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Abstract: TGF β Inducible Early Gene-1 (TIEG) was originally cloned from human osteoblasts (OB) and has been shown to play an important role in TGF β /Smad signaling, regulation of gene expression and OB growth and differentiation. To better understand the biological role of TIEG in the skeleton, we have generated congenic TIEG-null (TIEG $^{-/-}$) mice in a pure C57BLK6 background. Through the use of DXA and pQCT analysis, we have demonstrated that the femurs and tibias of two-month-old female TIEG $^{-/-}$ mice display significant decreases in total bone mineral content, density, and area relative to WT littermates. However, no differences were observed for any of these bone parameters in male mice. Further characterization of the bone phenotype of female TIEG $^{-/-}$ mice involved mechanical 3-point bending tests,

micro-CT, and histomorphometric analyses of bone. The 3-point bending tests revealed that the femurs of female TIEG^{-/-} mice have reduced strength with increased flexibility compared to WT littermates. Micro-CT analysis of femurs of two-month-old female TIEG^{-/-} mice revealed a highly significant decrease (9%) in total femur bone mass (cortical + cancellous bone) compared to WT littermates. Histomorphometric evaluation of the distal femur revealed that female TIEG^{-/-} mice also display a 31% decrease in cancellous bone area, which is primarily due to a 22% decrease in trabeculae number. At the cellular level, female TIEG^{-/-} mice exhibit a 42% reduction in bone formation rate which is almost entirely due to a reduction in double labeled perimeter. Differences in the mineral apposition rate were not detected between WT and TIEG^{-/-} mice. Taken together, these findings suggest that female TIEG^{-/-} mice are osteopenic mainly due to a decrease in the total number of functional/mature OBs and support a critical role for TIEG in OB differentiation as well as skeletal development and/or homeostasis.

We are submitting the enclosed paper entitled: "TIEG-Null Mice Display a Severe Osteopenic Gender-Specific Phenotype Characterized by Abnormal Cancellous Bone Microarchitecture" by Subramaniam et al., for consideration for publication as an original basic science study in the journal "Bone". We feel that this paper will be of interest to the readers of "Bone" as it describes the skeletal phenotype of our congenic TIEG knockout mice. TIEG is a transcription factor that has previously been implicated in osteoblast growth and differentiation. These studies expound on our previous work through the use of static and dynamic histomorphometry as well as pQCT, DXA, micro-CT and bending tests to carefully define the basis for the observed bone phenotype. Also of interest is the fact that this bone phenotype is only observed in female animals with no differences present in male mice.

TIEG-Null Mice Display a Severe Osteopenic Gender-Specific Phenotype Characterized by Abnormal Cancellous Bone Microarchitecture.

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Abstract

TGF β Inducible Early Gene-1 (TIEG) was originally cloned from human osteoblasts (OB) and has been shown to play an important role in TGF β /Smad signaling, regulation of gene expression and OB growth and differentiation. To better understand the biological role of TIEG in the skeleton, we have generated congenic TIEG-null (TIEG^{-/-}) mice in a pure C57BLK6 background. Through the use of DXA and pQCT analysis, we have demonstrated that the femurs and tibias of two-month-old female TIEG^{-/-} mice display significant decreases in total bone mineral content, density, and area relative to WT littermates. However, no differences were observed for any of these bone parameters in male mice. Further characterization of the bone phenotype of female TIEG^{-/-} mice involved mechanical 3-point bending tests, micro-CT, and histomorphometric analyses of bone. The 3-point bending tests revealed that the femurs of female TIEG^{-/-} mice have reduced strength with increased flexibility compared to WT littermates. Micro-CT analysis of femurs of two-month-old female TIEG^{-/-} mice revealed a highly significant decrease (9%) in total femur bone mass (cortical + cancellous bone) compared to WT littermates. Histomorphometric evaluation of the distal femur revealed that female TIEG^{-/-} mice also display a 31% decrease in cancellous bone area, which is primarily due to a 22% decrease in trabeculae number. At the cellular level, female TIEG^{-/-} mice exhibit a 42% reduction in bone formation rate which is almost entirely due to a reduction in double labeled perimeter. Differences in the mineral apposition rate were not detected between WT and TIEG^{-/-} mice. Taken together, these findings suggest that female TIEG^{-/-} mice are osteopenic mainly due to a decrease in the total number of functional/mature OBs and support a critical role for TIEG in OB differentiation as well as skeletal development and/or homeostasis.

Introduction

TGF β inducible early gene-1 (TIEG) was originally discovered in our laboratory as an early response gene following TGF β treatment of human osteoblasts (OB) [1]. TIEG is expressed in numerous tissues [2-7] and is a member of the Krüppel-like family of transcription factors (KLF-10) which are known to be involved in antiproliferative and apoptotic inducing functions similar to those initiated by TGF β action [8-10]. These factors bind to Sp-1-GC rich DNA elements via their zinc fingers and regulate the expression of genes involved in cell growth, differentiation and apoptosis [11-13]. The TIEG gene encodes a 480 amino acid (72 kDa) protein and has previously been shown to both activate and repress the transcription of a number of genes [14-16].

Previous studies by our laboratory have demonstrated that TIEG plays an important role in mediating TGF β signaling. TIEG directly represses the expression of the inhibitory Smad 7 gene by binding to a Sp-1-like GC-rich sequence in the proximal promoter [14]. Additionally, TIEG induces the expression and activity of Smad 2 [17]. Further, we have demonstrated that overexpression of TIEG enhances the TGF β activation of a Smad binding element reporter construct and increases the transcription of known TGF β target genes including p21, PAI-1, and Smad 2 [14]. Overall, TIEG expression results in activation of the Smad signaling pathway and mimics the functions of TGF β in multiple cell types.

TGF β is known to be an important signaling molecule in bone and has significant effects on osteoblast growth and differentiation. Despite conflicting results, the majority of studies indicate that TGF β increases bone formation by recruiting OB progenitors and stimulating their proliferation resulting in an increased number of cells committed to OB lineage. More specifically, two studies revealed that TGF β promotes early stages of OB differentiation, while it blocks later stages of differentiation and mineralization [18,19]. The TGF β family consists of three closely related isoforms: TGF β 1, β 2, and β 3, and all

three isoforms have been shown to induce TIEG expression [20]. The major isoform found in bone is TGF β 1 which accounts for about 90% of all TGF β in the bone microenvironment. The *in vivo* functions of the three TGF β isoforms are highly divergent as determined by gene knockouts [21].

Since TGF β is known to play important roles in multiple facets of bone biology, and since we have shown that TIEG is an important regulator of TGF β signaling in OBs and can mimic TGF β action when overexpressed, we developed a TIEG knockout mouse model (TIEG^{-/-}) [22]. These animals were originally developed in a C57BLK6/129 mixed breed genetic background. Calvarial OBs, isolated from the TIEG^{-/-} mice revealed decreased expression of important OB marker genes, including osteocalcin, osterix, and alkaline phosphatase [22]. Co-culture studies with osteoclast (OC) precursor cells demonstrated that fewer mature OCs developed when TIEG^{-/-} OBs were used to support OC differentiation compared to that of wild-type OBs [22]. Gene expression analysis of TIEG^{-/-} OBs revealed a decrease in the expression of RANKL and an increase in the expression of OPG relative to wild-type OBs, which could well explain the decreased support of OC differentiation by TIEG^{-/-} OBs [22].

Further characterization of these animals, through the use of pQCT and micro-CT, revealed a number of bone/skeletal defects [23]. However, since the skeletons of mixed breed knockout mice can mask a number of bone defects, and since there are significant baseline differences between the skeletons of C57BLK6 and 129 mouse strains, as well as quantitative differences in their skeletal responses to ovariectomy [24], we sought to create a congenic line of TIEG^{-/-} mice before further analyzing the basis for this bone phenotype. Our original mixed-breed (C57BLK6/129) TIEG^{-/-} mice have now been crossed with C57BLK6 wild-type mice for more than 10 generations. This manuscript describes a comprehensive analysis of the bone defects resulting from loss of TIEG expression in these congenic animals using micro-CT, DXA, pQCT, and

histomorphometric techniques. These studies demonstrate that TIEG^{-/-} mice display a severe osteopenic phenotype in a gender-specific manner likely as a result of decreased OB number. These studies further define the biological role of TIEG in bone and demonstrate that TIEG is critical for normal bone formation and/or maintenance.

Materials and Methods

Animals. TIEG^{-/-} mice were originally developed in a C57BLK6/129 mixed breed background as described previously [22]. To derive TIEG^{-/-} mice in a congenic background, we bred our mixed breed TIEG^{-/-} animals against C57BLK6 wild-type mice for at least 10 generations. Ten wild-type, male and female, and ten TIEG^{-/-}, male and female, 3-month-old mice were used for the 3-point bending studies. Twelve wild-type male and female, and twelve TIEG^{-/-} male and female 2 month-old mice were used for the DXA and pQCT studies. Ten wild-type and ten TIEG^{-/-} 2-month-old female mice were used for micro-CT and histomorphometric analysis. All mice were housed in a temperature controlled room (22 ± 2°C) with a light/dark cycle of 12 hours. All mice had free access to water and were fed standard laboratory chow (Laboratory Rodent Diet 5001; PMI Feeds, Richmond, VA) ad libitum. To reduce variability among experiments, wild-type and TIEG^{-/-} littermates were utilized in all of the experiments performed in these studies. The Institutional Animal Care and Use Committee (IACUC) approved all animal care and experimental procedures.

Peripheral quantitative computed tomography (pQCT). pQCT measurements were performed on tibias of 12 wild-type and 12 TIEG^{-/-} male and female mice at 2 months of age. The mice were anesthetized and placed in a supine position on a gantry using the Stratec XCT Research SA Plus using software version 5.40 (Norland Medical Systems, Fort Atkinson, WI). Slice images were measured at 1.9 mm (corresponding to

the proximal tibial metaphysis) and at 9 mm (corresponding to the tibial diaphysis) from the proximal end of the tibia as described previously [23].

Dual-energy x-ray absorptiometry (DXA). The same mice that were used for pQCT analysis were used in the DXA scans. Mice were anesthetized and placed on the Lunar PIXImus densitometer (software version 1.44.005; Lunar Corp, Madison, WI). Calibration of the machine was performed before scanning with the hydroxylapatite phantom provided by the manufacturer.

Micro-CT. Ten wild-type female mice and ten TIEG^{-/-} female mice were sacrificed using CO₂ and whole femurs were removed. Entire femurs were scanned at a voxel size of 12 x 12 x 12 μm using a Scanco Medical μCT 40 machine and data were processed on an HP AlphaStation DS15 (Scanco Medical AG, Basserdorf, Switzerland). The threshold for analysis was determined empirically and set at 235 (grey scale 0-1,000) for all bones.

Mechanical testing. Three-point bending tests were performed on the left femurs of 10 wild-type male and female and 10 TIEG^{-/-} male and female mice at 3 months of age. The mice were sacrificed using CO₂ and the femurs dissected out and cleaned to be free of ligaments, muscle, and tendons. The three-point bending tests were performed on cleaned femurs as previously described [23]. Briefly, femurs were placed in the anteroposterior direction on a metal support and a load was applied in the middle of the femoral shaft. The ultimate force, stiffness and work to failure were measured for each femur.

Calcein labeling. Ten wild-type and ten TIEG^{-/-}, 2-month-old female mice were injected with Calcein (15 mg/kg) subcutaneously 5 days and 2 days before sacrificing. Mice were sacrificed using CO₂ and femurs were harvested, as described above, and fixed in 10% neutral formalin overnight. Femurs were switched to 70% ethanol and stored at 4°C until time for histomorphometric analysis.

Histomorphometric analysis. After fixation in 70% ethanol, distal femurs were dehydrated in graded ethanols and xylene, and embedded, undecalcified, in modified methyl methacrylate for histomorphometric evaluation of cancellous bone. Longitudinal sections (4 μm thick) were cut with a vertical bed microtome (Leica 2065) and affixed to slides pre-coated with a 1% gelatin solution. One femoral section per animal was stained according to the von Kossa method with a Tetrachrome counterstain (Polysciences, Warrington, PA) and used for determining cancellous bone area and cellular endpoints. A second femoral section was left unstained and used for assessing fluorochrome labeling and dynamic measurements of bone formation. Histomorphometric data was collected with the OsteoMeasure System (OsteoMetrics, Inc., Atlanta, GA). The measurement area consisted of secondary spongiosa at distances greater than 0.25 mm from the growth plate. On average, 1.25 mm² of tissue (marrow and bone) was evaluated in each section. Cancellous bone area was measured and expressed as a percentage of tissue area. Osteoblast perimeter was also measured and expressed as a percentage of cancellous bone perimeter. Trabecular thickness, trabecular number, and trabecular separation were calculated based on measures of perimeter and area [25]. Measured fluorochrome-based indices of bone formation included double labeled perimeter, expressed as a percentage of total perimeter, and mineral apposition rate. Bone formation rate was calculated by multiplying double labeled perimeter by mineral apposition rate and normalized to bone perimeter, bone area, or tissue area. All histomorphometric data are reported in accordance with standard bone nomenclature [26].

Statistical Analysis. Data were analyzed by unpaired t-tests. Differences were considered significant at p-values < 0.05. All data are expressed as mean \pm standard error.

Results

pQCT DXA and micro-CT analysis of wild-type and TIEG^{-/-} mice. As an initial approach to determine if significant differences in specific bone parameters exist between wild-type and TIEG^{-/-} mice, we performed pQCT analysis on left tibiae of twelve wild-type male and female mice and twelve TIEG^{-/-} male and female mice at 2 months of age. As shown in Table 1, the tibiae of 2-month-old female TIEG^{-/-} mice exhibited significant decreases in total bone content, density, area; cortical bone content, density and area; and cortical bone thickness relative to wild-type littermates. The differences in these parameters ranged between 3% and 11%. In contrast, the tibiae of male TIEG^{-/-} mice did not show any significant difference in these bone parameters compared to wild-type littermates (Table 1). To determine the total body bone mineral density and content, we performed DXA analysis on ten wild-type male and female mice and ten TIEG^{-/-} male and female mice. As shown in Table 1, only the female TIEG^{-/-} mice exhibited statistically significant decreases in total, lumbar, femur and tibia bone mineral density as well as total bone mineral content, when compared to wild-type littermates. Since multiple bone parameters exhibited statistically significant decreases in the bones of female TIEG^{-/-} mice, we performed micro-CT analysis on ten femurs isolated from wild-type and TIEG^{-/-} female animals. As shown in Figure 1, femoral bone mass (cortical and cancellous bone) was significantly reduced by 9% in TIEG^{-/-} mice relative to wild-type controls. Taken together, these data clearly indicate that TIEG^{-/-} mice exhibit a significant bone phenotype characterized as osteopenic which is only evident in female mice.

Mechanical testing. Since a significant decrease in bone mass was detected in the femurs of female TIEG^{-/-} mice, 3-point bending assays were used to measure femoral strength. This analysis was performed on ten wild-type male and female mice, and ten TIEG^{-/-} male and female mice at 3 months of age. As shown in Figure 2, and

consistent with the pQCT, DXA and micro-CT results, the force to failure and stiffness were decreased only in female TIEG^{-/-} mice relative to wild-type littermates.

Interestingly, the work to failure is actually greater for femurs isolated from female TIEG^{-/-} mice suggesting that these bones may be more flexible than those of wild-type animals, possibly as a result of decreased bone mineral content and bone mineral density. In any event, these data indicate that the mechanical properties of bones isolated from TIEG^{-/-} female, but not male, mice differ greatly from those of wild-type animals.

Histomorphometric analysis of 2-month-old wild-type and TIEG^{-/-} female mice.

Based on the observation of decreased bone content, density, and strength only in female TIEG^{-/-} mice, it was of interest to further examine this bone phenotype at the cellular level. Histomorphometric analysis was performed on femurs isolated from ten wild-type female mice and ten TIEG^{-/-} female mice at 2 months of age. As shown in Figure 3, histological analysis of sections from the distal femur metaphysis reveal that the trabeculae (black staining) number and connectivity is severely reduced in TIEG^{-/-} mice with a concomitant increase in trabecular separation. Additionally, the growth plate and hypertrophied chondrocytes of these animals appear normal (Figure 3); indicating that deletion of TIEG expression does not have global consequences on all facets of bone growth and turnover. These studies clearly demonstrate that there is a significant defect in the cancellous bone of TIEG^{-/-} mice relative to wild-type littermates.

In light of the striking defects observed in the femurs of female TIEG^{-/-} mice with regard to trabeculae number and connectivity, it was of interest to determine if this defect was at least in part due to differences in the number of functioning osteoblasts lining the bone surface of these animals. As shown in Figure 4, these studies demonstrate that there is a highly significant decrease ($p < 0.01$) in the osteoblast perimeter of TIEG^{-/-} mice ($12.4 \pm 2.5\%$) relative to wild-type littermates ($30.0 \pm 5.6\%$). Additionally, as can be seen in Figure 4, the osteoid thickness is also reduced in TIEG^{-/-}

mice indicating that there is a reduction in matrix synthesis in these animals. These results could be due to decreased osteoblast activity in TIEG^{-/-} mice or simply reflective of the differences in total osteoblast number. These possibilities were explored by performing dynamic histomorphometry.

As shown in Figure 5, this analysis revealed that TIEG^{-/-} mice display a 31% decrease in cancellous bone area (BA/TA) which is primarily due to a 22% decrease in trabeculae number (Tb.N). There is also a trend toward decreased trabeculae thickness (Tb.Th) in female TIEG^{-/-} mice (-10%, $p \approx 0.1$). In accordance with a reduction in trabecular number in female TIEG^{-/-} mice, there is a 32% increase in the trabecular separation in the femurs of these animals (Figure 5). At the cellular activity level, female TIEG^{-/-} mice display a 42% reduction in bone formation rate (BFR/B.Pm and BFR/B.Ar) which is almost entirely due to a 40% reduction in double labeled perimeter (dL.Pm) (Figure 6). Additionally, the bone formation rate relative to tissue area (BFR/T.Ar) is drastically reduced in female TIEG^{-/-} mice (59%) (Figure 6). Finally, although there is a trend towards decreased mineral apposition rate (MAR) in the female TIEG^{-/-} mice, this difference is not statistically significant. Overall, these findings suggest that female TIEG^{-/-} mice are osteopenic mainly due to a significant decrease in total osteoblast number and not likely the result of large differences in osteoblast activity.

Discussion

Our original TIEG^{-/-} mice were developed in a C57BLK6/129 mixed breed background. Characterization of bones isolated from these mixed breed animals revealed several bone defects including decreases in bone content, density, and size [23]. Micro-CT analysis of the femoral head and vertebrae indicated increases in trabecular separation and decreases in cortical bone thickness and vertebral bone volume in the TIEG^{-/-} animals [23]. Additionally, transmission electron microscopy

indicated a significant decrease in osteocyte number in the femurs of TIEG^{-/-} mice implying a potential role for TIEG in OB maturation/differentiation [23]. However, since the skeletons of mixed breed knockout mice can mask a number of bone defects, and since there are significant baseline differences between the skeletons of C57BLK6 and 129 mouse strains, complete characterization of the TIEG^{-/-} mouse bone phenotype was not carried out in these animals.

In order to further elucidate the biological functions of TIEG in bone *in vivo*, and to better characterize the bone phenotype of TIEG^{-/-} mice, we have now developed a purebred line of animals in a pure C57BLK6 genetic background. Compared to our earlier work using only female mice with a mixed background, analysis of congenic TIEG^{-/-} animals, as described in this paper, has revealed an even more striking bone defect. Interestingly, this defect is only observed in female mice as no changes in any of the bone parameters analyzed to date are present in male animals. Through the use of static and dynamic histomorphometry, we have now characterized this bone defect at the cellular level. Specifically, femurs and tibias isolated from 2-month-old female TIEG^{-/-} mice display significant decreases in multiple bone parameters relative to wild-type littermates including decreased bone content, density, and thickness. Three-point bending tests revealed that the femurs of female TIEG^{-/-} mice are much weaker compared to wild-type animals. Dynamic histomorphometric analysis of the distal femur revealed that female TIEG^{-/-} mice display a decrease in cancellous bone area associated with a decrease in trabeculae number. At the cellular level, TIEG^{-/-} mice exhibit a reduction in osteoblast perimeter and bone formation rate. These findings indicate that female TIEG^{-/-} mice are osteopenic mainly due to a decrease in total OB number and not significant decreases in osteoblast activity *in vivo*.

As described in the Introduction, we have demonstrated that OBs isolated from mixed background TIEG^{-/-} mice exhibit decreased expression levels of important OB

marker genes including osteocalcin, osterix, and alkaline phosphatase [22]. Additionally, these TIEG^{-/-} OBs exhibit a delayed rate of mineralization relative to wild-type controls. The reduced expression of these bone related genes in TIEG^{-/-} cells could in part explain the osteopenic phenotype observed in the present studies.

We have previously demonstrated that TIEG plays an important role in TGF β mediated Smad signaling by inducing the expression of Smad 2 and repressing the expression of Smad 7 [14,17]. Additionally, we have shown that overexpression of TIEG in osteoblasts mimics the actions of TGF β 1 by decreasing cellular proliferation and inducing the expression of alkaline phosphatase [4]. Thus, we hypothesized that TIEG plays an important role in osteoblast and skeletal biology since: 1). TGF β is a major regulator of OB growth and differentiation and plays a critical role in normal bone formation and maintenance; 2). TGF β increases bone formation by recruiting OB progenitors and stimulating their proliferation, resulting in an increased number of cells committed to the OB lineage and; 3). TGF β promotes early stages of OB differentiation (bone matrix production), while it blocks later stages of differentiation and mineralization [18,19].

The TGF β family consists of three highly homologous isoforms classified as TGF β 1, β 2, and β 3. Mice deficient for TGF β 1, β 2, and β 3 have all been shown to develop severe bone defects [21]. Specifically, TGF β 1 KO mice display decreased bone mineral content, with a near absence of OBs in trabecular bone, resulting in an osteopenic phenotype [27]. TGF β 2 null mice contain numerous bone defects (bone loss) in the rib, sternum, vertebrae and long bones [28]. TGF β 3 null mice also exhibit loss of bone [29,30]. There are currently no data available concerning the effects of TGF β 1 or TGF β 3 overexpression in bone. However, overexpression of TGF β 2, under the control of the osteocalcin promoter, results in an age-dependent loss of bone mass resembling osteoporosis [31]. These results are likely explained by the fact that

osteocalcin is expressed at late stages of differentiation and the fact that TGF β inhibits OB differentiation at later stages.

In addition to TGF β KO mice, disruption of TGF β signaling has also been shown to have a significant impact on bone. Overexpression of a truncated TGF β type II receptor, which is incapable of mediating TGF β signaling in mice, under the control of the osteocalcin promoter, results in an age-dependent increase in trabecular bone mass [32]. As discussed above, it is expected that the disruption of TGF β signaling during late stages of OB differentiation would result in increased bone formation. Finally, disruption of TGF β signaling through deletion of Smad 3 results in an osteopenic phenotype [33]. The present study demonstrates that female TIEG^{-/-} mice exhibit a bone phenotype similar to that observed in TGF β 1 knockout animals, further implying an important role for TIEG in mediating the actions of TGF β 1 in bone.

Based on the observed gender specific osteopenic phenotype (females only), it is possible that E₂ is involved in mediating this phenomenon. The skeleton is one of the main targets of E₂ action in the body as it regulates bone growth and remodeling. Decreased E₂ levels are known to be one of the major causes of osteoporosis and play a critical role in regulating bone metabolism and homeostasis [34-36]. Interestingly, studies from our laboratory indicate that E₂ induces the expression of TIEG in osteoblasts [7]. Estrogen receptor α and β double knockout mice display profound decreases in trabecular bone volume only in female animals with no differences in male mice [37]. The bone phenotype of these mice closely resembles that of our TIEG^{-/-} mice suggesting that TIEG may play an important role in mediating the effects of E₂ in bone.

In summary, histomorphometric analysis of femurs isolated from congenic TIEG^{-/-} female mice reveals major defects in multiple cancellous bone parameters including decreased trabecular number and size as well as increased trabecular separation. It appears that the major basis for this osteopenic phenotype is primarily due to a

reduction in OB number and not OB activity, since the osteoblast perimeter is significantly decreased while the mineral apposition rate is not affected. Even though our TIEG^{-/-} mice are deficient for TIEG expression in all cell types, it is important to note that the growth plate and hypertrophied chondrocytes appear normal in these animals, suggesting a specific role for TIEG in OB commitment and/or differentiation. Taken together, these data demonstrate that loss of TIEG expression results in a severe osteopenic phenotype only in female mice, suggesting a potential role for TIEG in mediating E₂ (as well as TGFβ) actions in bone.

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Figure 1. Total bone mass in the femurs of wild-type and TIEG^{-/-} female mice as determined by micro-CT. Data is presented as the average of 10 wild-type and 10 TIEG^{-/-} 2-month old littermates. Error bars represent standard error of the mean. Asterisks denote significance at the $p < 0.05$ level.

Figure 2. Bone strength and stiffness as determined by 3-point bending tests. The femurs of ten wild-type, male and female mice, and ten TIEG^{-/-}, male and female mice were subjected to 3-point bending tests at 3-months of age. Error bars represent standard error of the mean. Asterisks denote significance at the $p < 0.05$ level.

Figure 3. Histological sections of the distal femur metaphysis of representative wild-type and TIEG^{-/-} female mice. Four micron thick sections were stained using the von Kossa method with a tetrachrome counterstain and viewed using a 10X objective. Note the decrease in trabecular number (black staining) and connectivity in the TIEG^{-/-} female mice relative to wild-type littermates.

Figure 4. Histological sections of the distal femur metaphysis of representative wild-type and TIEG^{-/-} female mice. **A).** Four micron thick sections were stained using the von Kossa method with a tetrachrome counterstain and viewed using a 56X objective. Note the decrease in osteoblast number and unmineralized matrix (osteoid) lining the trabecular perimeter of TIEG^{-/-} mice relative to wild-type littermates. **B).** Osteoblast perimeter as determined by histomorphometric analysis of wild-type and TIEG^{-/-} female mouse femurs at 2 months of age. The percent difference between wild-type and TIEG^{-/-} mice is depicted below the graph. Error bars represent standard error of the mean. Asterisks denote significance at the $p < 0.05$ level.

Figure 5. Static histomorphometric analysis of wild-type and TIEG^{-/-} female mouse femurs. The femurs of ten 2-month old female wild-type and TIEG^{-/-} littermates were dissected, fixed and processed for static histomorphometric analysis of cancellous bone. Bones were analyzed for indicated parameters and the percent difference between wild-type and TIEG^{-/-} mice is depicted below each graph. Error bars represent standard error of the mean. Asterisks denote significance at the $p < 0.05$ level.

Figure 6. Dynamic histomorphometric analysis of wild-type and TIEG^{-/-} female mouse femurs. The femurs of ten 2-month old female wild-type and TIEG^{-/-} littermates were dissected, fixed and processed for dynamic histomorphometric analysis of cancellous bone. Bones were analyzed for indicated parameters and the percent difference between wild-type and TIEG^{-/-} mice is depicted below each graph. Error bars represent standard error of the mean. Asterisks denote significance at the $p < 0.05$ level.

Table 1						
2 Month Congenic Diaphysis Measurements						
Parameter	Female TIEG ^{+/+}	Female TIEG ^{-/-} *=P<0.05	Female % Difference	Male TIEG ^{+/+}	Male TIEG ^{-/-} *=P<0.05	Male % Difference
pQCT: Total Content (mg)	0.81 ± 0.01	0.73 ± 0.02*	9.90%	1.02 ± 0.03	0.99 ± 0.03	2.94%
pQCT: Total Density (mg/cm ³)	669.33 ± 7.28	643.45 ± 5.66*	3.87%	683.89 ± 7.81	688.05 ± 6.37	0.60%
pQCT: Total Area (mm ²)	1.21 ± 0.02	1.14 ± 0.02*	5.79%	1.48 ± 0.04	1.44 ± 0.03	2.70%
pQCT: Cortical Content (mg)	0.59 ± 0.01	0.52 ± 0.01*	11.86%	0.76 ± 0.03	0.75 ± 0.02	1.32%
pQCT: Cortical Density (mg/cm ³)	1044.82 ± 8.12	1012.65 ± 4.92*	3.08%	1078.02 ± 9.15	1079.67 ± 7.49	0.15%
pQCT: Cortical Area (mm ²)	0.56 ± 0.01	0.51 ± 0.01*	8.93%	0.70 ± 0.02	0.69 ± 0.02	1.43%
pQCT: Cortical Thickness (mm)	0.17 ± 0.003	0.15 ± 0.003*	11.76%	0.188 ± 0.004	0.188 ± 0.003	0.00%
DXA: Total BMD (gm/cm ²)	0.043 ± 0.0004	0.041 ± 0.0005*	4.65%	0.045 ± 0.0005	0.045 ± 0.0004	0.00%
DXA: Lumbar BMD (gm/cm ²)	0.049 ± 0.001	0.046 ± 0.001*	6.12%	0.049 ± 0.001	0.051 ± 0.0005	3.92%
DXA: Femur BMD (gm/cm ²)	0.055 ± 0.001	0.052 ± 0.001*	5.45%	0.062 ± 0.0015	0.064 ± 0.001	3.13%
DXA: Tibial BMD (gm/cm ²)	0.044 ± 0.0005	0.041 ± 0.0005*	6.80%	0.047 ± 0.0004	0.045 ± 0.0005	4.25%
DXA: Total BMC (gm)	0.334 ± 0.005	0.303 ± 0.006*	9.20%	0.367 ± 0.01	0.371 ± 0.006	1.08%

Figure 1

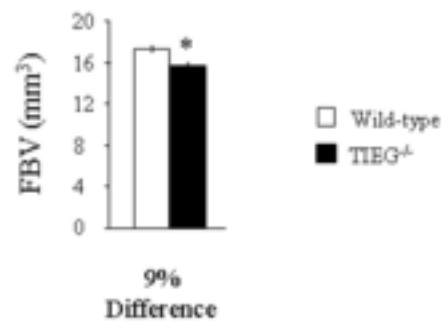


Figure 2

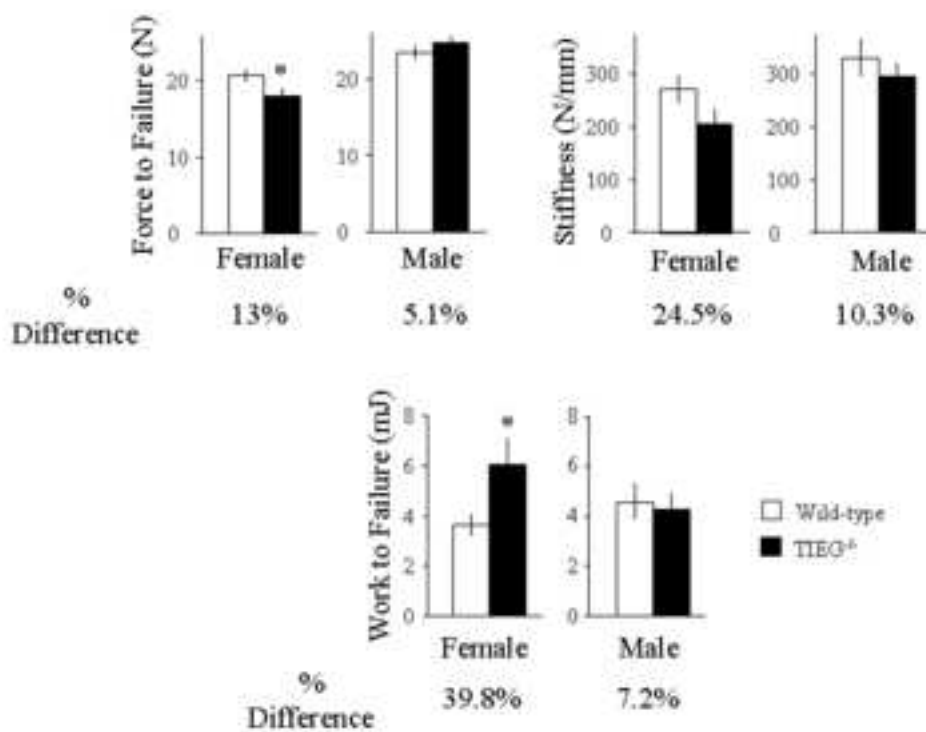


Figure 3

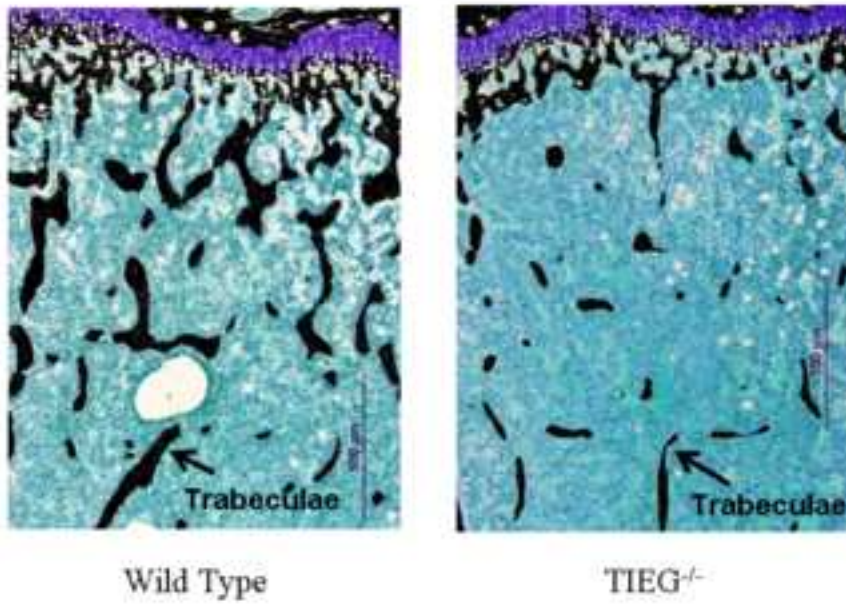


Figure 4

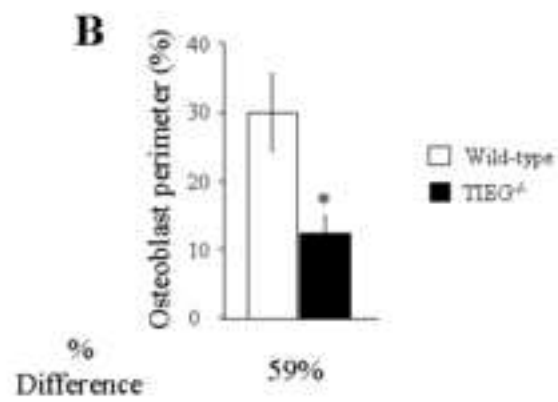
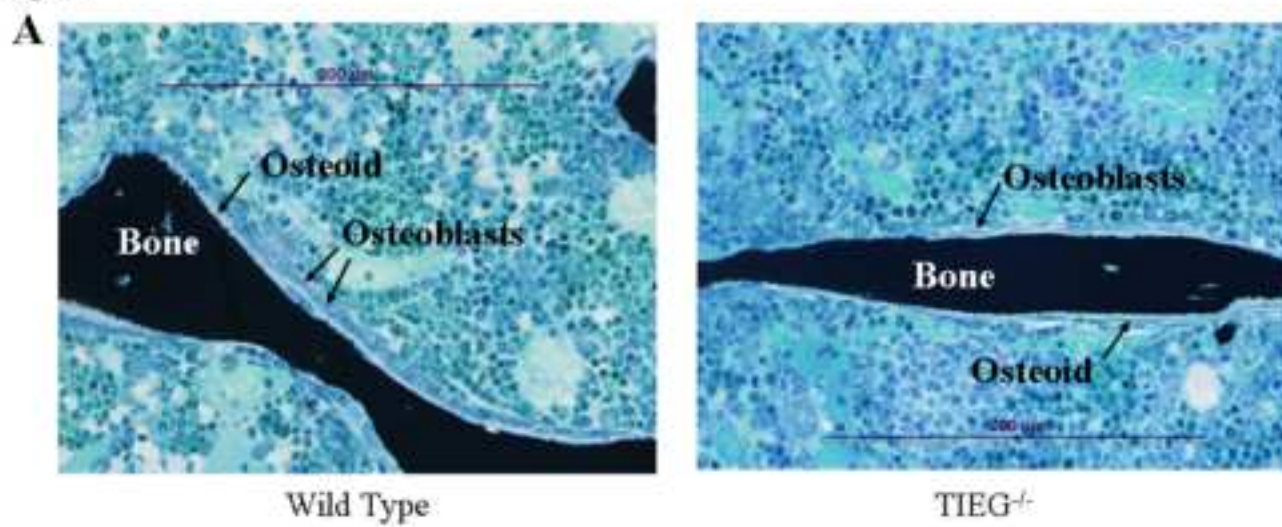


Figure 5

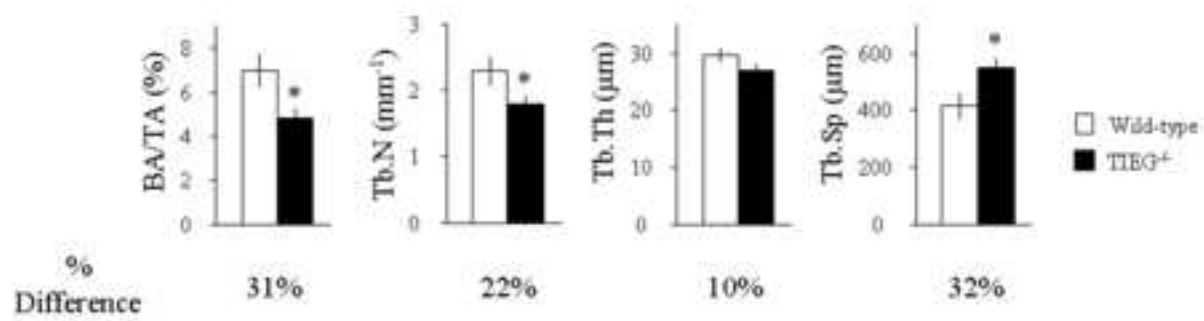


Figure 6

