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Tgfr2 is required in Osterix expressing cells for postnatal skeletal development

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Abstract

Transforming growth factor β (TGF β) is known to play an important role in early skeletal development. We previously demonstrated that loss of TGF β receptor II (Tgfr2) in Prx1-Cre-expressing mesenchyme results in defects in long bones, joints, and the skull vault in mice resulting from reduced naïve mesenchymal proliferation and condensation that interrupted osteoblast differentiation. In contrast, others have shown that the loss of Tgfr2 in fully differentiated mature osteoblasts results in increased bone volume. To study the role of Tgfr2 in immature osteoblasts, we generated Osx-Cre;Tgfr2^{fl/fl} mice and found defects in the postnatal development of the skull vault and long bones as compared to controls. No discernible skeletal defects were observed in newborn mice; however, at postnatal day 24 (P24), Tgfr2-deleted mice demonstrated short stature that correlated with reduced proliferation in the growth plate. X-ray and microCT analysis of long bone and skull from P24 mice showed reduced bone volume. Histomorphometry indicated reductions in osteoblast number but not osteoclast number. Quantitative real-time PCR demonstrated mRNA levels for the osteoblast marker, Runx2 were not altered but mRNA levels of a marker for mature osteoblasts, Bglap, were down in mutant calvaria relative to controls. The mRNA of a proliferation marker, proliferative nuclear cell antigen (PCNA), was also reduced whereas the ratio of Bax2:Bcl2 was unaltered to demonstrate no change in apoptosis. These results suggest proliferation and maturation of immature osteoblasts requires Tgfr2 signaling and that decreased bone volume in Osx-Cre;Tgfr2^{fl/fl} mice is likely because there are very few mature osteoblasts.

Keywords

Tgfr2; osteogenesis; proliferation; osteoblast differentiation; calvaria; long bone

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7.0 Disclosures

There are no conflicts of interest.

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1. Introduction

Bone growth and homeostasis involve three cell types: osteoblasts, osteocytes, and osteoclasts. Mesenchymally-derived osteoblasts lay down bone matrix and secrete factors that control osteoclast function. Osteoclasts, derived from the hematopoietic lineage, resorb bone to allow remodeling. Osteocytes, which make up 90–95% of adult bone cells [1], are derived from mature osteoblasts and regulate both osteoblast and osteoclast function. Bone formation occurs through two processes: endochondral ossification, involving a cartilaginous tissue intermediary that is later replaced with bone, and intramembranous ossification in which bones are formed directly without the cartilage intermediate. Most of the skull vault and the cortical bone in the limbs are made through the process of intramembranous bone formation while trabecular bone in the limbs is derived through endochondral bone formation.

Osterix/SP7 (Osx) is a zinc-finger-containing transcription factor that is downstream of and regulated by Runx2. It is required for osteoblast differentiation and bone formation [2–4]. It is first expressed in committed immature osteoblasts [2,4]. Expression has also been observed in prehypertrophic and hypertrophic chondrocytes during embryonic and postnatal development. The Cre/loxP system has been commonly used to generate conditional gene knockout mice. Osx-GFP::Cre (Osx-Cre) mice have been used to target skeletal tissues with a GFP/Cre fusion protein driven by the Osx promoter. Osx-Cre activity was first detected at embryonic day 12.5 (E12.5) in newly forming cortical bone [5, 7]. Expression was maintained and traced into the osteoblast lineages through embryonic and post-natal development using several mouse reporter lines [5–7].

Activity was also observed sporadically in chondrocytes of E12.5 day embryos in a pattern more restricted than that observed for Collagen2a expressing cells [5, 7]. In the postnatal growth plate, reporter activity was traced to hypertrophic chondrocytes as well as a subset of prehypertrophic cells [6]. Breeding the Osx-Cre mice with mice containing a target gene flanked by loxP sites creates a mouse with the target gene deleted in Osx expressing cells.

Growth factor signaling, such as BMP-, FGF-, and TGF β -receptor mediated signaling, is known to regulate skeletal development and homeostasis [3,8–11]. Transforming growth factor- β (TGF β) is part of a superfamily of proteins that mediate a wide range of biological activities including proliferation, differentiation, and formation of the extracellular matrix [12–15]. Disruption of TGF β signaling can cause skeletal and connective tissue disorders, including Marfan syndrome, Camurati-Engelmann disease, Cleidocranial Dysplasia and Loeys Dietz Syndrome [16–25]. TGF β ligands initiate signaling by binding to the TGF β type II receptor (Tgfr2), which then recruits the TGF β type I receptor (Tgfr1) and consequently forms a heterotetrameric complex with two Tgfr2 receptors and two Tgfr1 receptors. Tgfr2, which is a constitutively active serine/threonine kinase, then phosphorylates Tgfr1 to activate the type I serine/threonine kinase and initiate downstream signaling. The signal is transduced to the nucleus to trigger transcriptional activation of tissue specific differentiation factors [26].

Tgfr2 deletion in cranial neural crest cells and early limb mesenchyme during embryonic development caused reductions in cell proliferation and osteoblast differentiation leading to subsequent defects in bone development [27–32]. These studies indicated that Tgfr2 signals are crucial to promote osteoprogenitor maturation from mesenchymal precursors. Deletion of Tgfr2 in this early mesenchyme resulted in embryonic lethality so the role of Tgfr2 in postnatal bone growth and mineralization was not investigated [27,28,33–36]. In contrast, overexpression of a cytoplasmically truncated Tgfr2, which acts as a dominant-negative mutant (DNIIR), in mature osteoblasts driven by the Bone gamma-carboxyglutamate Protein/Osteocalcin (Bglap) promoter caused an increase in trabecular bone mass [37], which was similarly seen using Bglap-Cre mediated conditional deletion of Tgfr2 [38]. These studies indicate a dynamic role of Tgfr2 in bone development, maintenance, and remodeling that is stage-dependent.

The role of Tgfr2 in committed but immature osteoprogenitors has not been previously addressed in vivo. Commitment to the osteoblast lineage occurs simultaneous to the expression of Osx [5]. Therefore, we generated mice with conditional deletion of Tgfr2 in immature osteoblasts by crossing mice containing floxed alleles of Tgfr2 [39] with mice that express the Osx promoter driven Cre recombinase [40–43]. These mice survived postnatally but developed skeletal defects including runted growth and reduced bone volume by postnatal day 24 (P24). In addition to alterations in the length of the growth plate, histomorphometric analysis indicated that Tgfr2 deletion resulted in reduced numbers of osteoblasts while osteoclast numbers were not affected.

Expression of Proliferating Cell Nuclear Antigen (PCNA) and Bglap, a marker of mature osteoblasts, were reduced in Tgfr2-deleted calvaria indicating reduced proliferation and fewer mature osteoblasts. The results indicate that loss of Tgfr2 in immature osteoblasts results in reduced proliferation likely resulting in fewer mature osteoblasts and reduced bone volume.

2. Materials and methods

2.1 Mice

All experiments with mice were approved by the UAB institutional animal care and use committee. The generation of Tgfr2^{fl/fl} [39] and Osx-Cre [43] mice were previously described. Both lines are in the C57BL/6 background strain. Osx-Cre mice were crossed to the Tgfr2^{fl/fl} mice to generate Osx-Cre;Tgfr2^{fl/wt} mice, which were used as controls or crossed to Tgfr2^{f/f} mice to get Osx-Cre;Tgfr2^{fl/fl} as the experimental group. Wild type mice were also crossed to Osx-Cre;Tgfr2^{wt/wt} mice to generate Osx-Cre control mice. Together both wild type and Osx-Cre controls were generated. We previously showed that tooth eruption is delayed in Osx-Cre;Tgfr2^{fl/fl} mice [11] and that the mutant mice die after weaning between 4 and 6 weeks of age; therefore, Osx-Cre;Tgfr2^{fl/fl} and control pups were housed with the lactating mothers until the skeletons were characterized at P24 days. Both male and female mice were used for all of the analyses. Previous studies have not detected differences in the size of male and female mice up to P24 days [44–46].

2.2 Alizarin red and Alcian blue staining

Whole mount Alizarin red/Alcian blue stains were used to determine skeletal structures at postnatal day zero (P0). Skeletons from wild type, *Osx-Cre*, and *Osx-Cre;Tgfb β 2^{fl/fl}* P0 mice were dissected and double-stained for cartilage and bone with Alcian blue and Alizarin red [47]. All carcasses were eviscerated and skinned and fixed in 95% ethanol for at least 24 hours. Samples were then placed in 95% ethanol-Alcian blue for 8–12 hours, rinsed in 100% ethanol for 24 hours, and cleared with 1% KOH for 24 hours. Bones were counterstained with Alizarin red in 1% KOH for 12 hours, with subsequent clearing with 1% KOH/20% glycerol for 24 hours at 4°C. Samples were stored in 1:1 glycerol:ethanol.

To detect mineralization in primary cell cultures, cultures were fixed for 30 minutes in 4% paraformaldehyde, rinsed with dH₂O, and stained with Alizarin Red, pH 4.3, for 45 minutes. The alizarin red was aspirated and cells were washed several times with dH₂O, dried, and imaged to include an entire well from a 6 well plate. All stains were performed in triplicate.

2.3 X-ray analysis and micro-computed tomography

P24 mice from experimental and control groups were sacrificed and the calvaria and tibia were dissected and fixed in 70% EtOH at room temperature. Samples were radiographed using a Faxitron radiograph inspection unit. Calvarial x-rays taken at the same magnification with images of equal sizes were generated and length and width was measured using the ruler tool in Photoshop to provide equivalent unit comparisons. A Two-way ANOVA test with significance levels set to $P < 0.05$ was used to determine differences between lengths and widths. A MicroCT-40 computed tomography system (Scanco Medical, Basserdorf, Switzerland) with analysis software was used to analyze the bone and tissue volume as well as trabecular number for the calvaria, cortical and trabecular bones. These analyses were performed by the UAB Small Animal Imaging Shared Facility according to standard guidelines [48]. Unpaired t-tests were performed at a confidence interval of 95%, with significance levels set to $P < 0.05$.

2.4 Immunofluorescence

Tibia from P24 mice were fixed in 4% paraformaldehyde for one hour followed by decalcification in EDTA buffer for 7 days. The tissue was then embedded in optimal cutting temperature (OCT) compound, snap frozen, and sectioned with a cryostat at 10 μ m. Sections were permeabilized with cold acetone at -20°C for 10 minutes. After 2 rinses with PBS, the sections were blocked with 5% BSA, 5% goat serum in PBS for 1 hour and incubated with Ki67 (1:100, Abcam) overnight at 4°C. Section were then rinsed and incubated with goat-anti-rabbit IgG (1:200, Invitrogen) and DAPI for 4 hours at room temperature. Sections were rinsed, dehydrated, and plated. Three sections from each tissue sample were on each slide to provide duplicates and a negative control that was not incubated with the primary antibody for each sample. Images were taken using an Olympus BX-51 microscope with an Olympus Q Color5 camera and Cell Sense software. An area was drawn around the growth plate for images stacked and aligned in Fiji software to provide equivalent areas of quantification. Fluorescence was thresholded to Ki67positive cells or DAPI for total cells and pixel density was calculated using Fiji software. The percent Ki67 positive cells was determined as the average pixel density for the Ki67 channel over that for DAPI. The results were analyzed

with Prism software using an unpaired t-tests. n=3 biological replicates for Osx-Cre, and Osx-Cre;Tgfb β 2^{fl/fl}. Significance was determined as *P<0.05.

2.5 Histomorphometry

P24 femurs from wild type, Osx-Cre;Tgfb β 2^{fl/wt}, and Osx-Cre;Tgfb β 2^{fl/fl} mice were fixed in 70% ethanol and undecalcified bones were embedded in plastic, sectioned, and stained with Trichrome. Sections were then analyzed with standard bone histomorphometry as previously described [49]. Osteoblast and osteoclast numbers were calculated using Bioquant Image Analysis software (R&M Biometrics, Nashville, TN) from two dimensional histologic sections displaying profiles of three-dimensional structures. These analyses were performed by UAB Histomorphometry and Molecular Analysis Core. A Two-way ANOVA test with significance levels set to P<0.05 was used to determine differences in osteoblast and osteoclast numbers between groups.

Growth plate length was measured from Trichrome stained sections. Images were taken using an Olympus BX-51 microscope with an Olympus Q Color5 camera and Cell Sense software. The length of the growth plate and the length of the hypertrophic zone were measured along three different locations in each image using the ruler tool in Photoshop. Unit length was then converted to microns using the calibrated scale bar included in the Cell Sense imaging software. An unpaired T-test with significance levels set to P<0.05 was used to determine the significance of differences measured between control and experimental groups.

2.6 Quantitative and semi-quantitative real-time RT PCR

RNA was isolated from calvarial bone and cell cultures using the standard Trizol method [50]. RNA concentration was determined with a Nanodrop Spectrophotometer. QuantiFast SYBR Green RT-PCR Kit (Qiagen) was used for quantitative real-time RT-PCR (QPCR) analysis with specific primers, as listed in Table 1. Data was normalized to hypoxanthine-guanine phosphoribosyltransferase (Hprt) [51]. Primers were designed using NCBI Primer Blast. We used 6 samples of calvarial mRNA of all genotypes to analyze proliferation and differentiation markers, and 4 samples all genotypes for apoptosis markers. For cell culture, we compared cells from two separate biological experiments (tissue from separate litters), plated in triplicate. Technical triplicates from each plate were used in QPCR assays. For apoptosis markers, Bax2 and Bcl2, we performed unpaired t-tests with significance levels set to P<0.05 to determine significance of the ratios of Bax2:Bcl2 between control and experimental groups. For proliferation (PCNA) and differentiation (Runx2, Bglap, and Osteonectin), data analysis was performed with REST software [52]. REST performs efficiency correction and statistical testing for non-parametric RNA expression data. A pair-wise fixed reallocation randomization test is used to determine significance and confidence intervals.

To show knock down of the Tgfb β 2 receptor we used a semi-quantitative real time RT-PCR (SQPCR) assay that we previously developed [27]. RNA (80ng) isolated as described above was utilized to synthesize cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad). Next, 20–100 ng of the cDNA template was amplified for 15, 20, 25 and 30 cycles to ensure product

formation was in the linear range (data not shown). Beta-2-microglobulin was used as a normalization control. Primer sequences are shown in Table 1.

2.7 Primary osteoblast culture

Calvarial osteoblast cultures were set up essentially according to [53–55] with a few modifications. Calvarium were dissected and separated from *Tgfr2^{fl/fl}* mice on postnatal day 3. Sutures and stromal tissue was removed before tissue digestion. Tissue pieces were incubated at 37°C for 10 minutes with 0.25% trypsin-EDTA, aspirated, and further incubated with 0.2% collagenase II in Hank's Balanced Salt Solution for 30 minutes and aspirated to remove fibroblasts and other non-osteoprogenitor cells. A single cell suspension was obtained after a final 60 minute incubation with 0.2% collagenase II solution. The isolated cells were plated into 75 cm² flasks for expansion in growth media (alpha-MEM containing 10% fetal bovine serum (FBS), antibiotics and fungizone). At confluence, cells were lifted and plated to confluence on tissue culture treated 6 well plates in osteogenic media (alpha-MEM with 10% FBS, 50 µg/ml ascorbate, and 10 mM β-glycerophosphate, antibiotics and fungizone) at 37°C in a CO₂ incubator. On day 6, the osteogenic media was aspirated and replaced with media without antibiotics, containing Adeno-Cre-EGFP virus or Adeno-EGFP virus. After 48 hours of infection, the media containing virus was removed and replaced with osteogenic media and the cells were incubated for another 5 days for a total of 12 days culture. Medium was replaced every 2 days. At the end of the culture period, mRNA was isolated for QPCR and SQPCR analysis or cells were fixed and stained with Alizarin red.

3. Results

3.1 *Tgfr2* in *Osx* expressing cells is not required for prenatal development

In contrast to *Prx1-Cre;Tgfr2^{fl/fl}* mice, which die at birth due to lack of skull and sternum, *Osx-Cre;Tgfr2^{fl/fl}* mice survived postnatally to weaning and were visually indistinguishable from control littermates at birth (P0). To determine if loss of *Tgfr2* in *Osx-Cre* expressing cells affected development of the skeleton in newborn mice, we compared Alizarin red/Alcian blue skeletal preparations made from P0 wild type, *Osx-Cre*, and *Osx-Cre;Tgfr2^{fl/fl}* mice (Figure 1). Previous research indicated the *Osx-Cre* gene insertion causes a delay in calvarial ossification [41,42] so *Osx-Cre* mice were included as a control. We found no discernible differences in the limbs or trunks between any of the genotypes compared. Staining in the *Osx-Cre;Tgfr2^{fl/fl}* skull and the *Osx-Cre* control skull were comparable, suggesting *Tgfr2* deletion in *Osx-Cre* expressing cells did not affect skeletal development before birth. As previously reported, skulls from *Osx-Cre* mice were slightly hypomineralized as compared to wild type controls.

3.2 Postnatal skeletal defects in *Osx-Cre;Tgfr2^{fl/fl}* mice

We previously reported delayed molar tooth eruption in *Osx-Cre;Tgfr2^{fl/fl}* mice [11]. The first molars usually erupt around P20 [56]. For this study, mice were characterized at P24 to avoid secondary phenotypes associated with delayed tooth eruption. In addition, all pups, controls and mutants, were housed with the lactating mothers until they were characterized at P24 days. Furthermore, it was previously shown that mice with the *Osterix-Cre* insertion

are initially small but recover as they age [40–42] so we included mice with the Cre insertion alone (Osx-Cre) as controls. By P24 days of age Osx-Cre;Tgfr2^{fl/fl} mice appeared significantly smaller than wild type and Osx-Cre mice. Osx-Cre;Tgfr2^{fl/wt} mice appeared similar to Osx-Cre controls. This initial observation suggested that loss of Tgfr2 affected postnatal development of the skeleton.

To further characterize changes in the postnatal skeleton in Osx-Cre;Tgfr2^{fl/fl} mice, we collected calvaria and femurs at P24 days for radiographic imaging (Figure 2A–C). Radiographic images indicated decreased length and mineralization of the femur in Osx-Cre;Tgfr2^{fl/fl} mice compared to controls. In addition, it appeared as if the skull and calvaria in the Osx-Cre;Tgfr2^{fl/fl} mice were smaller and hypomineralized relative to the wild type and Osx-Cre controls. The length and width of the skulls were measured (Figure 2D). Length was measured from the posterior tip of the nasal bone to the posterior tip of the interparietal bone at the point of greatest distance. Width was measured through the widest section of the frontal bones. Both the length and width of Osx-Cre;Tgfr2^{fl/fl} skulls were significantly shorter than that measured in either of the control groups.

Next, we confirmed using SQPCR [27,28] that Tgfr2 mRNA was knocked down in the calvaria from Osx-Cre;Tgfr2^{fl/fl} mice (Figure 2E). Calvarial bones from P24 mice were harvested and RNA was extracted and converted to cDNA. Primers spanning the deleted exon 2 of Tgfr2 were used in subsequent PCR reactions. The level of Beta-2-microglobulin was used as a normalization control. Varying cycle numbers were used to ensure that product formation was in the linear range (not shown). Tgfr2 mRNA levels were reduced in calvaria relative to that seen in the Cre+ controls. Residual Tgfr2 mRNA in the samples from Osx-Cre;Tgfr2^{fl/fl} skulls likely comes from mesenchyme associated with the skull that does not express the Cre transgene.

3.3 Alterations in the growth plate of Osx-Cre;Tgfr2^{fl/fl} mice

The growth plate determines the length of bones. It was previously shown that Osx-Cre can target hypertrophic chondrocytes as well as prehypertrophic cells near the hypertrophic zone [6]. We compared Trichrome stained sections of from wild type, Osx-Cre;Tgfr2^{fl/wt}, and Osx-Cre;Tgfr2^{fl/fl} mice to determine if there were any alterations in the structure of the growth plate that would account for short stature in the Osx-Cre;Tgfr2^{fl/fl} mice (Figure 3). Growth plates from wild type control and Osx-Cre;Tgfr2^{fl/wt} mice had comparable growth plate histology (Figure 3A, B). In contrast, growth plates from Osx-Cre;Tgfr2^{fl/fl} mice appeared shorter (Figure 3A, B, C). Next, we compared the length of the total growth plate and the length of the hypertrophic zone from images of wild type, Osx-Cre;Tgfr2^{fl/wt}, and Osx-Cre;Tgfr2^{fl/fl} growth plates (Figure 3D). The total length of the growth plate was similar in wild type control and Osx-Cre;Tgfr2^{fl/wt} mice (wild type 178.8 microns n=9; Het 175.1 n=3; T-test p=0.68). In contrast, the length of the Osx-Cre;Tgfr2^{fl/fl} growth plate was significantly shorter (133.2 microns n=6 T-test p=3.2 X 10⁻⁶). The length of the hypertrophic zone was not statistically different among all three groups (wild type 59.5 microns, Osx-Cre;Tgfr2^{fl/wt} 62.7, Osx-Cre;Tgfr2^{fl/fl} 59.3). The results suggested that the deficiency in the growth plate observed in the Tgfr2 deleted mice likely occurs in the prehypertrophic zones so we used Ki67 to measure proliferation in the growth plate (Figure

3E–G). Sections from *Osx-Cre* control and *Osx-Cre;Tgfr2^{fl/fl}* growth plates were immunostained for Ki67 and then counterstained with Dapi. Ki67 (green) and DAPI (blue) pixel densities were determined using Fiji software. The ratio of pixel density for Ki67 over DAPI was calculated for each image from three biological replicates and the average ratio and standard deviation were calculated and compared (Figure 3H). Ki67 staining was significantly less in growth plates from *Osx-Cre;Tgfr2^{fl/fl}* mice indicating reduced proliferation in the prehypertrophic zone of *Tgfr2* deleted growth plates, which would be expected to impact overall length of the bones.

3.4 Reduced bone volume and osteoblast number in long bones from *Osx-Cre;Tgfr2^{fl/fl}* mice

While examining the growth plate, we noticed that there was a decrease in the amount of trabecular bone under the growth plate of *Osx-Cre;Tgfr2^{fl/fl}* mice (Figure 3), which forms via endochondral ossification. To quantify changes in trabecular bone volume, we used microCT imaging (Figure 4). MicroCT imaging and analysis of the trabecular bone just under the growth plate in the distal femur indicated reduced bone volume over the total volume of the tissue (BV/TV) in *Osx-Cre* controls relative to the wild type controls, consistent with previous reports indicating an effect of the *Osx-Cre* insertion [41,42]. Nevertheless, there was a statistically significant reduction in BV/TV in the *Osx-Cre;Tgfr2^{fl/fl}* mice when compared to the *Osx-Cre* controls suggesting that *Tgfr2* is required for normal trabecular bone formation (Figure 4A, B). Likewise, trabecular thickness was reduced in the *Osx-Cre;Tgfr2^{fl/fl}* mice as compared to wild type and *Osx-Cre* controls (Figure 4C). Trabecular number and trabecular spacing were not different in *Osx-Cre;Tgfr2^{fl/fl}* and control mice (Figure 4D and data not shown). The results indicate the decrease in bone volume is due to a decrease in the thickness of the trabeculae, which could be a result of alterations in the amount of bone matrix that is laid down onto or resorbed from the nascent trabeculae.

Both osteoblasts and osteoclasts play an important role in bone formation and remodeling. It is well known that there is cross-talk between osteoblasts and osteoclasts that regulates bone remodeling [57]. Although *Osx-Cre* is not expressed in osteoclasts, it is possible that loss of *Tgfr2* in osteoblasts would have an effect on osteoclast activity due to cross talk. In addition, our previous research indicated reductions in osteoclast number and activity [11] in the alveolar bone surrounding the teeth, likely due to alterations in osteoblast activity. To determine the contribution of osteoblasts and osteoclasts to the phenotype observed in *Osx-Cre;Tgfr2^{fl/fl}* mice, we performed histomorphometric analysis on Trichrome stained sections from femurs of P24 wild type, *Osx-Cre;Tgfr2^{fl/wt}*, and *Osx-Cre;Tgfr2^{fl/fl}* mice (Figure 5A and B; [49] using Bioquant Image Analysis software (R&M Biometrics, Nashville, TN) to count osteoblasts (OB) and osteoclasts (OC) (Figure 5B). We could visually see similar reductions in trabecular bone volume in the *Osx-Cre;Tgfr2^{fl/fl}* mice as compared to controls that we saw in the microCT imaging (Figure 4A and Figure 5A). Histomorphometric analysis indicated that there was a decrease in the number of osteoblasts per unit bone surface (N.Ob/BS) in *Osx-Cre;Tgfr2^{fl/wt}* controls relative to the wild type controls and an additional and significant decrease in N.Ob/BS in *Osx-Cre;Tgfr2^{fl/fl}* relative to the *Osx-Cre;Tgfr2^{fl/wt}* controls suggesting that *Tgfr2* is required to maintain the

number of osteoblasts in the trabecular bone (Figure 5C). The number of osteoclasts per unit bone surface (N.Oc/BS) was comparable in all conditions tested. These results suggest that the decrease in trabecular bone volume detected in Tgfr2 deleted mice is primarily due to Tgfr2 effects on osteoblasts and not due to indirect effects on the osteoclasts.

The cortical bone forms directly from the perichondrium through a process like intramembranous bone formation. We also compared cortical bone volume over total volume (BV/TV) in wild type, Osx-Cre, and Osx-Cre;Tgfr2^{fl/fl} mice (Figure 6C). In contrast to what was observed in the trabecular bone, there was no significant difference in BV/TV in the cortical bone from the wild type and Osx-Cre controls; however, there was still a significant decrease in cortical bone volume in the Osx-Cre;Tgfr2^{fl/fl} mice (Figure 6A, C). This difference could also be seen in microCT images and Trichrome stained sections (Figure 6A, B). The results suggest that the Osx-Cre insertion in itself does not affect cortical bone as severely as it does trabecular bone and that Tgfr2 is required for development of the cortical bone as well as trabecular bone.

3.5 Tgfr2 deletion reduces calvarial bone volume, proliferation, and late stage osteoblast maturation

Radiographic images suggested defects in the formation of the calvaria in addition to the defects in the long bones (Figure 2). To quantify changes in bone volume in the calvaria we used MicroCT imaging (Figure 7A–C). While there was a small decrease in BV/TV in the calvaria of Osx-Cre control mice this was not statistically significant (Figure 7B, D). There was a visible reduction in bone mineralization in the Osx-Cre;Tgfr2^{fl/fl} calvaria as compared to both wild type and Osx-Cre controls and a statistically significant decrease in the BV/TV (Figure 7). The results suggest that Tgfr2 is required for the formation of the bone in the calvaria.

To examine the mechanism responsible for reduced bone volume, we compared levels of cellular apoptosis, proliferation and osteoblast differentiation in the calvarial bone by measuring differences in expression of specific marker genes (Figure 8). We first investigated the levels of Bax2 and Bcl2, two genes involved in the regulation of cellular apoptosis [58–60]. Previous research has indicated apoptotic susceptibility with increased Bax2/Bcl2 ratios. Generally, a ratio less than one indicates homeostasis, with increasing susceptibility to apoptosis as the ratio increases [61,62]. Both controls and Tgfr2-deleted mice demonstrated comparable Bax2:Bcl2 ratios of less than one (Figure 8A), indicating no change in apoptosis susceptibility in the absence of Tgfr2. In addition, no change in the relative levels of Bax2 or Bcl2 were seen individually in control versus Tgfr2-deleted mice (Figure 8B, C).

Proliferation was measured as expression of PCNA mRNA [63] using quantitative real-time PCR. We first compared wild type to Osx-Cre samples and showed there was no significant difference in PCNA expression between the controls (Figure 8B). However, there was a substantial reduction in PCNA expression in the Osx-Cre;Tgfr2^{fl/fl} mice as compared to Osx-Cre controls (Figure 8C) suggesting osteoblast proliferation is reduced in the Tgfr2 deleted mice. Reduced proliferation could account for the reduction in osteoblast number and reduced bone volume observed in the Tgfr2-deleted mice.

Differentiation was measured by expression of Runx2/Cbfa1 mRNA, a key transcription factor for osteoblast differentiation [64,65]. Late stage differentiation was measured as expression of a marker for mature osteoblasts, Osteocalcin/Bglap [66]. Runx2 and Bglap expression were similar in the wild type and Osx-Cre controls (Figure 8B). Runx2 expression was not affected by loss of Tgfr2; however, there was a substantial reduction in Bglap expression in the Osx-Cre;Tgfr2^{fl/fl} mice as compared to Osx-Cre controls (Figure 8C). Based on these results we propose that Tgfr2 is required for osteoblast proliferation and full maturation of osteoblasts. Fewer mature osteoblasts would be expected to result in reduced bone volume in mice.

Previously, it was shown that knockdown of Tgfr2 in mature Bglap expressing osteoblasts in mice resulted in a high bone phenotype [37,38], which is in contrast to what we observe when Tgfr2 is knocked down in immature osteoblasts. To address the issue of the role of Tgfr2 in mature osteoblasts, we used primary calvarial osteoblasts in which Tgfr2 was deleted in mature cells. We previously showed that deletion of Tgfr2 in primary mouse calvarial osteoprogenitor cells resulted in a failure of these cells to fully mature [28] consistent with the low bone phenotype observed in Osx-Cre;Tgfr2^{fl/fl} mice. Here, osteoblasts were isolated from mouse calvarial tissue at postnatal day 3 (P3). The cells were cultured under conditions of high cell density with osteogenic media to promote maturation. After 6 days of culture, when Bglap is reportedly expressed and osteoblasts begin in vitro maturation [55], the cells were infected with Ad-Cre-GFP virus to delete Tgfr2 or Ad-eGFP as a control (Figure 9). GFP was used to show cells were comparably infected with Ad-Cre-GFP and Ad-GFP and morphological differences were not apparent between Cre and GFP infected cells (Figure 9A). Ad-Cre-infected cells demonstrated knock down of Tgfr2 as measured by SQPCR (Figure 9B). Cells were cultured an additional 6 days after infection for a total of 12 days in culture. Expression of markers associated with mature osteoblasts and mineralization, Bglap and Osteonectin [63, 65–66], were measured using QPCR (Figure 9C). There was no statistically significant change in either Bglap or Osteonectin expression after Tgfr2 knockdown when compared to the GFP infected controls. To determine if mineralization was altered in mature osteoblasts in which Tgfr2 was knocked down, cultures were stained with Alizarin red (Figure 9D). No remarkable alteration in staining were observed. Based in these results, we propose that loss of Tgfr2 in mature calvarial osteoblasts in culture does not have major cell autonomous effects on further maturation or mineralization. We also propose that Tgfr2 has different roles in osteoblast biology that are dependent on the maturation status of the cells.

4.0 Discussion

In this study, we analyzed the effects of Tgfr2 deletion in immature osteoblasts using the Osx-Cre gene deletion model. Osx-Cre;Tgfr2^{fl/fl} mice did not demonstrate skeletal defects at birth and survived for several weeks with runted stature associated with reduced proliferation in the growth plate. Radiographic, microCT, and trichrome stained histological sections indicated reduced bone volume in calvaria and in both trabecular and cortical compartments of the long bone. Histomorphometric analysis indicated decreased osteoblast, but not osteoclast, numbers. Quantitative RT-PCR for PCNA mRNA indicated significant reductions in proliferation and the Bax2:Bcl2 ratio indicated no change in apoptosis in

calvaria bones. Reduced expression of *Bglap* suggested a reduction in mature osteoblasts. The results demonstrate a role for *Tgfr2* in proliferation and maturation of immature osteoblasts. We propose that the resulting reduction in mature osteoblasts would negatively affect overall bone volume.

Recent reports have shown that the *Osx-Cre* insertion alone results in delayed osteogenesis in calvaria and long bone that is overcome as the mice age [40–42]. Therefore, we included wild type and *Osx-Cre* controls to rule out developmental defects caused solely by the *Osx-Cre* gene insertion. *Osx-Cre;Tgfr2^{fl/fl}* mice generally die between 4 and 6 weeks of age after weaning most likely as a result of delays in tooth eruption [11]. To avoid secondary consequences of delayed tooth eruption, mice were characterized at P24 days and kept with the lactating mothers until that time. It is also possible that post weaning lethality could be due to mis-targeted *Tgfr2* deletion in other tissues. In-depth investigation of *Osx-Cre* activity in mice at 8 weeks of age using the R26-mT/mG reporter line showed that *Osx-Cre* is expressed in some stromal cells, adipocytes, perivascular bone marrow cells, olfactory glomerular cells and a subset of gastric and intestinal epithelium [6].

Osx-Cre;Tgfr2^{fl/fl} mice appeared normal at birth but demonstrated short limbs by P24. Postnatal longitudinal growth occurs through endochondral bone formation at the growth plate. Resting cells at the top of the growth plate undergo a complex program of proliferation and differentiation leading to the formation of hypertrophic chondrocytes. The hypertrophic chondrocytes lay down a mineralized matrix that is later remodeled by osteoblasts to form the trabecular bone. *Osx-Cre;Tgfr2^{fl/fl}* mice demonstrated a shorter growth plate at P24 than wild type and *Osx-Cre;Tgfr2^{fl/wt}* controls primarily due to reduced proliferation in the prehypertrophic zone of the growth plate, which would be expected to affect overall bone length. It has been shown that *Osx-Cre* is active primarily in hypertrophic chondrocytes, although a subset of prehypertrophic chondrocytes near the hypertrophic zone were also targeted [6,67]. We previously showed that loss of *Tgfr2* in limb mesenchyme via *Prx1-Cre* deletion results in reduced proliferation and accelerated hypertrophic differentiation of chondrocytes in embryonic growth plate with consequential short limbs at birth [27]. In this case, all of the cells in the growth plate and perichondrium were targeted with *Cre* early (E9.5 days) in limb development. In contrast, when *Tgfr2* was deleted using *Col2a-Cre*, which targets committed chondrocytes (E12.5 days), limb length was normal at birth but post-natal mice developed dwarfism [68]. The differences in the extent of the alterations in the growth plate in these mice can be attributed to differences in timing and localization of *Tgfr2* deletion. The results presented in this study further narrow down the population of chondrocytes that require *Tgfr2* for normal postnatal growth plate function.

TGF β signaling has well-recognized roles in skeletal development and maintenance that when disrupted, lead to disease phenotypes [11,27,28,69–71]. Conditional deletion of *Tgfr2* with *Wnt1-* or *Prx1-Cre* leads to prenatal or perinatal lethality [27,28,33,68,72–74], preventing the investigation of postnatal bone development. *Prx1-Cre;Tgfr2^{fl/fl}* mice demonstrated a failure in the formation of the skull due to reduced proliferation in uncommitted mesenchymal cells in addition to inhibition of early osteoblast differentiation [28]. In contrast, dominant-negative interference or deletion of *Tgfr2* in *Bglap* expressing

mature osteoblasts (Bglap-DNIIR and Bglap-Cre;Tgfr2^{fl/fl}) led to increased bone mass [37,38]. Osx-Cre;Tgfr2^{fl/fl} mice provided the ability to investigate the role of Tgfr2 in vivo at a stage in between.

Osx expression is first detected in immature osteoblasts [2]. Osx-Cre activity, as measured by ROSA26 mouse reporter lines, was first detected at E12.5 in the perichondrium where the cortical bone starts to form [5, 7]. Cre activity was then traced to all cells of the osteoblast lineage through embryonic and postnatal development [5–7]. In contrast, Bglap is expressed in mature osteoblasts [75]. Bglap-Cre activity was first detected in osteoblasts and osteocytes in ossifying centers of the calvaria at E17.5 days [76]. Furthermore, experiments to trace activity of Bglap-Cre using primary bone marrow cultures from Bglap-Cre; ROSA26 LacZ reporter mice indicated that Bglap-Cre was active only when the matrix started to mineralize [77]. The difference in phenotype between Osx-Cre;Tgfr2^{fl/fl} and Bglap-Cre;Tgfr2^{fl/fl} mice could be due to differences in when Tgfr2 is deleted during osteoblast differentiation. In this study, we report that Osx-Cre;Tgfr2^{fl/fl} mice demonstrate reduced trabecular, cortical, and calvarial bone volume associated with reduced proliferation and maturation of immature osteoblasts. In contrast, Bglap;Tgfr2^{fl/fl} mice demonstrated increased trabecular bone volume while cortical bone volume was decreased [38]. Overall bone remodeling was increased as demonstrated by an increase in both osteoblast and osteoclast number and activity. Increased bone volume was also associated with an increase in PTH1R signaling, a known anabolic signal, in the mature osteoblasts. The results presented here indicate that there are few mature osteoblasts in Osx-Cre;Tgfr2^{fl/fl} mice, thus, we would not expect to see high bone volume since it is unlikely that even increased PTH1R signaling would be able to compensate for the reduced number of cells observed. In addition, it is not clear if immature cells that have lost Tgfr2 would have increased PTH1R activity or respond to the PTH1R signal in the same way as mature cells.

We previously showed that loss of Tgfr2 in calvarial mesenchyme grown in culture inhibits osteoblast differentiation independently of effects on proliferation. Here, we deleted Tgfr2 in cultures of mature Bglap expressing calvarial osteoblasts. Expression of markers for mature osteoblasts, Bglap and Osteonectin, were not affected by loss of Tgfr2. Furthermore, alizarin red staining was comparable in the control and Tgfr2-depleted cultures. This raises the possibility that the high bone phenotype seen in trabecular bone from Bglap;Tgfr2^{fl/fl} mice may not be completely cell autonomous. It was previously suggested that the high bone phenotype in Bglap-DNIIR mice was in part due to an imbalance in bone formation and resorption [37]. Furthermore, it has been suggested that the response of cells to TGF- β depends on their differentiation status [78]. Based on the comparison of phenotypes in mice deleted for Tgfr2 in Prx1-, Osx-, and Bglap-Cre expressing cells and cell culture experiments where Tgfr2 is deleted at different stages of osteoblast differentiation, we propose that Tgfr2 has varying roles on osteoblast biology depending on their stage of differentiation.

We conclude that Tgfr2 is required for the proliferation and subsequent maturation of immature osteoblasts in vivo. We propose that the resulting reduction in mature osteoblasts in these mice results in reduced bone volume.

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Abbreviations

DNIIR	Dominant negative Tgfr2
Osx	Osterix/Sp7
Bglap	Bone gamma-carboxyglutamate Protein/Osteocalcin
Tgfr2	TGF- β type II receptor
QPCR	quantitative real-time RT-PCR
SQPCR	semi-quantitative real time RT-PCR

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Highlights

- Tgfr2 is required in Osx-Cre expressing immature osteoblasts for postnatal development of trabecular, cortical, and calvaria bones.
- Limb length and the length of the prehypertrophic zones of the growth plate were reduced in Tgfr2-deleted mice.
- Deletion of Tgfr2 in immature osteoblasts resulted in reduced osteoblast proliferation and number during postnatal development.
- There was a reduction in Bglap, a marker for mature osteoblasts, in Tgfr2 deleted bone.
- Reduced bone volume in Tgfr2 deleted mice is likely due to a decrease in osteoblast proliferation leading to fewer mature osteoblasts.

Figure 1. Skeletal phenotype at P0

Representative skeletons of postnatal day 0 (P0) wild type (WT), *Osx-Cre* (Cre+) and *Osx-Cre;Tgfb2^{fl/fl}* (CKO) pups from left to right showing mineralized bones with Alizarin red and cartilaginous tissue with Alcian blue. No discernible difference is demonstrated between the *Osx-Cre;Tgfb2^{fl/fl}* mice and the *Osx-Cre* controls $n=7$ for all genotypes.

Figure 2. Skeletal phenotype of control and *Osx-Cre;Tgfr2^{fl/fl}* mice at P24

(A–C) Representative X rays of femur and calvaria at P24 days wild type (A, WT), *Osx-Cre* (B, Cre+), and *Osx-Cre;Tgfr2^{fl/fl}* (C, CKO). For long bone, WT (n=4), Cre+ (n=5) and CKO (n=4). For calvaria, WT (n=9), Cre+ (n=5) and CKO (n=4). (D) Quantification of calvarial length from the posterior tip of the nasal bone to the posterior tip of the interparietal bone and width across the frontal bone. Y-axis depicts units of distance generated by the ruler tool analyzed with images generated with equal magnification and image size. n= 4 for all genotypes. *p< 0.05, **p < 0.01, ***p<0.001, p = 0.0001 by two-way ANOVA. (E) RNA from P24 Cre+ and CKO calvaria isolated was isolated and used to generate cDNA to determine relative levels of *Tgfr2* using semi-quantitative RT-PCR. Product formation is shown within the linear range. Beta-2 microglobulin (B2MG) was used as a normalization control. Image depicts representative bands for n=4 of both genotypes.

Figure 3. Alterations in the growth plate of *Osx-Cre;Tgfb2^{fl/fl}* mice

Sections from P24 day wild type control (A, WT), *Osx-Cre;Tgfb2^{fl/wt}* (B, HET), and *Osx-Cre;Tgfb2^{fl/fl}* (C, CKO) distal femurs were stained with Trichrome and analyzed for growth plate histology. The secondary ossification center (2°OS) is on the top and the primary ossification center (1°OS) on the bottom. PHZ, prehypertrophic zones. HZ, hypertrophic zone. TB, trabecular bone. (D) Quantification of growth plate (GP) and hypertrophic zone lengths, demonstrating shorter growth plates in CKO samples as compared to both HET and WT samples. WT (n=9), HET (n=3), and CKO (n=6). ****p<0.0001 by unpaired t-test, C.I. of 95%. (E–F) Ki67 positive cells in the growth plate of Cre+ and CKO tibias, depicting fewer Ki67+ cells in the CKO mouse. (G) No primary control on Cre+ ankle growth plate. Scalebar = 50 μ m. (H) Quantification of Ki67 pixel density relative to DAPI in equivalent areas of growth plate, demonstrating significantly less Ki67 in the CKO. n=3 for all genotypes.

Figure 4. Alterations in trabecular bone at 24 days

(A) MicroCT images of femur trabeculae demonstrating reduced trabecular bone in *Osx-Cre;Tgfb β 2^{fl/fl}* (CKO) relative to both wild type (WT) and *Osx-Cre* (Cre+) controls. (B–D) Quantification of microCT data demonstrating reduced trabecular bone volume (B) in CKO mice relative to controls. Trabecular thickness (C), but not trabecular number (D), was also reduced in CKO mice relative to the WT control. *WT (n=10), Cre+ (n=8) and CKO (n=6). ** p<0.01, ***p < 0.001, ****p<0.0001 by unpaired t-test, C.I. of 95%.

Figure 5. Histomorphometric quantification of distal femora of mice at P24 days
 (A) Sections of wild type (WT), *Osx-Cre;Tgfr2^{fl/wt}* (HET) and *Osx-Cre;Tgfr2^{fl/fl}* CKO) mice where stained with Trichrome to highlight bone. Scalebar = 50 μ m. (B) Inset from trabecular region demonstrating osteoblasts (OB) and osteoclasts (OC). (C) Histomorphometric analysis demonstrated a reduction in the number of osteoblasts in CKO mice relative to WT and HET controls (left). Osteoclast numbers were not statistically different among the groups tested (right). n=12, 4, and 5 for WT, HET, and CKO, respectively. **p < 0.01, ***p<0.001 by two-way ANOVA analysis.

Figure 6. Defects in cortical bone in *Osx-Cre;Tgfb2^{fl/fl}* mice at P24 days

A) MicroCT images of cortical bone from femur demonstrating reduced bone volume in *Osx-Cre;Tgfb2^{fl/fl}* (CKO) relative to both wild type (WT) and *Osx-Cre* (Cre+) controls. B) Trichrome staining of sections of cortical bone from wild type (WT), *Osx-Cre;Tgfb2^{fl/wt}* (HET) and *Osx-Cre;Tgfb2^{fl/fl}* CKO) mice. Scalebar = 50 μ m. n=12, 4, and 5 for WT HET, and CKO, respectively. C) Quantification of microCT data demonstrating reduced cortical bone volume in CKO mice relative to WT and Cre+ controls. *WT (n=10), Cre+ (n=8) and CKO (n=6). ** p<0.01, ***p < 0.001, ****p<0.0001 by unpaired t-test, C.I. of 95%.

Figure 7. Reduced bone volume in calvaria from *Osx-Cre;Tgfb2^{fl/fl}* mice relative to controls at P24 days

MicroCT images of calvaria from wild type (A, WT), *Osx-Cre* (B, Cre+) and two representative *Osx-Cre;Tgfb2^{fl/fl}* (C, CKO) mice. (D) Results from the microCT analysis was quantified and shown in (C). Bone volume was reduced in CKO relative to both controls. *WT = (n=4), Cre+ (n=7) and CKO (n=5). ***p < 0.001, ****p < 0.0001 by unpaired t-test, C.I. of 95%.

Figure 8. Apoptosis, proliferation, and osteoblast maturation in P24 day calvaria

RNA was isolated from calvaria of wild type (WT), *Osx-Cre* (Cre+), and *Osx-Cre;Tgfr2^{fl/fl}* (CKO) mice. (A) Apoptosis was determined as the ratio of Bax2:Bcl2 mRNA measured by quantitative real time RT-PCR. All ratios were below 1. No significant change was detected by unpaired t-tests. $n = 4$ for all genotypes. (B) Bax2, Bcl2, PCNA, Runx2, and Bglap mRNA levels were measured by quantitative real time RT-PCR. Expression levels in Cre+ samples are shown relative to WT controls. (C) Bax2, Bcl2, PCNA, Runx2, and Bglap mRNA levels in CKO samples are shown relative to Cre+ controls. *Hprt* was used as the normalization control. $n = 6$ for all genotype for PCNA, Runx2 and Bglap. * $p < 0.05$ relative to controls by REST2009 using pair-wise fixed reallocation randomization tests to determine significance.

Figure 9. Tgfr2 knockdown in mature primary osteoblasts in culture

Calvarial osteoblasts from P3 Tgfr2^{f/f} skulls were cultured in osteogenic media for 6 days, infected with Adenovirus-eGFP (Ad-eGFP) or Adenovirus-Cre (Ad-Cre), and cultured an additional 6 days. (A) Brightfield and GFP images demonstrating similar morphology and comparably infected cells on day 12 of culture. Scalebar = 125 μ m. (B) SQPCR assay to show knock down of Tgfr2 expression. Image shows representative replicates. Beta-2 Microglobulin (B2MG) was used as a normalization control. (C) RNA was isolated from Ad-eGFP- and Ad-Cre-infected cells at day 12 of culture. Bglap and Osteonectin mRNA levels were measured by QPCR. NS, non-significant REST2009 pair-wise fixed reallocation

randomization test. (D) Alizarin red staining of mineralization in cultures on day 12. Images depict 2 representative wells from Ad-eGFP- and Ad-Cre-infected cells. n=2 separate experiments with triplicates of each sample, with additional technical triplicates for QPCR analysis.

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Table 1

Primers used in real-time PCR.

Gene name	Forward primer (5'-3')	Reverse primer (3'-5')
hPRT	TCA GTC AAC GGG GGA CAT AAA	GGG GCT GTA CTG CTT AAC CAG
B2MG	GCT ATC CAG AAA ACC CCT CAA	CAT GTC TCG ATC CCA GTA GAC
Tgfb β 2e2	TTA ACA GTG ATG TCA TGG CCA GCG	AGA CTT CAT GCG GCT TCT CAC AGA
Blap	ATA GCT CGT CAC AAG CAG GG	TGA CAA AGC CTT CAT GTC CA
Osteonectin	CCT TCG ACT CTT CCT GCC AC	GCG ATG TAT TTG CAT GGT CCG
Runx2	GCC TCC GCT GTT ATG AAA AA	TGG GGA GGA TTT GTG AAG AC
PCNA	TGT GCC CCT TGT TGT AGA GT	AAA GAC CTC AGG ACA CGC TG
Bax	AAA CTG GTG CTC AAG GCC C	CTT GGA TCC AGA CAA GCA GC
Bcl-2	AGG CTG GGA TGC CTT TGT GG	ACT TGT GGC CCA GGT ATG C