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TITLE: Targeting Transforming Growth Factor Beta to Enhance the Fracture Resistance of Bone

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Because aging can low	er the fracture resistance	ce of bone in ways that a	re independent of bone n	nass, the presen	t project investigated the possibility that		
transforming growth fac	ctor (TGF)-Beta inhibitio	n could improve multiple	measurements of fractur	e resistance in g	rowing, mature adult, and old rodents. TGF-		
Beta inhibition was achieved with a neutralizing antibody known as 1D11. When administered to growing mice for 4 weeks (13 to 17 weeks of age), 1D11							
substantially increased	trabecular bone volume	e traction. Moreover, trea	tment-related improveme	ents in trabecular	architecture and tissue mineral density		
translated to stronger vertebral bodies, when administered to adult (6 months) and old rats (22 months) for 6 weeks, 1011 did not have an appreciable effect							
hone in mice while it in	creased the structural st	trength of cortical hone in	old rats with no effects (	on hone in adult	rats. In comparison to control antibody		
treatment, 1D11 also in	creased the fracture to	Jahness of bone from arc	wing mice and old rats.	These effects ap	pear to be independent of bone mass, and		
identifying the cause (e	.g., changes in collager	rosslinking) is the next	step.		·····, ····, ·····, ······, ······, ······		
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### **INTRODUCTION:**

As women and men age, the likelihood of suffering a fracture increases [1]. Since this increase in fracture risk is not solely explained by an age- or menopausal-related decrease in bone mass [2,3], new anti-fracture therapies should also address other determinants of fracture resistance besides bone mass to be effective. One promising target is transforming growth factor-beta, a relatively abundant protein in bone that influences osteoblast differentiation as well as the extracellular matrix [4]. Thus, the goal of this CDMRP project, entitled *Targeting Transforming Growth Factor Beta to Enhance the Fracture Resistance of Bone* is to determine whether the suppression of TGF- $\beta$  activity improves the fracture resistance independent of effects on bone mass in rodents of varying age including very old rats with compromised bone quality.

### **BODY:**

### Preliminary work to establish effectiveness of neutralizing antibody

Prior to conducting the rat study, we needed to ensure that our batch of the anti-TGF- $\beta$  antibody (2G7) from the Vanderbilt Antibody and Protein Resource Laboratory sufficiently neutralized TGF- $\beta$  and caused the expected effect on bone. To do so, we conducted a head-to-head study in which 13-week-old male, FVB mice were treated for 4 weeks with intraperitoneal injections (i.p.) of either 2G7 at 1 of 2 doses (10 mg/kg and 20 mg/kg) or 1D11 from Genzyme Corporation (A Sanofi Company) at a dose previously determined to be effective in 13-week-old male, C57/BL6 mice (10 mg/kg) [5]. Respective control antibodies (12CA5 and 13C4) were included. We used *ex vivo* micro-computed tomography ( $\mu$ CT) scans at an isotropic voxel size of 12  $\mu$ m to assess the effect of treatment on the amount of trabecular bone (BV/TV) within the femur metaphysis. To our surprise, only 1D11 significantly increased BV/TV of the femur metaphysis (Figure 1). A subsequent *in vitro* 

TGF- $\beta$  reporter assay confirmed that 2G7 reduced SMAD2 signaling (downstream effector) in a transfected human cancer cell line by only 20%, whereas 1D11 reduced downstream signaling by 90%, further establishing that our 2G7 was ineffective. Therefore, in subsequent studies, we used 1D11, which was kindly donated by Genzyme through a Material Transfer Agreement between the company and Vanderbilt University. Unfortunately, getting the agreement in place delayed the rat experiment.



Figure 1. The locally supplied anti-TGF- $\beta$  antibody 2G7 did not increase trabecular bone fraction in the femur metaphysis (Left) as determined by a one-way ANOVA; whereas a commercially supplied antibody 1D11 did (Right) as determined by a two-sided Student's t-test. (mean ± SD)

### Establishing a tool for predicting strength of mouse vertebral bodies

Since the goal of this CDMRP project is to fully characterize fracture resistance, we used the 1D11-arm of the male mouse study to develop a non-destructive computational tool – known as  $\mu$ CT-derived finite element analysis (FEA) – for predicting vertebral body (VB) strength. CT-FEA can accurately assess the contribution of bone architecture to fracture resistance and is now being using in clinical studies of fracture [6]. Our  $\mu$ CT-FEA research will be presented at the upcoming 2013 Orthopaedic Research Society Meeting (see abstract in the Appendix for details on the technique and findings). Briefly, for the first time using mouse VBs, we showed that a simple modification to an existing elastic failure criteria [7] could predict VB strength, as determined by compression testing, and that 1D11 treatment increased VB strength primarily through increase in bone volume fraction but with some contribution from a treatment-related increase in tissue mineral density. This newly validated technique allows us and others to non-destructively determine the contribution of bone architecture and structure to whole bone strength such that the same specimens can subsequently be used for histology based mechanistic studies, thereby allowing more efficient and rapid progress in the field.

## *Effect of TGF-* $\beta$ *suppression on fracture resistance in rats*

To test our working hypothesis that an antibody targeted against TGF- $\beta$  increases the fracture resistance of young and aged bone by enhancing bone tissue-level properties, not just bone mass, young (6 months) or old

(22 months) male, Fischer F344 rats from the National Institute on Aging (NIA) were treated with the 13C4 control antibody or the 1D11 antibody at 1 or 5 mg/kg (1D11-1 or 1D11-5 groups, respectively; these recommended doses from Genzyme were based on their own ovariectomy rat studies) (Task 2). Injections were given i.p. at a frequency of 3 times per week for 6 weeks. Following an IACUCapproved protocol (Task 1), rats were euthanized, and all long bones were harvested. Throughout the 2-week acclimation period and 6-week treatment period, the young adult rats gained body weight while the old rats lost it. Per IACUC protocol, 3 of 36 rats were euthanized before treatment began because their body weight dropped below 20% of the baseline weight. After treatment commenced, 1 of 11 rats in the 13C4 group, 2 of 10 rats in the 1D11-1 group, and 2 of 12 rats in the 1D11-5 group were euthanized before completion of the study. The bones from these rats were not analyzed resulting in the following final n for each group: n=10 (13C4), n=8 (1D11-1), and n=10 (1D11-5). Since the young adult rats gained weight, none of these animals were removed, leaving the intended n=12 rats per treatment.  $\mu$ CT with 10  $\mu$ m voxel size (Task 3a) quantified cortical ( $I_{min}$ ) and trabecular (BV/TV) parameters as well as tissue mineralization density (TMD) [8], and three-point bend testing of un-notched radius (Task 3b) [9] and notched femur (Task 3d) quantified strength and fracture toughness of the long bones, respectively. For details on the notching method and fracture toughness calculation, see the abstract in the appendix that was presented at the 2012 American Society for Bone and Mineral Research (ASBMR) Annual Meeting.

Unlike the developing young, male FVB mice, 1D11 treatment did not have a demonstrative effect on the bone volume fraction (BV/TV) or trabecular architecture within the metaphysis of the femur from mature male F344 rats. Only when 2 outliers (2 standard deviations away from mean) were removed did a two-way ANOVA find a treatment effect (Figure 2). As expected, there was a clear difference in BV/TV between young and old rats, irrespective of treatment (ANOVA p<0.0001). While there was not much of a treatment effect on trabecular bone, suppression of TGF- $\beta$  by 1D11 increased the whole bone strength of the radius (Figure 3A). However, this increase in the peak force endured by the cortical bone was only statistically significant in old rats. This increase could be explained by a treatment-related increase in the moment of inertia (I<sub>min</sub>) of the radius mid-shaft (Figure 3B), where I<sub>min</sub> measures the distribution of tissue about the neutral bending axis (structure). Likewise, structural strength of the radius was likely higher for the old rats than for young rats because I<sub>min</sub> increased with aging. When we factored out the contribution of structure to whole bone strength using the flexure equation [8], there were no statistically significant differences in estimated material strength among the treatment groups



Figure 2. 1D11 at the higher dose only increased trabecular bone volume fraction in the femur metaphysis of young adult rats after removing outliers (mean ± SD with p-values adjusted by Holm-Sidak method for multiple pair-wise comparisons within age group; \*p<0.05).



Figure 3. 1D11 increased the whole bone strength in bending of the radius from only old rats (A), and this in explained in part by an increase in treatment-related increase in cortical bone structure (mean ± SD with p-values adjusted by Holm-Sidak method for multiple pair-wise comparisons within age group; \*\*p<0.01).

(AVOVA p=0.139; Figure 4A), this lack of a treatment effect could be due to a lack of a difference in tissue mineral density (TMD) among the treatment groups (ANOVA p=0.0896; Figure 4B). Interestingly, while TMD was clearly higher for old bone (Figure 4B), the bone from the old rats was not stronger than the bone from the young adult rats. Although 1D11 treatment had no effect on estimated material strength overall, it tended to reverse the age-related loss in strength, especially at the low dose (13C4-5 vs. 1D11-1,  $p_{adj.}$ =0.0522 within 22 months of age). However, this effect cannot be explained by treatment-related increase in TMD.

Besides structural and material strength, fracture resistance also depends on the ability of the tissue to dissipate energy during failure such that brittle bones have poor energy dissipation. Therefore, we tested for



Figure 4. Suppression of TGF- $\beta$  by 1D11 did not improved estimated material strength (A) or tissue mineral density (B) of the radius in either young or old rats, although there was a non-significant trend of 1D11 at the lower dose reversing age-related loss in strength. (mean ± SD).

differences in post-yield deflection (PYD: amount of deformation after yield or the onset of permanent damage), toughness (U<sub>t</sub>: energy-tofailure normalized to bone cross-sectional area), and fracture toughness (K<sub>c</sub>: resistance to crack propagation). PYD and U<sub>t</sub> was assessed from three point bending of the radii. As determined by a two-way ANOVA, there were no differences in post-yield deflection or in toughness among the treatment groups (PYD: p=0.996 and U<sub>t</sub>: p=0.763), but there were differences between the age groups (PYD: p<0.0001 and U<sub>t</sub>: p<0.0001). Thus, bones become brittle with age in the male F344 rats, and 6 weeks of 1D11 did not reverse this effect. Interestingly, when we created a notch in the femur and then tested the



Figure 5. When loading a notched femur in three-point bending, a crack propagates from the posterior to the anterior side of the femur mid-shaft (Above). By this test, 1D11 was found to increase fracture toughness in old rats. (mean ± SD with p-values adjusted by Holm-Sidak method for multiple pairwise comparisons within age group;

bone in three-point bending to propagate a crack, 1D11 treatment was found to increase the ability of the cortical tissue to resist crack propagation (Figure 5). However, as revealed in the post-hoc analysis, this effect primarily occurred in the old rats.

#### *Effect of TGF-\beta suppression on fracture resistance in female mice*

Since the suppression of TGF- $\beta$  activity by 1D11 did not affect the fracture resistance of young adult male rats as expected from our previously published research [5,10] and our unpublished work involving mice (see abstracts in appendix), we decided to verify that the new batch of 1D11 antibody from Genzyme was effective (i.e., not inadvertently denatured by freeze-thaw cycles or some unknown handling procedure). Therefore, we repeated the aforementioned 13 week, male FVB mice study (Figure 1) in 13 week, female FVB mice using the same aliquots of the antibody that was administered to the male rats. The reasons for testing the effect of 1D11 on bone in female FVB mice were four fold: the importance of preventing fractures among women; there is already published data on the effect of 1D11 on bone in male mice, but not in females; FVB mice have a higher baseline BV/TV and TMD than the C57BL6 strain [11]; and their femurs snap when subjected to bending making assessment of toughness possible, whereas C57BL6 bones exhibit excessive deformation and do not often snap.

To ensure sufficient power for detecting treatment effects on biomechanical properties of bone (not just BV/TV), we administered 13C4 at 10 mg/kg to 13 female mice and 1D11 at 10 mg/kg to 12 female mice, beginning at 13-weeks of age. As was done in the previous mouse studies, the i.p. injections were given 3 times per week for 4 weeks (Task 2). Thus, we euthanized the mice at 17 weeks of age, which corresponds to the end of the growth phase. The same methods were used to assess the effect of 1D11 on bone (Task 3).

Confirming that our batch of 1D11 is not necessarily ineffective, trabecular bone volume fraction was clearly greater in the femur metaphysis of 1D11-treated mice than that of 13C4-treated mice. Moreover, treatment improved trabecular architecture as determined by  $\mu$ CT-derived connectivity density (Conn.D) and TMD (Table 1). Since these changes, imply that the trabecular bone from treated mice is stronger than bone from non-treated mice, we also scanned the L6 vertebra (VB) by  $\mu$ CT and then tested them in compression to failure (monotonic loading). The same treatment effects occurred for the trabeculae of the VB: greater BV/TV,

Table 1. Selected properties (mean ± SD) of trabecular and cortical mouse bone

Property	Units	1:	3C4	ŀ	1D11 p-value	
Femur metaphysis		n=13		6	n=12	
BV/TV		0.092	±	0.017	0.195 ± 0.042 <.0001	
Conn.D.	mm⁻³	91.6	±	32.4	143.3 ± 30.6 0.0004	
Tb.TMD	mgHA/cm <sup>3</sup>	1015	±	12	1047 ± 7 <.0001	
Femur diaphysis						
I <sub>min</sub>	mm⁴	0.125	±	0.016	0.121 ± 0.012 0.553	
Ct.TMD	mgHA/cm <sup>3</sup>	1289	±	13	1286 ± 8 0.514	
Peak force	Ν	21.9	±	2.3	22.1 ± 1.5 0.784	
PYD	mm	0.187	±	0.082	0.158 ± 0.075 0.368	

Conn.D, and TMD with 1D11 treatment (data not shown). More importantly, the VB strength was greater for the 1D11 treated female mice than the 13C4 treated female mice (see Figure 6 as well as ASBMR abstract in appendix).

Unlike trabecular bone architecture, the cortical bone structure of the diaphysis was not different between the 13C4 and 1D11 groups

(Table 1). Being opposite of what we observed for the radius of the old rats, 1D11 treatment did not increase whole bone strength of the femur from young mice (Table 1) but did increase the estimated material strength (Figure 7). With regards to brittleness, there was no drug effect on PYD and toughness. However, the fracture toughness of femur mid-shaft was significantly, but marginally greater with 1D11 treatment ( $5.16 \pm 0.59$  vs.  $5.61 \pm 0.44$ ; p=0.044 from a two-sided t-test). Interestingly, when scanned at an isotropic voxel size of 6 µm, instead of 12 µm, the notched, contralateral femurs in the 1D11 group had greater TMD than did the femurs in the 13C4 group ( $1339 \pm 8$  vs.  $1329 \pm 12$ , p=0.028). Lastly, we cultured bone marrow stromal cells from the

humeri of all 25 female mice in osteogenic media. Providing some insight into the action of 1D11, the colony forming units expressing alkaline phosphatase (CFU-AP) was consistently lower for 1D11 treated mice than for the 13C4 treated mice. This suggests that 1D11 treatment depleted the osteoprogenitor pool to some extent as inhibition of TGF- $\beta$  activity *in vivo* may favor osteoblast differentiation over cell renewal. For more information on molecular actions of TGF- $\beta$ , see the article by my colleague Dr. Xiangli Yang and myself in the appendix.



Figure 6. 1D11 increased mouse VB strength (Two-sided Student's t-test. mean  $\pm$  SD), and this effect can be explained in part by an increase in trabecular bone volume fraction (Task 4c).

#### Unfulfilled tasks

While all demineralized tibia have been sectioned and stained (Task 3c) for bone histomorphometry, our previous, antiquated evaluation software kept crashing. Fortunately, after much waiting, the PI's local VA research office recently acquired a major upgrade to the microscope system and software (new camera, digital stage, and BIOQUANT software). Measurements of osteoblast and osteoclast numbers per bone surface are ongoing (Task 3f). The fatigue testing protocol of the ulna proved to be problematic (Task 3e). Following

published methods [9], we ran the dynamic, three point bending tests in stress control in which the target force was calculated using the  $\mu$ CT-derived structural parameters. To our surprise, the young bones had much lower fatigue life than the old bones (no treatment). Since the young bones have lower stiffness than old bones, they were actually subjected to greater initial strain for the given stress than were the old rodent bones. Therefore, we are implementing a new protocol in which all bones are subject to the same initial strain. There will be several stress ranges as well. Quantifying



Figure 7. 1D11 increased estimated material strength of mouse cortical at the femur mid-shaft (Two-sided Student's t-test; mean  $\pm$  SD). As a further check on this structure-independent effect, the y-intercept for the 1D11 regression line was greater than that for the 13C4 regression line (1 outlier removed).

collagen crosslinks by high performance liquid chromatography is the final unfilled task (3f). Despite performing this assay extensively as a postdoctoral fellow, the PI struggled mightily in setting up the assay at the VA with a different instrument. Despite this delay, we have observed that pentosidine, the non-enzymatic collagen crosslink biomarker, is higher in the old bone (24 month old rats) than in the young bone (6 month old rats). The next step is to determine whether 1D11 affects this crosslink as well as mature, enzymatic collagen crosslinks.

## Planned future work

The PI has other resources to complete the unfilled tasks and perform 1 more rat study without additional funding. The plan is to submit the female mouse study for publication prior to the male rat study since it is closer to completion. Then, to address potential species dependency and age dependency, young 13 week old, F344 male rats will be treated with either 13C4 or 1 of 2 doses of 1D11 (as was done for the 6 month and 22 month old rats). If 1D11 increases BV/TV, we can conclude that TGF- $\beta$  activity in trabecular bone is variable throughout the age of skeleton. If 1D11 does not increase BV/TV, then we can conclude that 1 mg/kg and 5 mg/kg are not particularly effective doses for affecting trabecular bone in rats, demonstrating an important species-specificity of this potential therapy. Alternatively, 6 weeks of treatment could be too long (mice were treated for only 4 weeks) or too short to observe an effect in rats. The effect of 1D11 on bone strength in young growing rats should also be equally interesting, and we can compare findings to what we have observed with our male and female mouse studies. Regardless of the outcomes, the work will be submitted to a journal.

## **KEY RESEARCH ACCOMPLISHMENTS:**

- TGF-β inhibition is a target for increasing fracture resistance with effects beyond changing bone mass including improved trabecular architecture and material properties of cortical bone.
- The effectiveness of a neutralizing antibody to inhibit TGF-β is context dependent with differential effects on bone between young developing, mature adult, and aged rodents.
  - Trabecular bone of developing rodents, namely mice, is highly responsive to the anti-TGF- $\beta$  antibody 1D11.
  - Whereas, 1D11 primarily affects cortical bone from aged rodents, namely rats.
- A tool for non-destructively assessing vertebral body strength was shown to reasonably match experimental measurements (destructive testing).

## **REPORTABLE OUTCOMES:**

- Three abstracts at national society meetings
  - 2013 Orthopaedic Research Society Annual Meeting. San Antonio, TX. January 26-29.
     J.S. Nyman, A. Makowski, S. Uppuganti, J.A. Sterling, and D.S. Perrien. Assessing the Strength of Mouse Vertebral Bodies with μCT-derived Finite Element Analysis.
  - 2012 American Society for Bone and Mineral Research Annual Meeting. Minneapolis, MN. October 12-15.

A.J. Makowski, S. Uppuganti, and **J.S. Nyman**. Measuring the Fracture Toughness of Mouse Bone.

A.J. Makowski, S. Uppuganti, B. Rowland, A. Merkel, D.S. Perrien, J.A. Sterling, and **J.S. Nyman**. TGF- $\beta$  Suppression with a Neutralizing Antibody Increases Vertebral Body Strength in Female Mice.

- Two publications related to the objective of the grant and published during the funding period
  - J.S. Nyman and A.J. Makowski. The Contribution of the Extracellular Matrix to the Fracture Resistance of Bone. *Current Osteoporosis Reports*. 10:169-77, 2012.
  - Lian, N., Lin, T., Liu, W., Wang, W., Li, L., Sun, S., Nyman, J.S. and X. Yang. Transforming Growth Factor β suppresses osteoblast differentiation via vimentin activating transcription factor 4 (ATF4) axis. *The Journal of Biological Chemistry*. 287:35975-84, 2012.
- Funding applied for based on work supported by this award
  - Assessing Bone Fracture Resistance with Reference Point Indentation. Vanderbilt's Institute for Clinical and Translational Research Grant. Objective: The objective is to determine if a local

measurement of bone quality by reference point indentation at a distal site is predictive of fracture resistance at a site that is prone to osteoporotic fracture.

- Role of Vascularity and Matrix in Bone Brittleness. Vanderbilt Orthopaedic Institute Pilot Grant. Objective: The objectives are to determine whether a difference in blood vessel volume within bone decreases with aging in rodents and to identify matrix factors that potentially explain the loss of bone toughness with the loss of MMP-9 and aging.
- This award provided training experience for a graduate student (Alexander J. Makowski) pursuing a Ph.D.

## **CONCLUSION:**

The inhibition of TGF- $\beta$  by a commercially available antibody can improve the fracture resistance of cortical bone from aged animals. This is important because aging lowers fracture resistance beyond age-related loss in bone mass. The effect of TGF- $\beta$  inhibition by antibody treatment is however complex as there were treatment differences in fracture resistance among different age groups of rodents. For example, 1D11 increased whole bone strength through an increase in structure when tested in old rats, but it only increased material strength (independent of structure) when tested in young growing mice. With the presented results, there is impetus to assess matrix proteins in order to identify the target of TGF- $\beta$  that affects the resistance to crack propagation with advanced age.

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## **APPENDICES:**

Appendix A: ORS Abstract. Strength of Mouse Vertebral Bodies with µCT-derived Finite Element Analysis. Pages 9-12.

Appendix B: ASBMR Abstract. Measuring the Fracture Toughness of Mouse Bone. Pages 13-14.

- Appendix C: ASBMR Abstract. TGF-β Suppression with a Neutralizing Antibody Increases Vertebral Body Strength in Female Mice. Pages 15-16.
- Appendix D: Journal Article. The Contribution of the Extracellular Matrix to the Fracture Resistance of Bone. Pages 17-27.
- Appendix E: Journal Article. Transforming Growth Factor β suppresses osteoblast differentiation via vimentin activating transcription factor 4 (ATF4) axis. Pages 28-37.

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TITLE: Assessing the Strength of Mouse Vertebral Bodies with µCT-derived Finite Element Analysis

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KEYWORDS: Bone Mechanics, Finite Element Analysis, Osteoporosis.

#### **ABSTRACT BODY:**

**Introduction:** Finite element analysis (FEA) can assess the strength of bone because quantitative computed tomography (qCT) scans can be converted to finite element (FE) models. The usefulness of qCT-FEA is evident in the growing number of clinical studies reporting that strength predictions derived from CT scans of hip, spine, or radius can differentiate fracture patients from non-fracture patients, though some overlap exists across the cohorts [1-2]. Much of the validation behind the failure criteria in these FE model predictions came from correlations with strength measurements as determined by whole bone testing of cadaveric tissue for which empirical relationships exist to convert mineral density to material properties. Given the relatively small size of mouse bones, especially vertebral bodies (VBs), no such relationships exist even though  $\mu$ CT-FEA could also be useful in pre-clinical and genetic studies involving mice. Since there is a dearth of evidence establishing the ability of  $\mu$ CT-FEA to assess mouse VB strength, we performed a parametric analysis to determine whether an elastic  $\mu$ CT-FEA with simple failure criteria could reasonably predict VB strength as

determined by compression tests.

**Methods:** Study design: We assessed the strength of VBs from 2 studies in which differences in BV/TV were expected from prior work: a TGF $\beta$ -neutralizing Ab (1D11) for 4 weeks increased trabecular bone volume fraction (BV/TV) [3] and mice lacking activating transcription factor 4 (ATF4) have a low BV/TV compared to wild-type mice [4]. Therefore, we treated 13-wk, male mice (FVB strain) with a control antibody (13C4, n=7) or 1D11 (n=8) for 4 weeks at the same dose (10 mg/kg 3x per wk). We aged Atf4+/+ (n=10) and Atf4-/- (n=10) mice (male and female on a FVB background) to 17 weeks. After euthanasia, L6 VBs were harvested and stored frozen in PBS.

µCT-FEA: All VBs were scanned at 12 μm voxel size using the same settings (μCT40, Scanco Medical) and hydroxyapatite (HA) phantom calibration. To calculate BV/TV, contours were manually drawn for the region between the end plates (Fig. 1), whereas to generate the FE model, a circle with a constant radius was copied into each image and positioned to transect the transverse processes, which do not bear load in the compression test (Fig. 2). For the VBs of the 1D11 study, a threshold of 421.4 mgHA/cm3 segmented bone from soft tissue and air. Then, using Scanco FEA elastic solver software (brick elements), the equivalent strains were determined for high-friction, axial compression loading of each VB at 1% apparent strain, elastic modulus (E) of 10 GPa, and υ of 0.03 for all elements. The reaction force was determined for initial failure criteria in which 2% of the model volume exceeds 0.7% equivalent strain (Base Model), which were the criteria in a validation study for human bone [5].

Parametric study: Keeping the failure criteria of the Base Model constant, the elastic modulus was increased to 18 GPa and 22 GPa. Next, E was held constant while varying the percentage of volume that must exceed 0.7% strain before failure. Instead of having one value for E across all VBs, modulus was also based on the tissue mineral density (TMD) of each VB using a conversion derived by Wagner et al. [7]. As one final change, we investigated the effect of increasing the threshold to 538.9 mgHA/cm3 (Fig. 2).

To verify applicability across studies, the strength of VBs from the ATF4 study were determined for a threshold of 450.8 mgHA/cm3, 18 GPa, 0.7% failure strain, and a critical volume of 2%.

Compression tests: Each hydrated VB was subjected to axial compression at 3 mm/min in which the supporting platen had a rough surface and a moment relief to minimize slippage and off-axis loading. Two VBs were removed due to translation.

Statistical analysis: The ability of µCT-FEA to predict mouse VB strength was ascertained by linear regression and the root mean squared error (RMSE).

**Results:** The Base Model FEA was not effective in predicting mouse VB strength (Fig. 3a) as the RMSE was rather high and the 95% confidence interval (CI) of the slope was above 1. However, increasing E of bone tissue reduced the error and led to a CI of 0.67 to 2.09, while not affecting the R<sup>2</sup> (Fig. 3b,c). Using an E based on mean TMD for each VB increased the ability of FEA to predict experimental strength (Fig. 3d). Varying the failure volume from 1% to 4% had modest effects on R<sup>2</sup> and RMSE: 1% (66.9% and 5.8), 2% (62.3% and 5.0), 3% (62.0% and 5.5), and 4% (61.9% and 6.3). Interestingly, whether using a constant E or VB-specific E, increasing the threshold to reduce the number of bony elements in the model improved the R<sup>2</sup> to 70.7% and 73.1%, respectively (Fig. 3b,e). Building confidence in this approach to  $\mu$ CT-FEA, the predicted strength also strongly correlated with experimental strength of the VBs from the Genetic study with low error (Fig. 3f). Even though the FE model includes the thin cortex, the ability of FEA to explain the variance in experimental strength was similar to that of trabecular BV/TV (1D11: R2 = 62.4% & Genetic: R2 = 83.6%).

**Discussion:** A µCT-derived elastic FEA can reasonably assess the strength of mouse VBs when the model has uniform isotropic material properties typical of bone (18 GPa) and uses simple failure criteria (2% of volume exceeds 0.7% equivalent strain). The choice of the threshold does influence the ability of µCT-FEA to predict VB strength, and as such, segmented images should be viewed for multiple VB scans across experimental groups. Using an E based on TMD can improve the prediction. Whether further improvements are gained with an inhomogeneous distribution of E and non-linear material behavior are yet to be determined. Compression tests of mouse VBs are prone to error owing to their small size and irregular shape.

As such,  $\mu$ CT-FEA is an attractive way to assess experimental effects on VB strength, especially if histology is desired. However, the predicted strengths should be interpreted in the context of the FEA assumptions, namely material behavior. If differences are expected at the material level, then compression tests should be carefully conducted.

**Significance:** This study provides evidence that µCT-FEA can predict mouse vertebral strength in pre-clinical studies assessing anti-fracture efficacy.

Acknowledgements: CDMRP Concept Award provided support.

**References:** [1] Orwoll et al. J Bone Miner Res 2009 [2] Vilayphiou et al. J Bone Miner Res 2011 [3] Edwards et al. J Bone Miner Res 2010 [4] Yang et al. Cell 2004 [5] Pistoia et al. Bone 2002 [6] Wagner et al. Bone 2011.

(No Table Selected)



Figure 1. Hand contour for measuring BV/TV (left). Circle contour to generate finite model of the VB excluding the transverse processes.



Figure 2. Segmentation of the  $\mu$ CT scans at 2 threshold values (above line). Compression test of the L6 vertebra, boundary conditions of the FE model of the VB, and equivalent strain distribution for compression (below line).



Figure 3. Linear regression with 95% confidence intervals for selected failure criteria and modulus assumptions (modulus of model, critical volume, failure strain, and threshold value is given in the title of each graph).

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Events Calendar	<b>Female Mice</b> Categories: Osteoporosis - Treatment (Preclinical) Bone Biomechanics and Quality (Basic) Osteoporosis - Assessment <i>Poster Sessions, Presentation Number: SU0069</i> Session: Poster Session II and Poster Tours Sunday, October 14, 2012 11:30 AM - 1:30 PM, Minneapolis Convention Center, Discovery Hall-Hall B * Alexander Makowski, Department of Veterans Affairs Vanderbilt University, USA, Sasidhar Uppuganti, Vanderbilt University, USA, Barbara Rowland, Vanderbilt University, USA, Alyssa Merkel, Vanderbilt University, USA, Daniel Perrien, Vanderbilt University Medical Center, USA, Julie Sterling, Department of Veterans Affairs (TVHS)/Vanderbilt University, Medical Center, USA, Jeffry Nyman, Vanderbilt University, Medical Center, USA Transforming growth factor beta (TGF-β) is a promising target for reversing the breast cancer-induced decrease in bone's resistance to fracture. Our previous work with a neutralizing antibody to the 3 isoforms of TGF-β (1D11) found that TGF-β suppression increased trabecular bone (BV/TV) in the long bones of both young female, tumor-bearing mice are treated with an inhibitor of the TGF-β type I receptor kinase for 6 weeks. To test the hypothesis that 1D11 could be effective in enhancing fracture resistance in female mice, we treated 13 wk old FVB mice for 4 weeks with either 13C4 (control antibody, n=5) or 1D11 (10 mg/kg, n=7) 3x per week. After euthanasia, the L6 VB was scanned at an isotropic voxel size of 12 µm with a µCT scanner calibrated to a hydroxyapatite phantom. Using the scanner's finite element (FE) model generator (voxel-to-brick element) and elastic solver, the failure load of each VB was subjected to raila compression at 1% apparent strain and using failure criteria in which 2% of the model volume exceeds 0.007 equivalent strain. We selected 2 cases of material properties (v = 0.03): constant modulus (CM) of 18 GPa and VB-specific modulus (SpM) derived from the mean tissue mineral density (TMD). Lastly, each VB was subjected to axial compression at 3 mm/min in				
	BV/TV with some contribution from an increase in TMD. Disclosures: J. Sterling, VA: Research Grants. J. Nyman, VA: Research Grants. * Presenting Authors(s): Alexander Makowski, Department of Veterans Affairs Vanderbilt University, USA				
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Figure 1. Vertebral body strength was greater for the 1D11-treated mice than control mice.	
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*The Contribution of the Extracellular Matrix to the Fracture Resistance of Bone* 

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SKELETAL REGULATIONS (D GADDY, SECTION EDITOR)

## The Contribution of the Extracellular Matrix to the Fracture Resistance of Bone

Jeffry S. Nyman · Alexander J. Makowski

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Abstract The likelihood of suffering a bone fracture is not solely predicated on areal bone mineral density. As people age, there are numerous changes to the skeleton occurring at multiple length scales (from millimeters to submicron scales) that reduce the ability of bone to resist fracture. Herein is a review of the current knowledge about the role of the extracellular matrix (ECM) in this resistance, with emphasis on engineering principles that characterize fracture resistance beyond bone strength to include bone toughness and fracture toughness. These measurements of the capacity to dissipate energy and to resist crack propagation during failure precipitously decline with age. An age-related loss in collagen integrity is strongly associated with decreases in these mechanical properties. One potential cause for this deleterious change in

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J. S. Nyman (⊠) Vanderbilt Orthopaedic Institute, Medical Center East, South Tower, Suite 4200, Nashville, TN 37232, USA e-mail: jeffry.s.nyman@vanderbilt.edu addition to postmenopausal osteoporosis, certain diseases such as diabetes [1] and chronic kidney disease [2] also increase fracture risk. While an age-related or diabetes-related propen-

Introduction

fracture risk. While an age-related or diabetes-related propensity to fall can certainly increase the chance of breaking a bone [3], the skeleton does undergo deleterious changes with aging and disease onset, and these changes reduce the ability of bone to resist fracture. One well-recognized change is a loss of bone mass, and as such, osteoporosis therapies are designed to prevent bone loss (eg, bisphosphonates [BisPs]) or promote bone gain (eg, intermittent parathyroid hormone). Moreover, assessment of bone loss as a T-score or Z-score derived from dual-energy X-ray absorptiometry (DXA) is used clinically to assess fracture risk.

The risk of suffering a bone fracture increases with age, and in

the ECM is an increase in advanced glycation end products,

which accumulate with aging through nonenzymatic collagen crosslinking. Potential regulators and diagnostic tools of the

ECM with respect to fracture resistance are also discussed.

Keywords Bone quality · Toughness · Strength · Fracture

Microdamage · Porosity · Biomechanics · Fracture risk ·

AGEs · Osteoporosis · Diabetes · Aging · Finite element

analysis · Noncollagenous proteins · Mineral · TGF-B ·

toughness · Collagen · Structure · Architecture ·

Crosslinking · Matrix metalloproteinase

Despite the effectiveness of antiresorptive therapy [4] and bone anabolic therapy [5] in increasing DXA-derived areal bone mineral density (aBMD) and more importantly reducing the incidence of fracture, certain individuals still sustain fractures with treatment. In addition, long-term BisP use has been associated with atypical, subtrochanteric fractures, although no causal link has been established to date [6]. Thus, fractures are not solely the result of low bone mass or density, and treatment-related reductions in fracture incidence are not solely explained by changes in aBMD [7].

As further evidence supporting the notion that bone mass is not the sole determinant of fracture resistance, aBMD is not a particularly accurate predictor of an individual's risk of fracture, with many fractures occurring in subjects with T-scores below -2.5 (the number of standard deviation below or above young adult normal mean aBMD) [8]. In effect, the agerelated increase in fracture risk is independent of the agerelated decrease in BMD [8, 9]. That is, a 70-year-old is at a much greater risk of breaking a bone than a 50-year-old with the same BMD, an observation first published in the 1980s [10]. In addition to aging, the diabetes-associated increased risk of bone fracture is disproportionate to differences in aBMD between diabetic and age-matched nondiabetic patients [11]. Type 2 diabetes (T2DM) is not necessarily associated with lower BMD [12], and, in several studies, aBMD was actually found to be higher in T2DM patients [11, 13] than in age-matched, nondiabetic patients. This inability of aBMD to predict individual fracture risk has various origins, but one indication is that aging and certain diseases affect bone in ways that are invisible to DXA-derived measures of bone mass (eg, collagen integrity [14]). Capturing the importance of non-BMD factors, bone quality was defined in a National Institutes of Health conference as "the sum total of characteristics of the bone that influence the bone's resistance to fracture" [15]. Those characteristics span the multiple length scales, from millimeters to submicrons, comprising the hierarchical organization of bone and include the extracellular matrix (ECM).

Besides bone mass, cortical bone structure and trabecular bone architecture are determinants of fracture resistance. Avoiding the DXA limitations of acquiring measurements from a two-dimensional projection, quantitative, X-ray computed tomography (QCT) provides both structural parameters and volumetric BMD (in equivalent density of a hydroxyapatite phantom). High-resolution peripheral CT (HR-pQCT) can provide additional architectural parameters such as trabecular thickness and connectivity density [16]. With regard to cortical bone structure, moment of inertia and cross-sectional area correlate with whole-bone strength as determined by biomechanical testing of cadaveric femurs and radii in simulated fall configurations (Fig. 1) [17, 18]. In clinical studies involving QCT, patients with a femoral neck or trochanteric fracture had a smaller cortical cross-sectional area and lower moment of inertia at the neck than age-matched non-fracture patients [19], indicating decreased bone strength. The importance of bone structure to fracture resistance has been further demonstrated in recent studies that used the finite element method (FEM) to predict bone strength from patients' CT scan of the hip and found strength was strongly associated with fracture risk [20,

21••]. In addition, QCT-FEM assessment of vertebral body strength had a greater ability of discriminating vertebral fractures than areal BMD or volumetric BMD in a cross-sectional study of postmenopausal women [22]. Nonetheless, given that fractures can arise from fatigue loading, and that the fracture process involves the growth of cracks through the bone tissue, bone strength is not likely the sole contributor to a fracture event. Fracture resistance of bone also depends on the inherent quality of the ECM as described herein.

#### Age-Related Changes in Bone Toughness

From the 1960s to the present, studies involving the mechanical testing of fresh frozen, cadaveric bone have identified numerous age-related determinants of bone's resistance to fracture (Table 1). In these cadaveric studies, bone samples are milled or cored to produce specimens with uniform geometry at the length scale of several millimeters (Fig. 1) [23], which is 10-20 times greater than the size of an osteon or trabecula. With respect to cortical bone, aging affects the ability of bone to dissipate energy during failure (bone toughness) to a greater extent than it does the material strength [24–27]. Basically, older bone is brittle while younger bone is ductile, and this age-related change is primarily due to a loss in the ability of aged bone to deform or stretch beyond the point at which damage begins to form in the ECM (ie, yield point or proportional limit) [27]. The exact mechanism causing a decrease in bone toughness with age still requires further investigation. However, it is known that the organic phase of the ECM, namely type 1 collagen, is primarily responsible for bone toughness. For example, in mechanical testing studies of cadaveric bone, an age-related decrease in the stability of collagen correlated with bone toughness [26] as did an agerelated decrease in collagen content [27]. In addition, manipulations to the collagen, such as thermal-induced collagen denaturation [28], formalin fixation [29], and high-energy, gamma or X-ray irradiation [30, 31], reduce the toughness of bone without necessarily affecting its material strength.

## Fracture Toughness as a Measurement of Fracture Resistance

In engineering mechanics, strength is not the only material property guiding design. Since all materials have flaws or void spaces, however small, there is potential for a crack to form during the service life of the material. If the crack reaches a critical size, failure of the material is inevitable. Therefore, there are mechanical tests that characterize the ability of a material to resist crack initiation and crack propagation (a.k.a. fracture toughness) in addition to those that measure strength (yield or peak stress) and toughness (area

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**Fig. 1** To fully characterize the contributors to the fracture resistance of bone, the tissue is tested at multiple length scales, which involves analyses at progressively smaller length-scales: **a**, macrostructure (> 10 mm), testing of a femur in a sideways fall configuration to quantify whole-bone strength; **b**, submacrostructure (2–5 mm), tensile mechanical testing of uniform cortical bone strips to quantify apparent material propeties; **c**, microstructure (150–350 µm), reference point indentation (RPI) to characterize in situ or in vivo damage resistance of the extracellular matrix, as shown in the scanning electron micrograph in the *lower portion of the panel*; **d**, submicrostructure (0.5–25 µm), Raman spectroscopy (RS) to assess compositional properties of the matrix such as mineral-to-collagen ratio and crystallinity and nanoindentation to acquire complementary properties of modulus and hardness

under the stress-vs-strain cure). Whether measured by the critical stress intensity factor [32, 33] or the strain energy release rate [34, 35], fracture toughness of human bone decreases during aging (Table 1). Moreover, healthy bone tissue possesses the ability to demand greater energy to continue propagating a crack as the crack grows in length (ie, R-curve behavior) [36, 37], and this behavior is lost or reduced with aging [38, 39].

The determinants of fracture toughness are rather complex, spanning multiple length scales (Table 1), and as such, clinical surrogates of fracture toughness are not obvious. With respect to mechanism, Vashishth et al. [40, 41] observed the formation of microscopic cracks within the ECM of bone-bovine, antler, and human-occupying "process zones" ahead and in the wake of a propagating crack. Nalla et al. [42] and Zimmermann et al. [43•] further observed unbroken "ligaments" of bone tissue along the path of the propagating crack. As explained by Ritchie [44], these processes contribute to toughening, with an intrinsic mechanism of formation of the frontal microdamage zone and extrinsic mechanisms of crack bridging within the ECM as well as crack deflection around osteons. The exact changes in the ECM affecting these mechanisms remain to be fully elucidated, but age-related increases in nonenzymatic collagen crosslinking are implicated [43•, 45, 46].

The aforementioned observations of toughening mechanisms were made through analyses of ex vivo bone samples using nonclinical instruments such as scanning electron microscopy and synchrotron micro-CT (SR-µCT). Perhaps the closest clinical measurement of bone's resistance to crack propagation is the indentation distance increase (IDI) that is quantified using reference point indentation (RPI). Unlike microindentation or nanoindentation in which a hard tip (eg, diamond) penetrates the material such that depth, force, and contact area are recorded to quantify hardness and modulus, RPI utilizes a reference probe (akin to a hypodermic needle) and a test probe that slides through the reference probe indenting the bone tissue (Fig. 1) [47]. Moreover, RPI is designed for clinical use. Specifically, the RPI reference probe engages the patient's bone under the periosteum of the tibial mid-shaft; the test probe performs 20 cycles of microindents into the tissue (50-100 µm in diameter x 100-200 µm in depth) in force control; and then, among other properties, the instrument



Table 1Age-related changes inbone at different length scaleshave been associated withchanges in fracture resistance

Length scale, $\mu m$	Bone tissue characteristic	Material property	Reference	
100	↑ Osteons per area	↓ Tensile strength	[23]	
100	↑ Percentage osteons	↓ Resistance to crack propagation	[34]	
10-50	↑ Porosity	↓ Post-yield toughness	[25]	
10-50	↑ Porosity	↓ Resistance to crack propagation	[35]	
1–5	↑ Microdamage	↓ Resistance to crack propagation	[33]	
0.1	↓ Collagen integrity	↓ Toughness	[26]	
0.1	↑ Collagen crosslinks	↓ Fracture toughness	[45]	
0.1	$\downarrow$ Collagen content	↓ Post-yield energy dissipation	[27]	

records IDI, the relative distance that the test probe travels into the ECM of bone. During indentation, microdamage forms below the indenter (Fig. 1), and presumably, bone matrix with less resistance to microdamage formation allows the test probe to penetrate deeper than bone matrix with high resistance. Recently, the IDI for 27 fracture patients was observed to be 47% greater than the IDI for 8 age-matched patients (P= 0.008) in the same hospital for non-fracture reasons [48••]. Using cadaveric bone from several donors, IDI was found to be inversely proportional to crack growth toughness [48••], thus linking local indents to apparent-level fracture toughness.

## Formation of Fatigue Microdamage in the Extracellular Matrix

Although the bone's ECM dissipates energy through the generation of microdamage as a means to improve its fracture toughness, bone must still minimize the accumulation of fatigue-induced microdamage in non-cracked regions. When subjected to repeated (ie, cyclic) loading over time at stresses well below the yield strength of bone, the bone matrix accumulates linear microcracks and diffuse patches of nanocracks. Among other functions, bone remodeling serves to remove this fatigue-induced microdamage [49, 50]. Nonetheless, the degree of microdamage in bone increases with age [51, 52], suggesting age-related changes in bone's ECM favors microdamage formation. Certainly, engineering measurements of fatigue properties such as fatigue life (number of cycles to failure, N<sub>f</sub>) and fatigue strength (y-intercept on an applied stress vs Nf semi-log curve) of cadaveric bone decrease with age [53, 54]. The changes in the ECM that lead to such declines are not fully understood, but fatigue microdamage is known to accumulate between osteons in the interstitial sites [55], and interstitial sites (ie, remnants of remodeled osteons) have an older tissue age with higher mineral-to-collagen ratio [56] and greater concentrations of mature collagen crosslinks [57]. Moreover, the number of lacunae in the ECM and the degree of mineralization in peri-lacunar space has been observed to decrease and increase, respectively, with age [58•]. Also, greater microdamage has been associated with a reduction

in osteocyte lacunar density [59]. Thus, a "brittling" of the ECM with human and tissue age lowers the tissue strain surrounding a crack, allowing it to propagate through microstructural barriers like cement lines, as recently observed for human cortical bone [60].

## Organic Contribution to Fracture Resistance: Collagen, AGEs, and NCPs

As previously discussed, type 1 collagen, which comprises 90% of the organic matrix, is the primary determinant of bone toughness. It is critical to overall fracture resistance, even though increasing mineral content is still the primary focus of drug discovery for fracture prevention. The classic example of collagen's importance in bone is osteogenesis imperfecta (OI), known as the brittle bone disease, in which mutations in the gene encoding collagen strands (collal) lead to various grades of severity in the loss of fracture resistance. OI mice have bones with less post-yield deformation (ie, more brittle) than do wild-type mice [61, 62], and there is greater microdamage formation with altered morphology in OI bones [63]. Beyond this defect in collagen that causes other changes in the matrix (eg, crystallinity), crosslinking is also important. For example, disrupting enzymatic collagen crosslinking by treating rats with a lysyl oxidase inhibitor reduces bone strength without affecting mineralization [64].

Increases in advanced glycation end products (AGEs) perhaps have garnered the greatest attention as a potential change in the organic matrix causing a decrease in fracture resistance of bone. Formed through a nonenzymatic reaction process involving sugar, these crosslinks of collagen increase in concentration with age and diabetes, even though bone turns over or remodels. Age-related increases in pentosidine, a quantifiable biomarker of AGEs, are associated with a decrease in post-yield energy dissipation and fracture toughness of bone [27, 45]. Clinically, serum or urine levels of pentosidine have been associated with higher incidence of fracture in postmenopausal women [65, 66••] and subjects with T2DM [67, 68]. The concentration of pentosidine is higher in rodents with diabetes relative to control bone [69], and given enough time for diabetes to progress in rodents, bones become brittle [70]. Although a mechanism is not fully delineated, one possibility is that AGEs stiffen the collagen matrix such that the fibrils dissipate less energy, allowing microdamage to more readily form and propagate through the ECM.

Recently, attention has also been given to noncollagenous proteins (NCPs) as contributors to the fracture resistance of bone. Initially, small-scale mechanics using atomic force microscopy demonstrated that phosphorylated proteins can bridge neighboring mineralized collagen fibrils forming so-called sacrificial bonds resisting fibril separation [71]. Subsequently, osteopontin (OPN) was one NCP observed to dissipate energy through this mechanism [72]. Mice lacking osteopontin (OPN-/- mice) have bones with 30% lower fracture toughness compared to wild-type bones. However, it should be noted that this difference may not solely be due to the role of OPN as a sacrificial bonding agent since OPN is involved in mineralization and OPN-/- bones exhibited greater tissue heterogeneity than OPN+/+ bones [73]. Interestingly, osteonal tissue has higher amounts of OPN and osteocalcin than does interstitial tissue [74], and as previously mentioned, interstitial sites are where microcracks are most often observed in cortical bone.

#### The Role of Water in Fracture Resistance

As an essential component of the ECM, water is bound to both collagen and mineral phases via hydrogen-hydrogen bonding. As the amount of water removal from human cortical bone increases with an increase in drying temperature (24°C to 50°C to 70°C), the toughness of bone significantly decreases, suggesting loosely and tightly bound water contribute to fracture resistance [75]. Using solid-state nuclear magnetic resonance (NMR), a structural layer of water was observed between the collagen and mineral phases of bone [76] and could act as another sacrificial layer protecting collagen fibrils from shear forces [76]. Measurements of bound water using <sup>1</sup>H NMR spectroscopy have been correlated with the mechanical properties of human cortical bone [77, 78], and if successfully translated to clinical imaging (magnetic resonance imaging), bound water could potentially be an important indicator of fracture resistance.

## Spectroscopic Characterization of Bone Tissue with Respect to Fracture Resistance

Fourier transform infrared (FTIR) and Raman spectroscopy (RS) are two complementary optical techniques widely used to gain insight into the biophysical nature of bone's ECM as it relates to fracture resistance. In brief, when light impinges on chemical bonds, energy is gained or lost depending on the light wavelength and molecular vibrations of the chemical moieties in the tissue. The intensity spectrum of collected light (Fig. 1) corresponds to the biochemical distribution of the bone matrix. FTIR indirectly measures differential absorption of light by anti-symmetric vibrations through the attenuation of transmitted infrared wavelengths. Due to water absorption, FTIR usually requires thin, dehydrated ex vivo samples [79]. RS utilizes direct reflectance measures of differential scattering off symmetric vibrations, shifted relative to the input wavelength. Despite added complications from concurrent fluorescence, RS with its reflectance design has clinical potential as a noninvasive instrument for bone matrix measures in situ and even diagnostic capability through skin [80, 81].

While the potential for optical spectroscopy to assess fracture resistance clinically is nascent, many studies into the ECM of bone have already contributed to our understanding of fracture resistance. Investigations utilizing optical spectroscopies have demonstrated the following: FTIR sensitivity to many processes involved in matrix mineralization [82]; Raman sensitivity to damage and defects in the bone matrix [83]; and Raman spectral changes in the ECM related to agerelated changes in the mechanical properties of cortical bone [84, 85]. Building upon established Raman markers of bone quality [86], we recently demonstrated consistent Raman measurements of compositional differences between osteonal and interstitial tissue from human cortical bone across bone orientation and processing conditions [56].

Recently, there have been efforts to determine whether spectroscopy can assess fracture risk and treatment-related changes to the ECM. Expanding upon evidence for a spectroscopic profile of osteoporosis [87], Boskey et al. [88] analyzed iliac crest biopsies with FTIR. They correlated matrix turnover rates of osteoporotic bone with relative tissue homogeneity and specifically demonstrated globally decreased mineral-tomatrix ratio and increased crystal size in tissue from patients with osteoporosis. In another FTIR study, Gourion-Arsiquaud et al. [89] showed that fragility fractures were associated with locally increased mineral content, carbonate substitution, and collagen crosslinking. Interestingly, carbonate substitution provided the greatest difference between fracture and non-fracture patients. Moreover, the associations of the FTIR-derived properties of mature-to-immature collagen crosslinking ratio and mineral-to-collagen ratio with fracture risk were independent of aBMD. Further insight into the effects of BisPs on bone matrix was provided by Gourion-Arsiguaud et al. [89]. They examined the tibia of beagles after 1 year of BisP treatment and found increased mineral content and increased collagen crosslinking maturity that was also more homogenous than bone from untreated controls [90]. Then, in an FTIR analysis of iliac crest samples obtained from patients enrolled in the Fracture Prevention Trial, Paschalis et al. [91] showed a spectral profile of teriparatide (recombinant human parathyroid hormone)-

SKELETAL REGULATIONS (D GADDY, SECTION EDITOR)

## The Contribution of the Extracellular Matrix to the Fracture Resistance of Bone

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Abstract The likelihood of suffering a bone fracture is not solely predicated on areal bone mineral density. As people age, there are numerous changes to the skeleton occurring at multiple length scales (from millimeters to submicron scales) that reduce the ability of bone to resist fracture. Herein is a review of the current knowledge about the role of the extracellular matrix (ECM) in this resistance, with emphasis on engineering principles that characterize fracture resistance beyond bone strength to include bone toughness and fracture toughness. These measurements of the capacity to dissipate energy and to resist crack propagation during failure precipitously decline with age. An age-related loss in collagen integrity is strongly associated with decreases in these mechanical properties. One potential cause for this deleterious change in

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J. S. Nyman (⊠) Vanderbilt Orthopaedic Institute, Medical Center East, South Tower, Suite 4200, Nashville, TN 37232, USA e-mail: jeffry.s.nyman@vanderbilt.edu addition to postmenopausal osteoporosis, certain diseases such as diabetes [1] and chronic kidney disease [2] also increase fracture risk. While an age-related or diabetes-related propen-

Introduction

fracture risk. While an age-related or diabetes-related propensity to fall can certainly increase the chance of breaking a bone [3], the skeleton does undergo deleterious changes with aging and disease onset, and these changes reduce the ability of bone to resist fracture. One well-recognized change is a loss of bone mass, and as such, osteoporosis therapies are designed to prevent bone loss (eg, bisphosphonates [BisPs]) or promote bone gain (eg, intermittent parathyroid hormone). Moreover, assessment of bone loss as a T-score or Z-score derived from dual-energy X-ray absorptiometry (DXA) is used clinically to assess fracture risk.

The risk of suffering a bone fracture increases with age, and in

the ECM is an increase in advanced glycation end products,

which accumulate with aging through nonenzymatic collagen crosslinking. Potential regulators and diagnostic tools of the

ECM with respect to fracture resistance are also discussed.

Keywords Bone quality · Toughness · Strength · Fracture

Microdamage · Porosity · Biomechanics · Fracture risk ·

AGEs · Osteoporosis · Diabetes · Aging · Finite element

analysis · Noncollagenous proteins · Mineral · TGF-B ·

toughness · Collagen · Structure · Architecture ·

Crosslinking · Matrix metalloproteinase

Despite the effectiveness of antiresorptive therapy [4] and bone anabolic therapy [5] in increasing DXA-derived areal bone mineral density (aBMD) and more importantly reducing the incidence of fracture, certain individuals still sustain fractures with treatment. In addition, long-term BisP use has been associated with atypical, subtrochanteric fractures, although **Disclosures** Conflicts of interest: J.S. Nyman: has received grant support from National Science Foundation, Department of Defense, Veterans Affairs; and has a patent (planned, pending or issued) for Vanderbilt University and the Department of Veterans Affairs for U.S. Provisional Application No. 61/369,248: System and Method for Determining Mechanical Properties of Bone Structures; A.J. Makowski: none.

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## Transforming Growth Factor $\beta$ Suppresses Osteoblast Differentiation via the Vimentin Activating Transcription Factor 4 (ATF4) Axis<sup>\*</sup>

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**Background:** Transforming growth factor  $\beta$  (TGF $\beta$ ) inhibits *osteocalcin* (*Ocn*) transcription and osteoblast differentiation. **Results:** The inhibition of *Ocn* expression and osteoblast differentiation by TGF $\beta$  is blunted upon lack of *Atf*4 or *vimentin* knockdown.

**Conclusion:** ATF4 and vimentin are novel downstream targets of TGF $\beta$  in osteoblasts.

**Significance:** Understanding mechanisms by which transcription factors are regulated is crucial for developing effective anabolic drugs for bone.

ATF4 is an osteoblast-enriched transcription factor of the leucine zipper family. We recently identified that vimentin, a leucine zipper-containing intermediate filament protein, suppresses ATF4-dependent osteocalcin (Ocn) transcription and osteoblast differentiation. Here we show that TGFB inhibits ATF4-dependent activation of Ocn by up-regulation of vimentin expression. Osteoblasts lacking Atf4 ( $Atf4^{-/-}$ ) were less sensitive than wild-type (WT) cells to the inhibition by TGF $\beta$  on alkaline phosphatase activity, Ocn transcription and mineralization. Importantly, the anabolic effect of a monoclonal antibody neutralizing active TGF $\beta$  ligands on bone in WT mice was blunted in  $Atf4^{-/-}$  mice. These data establish that ATF4 is required for TGF<sub>β</sub>-related suppression of Ocn transcription and osteoblast differentiation in vitro and in vivo. Interestingly, TGF $\beta$  did not directly regulate the expression of ATF4; instead, it enhanced the expression of vimentin, a negative regulator of ATF4, at the post-transcriptional level. Accordingly, knockdown of endogenous vimentin in 2T3 osteoblasts abolished the inhibition of Ocn transcription by TGF $\beta$ , confirming an indirect mechanism by which TGF $\beta$  acts through vimentin to suppress ATF4-dependent Ocn activation. Furthermore, inhibition of PI3K/Akt/mTOR signaling, but not canonical Smad signaling, downstream of TGF $\beta$ , blocked TGF $\beta$ -induced synthesis of vimentin, and inhibited ATF4-dependent Ocn transcription in osteoblasts. Thus, our study identifies that TGFB stimulates vimentin production via PI3K-Akt-mTOR signaling, which leads to suppression of ATF4-dependent Ocn transcription and osteoblast differentiation.

Transforming growth factor  $\beta$  (TGF $\beta$ )<sup>2</sup> regulates many biological processes including patterning during development, cell proliferation, differentiation, apoptosis, and other physiological and pathological conditions such as wound healing, fibrosis, and cancer growth and metastasis. In the mammalian skeleton, TGF $\beta$  is one of the most abundant cytokines stored in bone matrix; and once activated from its latent form, it promotes osteoblast proliferation and mesenchymal stem cell recruitment to active bone remodeling sites (1-5), while inhibits osteoblast differentiation (6-8). The canonical Smad-dependent signaling has been identified as a mediator of TGF $\beta$  in skeletal cells as well as in many other cell types. In response to ligand binding, TGF $\beta$  receptors phosphorylate Smad2 and/or Smad3, which in turn bind to Smad4 to induce translocation into the nucleus (9), where the Smad complex either binds directly to DNA or indirectly to other transcription factors to regulate gene transcription (10).

It has become clear now that multiple Smad-independent or noncanonical signaling pathways are activated in response to TGF $\beta$  ligands in various cell types. These include: MAP kinase (MAPK) pathways in intestine, lung epithelial cells, and breast cancer cells; Rho-like GTPase signaling pathways during epithelial to mesenchymal transition (EMT) in epithelial cells and primary keratinocytes; and mammalian targets of rapamycin (mTOR) through phosphatidylinositol 3-kinase (PI3K) and Akt pathways during EMT in fibroblasts (11–18). Although each of these signaling pathways has been shown to play an important role in skeletal biology, whether TGF $\beta$  activates non-canonical signaling pathways in osteoblasts is unknown.

Activating transcription factor (ATF4) is a leucine zippercontaining transcription factor belonging to the CREB family



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<sup>&</sup>lt;sup>2</sup> The abbreviations used are: TGFβ, transforming growth factor β; EMT, epithelial to mesenchymal transition; ATF, activating transcription factor; mTOR, mammalian targets of rapamycin; *Ocn, osteocalcin*; μCT, microcomputed tomography; HA, hydroxyapatite; HDAC, histone deacetylase; PTH, parathyroid hormone.

## TGFβ Stimulates Vimentin via Non-Smad Signaling

and was originally identified as an osteoblast-specific transcription factor required for *osteocalcin* (*Ocn*) transcription and osteoblast differentiation (19). *Ocn* mRNA is exclusively expressed in differentiated osteoblasts hence it is often used as a marker gene of mature osteoblasts (20). Together with Runx2, the first reported osteoblast-specific transcription factor, ATF4 activates *Ocn* transcription *in vitro* and *in vivo* through direct binding to its cognate <u>osteoblast-specific element 1</u> (OSE1) of the *Ocn* promoter (19, 21, 22). In osteoblasts, TGF $\beta$  targets Runx2 via a canonical Smad signaling pathway to achieve its inhibition of both *Runx2* and *Ocn* transcription, thereby suppressing osteoblast differentiation (6). However, it is possible that TGF $\beta$  targets other effectors at the transcriptional level to inhibit *Ocn* transcription in osteoblasts.

We have recently identified that in osteoblasts vimentin binds directly to ATF4 through its first leucine-zipper domain, which prevents ATF4 from binding to its cognate DNA OSE1 on the Ocn promoter, leading to inhibition of ATF4-dependent Ocn transcription and osteoblast differentiation (23). Vimentin is a member of the intermediate filament protein family and the most widely accepted molecular marker of mesenchymal cells. Moreover, its mRNA is often up-regulated in response to  $TGF\beta$ during EMT and cancer progression (24, 25). Consistent with its inhibitory role during osteoblast differentiation, vimentin expression is down-regulated when osteoblasts progress toward a fully differentiated stage (23). Since this suggested that one component of vimentin regulation involves the regulatory control of its expression, we searched for the extracellular ligands that govern its expression. In doing so, we noticed that TGF $\beta$  stimulated *vimentin* mRNA in C2C12 myoblastic cells (26), a cell type of mesenchymal origin that can differentiate into chondrocytes and osteoblasts (27). Given that TGF $\beta$  and vimentin both negatively regulate Ocn transcription and osteoblast differentiation, we hypothesized that  $TGF\beta$  targets vimentin and ATF4 to suppress Ocn transcription and osteoblast differentiation.

Here we present evidence that TGF $\beta$  requires endogenous ATF4 to inhibit *Ocn* transactivation in primary osteoblasts and osteoblastic cell lines. With the delivery of a monoclonal anti-TGF $\beta$  antibody to mice, we show that ATF4 is also required for TGF $\beta$  to increase bone mass. Employing a series of molecular and biochemical approaches, we demonstrate that TGF $\beta$  directly up-regulates vimentin production at post-transcriptional level, via PI3K-Akt-mTOR signaling, but not Smad signaling, to achieve its inhibition of *Ocn* transcription. Therefore, our study identifies two novel effectors, vimentin and ATF4, that act downstream of TGF $\beta$  in the regulation of osteoblast differentiation via PI3K-Akt-mTOR signaling.

#### **EXPERIMENTAL PROCEDURES**

*Materials*—Tissue culture media and fetal bovine serum were purchased from Invitrogen. Anti-vimentin antibodies were from Santa Cruz Biotechnology and Biovision for V9 antirat vimentin and anti-mouse vimentin (#3634), respectively. Antibodies for ATF4 (C20) and Sp1 (PEP2) were from Santa Cruz Biotechnology, HA tag was from Abcam (ab9110); Flag tag was from Sigma (M2); and phospho-Smad2/3 was from Cell Signaling. Recombinant human (rh) TGF $\beta$ 1 is from R&D systems. All chemicals were from Sigma unless indicated otherwise.

Cell Culture—ROS17/2.8 rat osteosarcoma cells were grown in DMEM/F-12 medium containing 10% FBS. Mouse osteoblastic 2T3 and MC3T3-E1 cell lines were cultured in  $\alpha$ -Minimal Essential Medium ( $\alpha$ MEM) containing 10% FBS. C2C12 myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM) that contains 10% FBS and myoblastic DMEM or differentiation medium that contains 2% horse serum. COS1 monkey kidney cells were cultured in DMEM containing 10% FBS. All media were supplemented with 1% penicillin-streptomycin, and cells were passaged every 3 days.

Northern Blot Hybridization—Total RNA from indicated sources was isolated using TRIzol (Invitrogen) according to the manufacturer's protocols. Total RNA (5  $\mu$ g) was resolved in 1% agarose gel and transferred onto nylon membranes. After crosslinking with UV light, the membranes were hybridized in 6× SSC buffer at 60 °C overnight with the following probes: partial cDNA of mouse *vimentin* from 792 to 1218, mouse *Atf4* covering 287 nucleotides of 5'-untranslated region and 180 nucleotides of coding region, full-length mouse *Ocn gene 2*, and mouse *Gapdh* cDNAs.

*Establishment of Permanent Reporter Cells*—ROS17/2.8 cells were seeded at a density of  $5 \times 10^5$  cells/well in 6-well culture dishes, and reporter construct (1 µg) of p6×OSE1-Luc, p6×mOSE1-Luc, or negative control p3xAP1-Luc was cotransfected with pcDNA3.1(+) (20 ng) at 50:1 ratio using Lipofectamine (Invitrogen) 18 h later. Cells were then allowed to grow to confluence. Neomycin (G418)-resistant colonies were then selected with G418 (300 µg/ml)-containing culture medium and pooled for experimental use.

Transient DNA Transfection and Luciferase Assay—COS1 cells were seeded at a density of  $2.5 \times 10^5$ /well in 6-well culture dishes for 20 h and then transfected with 2 µg/well of Flag-ATF4 or HA-vimentin expression plasmid. For reporter assays, cells were plated in 24-wells at a density of  $2.5 \times 10^4$ /well. p6×OSE1-Luc (0.25 µg/well) together with 0.05 µg/well of  $\beta$ -galactosidase ( $\beta$ -gal) were transfected into COS1 cells using Lipofectamine (Invitrogen). Cells were lysed 24 h post DNA transfection, and the luciferase and  $\beta$ -gal activity was measured using cell lysate. Fold activation or inhibition of luciferase activity with  $\beta$ -gal activity. For vimentin knockdown, 2T3 osteoblastic cells were plated in 6-well culture dishes at  $2.5 \times 10^5$ /well and then transiently cotransfected with *psiRNA* (1 µg/well) empty vector or *psiRNA-Vim* with indicated reporter constructs (1 µg/well).

Primary Calvarial Osteoblast Isolation and Osteoblast Differentiation Assay—Calvariae were collected from neonatal (P3) pups, pressed on Science Brand Kimwipes to remove blood and surrounding tissues, cleaned with  $1 \times$  PBS, and then subjected to series of collagenase digestions as described previously (28). Cells released from the first 2 digestions were discarded to enrich the numbers of osteoblastic cells. Cells released from the third digestion were plated in  $\alpha$ MEM containing 10% FBS until confluence, which was defined as passage 0.

For differentiation assays, primary cells from passage 1 or osteoblastic cell lines were grown in 24-well plates in  $\alpha$ MEM supplemented with 5 mm  $\beta$ -glycerophosphate and 100  $\mu$ g/ml



ascorbic acid for either 2 or 12 days for alkaline phosphatase staining or von Kossa staining, respectively, as previously described (29).

2G7 Treatment—Ten pairs of 10-week-old WT and  $Atf4^{-/-}$  littermates were treated with anti-TGF $\beta$  monoclonal antibody 2G7 or control IgG at 10 mg/kg. The intraperitoneal injections of each antibody were administered 3 times per week for 4 weeks (30).

Micro-computed Tomography (µCT) Analysis—Mouse femurs were collected and fixed overnight in 4% PFA (pH 7.4) and then 70% ethanol. Trabecular bone of the metaphysis was evaluated using an ex vivo µCT imaging system (Scanco µCT40; Scanco Medical, Bassersdorf, Switzerland). Each femur was fit into a specimen tube and aligned with the scanning axis. Tomographic images were acquired at 55 kV and 145 mA with an isotropic voxel size of 12  $\mu$ m, and at an integration time of 250 ms with 500 projections collected per 180 °C rotation. For 100 slices proximal to the growth plate, contours were fit to the inner layer of the cortical bone to define the total tissue volume (TV). Applying a Gaussian noise filter with a variance of 0.8 and support of 1 as well as a threshold of 300 mg of hydroxyapatite (HA) per cm<sup>3</sup> to distinguish mineralized from non-mineralized tissue for each femur scan, we quantified the volume of trabecular bone tissue (BV) per TV (BV/TV) as well as volumetric density (in mgHA/cm3) of the bone-mineralized tissue (BMD) (31).

Western Blot Analysis—Total cell lysates from indicated cell types were isolated with radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, and protease inhibitors (Complete ULTRA Protease Inhibitor Mixture Tablets, Roche Applied Science). Nuclear extracts were isolated using high-salt nuclear extraction buffer on hypotonic buffer-swelled cells as described (32). Total cellular proteins or nuclear extracts (50  $\mu$ g per lane) were loaded onto 10% SDS-PAGE and Western blots were performed following standard protocols. Antibody concentrations were 0.5–1  $\mu$ g/ml.

TGF $\beta$  and Inhibitor Treatment—ROS17/2.8 cells, primary rat or mouse calvarial osteoblasts, in 60-mm plates at 90% confluent, were starved in serum-free media overnight. rhTGF $\beta$ 1 (0.2~2 ng/ml) or vehicle (4 mM HCl containing 1 mg/ml BSA) was then added to the cell culture medium containing 1% FBS, and cells were incubated for an additional 5–6 h. For inhibitor treatment, MG115 (Piptide Institute Inc. Japan), SB505124 (Sigma), cycloheximide (Sigma), wortmannin (EMD4Biosciences), or rapamycin (EMD4 Biosciences) at indicated concentrations (0.1 nM to 5 nM) were pre-incubated with cells for 30 min prior to adding TGF $\beta$ .

*Statistics*—Data are presented as mean  $\pm$  S.D. Statistical analyses were performed using Student's *t* test. All *in vitro* experiments were repeated at least three times.

### RESULTS

ATF4 Is Required for TGF $\beta$  to Inhibit Osteoblast Differentiation—To investigate whether ATF4 was downstream of TGF $\beta$ , we first established stable ROS17/2.8 cell lines carrying a reporter luciferase gene driven by 6 repeats of wild type (WT) ATF4 binding element OSE1, p6xOSE1-Luc. As controls, we also made two other stable cell lines carrying a luciferase gene driven by either 6 repeats of a mutant ATF4 binding element, p6xmOSE1-Luc to which ATF4 fails to bind (21), or 3 repeats of the activating protein 1 (AP1) binding element, p3xAP1-Luc. Treatment of these reporter cell lines with rhTGF $\beta$ 1 at various concentrations, ranging from 0.2 to 2 ng/ml, inhibited dose-dependently luciferase activity driven by 6xOSE1, but not by 6xmOSE1 (Fig. 1, *A* and *B*), indicating that the inhibitory effect of TGF $\beta$  on ATF4-dependent *Ocn* promoter activity relies on ATF4 binding to its cognate DNA. Furthermore, TGF $\beta$  inhibition is specific to the *Ocn* promoter and not a general phenomenon of leucine zipper-containing transcription factors since TGF $\beta$  failed to reduce luciferase activity in reporter cells driven by AP1 DNA binding element repeats (Fig. 1*C*).

To determine whether ATF4 was necessary for TGFβ to suppress endogenous Ocn expression and osteoblast differentiation, we utilized the *Atf4*-deficient ( $Atf4^{-/-}$ ) mouse model (33). Freshly isolated primary calvarial osteoblasts (passage 1) from wild type (WT) and  $Atf4^{-/-}$  pups were treated with rhTGF $\beta$ 1 (0.5 ng/ml) under osteogenic induction media for 10 days, and total RNA was collected for qRT-PCR analyses. Our data showed that TGF $\beta$  inhibited endogenous *Ocn* expression by 86% in WT calvarial osteoblasts but only by 43% in  $Atf4^{-/-}$ calvarial osteoblasts (Fig. 1D). Consistent with this observation, TGFB reduced alkaline phosphatase (ALP) activity in WT control cells by 46% but had no such effect in  $Atf4^{-/-}$  calvarial cells (Fig. 1E). Lastly, low doses of TGFB (0.1 and 0.2 ng/ml) decreased the number of mineralized nodule formation by 90% in WT but only 20% in  $Atf4^{-/-}$  calvarial osteoblasts (Fig. 1F). Collectively, these data indicate that lack of endogenous ATF4 attenuates the potency of TGF $\beta$  to inhibit endogenous Ocn expression, ALP activity and differentiation or mineralization of osteoblasts in vitro.

To address whether ATF4 was also required for TGF $\beta$  *in vivo* and because treatment of WT mice with 1D11 an anti-TGF $\beta$  monoclonal antibody blocking all three isoforms of TGF $\beta$  (34), increased trabecular bone volume fraction in mice (30), we treated WT and  $Atf4^{-/-}$  mice with 2G7, an monoclonal antibody with similar reactivity to the 1D11 (35). WT control and  $Atf4^{-/-}$  mice were injected intraperitoneally with a TGF $\beta$  monoclonal antibody 2G7 for 4 weeks. Consistent with the results reported previously using 1D11 (30),  $\mu$ CT analysis revealed that 2G7 treatment increased bone volume fraction in WT mice by more than 30%, however, it could not improve the low bone mass in  $Atf4^{-/-}$  mice (Fig. 1, *G* and *H*). Thus, these results confirmed that ATF4 is an important molecule downstream of TGF $\beta$  *in vivo*.

TGF $\beta$  Stimulates Vimentin Post-transcriptionally in Osteoblasts—To determine whether TGF $\beta$  suppressed ATF4-dependent activation of *Ocn* and osteoblast differentiation (Fig. 1) via a direct effect on ATF4, we then analyzed whether TGF $\beta$ inhibited ATF4 expression. Primary calvarial osteoblasts (passage 1) were treated for 10 days with rhTGF $\beta$ 1 at concentrations of 0.2, 1, or 2 ng/ml under osteogenic culture conditions, and then total RNA and protein were analyzed. As expected, endogenous *Ocn* expression decreased whereas ATF4 expression slightly increased, in response to TGF $\beta$  in a dose-depen-



### TGFβ Stimulates Vimentin via Non-Smad Signaling



FIGURE 1. **ATF4 is required for TGF** $\beta$  **to inhibit osteocalcin (Ocn) transcription.** *A*–*C*, TGF $\beta$  inhibits ATF4-dependent reporter activity. Luciferase activity is decreased by treatment of rhTGF $\beta$ 1 at indicated concentrations in stable ROS17/2.8 reporter cells. p6xOSE1-Luc, luciferase gene driven by 6 repeats of ATF4-binding elements reporter (*A*); p6xmOSE1-Luc, 6 repeats of mutant ATF4-binding element driving luciferase reporter (*B*); p3xAP1-Luc, 3 repeats of unrelated leucine-zipper protein AP1-binding site- driven reporter (*O*. *D*, inhibition of endogenous *Ocn* expression by TGF $\beta$  is attenuated in *Atf4<sup>-/-</sup>* calvarial osteoblasts. Quantitative RT-PCR (qRT-PCR) analysis of total RNA isolated from WT and *Atf4<sup>-/-</sup>* calvalrial osteoblasts treated with vehicle (–) or rhTGF $\beta$ 1 (+, 0.5 ng/ml) in osteogenic medium for 10 days. Note that TGF $\beta$  decreased endogenous *Ocn* mRNA level in WT calvarial osteoblasts. ALP assay of WT and *Atf4<sup>-/-</sup>* calvarial osteoblasts treated with vehicle (–) or rhTGF $\beta$  (+, 0.5 ng/ml) in osteogenic medium for 2 days. *F*, TGF $\beta$  inhibits mineralized nodule formation in WT but not in *Atf4<sup>-/-</sup>* osteoblasts. von Kossa staining of calvarial osteoblasts treated with rhTGF $\beta$ 1 (0.5 ng/ml) in osteogenic medium for 10 days. Note that TGF $\beta$  reduced the number of mineralized nodules (*black colonies*) dramatically (>90%) in the WT cultures but only sliphly in *Atf4<sup>-/-</sup>* osteoblasts (20%). *G*, ATF4 is required for the anabolic effect of anti-TGF $\beta$  antibody *in vivo*.  $\mu$ CT analysis of trabecular bones of WT and *Atf4<sup>-/-</sup>* femurs treated with control IgG antibody *in vivo*.  $\mu$ CT analysis of trabecular bones of WT and *Atf4<sup>-/-</sup>* femurs treated with control IgG antibody *in vivo*.  $\mu$ CT analysis of trabecular bones of WT and *Atf4<sup>-/-</sup>* femurs treated with control IgG antibody *in vivo*.  $\mu$ CT analysis of trabecular bones of WT and *Atf4<sup>-/-</sup>* femurs treated with control IgG antibody *in vivo*.  $\mu$ CT analysis of trabecular bones of WT and *Atf4<sup>-/-</sup>* femurs treated with contr

dent manner (Fig. 2, *A* and *B*). These results suggested that the suppression of ATF4 transcriptional activity by TGF $\beta$  is not due to a decrease in the level of ATF4 but may be via an indirect mechanism.

One of the candidates was vimentin, because we recently found that it acted as a suppressor of ATF4 in osteoblasts (23) and others have shown that its expression was stimulated by TGF $\beta$  (26). To address whether vimentin was the mediator linking TGF $\beta$  signaling and ATF4, we first treated ROS 17/2.8 cells with TGF $\beta$  and found it could induce vimentin protein expression dose-dependently. Surprisingly, however, TGF $\beta$  did not increase *vimentin* mRNA expression at all of the concentrations tested (Fig. 2, *C* and *D*). These data indicated that in osteoblasts TGF $\beta$  regulates vimentin expression at the protein level and not at the mRNA level, which was different from what had been observed in C2C12 cells previously (26). To further verify our findings, we also treated a panel of osteoblasts along with C2C12 cells (as a positive control) with low dose rhTGF $\beta$ 1 (0.2 ng/ml), which significantly suppressed WT primary osteoblast differentiation *in vitro* (Fig. 1*F*). The results indicated that TGF $\beta$  increased vimentin protein level but not its mRNA level in all tested cells, including mouse primary bone marrow stromal cells (BMSC), calvarial osteoblasts (POB), the 2T3 mouse preosteoblastic cells, and the MC3T3-E1 mouse committed osteoblasts. However and consistent with previous report (26), TGF $\beta$  in the C2C12 cells increased both protein and mRNA vimentin levels (Fig. 2, *E* and *F*). Thus, these data demonstrated that TGF $\beta$  up-regulates vimentin expression at the post-transcriptional level in osteoblasts.

Vimentin Is Required for TGF $\beta$  to Inhibit Ocn Expression— To understand whether vimentin played an essential role in mediating the inhibitory effect of TGF $\beta$  on ATF4 transcriptional activity and Ocn transcription, we knocked down endogenous vimentin expression in 2T3 mouse osteoblastic cells by transfection with siRNA-Vim (23) and the p6xOSE1-Luc as a readout for ATF4 transcriptional activity, and then tested their response to TGF $\beta$  by reporter assays. 2T3 mouse cells instead of primary osteoblasts were used to ensure the transfection effi-





FIGURE 2. **TGF** $\beta$  **inhibits** *Ocn* **expression and stimulates vimentin expression post-transcriptionally.** *A*, Northern blot analysis of total RNA from primary calvarial osteoblasts showing that TGF $\beta$  inhibits endogenous *Ocn* expression dose-dependently. *B*, Western blot analysis of total protein from primary calvarial osteoblasts. *C*, Northern blot analysis with indicated cDNA probes showing that TGF $\beta$  does not affect endogenous *vimemtin* mRNA level in calvarial osteoblasts. *D*, Western blot analysis showing that TGF $\beta$  stimulates vimentin protein in calvarial osteoblasts. *E*, Western blot analyses showing that rhTGF $\beta$ 1 (0.2 ng/ml) stimulates vimentin protein expression in the indicated primary osteoblasts and osteoblasts cell lines. *F*, Northern blot analyses showing that rhTGF $\beta$ 1 (0.2 ng/ml) does not affect endogenous *vimentin* mRNA level in the indicated primary osteoblasts and osteoblasts cell lines.

ciency. We also transfected 2T3 cells with siRNA empty vector to serve as negative control cells. Fig. 3A (lanes 1 and 2), rhTGFB1 (0.5 ng/ml) decreased luciferase activity by 36%, which was expected and consistent with what we observed previously in ROS17/2.8 reporter cells (Fig. 1A). Luciferase activity increased 2.7-fold in 2T3 cells transfected with siRNA-Vim, due to the knockdown of endogenous *vimentin* that removed its suppression of ATF4 transcriptional activity (23). When TGF $\beta$ was added to these cells, ATF4 reporter activity remained 2.5fold higher in cells containing siRNA-Vim than in cells containing siRNA control vector (Fig. 3A, lanes 3 and 4). The knockdown of endogenous vimentin was confirmed by the absence of vimentin mRNA in siRNA-Vim transfected cells, which could not be rescued by rhTGF\$1. Consistent with our previous finding (23) and transfection results shown in Fig. 3A, a low level of endogenous Ocn expression in 2T3 cells transfected with siRNA control vector was inhibited by rhTGF<sub>β1</sub> (0.5 ng/ml) treatment; whereas this inhibition was blunted in cells transfected with *siRNA-Vim* (Fig. 3B). qRT-PCR data indicated that TGFβ1 inhibited endogenous Ocn expression by 60% in cells transfected with empty siRNA vector. As expected, vimentin knockdown in 2T3 preosteoblasts by siRNA-Vim increased endogenous Ocn expression by 4-fold, which was not affected

by TGF $\beta$ 1 (Fig. 3*C*). Therefore, these data validated that endogenous *vimentin* is required for TGF $\beta$  to suppress ATF4-dependent *Ocn* transcription *in vitro*.

To determine whether vimentin protein level also fluctuated *in vivo* in response to TGF $\beta$  administration, we analyzed vimentin content in the long bones of 2G7 or control antibodytreated mice. Western blot showed that 2G7 treatment decreased the abundance of endogenous vimentin in long bone lysates compared with IgG control antibody treatment while ATF4 abundance remained constant in both control and 2G7 treated long bone samples (Fig. 3D). Thus, these results strongly suggested that TGF $\beta$  suppresses *Ocn* transcription and osteoblast differentiation via up-regulation of vimentin expression.

An increased protein level can be attributed to increased synthesis and/or decreased degradation. To determine which of these two possibilities contributed to the TGF $\beta$ -induced accumulation of vimentin protein in osteoblasts, we tested the effects of cycloheximide, a protein synthesis inhibitor, MG115, a proteasome inhibitor, on vimentin expression. We included Flag-ATF4 as a positive control, because it accumulates in many non-osteoblastic cell lines upon MG115 treatment (36). Analysis of nuclear extracts of COS1 cells transfected with HAvimentin or Flag-ATF4 expression vector showed that treat-





FIGURE 3. **Vimentin is required for the suppression of** *Ocn* **expression by TGF** $\beta$  **in osteoblasts.** *A*, transient DNA transfection in 2T3 preosteoblastic cells demonstrating that rhTGF $\beta$  (0.5 ng/ml) decreased luciferase activity (*lanes 1* and *2*). Note that luciferase activity increased when *siRNA-Vim* was co-transfected (*lanes 3* and *4*). *N.S.*, not statistically significant. *B*, Northern blot analysis of RNA from 2T3 osteoblastic cells transfected with *siRNA* vector or *siRNA-Vim* using indicated cDNA probes. Note that knockdown of endogenous *vimentin* by *siRNA-Vim* (*top panel*) blunted the inhibition of *Ocn* transcription by TGF $\beta$  (*middle panel*). *C*, qRT-PCR results confirming that *vimentin* knockdown by *siRNA-Vim* abolished the suppression of endogenous *Ocn* expression by rhTGF $\beta$  (0.5 ng/ml). *D*, Western blot analysis of long bone total protein extracts demonstrating that neutralizing the activity of TGF $\beta$  *in vivo* by anti-TGF $\beta$  decreases vimentin protein abundance. 10-week-old mice were treated with control antibody (*lgG*) or anti-TGF $\beta$  monoclonal antibody (*aTGF* $\beta$ ) neutralizing three forms of TGF $\beta$  ligand for 4 weeks. *E*, proteasomal degradation inhibitor MG115 (*MG*) stabilizes ATF4 but not vimentin. Western blot analysis of nuclear extracts of COS1 cells transfected with expression plasmids of HA-Vim or Flag-ATF4 using anti-HA or anti-Flag antibodies. Sp1, loading control of nuclear extracts. *F*, protein synthesis is required for TGF $\beta$  induce vimentin is not affected by proteasomal inhibitor, MG115 (*MG*), or lysosomal inhibitors, chloroquine (*ChlQ*, 100 mM) and ammonium chloride (*AC*, 50 mM), but is diminished by protein translation inhibitor cycloheximide (*CHX*).

ment of MG115 (10  $\mu$ g/ml) did not alter the abundance of HAvimentin but did, as expected, increase Flag-ATF4 considerably (Fig. 3*E*). To address whether endogenous vimentin in osteoblasts behaved similarly to HA-vimentin in COS1 cells, we cotreated ROS17/2.8 cells with rhTGF $\beta$ 1 (0.5 ng/ml), MG115 (10  $\mu$ g/ml), or two lysosomal inhibitors chloroquine and ammonium chloride. As can be seen in Fig. 3*F*, the TGF $\beta$ -induced increase in vimentin expression was not affected by MG115, chloroquine (100  $\mu$ M), or ammonium chloride (50 mM), but strongly decreased to basal level by cycloheximide (15  $\mu$ g/ml). Collectively, these data indicated that protein neosynthesis is required for TGF $\beta$  to stimulate vimentin expression in osteoblasts.

PI3K-Akt-mTOR Signaling Is Required for TGFB to Induce Vimentin Synthesis—Because TGF<sup>β</sup> has been shown to stimulate protein translation initiation via the PI3K-Akt-mTOR signaling pathway (Fig. 4A, adapted from Ref. 14), we then examined whether the PI3K-Akt-mTOR and/or the canonical Smad-signaling is responsible for TGF $\beta$  to induce vimentin in osteoblasts (Figs. 2 and 3). Wortmannin, an inhibitor of Akt phosphorylation by PI3K; rapamycin (1 and 5 nM), an inhibitor of S6K phosphorylation by mTOR (14, 37-39); and SB505124, an inhibitor of Smad2/3 phosphorylation downstream of the type I TGF $\beta$  receptor (40), were used to treat ROS17/2.8 cells. Western blot demonstrated that both wortmannin and rapamycin (1 and 5 nM) drastically reduced vimentin protein expression induced by TGF $\beta$ , which correlated with a strong decrease in Akt and S6K phosphorylation. SB505124 (0.1 and 0.2 nm) effectively decreased TGF<sub>β</sub>-induced Smad2 phosphorylation but not TGF $\beta$ -induced vimentin expression (Fig. 2, B-D). As expected, wortmannin and rapamycin (1 and 5 nm)

did not inhibit the TGF $\beta$ -induced Smad2 phosphorylation (Fig. 4, *B*--*E*), demonstrating that these inhibitors do not have an off-target effect at these concentrations. Moreover, overexpression of a dominant negative form of Akt, Akt-AA (38), blocked the increase in vimentin expression induced by TGF $\beta$ , which also correlated with an inhibition of S6K phosphorylation induced by TGF $\beta$  (Fig. 4*E*). Collectively, these data indicated that activation of PI3K-Akt-mTOR signaling, but not the canonical Smad signaling, is required for the stimulation of vimentin protein expression by TGF $\beta$ .

To further elucidate the functional relevance of this noncanonical signaling pathway in controlling vimentin expression on ATF4-dependent activation of *Ocn* expression, we treated ROS17/2.8 reporter cells containing p6xOSE1-Luc with various kinase inhibitors. Consistent with their effects in abolishing the effect of TGF $\beta$  on vimentin production, wortmannin and rapamycin both blunted the inhibition of luciferase activity by TGF $\beta$ , whereas SB505124 did not affect it (Fig. 4*F*). Taken together, these results strengthened the finding that PI3K-AktmTOR signaling, but not Smad signaling, acts downstream of TGF $\beta$  to mediate the inhibition of *Ocn* transcription.

#### DISCUSSION

In this study, we show that TGF $\beta$  up-regulates post-transcriptionally vimentin expression in osteoblasts through a PI3K-Akt-mTOR non-canonical TGF $\beta$  pathway to inhibit osteoblast differentiation. Thus, this study defines vimentin and ATF4 as downstream mediators of TGF $\beta$ , providing two additional modulation points of this ancient and important pathway.



## TGFβ Stimulates Vimentin via Non-Smad Signaling



FIGURE 4. **TGF**β **stimulates vimentin protein synthesis via PI3K-mTOR-Akt signaling but not Smad signaling**. *A*, schematic presentation of inhibitors that block canonical and noncanonical signaling pathways downstream of TGFβ. *B* and *C*, Western blot of ROS17/2.8 cells indicating that wortmannin (*B*) and rapamycin (*C*) dose-dependently blunted rhTGFβ1 (0.2 ng/ml) induced vimentin and phosphorylation of downstream targets Akt or S6K. *D*, Western blot analysis of ROS17/2.8 cells showing that SB505124 did not inhibit rhTGFβ1 (0.2 ng/ml) to stimulate vimentin expression. Note that SB505124 effectively inhibited Smad2 phosphorylation. *E*, Western blot analysis showing that overexpression of dominant negative form of Akt (*Akt-AA*) inhibited rhTGFβ1 (0.2 ng/ml)-induced vimentin protein level in ROS17/2.8. *F*, ATF4-dependent activation of *Ocn* transcription requires PI3K-Akt-mTOR signaling. Luciferase activity in ROS17/2.8 reporter cells containing p6xOSE1-Luc was effectively (30%) inhibited by rhTGFβ (1 ng/ml) and SB505124 (0.4 μm), which was blunted by wortmannin (5 nM) or rapamycin (5 nM). \*, *p* < 0.01.

ATF4 and Vimentin as Downstream Targets of TGF $\beta$  in Osteoblasts-The finding of the monoclonal TGFB-neutralizing antibody 1D11 to promote mechanical strength in young adult mice (30) motivated us to investigate the mechanism(s) whereby anti-TGFB treatment promotes bone mass and fracture resistance. Here, we provided evidence that the inhibition of Ocn expression, ALP activity, and mineralized nodule formation by TGF $\beta$  was attenuated by 50% in *Atf* $4^{-/-}$  osteoblasts compared with the WT osteoblasts. Moreover, the anabolic effect of 2G7 in WT mice (a 30% increase in trabecular bone volume fraction) is completely blunted in  $Atf4^{-/-}$  mice (Fig. 1). These data allow us to conclude that ATF4 is required for transmitting TGF $\beta$  signals in osteoblasts in both cultured primary cells and in vivo. Interestingly, TGFB did not decrease ATF4 expression but increased vimentin protein abundance in osteoblasts and bones in vivo (Figs. 2 and 3), which in turn led to the suppression of ATF4 transcriptional activity. Furthermore,

silencing endogenous *vimentin* by *siRNA-Vim* attenuated the effects of TGF $\beta$  (Fig. 3). Therefore, we conclude that both ATF4 and vimentin are downstream targets of TGF $\beta$ . Further investigations are necessary to confirm the *in vivo* roles that vimentin play in osteoblasts.

Studies using cell and transgenic mouse models have shown that vimentin promotes cell growth in Ras-transformed cells (41) but inhibits cell differentiation (42). Loss of vimentin  $(Vim^{-/-})$  in mice results in failures of vascular adaptation, which leads to pathological conditions such as reduced renal mass (43), glial cellmalformation (44), impaired wound healing (45), decreased arterial resistance to sheer stress (46), and disturbed leukocyte homing to lymph nodes (47). It will be of interest to investigate whether all or part of these defects are attributable to the essential role that vimentin plays in transmitting the signals of TGF $\beta$ , given the versatile and essential roles that TGF $\beta$  plays in many physiological and pathological



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conditions, including the ones affected in  $Vim^{-/-}$  mice. Furthermore, vimentin is often strongly expressed in undifferentiated mesenchymal cells. Overexpressing *vimentin* in mesenchymes under its own promoter inhibited cell differentiation (48, 49). Thus, it is plausible to view TGF $\beta$  as one of the local growth factors that is required to maintain the "stemness" of mesenchymal stem cells in bone through its up-regulation of vimentin in bone. Thirdly, as an intermediate filament (IF) protein, vimentin also undergoes spatial reorganization in a variety of cell types, in response to stimulation with physiological signals, including TGF $\beta$  (data not shown). It will be important to understand the molecular basis whereby vimentin, a molecule that mostly resides in cytosol, travels into the nucleus to modulate the activity of tissue-specific transcription factors.

Analogous to the signaling axis of TGF $\beta$ -vimentin-ATF4 is that of TGF<sub>B</sub>-Smad3-Runx2, which regulates Ocn transcription and osteoblast differentiation. In the latter pathway, upon TGF $\beta$  ligand stimulation, Smad3 is first phosphorylated by TGF $\beta$  receptors and then it binds to Smad4 and translocates into the nucleus, where the complex of Smad3/4 interacts with Runx2 (6), a master regulator of osteoblast differentiation (50-53). Subsequent studies demonstrated that the Smad3/4 complex also recruits histone deacetylases (HDAC) 4 and -5 to the Runx2-binding site of Ocn promoter to suppress Ocn transcription and osteoblast differentiation (54). It is currently unknown whether the vimentin-ATF4 complex can recruit additional repressors, such as HDAC family members, to the OSE1 binding site. More importantly, the relative contribution and potential interactions of TGFB-vimentin-ATF4 and TGFB-Smad3-Runx2 pathways remain to be studied.

The Role of PI3K-Akt-mTOR Signaling in Osteoblasts-It has previously been reported that specific inhibition of PI3K signaling with wortmannin stimulates human mesenchymal stem cells to differentiate into osteoblasts (55). Similarly, rapamycin also promotes human embryonic stem cells to differentiate into osteoblasts (56). A positive role of these kinase inhibitors of the PI3K-Akt-mTOR pathway in the regulation of osteoblast differentiation is substantiated by the evidence that both wortmannin and rapamycin blunted the inhibitory effect of  $TGF\beta$ on ATF4-dependent Ocn transcription in ROS17/2.8 cells containing the p6xOSE1-Luc reporter (Fig. 4). At odds with these results, a low concentration (0.1 nm) of rapamycin has been shown to inhibit Ocn expression and osteoblast differentiation by suppressing the expression of Runx2, in primary mouse bone marrow stromal cells and MC3T3-E1 mouse osteoblastic cells (57). It is currently unknown whether these conflicting observations are a reflection of difference in cell lines, *i.e.* human versus mouse cell lines, or in drug concentrations. Furthermore, it needs to be further evaluated the in vivo relevance of the newly discovered TGFB-PI3K-Akt-mTOR-vimentin-ATF4-Ocn axis. Regardless, our current findings represent a novel paradigm involving vimentin and ATF4 as downstream effectors of TGF $\beta$  signaling in the regulation of osteoblast differentiation.

This study does not exclude factors other than TGF $\beta$  acting as physiological upstream regulators of vimentin expression in osteoblasts. Indeed, parathyroid hormone (PTH), an important bone anabolic agent when used intermittently *in vivo*, has been reported to suppress the *de novo* biosynthesis of vimentin in human osteoblastic cells (58). Interestingly, PTH has also been shown to increase the expression and activity of ATF4 in osteoblasts (59). Thus, it is possible that PTH down-regulates vimentin expression in differentiating osteoblasts, which may counteract the action of TGF $\beta$  and lead to stimulation of ATF4's transcriptional activity and osteoblast differentiation. From this perspective, our study provides the first step toward to understanding the complexity of signaling cascades that control the anabolic action of TGF $\beta$  and PTH in bone. It is important to further determine whether vimentin is a convergent point that integrates both TGF $\beta$  and PTH signals to finely tune the differentiation process of osteoblasts.

TGF $\beta$  as a potent osteotropic factor has been extensively studied (60) and our understanding of how it regulates bone development and remodeling has continued to evolve (4, 5, 30, 61–63). It is encouraging that *in vivo* inhibition of TGF $\beta$  activity can stimulate bone formation (30). However, evaluating the long-term effects of TGF $\beta$  neutralizing antibodies on the skeleton will be important, since TGF $\beta$  promotes osteoblast proliferation and migration (63–65), two functions that are also required for bone formation. In addition, better understanding the molecular signaling pathways downstream of TGF $\beta$  should allow one to target selective molecules in osteoblast, thereby limiting off-target effects of general inhibition of TGF $\beta$  activity and the possible development of an immune response caused by the use of an antibody-based bone anabolic strategy.

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