Chapter 2

Snail in TGFβ and BMP-induced endothelial to osteoblast transdifferentiation

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To be submitted

Abstract

Endothelial cells were recently shown to be capable of differentiating into osteoblasts, and were also identified to function as osteo-precursor cells for ectopic bone formation in Fibrodysplasia Ossificans Progressiva patients. In this study, the cooperation between the TGFβ and BMP signalling pathways is investigated to determine how they mediate the transdifferentiation of endothelial cells into osteoblasts. Whereas pre-treatment of endothelial cells with TGFβ greatly potentiated the BMP-induced osteoblast differentiation in endothelial cells, BMP signalling was found to be required for this response. Co-treatment with TGFβ and BMP induced *snail* expression, while ectopic *snail* expression potentiated the BMP-induced osteoblast differentiation in endothelial cells.

Key words: BMP, differentiation, endothelial, osteoblast, signalling, *snail*, TGFβ,

Introduction

In 1965，Urist discovered a group of proteins isolated from demineralised bone matrix that showed the ability to induce the development of bone when injected subcutaneously under the skin of rodents, as a result, these proteins were named bone morphogenetic proteins (BMPs) [1]. BMP signalling is a critical pathway controlling osteoblast differentiation. The signal is initiated with the formation of a heteromeric complex between BMP type I and type II serine/theronine kinase receptors at the cell membrane. Upon ligand binding, the type I receptor, also termed the activin receptor-like kinase (ALK), is activated through phosphorylation in its glycine-serine (GS) residue rich juxtamembrane domains by the type II receptor [2]. Subsequently, the activated type I receptors phosphorylate the BMP receptor-regulate Smad, Smad1/5/8. In mammals, there are seven type I receptors. BMPs mainly utilizes ALK1/2/3/6 as type I receptors and Smad1/5/8 as R-Smads, whereas TGFβ mostly signals via ALK4/5/7 using Smad2/3 as R-Smads. In endothelial cells TGFβ could also signal via ALK1 with Smad1/5/8 as R-Smads [2, 3]. The activated Smad1/5/8 can form heteromeric complexes with Smad4, which accumulate in the nucleus, where they can act together with other transcriptional factors, co-activators and co-repressors, to regulate osteogenic gene expression [2].

Fibrodysplasia Ossificans Progressiva (FOP) is a rare heritable genetic disease in which acute inflammation enhances bone formation. The patients are born without aberrations except for deformed great toes. However, in the first decade of patient lives, painful inflammatory responses can gradually cause transformation of soft tissue into ectopic bone via mechanisms that are not well characterized [4]. The mutated gene causing the disease was identified as the BMP type I receptor ALK2 [5]. The classic FOP associated mutation, R206H, occurs in the GS domain of ALK2 that is required for binding of FKBP12, an intracellular inhibitor of BMP receptor signaling. Further studies indicated that the mutated ALK2 with defective FKPB12 binding confers elevated BMP signalling to cells [6-8]. Initially, mesenchymal stroma cells residing in the soft tissue, which have the potential to differentiate into multiple mesodermal cell types, were considered to be the osteo-progenitor cells for the ectopic bone tissues [9-12]. However, recent studies on ectopic bone formation in FOP patients and a transgenic mice model with constitutively active ALK2 have proposed an endothelial origin for heterotopic cartilage and bone. Briefly, it was demonstrated that under specific conditions the endothelial progenitor cells and the mature endothelial cells can be transdifferentiated into osteoblasts via one process called Endothelial Mesenchymal transition (EndoMT). [13, 14].

Medici and colleagues discovered that BMP4 and TGFβ2 mediate the transition of endothelial cells into osteoblasts [14]; Interestingly, Medici *et al.*also showed that the other BMPs such as BMP7 had no effect on this transition. Furthermore both ALK2 and ALK5 were demonstrated to be necessary for EndoMT, the initial step for transition of endothelial cells into osteoblasts [14]. However, the precise molecular mechanisms underlying the transition of endothelial cells into multipotent mesenchymal cells and the subsequent osteoblasts are still unclear.

In this study, the differential requirements for TGFβ and BMP in the EndoMT and subsequent osteoblast differentiation process have been investigated, and in

particular the effector role for the transcription factor *snail* in BMP-induced osteoblast differentiation in endothelial cells has been studied.

Materials and Method

Materials. Recombinant human BMP6 and BMP7 were obtained from R&D systems (Minnapolis, MN, USA). Recombinant human TGFβ3 was kindly provided by Dr K Iwata (OSI Pharmaceuticals, Melville, NY, USA). SB431542 (SB) was purchased from Tocris/Biotrend GmbH (Koln, Germany). LDN-194189 (LDN) was kindly provided by Herbert Y. Lin (Massachusetts General Hospital, Harvard Medical School, Boston, USA).

Cell culture. Mouse embryonic endothelial cells (MEECs), and human epidermal kidney cells HEK293T were maintained in a proliferation medium: DMEM (Gibco BRL, Carlsbad, CA, USA) supplemented with penicillin/streptomycin (Invitrogen, Carisbad, CA, USA) and 10% fetal bovine serum (FBS, Gibco, BRL, Carlsbad, CA, USA). MEECs were grown at 37°C in a humidified incubator 5% CO2. For the mineralization assay, MEECs were maintained in osteogenic medium: α modified essential medium (α-MEM, Gibco, BRL, Carlsbad, CA, USA) containing 10% FBS (Gibco), 0.2 mM L-ascorbic acid-2-phosphate (Sigma, St.Louis, MO) and 5 mM β-glycerophosphate (Sigma, St.Louis, MO)

Lentivirial transfection and construction of stable cell lines. Lentiviruses were produced by transfected MISSION® shRNA Plasmid DNA together with helper plasmids pCMV-VSVG, pMDLg-RRE and pRSV-REV into HEK293T. Cell supernatants were harvested 2 days after transfection and were used to infect MEECs. After infection, MEECs were maintained in medium containing 4 μg/ml puromycin medium.

Alkaline phosphatase assay. MEECs were cultured in proliferation medium in the absence or presence of BMP. Histochemical examination of alkaline phosphatase (ALP) activity was performed using naphtol AS-MX phosphate (Sigma, St.Louis, MO) and fast blue RR salt (Sigma, St.Louis, MO), as described previously [6]. For quantification of ALP activity, cells were cultured in 48-well plate. Alkaline phosphatase was induced upon BMP stimulation. Then cells were washed twice with phosphate buffered saline (PBS) and subsequently lysed by adding 80 μL of ALP lysis buffer [31].

Mineralization assay. MEECs were cultured in osteogenic medium in the absence or presence of BMP. Cells were stained with alizarin red S. Cells were washed with PBS and then fixed in 3.7% formaldehyde. The cells were then washed with PBS again and incubated with 2% alizarin red S solution (pH 5.4) for 1 min, and subsequently washed with distilled water.

Western blotting. Western blotting was performed as previously described [32]. The antibodies used for immunoblotting were as follows: P-Smad1/5/8 (Cell Signalling, USA) for detection of phosphoryalated Smad1/5/8 and GAPDH antibody (Sigma, St.Louis, MO) was used as the loading control.

RNA isolation and qRT-PCR. Total RNA was isolated using the NucleoSpin RNA II Kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's protocol. Subsequently, cDNA was reversed transcribed from 1 μg total RNA using the Revert

Aid protocol (Fermentas, St.Leon-Rot, Germany) with Oligo-dT primers (Fermentas). Quantitative Real-Time PCR analysis was performed using the Roche 480 lightcycler and the relative expression level of the gene of interest was determined in triplicate for each sample using the $2^{-\triangle\triangle CT}$ method. Values were normalized to GAPDH expression. Primers used for qRT-PCR were:

mouse BSP FW	5'-AGGGAACTGACCAGTGTTGG-3'
mouse BSP RV	5'-ACTCAACGGTGCTGCTTTTT-3'
mouse OSC FW	5'-AGACTCCGGCGCTACCTT-3'
mouse OSC RV	5'-CTCGTCACAAGCAGGGTTAAG-3'
mouse SNAIL FW	5'-TCCAAACCCACTCGGATGTGAAGA-3'
mouse SNAIL RV	5'-TTGGTGCTTGTGGAGCAAGGACAT-3'
mouse GAPDH FW	5'-AACTTTGGCATTGTGGAAGG-3'
mouse GAPDH RV	5'-ACACATTGGGGGTAGGAACA-3'
Mouse slug FW	5`-CACATTCGAACCCACACATTGCCT-3`
Mouse slug RV	5`-TGTGCCCTCAGGTTTGATCTGTCT-3`

Results

MEECs are able to mineralize under osteogenic conditions

Since it was previously shown that Mouse embryonic endothelial cells (MEECs) from different sources might undergo EndoMT [15] MEECs were chosen as the cell model to check whether MEECs can undergo osteoblast differentiation. As proposed by Medici *et al.* [14], MEECs were pre-treated with either TGFβ3 or BMP4 to induce EndoMT transition, in order to convert MEECs into mesenchymal-like stem cells. MEECs, when stimulated with TGFβ3, were found to undergo a morphological change and started to express αSMA, indicative for mesenchymal cells. However, BMP4 was not found to change the cells morphology or induce αSMA expression (Fig 1A). To verify whether MEECs can undergo osteoblast differentiation after EndoMT transition, MEECs were cultured under the osteogenic conditions as indicated in Fig 1B, and two marks for osteoblast differentiation: alkaline phosphatase (ALP) activity and mineralization were analyzed. The MEECs cultured under osteogenic condition were positive for both ALP activity and alizarin red staining (ARS), whereas the control MEECs grown in proliferation medium without TGFβ or BMP treatment were negative for both ALP and ARS (Fig1 B a-d). At the same time, the osteogenic gene expression was examined in both control MEECs and MEECs in osteogenic condition, and the osteogenic genes *OSC* and *BSP* were shown to be significantly elevated in MEECs cultured under osteogenic condition (Fig1 B). These results suggest that under specific conditions, osteoblast differentiation program can be activated in MEECs.

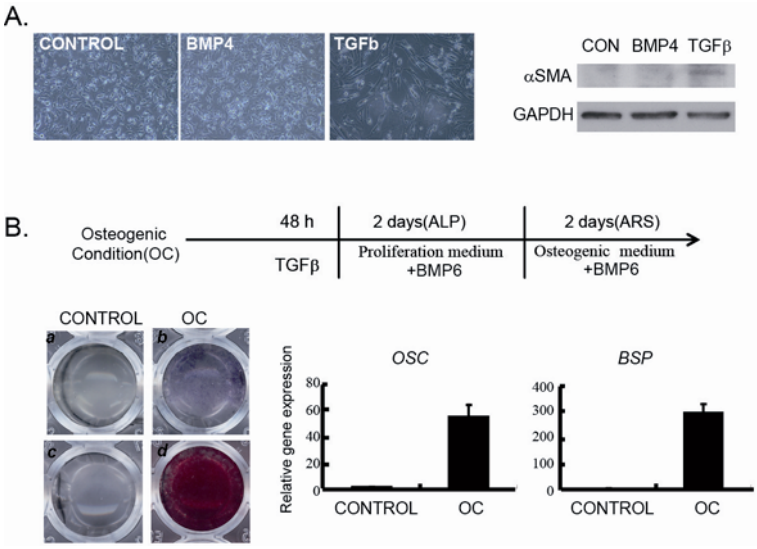

Fig1. MEECs were differentiated into osteoblasts after TGFβ induced Endo-MT transition. (A). MEECs were cultured in proliferation medium, and stimulated with 50 ng/ml BMP4, 5 ng/ml TGFβ or vehicle control. 2 days after stimulation, cell morphology was examined and pictures were taken. Total cell lysates were analyzed by immunoblotting with αSMA or GAPDH antibodies. (B). MEECs cells were cultured in proliferation medium, and stimulated with 5 ng/ml TGFβ for 2 days. Then cells were cultured in proliferation medium containing 100 ng/ml BMP6. ALP activity associated cells were measured histochemically 2 days after BMP6 stimulation (a, b). mineralization was measured 4 days after BMP6 stimulation (c, d) by performing alizarin red staining (ARS). 4 days after BMP6 stimulation, RNA was isolated and OSC and BSP transcripts were measured by qRT-PCR. GAPDH was used as the reference. The control sample is the cells cultured in proliferation medium without BMP or TGFβ treatment.

TGFβ induced EndoMT transition is not required for BMP induced osteoblast differentiation in MEECs

In previous studies, it has been demonstrated that osteoblast differentiation can be observed in MEECs cultured under osteogenic conditions. The next step was to check whether TGFβ–induced EndoMT transition is required for this process. Therefore MEECs were directly stimulated with BMP6 in osteogenic medium without TGFβ pretreatment. Unexpectedly, BMP signalling was found to be able to directly induce ALP activity and mineralization in MEECs even in the absence of TGFβ-induced EndoMT (Fig 2A-D). MEECs were stimulated with BMP6, BMP7, TGFβ or vehicle control in proliferation medium, after 3 days of stimulation, both BMP6 and BMP7 were able to induce ALP activity in MEECs, whereas TGFβ alone did not induce ALP activity at all. Interestingly, BMP6 was more potent than BMP7 with regards to the induction of ALP activity （Fig 2A）. Moreover, using an ARS

staining, we observed BMP6-induced mineralization after 6 days continuous stimulation in osteogenic medium, and this effect was completely abolished by treatment with the BMP receptor type I inhibitor LDN-193189 (LDN) (Fig 2D). Results also showed that BMP signalling is necessary for the induction of mineralization as LDN, the BMP inhibitor that efficiently represses BMP-induced Smad1/5/8 phosphorylation (Fig 2F), abolished BMP6 induced mineralization in MEECs (Fig 2D). QRT-PCR analysis confirmed the elevated expression of the osteogenic genes, *OSC* and *BSP* in MEECs stimulated with BMP6 for 6 days (Fig 2E), whereas LDN blocked the expression of *OSC* and *BSP* (Fig 2E). From these results, it can be concluded that activation of BMP signalling mediates osteoblast differentiation in MEECs.

Our results have shown that BMP4 cannot induce EndoMT-related morphological changes in MEECs. To test whether BMP6 or BMP7 can induce EndoMT transition in MEECs, MEECs were stimulated with BMP6, BMP7, TGFβ3 or vehicle control for 48 hours. TGFβ3 induced EndoMT transition, with the appearance of the mesenchymal marker α-SMA, and decrease of the endothelial marker PECAM-1(Fig 2C); however, neither BMP6 nor BMP7 showed any effect on the expression of α-SMA or platelet endothelial cell adhesion molecule (PECAM)-1 (Fig 2C) thus suggesting that BMP6 and BMP7 induced osteogenic differentiation requires mechanisms other than the previously reported EndoMT transition.

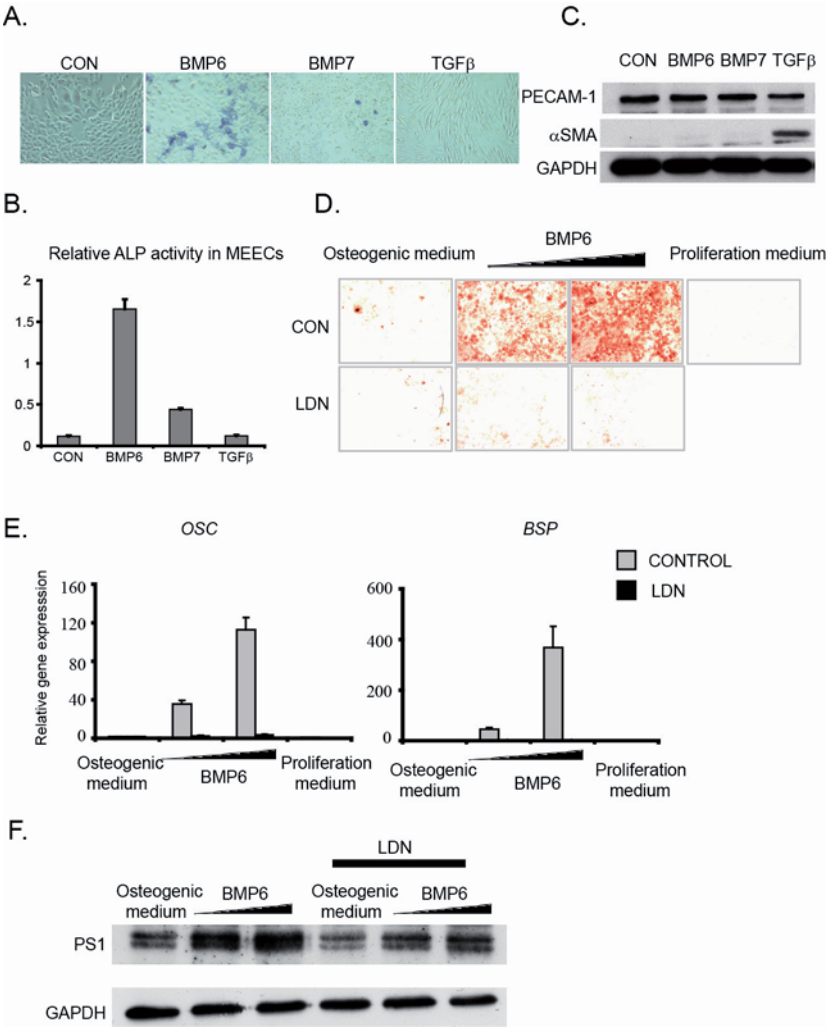

Fig2: BMP signaling directly induces MEECs differentiation into osteoblast. (A) Confluent MEECs were stimulated with 100ng/ml BMP6, 100 ng/ml BMP7 or 5 ng/ml TGFb for 3 days in proliferation medium; ALP activity associated with cells was measured histochemically. (B) Quantification of BMP6, BMP7 or TGFβ induced ALP activity in MEECs 3 days after stimulation. Values represent the average of three independent experiments corrected with total protein concentration. (C) Confluent MEECs were stimulated with 100 ng/ml BMP6, 100 ng/ml BMP7 or 5 ng/ml TGFβ for 3 days in proliferation medium, total cell lysates were analyzed by immunoblotting with aSMA, PECAM-1 or GAPDH. (D) Confluent MEECs were stimulated with 100 ng/ml BMP6, 200 ng/ml BMP6 or vehicle control in the presence or absence of BMP inhibitor LDN for 6 days in osteogenic medium. Mineralization was visualized histochemically with alizarin red staining. (E) Confluent MEECs were stimulated with 100ng/ml BMP6，

or 200ng/ml BMP6 or vehicle control in the presence or absence of BMP inhibitor LDN for 6 days in osteogenic medium, RNA was isolated, osteogenic gene osteocalcin(OSC) and bone sialoprotein (BSP) expression were measured by qRT-PCR. Gene expression was normalized with GAPDH and expressed as fold change relative to the control. (F) Confluent MEECs were stimulated with 100 ng/ml BMP6, 200ng/ml BMP6 or vehicle control for 2 days in the presence or absence of BMP inhibitor LDN in the proliferation medium. Cell lysates were analyzed by immunoblotting with phosphorylated Smad1/5/8 or GAPDH

TGFβ3 pre-treatment sensitized MEECs for BMP6-induced cell differentiation

Our data shows that EndoMT transition induced by TGFβ pretreatment is not required for BMP-induced mineralization in MEECs. Since EndoMT changes endothelial cells into a mesenchymal phenotype and BMP signaling is able to stimulate mesenchymal cells into bone, we investigated if TGFβ pretreatment would enhance mineralization. MEECs were stimulated with either TGFβ3 or vehicle control for 48 hours to induce EndoMT transition, followed by incubation in proliferation medium for 2 days, and osteogenic medium for 4 days. Consistent with previous results, ALP activity and mineralization was observed in the samples stimulated with BMP6 (Fig 3A, 3B), with elevated expression of osteogenic gene *OSC* and *BSP* (Fig 3C). TGFβ pre-treatment significantly enhanced BMP6-induced ALP activity and mineralization in MEECs (Fig 3A, 3B). The osteogenic genes *OSC* and *BSP* were also dramatically enhanced in the samples that were pre-treated with TGFβ3 (Fig 3C).

Previous studies suggested the necessary role of ALK5, the TGFβ type I receptor, in the conversion of endothelial cells into mesenchymal-like cells [14]. To study the possible involvement of endogenous TGFβ or ALK5 activity in the conversion of MEECs into osteoblasts, a kinase inhibitor for the ALK5, i.e. SB-431542 (SB), was used to repress the endogenous ALK5-mediated TGFβ activity. The SB had no effect on BMP6-induced Smad1/5/8 phosphorylation (Fig 3D), but efficiently repressed BMP6-induced ALP activity and mineralization in MEECs (Fig 3A, 3B). The osteogenic genes *OSC* and *BSP* were also repressed by the SB (Fig 3C). Consistent with the role of endogenous TGFβ activity, the presence of exogenous TGFβ activity was also shown to potentiate osteoblast differentiation in MEECs (Fig S1).

Another aim of this study was to determine why TGFβ3 pre-treatment is able to sensitize BMP6-induced osteoblast differentiation. One possibility is that TGFβ3 pretreatment might sensitize cells to BMP6 activity. To test this hypothesis, MEECs were pretreated with TGFβ3 for 48 hours, followed by BMP6 stimulation for 4 hours. BMP6-induced Smad1/5/8 phosphorylation was slightly decreased in the samples pre-treated with TGFβ3 (Fig 3D), suggesting that the cells do not show enhanced activation of BMP-mediated Smad pathway. Interestingly, the presence of continuous exogenous TGFβ3 activity was shown to slightly repress BMP6-induced Smad1/5/8 phosphorylation (Fig S1). Therefore we can not explain the TGFβ3 stimulated enhanced BMP-induced osteoblast differentiation in MEECs by increased activation of the BMP-Smad signaling pathway.

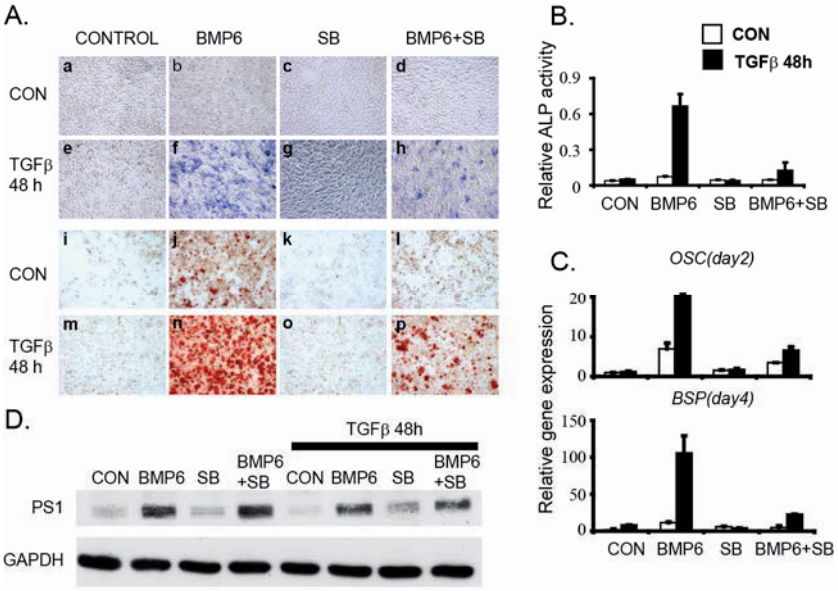

Fig3: TGFβ pretreatment sensitize MEECs to BMP6-induced osteoblast differentiation. (A) MEECs were cultured with or without 5 ng/ml TGFβ for 48 hours in the proliferation medium, and then switch cells into either proliferation medium(for ALP activity assay) or osteogenic medium(for mineralization assay). After medium refreshment, cells were stimulated with 100 ng/ml BMP6 in the presence or absence of 5 μM of TGFβ inhibitor SB431542. ALP activity associated cells were measured histochemically 2 days after BMP6 stimulation (a-h); mineralization was measured 4 days after BMP6 stimulation (i-p) by performing alizarin red staining. (B) Quantification of ALP activity for A(a-h); (C) MEECs were cultured with or without 5 ng/ml TGFb for 48 hours in the proliferation medium, and then switch cells into osteogenic medium. After medium refreshment, cells were stimulated with 100 ng/ml BMP6 in the presence or absence of 5 μM of TGFβ inhibitor SB431542 in the osteogenic medium. RNA was isolated at day 2 and day4 respectively. OSC (day2) and BSP(day4) transcripts were measured by qRT-PCR. GAPDH was used as the reference. (D) MEECs were cultured with or without TGFb for 48 hours in the proliferation medium, and then switch cells into proliferation medium. After medium refreshment, cells were stimulated with 100 ng/ml BMP6 or vehicle control in the presence or absence of SB431542 for 4 hours. Cell lysates were analyzed by immunoblotting with phosphorylation of smad1/5/8 and GAPDH.

TGFβ3 and BMP6 synergistically induce *snail* expression in MEECs

BMP-induced osteoblast differentiation and mineralization is already well studied in multiple mesoderm lineage cells, including mesenchymal stem cells derived osteo-progenitor cells, smooth muscle cells, myofibroblasts, and vascular pericytes [16-18]. TGFβ signalling pathway plays crucial roles in the induction of the mesoderm during embryogenesis [19], and BMP4 and BMP7 were also reported

to be function as mesoderm inducers [20] TGFβ signaling is even reported to be crucial for the maintenance of epithelial mesenchymal transition (EMT) [21]. Therefore we hypothesized that TGFβ signalling might induce and maintain the expression of specific factors leading to mesoderm-like differentiation, which could potentiate BMP-induced mineralizationin endothelial cells.*Snail* and *slug* are zinc finger proteins that are required for the induction of the mesoderm layer during embryonic development, and important for TGFβ induced EMT [22-25]. Furthermore recent studies have suggested that *snail* is crucial for TGFβ-induced EndoMT transition [24, 26]. While TGFβ3 was not able to induce the expression of *slug* it was increased upon BMP6 stimulation, and interestingly, TGFβ3 showed a repressive effect on the BMP6-induced *slug* expression (Fig 4). On the contrary, *snail* can be induced by both TGFβ3 and BMP6. Co-stimulation of MEECs with TGFβ3 and BMP6 enhance *snail* expression in MEECs, and inhibition of TGFβ and BMP pathways repressed either TGFβ3 or BMP6 induced *snail* expression in MEECs (Fig 4). Therefore *snail* might be the mesoderm inducer that is induced upon TGFβ3 stimulation thereby enhancing BMP-induced osteoblast differentiation in MEECs.

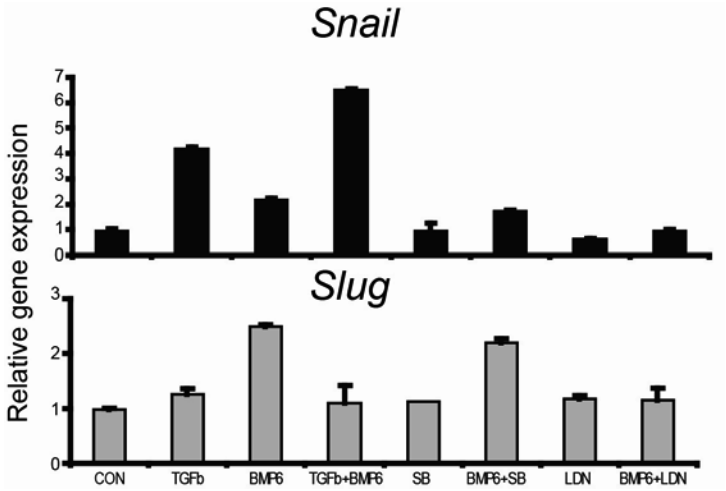

Fig4: Snail expression is induced by TGFβ and BMP6 in MEECs. MEECs were cultured in the proliferation medium with the indicated condition. 1 day after culture, RNA was isolated; snail and slug transcripts were measured by qRT-PCR, and corrected by GAPDH level.

Ectopic expression of *snail* potentiates BMP6-induced osteoblast differentiation in MEECs

To test the hypothesis that *snail* might potentiate BMP-induced osteoblast differentiation in MEECs, MEECs were stably transfected using a lentivirus expressing *snail*. Though ectopic expression of *snail* in epithelial cells can directly induce EMT transition [27], the EndoMT transition in stable MEEC cell lines with

ectopic expression of *snail* was not observed. *snail* over-expression did not induce any morphology changes nor did it induce the expression of the mesenchymal marker α-SMA (data not shown). The results obtained are consistent with a previous report showing that ectopic expression of *snail* in human umbilical vein endothelial cells (HUVEC) failed to induce EndoMT transition [15]. Therefore the enhanced osteogenic response upon pretreatment with TGFβ can not be explained by merely the induction of *snail* expression.

Next studies were performed to induce osteoblast differentiation in the stable cell line. As expected, BMP6-induced ALP activity and mineralization was dramatically enhanced in MEECs with ectopic expression of *snail* (Fig 5A, B), and the osteogenic genes *OSC* and *BSP* are also significantly elevated in MEECs with ectopic expression of *snail* (Fig 5C), supporting the hypothesis that *snail* expression potentiates BMP6-induced osteoblast differentiation in MEECs. Interestingly, the TGFβ inhibitor SB partially inhibited BMP6-induced ALP activity, mineralization and osteogenic gene expression even in the presence of ectopic expression of *snail* (Fig 5A, 5B, 5C). These results suggest that endogenous TGFβ is required but not sufficient for the regulation of s*nail* activity during BMP-induced osteoblast differentiation in MEECs.

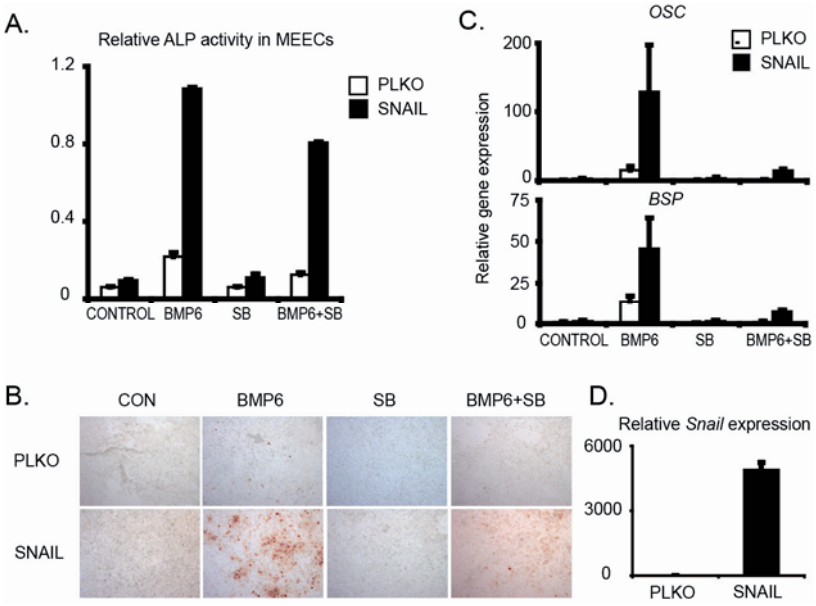

Fig5: Ectopic expression of snail sensitized BMP6 induced osteoblast differentiation in MEECs. (A) MEECs were transduced with lentivirus to generate stable cell lines overexpressing *Snail* (SNAIL) or the control cell lines (PLKO) infected with control virus. Then cells were stimulated with 100 ng/ml BMP6 in the presence of or in the absence of TGFβ inhibitor SB in proliferation medium for 3 days. ALP activity was measured. Values represent the average of three independent experiments corrected with total protein concentration. (B, C, D) MEECs stable cell lines with ectopic

expression of SNAIL or control cell lines PLKO were stimulated with 100 ng/ml BMP6 with or without TGFβ inhibitor SB for 6 days in osteogenic medium. Mineralization was visualized by alizarin red staining (B), RNA was isolated, OSC, BSP and SNAIL were measure by qRT-PCR with GAPDH as reference(C, D).

Discussion

Recently it has been demonstrated that endothelial cells are one of the sources for the osteo-progenitor cells differentiating towards osteoblasts contributing to the ectopic bone formation in FOP patients [14]. Furthermore, it was shown that endothelials undergoing a process called EndoMT upon TGFβ2 or BMP4 stimulation and the EndoMT transition induced by either TGFβ2 or BMP4 is required for the generation of multi-potent mesenchymal stem cells from endothelial cells, which subsequently can undergo osteoblast differentiation [14]. In this study we show that activation of BMP signalling is sufficient and necessary for induction of osteoblast differentiation in MEECs. Furthermore we showed that TGFβ-induced EndoMT transition, or the activation of TGFβ type I receptor might potentiate BMP-induced osteoblast differentiation in MEECs. Finally, this study shows for the first time that ectopic expression of *snail* can sensitize BMP-induced osteoblast differentiation in endothelial cells.

Snail is a zinc finger-containing transcriptional factor that potently represses the expression of the E-cadherin gene, which is essential for the maintenance of embryonic and postnatal epithelial tissue [22]. Mice mutants lacking the *snail* gene exhibit defects in the epithelial-mesenchymal transition required for the generation of the mesoderm cell layer [28]. Moreover, *snail* is an early responsive gene for the TGFβ signalling pathway, and is required for TGFβ-induced epithelial mesenchymal transition [24]. Ectopic expression of *snail* in epithelial cells can directly drive epithelial mesenchymal transition [22]. A recent study discovered that the ectopic expression of *snail* in breast epithelial cells can endow cells with mesenchymal stem cell traits [27]. Therefore *snail* is already well established for its function in the generation of mesoderm lineage cells from epithelial cells. However, the role of *snail* in the differentiation of endothelial cells is still not understood. The results presented here suggested that *snail* displayed different functions in endothelial cells compared to its role in epithelial cells. Ectopic expression of *snail* cannot induce Endo-MT transition in MEECs cells, supporting previous similar findings using HUVECs overexpressing *snail* [15]. The results of this study have shown for the first time that ectopic expression of *snail* can sensitize BMP-induced osteoblast differentiation in MEECs, suggesting an important role for *snail* in the ectopic bone formation originated in endothelial cells. Interestingly, sustained activation of *snail* in transgenic mice displayed deficient osteoblast differentiation, since *snail* can directly bind to the promoter region and repress the transcription of *Runx2*, which is a transcription factor that is necessary for further osteoblast differentiation [26]. However, it has been observed that over-expression of *snail* in endothelial cells enhanced *RunX2* expression as well as other osteogenic genes (Fig S2). These results implied that *snail* might have a different function in induction of osteogenic genes in endothelial cells.

Ectopic expression of *snail* could promote BMP induced mineralization in MEECs, suggesting TGFβ induced *snail* is a positive regulator for BMP induced mineralization in MEECs. However, knocking down of *snail* by siRNA did not alter sensitivity of MEECs undergoing TGFβ induced EndoMT or repress BMP-induced mineralization in MEECs (data not shown). It is possible that Snail levels that remain remain after knock down are still sufficient to mediate EndoMT-osteoblast differentiation, or that the function of snail is compensated by other related family members that are found frequently to be induced by TGF-β family members. Till now the exact role of *snail* in EndoMT is still elusive as *snail* showed multiple functions in regulating EndoMT in endothelial cells from different origins [15, 29, 30]. Our work for the first time proposed a novel function for *snail* in enhancing BMP induced mineralization in endothelial cells. The future work might focus on whether other mesoderm inducers including Twist/Zeb1 might be induced by TGFβ and function with *snail* to promote mineralization in MEECs and other endothelial cells. In conclusion, this study shows that TGFβ by inducing snail expression can enhance BMP-induced osteoblast differentiation of endothelial cells.

Acknowledgements
This work was supported by the Netherlands Organization for Scientific Research (NWO 918.66.066), IOP Genomics grant on "Towards broad clinical and technological application of gene expression engineering by exon skipping" and Centre for Biomedical Genetics.

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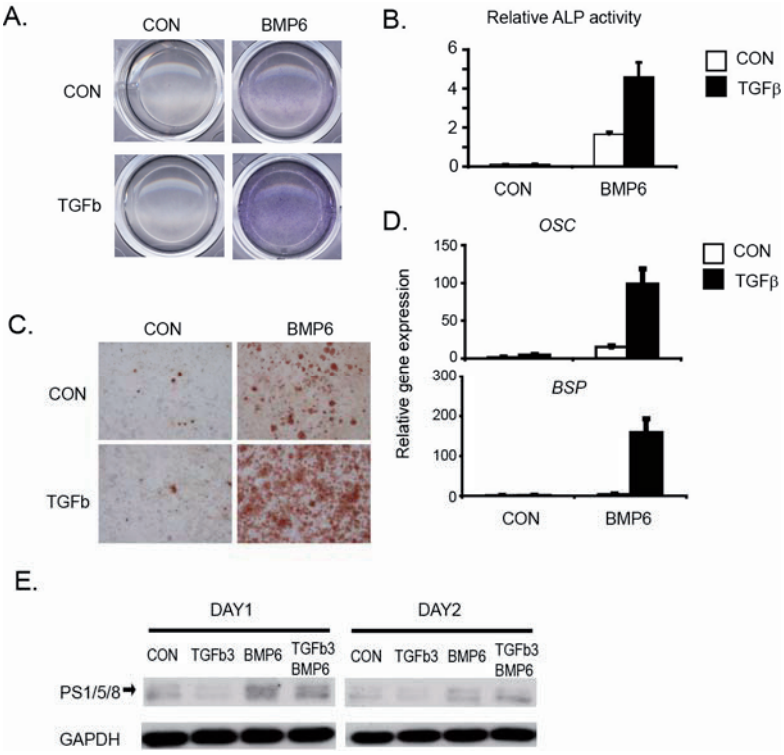
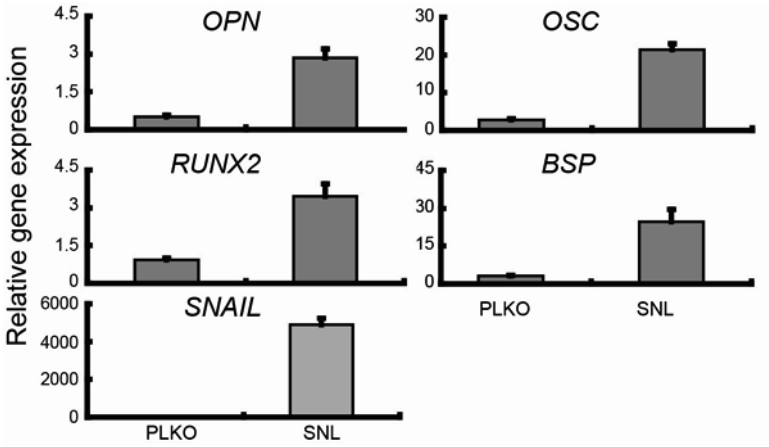


FigS1: TGFβ sensitizes MEECs to BMP6 induced osteoblast differentiation. (A) MEECs were stimulated with 100 ng/ml BMP6 or vehicle control in the presence or absence of 5 ng/ml TGFβ for 3 days in proliferation medium. ALP activity associated cells were visualized histochemically. (B) Quantification of ALP activity in (A). (C)(D) MEECs were stimulated with 100ng/ml BMP6 or vehicle control in the presence or absence of 5 ng/ml TGFβ for 6 days in osteogenic medium. Mineralization was visualized by alizarin red staining(C). RNA was isolated and osteogenic gene *OSC* and *BSP* was measured by qRT-PCR. (E) MEECs were stimulated with 100 ng/ml BMP6 in the presence or absence of 5 ng/ml TGFβ for 1 day and 2 days respectively. Cell lysates were analyzed by immunoblotting with phosphorylation of smad1/5/8 and GAPDH.



FigS2. Osteogenic gene expression in MEECs stable cells lines. MEEC stable cell lines with ectopic expression of snail or control cell lines were stimulated with 100 ng/ml BM6 in osteogenic medium for 6 days. Osteogenic genes were examined and compared through q-PCR. GAPDH was used as reference.