



TLE4 Is a Critical Mediator of Osteoblast and Runx2-Dependent Bone Development

Thomas H. Shin^{1,2}, Evangelos Theodorou¹, Carl Holland¹, Rae'e Yamin¹, Cathleen L. Raggio³, Philip F. Giampietro⁴ and David A. Sweetser^{1*}

¹ Department of Pediatrics, Center of Genomic Medicine, Divisions of Pediatric Hematology/Oncology and Medical Genetics, Massachusetts General Hospital, Harvard Medical School, Boston, MA, United States, ² Department of Molecular and Translational Medicine, Boston University School of Medicine, Boston, MA, United States, ³ Department of Pediatric Orthopedics, Hospital for Special Surgery, New York, NY, United States, ⁴ Department of Pediatrics, University of Illinois, Chicago, IL, United States

OPEN ACCESS

Edited by:

Cesare Indiveri,
University of Calabria, Italy

Reviewed by:

Bram van der Eerden,
Erasmus Medical Center, Netherlands
Dian Widya Damaiyanti,
Universitas Hang Tuah, Indonesia

*Correspondence:

David A. Sweetser
dsweetser@mgh.harvard.edu

Specialty section:

This article was submitted to
Cellular Biochemistry,
a section of the journal
Frontiers in Cell and Developmental
Biology

Received: 22 February 2021

Accepted: 14 July 2021

Published: 06 August 2021

Citation:

Shin TH, Theodorou E, Holland C, Yamin R, Raggio CL, Giampietro PF and Sweetser DA (2021) TLE4 Is a Critical Mediator of Osteoblast and Runx2-Dependent Bone Development.
Front. Cell Dev. Biol. 9:671029.
doi: 10.3389/fcell.2021.671029

Healthy bone homeostasis hinges upon a delicate balance and regulation of multiple processes that contribute to bone development and metabolism. While examining hematopoietic regulation by *Tle4*, we have uncovered a previously unappreciated role of *Tle4* on bone calcification using a novel *Tle4* null mouse model. Given the significance of osteoblasts in both hematopoiesis and bone development, this study investigated how loss of *Tle4* affects osteoblast function. We used dynamic bone formation parameters and microCT to characterize the adverse effects of *Tle4* loss on bone development. We further demonstrated loss of *Tle4* impacts expression of several key osteoblastogenic genes, including *Runx2*, *Oc*, and *Ap*, pointing toward a potential novel mechanism for *Tle4*-dependent regulation of mammalian bone development in collaboration with the RUNX family members.

Keywords: *Tle4*, osteoblast, *Runx2*, bone mineralization, *Tle4*-Runx axis, bone calcification

INTRODUCTION

Normal bone development is a dynamic process that depends on the balance between bone formation and bone resorption. These two processes are largely mediated by osteoblasts and osteoclasts, respectively. An imbalance of these two forces results in various bone pathologies, including osteopetrosis and osteoporosis (Cohen, 2006; Kaul et al., 2015). Osteoblasts are derived from mesenchymal cells that are triggered by Wnt signaling toward osteoblastic differentiation (Hill et al., 2005; Houschyar et al., 2019). Various factors, including Wnt, BMP signaling, and *Runx2*, have been found to play roles, not only in normal osteoblast function, but also maturation and viability (Cohen, 2006; Kozhemyakina et al., 2015). *Runx2* and *Osterix* regulate the differentiation of mesenchymal stem cells (MSCs) to osteoblastic lineages (Asada and Katayama, 2014). The loss of *Runx2*, a known interaction partner of *Tle* co-repressors, results in the absence of bone formation thought to be secondary to aberrant osteoblast differentiation in mice (Choi et al., 2001). Additionally, previous studies have shown that *Runx2* expression induces osteoblastic differentiation of mouse stromal cells (Baniwal et al., 2012). *Runx2* augments mesenchymal lineage proliferation while also assisting the commitment to osteoblasts by regulating a series of signaling pathways that include Wnt, FGF and PTH, as well as *Dlx5* (Komori, 2019). Moreover, *Runx2* enhances the expression of bone matrix protein genes including

Col1a1, *Spp1*, *Ibsp*, *Bglap2* and *Fin1* (Komori, 2019). *Smad* and p38 MAPK signaling pathways regulate *Runx2* promoting osteoblast and chondrocyte differentiation (Wu et al., 2016). Other key regulators of osteoblast differentiation include osteoprotegerin, osterix, and alkaline phosphatase, and osteopontin. These factors are expressed at different stages of osteoblast differentiation and regulate osteoblast precursor fate decisions, bone metabolism, and osteoclast induction (Cohen, 2006).

The Groucho/TLE family of proteins are intimately involved in the regulation of various signaling pathways critical to cell fate and development, including receptor tyrosine kinase/Ras/MAPK, Notch, and Wnt signaling (Zhang and Dressler, 2013; Chodaparambil et al., 2014). The Groucho/TLE family have been extensively studied as corepressors of various binding partners, including the RUNX/AML family through the C-terminal VWRPY Groucho recruitment motif (Levanon et al., 1998; Chen and Courey, 2000). In leukemia, we have shown *t*(8;21) leukemic cell viability and growth are sensitive to TLE4 levels and that loss of the TLE homolog in zebrafish, Gro3, cooperates with AML1-ETO to create a myeloid leukemia phenotype (AML) (Dayyani et al., 2008). Having identified the tumor suppressor role of *TLE4* in myeloid leukemias, we generated a novel *Tle4* knockout mouse model to better understand its role in mammalian development (Sweetser et al., 2005; Dayyani et al., 2008; Wheat et al., 2014). In addition to various hematopoietic abnormalities, we unexpectedly found a severe bone development defect in these mice leading to severe runting and decreased bone mineralization (Wheat et al., 2014). Similar dual functions have been described for other regulators of bone development that also can function as tumor suppressor genes including FoxO members and ARF which drives bone remodeling and osteosarcoma development in mice through both p53 independent and dependent mechanisms (Rauch et al., 2010; Le et al., 2018; Ma et al., 2018; Schmitt-Ney, 2020).

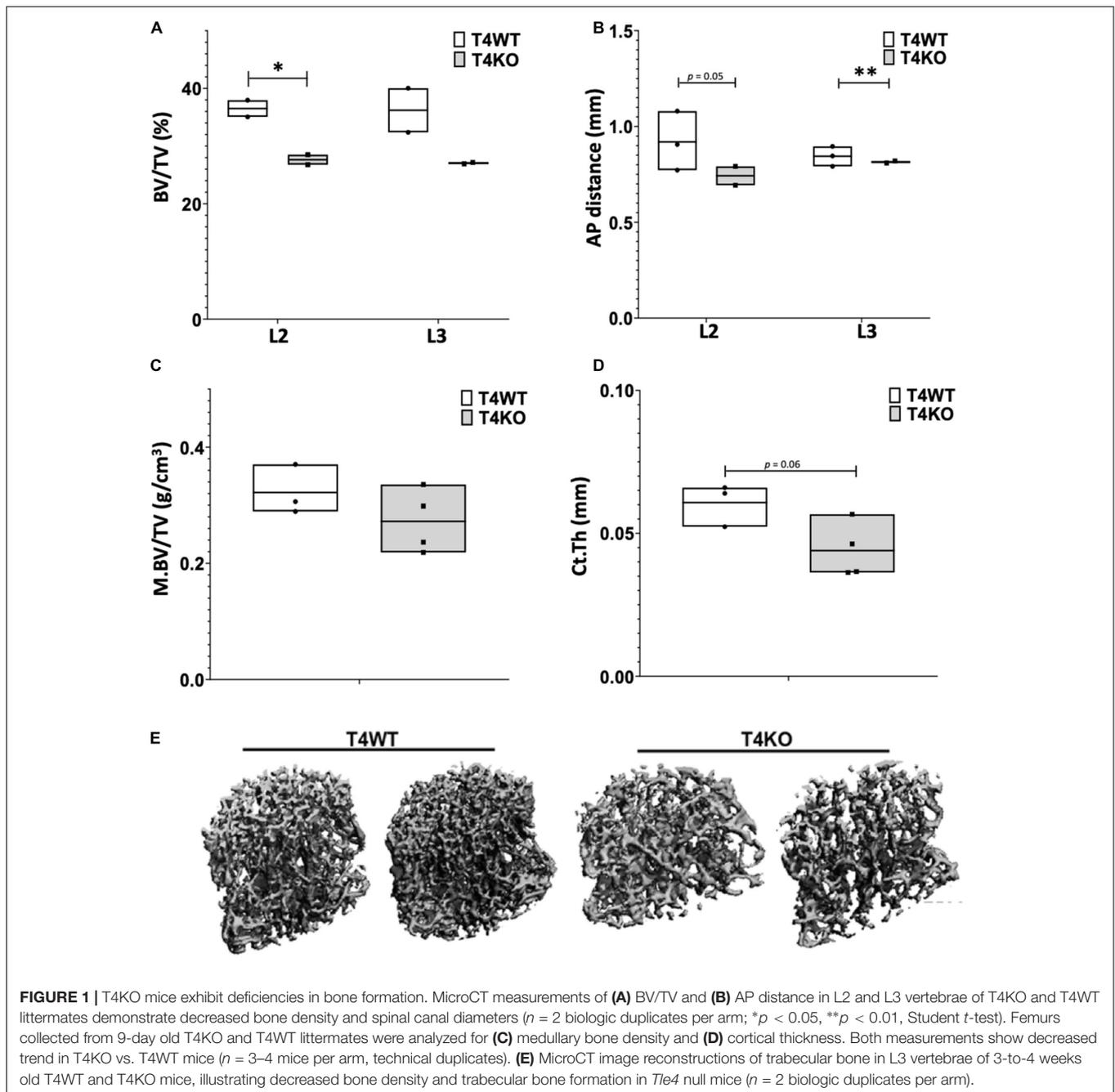
Hematopoiesis and normal bone development are intimately connected (Bianco, 2011; Despars and St-Pierre, 2011). In concert with other resident bone tissue cells, osteoblasts create and protect a hospitable hematopoietic stem cell (HSC) microenvironment (Cohen, 2006; Yin and Li, 2006). Co-cultures of MSCs with various leukemic cell lines increase osteoblastic markers such as *Runx2*, *Osx*, *Opn* (Le et al., 2018). Initial studies of hematopoiesis in the bone marrow found many HSCs in close proximity to the inner bone endosteal area, which has triggered much inquiry into the specific relevance of osteoblasts in HSC maintenance and niche (Yin and Li, 2006; Garcia-Garcia et al., 2015). Osteoblasts communicate with HSCs through direct receptor-ligand interactions (e.g., Ang1/Tie2 and TPO/MPL), to support HSC adhesion and residence in the niche, including interactions between N-cadherin/ β -catenin, and osteopontin/ β_1 integrin (Yin and Li, 2006; Le et al., 2018). In addition, osteoblasts secrete factors including G-CSF, hepatocyte growth factor and osteopontin that regulate the pool size of the CD34⁺ progenitor population (Le et al., 2018). Osteoblasts regulate HSC migration in and outside of the bone marrow through CXCL12/CXCR4 and VCAM-1/VLA-4 (Le et al., 2018). The constitutive activation of β -catenin in osteoblasts and

resultant expression of the Notch ligand Jagged-1 activates Notch signaling in HSC leading to the development of AML. This underscores the importance of normal regulation of mediators of osteoblast differentiation on normal hematopoiesis (Kode et al., 2014). *Dicer1* or *Ptfn11* deficient osteoprogenitor cells in mice display myelodysplastic syndrome and secondary acute myeloid leukemias, as well as juvenile myelomonocytic leukemia-like myeloproliferative neoplasms, respectively (Le et al., 2018). This is further demonstrated by studies that found HSC populations increased in parallel with expansion of osteoblasts due to parathyroid hormone treatment in mice (Calvi et al., 2003), while PTH activation enhances the migration of long-term repopulating HSCs (Even et al., 2021). Moreover, a previous study targeted ablation of osteoblasts in mice found that the loss of osteoblasts significantly reduced HSC and hematopoietic progenitor populations (Visnjic et al., 2004). Furthermore, animals deficient in Sca marker present bone abnormalities (Aguila and Rowe, 2005). Thus, osteoblast function intimately connects bone formation and hematopoiesis. Specifically Car/LepR⁺ CXCL12 expressing cells create a niche for HSCs cells while simultaneously give rise to osteoblasts (Galan-Diez and Kousteni, 2018). The similarity of *Tle4* null mice to *Runx2* null mice suggested the loss of *Tle4* might either impair the function or the expression of *Runx2*. To better characterize the nature of the defect in bone development and maintenance in *Tle4* null mice we have used *Tle4* null and conditional *Tle4* knockout mice and performed assays of osteoblast function and development in bone stromal cultures and mesenchymal bone marrow cell lines.

MATERIALS AND METHODS

Generation of *Tle4* Null and Conditional *Tle4* Knockout Mice

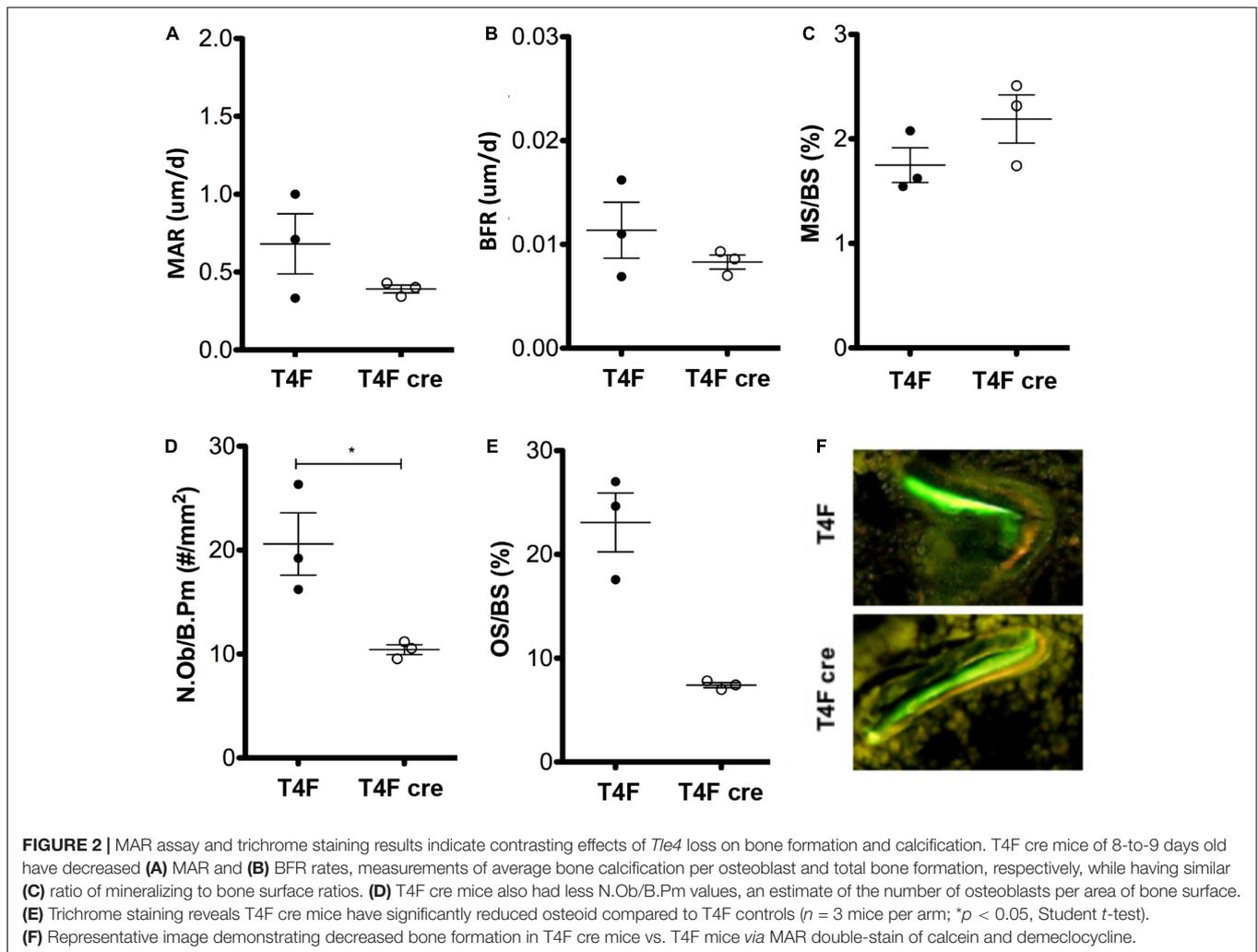
For these experiments we used *Tle4* null (T4KO) and *Tle4* conditional knockout mice generated in our laboratory as previously described (Wheat et al., 2014). Briefly, conditional mice were constructed by targeting LoxP sites to flank exon 2 of *Tle4* via homologous recombination using the 129S6/SvEvTac ES cell line (T4F). To generate T4KO, resultant mice were crossed with β -actin:Cre mice (gift of Dr. Susan Dymecki) to delete exon 2 in all tissues. Heterozygote mice were backcrossed to C57BL/6 background for over six generations and interbred to generate *Tle4* null mice. For conditional knockout of *Tle4*, homozygous T4F mice containing *Mx1*-Cre (T4F cre) were used. Excision of *Tle4* exon 2 was induced with three intraperitoneal injections of 15 mg/kg polyinosinic-polycytidylic acid (pIpC; Sigma) separated by 48 h. pIpC treatment induces interferon- γ signaling with activation of Cre expression and subsequent Cre recombinase excision of exon 2 of *Tle4*. This is predicted to cause a frameshift resulting in a premature stop codon and non-functional truncated *Tle4* protein (Wheat et al., 2014). Demonstration of T4F knockout efficiency by pIpC was performed by PCR using primers mT4WTvFlpR 5'- GGAGACTTGGAAAACGCTGA-3', mT4PcreF 5'- CAAAGGGCCCCAGAATCTT-3' and mT4PcreR 5'- CGACCGACTTGTAGCCATTT-3'. Mice were housed in



a specific pathogen-free environment with a 12-h light/dark cycle, 30–70% relative humidity and approximately 70°F ambient temperature, in groups not surpassing four adult animals. Mice had *ad libitum* access to tap water and standard rodent chow (Prolab® RMH 3500, Scotts Distributing, Hudson, NH, United States). For analysis mice were euthanized by inhalation of 100% CO₂. This study was carried out in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

Cell Culture, shRNA Construction, and Lentiviral Infection

ST2D cells (Generous gift of Dr. Baruch Frenkel) were generated by stably transforming mouse mesenchymal ST2 cells derived from bone marrow with a doxycycline-inducible *Runx2* expression vector (Baniwal et al., 2012). ST2D cells were cultured in RPMI-1640 (Lonza, Walkersville, MD, United States) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, United States) and 1% penicillin/streptomycin (Invitrogen, United States). Cells were maintained at 37°C and 5% CO₂. When indicated, cells were also cultured with 350 ng/mL



doxycycline (Sigma-Aldrich, United States) or DMSO (Sigma-Aldrich, United States). Non-targeting control (scr) and *Tle4*-specific shRNA constructs were developed using the lentiviral vector FUGW and delivered to cells *via* lentiviral delivery as previously described (Dayyani et al., 2008). The *Tle4* shRNA (sh*Tle4*) used has the following target sequence: AGTGATGACAACTTGGTGG and a control scrambled shRNA (scr) CAGTCGCCATTAGTTCAC. Infected cells were identified by GFP fluorescence detected using FACS LSRII or GFP-selected *via* cell sorting with FACS Aria (BD, United States).

Generation of Stromal Cultures

Stromal cultures were generated from bones of 1-week old T4WT or T4KO littermates as previously described (Mukherjee et al., 2008; Wheat et al., 2014). After harvesting femur and humeri, whole bones were crushed and plated on tissue culture plates with MEM α (Invitrogen, United States) supplemented with 20% FBS and 1% Penicillin/Streptomycin (Invitrogen, United States). After 3 days, non-adherent cells were removed and media was changed to osteogenic media containing 100 μ M β -glycerophosphate, 2.84 μ M ascorbic acid, and 10 nM dexamethasone. After 1 week

in osteogenic media, stromal cultures were either lysed with Trizol (Invitrogen, United States) for RNA or stained for alkaline phosphatase activity (Sigma).

In osteoblast function experiments, ST2D cells were treated with *Tle4*-specific (T4KD) or scramble control shRNA (SCR) *via* lentiviral expression. One week after spinoculation, GFP + ST2D cells were selected using FACS Aria (BD, United States) and cultured in 6-well plates. Upon reaching confluence, ST2D cells were cultured in osteogenic media, with or without 350 ng/mL doxycycline. After 2 days, ST2D cultures were lysed with Trizol (Invitrogen, United States) for RNA. In a separate experiment RNA was harvested from ST2D cells cultured after stimulation with 350 ng/mL doxycycline at 24, 48, and 72 h.

Expression Analysis via qRT-PCR

RNA was harvested from whole bone lysate, stromal cultures, or ST2D cell culture using Trizol (Invitrogen, United States). Expression levels of select differentially expressed genes and others of interest were performed *via* qRT-PCR as previously described (Wheat et al., 2014). Briefly, the RNA was reversed transcribed using the M-MLV Reverse transcriptase kit

(Invitrogen, United States), followed by the quantitative analysis using either SYBR Green system (Bio-Rad, United States) or predesigned TaqMan Gene Expression Assay (Applied Biosystems, United States). Primer sequences for SYBR Green assays are listed in **Supplementary Table 1**. *Tle4* expression was assayed using the TaqMan Gene Expression Assay (*Tle4*: Mm01196934). The expression levels of genes of interest were normalized to the expression levels of the 18S housekeeping gene.

Mineral Apposition Rate Assay

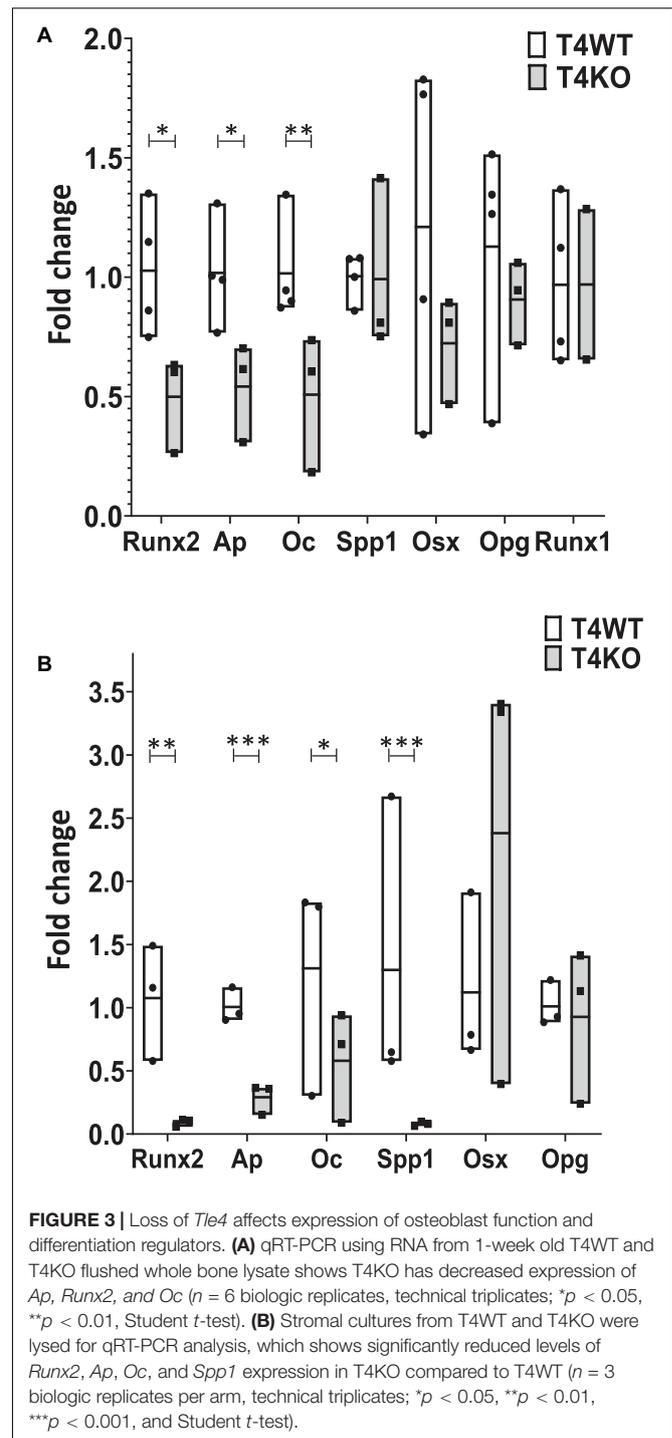
For mineral apposition assay, 2-month old T4F and T4F cre littermates were irradiated with two doses of 450cGy and subsequently transplanted with 1×10^4 Lineage- c-Kit + Sca-1 + (LKS) cells from 2-month old wild-type C57BL/6 mice *via* tail vein injection. These transplanted wild-type LKS cells were isolated using a FACS Aria (BD, United States). Eight weeks after transplant, *Tle4* excision was induced by three pIpC (Sigma) intraperitoneal injections at a dose of 15 mg/kg every 48 h. Four weeks after *Tle4* excision, recipient mice were pulsed with 20 mg/kg calcein (Sigma- Aldrich, United States) *via* intraperitoneal injection. After 1 week, recipients were given 30 mg/kg demeclocycline (Sigma- Aldrich, United States) *via* intraperitoneal injection. Femurs were harvested 3 days after demeclocycline injection and fixed in 70% ethanol. Femurs were processed for resin embedding for mineral apposition rate analysis and for immunohistochemistry staining of Runx2 (PA5-86506, ThermoFisher Scientific, Waltham, MA, United States), Oc (MBS2003553, MyBiosource, San Diego, CA, United States) and β -catenin (ab6302, Abcam, Cambridge, United Kingdom) (Bouxsein et al., 2010).

MicroCT Bone Analysis

Microcomputed tomographic analysis (microCT) was performed on a subset of lumbar vertebrae. The femora were scanned at a resolution of 6 μ m using a Scanco-35 microCT (Scanco United States, Inc., Southeastern, PA, United States). Each scan included a phantom containing air, saline and a bone reference material (1.18 g/cm³) for conversion of Hounsfield units to mineral density in g/cm³. Reconstruction of the individual projections to computed tomography volume data was performed using instrument software. Specimen-specific thresholds were determined by first selecting a volume of interest, generating the attenuation histogram, and determining the threshold that segments mineralized tissue from background. Properties determined included medullary bone mineral density (BMD), cortical bone thickness, AP distance, vertebral pedicle length, and trabecular bone volume fraction (trabecular bone volume to total volume ratio, BV/TV) (Bouxsein et al., 2010).

Statistics

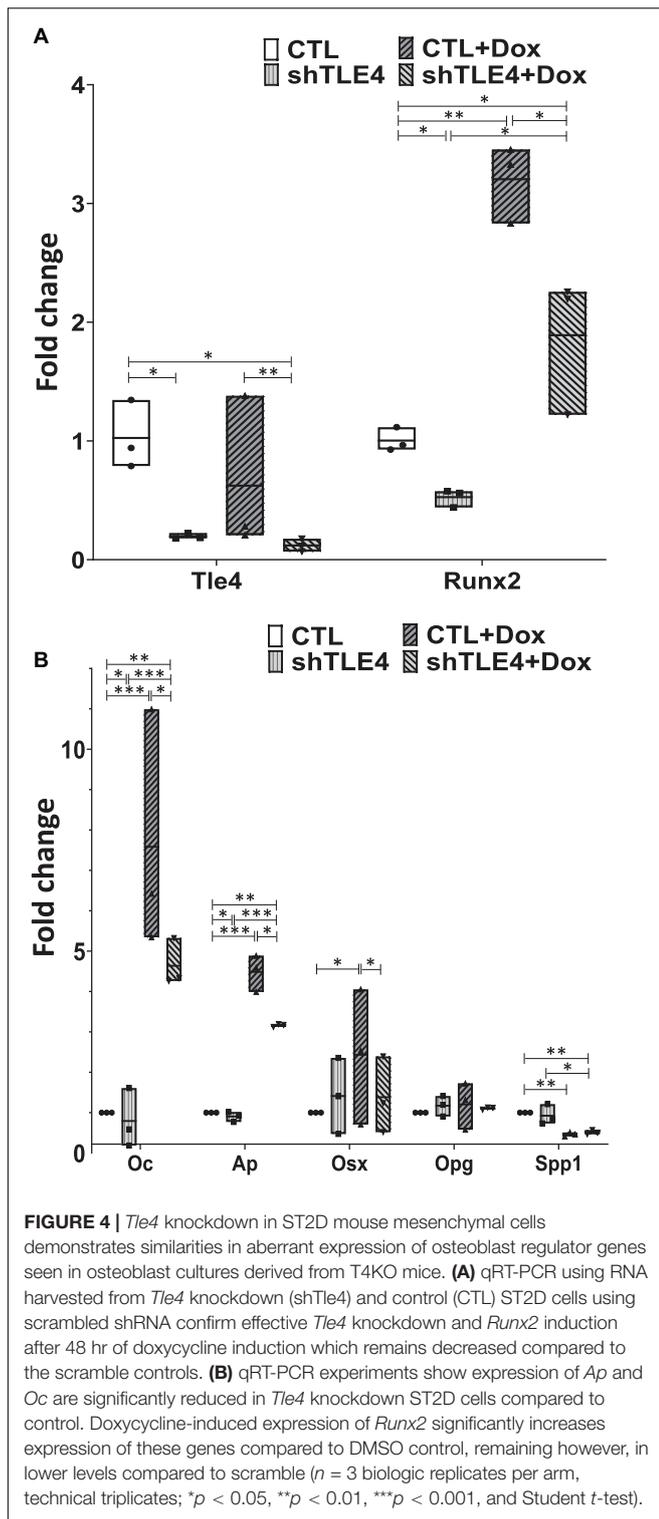
Analyses used student's unpaired *t*-test with Graphpad Prism for comparing two genotypes (Graph-pad Software, La Jolla, CA, United States). Data are presented as floating bars showing the minimum to maximum values or scatter plots, and values of $p < 0.05$ are considered statistically significant.



RESULTS

Loss of *Tle4* Leads to Defective Bone Development

To assess bone formation in *Tle4* null mice, we performed microCT analysis of lumbar vertebrae of 3-day old T4KO and wild-type (T4WT) littermates. T4KO mice exhibit decreased



trabecular bone volume fraction (BV/TV) and AP distance measurements compared to T4WT counterparts, suggesting loss of *Tle4* is associated with decreased bone density and vertebral pedicle length (Figures 1A,B). Additionally, femurs of 8 to 9-day-old T4KO and T4WT littermates were harvested for

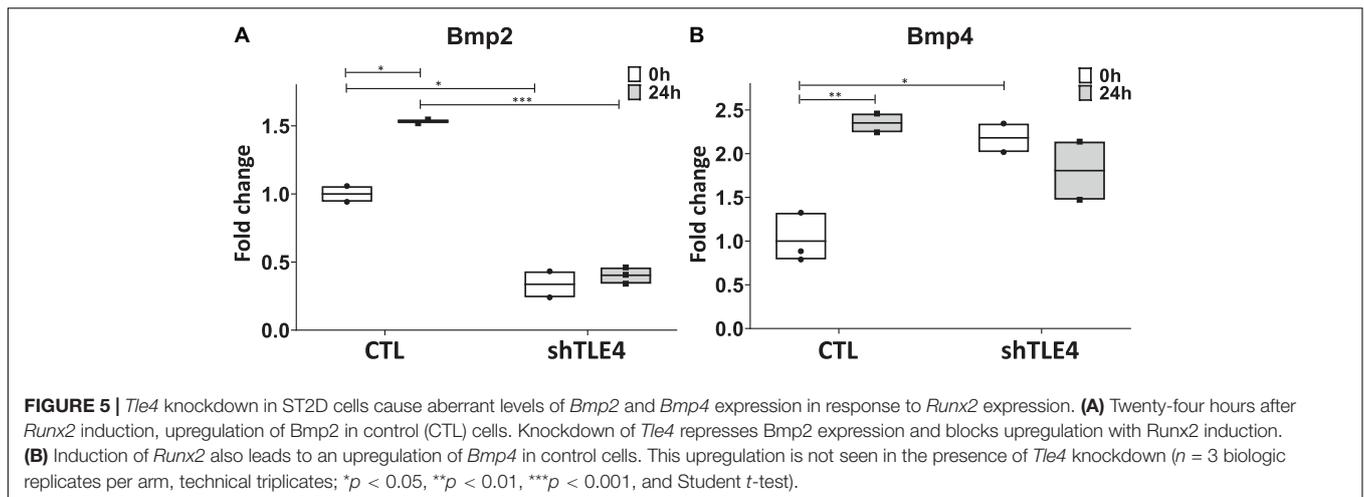
microCT analysis of medullary and cortical bone (Figures 1C,D). This analysis showed T4KO mice have reduced medullary bone density (M.BV/TV) and cortical bone thickness (Ct.Th). Moreover, microCT image reconstructions of trabecular bone in L3 vertebrae of 3 to 4-week old T4KO and T4WT littermates further illustrate decreased bone calcification and increased trabecular space in T4KO vertebrae compared to those of T4WT (Figure 1E). This is consistent with previous findings that loss of *Tle4* is associated with deficits in vertebral and long bone formation.

Dynamic Bone Formation and Osteoblast Deficiencies in Conditional *Tle4* Knockout Mouse

To further characterize the effects of *Tle4* loss on bone development, we pursued a dynamic double-label mineral apposition rate (Calvi et al., 2003) assay using mice that have *loxP* target sites flanking exon 2 of *Tle4* with or without Cre recombinase driven by the *Mx1* promoter (T4F cre and T4E, respectively). In order to isolate the effects of *Tle4* loss to bone marrow mesenchymal cells we replaced the bone marrow hematopoietic cells with wild type cells by bone marrow transplantation prior to knockout of *Tle4* by pIpC in these *Mx1*-Cre expressing mice. The high degree of knockout efficiency of exon 2 of *Tle4* in these conditional T4F cre mice was demonstrated in harvested bone marrow of similarly pIpC treated T4F-cre compared to T4F mice lacking *Mx1*-cre (Supplementary Figure 1). The MAR assay revealed T4F cre mice have multiple lower dynamic parameters of bone formation (Figures 2A–F). While ratios of mineralizing to bone surface areas (MS/BS) were similar, MAR and bone formation rates (BFR) were lower in T4F cre mice compared to their control T4F counterparts (Figures 2A–C). Additionally, T4F cre mice had lower numbers of osteoblasts per given bone perimeter area (Figure 2D). Combined with lower osteoid to bone surface area ratios (Figure 2E), these results point toward an association between decreased bone formation and conditional loss of *Tle4* in adult mice.

Bone Defects Due to *Tle4* Loss May Be Mediated Through Dysregulation of Canonical Regulators of Bone Development

Given the defective bone and bone marrow phenotype seen in T4KO mice, we hypothesized that loss of *Tle4* may affect osteoblast function and development. To assess this, we first harvested RNA from flushed whole bone lysates of 1-week-old T4KO and T4WT littermates. Expression analysis, using qPCR, revealed T4KO bone had significantly lower levels of *Ap*, *Runx2*, and *Oc* expression (Figure 3A). *Ap* is often used as a proxy for osteoblast function while *Runx2* and *Oc* have previously been connected to osteoblast maturation and bone anabolic regulation (Jang et al., 2012). To minimize bone cell heterogeneity, crushed 1-week-old T4KO and T4WT femurs were cultured in osteogenic media to generate osteoblast stromal cultures. T4KO stromal cultures demonstrated significant decreases in osteoblast genes,



including *Runx2*, *Ap*, *Oc*, and *Spp1* (Figure 3B). Moreover, alkaline phosphatase staining qualitatively showed a trend toward decreased alkaline phosphatase activity in T4KO stromal cells (Supplementary Figure 2).

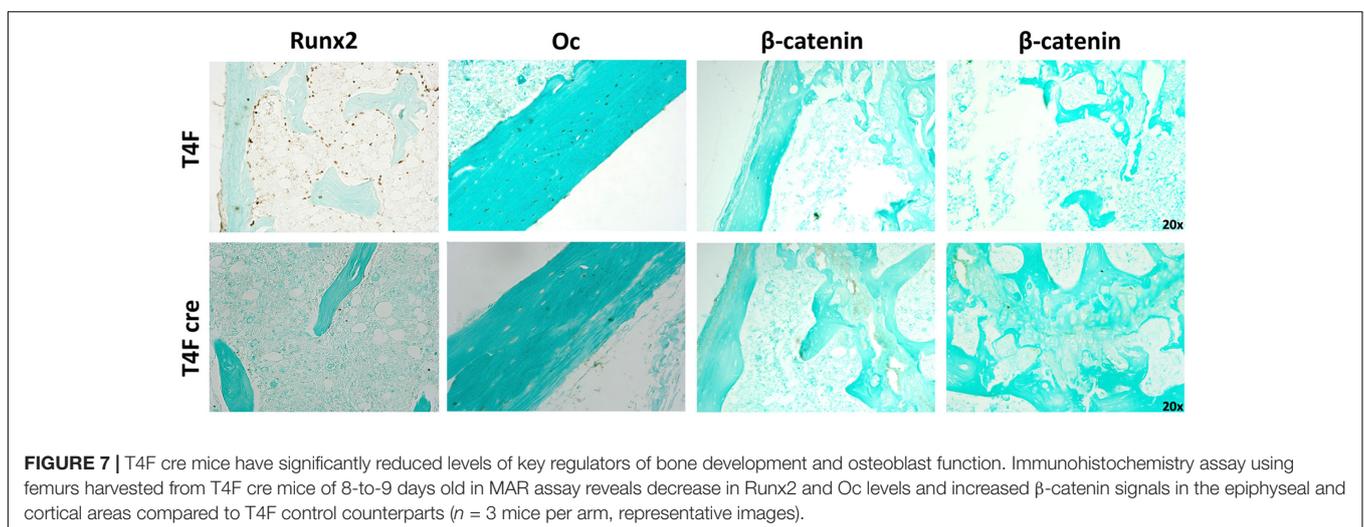
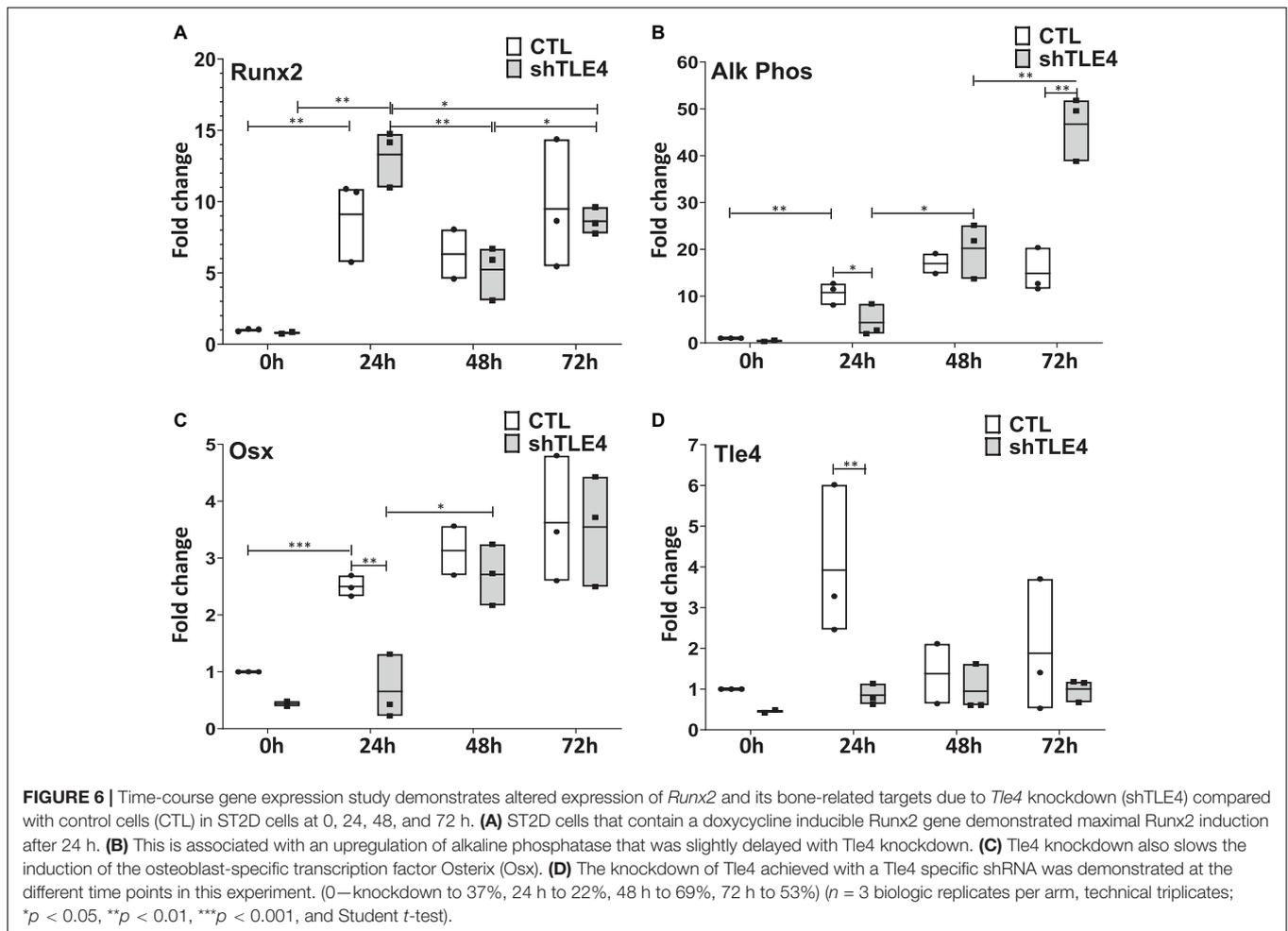
Our T4KO results revealed that absence of *Tle4* was associated with significant reductions of *Runx2*, suggesting T4KO-associated bone abnormalities might be due to decreased *Runx2* expression. To further elucidate the time course of *Tle4*-mediated effects on bone development factors through the Runx axis and considering the recent data on the Runx1 involvement in bone development (Tang et al., 2020), we assessed the expression levels of *Runx1* in the 1-week-old T4KO and T4WT littermates, and found that the absence of *Tle4* does not affect the expression levels of *Runx1* (Figure 3A) suggesting the calcification defects in *Tle4* KO mice are more likely explained by *Runx2* inhibition.

To better understand the mechanisms underlying *Tle4* effect on bone development and the interplay with *Runx2*, we turn to an *in vitro* system using mouse mesenchymal ST2 cells stably transformed with a doxycycline-inducible *Runx2* vector (ST2D) (Dayyani et al., 2008; Baniwal et al., 2012). In these ST2D cells the addition of doxycycline leads to an induction of *Runx2* expression (Figure 4A). In this system we evaluated the effects of knocking down *Tle4* expression via lentiviral delivery of *Tle4*-specific shRNA. Expression analysis using qPCR demonstrated over 80% reduction in *Tle4* message via shRNA in both doxycycline and DMSO cultures and showed that ectopic expression of *Runx2* in the absence of *Tle4* shRNA did not significantly affect *Tle4* expression levels (Figure 4A). In this system we demonstrated *Tle4* knockdown reduced endogenous *Runx2* expression levels by approximately 50% in ST2D cells cultured in control DMSO media. This result correlated nicely with that found *in vivo*. The addition of doxycycline significantly increased *Runx2* expression levels. *Tle4* knockdown via shRNA was not able to prevent this increase driven from a lentiviral promoter, though the increase was blunted, likely reflecting repression of *Runx2* from the endogenous promoter. Induction of *Runx2* by doxycycline resulted in a significant increase in the expression of *Oc*, *Ap*, and *Osx* after 48 h, and this increase was significantly

decreased in *Tle4* knockdown arms (Figure 4B). To further understand the relationship between *Tle4* and *Runx2*, we queried expression levels of *Bmp2* and *Bmp4*, given their previously described roles as regulators of *Runx2* and normal skeletal development (Bandyopadhyay et al., 2006; Krishnan et al., 2006). We demonstrated that loss of *Tle4* caused a significant reduction of *Bmp2* expression at baseline and prevented upregulation in response to *Runx2* induction. Interestingly, levels of *Bmp4* were increased with *Tle4* knockdown, but the upregulation seen with *Runx2* expression was blocked (Figure 5).

Extended time-course experiments demonstrate that *Tle4* knockdown creates an initial surge of *Runx2* expression by 24 h of knockdown compared to control, followed by decreased *Runx2* levels at 48 and 72 h; consistent with the above observations (Figure 6). However, we found decreased expression levels of *Runx2*-mediated regulators of bone, including *Alp* and *Osx* at 24 h. This suggests the loss of *Tle4* blunted the ability of *Runx2* to upregulate *Osx* and *Alp* expression at early time points. By 72 h after *Tle4* knockdown, the differential expression of these genes is lost, suggesting a time-delay of *Alp* and *Osx* induction due to *Tle4* knockdown. These experiments support a role of *Tle4* in regulating *Runx2* and, subsequently, its target genes canonically associated with osteoblast differentiation and function.

Immunohistochemistry using femurs from mice used in MAR assay revealed decreased amounts of *Runx2* and Osteocalcin in T4F cre mice compared to control (Figure 7). In T4F mice, *Runx2* positivity can be seen in numerous osteoid-lining cells, suggestive of *Runx2*-positive periosteal osteoblasts. The frequency and localization of these *Runx2*-positive cells dramatically drops in T4F cre mice. Similarly, osteocalcin positivity in cortical bone seen in T4F mice is not evident in the conditional *Tle4* knockout mice. In addition to *Runx2*, β -catenin-mediated Wnt signaling has also been previously described as an important mediator of bone development and osteoblast differentiation (Hill et al., 2005; Cohen, 2006; Rodda and McMahon, 2006; Kook et al., 2015). Given previous reports implicating *Tle4* as a negative regulator of Wnt signaling, we queried β -catenin levels via immunohistochemistry in bones of the T4F and T4F



cre mice used in the above MAR assay as an exploratory objective. T4F cre femurs showed increased β -catenin signals in the epiphyseal and cortical areas. While T4F cre and T4KO mice display bone calcification and osteoblast function defects,

loss of *Tle4* in bone leads to increased β -catenin levels, which is consistent with previous reports implicating *Tle4* as a repressor of Wnt signaling. However, using the above-described ST2D system, we were not able to find a *Tle4* knockdown-dependent

change in expression of canonical *Wnt* target gene expression (data not shown).

DISCUSSION

The Groucho/TLE family of proteins has been extensively studied in *Drosophila* where it has been termed a master regulatory gene in development *via* its interaction with a number of important signaling pathways including Notch and Wnt and also can be recruited by transcription factors members of Hex, Runx, Nkx, Lef1/Tcf, Pax, Six and c-myc (Jennings and Ish-Horowicz, 2008; Agarwal et al., 2015). Our understanding of the roles of this protein family in vertebrate development is limited. The novel *Tle4* null mouse provides valuable insight into the previously unappreciated roles of *Tle4* in mammalian vertebrates regarding bone maturation, medullary hematopoiesis and HSPC maintenance. One of the striking abnormalities in the T4KO mice is a decreased calcification of the skeleton (Wheat et al., 2014). This impaired ossification is apparent in both membranous and endochondral bones by birth. The above phenotypes are more intense and progressive in an age-dependent manner in T4KO mice than in *Grg5* null mice lacking a truncated member of the Groucho/TLE family (Wheat et al., 2014). Our earlier characterization of *Tle4* KO mice suggested *Tle4* also affects osteoclast function as demonstrated by an increase in osteoclasts by tartrate-resistance acid phosphatase (TRAP) staining (Wheat et al., 2014). In the current study microCT and MAR assay measurements indicate loss of *Tle4* impaired bone formation, calcification, and osteoid production. The lethality of *Tle4* null mice and the effects in hematopoietic cells and observed degradation of the bone marrow niche made it technically challenging to isolate the effect on osteoblasts in germline knockout mice. Conditional *Tle4* mice transplanted with normal bone marrow hematopoietic cells served as a proxy for osteoblast-specific effects of *Tle4* loss. *Mx1*-cre system has been demonstrated as one of the most commonly “deleter strain” in experimental hematology (Velasco-Hernandez et al., 2016). Previous work by Park et al. (2012) have demonstrated the contributory role of *Mx1*-expressing bone mesenchymal cells toward the generation of new osteoblasts responsible for new bone formation, supporting the use of the *Mx1*-Cre model in our experiments to examine the role of *Tle4* loss in osteoblast function (Park et al., 2012). The concordance of observations seen in these models and studies strongly suggest that *Tle4* may affect osteoblasts and other periosteal cells that are responsible for bone production and maintenance.

In the context of our previous work identifying hematopoietic defects in *Tle4* null mice, we have attempted to determine whether the bone abnormalities due to *Tle4* loss can be attributable to dysfunctional osteoblasts. We had shown *Tle4* loss significantly impairs LSK differentiation into granulocyte, monocyte, macrophage progenitors and LSK self-renewal and adversely affects the integrity of bone marrow niche and stroma (Wheat et al., 2014). Osteoblasts are known to play a critical role in maintaining the bone marrow niche (Calvi et al., 2003; Asada et al., 2013; Fulzele et al., 2013;

Even et al., 2021). In this current work qRT-PCR analysis using T4KO mouse samples from flushed whole bones and cultured stromal cells revealed significantly decreased expression of various transcription factors and regulators responsible for osteoblast function and differentiation, including *Ap* and *Oc*—both frequently used proxies for describing osteoblast function. Interestingly, osteocalcin is one of the main components of ground substance that, together with Type 1 collagen, constitute the bone matrix (Hill et al., 2005; Asada et al., 2013; Fulzele et al., 2013). *Osx* demonstrates a multifunctional role on osteoblast differentiation, growth and homeostasis, since its deletion in several time points postnatally in growing and adult bones causes defects in maturation, morphology and function of osteocytes (Zhou et al., 2010; Liu et al., 2020). Decreased *Oc* expression may lend insight into a physiologic basis for the *Tle4* knockout-induced bone phenotype.

Wnt signaling has been described as a central mediator of bone formation (Hill et al., 2005; Cohen, 2006; Rodda and McMahon, 2006; Kook et al., 2015). Surprisingly, we observed defective bone formation and calcification in T4KO mice, in which Wnt signaling is expected to be activated especially given higher levels of β -catenin signal as determined by immunohistochemistry (Kronenberg, 2003; Chodaparambil et al., 2014). However, previous studies have shown that there is an intimate relationship between the timing of Wnt signaling and normal osteoblast differentiation; and thus, constitutive or increased Wnt signaling at an inappropriate stage of osteoblast development may be detrimental to normal bone growth (Rodda and McMahon, 2006; Janeway and Walkley, 2010). Alternatively, the detrimental effects of *Tle4* loss on Runx2 activity might outweigh the effects of Wnt activation on osteoblast differentiation and calcification. Our results do not preclude the possibility that loss of *Tle4* is responsible for the bone defects through other mechanisms and the potential effects of paracrine hormonal or *Tle4* levels in other non-*Mx1*-expressing compartments.

The ST2D system provided insight into potential molecular mechanisms that may explain our findings, including not only Wnt signaling but also dysregulation of Bmp signaling and consequently *Runx2* expression. Previous studies have demonstrated loss of function of *Bmp2* and *Bmp4* impair bone condensation and skeletal development (Bandyopadhyay et al., 2006; Krishnan et al., 2006; Wu et al., 2016). There is an interplay between BMP and RUNX2 in regulating osteoblast differentiation (Lowery and Rosen, 2018). BMP signaling is required for transcriptional activity of Runx2 and Runx2 enhances the sensitivity of cells to BMPs (Phimphilai et al., 2006).

Our experiments indicate there is a decrease in *Runx2* expression in T4KO bone, T4KO stromal cells, and in ST2D cells with *Tle4* knockdown in a time dependent manner. Previous studies have shown that TLE proteins are capable of interacting with Runx2, a critical regulator of bone development and maturation (McLarren et al., 2000; Choi et al., 2001; Kaul et al., 2015). The RUNX protein family is known to form co-repressor complexes with TLE proteins (Javed et al., 2000). Thus, the TLE proteins might affect both *Runx2* expression as well as the activity of the Runx2 protein. The TLE effect on *Runx2* expression could reflect interference of Runx2 transcriptional

autoregulation (Drissi et al., 2000). In our studies, even if the experiments do not distinguish whether the blunting in the expression of these osteoblast genes with *Tle4* knockdown is due to decreased *Runx2* expression or decreased function of the Runx2 protein in the absence of *Tle4*, the differential expression levels of *Runx2*-mediated regulators of bone development are most likely a downstream effect consequent of the decreased endogenous *Runx2* expression after *Tle4* knockdown. *Runx2* null mice demonstrate bone phenotypes similar, but more severe than our T4KO mouse: expiring at birth and completely missing skeletal and bone development, owing to defective osteoblast maturation (Komori et al., 1997; Otto et al., 1997; Okura et al., 2014). Previous studies have shown the importance of *Runx2* in normal bone development, as *Runx2* null mice lack bone ossification and osteoblast differentiation (Cohen, 2006). The similarity of *Tle4* null mice to *Runx2* null mice suggested the loss of *Tle4* might either impair the function or the expression of *Runx2*. The less severe effect observed with *Tle4* knockout could reflect complementary effects from the expression of other *Tle* family members along with *Runx2* residual expression which thus induce the osteoblastic genes expression later during bone development in the *Tle4* loss background, in contrast to the early in time severe effects of *Tle4* loss in bone development. Additional studies are required to further characterize the interaction and potential regulatory role of *Tle4* on *Runx2* expression levels and function as it may relate to the defective bone development in the absence of *Tle4*. While direct functional interactions between RUNX and TLE have been described (Westendorf, 2006) and a possibility of the requirement of such a direct active interaction in the early osteoblastic development is suggested in our time dependent experiments, additional experiments may reveal further insight into whether TLE exerts a direct regulatory effect on RUNX transcription, stability or targets.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Guide for the Care and Use of Laboratory Animals of the National Institutes

REFERENCES

- Agarwal, M., Kumar, P., and Mathew, S. J. (2015). The Grp78/Transducin-like enhancer of split protein family in animal development. *IUBMB Life* 71: 1824.
- Aguila, H., and Rowe, D. (2005). Skeletal development, bone remodeling, and hematopoiesis. *Immunol. Rev.* 208, 7–18. doi: 10.1111/j.0105-2896.2005.00333.x
- Asada, N., and Katayama, Y. (2014). Regulation of hematopoiesis in endosteal microenvironments. *Int. J. Hematol.* 99, 679–684. doi: 10.1007/s12185-014-1583-1

of Health and approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

DS, TS, and ET: study conception and design, writing manuscript, and study supervision. TS, ET, CH, RY, CR, and PG: study conduct and data acquisition. TS, ET, CR, PG, and DS: methodology development and data analysis and interpretation. ET and DS: revising manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported in part by the National Institutes of Health Grant R01 CA115772 (DS), Swim Across America (TS and DS), The Hill Family Fund for the Diagnosis and Management of Rare and Undiagnosed Diseases at Mass General (DS and ET), the Eunice Kennedy Shriver National Institute of Child Health and Human Development of the National Institutes of Health under Award Number R03HD099516 (PG), and The Malika Ray, Asok K. Ray, M.D., FRCS/(Edin) Initiative for Child Health Research (PG).

ACKNOWLEDGMENTS

The authors would like to thank Baruch Frenkel and Jiali Yu for generous gift of ST2D cells and scientific discourse. We would also like to thank Nicolas Severe, Janaina Da Silva, and Marie Demay for guidance with mineral apposition rate assay and bone histology. We would also further like to thank Meredith Weglarz, Amy Galvin, and Maris Handley for assistance with flow cytometry and cell sorting. The authors would also like to thank Joanna Yeh, Yiyun Zhang, Matthew Jones, George Murphy, and Bob Varelas for their guidance, comments, and discourse. The authors gratefully acknowledge the support of the Malika Ray, Asok K. Ray, FRCS/(Edin) Initiative for Child Health.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2021.671029/full#supplementary-material>

- Asada, N., Katayama, Y., Sato, M., Minagawa, K., Wakahashi, K., Kawano, H., et al. (2013). Matrix-embedded osteocytes regulate mobilization of hematopoietic stem/progenitor cells. *Cell Stem Cell* 12, 737–747. doi: 10.1016/j.stem.2013.05.001
- Bandyopadhyay, A., Tsuji, K., Cox, K., Harfe, B. D., Rosen, V., and Tabin, C. J. (2006). Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis. *PLoS Genet.* 2:e216. doi: 10.1371/journal.pgen.0020216
- Baniwal, S. K., Shah, P. K., Shi, Y., Haduong, J. H., Declerck, Y. A., Gabet, Y., et al. (2012). Runx2 promotes both osteoblastogenesis and novel osteoclastogenic signals in ST2 mesenchymal progenitor

- cells. *Osteoporos. Int.* 23, 1399–1413. doi: 10.1007/s00198-011-1728-5
- Bianco, P. (2011). Bone and hematopoietic niche: tale of two stem cells. *Blood* 117, 5281–5288. doi: 10.1182/blood-2011-01-315069
- Bouxsein, M. L., Boyd, S. K., Christiansen, B. A., Guldberg, R. E., Jepsen, K. J., and Müller, R. (2010). Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J. Bone Miner. Res.* 25, 1468–1486. doi: 10.1002/jbmr.141
- Calvi, L., Adams, G., Weibrecht, K., Weber, J. M., Olson, D. P., Knight, M. C., et al. (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* 425, 841–845. doi: 10.1038/nature02040
- Chen, Q., and Courey, A. (2000). Groucho/TLE family proteins and transcriptional repression. *Genes Chromosomes Cancer* 29, 1–16. doi: 10.1016/s0378-1119(00)0161-x
- Chodaparambil, J. V., Pate, K. T., Hepler, M. R., Tsai, B. P., Muthurajan, U. M., Luger, K., et al. (2014). Molecular functions of the TLE tetramerization domain in Wnt target gene repression. *EMBO J.* 33, 719–731. doi: 10.1002/embj.201387188
- Choi, J. Y., Pratap, J., Javed, A., Zaidi, S. K., Xing, L., Balint, E., et al. (2001). Subnuclear targeting of Runx/Cbfa/AML factors is essential for tissue-specific differentiation during embryonic development. *Proc. Natl. Acad. Sci. U.S.A.* 98, 8650–8655. doi: 10.1073/pnas.151236498
- Cohen, M. M. Jr. (2006). The new bone biology: pathologic, molecular, and clinical correlates. *Am. J. Med. Genet. A* 140, 2646–2706. doi: 10.1002/ajmg.a.31368
- Dayyani, F., Wang, J., Yeh, J. R., Ahn, E. Y., Tobey, E., Zhang, D. E., et al. (2008). Loss of TLE1 and TLE4 from the del(9q) commonly deleted region in AML cooperates with AML1-ETO to affect myeloid cell proliferation and survival. *Blood* 111, 4338–4347. doi: 10.1182/blood-2007-07-103291
- Despars, G., and St-Pierre, Y. (2011). Bidirectional interactions between bone metabolism and hematopoiesis. *Exp. Hematol.* 39, 809–816. doi: 10.1016/j.exphem.2011.04.008
- Drissi, H., Luc, Q., Shakoori, R., Chuva De Sousa Lopes, S., Choi, J. Y., Terry, A., et al. (2000). Transcriptional autoregulation of the bone related Cbfa1/RUNX2 gene. *J. Cell. Physiol.* 184, 341–350. doi: 10.1002/1097-4652(200009)184:3<341::aid-jcp8>3.0.co;2-z
- Even, J., Yi, L., Chang, C. K., and Rossi, F. M. V. (2021). The parathyroid hormone-dependent activation of osteoblasts enhances hematopoietic stem cell migration and reduces their engraftment abilities. *bioRxiv* [Preprint]. doi: 10.1101/2021.03.04.433901
- Fulzele, K., Krause, D. S., Panaroni, C., Saini, V., Barry, K. J., Liu, X., et al. (2013). Myelopoiesis is regulated by osteocytes through Gsalpha-dependent signaling. *Blood* 121, 930–939. doi: 10.1182/blood-2012-06-437160
- Galan-Diez, M., and Kousteni, S. (2018). A bone marrow niche-derived molecular switch between osteogenesis and hematopoiesis. *Genes Dev.* 32, 324–326. doi: 10.1101/gad.314013.118
- García-García, A., de Castillejo, C. L., and Mendez-Ferrer, S. (2015). BMSCs and hematopoiesis. *Immunol. Lett.* 168, 129–135. doi: 10.1016/j.imlet.2015.06.020
- Hill, T. P., Später, D., Taketo, M. M., Birchmeier, W., and Hartmann, C. (2005). Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes. *Dev. Cell* 8, 727–738. doi: 10.1016/j.devcel.2005.02.013
- Houschyar, K. S., Tapking, C., Borelli, M. R., Popp, D., Duscher, D., Maan, Z. N., et al. (2019). Wnt pathway in bone repair and regeneration-what do we know so far. *Front. Cell Dev. Biol.* 6:170. doi: 10.3389/fcell.2018.00170
- Janeway, K. A., and Walkley, C. R. (2010). Modeling human osteosarcoma in the mouse: from bedside to bench. *Bone* 47, 859–865. doi: 10.1016/j.bone.2010.07.028
- Jang, W. G., Kim, E. J., Kim, D. K., Ryoo, H. M., Lee, K. B., Kim, S. H., et al. (2012). BMP2 protein regulates osteocalcin expression via Runx2-mediated Atf6 gene transcription. *J. Biol. Chem.* 287, 905–915. doi: 10.1074/jbc.m111.253187
- Javed, A., Guo, B., Hiebert, S., Choi, J. Y., Green, J., Zhao, S. C., et al. (2000). Groucho/TLE/R-esp proteins associate with the nuclear matrix and repress RUNX (Cbfa/AML/PEBP2a) dependent activation of tissue-specific gene transcription. *J. Cell Sci.* 113, 2221–2231. doi: 10.1242/jcs.113.12.2221
- Jennings, B. H., and Ish-Horowicz, D. (2008). The Groucho/TLE/Grg family of transcriptional co-repressors. *Genome Biol.* 9:205. doi: 10.1186/gb-2008-9-1-205
- Kaul, H., Hall, B. K., Newby, C., and Ventikos, Y. (2015). Synergistic activity of polarised osteoblasts inside condensations cause their differentiation. *Sci. Rep.* 5:11838. doi: 10.1038/srep11838
- Kode, A., Manavalan, J. S., Mosialou, I., Bhagat, G., Rathinam, C. V., Luo, N., et al. (2014). Leukaemogenesis induced by an activating beta-catenin mutation in osteoblasts. *Nature* 506, 240–244. doi: 10.1038/nature12883
- Komori, T. (2019). Regulation of proliferation, differentiation and functions of osteoblasts by Runx2. *Int. J. Mol. Sci.* 20:1694. doi: 10.3390/ijms20071694
- Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., et al. (1997). Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 89, 755–764. doi: 10.1016/s0092-8674(00)80258-5
- Kook, S. H., Heo, J. S., and Lee, J. C. (2015). Crucial roles of canonical Runx2-dependent pathway on Wnt1-induced osteoblastic differentiation of human periodontal ligament fibroblasts. *Mol. Cell. Biochem.* 402, 213–223. doi: 10.1007/s11010-015-2329-y
- Kozhemyakina, E., Lassar, A., and Zelzer, E. (2015). A pathway to bone: signaling molecules and transcription factors involved in chondrocyte development and maturation. *Development* 142, 817–831. doi: 10.1242/dev.105536
- Krishnan, V., Bryant, H. U., and Macdougald, O. A. (2006). Regulation of bone mass by Wnt signaling. *J. Clin. Invest.* 116, 1202–1209. doi: 10.1172/jci28551
- Kronenberg, H. (2003). Developmental regulation of the growth plate. *Nature* 423, 332–335. doi: 10.1038/nature01657
- Le, P. M., Andreeff, M., and Battula, V. L. (2018). Osteogenic niche in the regulation of normal hematopoiesis and leukemogenesis. *Haematologica* 103, 1945–1955. doi: 10.3324/haematol.2018.197004
- Levanon, D., Goldstein, R., Bernstein, Y., Tang, H., Goldenberg, D., Stifani, S., et al. (1998). Transcriptional repression by AML1 and FEF-1 is mediated by the TLE/Groucho corepressors. *Proc. Natl. Acad. Sci. U.S.A.* 95, 11590–11595. doi: 10.1073/pnas.95.20.11590
- Liu, Q., Li, M., Wang, S., Xiao, Z., Xiong, Y., and Wang, G. (2020). Recent advances of osterix transcription factor in osteoblast differentiation and bone formation. *Front. Cell Dev. Biol.* 8:601224. doi: 10.3389/fcell.2020.601224
- Lowery, J., and Rosen, V. (2018). The BMP pathway and its inhibitor in the skeleton. *Physiol. Rev.* 98, 2431–2452. doi: 10.1152/physrev.00028.2017
- Ma, X., Liu, Y., Liu, Y., Alexandrov, L. B., Edmonson, M. N., Gawad, C., et al. (2018). Pan-cancer genome and transcriptome analyses of 1,699 paediatric leukaemias and solid tumours. *Nature* 555, 371–376. doi: 10.1038/nature25795
- McLarren, K., Lo, R., Grbavec, D., Thirunavukkarasu, K., Karsenty, G., and Stifani, S. (2000). The mammalian basic helix loop helix protein HES-1 binds to and modulates the transactivating function of the Runx-related factor Cbfa1. *J. Biol. Chem.* 275, 530–538. doi: 10.1074/jbc.275.1.530
- Mukherjee, S., Raju, N., Schoonmaker, J. A., Liu, J. C., Hideshima, T., Wein, M. N., et al. (2008). Pharmacologic targeting of a stem/progenitor population *in vivo* is associated with enhanced bone regeneration in mice. *J. Clin. Invest.* 118, 491–504.
- Okura, H., Sato, S., Kishikawa, S., Kaneto, S., Nakashima, T., Yoshida, N., et al. (2014). Runx2-I isoform contributes to fetal bone formation even in the absence of specific N-terminal amino acids. *PLoS One* 9:e108294. doi: 10.1371/journal.pone.0108294
- Otto, F., Thornell, A., Crompton, T., Denzel, A., Gilmour, K. C., Rosewell, I. R., et al. (1997). Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell Stem Cell* 89, 765–771. doi: 10.1016/s0092-8674(00)80259-7
- Park, D., Spencer, J. A., Koh, B. I., Kobayashi, T., Fujisaki, J., Clemens, T. L., et al. (2012). Endogenous bone marrow MSCs are dynamic, fate-restricted participants in bone maintenance and regeneration. *Cell Stem Cell* 10, 259–272. doi: 10.1016/j.stem.2012.02.003
- Phiphilai, M., Zhao, Z., Boules, H., Roca, H., and Franceschi, R. T. (2006). BMP signaling is required for RUNX2-dependent induction of the osteoblast phenotype. *J. Bone Miner. Res.* 21, 637–646. doi: 10.1359/jbmr.060109
- Rauch, D. A., Hurchla, M. A., Harding, J. C., Deng, H., Shea, L. K., Eagleton, M. C., et al. (2010). The ARF tumor suppressor regulates bone remodeling and osteosarcoma development in mice. *PLoS One* 5:e15755. doi: 10.1371/journal.pone.0015755

- Rodda, S. J., and McMahon, A. P. (2006). Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development* 133, 3231–3244. doi: 10.1242/dev.02480
- Schmitt-Ney, M. (2020). The FOXO's advantages of being a family: considerations on function and evolution. *Cells* 9:787. doi: 10.3390/cells9030787
- Sweetser, D. A., Peniket, A. J., Haaland, C., Blomberg, A. A., Zhang, Y., Zaidi, S. T., et al. (2005). Delineation of the minimal commonly deleted segment and identification of candidate tumor-suppressor genes in del(9q) acute myeloid leukemia. *Genes Chromosomes Cancer* 44, 279–291. doi: 10.1002/gcc.20236
- Tang, J., Xie, J., Chen, W., Tang, C., Wu, J., Wang, Y., et al. (2020). Runt-related transcription factor 1 is required for murine osteoblast differentiation and bone formation. *J. Biol. Chem.* 295, 11669–11681. doi: 10.1074/jbc.ra119.007896
- Velasco-Hernandez, T., Sawen, P., Bryder, D., and Cammenga, J. (2016). Potential pitfalls of the Mx1-cre system: implications for experimental modeling of normal and malignant hematopoiesis. *Stem Cell Rep.* 7, 11–18. doi: 10.1016/j.stemcr.2016.06.002
- Visnjic, D., Kalajzic, Z., Rowe, D. W., Katavic, V., Lorenzo, J., and Aguila, H. L. (2004). Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood* 103, 3258–3264. doi: 10.1182/blood-2003-11-4011
- Westendorf, J. J. (2006). Transcriptional co-repressors of Runx2. *J. Cell. Biochem.* 98, 54–64. doi: 10.1002/jcb.20805
- Wheat, J. C., Krause, D. S., Shin, T. H., Chen, X., Wang, J., Ding, D., et al. (2014). The corepressor Tle4 is a novel regulator of murine hematopoiesis and bone development. *PLoS One* 9:e105557. doi: 10.1371/journal.pone.0105557
- Wu, M., Chen, G., and Li, Y. P. (2016). TGF- β and BMP signaling in osteoblast, skeletal development, and bone formation, homeostasis and disease. *Bone Res.* 4:16009.
- Yin, T., and Li, L. (2006). The stem cell niches in bone. *J. Clin. Invest.* 116, 1195–1201.
- Zhang, P., and Dressler, G. R. (2013). The Groucho protein Grg4 suppresses Smad7 to activate BMP signaling. *Biochem. Biophys. Res. Commun.* 440, 454–459. doi: 10.1016/j.bbrc.2013.09.128
- Zhou, X., Zhang, Z., Feng, J. Q., Dusevich, V. M., Sinha, K., Zhang, H., et al. (2010). Multiple functions of Osterix are required for bone growth and homeostasis in postnatal mice. *Proc. Natl. Acad. Sci. U.S.A.* 107, 12919–12924. doi: 10.1073/pnas.0912855107

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Shin, Theodorou, Holland, Yamin, Raggio, Giampietro and Sweetser. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.