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BEST3-Mediated Promotion of RANKL Expression in ST2 Cells

Kazutake Kosuda[1], Toyonobu Maeda[2], Shinya Yamanobe[1], Hitoshi Kawanabe[1]

[1]Division of Orthodontics and Dentofacial Orthopedics, Department of Oral Growth and Development, Graduate School of Dentistry, Ohu University, Koriyama, Japan
[2]Department of Oral Function and Molecular Biology, School of Dentistry, Ohu University, Koriyama, Japan
Email: kossue1214@gmail.com

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Abstract

Objective: Skeletal mandibular protrusion, is prevalent in the Japanese population. Despite therapeutic interventions during the developmental stages, some individuals experience recurrence from mandibular overgrowth. Therefore, it is imperative to identify the factors contributing to mandibular overgrowth. Recent studies have identified nonsynonymous mutations in *BEST3* in Japanese patients with mandibular prognathism. However, the specific role of *BEST3* in bone metabolism remains unclear. This study aimed to investigate the effect of *BEST3* on bone differentiation. Mouse mesenchymal-derived ST2 cells were transfected with *BEST3*, and the expression of genes associated with mineralization, osteoblast differentiation, and osteoclast differentiation was analyzed. Methods: Mouse ST2 cells were maintained in RPMI1640 medium. Osteoblastic differentiation was induced using α-MEM supplemented with ascorbic acid and β-glycerophosphate. Mineralization was assessed by Alizarin Red S staining. A *BEST3* expression vector (*pBEST3*) was constructed using cDNA derived from HEK293 cells and transfected with Xfect for transient overexpression. Gene expression was evaluated using RT-qPCR of total RNA, and RANKL expression was quantified by ELISA. Results: The overexpression of *BEST3* did not influence osteoblast differentiation or mineralization. Furthermore, the expression of bone formation-related genes, including *Bmp2*, *Bmp4*, *Alp*, *Col1a1*, and *Bglap*, remained largely unaffected despite the overexpression of *BEST3*. In contrast, there was a significant upregulation in the expression of osteoclast differentiation factors, notably RANKL (*Tnfsf11*) and M-CSF (*Csf1*). Overexpression of *BEST3* was correlated with increased RANKL protein levels. Conclusion: Overexpression of ectopic *BEST3* in osteoblast-like ST2 cells promotes RANKL gene and protein expression.

Keywords

Malocclusion, Angle Class III, *BEST3*, Osteoblasts, RANKL

1. Introduction

Skeletal mandibular prognathism is characterized by mesial positioning the mandibular first molar relative to the maxillary first molar. Angle Class III malocclusion is associated with numerous combinations of skeletal and dental morphological variations [1]. The prevalence of Angle Class III malocclusion in Caucasians ranges from 0.48% to 4% [2]; however, it exceeds 10% in Japanese individuals [3]. Angle Class III malocclusion can cause speech disorders and impaired mastication. Among adult patients with mandibular prognathism, occasional cases require orthodontic treatment alone, as well as orthognathic surgery [4]. Orthodontic treatment is frequently performed in adult patients with mandibular prognathism. For younger patients with mandibular prognathism, orthopedic treatment using appliances to restrict mandibular growth during the growth phase has been performed. However, in recent years, growth-inhibiting orthodontic treatment has been discouraged because of concerns regarding its impact on temporomandibular joint disorders. Due to genetic factors and other causes, the mandible may grow excessively, leading to the recurrence of mandibular prognathism in the late growth phase. Consequently, some patients may require orthognathic surgery. If orthodontists can predict whether a patient has strong risk factors for excessive mandibular growth, they may be able to select strategies for more effective treatment of mandibular prognathism [5]-[8].

Best vitelliform macular dystrophy (BVMD Best disease) is an autosomal dominant hereditary disorder discovered by Friedrich Best in 1905 and is characterized by the abnormal accumulation of lipofuscin within and beneath retinal pigment epithelial cells. Petrukhin *et al.* analyzed the best disease families and discovered in 1998 that the cause lies in the retina-specific gene VMD2, located on chromosome 11q13 [9]. VMD2 was renamed Bestrophin-1 (BEST1), and BEST2, *BEST3*, and BEST4 were identified through homology searches and organized into the Bestrophin family. The Bestrophin family comprises intracellular proteins and proteins detectable in the plasma membrane. They share a common structure, featuring a consensus sequence containing four transmembrane domains, and form pentamers that function on the membrane. Their common function is to act as Ca^{2+}-dependent anion channels (e.g., for Cl^{-}) [10]-[12].

BEST1 is an integral membrane protein primarily expressed in the retinal pigment epithelium (RPE) and localized in the basal lamina [13]. Within the RPE, BEST1 functions as both an anion channel and a regulator of intracellular calcium signaling [10]. Additionally, BEST1 is expressed in the brain and is involved in the transport of glutamate and GABA [14] [15]. On the other hand, BEST2 is known to be involved in the sweating mechanism [16] and the maintenance of intraocular pressure [17].

The *BEST3* gene (NM_032735.3) is located on chromosome 12, consisting of 10 exons and 9 introns [18]. The *BEST3* protein (NP_116124.2) translated from this gene is approximately 76 kDa and has been confirmed to be expressed in many organs, including skeletal muscle, the brain, the thymus, the adrenal glands, and the stomach [19]. *BEST3* differs from other members of the Bestrophin family in that it possesses a long intracellular C-terminus containing a region with a protein kinase G consensus sequence [10] and is ubiquitously distributed throughout various organs. In addition to its previously reported function as a Ca^{2+}-dependent anion channel, *BEST3* has been found to possess anti-apoptotic functions in arterial smooth muscle cells [20] and anti-inflammatory effects in endothelial cells [21]. Recently, whole-exome analysis of Japanese patients with mandibular prognathism revealed a non-synonymous single nucleotide variant (SNV) in the *BEST3* gene [22]. However, the specific function of *BEST3* in bone differentiation and metabolism remains unclear. Therefore, in this study, we investigated the effects of the *BEST3* gene on the differentiation of undifferentiated mesenchymal cells using cultured mesenchymal cells as a model system.

2. Materials and Methods

2.1. Reagents

RPMI 1640 medium, simvastatin, and p3xFLAG-CMV-10 were purchased from Sigma-Aldrich (MO, USA); Minimum Essential Media Alpha (α-MEM) was purchased from MP Bio (CA, USA); and ascorbic acid and glycerol-2-phosphate were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Hyclone (UT, USA); Prime STAR GXL DNA Polymerase and Xfect Transfection Reagent were obtained from Takara Bio (Tokyo, Japan); High Capacity RNA-to-cDNA was obtained from Thermo Fisher Scientific (MA, USA); and GoTaq qPCR Master Mix was obtained from Promega (WI, USA).

2.2. Cell Culture and Differentiation Induction

Mouse ST2 cells were maintained in basic culture at 37˚C and 5% CO_2. This culture was performed in 10 cm dishes, maintaining a cell density not exceeding 2×10^6 cells [23]. The medium consisted of RPMI 1640 supplemented with 10% FBS and was changed every 2 - 3 days. For osteoblast differentiation, α-MEM medium supplemented with 10% FBS, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate was used, with 1 μM simvastatin added as a calcification promoter [24].

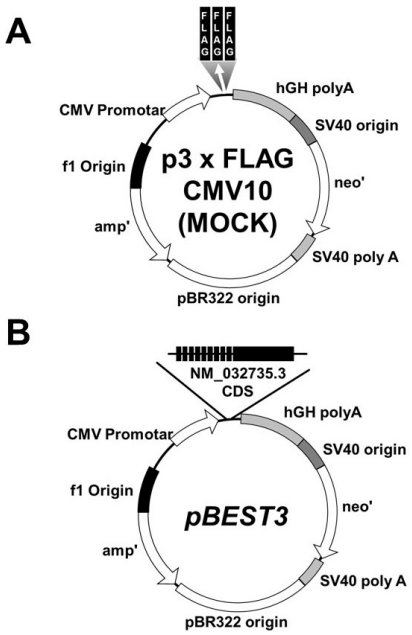
2.3. Measurement of Calcification

1×10^5 cells were seeded per well in a 24-well plate. After reaching confluence, the medium was changed to α-MEM and cultured further. After removing the medium, ST2 cells, were washed with Mg^{2+}/Ca^{2+}-free Dulbecco's phosphate-buffered saline (PBS) and fixed in 70% ethanol for 1 hour. After washing three times with distilled water, the cells were stained with 40 mM Alizarin Red S (pH 4.2). The red-stained areas were identified as calcified deposits [25]. Two independent bio-

logical experiments were performed, and each biological replicate was analyzed using four technical replicates.

2.4. Construction of the *BEST3* Expression Vector and Transfection

HEK293 cells were washed with PBS and then lysed inguanidine lysis buffer (4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sodium N-lauroylsarcosylate, 100 mM mercaptoethanol. Total RNA was extracted using the Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction (AGPC) method). This RNA was used to synthesize cDNA corresponding to the coding region of *BEST3* (NM_032735.3) via reverse transcription using SuperScript IV Reverse Transcriptase. Following amplification with Prime STAR GXL DNA Polymerase, this was used as a template for cloning. This PCR product was cloned into p3 × FLAG-CMV-10 using the In-Fusion® Cloning Kit (Takara Bio) via the SLiCE (Seamless Ligation Cloning Extract) method to create the *BEST3* expression vector (p*BEST3*) (Figure 1). An empty vector without the insert was used as the control (MOCK). Cell transfection was performed using the lipofection method with Xfect™ Transfection Reagent. Furthermore, all experiments were conducted using transient expression systems without selecting stable vector-transfected cells [26].



3 × FLAG CMV10 possesses a CMV promoter that drives the transcription of downstream sequences in mammalian cells. We removed the 3 × FLAG sequence from this vector and ligated a 2,007 bp coding region of the human *BEST3* gene (NM_032735.3) to generate p*BEST3* (A), which was used in the experiments. Additionally, a construct derived from 3 × FLAG CMV10, with the 3 × FLAG sequence removed, was used as a control (MOCK [B]). The 3 × FLAG CMV10 vector contains a neomycin resistance cassette (Neo) for the selection of stably expressing cells. In this study, G418 antibiotics were not used and no specific cells were selected.

Figure 1. Vector map used for gene introduction.

2.5. Reverse-Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

After reverse transcription of total RNA using the High Capacity RNA-to-cDNA Kit, qPCR was performed using GoTaq qPCR Master Mix with the respective primers (Table 1). Detection was performed using a Thermal Cycler Dice Real Time System TP951 (Takara Bio) [27]. Three independent biological experiments were performed, and each biological replicate was analyzed using three technical replicates.

Table 1. Specific primers used for RT-qPCR.

Gene		Sequence (5'->3')	Product
BEST3	Forward	AGCTGCTGAC TACTGCATAC CCTCATTTC	247 bp
(NM_032735.3)	Reverse	GGCTGGGCTGAGGTCATCTCG	
Bmp2	Forward	TGACTGGATCGTGGCACCTC	112 bp
(NM_007553.3)	Reverse	CAGAGTCTGCACTATGGCATGGTTA	
Bmp4	Forward	AGCCGAGCCAACACTGTGAG	68 bp
(NM_007554.2)	Reverse	TCACTGGTCCCTGGGATGTTC	
Bglap3	Forward	CTGAGTCTGACAAAGCCTTCA	137 bp
(NM_001305448.1)	Reverse	AGCAGGGTCAAGCTCACATA	
Alp	Forward	AGCAGGGTCAAGCTCACATA	192 bp
(NM_007431.3)	Reverse	ATGGCCTGGTCCATCTCCAC	
Col1a1	Forward	GACATGTTCAGCTTTGTGGACCTC	119 bp
(NM_007742.4)	Reverse	ATGGCCTGGTCCATCTCCAC	
Col3a1	Forward	TGACTGTCCCACGTAAGCAC	105 bp
(NM_009930.2)	Reverse	GAGGGCCATAGCTGAACTGA	
Tnfsf11	Forward	GTACTTTCGAGCGCAGATGGA	103 bp
(NM_011613.4)	Reverse	CGAGTCCTGCAAATCTGCGT	
Tnfrsf11b	Forward	CACATTTGGCCTCCTGCTAATTC	107 bp
(NM_008764.4)	Reverse	ATGGCCTGGTCCATCTCCAC	
Csf1	Forward	TAGACCAGGAACAGCTGGATGAT	111 bp
(NM_007778.4)	Reverse	TAGCATTGGGGGTGTTGTCTTTA	
Actb	Forward	CATCCGTAAAGACCTCTATGCCAAC	171 bp
(NM_007393.5)	Reverse	ATGGAGCCACCGATCCACA	

2.6. ELISA

ST2 cells, seeded at 4×10^5 cells per well in a 6-well plate and cultured for a specified period after transfection, were resuspended in 200 μL of RIPA buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.5% sodium deoxycholate; 0.1% sodium do-

decyl sulfate; 1.0% NP-40). The lysate was sonicated on ice, centrifuged at 2,000 × g at 4˚C for 1 minute, and the supernatant was used as the sample. The RANKL concentration in these samples was measured using a sandwich ELISA kit (Proteintech, Shanghai, China) [28]. ELISA was performed in two independent biological experiments, with each biological replicate analyzed in triplicate technical replicates.

2.7. Statistical Analysis

Statistical analyses were performed separately at each culture time point using one-way analysis of variance (ANOVA) followed by Scheffe's post hoc test to compare the experimental groups. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effect of Calcification Granule Accumulation on Ectopic Overexpression of *BEST3*

We verified whether *BEST3* is expressed in ST2 cells transfected with p*BEST3*. ST2 cells transfected with MOCK or p*BEST3* were cultured for 48 h. Total RNA was extracted, and RT-qPCR was performed. Human *BEST3* was not detected in MOCK-transfected cells. In cells transfected with p*BEST3*, amplification was observed, with a CT value of 20.05 ± 1.034. Since this value is approximately 2.6 times higher than the Actb CT value of 17.37 ± 0.241, it was determined that the gene expression level was approximately one-sixth that of Actb (Table 2 upper panel).

Table 2. Ct values for *BEST3* in RT-qPCR. upper panel.

upper panel		
CT value	MOCK	p*BEST3*
BEST3	—	20.05 ± 1.034
Actb	17.49 ± 0.806	17.37 ± 0.241

lower panel

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NM_032735.3 (hBEST3)   1154'  ACACATTGGC AGCTGCTGAC TACTGCATAC CCTCATTTCT GGGGTCAACA GTCCAGATGG
                               ***** ****  ***** *** ********** * ** ***** *** *****   **** ****
NM_001007583.1(mBest3) 1555"  ACACACTGGC GGCTGCCGAC TACTGCATAC CGTCCTTTCT GGGCTCAACG ATCCAAATGG

                       1214'  GGCTGTCTGG GTCCGACTTT CCTGACGAGG AGTGGCTGTG GGATTATGAG AAGCATGGCC
                               ******* ** **** ****  ** *  **** * ******** * * ** *** ** ** **
                       1615"  GGCTGTCCGG GTCCAACTTC CCCGCAGAGG ACTGGCTGTG GAACTACGAG AAACACGGAA

                       1274'  ATCGGCATTC CATGATAAGA AGAGTCAAGC GGTTCCTGAG TGCCCACGAA CACCCCTCCA
                               * ***** **   **** **  ** ******* * ******** * *******  ** **   **
                       1675"  ACCGGCACTC AGTGATGAGG AGGGTCAAGC GCTTCCTGAG TACCCACGAG CATCCTGGCA

                       1334'  GCCCC---AG AAGAAGAAGC TACAGGAGGC AGACAAGTGA CAGCTCCATG TTCTTACCCC
                               *****   **  ** ** *** * * * **** ** * ** ** ********** **********
                       1735"  GCCCCAGGAG GAGGAGGAGC TTCGGCAGGC AGGCCAGCGA CAGCTCCATG TTCTTACCCC

                       1391'  GAGATGACCT CAGCCCAGCC AGGGACCTAC TGGATGTGCC CTCAAGAAAC CCCCCCAGGG
                                        ********  **** ** * **********  ** ** *** **** * * *
                       1795"  ----------C AAGCCCAGCT CGGGATCTGC TGGATGTGCC GTCCAGGAAC CCCCACCGAG
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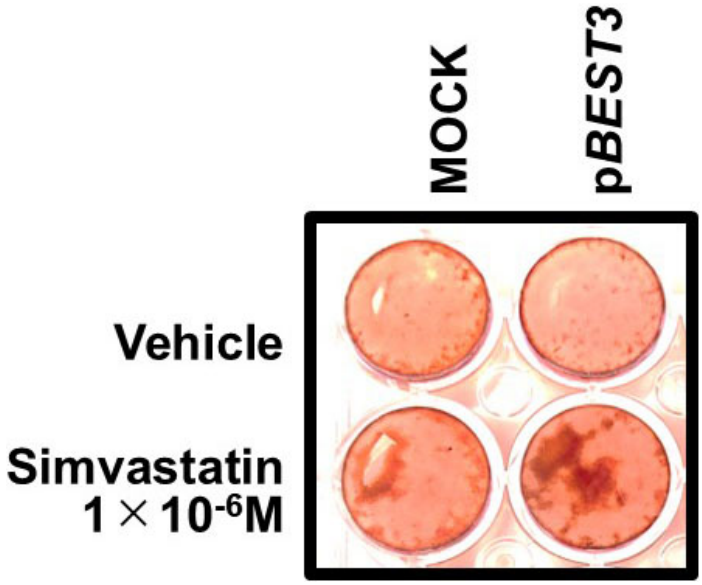
These cells were seeded at 1×10^5 cells per well in a 24-well multi-plate, and β-glycerophosphate and ascorbic acid were added, with simvastatin used as a calcification promoter. Alizarin Red S staining was performed after 21 days. In the absence of simvastatin, almost no calcified particle accumulation was observed in the MOCK-transfected cells. No accumulation of calcified particles was observed in cells expressing *BEST3*. The addition of 10^{-6} M simvastatin increased the number of calcification particles stained with Alizarin Red S. However, no significant change in the accumulation of calcification particles was observed in p*BEST3*-transfected cells compared to MOCK-transfected cells (Figure 2).

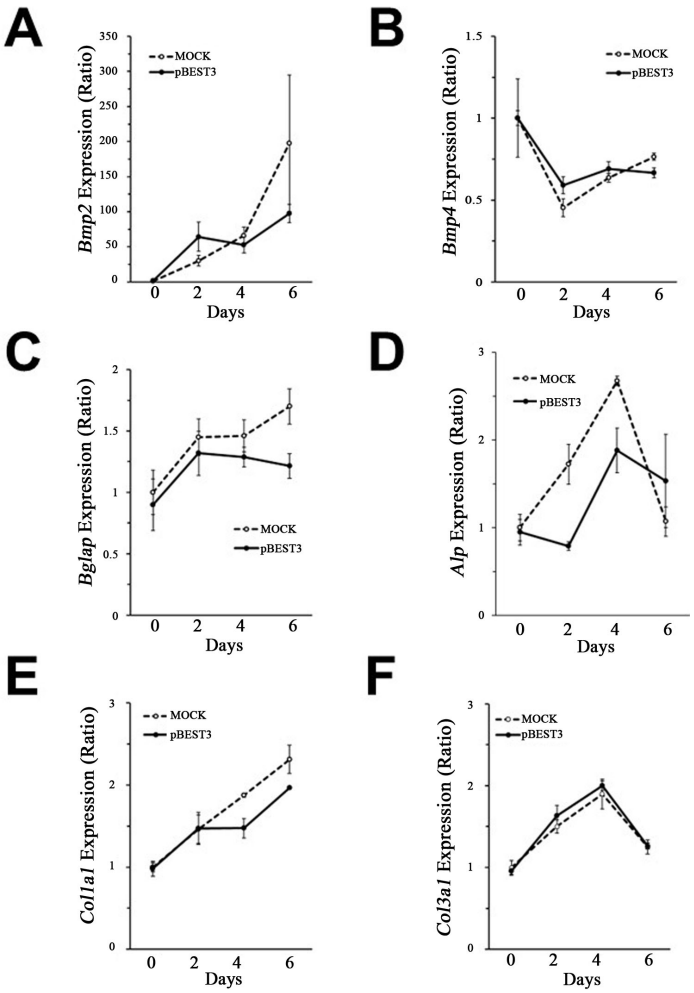


ST2 cells were cultured in a 24-well plate, and either MOCK or p*BEST3* cells were introduced for 21-day culture period. During the 21-day culture period, 1×10^{-6} M simvastatin or solvent (70% ethanol) was added.

Figure 2. Alizarin Red S staining image on day 21 of culture.

3.2. Effects of *BEST3* on the Expression of Early Osteoblast Differentiation Markers

The following experiment was conducted to verify whether the ectopic overexpression of *BEST3* directly affected the early stages of osteoblast differentiation. ST2 cells were seeded at a density of 4×10^5 cells per well in a 6-well multi-plate. When cells reached 50% confluence, p*BEST3* or MOCK (7.5 μg/well) was introduced. After reaching confluence, the medium was replaced with a medium containing β-glycerophosphate and ascorbic acid, and the cells were cultured for 6 days, with samples collected every 2 days. Total RNA was extracted from the samples using the AGPC method and subjected to qPCR analysis. In MOCK-transfected cells, *Bmp2* gene expression increased approximately 200-fold by day 6 compared to day 0. However, *BEST3* overexpression did not affect this increase (Figure 3A). *Bmp4* expression showed no significant changes in MOCK-transfected cells until day 6. This was also observed in the cells transfected with p*BEST3* (Figure 3B).



ST2 cells were cultured in 6-well plates and treated with MOCK or p*BEST3* for 6 days. Cells were harvested every 2 d, and gene expression levels were detected by RT-qPCR. Results were normalized to *Actb* expression (n = 4). Statistical significance was set at $P < 0.05$.

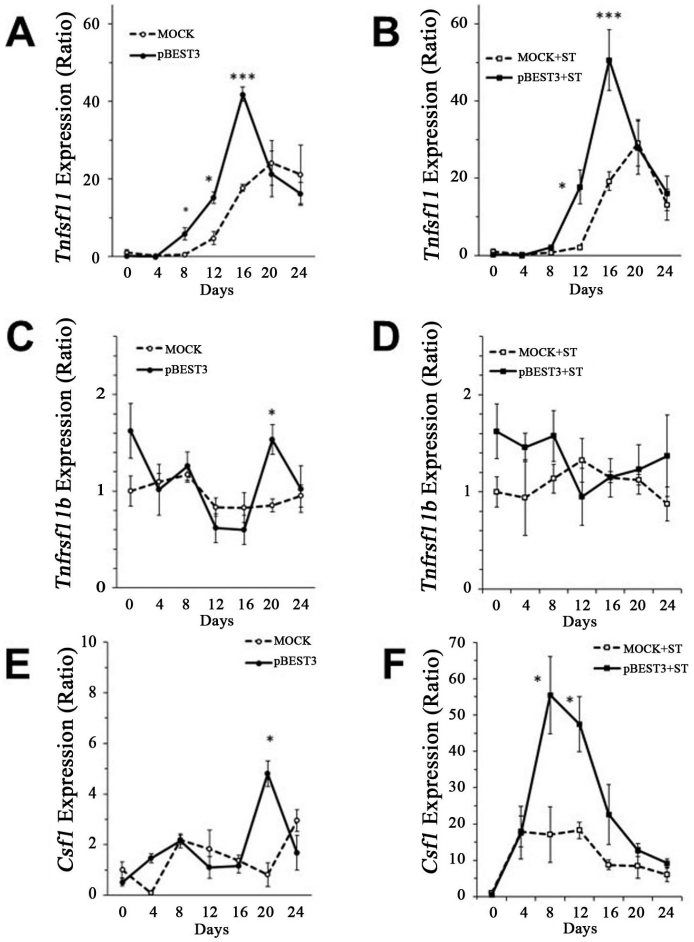
Figure 3. Time-course gene expression changes of bone metabolism markers during early culture.

3.3. Effects of *BEST3* on Gene Expression in the Bone Matrix

Gene expression of osteocalcin (*Bglap3*), a protein specific to hard tissues, increased in MOCK-transfected cells up to day 6 of culture. p*BEST3* transfection did not significantly affect this increase (Figure 3C). Gene expression of tissue-non-specific alkaline phosphatase (Alp) was significantly increased in MOCK-transfected cells on day 4 compared with that on day 0. In contrast, *BEST3* overexpression did not result in a significant change compared to that on day 0, even on day 4 (Figure 3D). The expression of the gene for the pro-α1 chain of type I collagen (*Col1a1*), a major fibrous protein in bone, increased as culture progressed. Even when p*BEST3* was introduced, there was no significant difference compared to the MOCK group (Figure 3E). Similarly, for the gene encoding the pro-α1 chain of type III collagen (Col3a1), *BEST3* overexpression did not affect its expression levels (Figure 3F).

3.4. Effects of *BEST3* on the Expression of Osteoclast Differentiation Markers

Overexpression of *BEST3* significantly increased the expression level of the RANKL gene (*Tnfsf11*), an osteoclast differentiation and activation factor, up to day 16 of culture (Figure 4A). This change was maintained even after the addition of statins (Figure 4B). Expression of OPG (*Tnfrsf11b*), a decoy receptor for RANKL, was not affected by *BEST3* overexpression (Figure 4C, Figure 4D). The expression level of the M-CSF gene significantly increased from day 4 to day 16 of culture following simvastatin addition, and *BEST3* overexpression further enhanced this increase (Figure 4E, Figure 4F).

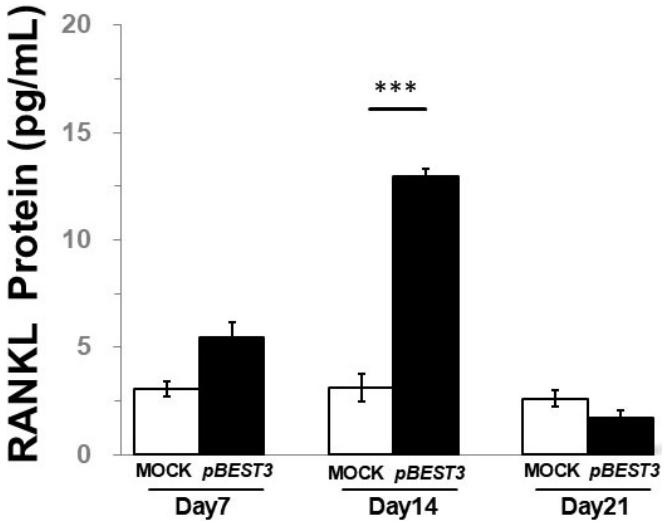


ST2 cells were cultured and treated with MOCK or p*BEST3* for 24 days. Cells were harvested every 4 days, and gene expression levels were detected by RT-qPCR. Gene expression levels were normalized against *Actb* expression (n = 3). A - B: RANKL gene (*Tnfsf11*) expression; A: No simvastatin 1×10^{-6} M (Vehicle); B: Simvastatin 1×10^{-6} M (ST). C-D: OPG gene (*Tnfrsf11b*) expression; C: No simvastatin 1×10^{-6} M (Vehicle); D: Simvastatin 1×10^{-6} M (ST). E-F; M-CSF gene (*Csf1*) expression, E; No simvastatin 1×10^{-6} M added (Vehicle), F; Simvastatin 1×10^{-6} M added (ST). ***indicates $p < 0.001$ compared to MOCK, and * indicates $p < 0.05$ compared to MOCK (n = 3).

Figure 4. Time-course gene expression changes of factors influencing osteoclast differentiation following p*BEST3* introduction.

3.5. Effect of *BEST3* on RANKL Protein Expression

Finally, ELISA was performed to confirm whether *BEST3*-induced Tnfsf11 expression altered the protein translation levels. Consistent with the changes in mRNA levels, the introduction of p*BEST3* significantly increased total cellular RANKL by approximately fourfold by day 14 of culture (Figure 5).



ST2 cells were cultured in 6-well multi-plates and transfected with MOCK or p*BEST3*, then cultured for 21 days. Cells were harvested every 7 days, washed with PBS, lysed in 200 μL of RIPA buffer, sonicated, and the supernatant was used for RANKL ELISA. ***indicates $p < 0.001$ compared to MOCK ($n = 4$).

Figure 5. Effect of p*BEST3* transfection on RANKL protein expression.

4. Discussion

A whole-exome analysis of Japanese patients with skeletal mandibular prognathism reported the presence of a nonsynonymous single-nucleotide variant of *BEST3* [22]. *BEST3* mRNA undergoes various alternative splicing processes [18] [21] [29]-[32]. Among these variants, some lack an ion-conducting domain and their functions remain unknown. Thus, the functional implications of the L606I variant discovered by Kajii *et al.* [22] remain unclear. Therefore, we conducted this study to test the hypothesis that *BEST3* itself may exert some influence on bone formation and metabolism. Human *BEST3* cDNA was overexpressed in mouse ST2 cells, because few established human mesenchymal cell lines reproducibly undergo osteoblastic differentiation and mineralization while retaining the characteristics of multipotent undifferentiated mesenchymal cells.

Transient forced expression of p*BEST3* leads to ectopic expression of *BEST3*. This represents approximately one-sixth of the expression level of *Actb,* which is known to be expressed at relatively high levels, indicating that *BEST3* was ectopically expressed at a lower level than *Actb* (Table 2). One possible concern was that the detected *BEST3* amplification reflected amplification of endogenous mouse *Best3*. This possibility was considered unlikely, because no amplification was ob-

served in the MOCK control group, even after 65 cycles of qPCR. Moreover, the antisense primer shared only 42.9% sequence homology with mouse *Best3*, making amplification of the endogenous mouse transcript highly improbable (**Table 2**, lower panel). Overexpression of *BEST3* neither promoted calcification in ST2 cells cultured for 3 weeks in α-MEM medium supplemented with β-glycerophosphate and ascorbic acid nor suppressed the promotion of calcification induced by simvastatin (**Figure 2**). These results suggest several possible explanations. (1) *BEST3* drives factors that both induce and suppress calcification accumulation. (2) Although *BEST3* induces (or suppresses) the expression of osteoblast differentiation factors, its transient expression prevents maintenance of this phenotype until calcified granules accumulate. (3) *BEST3* did not affect ST2 differentiation. To investigate these possibilities, we monitored the gene expression of BMP-2 and BMP-4 [33], proteins that play central roles in bone differentiation, every other day until day 6 of culture—a time point known to maintain sufficient expression even with transient gene transfection (**Figure 3A**, **Figure 3B**). The results showed that *BEST3* did not cause any significant changes in the expression of *Bmp2* or *Bmp4*. This was also true for the gene expression of osteocalcin [34], a bone-specific non-fibrillar protein (**Figure 3C**), alkaline phosphatase (**Figure 3D**), and the pro-α-chain genes of type I and type III collagen (**Figure 3E**, **Figure 3F**). These results suggest that the ectopic overexpression of *BEST3* does not affect ST2 cell differentiation.

We investigated how *BEST3* expression affects the differentiation and activation of osteoclasts, which regulate bone mass changes *in vivo* [35]. *BEST3* overexpression in ST2 cells significantly induced the expression of *Tnfsf11*, a gene encoding RANKL (**Figure 4A**, **Figure 4B**). However, it had little effect on the expression of *Tnfrsf11b*, which encodes OPG (**Figure 4C**, **Figure 4D**). Even after the addition of simvastatin, no significant changes in *Tnfsf11* and *Tnfrsf11b* expression were observed. In contrast, the gene expression of Csf1, the gene encoding M-CSF, showed almost no change with p*BEST3* introduction in the absence of simvastatin (**Figure 4E**), but increased significantly on days 8 - 12 in the presence of simvastatin compared to the absence of simvastatin, and p*BEST3* introduction further accentuated this (**Figure 4F**). A possible explanation for this is that *BEST3*, like simvastatin, may inhibit the prenylation of Rho to the cell membrane [36]; however, since it did not promote *Bmp2* expression (**Figure 3A**), this possibility appears extremely unlikely. Furthermore, changes in *Tnfsf11* and *Csf1* expression induced by *BEST3* overexpression appeared from day 8 of culture. This suggests the following possibilities: 1: Ectopically overexpressed *BEST3* acts on something, which indirectly promotes the expression of *Tnfsf11* and *Csf1*. 2: The decline in transiently overexpressed *BEST3* triggers the promotion of *Tnfsf11* and *Csf1* expression. These mechanisms remain unclear. However, it is clear that *BEST3* expression in osteoblasts promotes, at a minimum, the expression of RANKL. Importantly, the increase in *Tnfsf11* expression without a corresponding increase in *Tnfrsf11b* expression is expected to increase the functional RANKL/OPG ratio, thereby favoring osteoclast differentiation and activation. Furthermore, the con-

comitant increase in *Csf1* expression would be expected to further promote osteoclastogenesis by supporting the survival, proliferation, and differentiation of osteoclast precursor cells. Taken together, these findings suggest that, if similar changes occur *in vivo*, *BEST3* overexpression may create a microenvironment favorable for osteoclast formation and bone remodeling. Further research is needed to determine whether this is associated with the genetic factors underlying skeletal mandibular prognathism.

The gene transfer method used in this study was transient transfection rather than stable transfection. Successful *BEST3* overexpression was confirmed by RT-qPCR at 48 h after transfection. In proliferating cells, transiently transfected plasmids generally produce maximal mRNA and protein expression within 24 - 72 h after transfection, after which expression gradually declines and is typically lost within approximately 7 days as the plasmid is diluted during cell division [37]. Because we did not directly monitor the duration of *BEST3* overexpression throughout the differentiation period, the persistence of transgene expression beyond the early post-transfection phase remains unknown and should be considered a limitation of this study. Nevertheless, the delayed changes in *Tnfsf11* and *Csf1* expression observed in the present study suggest that transient *BEST3* overexpression may initiate downstream regulatory events rather than directly maintaining their expression.

5. Conclusion

Overexpression of ectopic *BEST3* in osteoblast-like ST2 cells promotes RANKL gene and protein expression.

Acknowledgements

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This study was approved by the Ohu University Genetic Recombination Safety Committee (2022001-3) and was conducted in accordance with the Ohu University Regulations on the Safety Management of Genetic Recombination Experiments.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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