI and nanoindentation provide information on local mineral content and material properties. Although local elastic modulus typically correlates with the local mineral concentration, the decreased elastic modulus in

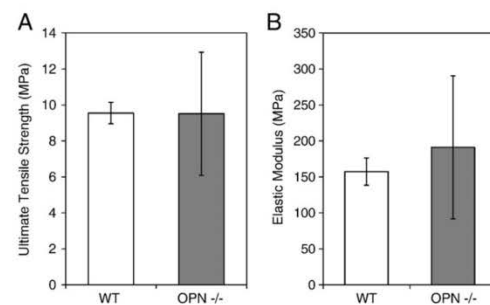


Fig. 7. OPN deficiency does not compromise the tensile strength of demineralized bone matrix. Tensile testing of specimens of demineralized femoral bones showed no difference in tensile strength (A) or elastic modulus (B).

Table 1
Quantitative analysis of apatite and collagen by transmission electron microscopy.

Variable (nm)	Wild-type	OPN-deficient
Apatite thickness	2.26 ± 0.38	2.16 ± 0.48
Collagen fibril width	79.14 ± 2.20	78.8 ± 2.33
Collagen banding	67.3 ± 2.42	65.1 ± 2.67

OPN-deficient bone matrix was not accompanied by a reduction in calcium concentration. Instead, QBEI detected a significant increase in calcium variability in OPN-deficient bone matrix. Interestingly, this increased heterogeneity of OPN-deficient bone corresponds to recent observations by Busse et al. [18]. These authors investigated the properties of single trabeculae from bone biopsies of healthy and osteoporotic patients. They reported an increase in mean calcium concentration and heterogeneity in osteoporotic trabeculae, which was accompanied by a decrease in elastic modulus and energy to failure, as determined from three-point bending tests. Furthermore, Grynpas et al. observed a marked decrease in certain noncollagenous protein fractions in osteoporotic bone [64]. The extent to which changes in osteoclast activity in OPN-deficient mice, or in osteoporosis, are responsible for the altered bone matrix material properties remains unclear and is an important area for future research. Collectively, current studies suggest that loss of noncollagenous proteins such as OPN may contribute to osteoporosis by compromising bone matrix material properties.

Although fracture toughness can be affected by matrix material properties, bone structure, porosity, matrix mineralization or organization [12,52,65], this study shows that these leading potential mechanisms are insufficient to explain the reduced fracture toughness in OPN-deficient mice. An attractive area for future research will be investigating the role of cross-linking of collagenous and noncollagenous extracellular matrix proteins in the reduced fracture toughness of OPN-deficient mouse bone. Studies on diabetic, osteoporotic and aging bone highlight the potential role of collagen cross-linking in fracture toughness [9]. Transglutaminase 2 cross-links several noncollagenous proteins in bone, including osteopontin [66,67]. However, the extent to which defects in matrix protein cross-linking contribute to the reduced fracture toughness of OPN-deficient mouse bone, or in the altered size-independent mechanical properties of bones from mice exposed to physical exercise or high-fat diet [68–70], remains to be fully elucidated.

We hypothesized that the ability of OPN to form self-healing protein networks [28,60] may contribute to fracture toughness. OPN and other noncollagenous proteins, are abundant at interfibrillar interfaces and lamellar/cement lines [31–34]. Microscopic and mechanical investigations have shown that bone generally fails at these well defined interfaces, either on the single mineralized collagen fibril level or at the lamellar/cement line [35–37,39,52,71]. Therefore it can be argued that variability, such as compositional variations, at these interfaces may influence fracture behavior and hence fracture toughness. The possibility that OPN deficiency causes interfacial weakening and a lowered fracture toughness merits further investigation. Such a mechanistic analysis of fracture behavior could be achieved using a combination of imaging and fracture toughness testing, as in two recent studies [52,63]. In this light, it might have also been beneficial to perform tension tests perpendicular to the long axis of the bone. Due to technical limitations of sample preparation, however, this was not possible. Consequently, this study does not exclude a direct role for OPN in influencing the fracture toughness. More advanced sample preparation methods, such as laser microdissection of bone specimens [72], may enable analysis of specific bone interfaces. In addition, more advanced multiscale indentation testing, previously reported on cartilage [73], might enable the investigation of fibrillar and interfibrillar mechanical properties in demineralized bone samples.

The expression of OPN and other noncollagenous proteins, such as osteocalcin, is highly regulated throughout osteoblast differentiation. Both factors that regulate bone matrix material properties, TGF- β and glucocorticoids, also regulate OPN expression *in vitro* [74]. We hypothesized that OPN may be a downstream target of TGF- β in the control of bone matrix material properties. However, the expression of OPN was not significantly altered in neonatal, 2-month old, or 6-month old transgenic mice (data not shown) that express a dominant negative

TGF- β type II receptor and have increased bone matrix material properties. The downstream targets of the TGF- β and glucocorticoid pathways that are responsible for the changes in bone matrix material properties remain unknown. Since these properties are biologically regulated, anatomically distinct, and important aspects of bone quality, identifying the mechanisms by which they are established is a critical area for future investigation.

In conclusion, we find that OPN deficiency significantly impairs the ability of bone to resist the propagation of incipient cracks, accompanied by a decrease in matrix elastic modulus and increased calcium variability. Whether the interfacial strength of micro- and nanostructural features such as cement lines, interlamellar boundaries or mineralized collagen fibrils is also impaired can neither be proven nor falsified at this stage and requires further study. Importantly, our results suggest that osteoporotic and OPN-deficient bone share some common hallmarks; in both cases, local mineral distribution is altered and leads to increased heterogeneity. Hence, the possibility of using noncollagenous proteins such as OPN as biomarkers for the mechanical competence of bone should be further investigated.

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Tissue-specific calibration of extracellular matrix material properties by transforming growth factor- β and Runx2 in bone is required for hearing

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Physical cues, such as extracellular matrix stiffness, direct cell differentiation and support tissue-specific function. Perturbation of these cues underlies diverse pathologies, including osteoarthritis, cardiovascular disease and cancer. However, the molecular mechanisms that establish tissue-specific material properties and link them to healthy tissue function are unknown. We show that Runx2, a key lineage-specific transcription factor, regulates the material properties of bone matrix through the same transforming growth factor- β (TGF β)-responsive pathway that controls osteoblast differentiation. Deregulated TGF β or Runx2

function compromises the distinctly hard cochlear bone matrix and causes hearing loss, as seen in human cleidocranial dysplasia. In Runx2^{+/-} mice, inhibition of TGF β signalling rescues both the material properties of the defective matrix, and hearing. This study elucidates the unknown cause of hearing loss in cleidocranial dysplasia, and demonstrates that a molecular pathway controlling cell differentiation also defines material properties of extracellular matrix. Furthermore, our results suggest that the careful regulation of these properties is essential for healthy tissue function.

Keywords: elastic modulus; Runx2; hearing; TGF- β ; bone quality

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INTRODUCTION

Tissues have characteristic material properties that arise largely from their extracellular matrices (ECMs), ranging from stiff bone and enamel (approximately 10⁷ kPa) to compliant brain and skin (approximately 0.1–250 kPa). In addition to their structural roles, the material properties of an ECM are powerful determinants of cell function. Similarly to biochemical signals, properties such as the elastic modulus direct cell differentiation and gene expression. Conversely, subtle changes in material properties of a tissue's ECM disrupt cell proliferation and drive disease progression (Butcher *et al*, 2009). Mechanisms that

regulate ECM material properties are, therefore, important in development and disease, but very little is known about these mechanisms, or the functional roles of ECM material properties in healthy tissues.

The skeleton is an ideal model system in which to define mechanisms that establish ECM material properties and to understand their relationship with tissue-specific function. Healthy bone exhibits a variety of material properties depending on its skeletal location (Currey, 1999). Furthermore, factors that regulate ECM material properties were first discovered in the skeleton, including transforming growth factor- β (TGF β). Alteration of TGF β signalling in mice severely disrupts bone mass and the material properties of bone matrix (Balooch et al, 2005)—including elastic modulus and hardness—which determine the ability of bone matrix to resist deformation. Although the effects of TGF β on bone mass are due to its regulation of osteoblasts and osteoclasts (Mohammad et al, 2009), the cellular and molecular targets of TGF β in the control of bone matrix material properties are not known.

TGF β regulates osteoblast differentiation by targeting the lineage-specific transcription factor Runx2. *In vitro*, TGF β represses Runx2 expression and function through a Smad3-dependent pathway that is essential for TGF β to inhibit osteoblast differentiation (Alliston et al, 2001). The fact that TGF β also represses Runx2 function *in vivo* is supported by the appearance of dysplastic clavicles and open cranial sutures in both *Runx2*^{+/-} mice and in mice that overexpress TGF β 2 in osteoblasts (Erlebacher & Derynck, 1996; Otto et al, 1997). These are characteristics of the human genetic bone syndrome cleidocranial dysplasia (CCD), which results from *RUNX2* haploinsufficiency (Cooper et al, 2001).

Patients with CCD also exhibit hearing loss (Visosky et al, 2003). Hearing loss in human CCD can be sensorineural (SNHL), conductive (CHL) or mixed. CHL occurs when sound cannot reach the cochlea in the inner ear due to problems in the external ear, tympanic membrane or middle ear. By contrast, SNHL results from cochlear, auditory nerve or central nervous system (CNS) defects. In many bone diseases, however, these clinical definitions blur, as in CCD, cochlear otosclerosis, Paget disease and osteogenesis imperfecta (Chole & McKenna, 2001; Hartikka et al, 2004; Monsell, 2004). For example, some cochlear otosclerosis patients exhibit SNHL without obvious damage to the organ of Corti or auditory nerve. Although these diseases highlight a role for bone in normal cochlear function, the mechanisms by which bone abnormalities cause SNHL are poorly understood.

Owing to its specialized structure and function, cochlear bone presents a unique opportunity to study the regulation and role of bone matrix properties. Cochlear bone is protected from normal bone remodelling (Sorensen, 1994) and has been suggested to be the hardest bone in the body, although this assertion is based on clinical experience and intriguing studies of whale ear bone (Currey, 1999). Thus, we hypothesize that the matrix properties of cochlear bone are precisely calibrated and required for normal hearing. To test this hypothesis, we evaluated bone matrix material properties and hearing in two mouse models of CCD, namely *Runx2*^{+/-} mice and D4 mice with increased TGF β expression in osteoblasts. This study has broad implications for other tissues because it allows exploration of the mechanisms by which tissue-specific ECM material properties are established and linked to healthy tissue function.

RESULTS AND DISCUSSION

Bone matrix material properties are anatomically distinct

Although mouse models provide powerful tools with which to investigate the control of ECM material properties, most mouse bones are too small for macromechanical analyses. Nanoindentation measures material properties in the sub-micrometre range independently of bone size, porosity or shape (Rho et al, 1997). Using nanoindentation, we observed significant differences in the matrix elastic modulus of several mouse bones, ranging from the 30-GPa cochlear bone matrix to the 14-GPa calvarial bone matrix (Fig 1A). These data conclusively show that the cochlear capsule and ossicles are stiffer than other bones—as has been postulated previously (Currey, 1999)—and that ECM material properties depend on the anatomical identity of the bone.

Loss of cochlear bone material properties and hearing

How are such differences in ECM material properties established and maintained? We previously identified TGF β as a key regulator of bone matrix material properties (Balooch et al, 2005), but whether TGF β also regulates bone quality in the unusual cochlear bone was unknown. Nanoindentation was used to evaluate cochlear bone from D4 mice that express an activated form of human TGF β 2 in osteoblasts under the control of osteocalcin promoter (Erlebacher & Derynck, 1996). The D4 cochlear bone matrix had decreased elastic modulus and hardness (Fig 1B,C), despite the lack of bone remodelling in the cochlea (Sorensen, 1994).

Presumably, tissue-specific material properties arise under selective pressure. Their functional importance has long been suggested (Currey, 1999), but is difficult to discern independently from other aspects of bone quality. To determine whether the decrease in cochlear bone hardness in D4 mice compromised cochlear function, we tested hearing in D4 and wild-type (WT) mice using the auditory brainstem response (ABR). An elevated auditory threshold, the lowest volume that elicits an ABR, indicates hearing loss. Stimuli included a multi-frequency click and pure tones at 8, 16 and 32 kHz. The D4 mice had significantly elevated ABR thresholds compared with WT (Fig 1D), with mean differences ranging from 13 to 16 dB. Therefore, increased TGF β signalling in bone impaired both the material properties of cochlear bone and hearing, suggesting that the unique material properties of this tissue might be required for function.

Runx2 insufficiency impairs hearing

These observations raised two key questions. First, are defective bone matrix material properties responsible for hearing loss in D4 mice or in human disease? Second, what are the mechanisms by which TGF β defines matrix material properties and hearing? A major clue came from the fact that both D4 and *Runx2*^{+/-} mice have characteristics of CCD; a syndrome linked to loss of Runx2 function (Fig 2A). Another feature of human CCD is hearing loss (Cooper et al, 2001; Visosky et al, 2003), although hearing in *Runx2*^{+/-} mice has not been evaluated. In this study, we observed that *Runx2*^{+/-} mice have significantly higher ABR thresholds than WT mice (Fig 2B), similar to D4 mice. Compound action potentials (CAPs) were recorded to discriminate involvement of the CNS in *Runx2*^{+/-} hearing loss. The elevated CAP thresholds in *Runx2*^{+/-} mice revealed that the CNS is not involved, thereby isolating hearing loss to the peripheral auditory system (Fig 2C).

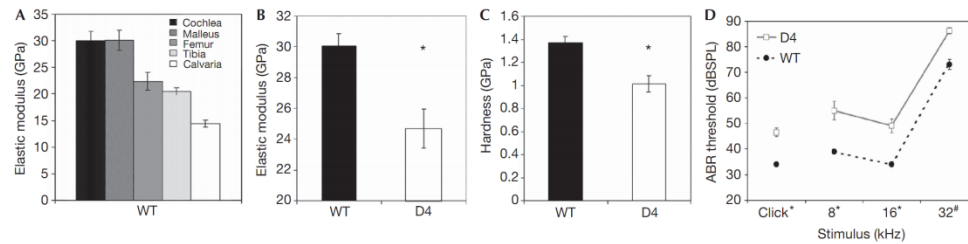


Fig 1 | Transforming growth factor- β overexpression disrupts cochlear bone matrix material properties and hearing. (A) The elastic modulus (GPa) of each bone is distinct. In the cochlear capsule, the (B) elastic modulus and (C) hardness of D4 cochlear bone matrix are reduced relative to WT (* $P < 0.01$). (D) D4 mice (open squares) have elevated ABR thresholds for click and pure tone stimuli relative to WT (filled circles) (* $P < 0.001$; * $P < 0.005$). All error bars represent s.d. values. ABR, auditory brainstem response; TGF β , transforming growth factor- β ; WT, wild type.

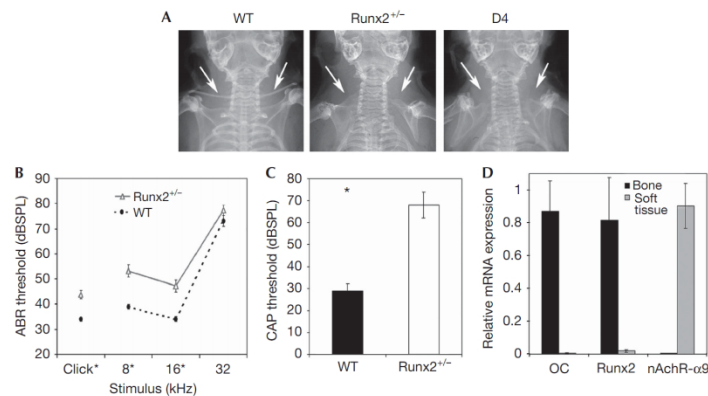


Fig 2 | Runx2 insufficiency in mouse models of cleidocranial dysplasia impairs hearing. (A) X-ray confirmed absent clavicles (arrows) in *Runx2*^{+/-} and D4 mice. (B) *Runx2*^{+/-} mice (open triangles) exhibit higher ABR thresholds than WT (filled circles) (* $P < 0.001$). (C) CAP thresholds (click stimuli) were elevated in *Runx2*^{+/-} mice. (D) Expression of Runx2, OC and nAChR- $\alpha 9$ mRNA in cochlear bone or soft tissue. ABR, auditory brainstem response; CAP, compound action potential; mRNA, messenger RNA; OC, osteocalcin; WT, wild type.

Although Runx2 is typically restricted to mineralizing tissues, Runx2 has also been detected in neurons (Benes *et al*, 2007) and its expression in the cochlea is unknown. Unlike the hair-cell-specific nicotinic acetylcholine receptor $\alpha 9$, Runx2 messenger RNA was expressed only in microdissected cochlear bone or in the developing cochlear capsule (Fig 2D; supplementary Fig S1 online). Runx2 was not expressed by cells that give rise to the supporting or neural structures of the cochlea, demonstrating that the effects of Runx2 haploinsufficiency on hearing loss were due to a bone-intrinsic defect.

Sensorineural and bone anatomy of ears are normal

The CCD-like *Runx2*^{+/-} and D4 mice are valuable tools to study the unexplained cause of SNHL in CCD and other bone diseases. Defects in the organ of Corti structure, hair cells, ganglion cells or other supporting cochlear structures can cause SNHL. However,

no differences in the sensorineural structures of the inner ear were observed in either *Runx2*^{+/-} or D4 mice (Fig 3A,B; supplementary Fig S2 online). Together, three functional hearing tests (ABR, CAP and distortion product otoacoustic emission) localized the hearing defect to the peripheral auditory system and showed that conductive hearing remains largely intact, strongly suggesting a cochlear aetiology for hearing loss (Fig 2B,C; supplementary Fig S3 online).

As bone-intrinsic defects could cause hearing loss, we examined the outer, middle and inner ears of WT, *Runx2*^{+/-} and D4 mice anatomically, radiologically and histologically. For example, *OPG*^{-/-} mice exhibit gross ear bone defects, due to hyperactive osteoclasts, which cause hearing loss similar in magnitude to that in D4 and *Runx2*^{+/-} mice (Kanzaki *et al*, 2006; Zehnder *et al*, 2006). However, our studies found no such defects (Fig 3C,D; supplementary Fig S2 online). Therefore, hearing loss in mouse

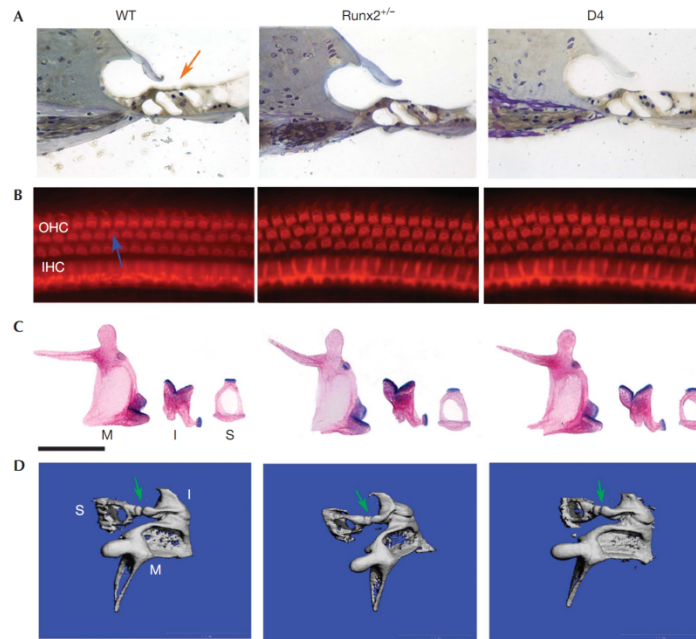


Fig 3 | Mouse models of cleidocranial dysplasia lack discernible defects in cochlear structures. (A) No differences between WT, *Runx2*^{+/-} and D4 cochleae were observed in the organ of Corti (orange arrow), spiral ligament, stria vascularis and spiral ganglia. (B) Phalloidin-stained organ of Corti showed normal organization with one IHC row and 3–4 OHC rows. Stereocilia (blue arrow) are normal in each strain. (C) Dissected ossicles (malleus (M), incus (I), stapes (S)) stained for bone and cartilage showed normal development and mineralization at 6 days of age. (D) Micro-CT shows that each ossicle and the joints (green arrow) between them are intact. Apparent differences are artefacts of imaging at scanner resolution limits. CT, computed tomography; IHC, inner hair cell; OHC, outer hair cell; WT, wild type.

models of CCD is not explained by overt developmental or anatomical defects in the skeletal or sensorineural structures of the ear.

Runx2 regulates ECM material properties and mineralization

Next, we sought to determine whether hearing loss in *Runx2*^{+/-} mice was due to the altered material properties of cochlear bone. Although TGF β regulates bone material properties (Fig 1B,C), the underlying mechanisms for this are unknown. Furthermore, a role for Runx2 or any other transcription factor in the control of ECM material properties has not been reported. Nanoindentation revealed a reduction in the hardness and elastic modulus of *Runx2*^{+/-} cochlear bone, relative to WT (Fig 4A,B). A major determinant of bone matrix material properties is the mineral content, which can be quantified using X-ray tomography (XTM). Using XTM, a shift was shown in the distribution of mineral content of *Runx2*^{+/-} bone matrix relative to WT mice (Fig 4C). The decreased mineralization correlated with the decreased material properties of *Runx2*^{+/-} bone matrix and resembled the

reduced mineral content in D4 bone matrix (Balooch *et al*, 2005). We therefore concluded that, the osteoblast transcription factor Runx2 is a crucial regulator of the material properties and mineralization of bone matrix.

Loss of cochlear ECM quality accounts for hearing loss

Loss of the distinctive hardness of cochlear bone matrix in D4 and *Runx2*^{+/-} mice was the only observed defect that could account for hearing loss in both strains, suggesting that the unique material properties of this tissue might directly affect its function. This was confirmed by a statistical analysis that related the auditory threshold to the elastic modulus of the cochlear bone matrix for the same ear. The elastic modulus correlated with ABR thresholds at 8 kHz, such that for every 1-GPa increase in elastic modulus, ABR thresholds dropped by 1.84 dB (sound pressure level; Fig 4D; $P < 0.01$; $R^2 = 0.77$). This relationship was independent of genotype. Therefore, the defective material properties of D4 and *Runx2*^{+/-} bone matrix correlate significantly with hearing loss.

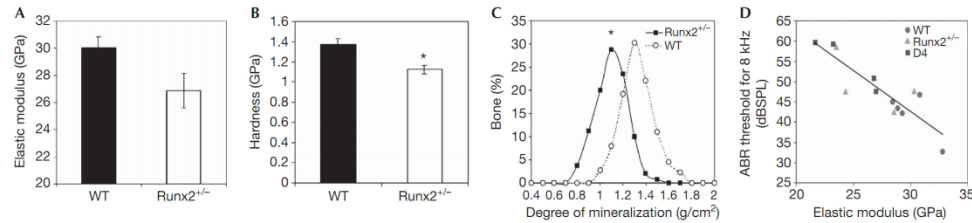


Fig 4 | Cochlear bone matrix material properties are regulated by Runx2 and are essential for hearing. The (A) elastic modulus and (B) hardness of cochlear bone matrix from *Runx2*^{+/-} mice is reduced relative to WT (**P* < 0.01). (C) Reduced mineralization of *Runx2*^{+/-} tibial bone as assessed by XTM. (D) Multiple linear regression analysis comparing elastic modulus with ABR thresholds for each ear shows that elastic modulus is a critical determinant of auditory function (*P* < 0.01, *R*² = 0.77). ABR, auditory brainstem response; WT, wild type; XTM, X-ray tomography.

These results, to the best of our knowledge, provide the first direct evidence that ECM material properties are calibrated for their tissue-specific function. Furthermore, our results strongly suggest that changes in the specialized ear bone matrix material properties account for the unexplained SNHL in CCD. Although additional study is needed to elucidate the precise mechanisms for this, our findings might extend to hearing loss in Camurati-Engelmann disease, osteogenesis imperfecta and Paget disease, diseases which also result from defects in factors linked to bone quality (TGF β , collagen and mineral, respectively; Higashi & Matsuki, 1996; Hartikka et al, 2004; Monsell, 2004; Balooch et al, 2005).

TGF β represses Runx2 to regulate ECM material properties

The similarity of the D4 and *Runx2*^{+/-} bone phenotypes suggests that TGF β and Runx2 act via the same pathway. *In vitro*, TGF β represses Runx2 (Alliston et al, 2001). Although Runx2 mutations that ablate its binding to bone morphogenetic protein- or TGF β -activated Smads cause CCD (Zhang et al, 2000), the regulation of Runx2 by TGF β *in vivo* remains unclear. As shown in Fig 5A, TGF β repressed the expression of the Runx2-regulated genes, *osteocalcin* and *Runx2*, by 50% in D4 calvarial bone, relative to WT. Conversely, decreased TGF β signalling in calvariae from DNT β RII mice—which express a dominant-negative TGF β type II receptor in osteoblasts (Filvaroff et al, 1999)—increased *Runx2* and *osteocalcin* messenger RNA levels (Fig 5B; supplementary Fig S4 online). These results indicate that TGF β represses Runx2 function *in vivo* and *in vitro*.

Although transcription factors respond to growth factors in order to regulate gene expression and differentiation, their ability to control ECM material properties has not been explored. To determine whether Runx2 functions downstream from TGF β in the control of bone matrix material properties, *Runx2*^{+/-} mice were crossed with DNT β RII mice. We hypothesized that the *Runx2*^{+/-} phenotype would be rescued by the inhibition of TGF β function. In DNT β RII;*Runx2*^{+/-} mice, TGF β inhibition did not rescue the clavicle dysplasia of *Runx2*^{+/-} mice (Fig 5C). However, the reduced elastic modulus and hardness of *Runx2*^{+/-} tibial bone matrix were rescued by inhibition of TGF β signalling in osteoblasts (Fig 5D). Blocking the ability of autocrine TGF β to repress Runx2 function was also sufficient to rescue the defective mineralization of *Runx2*^{+/-} bone. Unmineralized patches, apparent only in *Runx2*^{+/-} bone, were absent in DNT β RII;*Runx2*^{+/-} bone matrix (Fig 5E). Therefore, TGF β regulates

mineralization and matrix material properties through the same pathway that it uses to control cellular differentiation (Alliston et al, 2001), by repressing the activity of the lineage-specific transcription factor Runx2.

Rescue of CCD-associated hearing loss

To determine whether the rescue of the defective material properties of *Runx2*^{+/-} bone was sufficient to restore hearing, we examined the hearing of DNT β RII;*Runx2*^{+/-} mice. As expected, ABR thresholds for *Runx2*^{+/-};WT mice were higher than those for DNT β RII;WT or WT;WT mice (Fig 5F). Importantly, inhibition of TGF β signalling in osteoblasts of DNT β RII;*Runx2*^{+/-} mice rescued the hearing loss in *Runx2*^{+/-} mice, such that ABR thresholds were indistinguishable from WT. The ability of an osteoblast-specific decrease in TGF β function to rescue the *Runx2*^{+/-} hearing loss further supports the bone origin of hearing loss associated with CCD. Furthermore, the rescue of hearing loss by restoration of bone matrix material properties supports our conclusion that precise regulation of ECM material properties is essential for healthy tissue function.

Tissue-specific calibration of ECM material properties

We investigated the mechanisms by which the distinctive material properties of a tissue are established and linked with its physiological function. Using bone matrix as a model, we identified Runx2 as a lineage-specific transcription factor that establishes ECM material properties through the same TGF β pathway that controls osteoblast differentiation. This role of TGF β and Smad3 extends beyond mineralized tissues, as Smad3 also controls skin ECM properties (Arany et al, 2006). Just as TGF β regulates Runx2 function, it regulates other lineage-specific transcription factors to direct the expression of tissue-specific ECM proteins, which in turn might influence ECM material properties. Therefore, this study elucidates the control of bone matrix quality and the role of bone in hearing. It also suggests a new paradigm in which signalling pathways and lineage-specific transcription factors cooperate to define the functionally essential ECM material properties of a specific tissue.

METHODS

Mice. The generation and skeletal abnormalities of D4, *Runx2*^{+/-} and DNT β RII mice have been described previously (Erlebacher & Derynck, 1996; Otto et al, 1997; Filvaroff et al, 1999). Mice

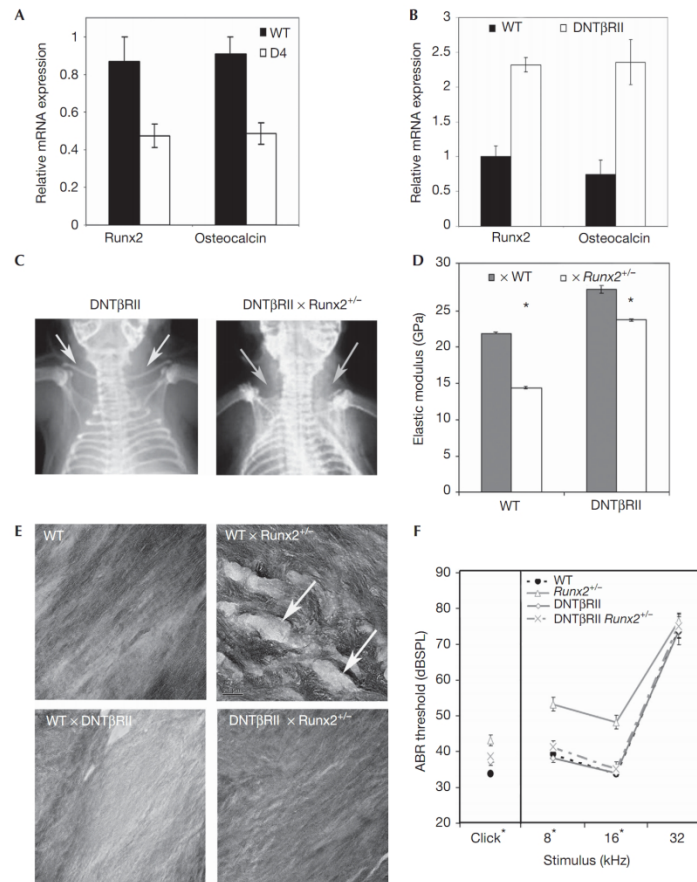


Fig 5 | Defective *Runx2*^{+/-} bone matrix material properties, mineralization and hearing are rescued by inhibition of transforming growth factor- β . Quantitative reverse transcriptase-PCR shows the effect of (A) elevated TGF β in D4 mice and (B) TGF β inhibition in DNT β RII mice on *Runx2*-target gene expression in calvarial bone. X-rays, nanoindentation and TEM show that (C) clavicle dysplasia is not, but (D) elastic modulus ($*P < 0.001$) and (E) mineralization are rescued in DNT β RII;*Runx2*^{+/-} mice. (E) Arrows show sites lacking mineralization as detected through TEM. (F) ABR thresholds for *Runx2*^{+/-} (triangles), DNT β RII (diamonds) and DNT β RII;*Runx2*^{+/-} (crosses), or WT mice (filled circles) are shown ($*P < 0.05$). ABR, auditory brainstem response; TEM, transmission electron microscopy; TGF β , transforming growth factor- β ; WT, wild-type.

were bred on a B6D2 $\times$ C57/Bl6 background. WT littermates were used as controls. Procedures were approved by the University of California San Francisco Institutional Animal Care and Use Committee.

Auditory function. Evoked auditory brainstem response thresholds, compound action potentials and distortion product otoacoustic emissions were measured in 2-month-old male mice ($n \geq 4$; Akil *et al*, 2006; Seal *et al*, 2008). Statistical analysis used one-way analysis of variance ($P < 0.05$).

Histology and analysis of gene expression. Cochlear histology studies were carried out on $n \geq 3$ mice (Akil *et al*, 2006). Qualitative hair cell analysis was performed on dissected cochleae by microscopic visualization of rhodamine-phalloidin staining ($n \geq 3$; Seal *et al*, 2008). Dissected ossicles from 1- and 6-day-old mice were stained with Alizarin red and Alcian blue to visualize bone and cartilage, respectively ($n \geq 3$; Otto *et al*, 1997). Gene expression was assessed using RNA isolated from dissected calvariae or cochlear bone or soft tissue ($n \geq 4$; Seal *et al*,

2008; Mohammad et al, 2009). Using quantitative reverse transcriptase-PCR, amplification of Runx2, osteocalcin and nAchR- α 9 were normalized to ribosomal protein L19. Statistical differences were calculated by Student's *t*-test ($P < 0.05$).

Micro-computed tomography. Temporal bones, dissected with intact bulla to preserve the ossicular chain, were scanned by micro-computed tomography ($n = 3$; VivaCT-40, Scanco). Image acquisition consisted of 418 slices encompassing the cochlea at 10.5- μ m voxel size. For each 180°, 1,000 projections were taken. The integration time was 346 ms, with potential of 55 kVp and current of 145 μ A. The images were segmented using a low pass filter, and a threshold (0.7/1/270) was uniformly applied. For three-dimensional reconstruction, cochlear bone was excluded to visualize the ossicular chain in 200–220 slices.

Nanoindentation, XTM and transmission electron microscopy. For nanoindentation, cochleae were cut along the modiolar axis with a scalpel before mounting in epoxy resin. The malleus was mounted with epoxy without cutting or polishing. Using a saw, tibiae and femora were cut to expose mid-diaphyseal cortical bone, whereas calvariae were cut sagittally. Polished sections were nanoindented in load-control by using a Nanoscope III atomic force microscope (Veeco) with a Triboscope indenter head with a Berkovich tip (Hysitron; Mohammad et al, 2009). Indentations in three 30- μ m lines with a 2- μ m step size were placed across the cortical bone surfaces near the apex of the cochlea. Malleus material properties were determined using 10 indents per bone. Loading and unloading rates were 100 μ N/s with a 10-s dwell time. For each bone, calculated elastic modulus or hardness values were averaged (Rho et al, 1997). Statistical analysis used one-way analysis of variance ($P < 0.05$). XTM and transmission electron microscopy were performed as described previously (Mohammad et al, 2009; Thurner et al, 2010).

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Appendix B: Hysitron Nanoindenter Protocol for Measuring Mouse Bones

Sample Preparation

I. Embedding

Materials

- Stycast 1266 (Emerson Cuming)
- Wooden tongue depressors
- Plastic droppers
- Plastic medicine cups
- 22 x 22 mm peel away embedding molds

Procedure

1. Isolate tibiae and store in HBSS with 0.05% sodium azide at 4°C.
2. Weigh out 10 g Stycast 1266 Part A with a wooden tongue depressor into a medicine cup and then add 2.8 g Stycast 1266 Part B with a plastic dropper. This is enough for about 10 tibiae.
3. Mix part A and part B very slowly so as not to introduce bubbles. Mix for at least 1 minute.
4. Pour a layer approximately 3-5 mm deep into the peel away molds and let set overnight, covered with Kimwipes to prevent contamination.
5. The next day, mix up a new batch of Stycast. 10 g part A is enough for 3 tibiae. After mixing, let sit at room temperature for about 45 minutes until it becomes somewhat more viscous to prevent excessive infiltration.
6. Meanwhile, take tibiae out of storage fluid and let dry for about 30 minutes.
7. When the Stycast epoxy is ready, arrange tibiae on previously poured layer of epoxy, being careful to be uniform about positioning, and pour a new layer of epoxy so that the bone is completely covered.

8. Use needles to dislodge bubbles from around the bone – this is to ensure that the bone is securely embedded and that there are no air pockets. Cover with Kimwipes and let set overnight.

II. Sectioning

Materials

- Buehler Isomet low speed saw
- Buehler Diamond wafering blade, catalog no: 11-4245
- Wooden tongue depressors, cut into thirds
- Glue gun
- Dental grinder/polisher
- AFM metal specimen discs (Ted Pella)
- Cyanoacrylate

Procedure

1. Peel the molds off the embedded tibiae and use the glue gun to affix to the end of a wooden tongue depressor so that it can be clamped to the low speed saw. Make sure the position is such that the blade can make cross-section cuts at the mid-diaphysis.
2. Fill the well with water so that the blade is lubricated and cooled.
3. Turn on the saw at a low speed setting, 2-3, and slowly lower the epoxy embedded specimen onto the moving blade. Never start the blade with the specimen on it, as this can cause the blade to warp and chip.
4. Once a small groove has been made in the specimen, the speed of the saw may be slowly increased and small weights may be placed on the specimen chuck to speed the sectioning process.
5. After one cut is made all the way through the block, the micrometer can be used to move the specimen so that a parallel cut may be made. For nanoindentation, a good thickness is 3-5 mm. Make the second cut in the same way as the first.

6. After two parallel cuts have been made, remove the block from the wooden tongue depressor. 6 well cell culture plates are convenient to label and store the cut pieces.
7. The cut section will be too large to mount on the specimen disc, so use the dental grinder to sand away the excess epoxy, being careful not to sand too close to the specimen (or your fingers).
8. Label the back of an AFM specimen disc. Use a very thin layer of epoxy to glue the section, indentation surface up, onto the disc. Use pressure to hold in place for several seconds and then release. The specimen is now ready for polishing. 12 well plates are convenient for labeling and storing the specimens at this point.

III. Polishing

Materials

- 10 glass platens, 1 for each grit and 1 for each diamond suspension
- Buehler 240, 320, 400, 600, 800 and 1200 grit CarbiMet abrasive discs, PSA backed
- Buehler TexMet polishing cloths (1 for each diamond suspension)
- Buehler MetaDi Supreme Diamond Suspensions: 6 μm , 3 μm , 1 μm , and 0.25 μm
- Ultrasonicator
- Squeeze bottle with deionized water
- Dissecting microscope

Procedure

1. Adhere the abrasive discs and four polishing cloths to the glass platens. You should have 10 total.
2. Start with the roughest grit, 240 and squeeze a small puddle of deionized water onto the surface of the disc. Use a figure eight motion and an extremely light touch to sand the nanoindentation surface of the specimen. Too much pressure will cause grooves to form on the surface, which are very hard to polish away.
3. After approximately a minute, use a dissecting microscope to make sure no deep grooves have formed and the surface appears smooth. Rinse the specimen with deionized water, using bare fingers to sweep away remaining grit.

4. Clean the working surface to prevent cross contamination from larger particles, and repeat the polishing with the next roughest abrasive disc, 320 grit.
5. Repeat with all the abrasive discs, checking periodically to ensure that the specimen appears smooth, with large scratches.
6. Ultrasonicate the glass platens with polishing cloths (4) and the specimens. The platens should be sonicate for at least a minute or two, and the specimens may be dipped into the ultrasonicator one at a time for several seconds each. This is to ensure no cross contamination so as to prevent scratches. Make sure the work area is again cleaned and wiped down.
7. Spray the 6 μm diamond suspension onto one glass platen with polishing cloth. Polish the specimen in a figure eight motion with very light pressure. You should be able to do so without feeling the specimen “catch.” If it does not glide smoothly, check for scratches under a dissecting scope and/or decrease the pressure on the specimen. If there are scratches, go back to the abrasive discs and polish until they disappear.
8. Continue with the remaining diamond suspensions, making sure to both rinse with deionized water and ultrasonicate between diamond sizes. At the end, the surface of the specimen should appear shiny and smooth. Make sure there are no remaining scratches using a dissecting microscope.
9. Rinse and ultrasonicate each specimen one final time, dry carefully with a Kimwipe, and store. The specimens are now ready for nanoindentation.

Nanoindentation of Mouse Tibiae

I. Putting in a new tip

Materials

- Spring wrench (comes with Triboindenter)
- Berkovitch indenter tip
- Flat head screwdriver (comes with Triboindenter)

Procedure

1. Before installing the tip, clean carefully by swabbing gently with a fluffed up cotton swab. Do not apply pressure.
2. Turn the tool and controller off.
3. Unplug wire from controller to transducer (in the back).
4. Unplug left plug on transducer. Leave right plug.
5. Hold transducer and loosen right side screw. This is a dovetail connection; the transducer assembly slides out by lifting up.
6. Very carefully place transducer on table with dovetail connection on the surface of the table.
7. While holding the transducer with your left hand and applying slight pressure, carefully use one finger and right hand to guide spring wrench onto tip. Unscrew carefully.
8. Install and tighten the Berkovitch tip in the same manner.
9. Put transducer back – plug in two plugs disconnected in steps 2 and 3, lift halfway (for more clearance to accommodate taller samples) and screw in TIGHTLY.
10. Turn controller and tool on and re-zero load.

II. **Calibrating the Triboindenter**

Materials

- Fused quartz sample
- Single crystal aluminum sample

Procedure

A. Advanced Z-axis Calibration

1. Do advance z-calibration twice by following the instructions on the software.

Before beginning, make sure the displacement gain is set to 100 on both the controller and the software. (On the Pruitt lab Triboindenter, the microscope feedback gain must be set to 100 as well.) After each iteration, check to make sure the fit for the electrostatic force constant is reasonable.

2. Before continuing, the displacement and microscope feedback gains must be set back to 1000. This is what the setting should be for the remaining calibrations. For shallow indents, on the order of nanometers, the displacement gain should be set at 1000. For deeper indents on the order of micrometers, like in soft materials, the displacement gain should be set at 100. Make sure that both the software and the controller are set to the same value.

B. Tip-Optics Calibration

1. Place the single crystal aluminum sample on the stage.
2. Go to file → safety limits and define the safety limits for a new sample (aluminum) by placing points around the edges of the sample.
3. Go to set up → calibrate optics → stage optics and then click on New “H” Pattern.
4. Focus on the location on the aluminum standard to place the calibration pattern. Any relatively clean area will work.
5. The software will prompt you to set up a load function. Set up triangle load function with a peak load of 8000-10,000 μN to make a visible indent on the aluminum.
6. The Triboindenter automatically moved the tip to the location specified and then prompts the user to lower the tip manually until it is within 1 mm away from the surface. Be very careful when moving the tip manually, since the safeties are disabled for this calibration.
7. The Triboindenter then makes seven indents automatically. This can take ~ 20 minutes.
8. Once the indents are complete, the Triboindenter moves so that the “H” pattern is visible under the optics. Focus the optics, place the crosshairs on the center indent of the “H” pattern and click “OK.”

C. Machine Compliance

1. Place fused quartz sample on stage.

2. Define the safety limits and do a quick approach. Note: never do a quick approach unless the tip-optics offset has been calibrated. Neglecting to do so may cause the tip to crash and irreversible damage to occur.
3. Make 15 indents on the sample using maximum loads from 9000-5000 μN . Always start with the highest load to minimize thermal drift errors. The load function should have a 10 second loading time, a 3 second dwell, and a 10 second unloading time.
4. Run Multiple File Analysis on the data set taken.
5. Open the text file created in Excel. Delete columns E-L.
6. In column E, create $1/\sqrt{\text{load}}$, the load data is in column C.
7. In column F, create the measured compliance data, which is the inverse of the data in column D.
8. Select columns E and F and insert an XY Scatter chart. Add a linear trendline and display the equation. The y-intercept is the compliance in $\mu\text{m/mN}$. Multiply this value by 1000 to get the compliance in nm/mN , which can be entered in the transducer constants page in the software.

D. Tip Area Calibration

1. Use the same sample used for machine compliance calibration.
2. Make 100 indents on the sample using loads from 9000-100 μN , using the same loading function as used in the machine compliance calibration. This takes several hours, so it is best to run it overnight.
3. Run Multiple File Analysis on the data set taken, omitting the first and last five data points.
4. Select Calculate Area Function from the analysis menu and select the text file created from the data when prompted and press "Load."
5. Enter the reduced modulus of fused quartz when prompted, 69.6 GPa, and press "OK."

6. A screen with the tip area function data points will appear. Use the blue and red cursors to select the portion of the graph to fit and then click “Execute Area Fit.”
An overlay of the area function will appear on the graph with the data points.
7. Click the icon below “Save Area Function” and it will automatically be input into the software and is ready to be used to analyze data.

III. Quasi-Static Measurements of Mouse Tibiae

Materials

- Embedded, sectioned and polished samples

Procedure

1. Define safety limits and do a quick approach. Focus on the area to indent.
2. Use imaging controls to make a scan of the region of interest. Engage the indenter tip and make a 50 μm scan.
3. Select Piezo Automation and click on “Click Script.” Use the mouse to select locations to place indents. Look for areas that are flat and smooth with no lacunae. Leaving $\sim 5 \mu\text{m}$ between each indent ensures that the stress fields do not overlap.
4. For bone, use the load control mode to specify the load function. The load function used in these studies is 100 $\mu\text{N/s}$ load and unload rates and 10 second dwell, with a maximum load of 600 μN .
5. Using Multiple Curve Analysis to analyze segment 3 (unloading portion) of the data to generate a text file with indent depths, reduced moduli, and hardness values. Calculations and theory are detailed in Chapter 3, Nanoindentation.

Appendix C: Asylum MFP3D-BIO AFM Protocol for Force Curve Measurements

I. Calibration for force measurements – use a clean glass slide

A. Engage (contact mode engage)

1. Switch the imaging mode to contact.
2. Enter the set point, 1V works for most tips.
3. Click on engage.
4. Watch the Z-voltage (should rail at +150V) and slowly move the front wheel on the head down (counter-clockwise as viewed from above) until you hear a beep and the Z-voltage starts to move. Keep moving the front wheel down slowly until the Z-voltage is somewhere near +70.
5. Click on the withdraw button to move your tip back away from the surface.

B. Setting the InvOLS – scales deflection to distance

1. Set force distance to $\sim 1 \mu\text{m}$. This will depend on how sticky the surface and tip are. You want to have the distance large enough so that you can pull the tip off the surface. In air, you may want to use 3-4 μm .
2. Set the trigger channel to deflection volts.
3. Set the trigger point to +2V, click positive slope and absolute.
4. Use the photodiode knob so that the deflection away from the surface reads -2V. This is done because you want to have a nice linear region around 0 volts to fit to a straight line. The photodiode is most linear around 0 V, which is where the thermal data is collected. Close and latch the isolation enclosure.
5. Go to the Force tab and do a single force with a tip velocity of 2 $\mu\text{m/s}$. You should see that the force plot hits the surface.
6. On the deflection volts vs. LVDT graph, move the cursors so they are in the contact region of the force plot at about -1V to 1V
7. Make sure they are either both on the extend or retract portion of the curve. Hit the left or right arrow key, both cursors should move the same direction. If they

move towards or away from each other, they are not on the same part of the curve. Zoom in on one of them and move one of the cursors so it is on the same part of the curve.

8. Go to Force → Calibration → Set Sensitivity → InvOLS.
9. Make sure the black line on the deflection trace is a decent fit.

C. Setting the spring constant: Thermal Method

1. Click on the withdraw button.
2. Use the photodiode knob to zero the deflection.
3. Click on “Do Thermal.”
4. Wait awhile for some data to collect, about 50 curves.
5. Click on stop thermal.
6. On the thermal graph, draw a box around the thermal peak. It is generally the highest and first (low frequency) peak. In liquids, this peak can be quite broad. Right click in the box you dragged and click “ZoomZoom”.
7. Click “Fit Guess.” This should show you the a blue “fit guess” line. This should roughly describe the data, with the peak at the right frequency and the width and amplitude close to the actual.
8. Click “Try Fit” next to actually fit the data. This should then make the blue line fit with the collected data. If the fit does not describe the data well, click the button a few times to see if the fit is any closer. If the fit does not describe the data well at all, go to the thermal panel and change the fit width to something that only includes the peak and try the “Fit Guess” and “Try Fit” again.

II. Force measurements in wet conditions

A. Making an indent

1. Do InvOLS and thermal in air as described above to get the spring constant and then do InvOLS again in fluid (the laser path changes due to diffraction, so the InvOLS needs to be calibrated in fluid) to get accurate depth measurements.

2. If cantilever is dry and sample is wet, pick up the AFM head and place a few drops of fluid around the cantilever cantilever and then carefully invert on pad to prevent drips. Next, place tip onto sample with the tip up. This will prevent the bending of the tip when it goes into fluid. For example, biolevers, often used for soft hydrated samples, have a very spring constant and are very flexible. Thus, they will often fold when immersed into water unless wetted first.
3. Engage the tip as described above.
4. For the polyacrylamide gels used in our studies, we used 10 nN trigger and 1 $\mu\text{m/s}$ for both the approach and retract velocities.

B. Data analysis

1. Data analysis modules are included in the software. Click review and select the data set for the desired indent.
2. Select “Force” for the y-axis and “Ind” for the x-axis to get a load-displacement plot. Go to the Analysis tab and use click-shift to choose the locations where you want to fit the Hertz equation on the extend portion of the plot.
3. Go to the Elastic tab and then to the Hertz tab. Fill in the tip geometry parameters: for the polyacrylamide gels, we selected a spherical indenter with a radius of 2.5 μm . Then click “Fit” and the module will output the elastic modulus.

Appendix D: Primary Tendon Fibroblast Isolation Protocol

Materials and Preparation

- Coat 6-well cell culture plate with **rat tail type I collagen** [0.15 mg/mL in 0.02M acetic acid] (BD BioSciences).
 - Add enough to cover the bottom of the well (~1mL), swirl to ensure coverage, and let sit with lid on for 1 hour in tissue culture (TC) hood.
 - After 1 hour, aspirate wells with pipetaid. The collagen can be reused up to 6x. Store collagen in 4°C.
 - Wash 2x with **D-PBS CMF** (w/o calcium and magnesium salts), aspirate.
- Thaw/Warm
 - **FBS** (4 ml)
- Digestion medium (4 ml)
 - **collagenase P** [0.1 mg/mL] (Roche)
 - **trypsin/EDTA** [2.5%, Type IX-S, from Porcine pancreas] (Sigma)
 - **αMEM**
- Dissection
 - 2 60 mm cell culture dishes with **tendon cell culture media** (αMEM w/L-glutamine, 20% FBS, 1% penicillin:streptomycin (pen-strep)) on ice
 - 2 60 mm cell culture dishes with **D-PBS with pen-strep** (w/o calcium and magnesium salts) on ice
 - 1 cell culture dish for fluffing and cutting tendons
 - 1 - 1.5mL eppendorf with 1mL tendon cell culture media for tendon storage on ice
 - Surgical tools in 70% EtOH: #11 scalpel, curved dissecting scissors, spring-loaded scissors, forceps

Procedure

- Tendon Isolation

- Sacrifice mouse and obtain hind FDL tendons. Once isolated from the mouse, place tendon in tendon cell culture media in 60 mm cell culture dish for temporary storage. When finished isolating all tendons, transfer to clean 60 mm cell culture dish with tendon cell culture media, and under a dissecting microscope, clean tendon of epitenon and excess tissue. After cleaning, swirl tendon in D-PBS w/pen-strep (2x) to rinse clean.
- Place tendon in microcentrifuge tube with 1 ml tendon cell culture media; place all cleaned tendons in this tube.
- Once all tendons have been isolated, dump the contents of the microcentrifuge tube into a clean 60 mm cell culture dish. Under dissecting microscope, slice tendons into smaller pieces and slice longitudinally to “fluff” them up. This allows cells to escape matrix more easily. Tendons will resemble small pieces of cotton after slicing and dicing.
- In the TC hood, transfer fluffed tendons to 15mL conical tube using a p1000 pipette with pipette tip end cut with sterile scissors to allow a larger opening (avoids pieces of tendon getting stuck in pipette tip).
- Centrifuge 30 seconds @ 1000 RPM. In TC hood, pipette off clear media from top, being careful to avoid taking up tendon pieces, and add 4 ml digestion media, swirl to mix.
- Tendon Digestion
 - Leave digestion tube at room temperature and agitate by inverting the tube several times every 15 minutes. Do this for 2 hours.
- Plating
 - After 2 hours of digestion, add 4 ml FBS to quench the digestion reaction enzymes. Centrifuge tube for 5 minutes @ 1000 RPM.
 - Aspirate with pipetaid, being careful to avoid disturbing the pellet.
 - Wash and resuspend with PBS. Centrifuge for 5 minutes @ 1000 RPM.
 - Aspirate with pipetaid, do not disturb pellet.

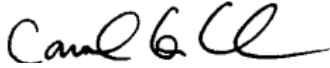
- Resuspend with 2 ml tendon cell culture media and plate in 1 well of the collagen-treated 6-well cell culture plate.
- Maintenance
 - Media should be replaced every 4-5 days.

Publishing Agreement

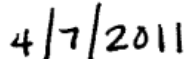
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