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TIEG1 enhances Osterix Expression and Mediates its Induction by TGF β and BMP2 in Osteoblasts

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Abstract

Deletion of TIEG1/KLF10 in mice results in an osteopenic skeletal phenotype with significant decreases in both bone mineral density and content throughout the skeleton. Calvarial osteoblasts isolated from TIEG1 knockout (KO) mice display numerous changes in gene expression and exhibit significant delays in their mineralization rates relative to wild-type (WT) controls. Here, we demonstrate that loss of TIEG1 expression in osteoblasts results in decreased levels of Osterix mRNA. Suppression of TIEG1 expression in WT osteoblasts leads to decreased Osterix expression while restoration of TIEG1 expression in TIEG1 KO osteoblasts results in increased levels of Osterix. Transient transfection and chromatin immunoprecipitation assays reveal that TIEG1 directly binds to and activates the Osterix promoter and demonstrate that the zinc finger containing DNA binding domain of TIEG1 is necessary for this regulation. Furthermore, we reveal that TIEG1 expression is essential for the induction of Osterix expression by important bone-related cytokines such as TGF β and BMP2 in osteoblast cells. Taken together, these data implicate an important role for TIEG1 in regulating the expression of Osterix, a master regulator of osteoblast differentiation and bone formation, and suggest that decreased expression of Osterix, as well as impaired TGF β and BMP2 signaling, contribute to the observed osteopenic bone phenotype of TIEG1 KO mice.

Keywords

TIEG1; KLF10; Osterix; Osteoblast; TGF β ; BMP2

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Conflict of Interest

None

Introduction

TGF β Inducible Early Gene-1 (TIEG1), also referred to as KLF10, was originally cloned from human osteoblasts as a primary response gene following TGF β and BMP2 treatment[1]. TIEG1 encodes a 480 amino acid protein that contains three Zinc finger domains at the C-terminus and several proline rich Src homology and protein/protein interacting domains at the N-terminus[1,2,3,4]. Overexpression of TIEG1 has been shown to enhance cellular differentiation, suppress cell proliferation and induce apoptosis in a cell type dependent manner, effects that mimic that of TGF β treatment in the same model systems[2, 3, 5]. We have also demonstrated that TIEG1 plays an important role in mediating Smad signaling in multiple cell types by inhibiting expression of the inhibitory Smad7 gene and inducing the expression of the Smad2 gene[6, 7].

Through the development of TIEG1 knockout (KO) mice[8], we have revealed important roles for this transcription factor in multiple cell and tissue types[2, 3]. With regard to bone, initial characterization of the skeletal system revealed that osteoblasts isolated from TIEG1 KO mice exhibit defects in mineralization[8], a phenomenon that we have partially explained by reduced Runx2 expression and activity[9]. TIEG1 KO osteoblasts also display reduced expression of RANKL and increased expression of OPG resulting in a decreased capacity to support osteoclast differentiation[8]. Subsequent studies have demonstrated that TIEG1 regulates the expression of OPG by directly binding to its promoter[10].

Detailed characterization of the intact skeleton has revealed that TIEG1 KO mice display a female specific osteopenic phenotype characterized by decreased bone mineral density and content, in both trabecular and cortical compartments, compared to wild-type (WT) littermates[11, 12]. Mechanical analysis of TIEG1 KO long bones using 3-point bending tests revealed significant decreases in their mechanical properties and strength relative to WT littermates[11, 12]. Further investigation into the issue of the observed gender specific phenotype has demonstrated that TIEG1 expression is enhanced by estrogen[13] and is necessary to mediate maximal estrogen signaling throughout the mouse skeleton[14].

Through more recent global gene expression studies on TIEG1 KO osteoblast cells, we observed significant decreases in the expression of Osterix. Osterix is also a zinc finger-containing transcription factor that was originally identified as a BMP2 regulated gene in osteoprogenitor cells[15]. Subsequent analysis of Osterix function revealed that it is a master regulator of bone formation since complete ablation of this gene in mice inhibits skeletal development[15]. Osterix has also been shown to regulate the expression of down-stream markers of mature osteoblasts including Collagen 1, Secreted Protein Acidic Cysteine-Rich, Osteopontin, Bone Sialoprotein, Osteocalcin and Alkaline Phosphatase[16]. Given its essentiality in skeletal development and bone formation, significant attention has been devoted to understanding the transcription factors and signaling pathways that are responsible for regulating Osterix expression in osteoblasts.

Since our gene expression studies revealed decreased expression of Osterix in TIEG1 KO calvarial osteoblasts, and since both TIEG1 and Osterix are regulated by similar growth factors, cytokines and signaling pathways, we sought to determine if TIEG1 functions

upstream of Osterix to enhance its expression in osteoblast cells. In this study we provide evidence that osteoblasts derived from TIEG1 KO mice exhibit decreased Osterix gene expression and that manipulation of TIEG1 expression levels directly correlate with Osterix expression. Further, we provide evidence that TIEG1 functions upstream of Osterix, directly binds to the Osterix promoter to regulate its activity and is necessary for BMP2 and TGF β mediated induction of Osterix expression.

Materials and Methods

Calvarial osteoblast isolation and maintenance

Calvarial osteoblasts were isolated from 3 day old WT or TIEG1 KO neonatal pups obtained from C57BL/6 heterozygous breeding pairs as described previously[8, 10]. Following isolation, cells were maintained in α -MEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA) and 1% antibiotic/antimycotic (ThermoFisher Scientific, Waltham, MA) in a humidified 37°C incubator with 5% CO₂. All experiments involving calvarial osteoblasts were conducted within the first three passages from the time of isolation. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Mayo Clinic Institutional Animal Care and Use Committee (Permit Number: A9615). U2OS cells were purchased from ATCC and were cultured in phenol red-free Dulbecco's modified Eagle's medium/F12 medium (DMEM/F12) (ThermoFisher Scientific) containing 10% FBS and 1% antibiotic/antimycotic.

RNA isolation and Real-time PCR

Wild-type and TIEG1 KO calvarial osteoblasts were plated at a density of approximately 50% in 12 well plates. All studies were performed in triplicate using cells isolated from at least 3 independent pups. Cells were allowed to proliferate until they reached 70% confluence at which time total RNA was extracted using Trizol reagent (ThermoFisher Scientific). One μ g of RNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA) and real-time PCR was performed in triplicate using a Bio-Rad iCycler and a PerfeCTa™ SYBR Green Fast Mix™ for iQ real-time PCR kit (Quanta Biosciences, Gaithersburg, MD) as specified by the manufacturer. Cycling conditions were as follows: 95°C for 30 seconds followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Melt curves were generated to ensure amplification of a single PCR product. Quantitation of the PCR results was calculated based on the threshold cycle (C_T) following normalization to β -tubulin and averaged across genotypes.

All PCR primers were designed using Primer3 software (<http://frodo.wi.mit.edu/primer3/>) and were purchased from Integrated DNA Technologies (Coralville, IA). Primer sequences were as follows. TIEG1 F: GTGACCGTCGGTTTATGAGG; TIEG1 R: ACTTCCATTGTCAGTTTGG; Osterix F: GGAGGTTTCACTCCATTCCA; Osterix R: TAGAAGGAGCAAGGGGACAGA; β -Tubulin F: CTGCTCATCAGCAAGATCAGAG; β -Tubulin R: GCATTATAGGGCTCCACCACAG.

TIEG1 siRNA

A TIEG1 specific siRNA (AAUGGAACUAAUUUCUGAA)d(TT) and a scrambled TIEG1 siRNA (non-sense) were custom designed and purchased from Dharmacon (Lafayette, CO). Three independent WT calvarial osteoblast lines were cultured as above and transfected with siRNAs as previously described[9]. Forty eight hours post-transfection, TIEG1 and Osterix expression levels were determined via real-time PCR.

TIEG1 adenovirus

A mouse TIEG1 and control adenovirus was produced by Vector Biolabs (Philadelphia, PA) as previously described[9, 17]. Three independent TIEG1 KO calvarial osteoblasts were cultured as above and infected at a multiplicity of infection (MOI) of 10 for 24 hours at which time TIEG1 and Osterix expression levels were determined.

Osterix promoter constructs

A 2Kb fragment immediately upstream of the transcriptional start site of the mouse Osterix gene was cloned into the pGL3-basic luciferase reporter construct as previously described[18]. Additional 5'- deletions of the 2 Kb promoter were generated using a PCR based approach as previously described[18].

Luciferase assays

U2OS cells were plated in 12 well plates at approximately 50% confluence. Cells were transfected with 250 ng of indicated Osterix promoter-reporter constructs along with 250 ng of either pcDNA4/TO empty vector, pcDNA4/TO-TIEG1 1-480 or pcDNA4/TO-TIEG1 1-370. The TIEG1 1-480 construct represents the entire coding sequence of the TIEG1 gene while the 1-370 construct is missing the DNA binding domain. Transient transfections were performed using Eugene-6 (Roche, Indianapolis, IN) as specified by the manufacturer. Twenty four hours following transfection, cells were lysed, protein lysates were quantitated and equal amounts were used to measure luciferase activity using Luciferase Assay Reagent (Promega, Madison, WI) and a Glomax-Dual luminometer (Promega).

Chromatin immunoprecipitation (ChIP) assays

U2OS cells were plated in 100 mm petri dishes at a confluency of approximately 70% and were subsequently transfected with 5 µg of the Osterix 0.7Kb reporter construct along with 5 µg of empty vector or TIEG1 1-480 expression construct. Following 24 hours of transfection, ChIP assays were performed as previously described[13].

Immunoprecipitations were carried out using 0.5 µg of the M2-Flag specific monoclonal antibody or an IgG control antibody (Sigma-Aldrich, St. Louis, MO). Quantitative Real-Time PCR was conducted in triplicate on all samples. Primers used in the PCR were as follows: Osterix promoter ChIP F: GACTGCCACAGATGCCTAT; Osterix promoter ChIP R: GGGCAAGTTGTCAGAGCTTC. Quantitative PCR values were calculated based on the threshold cycle (C_t), normalized to input controls and compared to IgG immunoprecipitated samples.

TGF β 1 and BMP2 treatments

Three independent WT and TIEG1 KO calvarial osteoblast cell lines were plated in triplicate in 12-well tissue culture plates at a density of approximately 50%. The following day, cells were treated with vehicle (0.25% BSA in 1X PBS), TGF β 1 (2 ng/mL) or BMP2 (200 ng/mL) for a time course ranging from 2 hours to 4 days. TGF β 1 was purchased from Austral Biologicals (San Ramon, CA) while BMP2 was purchased from R&D Systems (Minneapolis, MN). Following indicated treatment times, total RNA was harvested and Osterix expression was determined.

Statistical analyses

All graphs depict mean $\pm$ SE of at least three independent experiments. Statistical analyses were conducted using either a two-tailed Students T-test or a two-way ANOVA. P-values less than 0.05 were considered statistically significant.

Results

TIEG1 modulates Osterix expression in osteoblasts

We have previously demonstrated that osteoblasts isolated from TIEG1 KO mice display defects in mineralization and support of osteoclast differentiation[8]. To understand the molecular mechanisms behind these defects, we performed gene expression profiling on RNA isolated from WT and TIEG1 KO calvarial osteoblasts. Interestingly, a number of important osteoblast marker genes exhibited decreased expression, including Osterix which was decreased by approximately 2.5 fold in TIEG1 KO cells (Figure 1A). To further confirm a role for TIEG1 in regulating Osterix expression, we suppressed TIEG1 expression in WT calvarial osteoblasts using a TIEG1 specific siRNA. Suppression of TIEG1 in WT cells was associated with a 2-fold decrease in Osterix expression relative to scrambled siRNA control transfected cells (Figure 1B). Conversely, adenoviral mediated overexpression of TIEG1 in TIEG1 KO calvarial osteoblasts resulted in a significant increase in Osterix expression levels (Figure 1C). These data demonstrate that TIEG1 modulates Osterix expression levels in osteoblast cells.

TIEG1 binds to the Osterix promoter and regulates its activity

Based on our observation that TIEG1 modulates Osterix expression levels in osteoblasts, we sought to determine if this regulation occurred through direct binding of TIEG1 to the Osterix promoter. Towards this goal, we utilized luciferase reporter constructs containing a 2.0 Kb fragment, or serial 5'-deletions, of the mouse Osterix promoter[18]. As shown in Figure 2A, TIEG1 slightly suppressed the activity of the -2.0 Kb Osterix promoter construct in U2OS cells. However, TIEG1 substantially increased the activity of the -1.3 Kb, -0.7 Kb and -0.5 Kb Osterix promoter fragments suggesting that repression domains exist in the upstream regions of the full-length promoter construct (Figure 2A). A significant loss in TIEG1 mediated Osterix promoter activity was also observed for the -0.5 Kb fragment suggesting that a TIEG1 regulatory site lies between -0.7 Kb and -0.5 Kb upstream of the transcriptional start site (Figure 2A). An additional TIEG1 regulatory site likely exists in the

-0.5 Kb fragment since TIEG1 significantly modulated activity of this promoter construct, albeit to a lesser degree (Figure 2A).

We next utilized the smallest Osterix promoter construct with maximal TIEG1 mediated activity (-0.7 Kb) to determine if DNA binding by TIEG1 was essential for modulation of promoter activity. Transfection of a TIEG1 expression construct in which the zinc-finger DNA binding domain has been deleted (TIEG1 1-370)[6, 19] substantially inhibited the ability of TIEG1 to induce Osterix promoter activity compared to the full-length TIEG1 1-480 construct suggesting that the zinc finger DNA binding domain, and therefore DNA binding, are essential (Figure 2B). We next performed transient ChIP assays to determine if TIEG1 protein associates with the Osterix promoter. As shown in Figure 2C, substantial binding of the full-length TIEG1 protein was observed on the -0.7 Kb fragment of the Osterix promoter relative to U2OS cells which were transfected with an empty vector expression plasmid. Taken together, these data demonstrate that TIEG1 directly binds to and enhances Osterix promoter activity.

TIEG1 mediates TGF β and BMP2 induction of Osterix expression

Since TIEG1 was originally identified as a TGF β and BMP2 induced early response gene[1], and since Osterix is also known to be induced by these two cytokines [15, 20, 21], we sought to determine if TIEG1 expression was necessary for Osterix gene induction following TGF β or BMP2 treatment. Therefore, Osterix expression was analyzed following treatment of WT or TIEG1 KO calvarial osteoblasts with TGF β or BMP2 for 0, 2, 4, 7, 24, 48, 72 and 96 hours. As shown in Figure 3, TGF β and BMP2 significantly induced Osterix mRNA expression in WT calvarial osteoblasts following 24 hours of cytokine treatment. No significant increases in Osterix expression were observed at any time point in the TIEG1 KO cells indicating that TIEG1 expression is essential for TGF β and BMP2 mediated induction of Osterix mRNA (Figure 3).

Discussion

In this study, we provide evidence that osteoblasts derived from TIEG1 KO mice exhibit decreased Osterix expression compared to WT calvarial osteoblasts. We also demonstrate that suppression of TIEG1 in WT cells leads to decreased levels of Osterix while overexpression of TIEG1 in KO cells increases Osterix expression. TIEG1 appears to directly regulate the expression of Osterix through direct DNA binding to its proximal promoter. Finally, we demonstrate that expression of TIEG1 is necessary for the induction of Osterix by important bone-related cytokines such as BMP2 and TGF β . A model summarizing these findings is shown in Figure 4.

Osterix is known to be a critical bone-related transcription factor as homozygous deletion of this gene in mice is lethal and results in the formation of an unmineralized skeleton in embryos and neonates[15]. Despite the essential nature of Osterix for skeletal development, little remains known about the mechanisms responsible for controlling its expression in bone. Thus far, only a handful of transcription factors, including Runx2[15, 18] and β -catenin[22] or growth factors/cytokines such as BMP2[15, 21], TGF β [20], fibroblast growth factor[22] and Wnt ligands [22], have been shown to induce Osterix expression. This study

identifies TIEG1 as a novel factor that is necessary for optimal expression of Osterix in osteoblast cells. Our results also suggest that TIEG1 directly regulates the expression of Osterix by binding to its proximal promoter. Based on our luciferase reporter construct assays, it appears that there are at least two important TIEG1 binding sites in the Osterix promoter, one residing between 700 and 500 bps upstream of the transcriptional start site and another between 0 and 500 bps upstream of the start of transcription. DNA binding by TIEG1 to the Osterix promoter appears to be essential for regulation of Osterix expression as deletion of the TIEG1 DNA binding domain rendered it incapable of enhancing Osterix promoter activity. These findings correlate well with a previous publication which identified two putative TIEG1 binding sites in the mouse Osterix promoter located approximately 40 and 700 bps upstream of the transcriptional start site[18].

Our laboratory originally cloned TIEG1 from human osteoblasts and identified it as an early response gene following TGF β treatment[1]. Further studies demonstrated that TIEG1 enhances TGF β signaling through suppression of Smad7[23] and induction of Smad2[7] and also revealed that TIEG1 transcription is enhanced by BMP2[1]. Interestingly, Osterix was originally identified as a BMP2 regulated gene[15] and was later shown to be regulated by TGF β [20]. We have previously demonstrated that TIEG1 is an immediate early response gene following TGF β and BMP2 treatment[1] and have shown that protein synthesis is not required for the induction of TIEG1 by these two cytokines[1]. However, previous studies have suggested that this is not the case for Osterix since pretreatment of cells with cycloheximide prevents the induction of Osterix expression by BMP2[21] demonstrating that *de novo* protein synthesis is required. These findings correlate well with our present results given that Osterix expression was not increased until 7 hours post TGF β and BMP2 treatment. Additionally, we did not observe any induction of Osterix expression by these two cytokines in TIEG1 KO cells further suggesting that TIEG1 is a key mediator of this response.

In summary, we have demonstrated that TIEG1 directly regulates Osterix expression in osteoblasts and is essential for the induction of Osterix by important bone-related cytokines such as TGF β and BMP2. Among three osteogenic master transcription factors (Runx2, Osterix and Dlx5) known to be regulated by cytokines such as BMP2[15, 24, 25, 26, 27], we have demonstrated that TIEG1 is at least partially responsible for mediating this response for Runx2[9] and now Osterix. Interestingly, a recent meta-analysis of gene expression datasets has identified TIEG1 as one of only three direct BMP target genes that play critical roles in mediating BMP signaling in developing long bones[28]. These studies add to our understanding of the mechanisms by which Osterix is transcriptionally controlled, further defines TIEG1 as an essential modulator of cytokine signaling and gene expression in osteoblast cells and sheds additional light on the molecular basis of the observed osteopenic bone phenotype of TIEG1 KO mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Subramaniam M, Harris SA, Oursler MJ, Rasmussen K, Riggs BL, Spelsberg TC. Identification of a novel TGF-beta-regulated gene encoding a putative zinc finger protein in human osteoblasts. *Nucleic Acids Res.* 1995; 23:4907–4912. [PubMed: 8532536]
2. Subramaniam M, Hawse JR, Johnsen SA, Spelsberg TC. Role of TIEG1 in biological processes and disease states. *Journal of cellular biochemistry.* 2007; 102:539–548. [PubMed: 17729309]
3. Subramaniam M, Hawse JR, Rajamannan NM, Ingle JN, Spelsberg TC. Functional role of KLF10 in multiple disease processes. *BioFactors (Oxford, England).* 2010; 36:8–18.
4. Kim J, Shin S, Subramaniam M, Bruinsma E, Kim TD, Hawse JR, Spelsberg TC, Janknecht R. Histone demethylase JARID1B/KDM5B is a corepressor of TIEG1/KLF10. *Biochemical and biophysical research communications.* 2010; 401:412–416. [PubMed: 20863814]
5. Song KD, Kim DJ, Lee JE, Yun CH, Lee WK. KLF10, transforming growth factor-beta-inducible early gene 1, acts as a tumor suppressor. *Biochemical and biophysical research communications.* 2012; 419:388–394. [PubMed: 22349513]
6. Johnsen SA, Subramaniam M, Janknecht R, Spelsberg TC. TGFbeta inducible early gene enhances TGFbeta/Smad-dependent transcriptional responses. *Oncogene.* 2002a; 21:5783–5790. [PubMed: 12173049]
7. Johnsen SA, Subramaniam M, Katagiri T, Janknecht R, Spelsberg TC. Transcriptional regulation of Smad2 is required for enhancement of TGFbeta/Smad signaling by TGFbeta inducible early gene. *J Cell Biochem.* 2002b; 87:233–241. [PubMed: 12244575]
8. Subramaniam M, Gorny G, Johnsen SA, Monroe DG, Evans GL, Fraser DG, Rickard DJ, Rasmussen K, van Deursen JM, Turner RT, Oursler MJ, Spelsberg TC. TIEG1 null mouse-derived osteoblasts are defective in mineralization and in support of osteoclast differentiation in vitro. *Mol Cell Biol.* 2005; 25:1191–1199. [PubMed: 15657444]
9. Hawse JR, Cicek M, Grygo SB, Bruinsma ES, Rajamannan NM, van Wijnen AJ, Lian JB, Stein GS, Oursler MJ, Subramaniam M, Spelsberg TC. TIEG1/KLF10 modulates Runx2 expression and activity in osteoblasts. *PloS one.* 2011; 6:e19429. [PubMed: 21559363]
10. Subramaniam M, Hawse JR, Bruinsma ES, Grygo SB, Cicek M, Oursler MJ, Spelsberg TC. TGFbeta inducible early gene-1 directly binds to, and represses, the OPG promoter in osteoblasts. *Biochemical and biophysical research communications.* 2010; 392:72–76. [PubMed: 20059964]
11. Hawse JR, Iwaniec UT, Bensamoun SF, Monroe DG, Peters KD, Ilharreborde B, Rajamannan NM, Oursler MJ, Turner RT, Spelsberg TC, Subramaniam M. TIEG-null mice display an osteopenic gender-specific phenotype. *Bone.* 2008; 42:1025–1031. [PubMed: 18396127]
12. Bensamoun SF, Hawse JR, Subramaniam M, Ilharreborde B, Bassillais A, Benhamou CL, Fraser DG, Oursler MJ, Amadio PC, An KN, Spelsberg TC. TGFbeta inducible early gene-1 knockout mice display defects in bone strength and microarchitecture. *Bone.* 2006; 39:1244–1251. [PubMed: 16876494]
13. Hawse JR, Subramaniam M, Monroe DG, Hemmingsen AH, Ingle JN, Khosla S, Oursler MJ, Spelsberg TC. Estrogen receptor beta isoform-specific induction of transforming growth factor beta-inducible early gene-1 in human osteoblast cells: an essential role for the activation function 1 domain. *Molecular endocrinology (Baltimore, Md.).* 2008; 22:1579–1595.
14. Hawse JR, Pitel KS, Cicek M, Philbrick KA, Gingery A, Peters KD, Syed FA, Ingle JN, Suman VJ, Iwaniec UT, Turner RT, Spelsberg TC, Subramaniam M. TGFbeta inducible early gene-1 plays an important role in mediating estrogen signaling in the skeleton. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research.* 2014; 29:1206–1216.
15. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrughe B. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell.* 2002; 108:17–29. [PubMed: 11792318]

16. Stein GS, Lian JB, Stein JL, Van Wijnen AJ, Montecino M. Transcriptional control of osteoblast growth and differentiation. *Physiological reviews*. 1996; 76:593–629. [PubMed: 8618964]
17. Cicek M, Vrabel A, Sturchio C, Pederson L, Hawse JR, Subramaniam M, Spelsberg TC, Oursler MJ. TGF-beta inducible early gene 1 regulates osteoclast differentiation and survival by mediating the NFATc1, AKT, and MEK/ERK signaling pathways. *PloS one*. 2011; 6:e17522. [PubMed: 21423731]
18. Nishio Y, Dong Y, Paris M, O'Keefe RJ, Schwarz EM, Drissi H. Runx2-mediated regulation of the zinc finger Osterix/Sp7 gene. *Gene*. 2006; 372:62–70. [PubMed: 16574347]
19. Noti JD, Johnson AK, Dillon JD. The leukocyte integrin gene CD11d is repressed by gut-enriched Kruppel-like factor 4 in myeloid cells. *J Biol Chem*. 2005; 280:3449–3457. [PubMed: 15561714]
20. Rich JT, Rosova I, Nolte JA, Myckatyn TM, Sandell LJ, McAlinden A. Upregulation of Runx2 and Osterix during in vitro chondrogenesis of human adipose-derived stromal cells. *Biochemical and biophysical research communications*. 2008; 372:230–235. [PubMed: 18482578]
21. Lee MH, Kwon TG, Park HS, Wozney JM, Ryoo HM. BMP-2-induced Osterix expression is mediated by Dlx5 but is independent of Runx2. *Biochem Biophys Res Commun*. 2003; 309:689–694. [PubMed: 12963046]
22. Felber K, Elks PM, Lecca M, Roehl HH. Expression of osterix Is Regulated by FGF and Wnt/beta-Catenin Signalling during Osteoblast Differentiation. *PloS one*. 2015; 10:e0144982. [PubMed: 26689368]
23. Johnsen SA, Subramaniam M, Monroe DG, Janknecht R, Spelsberg TC. Modulation of transforming growth factor beta (TGFbeta)/Smad transcriptional responses through targeted degradation of TGFbeta-inducible early gene-1 by human seven in absentia homologue. *J Biol Chem*. 2002c; 277:30754–30759. [PubMed: 12072443]
24. Miyama K, Yamada G, Yamamoto TS, Takagi C, Miyado K, Sakai M, Ueno N, Shibuya H. A BMP-inducible gene, dlx5, regulates osteoblast differentiation and mesoderm induction. *Developmental biology*. 1999; 208:123–133. [PubMed: 10075846]
25. Lee MH, Kim YJ, Kim HJ, Park HD, Kang AR, Kyung HM, Sung JH, Wozney JM, Ryoo HM. BMP-2-induced Runx2 expression is mediated by Dlx5, and TGF-beta 1 opposes the BMP-2-induced osteoblast differentiation by suppression of Dlx5 expression. *The Journal of biological chemistry*. 2003; 278:34387–34394. [PubMed: 12815054]
26. Lee MH, Javed A, Kim HJ, Shin HI, Gutierrez S, Choi JY, Rosen V, Stein JL, van Wijnen AJ, Stein GS, Lian JB, Ryoo HM. Transient upregulation of CBFA1 in response to bone morphogenetic protein-2 and transforming growth factor beta1 in C2C12 myogenic cells coincides with suppression of the myogenic phenotype but is not sufficient for osteoblast differentiation. *Journal of cellular biochemistry*. 1999; 73:114–125. [PubMed: 10088730]
27. Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, Wozney JM, Kim EG, Choi JY, Ryoo HM, Bae SC. Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. *Mol Cell Biol*. 2000; 20:8783–8792. [PubMed: 11073979]
28. Prashar P, Yadav PS, Samarjeet F, Bandyopadhyay A. Microarray meta-analysis identifies evolutionarily conserved BMP signaling targets in developing long bones. *Developmental biology*. 2014; 389:192–207. [PubMed: 24583261]

Highlights

- Loss of TIEG1 in osteoblasts results in decreased Osterix gene expression.
- TIEG1 directly binds Osterix promoter and regulates its activity.
- BMP2 and TGF β mediated induction of Osterix in osteoblasts is dependent on TIEG1.

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Figure 1.

TIEG1 regulates Osterix gene expression in osteoblasts. (A) Expression of Osterix mRNA in WT and TIEG1 KO calvarial osteoblasts as detected by real-time PCR analysis. The results are depicted as relative expression levels compared to WT cells. (B) TIEG1 and Osterix mRNA expression in WT calvarial osteoblasts transfected with a scrambled control siRNA (siCon) or a TIEG1 specific siRNA (siTIEG1). (C) Expression of TIEG1 and Osterix in TIEG1 KO calvarial osteoblasts infected with a control (ad. Con.) or TIEG1 (ad. TIEG1) adenovirus. Asterisks denote significance at the $p < 0.05$ level compared to indicated controls.

Figure 2.

Regulation of Osterix promoter activity by TIEG1 in osteoblasts. **(A)** U2OS cells were transiently transfected with empty vector (pcDNA4/TO) or TIEG1 expression constructs in combination with indicated Osterix promoter fragments fused to a luciferase reporter (pGL3-basic). **(B)** U2OS cells were transiently transfected with empty vector (pcDNA4/TO), full-length (1-480) TIEG1 or a TIEG1 DNA binding domain (1-370) expression constructs in combination with the Osterix -0.7 Kb promoter fragment reporter construct. Luciferase activity was determined and values are reported as relative fold change compared

to controls following normalization to total protein levels. (C) Transient chromatin immunoprecipitation (ChIP) assays were performed in U2OS cells transfected with a Flag-tagged TIEG1 full-length expression vector and the Osterix -0.7 Kb promoter fragment reporter construct. Data are expressed as the abundance of the Osterix promoter relative to cells transfected with an empty vector control expression construct. All data were normalized using input samples. *Asterisks* denote significance at the $p < 0.05$ level compared with empty vector controls. δ denotes significance at the $p < 0.05$ level between indicated promoter constructs.

Figure 3.

TIEG1 mediates Osterix expression in calvarial osteoblasts following TGF β and BMP2 stimulation. Wild-type (WT) and TIEG1 knockout (KO) cells were treated with vehicle, TGF β 1 (**A**) or BMP2 (**B**) for 0, 2, 4, 7, 24, 48, 72 and 96 hours. Osterix expression levels were monitored by real-time PCR and the results are expressed as relative fold change compared to vehicle treated cells at each time point. Asterisks denote significance at the $p < 0.05$ level compared to vehicle controls.

Figure 4. Model depicting the role of TIEG1 in regulating Osterix expression and response to TGFβ/BMP2 signaling in osteoblast cells.