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Profilin Expression is Regulated by Bone Morphogenetic Protein (BMP) in Osteoblastic Cells

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ABSTRACT

Profilin 1 (Pfn1) regulates cytoskeletal reorganization and migration, but its role in osteoblasts is not known. BMP (bone morphogenetic protein) is a multifunctional cytokine involved in osteoblastic differentiation and promotes bone regeneration and repair. Although several molecules are known to modulate BMP signaling, mechanisms that determine the levels of BMP action in osteoblastic function are still incompletely understood. We therefore examine the expression of Pfn1 in osteoblasts and its role in BMP-induced differentiation in osteoblasts. In osteoblastic MC3T3-E1(MC) cells, Pfn1 mRNA is expressed constitutively and its expression levels are declined during the culture in a time dependent manner in contrast to the increase in alkaline phosphatase activity revealing that Pfn1 expression is down regulated along with differentiation. To test the effects of osteoblastic differentiation on Pfn1expression further, MC cells are treated with BMP. BMP treatment suppresses the levels of Pfn1 mRNA. This suppressive effect of BMP is time dependent and further down regulation of Pfn1 mRNA levels is observed when the BMP treatment is continued for a longer period of time. Pfn1mRNA knock down (KD) by siRNAs enhances BMP-induced increase in alkaline phosphatase (Alp) activity in MC cells. To analyze the regulatory mechanism, Alp mRNA levels are examined and Pfn1 KD enhances the BMP-induced increase in the levels of Alp mRNA expression. Furthermore, Pfn1 KD enhances BMP-induced transcriptional expression of luciferase reporter activity via BMP response element in osteoblasts. These data indicate that Pfn1 is a novel target of BMP and suppresses BMP-induced differentiation of osteoblasts at least in part via transcriptional event. J. Cell. Biochem. 117: 621–628, 2016.

KEY WORDS: PROFILIN; BMP; OSTEOBLAST

Osteoporosis is one of the most prevalent diseases in the world and affects approximately 20 million patients in the United States [Cauley et al., 2014; Crandall et al., 2015; Jha et al., 2015]. This disease causes a significant mortality in the elderly patients and a certain fraction of patients would not survive more than one year once femoral neck fractures occur. As aged population is soaring worldwide, patients with low physical activities or bed-ridden patients are increasing due to muscular weakness and/or a higher prevalence rate in age-associated diseases such as neuronal, cardiovascular, and malignant diseases. These frail patients or bed ridden patients are prone to suffer from disuse osteoporosis since bone loss proceeds rapidly upon loss of mechanical stress [Alexandre and Vico, 2011; Lau and Guo, 2011; Armas and Recker, 2012].

Disuse osteoporosis is due to suppression of osteoblastic bone formation and enhancement of osteoclastic bone resorption and these are resulting in a simultaneous exacerbation in both of the two arms of bone metabolism [Conover et al., 2002; Li et al., 2005; Squire et al., 2008]. This situation is different from postmenopausal osteoporosis in that bone formation is enhanced. In contrast, it is significantly suppressed in disuse osteoporosis. However, how loss of mechanical stress affects bone formation by osteoblasts is still incompletely understood [Ishijima et al., 2001; Nagao et al., 2011;

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Chen and Jacobs, 2013; Klein-Nulend et al., 2013]. In search for molecular bases that would be involved in mechanical stress regulation of osteoblastic function, we focused on profilin as it is a critical regulator of cytoskeleton [Dominguez, 2009; Courtemanche et al., 2013] that could be involved in mechanical response in osteoblasts.

Profilin is known to control cytoskeleton by promoting actin polymerization and migration in many cell types [Witke, 2004; Yarmola and Bubb, 2009; Ding et al., 2012]. There are four profilin isoforms and among those profilin 1 is ubiquitously expressed and is a major form that regulates cytoskeleton. Profilin binds to monomeric G-actin and this complex adds new actin monomer to the barbed end of actin filaments. Thus, profilin is one of the major regulators of cytoskeletal reorganization in the cells. Cytoskeletal system renders migration property, structure of the cells under certain mechanical environments and supports the attachment of the cells to their substrates. Through these activities, cytoskeleton is involved in cellular response to mechanical stress [Snider and Omary, 2014; Liu and Lee, 2014; Stachowiak et al., 2014; Maier et al., 2015].

Bone formation is based on osteoblastic activity and these osteoblasts are sensitive to mechanical stress [Aryal et al., 2013; Suzuki et al., 2013; Shirakawa et al., 2014]. The loss of stress is known to lead to reduction in bone formation and disuse osteoporosis [Lau and Guo, 2011; Nagao et al., 2011; Armas and Recker, 2012]. Animals subjected to experimental model of disuse osteoporosis, such as hind limb suspension or tail suspension, lose bone rapidly due to suppression of bone formation and enhancement of bone resorption [Kondo et al., 2005, Dickinson et al., 2004, Mizoguchi et al., 2008, Hino et al., 2007 and Robling and Turner, 2009]. Regarding suppression of bone formation, loss of mechanical stress suppresses osteoblastic bone formation rate, mineral apposition rate, and mineralization surface. These suppressed-features are reduced by deficiency of osteopontin, a protein related to cell attachment to the substrates and bone matrix. Though osteoblastic attachment via osteopontin is based on integrins that signal to cytoskeleton and gene expression, molecular bases connecting cytoskeleton and ostoblastic function are not well understood.

Bone formation requires appropriate mechanical environment where it needs osteblastic differentiation and migration, both of which are controlled by BMP [Canalis, 2009; Lin and Hankenson, 2011; Chen et al., 2012]. Thus, certain controls for cytoskeleton and those for BMP may be related to each other. However, the link between cytoskeleton and BMP is still incompletely understood. We therefore examine the expression levels of profilin and its functional relationship to BMP actions in osteoblasts.

MATERIALS AND METHODS

CELL CULTURE

The osteoblast cell line MC3T3-E1 (Riken, Saitama, Japan) were cultured in alpha-minimum essential medium (Gibco, Life technologies, USA) supplemented with 10% fetal bomine serum (FBS; Gibco) and 1% antibiotic-antimycotic (Gibco; 1078517) in a 5% CO_2 humidified incubator at 37°C. The cells were passaged when they were about 80% confluent, and were lifted using a trypsin (0.25%), EDTA (1 mM) (Invitrogen) solution and subcultured in 10 cm dishes. The medium was changed twice a week. For BMP treatment, human recombinant BMP2 provided by Yamanouchi Pharmaceuticals Co. was used.

QUANTITATIVE RT-PCR ANALYSIS

Total RNA was extracted from cultured cells using TRIzol regent (Life technologies) according to manufacturer's instruction. 1 μg of total DNA free-RNA was used to synthesize cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA). cDNA equivalent to 10 ng of total RNA was used for real-time PCR reaction with Applied Biosystems StepOne Real-time PCR system "Step One" (Life technologies). RT-qPCR analyses were performed to detect Pfn-1, and alkaline phophatase (ALP) mRNA expression, and GAPDH was used as an internal control. The primers employed in assays were as follows: Pfn-1 forward 5′-TCTTTGCCTACCAGGACACC-3′, Pfn-1 reverse 3′-TTCCCCTCTTTGCTTCTGC-5′, ALP forward 5′-GCTATC TGCCTTGCCTGTATCTG-3′, ALP reverse 3′-AGGTGCTTTGGGAA TCTGTGC-5′, GAPDH forward 5′-AGAAGGTGGTGAAGCAGG-CATC-3′, GAPDH reverse 3′- CGAAGGTGGAAGAGTGGGAGTTG-5′.

siRNA TRANSFECTION

MC3T3-E1 cells were plated on at the density of 10,000 cells/cm^2. 24 h later the cells were transfected with control or Pfn1 siRNAs (Invitrogen, #4390843 and #s71525) using RNAi MAX reagent (Invitrogen). The transfected cells were cultured at standard condition for about 72 h.

ALKALINE PHOSPHATASE ASSAY

The levels of alkaline phosphatase (Alp) activity and protein were determined according to PNPP method. For this assay, the cells were rinsed twice with PBS and scraped into 10 mM Tris-HCl containing 2 mM $MgCl_2$ and 0.05% Triton X-100 at pH 8.2 to prepare cell lysates. The cell lysates were frozen and stored at −20°C. After thawing, aliquots of cell lysates were mixed with an aliquots of assay buffer containing 10 mM p-nitrophenyl phosphate in 0.1 M sodium carbonate, pH10 supplemented with 1 mM $MgCl_2$. This mixture was incubated at 37°C for 30 min. After adding 1 M NaOH, amounts of p-nitrophenol released in the assay mixtures were determined using a spectrophotometer.

LUCIFERASE ASSAY

MC3T3-E1 cells were plated at a density of 10,000 cells/cm^2 in α-MEM supplemented with 10% FBS and cultured overnight. These cells were transfected with reporter plasmid constructs containing BRE (BMP response element) linked to luciferase using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). After transfection, the cells were treated with BMP for 24 h. Renilla expression vector was also co-transfected to monitor transfection efficiency. The cells were then harvested, and luciferase activities in the cell extracts were measured based on light using Lumat LB9507 (Berthold).

STATISTICAL ANALYSIS

The data was expressed as mean ± SEM (standard error of a mean) for each group. Differences between the groups were analyzed based on Student's *t-test*. For evaluation of interaction, two way analysis of variance (ANOVA) was applied. *P* values less than 0.05 or 0.01 were considered to be statistically significant.

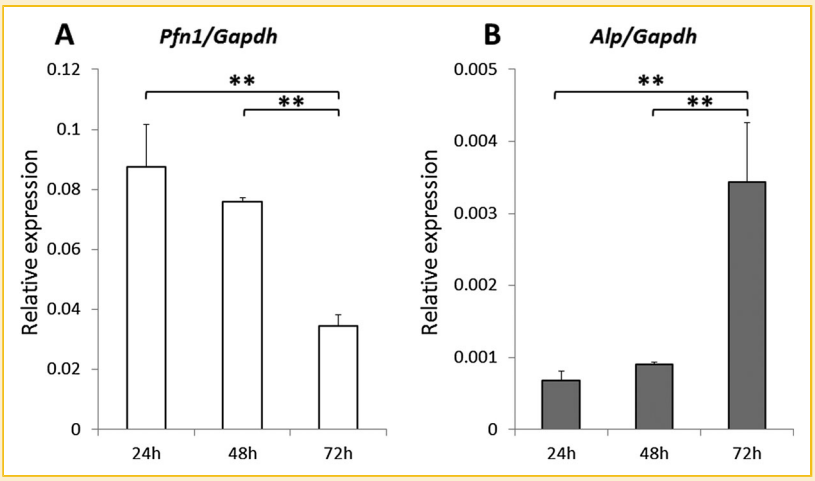

Fig. 1. Profilin 1 is expressed in osteoblastic MC3T3-E1 cells and its levels are regulated in a direction opposite to alkaline phosphatase expression along with time in culture. Osteoblastic MC3T3-E1 cells were plated at 10,000 cells per cm^2 and were cultured as described in materials and methods for the indicated period of time. Expression levels of profilin 1 (A) and alkaline phosphatase mRNA (B) were examined based on real time RT-PCR. **, $P < 0.01$. Number of samples = 4.

RESULTS

PROFILIN 1 IS EXPRESSED IN OSTEOBLASTIC MC3T3-E1 CELLS AND ITS LEVELS ARE REGULATED IN A DIRECTION OPPOSITE TO ALKALINE PHOSPHATASE EXPRESSION ALONG WITH TIME IN CULTURE

To address whether profilin 1 (Pfn1) is expressed in osteoblasts, MC3T3-E1 cells were subjected to mRNA expression analysis. Pfn1 mRNA is expressed in MC3T3-E1 cells constitutively (Fig. 1A). During the culture period of 72 h, there is a gradual down regulation in the levels of Pfn1 mRNA (Fig. 1A). Such gradual down regulation of Pfn1 mRNA levels along with time is opposite to the time dependent increase in alkaline phosphatase (Alp) mRNA levels (Fig. 1B). These observations suggest that Pfn1 is not only expressed in osteoblasts, but its expression levels are regulated as a function of time.

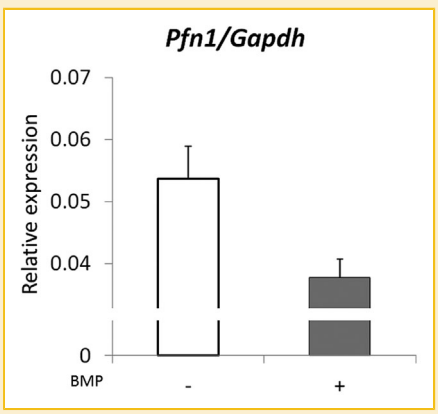

Fig. 2. BMP treatment suppresses Profilin 1 mRNA expression levels in osteoblastic MC3T3-E1 cells. Osteoblastic MC3T3-E1 cells were plated at 10,000 cells per cm^2 and were cultured as described in materials and methods for 48 h in the presence or the absence of 100 ng/ml BMP2. Expression levels of profolin 1 mRNA were examined based on real time RT-PCR. *, $P < 0.05$. Number of samples = 20–21.

BMP TREATMENT SUPPRESSES PROFILIN 1 mRNA EXPRESSION LEVELS IN OSTEOBLASTIC MC3T3-E1 CELLS

Osteoblastic MC3T3-E1 cells are known to differentiate along with time in culture. As the direction of time-dependent change in Pfn1 expression level is opposite to that of alkaline phosphatase (Alp), a differentiation marker of osteoblasts, it appears that Pfn1 expression is down regulated when the osteoblastic cells are differentiated. To this end, we examined the effects of BMP (that promotes osteoblastic differentiation) on Pfn1 expression. We found that BMP treatment suppresses Pfn1 mRNA expression in osteoblastic MC3T3-E1 cells (Fig. 2). Thus, Pfn1 is down regulated at least under two conditions, time and BMP, that promote differentiation of osteoblasts.

PROFILIN 1 mRNA LEVELS ARE DOWN REGULATED BY siRNA IN OSTEOBLASTIC MC3T3-E1 CELLS UNDER VARIOUS TIME AND siRNA CONCENTRATION CONDITIONS

Pfn1 mRNA is expressed in osteoblasts and down regulated time-dependently as well as by an osteoblastic differentiation stimulator, BMP, while the biological significance of such down regulation is not known. Therefore, we examined the role of Pfn1 in osteoblasts by knocking down Pfn1 mRNA using siRNAs. Transfection of siRNA for Pfn1 down regulates the levels of Pfn1 by 60–80% (Fig. 3A). Such down regulation by siRNA could be observed up to 96 h of transfection (Fig. 3A). As both 5 nM and 10 nM siRNA concentrations reveal similar knock down efficiency when normalized against either GAPDH (Fig. 3B) or β-actin (Fig. 3C), we use 5 nM for following experiments.

PROFILIN 1 mRNA KNOCK DOWN ENHANCES BMP-INDUCED INCREASE IN ALKALINE PHOSPHATASE ACTIVITY IN OSTEOBLASTIC MC3T3-E1 CELLS IN A SERUM CONCENTRATION DEPENDENT MANNER

Using the optimum knockdown condition by siRNA for Pfn1 in osteoblastic cells, Pfn1 function during BMP-induced differentiation was examined. Treatment with BMP for 48 h increases

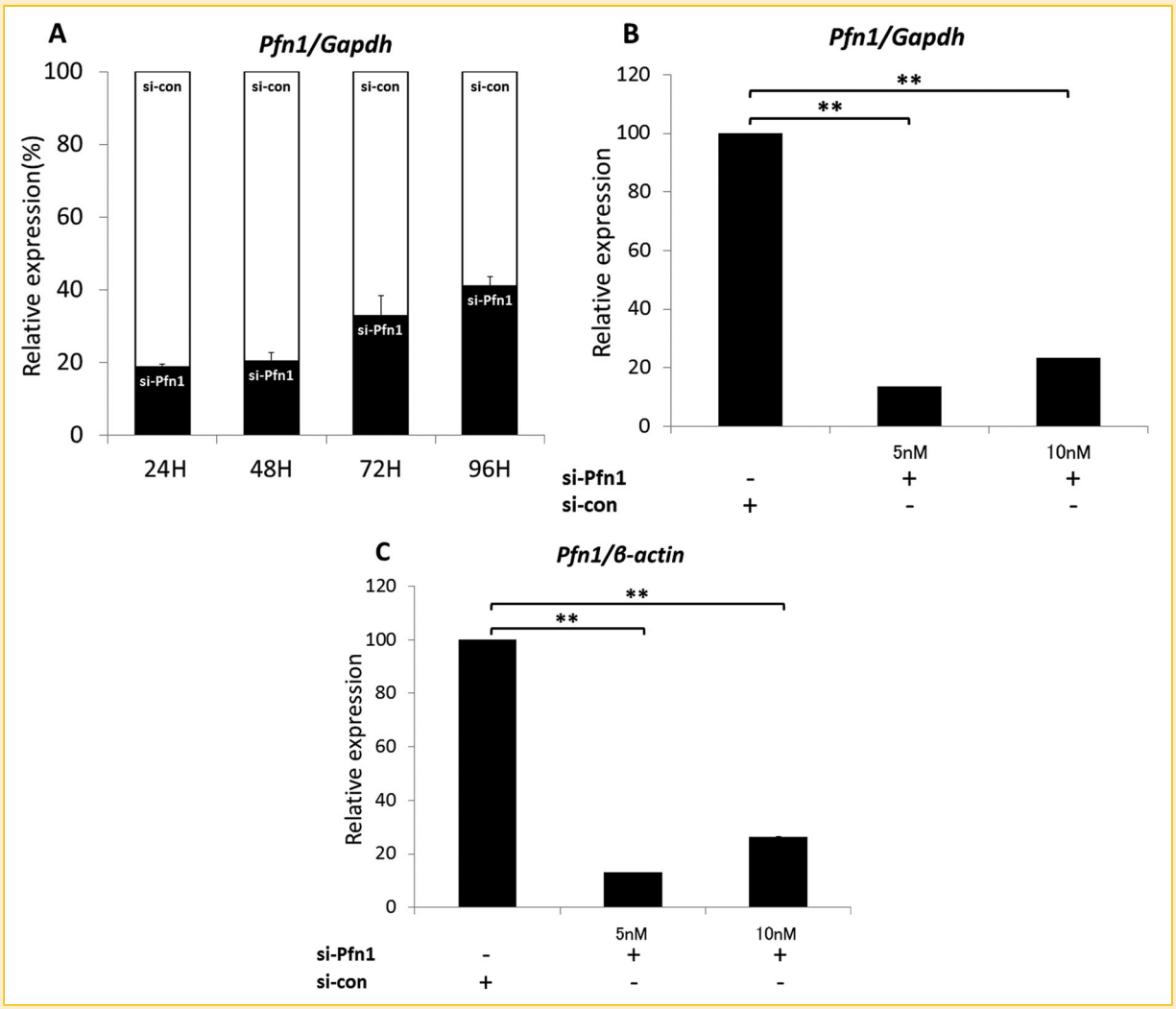

Fig. 3. Profilin 1 mRNA levels are down regulated by siRNA in osteoblastic MC3T3-E1 cells under various time and serum concentration conditions. Osteoblastic MC3T3E-1 cells were plated at 10,000 cells per cm^2 and were cultured as described in materials and methods and the cells were transfected with either control siRNA or siRNA against profilin 1. Expression levels of profilin 1 as a function of time (A) and siRNA concentratins (5 nM and 10 nM) were examined using GAPDH (B) or beta actin (C) mRNA as denominators.

alkaline phosphatase activity in osteoblastic MC3T3-E1 cells as known before. Knock down of Pfn1 enhances BMP-induced increase in alkaline phosphatase activity (Fig. 4). Enhancing effects of Pfn1 knock down on BMP-induced alkaline phosphatase activity in osteoblastic MC3T3-E1 cells are dependent on serum concentrations as 5% FBS is optimum compared to lower serum concentrations (Fig. 4). Thus, Pfn1 is not only regulated by BMP but it also controls BMP actions on osteoblastic differentiation.

PROFILIN 1 mRNA KNOCKDOWN ENHANCES BMP-INDUCED INCREASE IN ALKALINE PHOSPHATASE ACTIVITY IN OSTEOBLASTIC MC3T3-E1 CELLS EVEN AT THE HIGHEST BMP CONCENTRATION

As base line BMP-induced increase in alkaline phosphatase activity in MC3T3-E1 cells is dependent on BMP concentrations and is reaching up to 20-fold at the highest tested concentration of BMP (200 ng/ml), Pfn1 knock down enhancement of BMP-induced increase in alkaline phosphatase may be masked by the higher concentration of BMP. To test this point, Pfn1 knock down effects are examined with respect to the dosages of BMP. Pfn1 knock down enhances BMP-induced increase in alkaline phosphatase activity at all the BMP concentrations tested (Fig. 5). Notably, combination of the highest tested concentration (200 ng/ml) of BMP and Pfn1 knock down results in the highest levels of alkaline phosphatase activity (Fig. 5). Thus, higher concentration of BMP does not mask the enhancing effects of Pfn1 knock down on the alkaline phosphatase activity in osteoblastic MC3T3-E1 cells. Interestingly, Pfn1 knock down slightly enhances Alp activity in the absence of BMP (see 0 ng/ml). We therefore applied two way ANOVA analyses and this indicates that the interaction between the effects of BMP and those of Pfn1 knock down is statistically significant. Thus, Pfn1 knock down significantly enhances BMP-induced enhancement of Alp activities furthermore than the base line increase (i.e. in the absence of BMP) in these osteoblastic cells.

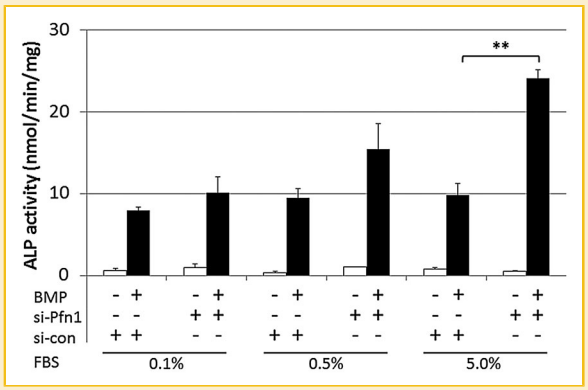

Fig. 4. Profilin 1 mRNA knock down enhances BMP-induced increase in alkaline phosphatase activity in osteoblastic MC3T3-E1 cells in a serum concentration dependent manner. Osteoblastic MC3T3-E1 cells were plated at 10,000 cells per cm^2 and were cultured as described in materials and methods for 48 h in the presence or the absence of 100 ng/ml BMP2 under the different levels of serum concentrations. Expression levels of alkaline phosphatase activity were examined based on PNPP assay. **, $PP < 0.01$.

PROFILIN 1 mRNA KNOCKDOWN ENHANCES BMP-INDUCED INCREASE IN ALKALINE PHOSPHATASE ACTIVITY IN OSTEOBLASTIC MC3T3-E1 CELLS EVEN IN A LONGER TERM CULTURE

Since alkaline phosphatase activity is increasing with time in culture in osteoblastic MC3T3-E1 cells both in the absence or the presence of BMP, long term cultures may also mask the enhancing effects of Pfn1 knock down on BMP-induced increase in alkaline phosphatase in osteoblastic MC3T3-E1 cells. To examine this point, time course of the effects of Pfn1 knock down is examined. During the time course experiments, combination of Pfn1 knock down and longest culture (72 h) in the presence of BMP results in the highest alkaline phosphatase activity in osteoblastic MC3T3-E1 cells compared to the shorter terms of cultures (24 or 48 h) (Fig. 6). Therefore, Pfn1 knock down enhances BMP-induced increase in alkaline phosphatase activity in osteoblastic MC3T3-E1 cells even in a longer term culture.

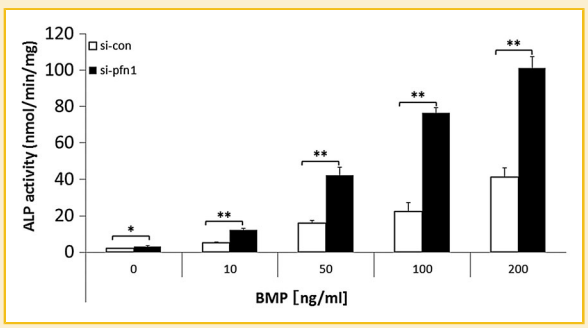

Fig. 5. Profilin 1 mRNA knockdown enhances BMP-induced increase in alkaline phosphatase activity in osteoblastic MC3T3-E1 cells even at the highest BMP concentration. Osteoblastic MC3T3-E1 cells were plated at 10,000 cells per cm^2 and were cultured as described in materials and methods for 48 hours in the presence or the absence of indicated concentrations of BMP2. Expression levels of alkaline phosphatase activity were examined based on PNPP assay. *, $P < 0.05$, **, $P < 0.01$. Number of samples = 6.

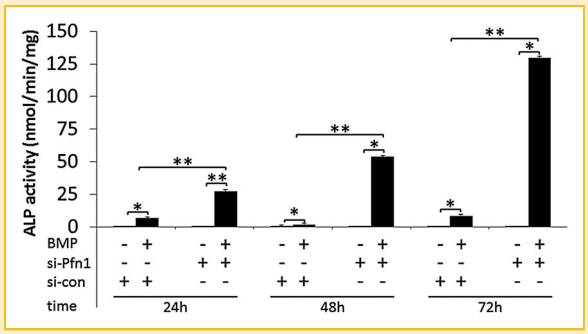

Fig. 6. Profilin 1 mRNA knockdown enhances BMP-induced increase in alkaline phosphatase activity in osteoblastic MC3T3-E1 cells even in a longer term culture. Osteoblastic MC3T3-E1 cells were plated at 10,000 cells per cm^2 and were cultured and transfected as described in materials and methods followed by subsequent culture in the presence or the absence of 100 ng/ml BMP2. Expression levels of alkaline phosphatase activity were examined based on PNPP assay. *, $P < 0.05$, **, $P < 0.01$. Number of samples = 6.

PROFILIN 1 mRNA KNOCKDOWN ENHANCES BMP-INDUCED INCREASE IN ALKALINE PHOSPHATASE mRNA EXPRESSION IN OSTEOBLASTIC MC3T3-E1 CELLS

To address how Pfn1 knock down regulates BMP-induced increase in alkaline phosphatase activity, we examined the effects of Pfn1 knock down on the BMP-induced increase in the levels of alkaline phosphatase mRNA. In control siRNA transfected cells, BMP treatment increases alkaline phosphatase mRNA levels (Fig. 7) and Pfn1 knock down enhances the alkaline phosphatase mRNA levels while combination of BMP treatment and Pfn1 knock down further enhances the BMP-induced increase in alkaline phosphatase mRNA levels in osteoblastic MC3T3-E1 cells (Fig. 7). These data indicate that Pfn1 knock down enhances BMP-induced increase of alkaline phosphatase activity at least in part by regulation at the messenger RNA levels.

PROFILIN 1 mRNA KNOCKDOWN ENHANCES BMP-INDUCED INCREASE IN BMP-RESPONSE ELEMENT DEPENDENT TRANSCRIPTION OF LUCIFERASE GENE EXPRESSION IN OSTEOBLASTIC MC3T3-E1 CELLS

Since Pfn1 knock down enhances BMP-induced increase in the levels of alkaline phosphatase mRNA expression, the levels of Pfn1 knock down enhancement on BMP-induced events was examined by using BMP response element linked to luciferase as a reporter gene. BMP treatment increases the luciferase levels as known before. Under this condition, Pfn1 knock down enhances BMP-induced increase in luciferase activity in osteoblastic MC3T3-E1 cells (Fig. 8). These data indicate that Pfn1 down regulation modulates BMP-induced transcriptional events and such modulation is at least in part involved in Pfn1 regulation of BMP-induced increase in alkaline phosphatase activity in osteoblastic MC3T3-E1 cells.

DISCUSSION

In this study, we found that Pfn1 is expressed in osteoblastic MC3T3-E1 cells and that its expression levels are down regulated in a longer

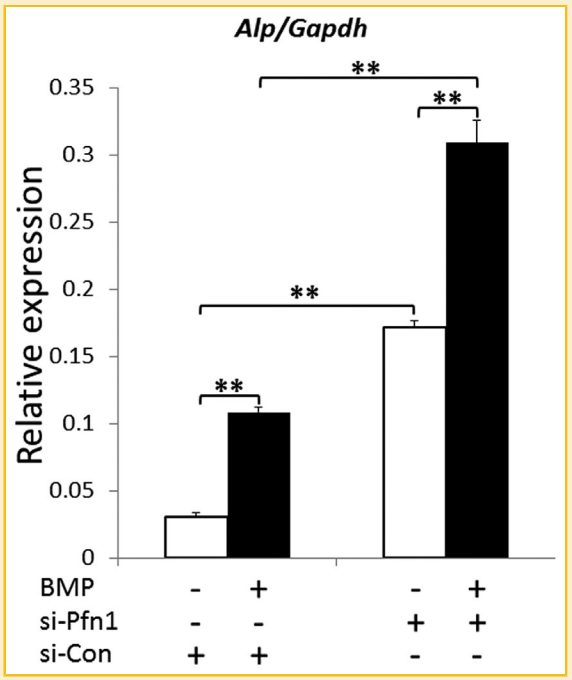

Fig. 7. Profilin 1 mRNA knockdown enhances BMP-induced increase in alkaline phosphatase mRNA expression in osteoblastic MC3T3-E1 cells. Osteoblastic MC3T3-E1 cells were plated at 10,000 cells per cm^2 and were cultured and transfected with siRNA as described in materials and methods followed by subsequent culture in the presence or the absence of 100 ng/ml BMP2. Expression levels of alkaline phosphatase mRNA were examined based on real time RT-PCR. **, $P < 0.01$. Number of samples = 6.

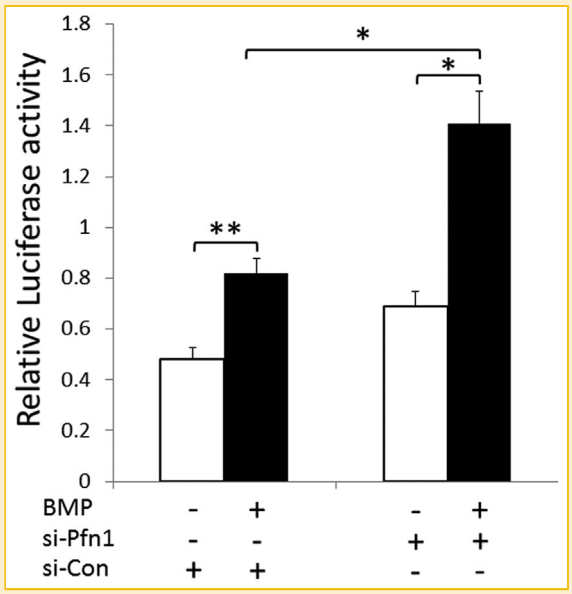

Fig. 8. Profilin 1 mRNA knockdown enhances BMP-induced increase in BMP-response element dependent transcription of luciferase gene expression in osteoblastic MC3T3-E1 cells. Osteoblastic MC3T3-E1 cells were plated at 10,000 cells per cm2 and were cultured and transfected with luciferase reporter and siRNAs as described in materials and methods followed by subsequent culture in the presence or the absence of 100 ng/ml BMP2. Expression levels of luciferase activity were examined based on luminometer system. Luciferase values were normalized against Renilla values. *, $P < 0.05$, **, $P < 0.01$. Number of samples = 6.

term culture when these cells are increasing their alkaline phosphatase activity, a marker of osteoblastic differentiation. Such inverse relationship between Pfn1 expression and alkaline phosphatase activity is further confirmed by our observation that BMP treatment suppresses Pfn1 expression in osteoblastic MC3T3-E1 cells in association with BMP-induced increase in alkaline phosphatase activity. These data reveal that Pfn1 is expressed in osteoblasts and its levels are regulated in association with osteoblastic differentiation.

As for the functional role of Pfn1 in osteoblasts, siRNA-based knock down was introduced and the effects of Pfn1 knock down on alkaline phosphatase expression were examined. Interestingly, Pfn1 knock down enhances BMP-induced increase in alkaline phosphatase activity in osteoblastic MC3T3-E1. Since BMP treatment increases the levels of osteoblastic differentiation and alkaline phosphatase activity is one of the representative markers of osteoblastic differentiation, Pfn1 would be a new member of BMP regulators that are involved in the control of osteoblastic regulation. BMP actions are down regulated by several molecules including secreted proteins such as noggin [Brazil et al., 2015], chordin [Rosen, 2006], follistatin [Sylva et al., 2013], cerberus [Tian and Meng, 2006] and dan [Nolan and Thompson, 2014]. Other inhibitory pathways for BMP actions include inhibitors of Smad signaling such as iSmad [Jeon and Jen, 2010] and Tob [Yoshida et al., 2000 and Usui et al., 2004]. Our discovery on Pfn1 suggests the presence of a unique regulatory pathway for BMP-induced differentiation as Pfn1 does not belong to the group of previously identified inhibitory molecules for BMP actions.

Intriguingly, although Pfn1 knock down enhances BMP-induced increase in alkaline phosphatase activity in osteoblasts, i.e. Pfn1 is a negative regulator of BMP, BMP treatment per se suppresses Pfn1 gene expression in osteoblastic MC3T3-E1 cells. This suggests that BMP suppresses the expression of inhibitor against itself and synergistically optimizes its action by forming a positive feedback line. Thus, this profilin-BMP relationship constitutes a novel regulatory mechanism underlying osteoblastic gene expression and differentiation. In many cases, BMP enhances the expression of its inhibitor such as noggin [Brazil et al., 2015] and iSmad [Jeon and Jen, 2010] and forming a negative feedback loop that would contribute to the maintenance of homeostasis in osteoblastic differentiation. In contrast, Pfn1 expression in osteoblastic MC3T3-E1 cells is down regulated by BMP and thus further enhances BMP actions. It is of interest how this new profilin-BMP relationship is balanced by previously known negative feedback loop of BMP signaling.

Analysis of alkaline phosphatase messenger RNA indicates that Pfn1 knock down enhance BMP-induced increase in alkaline phosphatase mRNA expression in osteoblastic MC3T3-E1 cells. Furthermore, Pfn1 knock down enhances BMP-induced increase in BMP-response element-based expression of luciferase reporter transcription. Therefore, Pfn1 action on BMP control would be at least in part via the regulation of BMP signaling possibly through Smad dependent transactivation of BRE. Pfn1 has been suggested to play some role in regulation of transcription but precise role of Pfn1

in transcriptional events is largely unknown. Our discovery on the enhancing effects of Pfn1 knock down on BMP-induced increase of alkaline phosphatase activity, as well as alkaline phosphatase mRNA levels and BRE- dependent luciferase would provide novel role of Pfn1 action in osteoblasts as the role of Pfn1 has not been shown in the regulation of these cells.

Although BMP has been attracted attentions regarding its application for treatment and has been tried to be used to enhance bone regeneration and repair, its application has been hampered by the fact that this is a protein and that development of such biological drug needs a huge cost that results in a large financial burden to the patients who need BMP as a drug. If simple chemicals could be developed to suppress Pfn1 expression or its activity, that may be beneficial for the medical cost and burden for the society and patients. Conversely, there are diseases where BMP actions are uncontrollably enhanced. Fibrodysplasia ossificancs progressiva (FOP) is such a disease whose prevalence is rare but the prognosis is quite poor. It is currently difficult to correct the positive balance of excessive bone formation activity induced by constitutively active BMP receptor mutation in these patients. It is still to be determined if Pfn1-BMP relationship is involved in the development of such disease. If so, question is whether any treatment could be possible by activation of Pfn1 to correct the abnormality in bone formation balance by suppression of BMP actions.

BMP action may be linked to cell attachment machinery such as cytoskeletons and integrins. We found that knock down of Pfn1 enhances BMP-induced differentiation in osteoblastic MC3T3-E1 cells that produce collagen type I. Tamura et al. have shown that FAK activation via the formation of focal adhesions is essential for BMP actions to stimulate the expression of genes encoding osteoblastic markers [Tamura et al., 2001]. This suggests that there may be communication between cell-matrix interactions and BMP signaling pathways. On the other hand, Pfn1 has been reported as a key player to control actin fiber structure which is involved in cellular functions such as proliferation and differentiation. Therefore, Pfn1 may play a role in connecting downstream signals of FAK. This possibility may be relevant if signaling in osteoblasts would have any similarity to the signaling reported in other cell system in that Pfn1 silencing decreases the expression of integrin β1 and inhibits the FAK signaling pathway for instance in gastric cancer [Cheng et al., 2015].

We observe that there is a slight increase in Alp activity by Pfn1 knockdown alone in the absence of BMP. As MC3T3-E1 cells produce BMP endogenously, modest base line increase would be due to an endogenous BMP in osteoblastic cells that is also affected by Pfn1 knock down.

In conclusion, we discovered that Pfn1 is regulated by BMP and it also controls BMP activity, forming Pfn1-BMP loop. This loop is a novel signaling pathway in osteoblastic MC3T3-E1 cells. This loop may function as one of the BMP regulatory systems contributing to the maintenance of homeostasis in osteoblastic differentiation.

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