Osteocyte intrinsic TGFβ signaling regulates bone quality through perilacunar/canalicular remodeling

Neha S. Dole[1], Courtney M. Mazur[1,2], Claire Acevedo[1,3], Justin P. Lopez[1,2], David A. Monteiro[1,2], Tristan W. Fowler[1], Bernd Gludovatz[3], Flynn Walsh[3], Jenna N. Regan[4], Sara Messina[3,5], Daniel S. Evans[6], Thomas F. Lang[7], Bin Zhang[8], Robert O. Ritchie[3,5], Khalid S. Mohammad[4], Tamara Alliston[1,2,*]

[1]Department of Orthopaedic Surgery, University of California San Francisco, San Francisco, CA, 94143
[2]UC Berkeley-UCSF Graduate Program in Bioengineering, San Francisco, CA, 94143

[3]Materials Science Division, Lawrence Berkeley National Laboratory, Berkeley, CA, 94720

[4]Department of Medicine, Indiana University School of Medicine, Indianapolis, IN, 46202

[5]Department of Materials Science and Engineering, University of California Berkeley, Berkeley, CA, 94720

[6]California Pacific Medical Center Research Institute, San Francisco, CA, 94107

[7]Department of Radiology and Biomedical Imaging, University of California San Francisco, San Francisco, CA, 94143

[8]Department of Genetics & Genomic Sciences, Icahn Institute of Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai, New York, NY, 10029

Corresponding Author:
Tamara Alliston, PhD
Department of Orthopaedic Surgery
University of California, San Francisco
513 Parnassus Avenue, S-1155
San Francisco, CA 94143
tamara.alliston@ucsf.edu
415-502-6523
@TamaraAlliston

*Lead Contact

Summary
Poor bone quality contributes to bone fragility in diabetes, aging, and osteogenesis imperfecta. However, the mechanisms controlling bone quality are not well understood, perpetuating the current lack of strategies to diagnose or treat bone quality deficits. TGFβ signaling is a crucial mechanism known to regulate the material quality of bone, but its cellular target in this regulation is unknown. Studies showing that osteocytes directly remodel their perilacunar/canalicular matrix motivated our hypothesis that TGFβ controls bone quality through perilacunar/canalicular remodeling (PLR). Using inhibitors and mice with an osteocyte-intrinsic defect in TGFβ signaling ($T\beta RII^{ocy-/-}$), we show that TGFβ regulates PLR in a cell-intrinsic manner to control bone quality. Altogether this study emphasizes that osteocytes are key in executing the biological control of bone quality through PLR, and thereby highlighting the fundamental role of osteocyte mediated perilacunar/canalicular remodeling in bone homeostasis and fragility.

Keywords

Osteocyte, TGFβ, bone quality, perilacunar/canalicular remodeling, bone fragility

Highlights

- TGFβ is an osteocyte-intrinsic regulator of perilacunar/canalicular remodeling (PLR)
- Osteocytes actively maintain bone quality through regulated control of PLR
- Osteocytic PLR is the cellular mechanism by which TGFβ controls bone quality. Defects in PLR cause severe bone fragility, even when bone mass is normal

eTOC Summary

Resistance to fracture requires healthy bone mass and quality. However, the cellular mechanisms regulating bone quality are unclear. Dole et al. show that osteocyte-intrinsic TGFβ signaling maintains bone quality through perilacunar/canalicular remodeling. Thus, osteocytes mediate perilacunar/canalicular remodeling and osteoclast-directed remodeling to cooperatively maintain bone quality and mass and prevent fragility.

Introduction

Bone fragility is determined by bone mass and quality. Bone quality encompasses parameters including bone geometry, porosity, trabecular microarchitecture, and bone extracellular matrix (ECM) material properties (Hernandez and Keaveny, 2006; Seeman, 2008). Historically the prognosis of fragility fractures has focused on bone mass but it is now known that compromised ECM properties plays a causal role in bone fragility in diabetes, aging, and osteogenesis imperfecta (OI) (Delmas and Seeman, 2004; Fleischli et al., 2006; Grafe et al., 2014; Lane et al., 2006; Nalla et al., 2004; Ott, 1993; Van Staa et al., 2003). Despite the clinical importance of this and other aspects of bone quality, the management of fragility currently focuses on improving bone mass. Overcoming this clinical gap in diagnosing and treating bone quality requires elucidation of mechanisms that orchestrate the biological control of bone quality in skeletal health and disease.

Currently, the transforming growth factor beta (TGFβ) pathway is one of the few signaling pathways known to regulate bone mass and quality (Alliston, 2014; Balooch et al., 2005; Mohammad et al., 2009; Chang et al., 2010; Edwards et al., 2010). In bone, TGFβ produced by bone forming osteoblasts is sequestered in the ECM in an inactive latent form (Sinha et al., 1998). When released upon osteoclastic resorption of the ECM, TGFβ exerts pleiotropic effects on osteoblasts, osteoclasts, and their progenitors to coordinate bone remodeling (Dallas, 2008; Tang and Alliston, 2013). Aberration in TGFβ signaling leads to altered bone mass and poor bone quality in multiple skeletal diseases including Camurati Engelman Disease (CED) and OI (Grafe et al., 2014; Kinoshita et al., 2000). While the pathogenesis of the poor bone quality associated with these diseases have been attributed to imbalanced osteoclast and osteoblast activity, not much is known about the causal role of osteocytes and osteocyte intrinsic TGFβ signaling in bone fragility.

In addition to regulating the activity of osteoclasts and osteoblasts, osteocytes also engage in perilacunar/canalicular remodeling (PLR), during which they directly resorb and deposit bone matrix surrounding their intricate lacuno-canalicular network (Qing and Bonewald, 2009). This process was originally called ‘osteocyte osteolysis’ when it was observed in pathologic conditions, or ‘perilacunar/canalicular remodeling’ in metabolically demanding situations such as lactation or hibernation (Haller and Zimny, 1977; Qing et al., 2012; Qing and Bonewald, 2009; Teti and Zallone, 2009; Wysolmerski, 2013). It is now clear that PLR is a homeostatic mechanism that helps to maintain mineral homeostasis and the lacuno-canalicular network. Several studies demonstrate the essential role in PLR of matrix metalloproteinases (Mmps, namely Mmp2, Mmp13 and Mmp14), cathepsin K (Ctsk), carbonic anhydrase 2, and tartrate resistant acid phosphatase (Acp5/ TRAP) (Kogawa et al., 2013; Qing et al., 2012; Qing and Bonewald, 2009; Wysolmerski, 2013). Through loss-of-function studies in mice, these genes were found to be essential for an intact lacuno-canalicular network, organization of collagen, and bone matrix mineralization (Holmbeck et al., 2005; Inoue et al., 2006; Kogawa et al., 2013; Kulkarni et al., 2012; Qing and Bonewald, 2009; Tang et al., 2012; Tang and Alliston, 2013; Wysolmerski, 2013), all of which contribute to bone quality. Macro-mechanical testing of MMP13-deficient bone revealed a correlation between the loss of PLR and bone fragility (Tang et al., 2012). Nonetheless, many questions remain about the relationship between osteocyte-mediated PLR and bone quality.

In an effort to elucidate the cellular and molecular mechanisms that control bone quality, we tested the hypothesis that TGFβ acts directly on osteocytes to control PLR, and that

this mechanism accounts for the TGFβ-dependent control of bone quality. Several lines of evidence support this model, including the ability of TGFβ to directly regulate the expression of several Mmps implicated in PLR (Krstic and Santibanez, 2014; Selvamurugan et al., 2004). To investigate this possible mechanism, we employed novel *in vivo* and *in vitro* models and pharmacologic TGFβ antagonists similar to those in human clinical trials for the treatment of bone fragility in OI. Using this approach, we uncovered the essential role of osteocyte-intrinsic TGFβ signaling in the control of PLR and fracture resistance and demonstrate the importance of PLR in bone fragility.

Results

Pharmacologic inhibition of TGFβ signaling dysregulates perilacunar/canalicular remodeling

We previously showed that pharmacologic inhibition of the TGFβ receptor type I (TβRI-inhibitor, SD-208) increases trabecular bone mass through stimulating osteoblastic bone formation and repressing osteoclastic resorption (Mohammad et al., 2009). However, the effect of SD-208 or other TβRI-inhibitory agents on osteocytes (OCY) is unknown. To investigate the role of TGFβ signaling in osteocytes, the most abundant bone cells in cortical bone (Franz-Odendaal et al., 2006), we examined histologic and molecular outcomes of osteocyte-mediated perilacunar/canalicular remodeling (PLR) in mice treated with SD-208. As expected, 6 weeks of TβRI-inhibitor treatment significantly increased trabecular bone mass (Fig. S1). Histologic analysis shows a dense and organized network of osteocyte canaliculi in cortical bone of vehicle-treated mice. However, TβRI-I treatment caused severe deterioration of the osteocyte canalicular network with a 50% reduction in canalicular length (Fig. 1A-B).

The dysregulated canalicular network in TβRI-I-treated bone resembles that seen in bones deficient in enzymes essential for PLR (Holmbeck et al., 2005; Inoue et al., 2006; Kulkarni et al., 2012; Tang et al., 2012). Therefore, we evaluated the effect of TβRI-inhibition on the expression of genes encoding PLR enzymes, including matrix metalloproteinases *Mmp2*, *Mmp13* and *Mmp14*, Cathepsin K (*Ctsk*), and tartrate resistant acid phosphatase (*Acp5*) in cortical bone. TβRI-I treatment coordinately reduced the level of mRNA encoding all five enzymes, relative to vehicle-treated controls (Fig. 1C). Expression of the ATPase *Atp6v0d*, is increased in TβRI-treated bone (Fig. S2). Moreover, TβRI-I treatment also causes a decline in osteocytic protein expression of MMP13, MMP14 and CTSK without impacting their viability (Fig. 1D-F). This strong, concerted repression of several genes required for osteocyte-mediated PLR upon TGFβ inhibition indicates the critical role of TGFβ in controlling osteocyte function.

TGFβ regulates perilacunar/canalicular remodeling in a cell intrinsic manner

While systemic inhibitors of TGFβ clearly impact lacuno-canalicular networks and the expression of genes associated with PLR, it was unclear if TGFβ exerts its effects on osteocytes directly or indirectly. Therefore, we examined the cell-intrinsic effects of TGFβ on MLO-Y4 osteocyte-like cells and OCY454 osteocytes, which more faithfully mimic osteocytic gene expression. Within 6 hours of treatment, TGFβ induced expression of *Mmp13, Mmp14* and *Ctsk* mRNA, as well as *Serpine1*, a well-known TGFβ-inducible gene in MLO-Y4 cells (Fig. 2A-B) (Graycar et al., 1989). TGFβ also induced expression of *Mmp13 and Ctsk,* but not *Mmp14,* in OCY454 cells (Fig.2 C-D). Further supporting the osteocyte intrinsic role of TGFβ, TGFβ induced the expression of

the osteocyte marker genes Sclerostin (*Sost)* and dentin matrix protein-1 *(Dmp1),* without affecting Phosphate Regulating Endopeptidase Homolog, X-Linked (*Phex*) (Fig.S3).

In addition to expressing PLR enzymes, osteocytes engaged in PLR acidify their microenvironment. Using the pH-sensitive dye 5-(and-6)-carboxy SNARF-1, AM, we examined the effect of TGFβ on MLO-Y4 cell acidification. As shown by others (Kogawa et al., 2013), recombinant sclerostin (rhSCL) induces PLR and lowers the intracellular pH (pHi) of MLO-Y4 cells. TGFβ treatment resulted in a larger acidification than sclerostin treatment. In contrast, blocking TGFβ signaling with an *in vitro* inhibitor of TβRI (SB-431542) relieved this acidification, such that pHi was equivalent to untreated cells (Fig. 2E-F). Altogether, our findings support the possibility that TGFβ induces PLR in an osteocyte-intrinsic manner.

Osteocyte-specific inhibition of TGFβ signaling impairs perilacunar/canalicular remodeling

To evaluate the osteocyte-intrinsic role of TGFβ signaling in vivo, TGFβ receptor II (TβRII) was deleted in osteocytes using DMP1-Cre mice, resulting in TβRII$^{ocy-/-}$ mice. We validated the specific reduction of TβRII expression in osteocytes (but not in other cell types) of TβRII$^{ocy-/-}$ bone relative to DMP1$^{Cre-/-}$; TβRII$^{fl/fl}$ (WT) littermate controls (Fig.3 A-B). Abrogation of TGFβ signaling in TβRII$^{ocy-/-}$ bone was validated by reduced *TβRII* and *Serpine1* gene expression (Fig. 3C). Furthermore, using primary bone marrow cultures from WT and TβRII$^{ocy-/-}$ mice, we verified the osteocyte-specific defect in TGFβ signaling by confirming that osteogenic gene expression is normal until after these cells differentiate into osteocytes (Fig S5A-D).

Since systemic inhibition of TGFβ signaling causes severe deterioration of the osteocyte canalicular network, and since TGFβ regulates osteocytic expression of PLR enzymes, we evaluated the lacuno-canalicular network in TβRII$^{ocy-/-}$ cortical bone. Upon osteocytic deletion of TβRII, the canalicular network was abrogated and visibly blunted. Relative to WT, the canalicular projections in TβRII$^{ocy-/-}$ bone were reduced by 50% and the total lacuno-canalicular area was reduced by 32% (Fig. 3D-E, Fig. S4A).

Among the panel of PLR genes, expression of *Mmp2, Mmp13, Mmp14, Ctsk*, and *Acp5* was downregulated in TβRII$^{ocy-/-}$ mice (Fig. 3F, Fig. S4B). In fact, the effect of osteocyte-intrinsic TβRII ablation on PLR gene expression was even more profound than that produced by TβRI-inhibitor treatment. Expression of osteocalcin (*Oc*) and bone sialoprotein (*Ibsp*), *Dmp1* and *Phex* genes the control systemic mineral homeostasis was unaffected by the absence of osteocytic TGFβ signaling. Expression of Sost, which is known to be induced by TGFβ (Loots et al., 2012; Nguyen et al., 2013), was downregulated in TβRII$^{ocy-/-}$ bones (Fig.3G). Protein expression of MMP13, MMP14 and CTSK in osteocytes of TβRII$^{ocy-/-}$ mice was also significantly reduced 27-40% compared to WT mice, without apparent changes in osteocyte number and viability as determined by H&E and TUNEL staining (Fig. 3H-I, Fig S4B-C). These findings corroborated the observations in the TβRI-I mouse model and revealed the direct role of osteocytic TGFβ signaling in the regulation of PLR.

To rigorously evaluate the effect of TβRII deletion on lacunar size, orientation and shape, we utilized synchrotron radiation micro-tomography (SRμT), which visualizes and quantifies the osteocyte lacunae in a 3-D space. In spite of the dramatic differences in

the canalicular network seen histologically, osteocyte lacunar volume, shape, and orientation relative to the long axis of the bone did not differ significantly between TβRII$^{ocy-/-}$ and WT cortical bone (Fig. 3J-M, Fig. S4E-H). Also using SRμT, we detected a 3% reduction (P=0.06) in peak bone mineral concentration in diaphyseal cortical bone of TβRII$^{ocy-/-}$ mice compared to WT (Fig. 3M, Fig. S6H). Therefore, osteocyte-intrinsic TGFβ signaling regulates the expression of enzymes required for PLR, and is essential for the integrity of the canalicular network and bone matrix mineralization. Not only does this reveal a novel role for TGFβ in osteocytes, but it also adds TGFβ to the short list of factors shown to regulate PLR.

Osteocyte-specific deletion of TβRII increases trabecular bone mass by inhibiting bone resorption

Because alterations in TGFβ signaling often impact bone mass (Balooch et al., 2005; Mohammad et al., 2009), we analyzed the impact of ablated osteocyte-specific TGFβ signaling on trabecular and cortical bone mass and geometry. Bones of TβRII$^{ocy-/-}$ mice showed no gross abnormalities relative to WT mice. Micro-computed tomography (μCT) analysis revealed a 35% increase in trabecular bone mass in 8-week old TβRII$^{ocy-/-}$ mice relative to WT littermates. This gain in mass was attributed to the corresponding increase in trabecular number (26%) and complementary decrease in trabecular spacing (25%) (Fig. 4A-D, Table 1). However, osteocytic deletion of TβRII did not affect cortical bone thickness or geometry (Fig. 4K-M). Cortical bone mineralization (Fig. 4N) of TβRII$^{ocy-/-}$ mice was reduced by 4.8%, consistent with the 3% decrease in peak bone mineral concentration detected by SRμT (Fig.3M). Therefore, osteocyte-intrinsic TGFβ regulates the mass and geometry of trabecular, but not cortical bone.

To understand the cellular mechanism underlying the elevated trabecular bone phenotype of TβRII$^{ocy-/-}$ mice, histomorphometry was performed. Neither static nor dynamic histomorphometric analyses revealed significant differences in osteoblast or bone formation parameters in TβRII$^{ocy-/-}$ bone (Fig. 4E-G, Table 1). On the other hand, measures of bone resorption implicate osteocyte-intrinsic TGFβ in the control of osteoclast function. Specifically, TRAP-positive osteoclasts were reduced by 40%, along with a 34% reduction in osteoclast surface in TβRII$^{ocy-/-}$ mice (Fig. 4H-I, Table 1). Furthermore, TβRII$^{ocy-/-}$ mice showed a substantial reduction in the ratio of RANKL/OPG mRNA expression due to low levels of the osteoclastogenic factor RANKL (*Rankl*), but unaffected levels of OPG (*Opg*), a RANKL antagonist (Fig. 4J, Fig. S5D-G). Together these results attribute the high trabecular bone mass phenotype of TβRII$^{ocy-/-}$ mice to decreased osteoclast function, which essentially results from decreased production of RANKL by TβRII-deficient osteocytes.

Osteocyte deletion of TβRII reduces fracture resistance of bone

Given that TβRII$^{ocy-/-}$ cortical bone mass and thickness are normal, evidence of bone fragility in these mice would be consistent with defects in bone quality. Despite our prior implication of TGFβ in bone quality regulation (Balooch et al., 2005; Chang et al., 2010; Mohammad et al., 2009), the cellular target responsible for the control of bone quality has since been elusive. The disruption of PLR and cortical bone mineralization in TβRII$^{ocy-/-}$ bone led us to hypothesize that TGFβ controls bone quality through regulation of osteocytic PLR. To test this hypothesis, we performed a series of tests to evaluate the macromechanical and material behavior of TβRII$^{ocy-/-}$ bone.

Macromechanical testing showed reduced fracture resistance of TβRII$^{ocy-/-}$ cortical bone. Using flexural testing, we found that TβRII$^{ocy-/-}$ femora exhibited a 26% decline in the bending modulus relative to WT bone, indicating a reduced capacity to resist elastic deformation (Fig. 5A). Similarly, the yield stress was reduced by 27% in TβRII$^{ocy-/-}$ bones (Fig. 5B). Using nanoindentation to examine the material properties of the TβRII$^{ocy-/-}$ bone revealed that the Young's modulus of TβRII$^{ocy-/-}$ bone matrix was significantly lower than for WT bone (Fig. 5C), a finding that is consistent with the reduced TβRII$^{ocy-/-}$ cortical bone mineralization (Fig. 4N). The most dramatic effects were observed in fracture toughness testing, in which notched TβRII$^{ocy-/-}$ cortical bone exhibited a 65% decrease in total work of fracture compared to WT bone (Fig. 5D). These findings are particularly remarkable, given that the severe fragility of TβRII$^{ocy-/-}$ bone could not be attributed to differences in cortical bone mass or geometry.

Accordingly, we sought to learn more about the material mechanisms responsible for TβRII$^{ocy-/-}$ bone fragility. For example, resistance to crack *initiation* is primarily imparted through intrinsic toughening mechanisms, representing a material's inherent resistance to microstructural damage. On the other hand, crack *growth* toughness stems from extrinsic toughening mechanisms, which act to shield the crack from the applied driving force to limit crack propagation (Launey and Ritchie, 2009).

To distinguish between the effects of osteocyte TβRII deficiency on crack initiation and crack growth, we conducted fracture toughness testing in a variable pressure scanning electron microscope to simultaneously visualize and quantify crack behavior. While crack initiation toughness could not be conclusively differentiated between genotypes, the shallow slope of the R-curve for TβRII$^{ocy-/-}$ bones is indicative of reduced crack growth toughness and a loss of extrinsic toughening mechanisms (Fig. 5E). *In situ* images of crack growth show evidence of extrinsic toughening by crack deflection and uncracked ligament bridging in WT bone (Fig. 5E i-iii). Conversely, the path of cracks in TβRII$^{ocy-/-}$ bone tended to be more linear and shorter relative to their profile extension (Fig. 5F-G). Therefore we conclude that TGFβ regulates bone quality in an osteocyte-intrinsic manner, specifically through extrinsic toughening mechanisms that limit crack growth. Identification of osteocytes as crucial cellular targets in the biological control of bone quality raises new questions about the role of osteocytes and PLR in human bone fragility.

Discussion

This study advances our understanding of bone homeostasis and fragility by revealing an osteocyte-intrinsic role for TGFβ signaling. Here we implicate TGFβ as a novel regulator of perilacunar/canalicular remodeling and pinpoint osteocytes as the cell type principally responsible for the biological control of bone quality. Either using pharmacologic TGFβ receptor type I kinase inhibitors or a genetic model of osteocyte-specific TGFβ receptor ablation, we demonstrate that suppression of TGFβ signaling causes a severe deterioration of osteocyte canalicular network, and dysregulates the expression of a host of PLR genes. Loss of osteocyte-intrinsic TGFβ signaling also reduces bone matrix mineralization (Fig. 6). Since TβRII$^{ocy-/-}$ cortical bone mass and geometry are normal, the profound fragility of these bones reveals that TGFβ controls bone quality through an osteocyte-intrinsic mechanism that relies on PLR. These findings strongly support the idea that PLR plays a fundamental role in bone homeostasis, specifically as the cellular mechanism responsible for the maintenance of the lacuno-canalicular network and bone quality.

Our findings revealed TGFβ signaling to be a cell-intrinsic regulator of perilacunar/canalicular remodeling. Osteocyte-specific inhibition of TGFβ signaling decreases the expression of several genes that have been functionally implicated in PLR, including *Mmp2, Mmp13, Mmp14, Ctsk* and *Acp5*. The coordinated regulation of these PLR genes by TGFβ is consistent with the effects of other PLR-regulatory pathways. Most of these genes are induced by PLR agonists, such as sclerostin and PTH, but repressed by PLR-antagonists such as glucocorticoids (Fowler et al., 2017; Kogawa et al., 2013; Qing et al., 2012). In each case, including in this study, these changes in gene expression correspond to alterations in the organization of the canalicular network. Interestingly, expression of vacuolar ATPases, that function in osteocyte acidification, is upregulated in TβRII$^{ocy-/-}$ bone, raising the possibility that a feedback loop compensates for the low level of proteases mediating PLR.

The effects of TGFβ and other PLR-regulatory pathways on the lacuno-canalicular network and on bone matrix differ in important ways. In addition to alterations in the canalicular network, lactation and glucocorticoid treatment cause changes in lacunar size (Fowler et al., 2017; Qing et al., 2012). Furthermore, collagen organization is disrupted in MMP13-deficient mice and in mice treated with glucocorticoids. In TβRII$^{ocy-/-}$ mice neither collagen organization nor lacunar volume, shape, and orientation was impacted. In this study, PLR mediated changes were observed at osteocyte canaliculi alone. Interestingly, emerging data from our lab and others (Fowler et al., 2017; Kaya et al., 2017; Tang et al., 2012) suggests that remodeling by osteocytes may be spatially defined, such that some circumstances favor remodeling at lacunae, whereas others will promote remodeling around canaliculi. Additional studies will be needed to determine the extent to which this is true.

The *in vitro* analysis of osteocyte acidification is a useful surrogate of PLR, but additional research is needed to better understand the cell biology of PLR. Nonetheless, TGFβ clearly acts directly on osteocytes to calibrate the extent of PLR and is required for the maintenance of the lacuno-canalicular network. Importantly, it is possible that the degenerated canalicular networks in our mouse models of impaired TGFβ signaling result from defective osteocyte integration into the bone matrix. A shorter time course or an inducible model would be needed to conclusively address this question. However, our previous studies have shown similar canalicular degeneration within 21 days of glucocorticoid treatment (Fowler et al., 2017) or a week of lactation (unpublished data)(Kaya et al., 2017; Qing et al., 2012; Qing and Bonewald, 2009; Wysolmerski, 2013), thereby indicating that changes in the canalicular network can occur rapidly in a manner that is independent of a maturation defect.

The critical role of TGFβ in osteocytes complements its actions in osteoblasts, osteoclasts, and their progenitors, where it couples bone formation to resorption (Dallas, 2008). Thus, it is not surprising that osteocyte-intrinsic ablation of TβRII would inhibit osteoclast function due to reduced levels of RANKL expression by osteocytes. Whether by systemic TβRI inhibition, expression of a dominant negative TGFβ type II receptor in osteoblasts, or in TβRII$^{ocy-/-}$ mice, trabecular bone mass is increased due to reduced RANKL expression and reduced osteoclastogenesis (Edwards et al., 2010; Filvaroff et al., 1999; Mohammad et al., 2009). Though we cannot completely exclude a causal role of TβRII$^{ocy-/-}$ osteocyte canalicular degeneration in the trabecular bone phenotype, our current and previous data suggest that TGFβ's regulation of RANKL expression is cell intrinsic. On the other hand, the complexity of TGFβ crosstalk in bone underlies the unique, and at times apparently contradictory, bone phenotypes that result from

manipulating TGFβ signaling in one cell type or another (Dallas, 2008; Tang and Alliston, 2013). Furthermore, in bone and in many other tissues, the effect of TGFβ is nonlinear; such that either increased and decreased TGFβ signaling can produce an osteoporotic phenotype (Balooch et al., 2005; Borton et al., 2001; Erlebacher and Derynck, 1996). Despite this known complexity, we were surprised by the low mineral concentration of TβRII$^{ocy-/-}$ bone, given that mineralization is increased by systemic post-natal TβRI-I treatment (Edwards et al., 2010; Mohammad et al., 2009). Given that osteocyte canaliculi are sites of secondary mineralization, it is possible that the reduced canalicular length in the TβRII$^{ocy-/-}$ bones reduces surface area available for mineralization. Moreover, the increased expression of vacuolar ATPase may create an acidic microenvironment that is unfavorable for mineralization. Additional studies will be needed to discern the mechanisms by which pharmacologic disruption of TGFβ signaling affects bone mineralization differently from genetic ablation of TβRII specifically in osteocytes.

Bone strength relies on bone mass and bone quality, both of which depend on the ability of TGFβ to coordinate the function of osteoblasts, osteoclasts and osteocytes. In spite of the fact that bone quality contributes to at least half of fractures in people with clinically normal bone mass (Schuit et al., 2004; Sornay-Rendu et al., 2007), the cellular mechanisms controlling bone quality have remained unclear. Understanding these mechanisms is a critical step in improving the diagnostics and therapeutics for fragility fractures (Hernandez and Keaveny, 2006; Seeman, 2008). This study represents the most definitive evidence so far implicating osteocyte-mediated PLR in the control of bone quality. Previous studies by our group and others show that bone quality is impaired following glucocorticoid treatment or systemic ablation of MMP13, and that PLR is impaired in each case (Fowler et al., 2017; Lane et al., 2006; Tang et al., 2012). Here we find that osteocyte-specific deletion of TGFβ signaling caused defects in PLR and in cortical bone mineralization, flexural strength, ECM material properties, and fracture toughness without impacting cortical bone mass. In diseases like Camurati Engelman syndrome and osteogenesis imperfecta, both of which are characterized by excessive TGFβ signaling (Grafe et al., 2014; Kinoshita et al., 2000; Tang et al., 2009), deregulation of osteocyte-mediated remodeling may contribute to the bone fragility in these diseases. Similarly the extent to which dysregulated PLR contributes to the fragility in other skeletal diseases, including renal osteodystrophy, secondary hyperparathyroidism, and glucocorticoid-induced osteoporosis, is an important area of further investigation. If so, PLR could be an attractive therapeutic target for improving fracture resistance in many conditions.

In conclusion, this study emphasizes the need to identify the cellular and molecular mechanisms regulating bone quality to develop new therapies to address the significant unmet clinical need for the treatment of bone fragility. Current therapeutics can improve 70% of trabecular fractures but only 20-40% of cortical bone fractures, which is precisely where PLR dysregulation is most profound (Ahmed et al., 2015; Chen and Sambrook, 2011; Rivadeneira and Mäkitie, 2016). A combination of systems analysis of GWAS data from clinical cohorts, along with functional in vivo and in vitro studies, can shed light on new molecular targets to control bone fragility and expand the pool of genetic markers needed for fracture risk assessment and prevention.

Experimental Procedures

Mice

To block TGFβ signaling systemically, 5-week old equally weighing C57BL/6 male mice were administered either vehicle (1% methylcellulose) or a specific inhibitor of the TGFβ type I receptor (TβRI-I), (SD-208, 60 mg/kg twice daily by oral gavage) for 6 weeks (Mohammad et al., 2009). We also generated mice with osteocyte-specific ablation of TGFβ type II receptor (TβRII), which effectively blocks osteocyte sensitivity to TGFβ ligand. Homozygous TβRII-floxed mice that possess *loxP* sites flanking exon 4 of the targeted gene were backcrossed for 3 generations into a C57BL/6 background and subsequently bred with hemizygous -10kb-DMP1-Cre$^{+/-}$ mice, which express Cre recombinase primarily in osteocytes (Leveen et al., 2002; Lu et al., 2007). Half of the mice from the resulting cross were DMP1-Cre$^{+/-}$;TβRII^{flfl} (named TβRII$^{ocy-/-}$ mice) and half were DMP1-Cre$^{-/-}$;TβRII$^{fl/fl}$ littermate controls (named Wild-type (WT) mice), as confirmed by PCR genotyping. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of California San Francisco and the Indiana University School of Medicine.

Morphological analysis.
For skeletal phenotyping, femurs harvested from 8-week old male mice (n=8 mice per group) were cleaned of soft tissue and fixed in 10% neutral buffered formalin for micro-computed tomography (microCT, μCT). For μCT, fixed femurs were stored in 70% ethanol. For histomorphometry, 7-week old male mice were administered with two intraperitoneal injections of calcein (20 mg/kg body weight, Sigma) 10 and 3 days prior to euthanasia and the harvested femurs were fixed in 10% neutral buffered formalin for 48 hours, processed and embedded in a MMA plastic resin. Additional details of μCT and histomorphometry procedures are described in the Supplemental Data.

Quantitative RT-PCR analysis
We purified RNA from cells in culture and from bones dissected from soft tissues using the miRNeasy mini kit (Qiagen, Valencia, CA), following the manufacturer's protocol. In vitro results are representative of n=3 replicates/ group and 3 independent experiments. For bones (humeri from n≥8 mice/ group), proximal and distal regions were cut off and marrow was removed by centrifugation before RNA extraction. The majority of RNA obtained from bone using this method is osteocyte derived, with very little contribution from osteoblasts (Halleux et al., 2012). Additional details of quantitative RT-PCR analysis are described in the Supplemental Data).

Immunohistochemistry
For immunohistochemistry, paraffin embedded (7μm thick) sections were incubated with primary antibodies for anti-MMP13 (1:100; Abcam, ab39012); anti-MMP14 (1:100; Abcam, ab38971); anti-CTSK (1:75; Abcam, ab19027), or anti-TβRII (1:500; Abcam, ab186838). This was followed by incubation with corresponding biotinylated secondary antibody, avidin-conjugated peroxidase, and diaminobenzidine substrate chromogen system (Innovex Universal Animal IHC kit). Corresponding nonimmune IgGs were used as negative controls. Hematoxylin and eosin (H&E) and TUNEL-DAPI staining were performed to visualize osteocyte number and apoptosis. Ploton silver staining (Jauregui et al., 2016; Ploton et al., 1986) was performed for visualization of the osteocyte lacuno-canalicular network. Images were were acquired using a Nikon Eclipse E800 bright-field microscope and analyzed with ImageJ. Sections were evaluated for one femur from each of n≥4 mice/ group. Additional details of immunohistochemistry and image analysis are described in the Supplemental Data.

Cell culture

The MLO-Y4 osteocyte-like cell line (generously provided by L. Bonewald) was maintained in α-MEM supplemented with 2.5% fetal bovine serum, 2.5% bovine calf serum, and 1% penicillin-streptomycin. The OCY454 osteocyte cell line (generously provided by P. Divieti-Pajevic) was cultured in α-MEM supplemented with 10% fetal bovine serum and 1% antibiotic/ antimycotic (Gibco). For treatment, cells were cultured in α-MEM containing 0.5-1% fetal bovine serum, supplemented with 5 ng/ml TGFβ1 (Humanzyme, HZ-1011), 10 μM SB431542 (Sigma, S4317) or 10 ng/ml recombinant human sclerostin (rhSCL, R&D Systems) for the indicated times.

Intracellular pH assay

Intracellular pH (pHi) was measured in transfected or untransfected MLO-Y4 cells treated with TGFβ1, SB431542 or rhSCL using the pH-sensitive fluorescent dye, 5-(and-6)-carboxy SNARF-1, AM (Molecular Probes, Inc.) as described (Kogawa et al., 2013). Briefly, after 3 days of culture in the indicated conditions, cells were washed with PBS and loaded with 5-(and-6)-carboxy-SNARF-1, AM at 37°C for 30 minutes, at a final concentration of 10 μM and visualized under a Leica TCS SPE confocal microscope. n=4 replicates/ group and 3 independent experiments. Additional details of the procedure are provided in the Supplemental Data.

Synchrotron radiation Micro-Tomography (SRμT)

SRμT studies were used to assess the degree of mineralization of bone as well as the volume and degree of anisotropy of osteocyte lacunae. The mid-diaphysis of 8-week old male mouse femurs were scanned with 20 keV x-ray energy, with a 300 ms exposure time, using a 5X magnifying lens for a spatial resolution of 1.3 μm (N=3-4 mice/ group). Additional details of the SRμT procedure are described in the Supplemental Data.

Mechanical tests

To measure bone quality, we assessed the macromechanical properties and the bone matrix material properties using flexural strength tests, *in situ* fracture toughness tests, and nanoindentation. Briefly, from 8-week old $T\beta RII^{ocy-/-}$ and WT mice (n=3-5 mice/ group) intact femurs were isolated, cleaned of soft tissue and stored in Hanks' Balanced Salt Solution (HBSS). Details of the flexural strength tests, *in situ* fracture toughness tests, and nanoindentation procedures are described in the Supplemental Data.

Statistical Analysis

We expressed all values as mean ± S.E.M or mean ± S.D as appropriate for each assay. Group sizes were determined by power calculations providing 80% probability of detecting a significant difference ($p \leq 0.05$). Group size "n" is denoted in figure legends. For *in vivo* data, n refers to the number of mice analyzed per group. For *in vitro* data, n refers to the number of independent experiments performed. Unpaired two-tailed Student's *t*-test was used to compare the means of two groups using GraphPad Prism (GraphPad Software). Data points falling more than 2 standard deviations from the mean were excluded. Variances ranged from 12.5% to 20% and were similar between groups. No blinding was used during analysis. In all figures, $p \leq 0.05$ was considered statistically significant.

Author Contributions

Conceptualization, N.S.D, T.W.F., K.S.M., T.A.; Investigation, N.S.D., C.M.M., C.A., J.P.L., D.A.M., B.G., J.N.R., F.W., D.E., T.F.L., B.Z.; Data Curation, S.M.; Analysis, all authors; Writing – Original Draft, N.S.D.; Writing – Review & Editing, all authors; Visualization, N.S.D., C.M.M., T.A.; Supervision, R.O.R., K.S.M., T.A.; Project Leadership, N.S.D., T.A.; Funding Acquisition, T.A.

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Figure and Table Legends

Figure 1. Pharmacologic TβRI inhibition impairs perilacunar remodeling (PLR)

(A, B) Silver nitrate stained images of femoral cortical bone from vehicle and TGFβ receptor I kinase inhibitor (TβRI-I, SD-208) treated mice show osteocyte lacuno-canalicular network (A) and canalicular length (B). Scale bar, 20 μm (n=6 mice/group). (C) qPCR analysis of PLR genes *Mmp2, Mmp13, Mmp14, Ctsk,* and *Acp5* in bones from vehicle and TβRI-I treated animals (n=8 mice/group). (D-F) Immunohistochemistry (IHC) of MMP13, MMP14 CTSK and H&E staining of femoral cortical bone of vehicle and TβRI-I treated animals. Arrows in the image indicate positively stained osteocytes that were quantified and normalized to total bone area. Scale bar, 50 μm (n=4 mice/group). Error bars indicate mean ± SEM, *p<0.05 compared to vehicle from Student's t test.

Figure 2. TGFβ promotes cell intrinsic osteocytic perilacunar remodeling
(A-D) qPCR analysis of PLR genes *Mmp13, Mmp14* and *Ctsk* and *Serpine1* upon TGFβ (5ng/mL) treatment in MLO-Y4 (A,B) and OCY454 (C,D) cells. (n=3 replicates/group). (E, F) Intracellular pH (pHi) of MLO-Y4 cells after 3 days of TGFβ (5ng/ml), TβRI inhibitor SB-431542 (10 μM), or recombinant sclerostin (rhSCL, 10 ng/ml). The representative image (E) shows the shift in the emission peak from 580 nm to 640 nm after TGFβ treatment of MLO-Y4 cells. Scale bar, 100 nm). TGFβ-induced acidification is blocked by SB-431542 (F) (n=4 replicates/group). Error bars indicate mean ± SD of 3 independent experiments, *p<0.05 different from control mRNA, a-p<0.05 different from control pHi, b-p<0.05 different from TGFβ pHi, and c-p<0.05 different from rhSCL pHi. Statistics calculated from Student's t test.

Figure 3. Osteocytic deletion of TβRII dysregulates perilacunar remodeling.
(A, B) TβRII-stained osteocytes (A) (arrow, scale bar, 50 μm) in the femoral cortical bone from WT and TβRII$^{ocy-/-}$ mice (8-week old males) were quantified as percentage of positively stained osteocytes normalized to total bone area (B) (n=5 mice/group) (C) qPCR analysis of TβRII and Serpine1 in WT and TβRII$^{ocy-/-}$ femoral bones. (n=8-10 mice/ group). (D, E) Silver nitrate stained images of WT and TβRII$^{ocy-/-}$ femoral cortical bone shows the osteocyte lacuno-canalicular network (D) and canalicular length (E) (scale bar, 20 μm, n=5 mice/group). (F, G) qPCR analysis of PLR genes, *Mmp2, Mmp13, Mmp14, Ctsk,* and *Acp5* (F) and OCY-specific genes, *Sost, Dmp1* and *Phex* (G) in the WT and TβRII$^{ocy-/-}$ bones (n=8-10 mice/group) (H, I) IHC of MMP13, MMP14, CTSK and H&E staining of WT and TβRII$^{ocy-/-}$ femoral cortical bone. Arrows in the image indicate positively stained osteocytes (H) that were quantified and normalized to total bone area (I), (n=4 mice/group).(J-M) SRμT shows volume (J), degree of anisotropy (K), orientation (L) and mineralization (N) of osteocyte lacunae of WT and TβRII$^{ocy-/-}$ bone (n=3-4 mice/group). Error bars indicate mean ± SEM with *p<0.05 compared to WT from Student's t test.

Figure 4. Osteocytic deletion of TβRII increases trabecular bone mass but does not affect cortical bone mass.
(A-D) μCT analysis of femur from WT and TβRII$^{ocy-/-}$ mice (8-week old males), Representative μCT reconstructions of trabecular bone (A) from mice and trabecular bone parameters: trabecular bone volume fraction (BV/TV) (B), trabecular number (Tb.N.) (C), and separation (Tb.Sp.) (D). Scale bar, 100 μm (n=10-11 mice/group)(E-I) Histomorphometric analysis of femur from WT and TβRII$^{ocy-/-}$ mice (8-week old males) measures osteoblast number normalized to bone surface (N.Ob/BS), bone formation rate (BFR) and percent mineralizing bone surface per bone surface (MS/BS), osteoclast number normalized to bone surface (N.Oc/BS), osteoclast suface normalized to bone

surface (Oc.S/BS) (n=6-7 mice/group). (J) qPCR analysis of mRNA harvested from WT and TβRII$^{ocy-/-}$ bones shows the *Rankl/Opg* ratio (n=8-10 mice/group).(K-N) Representative µCT reconstructions of femoral cortical bone femur from WT and TβRII$^{ocy-/-}$ mice (8-week old males) (K) and cortical bone parameters: cortical area fraction (Ct. BA/TA) (L), cortical thickness (Ct. Th) (M), and cortical mineralization (Ct. Min) (N). Scale bar,100 µm (n=10-11 mice/group). Data are presented as mean ± SEM and *p<0.05 compared to WT from Student's t test.

Figure 5. Osteocytic deletion of TβRII reduces bone material properties at multiple length scales.
Mechanical testing on femurs from WT and TβRII$^{ocy-/-}$ mice (8-week-old males). (A-B) Flexural tests of intact femurs shows bending modulus (A) and yield stress (B) (n=5 mice/group). (C) Nanoindentation of mid-diaphyseal femoral bone shows that tissue elastic modulus (n=3 mice/group). (D-E) *In situ* fracture toughness testing of notched femurs subjected to 3-point bending in a variable pressure SEM shows total work of fracture (WoF) (D) (n=5 mice/group), WoF R-curves, produced by calculating WoF at each instance of crack propagation (E) (n=3 mice/group). Three stages of crack growth are shown for a WT sample (i-iii), with pre-existing notch or crack in black and new crack extension in red, and the corresponding points are indicated in the R-curve. (F-G) The decrease in TβRII$^{ocy-/-}$ bone of the extrinsic toughening mechanism, crack deflection, is readily seen in two representative samples (F) (scale bar = 100 µm) and in quantification of the ratio of total crack length to crack extension (G) (N=5 mice/group). Data are presented as mean ± SD. 95% confidence intervals in (E) are calculated based on a power fit. *p<0.05 different from WT group.

Figure 6. TGFβ regulates bone fragility
A schematic summarizes the role of osteocytic TGFβ signaling in PLR and bone quality.

Table 1. Skeletal phenotyping of 8-week old WT and TβRII$^{ocy-/-}$ mice
µCT and histomorphometry analysis revealed significant differences in trabecular bone phenotype, cortical mineralization, and osteoclast behavior between wildtype and TβRII$^{ocy-/-}$ mice, but no differences were observed in cortical bone volume or osteoblast behavior. Bone parameters measured by microCT include trabecular bone volume fraction (TBV); connectivity density (Conn D); trabecular number (Tb. N); trabecular thickness (Tb. Th); trabecular separation (Tb. Sp); structural model index (SMI); trabecular mineralization (Tb. Min); cortical bone volume fraction (Ct. BV/TV); cortical thickness (Ct. Th); cortical SMI, and cortical mineralization (Ct. Min), with N=10-11 mice per group. Histomorphometry parameters measured include osteoid volume (OV/BV); osteoid surface (OS); osteoid width (O. Wi); osteoblast number (N. Ob); osteoblast surface (N. Ob/BS); osteoclast surface (Oc.S); eroded bone surface (Oc.S/BS); osteoclast number (N. Oc); osteoclast number (N.Oc/BS); mineralization surface (MS/BS); bone formation rate (BFR); and mineral apposition rate (MAR), with N=6-7 mice per group. Data are presented as mean ± SEM with *p < 0.05, # p = 0.06, and † p = 0.07 different from WT group.

MicroCT	WT	TβRII$^{ocy-/-}$
Distal Femur		
TBV (BV/TV) (%)	0.192 ± 0.016	0.259 ± 0.028 *
Conn D (1/mm^3)	377.74 ± 29.29	493.37 ± 71.58
Tb. N (1/mm)	6.275 ± 0.215	7.557 ± 0.542 *
Tb. Th (μm)	0.043 ± 0.002	0.043 ± 0.001
Tb. Sp (mm)	0.155 ± 0.006	0.126 ± 0.009 *
SMI	1.720 ± 0.184	1.063 ± 0.287 #
Tb. Min (mg HA/cm^3)	1020.98 ± 9.31	1014.23 ± 13.36
Midshaft Femur		
Ct. BV/TV (%)	0.437 ± 0.006	0.445± 0.008
Ct. Th (μm)	0.188 ± 0.004	0.189 ± 0.003
Ct. SMI	-0.442 ± 0.239	-1.066 ± 0.339
Ct. Min (mg HA/cm^3)	1279.69 ± 15.30	1217.59 ± 16.136 *
Static parameters		
OV/BV	0.007 ± 0.001	0.004 ± 0.001
OS	0.14 ± 0.02	0.12 ± 0.04
OS/BS (%)	0.04 ± 0.01	0.03 ± 0.01
O. Wi (μm)	3.28 ± 0.21	2.79 ± 0.36
N.Ob	61.00 ± 5.61	60.94 ± 8.81
N.Ob/BS (/mm)	19.01 ± 2.57	18.24 ± 2.38
Oc.S	0.83 ± 0.07	0.68 ± 0.01 †
Oc.S/BS (%)	0.12 ± 0.01	0.08 ± 0.01 *
N.Oc	39.57 ± 3.10	30.17 ± 1.66 *
N.Oc/BS (/mm)	5.63 ± 0.44	3.40 ± 0.28 *
Dynamic parameters		
MS/BS (%)	0.15 ± 0.01	0.16 ± 0.01
MAR (μm/d)	1.84 ± 0.13	1.89 ± 0.17
BFR/BS (μm^2. μm^3. d)	0.28 ± 0.03	0.30 ± 0.03