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TLE3, transducing-like enhancer of split 3, suppresses osteoblast differentiation of bone marrow stromal cells

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ABSTRACT

In senile osteoporosis the balance of adipogenesis and osteoblastogenesis in bone marrow stromal cells (BMSCs) is disrupted so that adipogenesis is increased with respect to osteoblastogenesis, and as a result, bone mass is decreased. While the molecular mechanisms controlling the balance between osteoblastogenesis and adipogenesis are of great interest, the exact nature of the signals regulating this process remains to be determined.

In general, adipogenesis is a reciprocal relationship with osteoblastogenesis in BMSCs. Recently transducin-like enhancer of split 3 (TLE3), was reported to enhance adipogenesis in pre adipocytes. However, the effect of TLE3 on osteoblast differentiation of BMSCs is completely unknown. Here we report that TLE3 not only enhances adipocyte differentiation in BMSCs but also suppresses osteoblast differentiation.

Firstly we examined the expression and localization of TLE3. We found that TLE3 is expressed in the nucleus of bone marrow stromal cells and that over-expression of TLE3 induced adipocyte differentiation and suppressed ALP activity induced by treatment with BMP2 in these cells. In contrast, adipocyte differentiation was decreased and ALP activity increased when endogenous TLE3 was knocked down by shRNA in BMSCs. To examine the mechanism by which TLE3 is able to suppress osteoblast differentiation, we focused on Runx2, a transcription factor essential for osteoblast differentiation. We found that TLE3 strongly suppressed ALP activity and OSE2-luciferase activity induced by Runx2 and this repression of Runx2 by TLE3 occurs via HDACs because treatment with TSA, a class I and II HDAC inhibitor, rescued this repression.

In conclusion, we identify TLE3 as a suppressor of BMSC differentiation in osteoblast lineage cells *in vitro*. Our data suggest that TLE3 activity may be a key in balancing adipocyte and osteoblast differentiation in the adult bone marrow microenvironment.

1. Introduction

Osteoblast lineage cells and marrow adipocytes are derived from common progenitors, which are bone marrow stromal cell (BMSC)s. In a senile osteoporosis, the balance of adipocyte and osteoblast differentiation is disrupted in this cell population so that adipocyte differentiation is increased relative to osteoblast differentiation, and as a consequence, bone mass is reduced, resulting in increased bone fragility and susceptibility to fracture [1,2]. In 2004, 10 million Americans over age 50 had osteoporosis with another 34 million Americans at risk for the disease. In this population it is estimated that 1.5 million fragility fractures occur each year, with an annual health care cost of $18 billion dollars [3]. By 2025, the health care expenditures for osteoporotic fractures will approach 25.3 billion dollars [4,5]. Therefore, the molecular mechanisms controlling the balance between osteoblastogenesis and adipogenesis in adult bone are of great significance; however, the precise mechanisms of regulating this process remains to be determined.

Adipogenesis is driven by a complex and well-orchestrated signaling cascade composed of several key transcription factors, most notably proliferator-activated receptor (PPAR)γ and several members of the CCAAT/enhancer-binding family of proteins (C/EBPs)

Abbreviations: TLE, transducing-like enhancer of split; ALP, alkaline phosphatase; PPARγ, proliferator-activated receptor γ; BMSC, bone marrow stromal cell; HDAC, histone deacetylase.

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[6]. PPARγ is commonly referred to as the master regulator of adipogenesis, because no factor has yet been identified that can induce normal adipogenesis in its absence [7].

BMP-Smad signaling plays important role in osteoblast differentiation of BMSCs. In response to BMP signaling, several critical transcription factors for osteoblast differentiation, such as Runx2, Osterix, Dlx2, Dlx5 are induced in target cells [8–11]. In particular, Runx2 is essential for the commitment of mesenchymal cells to the osteoblast lineage. Homozygous deletion of Runx2 in mice resulted in a complete lack of osteoblasts [12,13], while haploinsufficiency of Runx2 in mice or Runx2 in humans led to hypoplastic clavicles and delayed closure of the fontanelles, defects that are characteristic of cleidocranial dysplasia in humans [14,15]. The expression of Runx2 is regulated, in part, by BMP signaling [8] and Runx2 controls osteoblast-related genes such as Osterix, collagen I, and osteocalcin [9,16] including the Runx2 gene itself [17].

A variety of studies have shown that adipogenesis and osteoblastogenesis are reciprocally regulated in BMSCs. Several proteins such as tafazzin, Wnt 5a, Wnt 10b, Msx2, C/EBPβ and basic helix-loop-helix (bHLH) family member e40 (Bhlhe 40), and ID4 have been identified as regulators of this balance [18–21]. Recently transducin-like enhancer of split 3 (TLE3), one of the Groucho/TLE proteins, was reported to induce adipogenesis in pre adipocytes [22]. However, the effect of TLE3 on osteoblast differentiation of BMSCs is completely unknown. In this study, we report that TLE3 not only enhances adipocyte differentiation in BMSCs but also suppresses osteoblast differentiation by repressing Runx2 transcriptional activity via class I and/or II HDACs.

2. Materials and methods

2.1. Plasmids

Mouse TLE3 (accession number NM_001083927), and mouse PPARγ (accession number NM_011146.3) were obtained by a standard RT-PCR technique using PrimeSTAR HS DNA polymerase (TaKaRa, Ohtsu, Japan) and cloned into pcDNA3.1/V5-His expression vector (Invitrogen) or a pcDEF3 expression vector. aP2-luc was constructed by subcloning from −5.4 kb to −4.9 bp fragment of the 5′flanking region of the aP2 gene [23] into the pGL4-basic vector. TLE3 Point mutant TLE3, TLE3 (V726D) was generated from wild-type TLE3 by replacing valine residues at position 726, which was equivalent to 708 in TLE3 variant 3, with aspartic acid, using sets of mutated PCR primers [22]. Mouse Runx2 and 6 × OSE2-luc[8] were kindly provided by Dr. Toshihisa Komori (Nagasaki University) [24].

2.2. Cell culture and transfection

W-20-17 cells and HEK 293T cells were cultured and maintained as described previously [25,14]. Cells were treated with 25 ng/ml rhBMP2 (R&D Systems) or 1 μM Trichostatin A (TSA) (Sigma). Cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen) [26].

2.3. ALP and luciferase assays

ALP activity was measured as a marker of osteoblast differentiation. Cells treated with an acetone/ethanol (50:50) mixture were incubated with a substrate solution composed of 0.1 M diethanolamine, 1 mM $MgCl_2$ and 1 mg/ml *p*-nitrophenylphosphate, the reaction was terminated by adding 3 M NaOH, and absorbance values were measured at 405 nm [27]. Luciferase assays were performed using OSE2-luc and phRL-SV40 (Promega) with the Dual-Glo Luciferase Assay System (Promega) [28].

2.4. Adipogenic medium and oil red O staining

For *in vitro* adipocyte differentiation, cells were treated with adipogenic medium (10 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 μg/ml insulin)[29] and along with 10 μM Rosiglitazone. On 8 days adipogenic cultures of W-20-17 cells were rinsed twice with PBS, fixed in 10% buffered formaldehyde, stained with oil red O (Sigma) for 10 min at room temperature.

2.5. Immunohistochemistry and western blot analysis

The following antibodies were used for Immunohistochemistry and western blot analysis: anti-TLE3 polyclonal antibody (M-201; sc-9124)(Santa Cruz), anti-phosphorylated Smad1/5/8 polyclonal antibody, anti-Smad1 polyclonal antibody (Cell Signaling), anti-V5 (Sigma), and anti-β-actin mouse monoclonal antibody (Sigma). The target proteins were detected using a horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (Cell Signaling). For immunohistochemical tissue analysis, femur bones were collected from 10 weeks male mice. Mouse femurs were fixed in 4% paraformaldehyde, decalcified in Tris buffer containing 10% EDTA (pH 7.5) and embedded in paraffin [30]. Sections (5 μm thick) were heated in a vegetable steamer for 25 min in citrate antigen retrieval buffer (Biogenex Fremont CA). Primary antibodies were detected with ABC amplification using horseradish peroxidase (HRP) conjugated streptavidin (Vector Laboratories Inc) and diamino benzidine (Sigma) and counterstained with Mayer's hematoxylin (Sigma) [31]. For fluorescent immunohistochemical analysis, target proteins were visualized using an Alexa488- or Alexa594-conjugated secondary antibody (Invitrogen). Digital images were obtained using a Zeiss AxioImager MI Microscope fitted with an AxioCam HR digital camera Zeiss AxioVision imaging software (Carl Zeiss). For western blot analyses, the target proteins were detected using HRP conjugated anti-mouse or anti-rabbit IgG antibody (Cell signaling).

2.6. Reverse transcription and quantitative PCR analysis

Total RNA was isolated from W-20-17 cells by using Trizol (Invitrogen) and then reverse-transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche). SYBR green-based quantitative real-time PCR was performed in 96-well plate using Fast Start Universal SYBR Green Master (Roche) with iCycler Multicolor Real-Time PCR Detection system (BIO-RAD). Values were normalized to β-actin using the $2-\Delta\Delta$Ct method [32]. Detailed primer sequences are described as Supplementary information.

2.7. shRNA Plasmid

TLE3 shRNA constructs were designed using BLOCK-IT RNAi designer tool (Invitrogen), Sense and Antisense oligos were annealed and cloned into pcDNA 6.2-GW/miR (Invitrogen). More information is described as Supplementary information.

2.8. Statistical analysis

Comparisons were made using an unpaired Student's *t*-test; the results are shown as means ± S.D. Statistical significance is indicated as $^{*}P < 0.05$ and $^{**}P < 0.01$.

3. Results

3.1. TLE3 is expressed by murine bone marrow stromal cells

We examined the expression and localization of TLE3 in bone and found that TLE3 expressed by cells in bone marrow of 10-week-old male mice (Fig. 1A). TLE3 is also expressed and localized in nuclei of primary cultured BMSCs and W20-17 cells (Supplementary Fig. 1 and Fig. 1B), a mouse bone marrow stromal cell line that differentiates into osteoblasts in response to osteogenic stimuli, including BMPs [25,33]

3.2. TLE3 enhances adipocyte differentiation of BMSCs

TLE3 is reported to enhance adipogenesis in pre adipocytes [22]. We next examined the effect of TLE3 on adipogenesis in BMSCs. Over-expression of TLE3 promoted adipocyte differentiation of W20-17 cells under adipogenic condition as assessed by oil red O staining (Fig. 2A). TLE3 also enhanced the promoter activity of the aP2 gene, a typical adipogenic marker gene induced by PPARγ (Fig. 2B). In contrast, mRNA levels of aP2, induced under adipogenic culture conditions, was decreased when endogenous TLE3 was knocked down by short hairpin RNA (shRNA) against TLE3 of W20-17 cells (#1700 and #1791) (Fig. 2C and Supplementary Fig. 4A).

3.3. TLE3 suppresses osteoblast differentiation of BMSCs

We previously reported that W20-17 cells differentiate into osteoblast-like cells in response to BMPs [25,33]. We induced osteoblastogenesis in W20-17 by adding BMP2. Observed that over-expression of TLE3 suppressed ALP activity, and mRNA levels of Osteocalcin (Fig. 3A, Supplementary Fig. 3 and Fig. 3B). By contrast, induction of ALP activity by BMP2 increased when TLE3 is silenced by shRNA #1700 and #1791 (Fig. 3C). Thus, TLE3 firmly suppressed osteoblast differentiation of BMSCs induced by BMP2. However, TLE3 did not reduce phosphorylation of Smad1/5/8 induced by BMP2 (Supplementary Fig. 2A) nor did it suppress

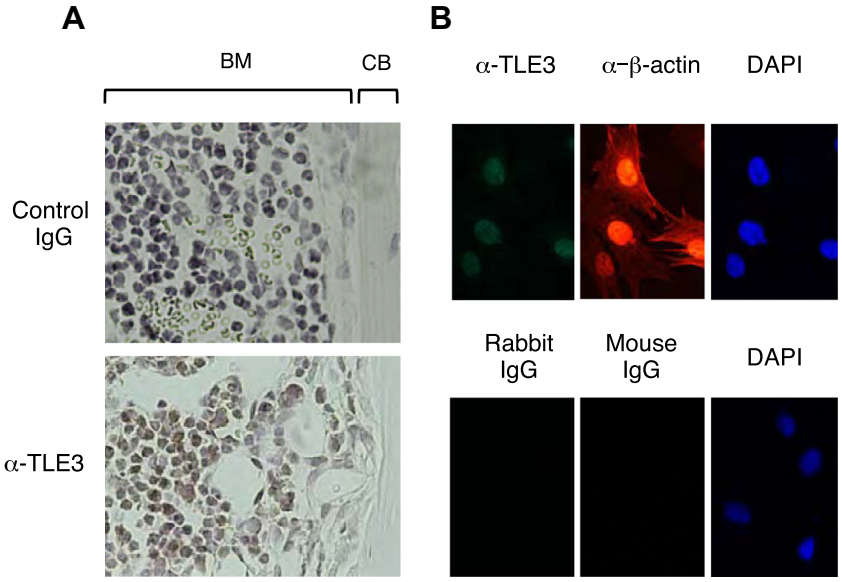

Fig. 1. TLE3 expresses by murine BMSCs. Immunohistochemistry based detection of TLE3 in femur bone from 10-wk-old male mice. These sections were stained with control Rabbit IgG (upper panel) or an anti-TLE3 antibody (lower panel. BM: Bone marrow, CB: Cortical bone. Original magnification 20× (A). Immunohistochemical analysis of TLE3 or β-actin in W20-17 cells. Original magnification 40× (B).

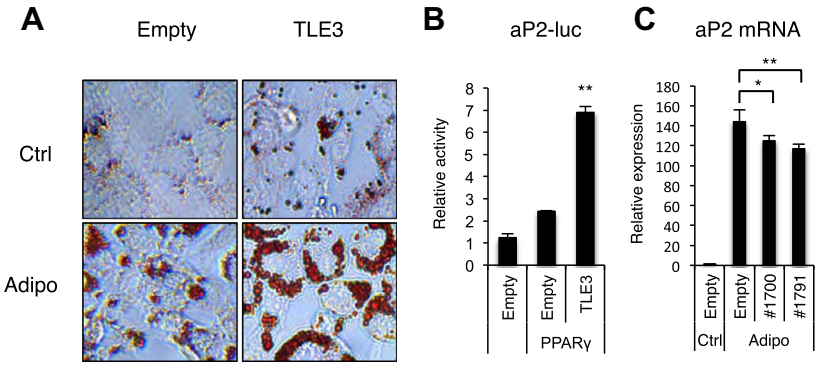

Fig. 2. TLE3 enhances adipogenesis of BMSCs. W20-17 cells were transfected with TLE3 or an empty vector along with adipogenic medium. Adipocytes were stained with oil red O on day 8 (A). W20-17 cells were transfected with TLE3 or an empty vector along with aP2-luciferase vector and adipogenic medium. Luciferase activity was determined on day 1 (B). W20-17 cells were transfected with control shRNA vector, shRNA (#1700), or shRNA (#1791) along with adipogenic medium. mRNA level of aP2 was determined by real-time PCR on day 2 (C). The data are shown as the mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ in comparison with transfected with empty vector or control shRNA vector. (For interpretation of color in this Figure, the reader is referred to the web version of this article.)

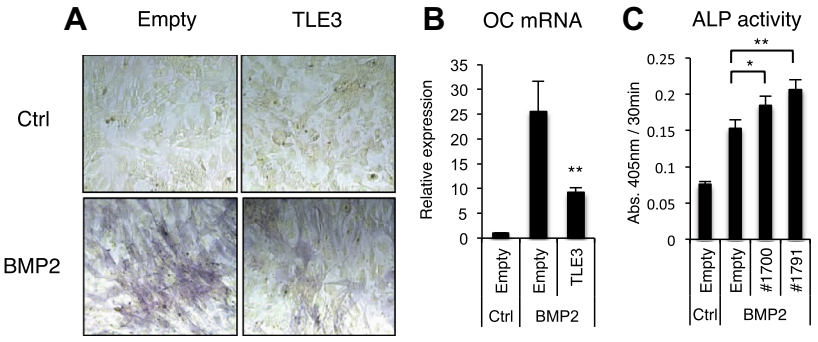

Fig. 3. TLE3 suppresses osteoblast differentiation. (A and B) W20-17 cells were transfected with an empty vector or TLE3 along with or without BMP2. ALP activities were determined on day 2 by staining (A), and mRNA level of OC was determined on day 3 by real-time PCR (B). W20-17 cells were transfected with control shRNA, shRNA (#1700), or shRNA (#1791) along with or without BMP2. ALP activities were measured on day 2 (C). The data are shown as the mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ in comparison with transfected with an empty vector or control shRNA vector.

BMP-early responsible genes such as Id1 or Id2 (Supplementary Fig. 2B and C), suggesting that TLE3 acts on more downstream effectors of BMP signaling.

3.4. TLE3 represses transcriptional activity of Runx2

Runx2 has a critical role in osteoblastogenesis and is a downstream effector of BMP-Smad axis [8]. Runx2 is known to interact with several proteins including Groucho/TLEs [34]. In turn, Groucho/TLE proteins work as co-repressors of Runx2 [35]. TLE1 and TLE2 also repress Runx2 dependent activation of Osteocalcin gene transcription [36]. Thus, we focused on Runx2 as a target of TLE3.

Over-expression of Runx2 was able to induce ALP activity in W20-17 cells, and co-transfection of TLE3 reduced this activation of ALP (Fig. 4A). However, over-expression of TLE3 did not affect the expression level of Runx2 (Fig. 4B), leading us to examine the effect of TLE3 on Runx2 transcriptional activity as evaluated by the 6 × OSE2-luc reporter. Over-expression of TLE3 completely suppressed OSE2-luc activity induced by Runx2 in W20-17 cells. Prior studies have identified loss-of-function of TLE3 by a point mutation [22,36]. Introduction of a point mutation in the WD40 domain of TLE3 (TLE3 (V726D)) diminished the ability of TLE3 (Fig. 4C), indicating that W40 domain of TLE3 plays important role in this repression.

Histone deacetylases (HDACs) are involved in one of the suppressive mechanisms of Groucho/TLE proteins [37], so we used W20-17 cells to investigate whether HDACs are involved in this suppressive mechanism. When the cells were treated with TSA, the suppressive effect of TLE3 on OSE2-luc activity was diminished (Fig. 4D). Taken together, these data suggested that TLE3 represses Runx2 transcriptional activity via Class I and/or Class II HDACs.

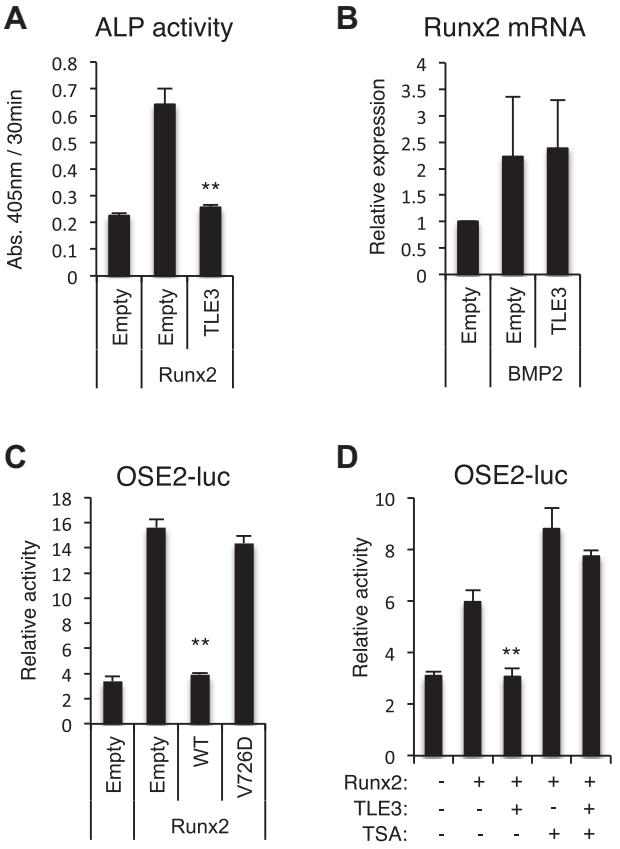

Fig. 4. TLE3 suppresses transcriptional activity of Runx2 via recruitment of HDACs. W20-17 cells were co-transfected with TLE3 or an empty vector along with OSE2-luc and Runx2. Over-expressed TLE3 suppressed ALP activity stimulated by over-expression of Runx2 on day 3 (A). Over-expression of TLE3 did not affect mRNA levels of Runx2 induced by treatment with BMP2 on day 1 (B). W20-17 cells were co-transfected with an empty vector, wild-type TLE3 (WT), or mutant-TLE3 (V726D) along with OSE2-luc and Runx2 (C). W20-17 cells were co-transfected with an empty vector or TLE3 along with OSE2-luc and Runx2. Treatment of 1 μM TSA counteracts the suppressive effect of TLE3 on OSE2-luc activity induced by transfection of Runx2 (D). $^{**}P < 0.01$ in comparison with transfected with a control.

4. Discussion

Both osteoblast and bone marrow adipocytes originate from BMSCs. While several proteins have been reported to regulate this process [19,21,38], the exact nature of the signals regulating the balance between osteoblast and adipocyte formation within the bone marrow space remains to be determined. Here we suggest a novel model by which HDACs repress transcriptional activity of Runx2 via TLE3 and promote adipogenesis by BMSC. This finding may shed light on the nature of osteoblast differentiation since transactivation of Runx2 is essential for this process.

HDACs deprive acetyl groups from lysine residues on histones. Removing the acetyl group alters chromatin structure by facilitating chromatin condensation to promote transcriptional repression [39]. Several HDACs have been reported to reduce Runx2 transcriptional activity [40–44]. Indeed, our data show that treatment TSA (Class I and II HDAC inhibitor) increased Runx2 transcriptional activity, indicating that HDACs are involved in repression of Runx2 activity at the basal level in target cells (Fig. 4C).

TLE3 induces adipogenesis in pre adipocytes by enhancing transcriptional activity of PPARγ, a master regulator of adipogenesis

[22]. Consistent with this report, we confirmed and showed that TLE3 enhance adipogenesis of BMSCs by enhancing PPARγ transcriptional activity. In addition, TLE3 suppressed osteoblastogenesis of BMSCs by reducing Runx2 transcriptional activity. Taken together, TLE3 activity may be key in balancing adipocyte and osteoblast differentiation in the adult bone marrow microenvironment.

In these experiments we used W20-17 cells, a bone marrow stromal cell line as a model of primary adult bone marrow mesenchymal progenitors. Like their primary cell counterpart, W20-17 cells differentiate into osteoblast-like cells in response to BMP treatment [25,33] and have the ability to differentiate into adipocytes (Fig. 2), indicating that W20-17 cells are very useful for examining the cell fate determination between osteoblast and adipocyte. While our *in vitro* studies will require confirmation with in vivo analyses, knockout of TLE3 in mice is embryonic lethal and at present no highly specific Cre-promoter mouse that targets BMSCs is available.

In conclusion, TLE3 suppresses osteoblast differentiation of BMSCs by suppressing Runx2 transcriptional activity via HDACs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.054.

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