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The role of focal adhesion kinase in BMP4 induction of mesenchymal stem cell adipogenesis

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ABSTRACT

Obesity is characterized by excessive adipocytic number growth and resultant adipose tissue hyperplasia. However, molecular mechanisms of abnormal recruitment of new adipocytes from precursor cells are not fully known. Several studies showed that bone morphogenetic proteins (BMPs) also play a role in inducing mesenchymal stem cells (MSCs) to commit to adipocytes. We tested the hypothesis that focal adhesion kinase (FAK), one of the vital focal adhesion signaling molecules, is required for BMP4 induction of MSC adipogenesis. BMP4 exposure triggered FAK activation at pY397 auto-phosphorylation site in murine C3H10T1/2 MSCs. Interestingly, silencing FAK by small hairpin RNA (shRNA) significantly suppressed BMP4 induction of MSC adipogenic activities, including lipid accumulation and expression of key adipogenic genes (C/EBPα, PPARγ, aP2), as relative to shRNA vector control. As a potential molecular mechanism, BMP4-triggered phosphorylation in Smad1/5/8 and p38 was significantly downregulated by shRNA-FAK. Pharmacological FAK inhibitor 14 provided similar results in BMP4-mediated MSC adipogenesis and Smad/p38 signaling. Our data clearly suggest a link between FAK and BMP4 induction of MSC adipogenesis, and may indicate a potential therapeutic approach targeting FAK for dealing with obesity.

1. Introduction

Obesity occurs through excessive adipocyte growth and adipose tissue hyperplasia. Adipocytes control energy storage/balance and also play a role in endocrine functions by secreting soluble factors to mediate immune response, insulin sensitivity, and angiogenesis [1]. However, pathological recruitment of new adipocytes to form adipose tissue expansion induces obesity, increasing risk factors for metabolic syndrome such as hypertension, heart disease, stroke, diabetes, and insulin resistance [2]. One of the main causes of adipocyte number growth is through the recruitment of newly formed preadipocytes from mesenchymal stem cell (MSC) sources and the mitotic clonal expansion of preadipocytes [3,4]. Since MSCs have plasticity to commit and differentiate toward several lineages including adipocytes, osteocytes, and myocytes, it is of significant interest for obesity study to understand the molecular mechanisms responsible for directing MSC commitment into adipocytic fate. We have reported data demonstrating the inhibition of MSC adipogenesis by soluble (retinoic acid) and mechanical (cell stretch) signals [5,6]. Here we test the potential molecular mechanism governing MSC adipogenesis.

Bone morphogenetic proteins (BMPs) belong to transforming growth factor β (TGFβ) superfamily and regulate the development of various tissues and organs including heart, central nervous system, and post-natal bone [7]. Interestingly, several studies revealed that BMPs are also involved in MSC commitment into adipocytes [8–11]. If C3H10T1/2 MSCs are pretreated with BMP4 during the proliferation stage, MSCs show significant upregulation in preadipocytic commitment by responding to a greater degree to adipogenic hormonal inducers. Even though molecular mechanisms of such BMP4 functioning have not been fully understood, it is now established BMPs can provide guidance signals for MSC preadipocytic commitment.

During the adipocytic differentiation *in vivo*, significant changes occur in extracellular matrix composition and organization, which in turn mediate the remodeling of cell morphology and cytoskeleton structure [12]. A consensus exists that such processes in general are governed by focal adhesion and related signaling [13]. However, very few studies investigated the role of focal adhesion signaling in adipogenic cellular activities and no study tested its relevance to BMP4-induced MSC adipogenesis. We examined the role of focal adhesion kinase (FAK), one of the forceful focal adhesion signaling cascades, in BMP4 induction of MSC adipogenesis. We showed for the first time that FAK is required for BMP4 triggering of MSC adipogenic activities and this may be governed by FAK involvement in BMP4-Smad/p38 signaling.

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2. Materials and methods

2.1. MSCs with FAK small hairpin RNA (shRNA)

shRNA was used to establish MSCs displaying stable FAK knockdown. C3H10T1/2 murine MSCs (ATCC, CCL-226) were transfected with FAK shRNA plasmid (Santa Cruz, sc-35353-SH) using lipofectamine 2000 transfection reagent. After 24 h of transfection, the medium was replaced with the selection medium containing puromycin (2 μg/ml). Puromycin-resistant cells were selected and further cloned to produce stable cell line displaying shRNA-FAK. The same transfection was repeated but with control shRNA plasmid (Santa Cruz, sc-108060) to provide shRNA vector control. Medium with puromycin was used to maintain cells with shRNA-FAK and vector control before tests. FAK interference by shRNA was tested by immunoblotting (Fig. 2A). Sustainable FAK interference after subcultures was also confirmed (not shown).

2.2. BMP4 induction of MSC adipogenesis

BMP4 induction of MSC adipogenesis was performed as published protocols [5,6]. Briefly, MSCs were cultured in 6-well plate (5×10^4 cells/well) using growth media composed of Dulbecco's modified Eagle's medium, 10% fetal bovine serum, and 1% penicillin–streptomycin, and grown for 4 days. During the 4 days of growth, cells were exposed to recombinant BMP4 (R&D Systems, 5020-BP-010) at 50 ng/ml. Both cultures with shRNA-FAK and vector control became confluent after 4 days of growth. Cells were then treated with adipogenic induction media (growth media further supplemented with 10 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM methylisobutylxanthine) for 2 days and kept with adipogenic maintenance media (growth media with 10 μg/ml insulin) for additional 6 days. Media were changed every two days.

2.3. RT-PCR and oil red O staining

At the end of adipogenesis protocol, MSC adipogenesis was evaluated using RT-PCR and oil red O staining, following previous protocols [5,6]. CCAAT/enhancer binding protein (C/EBP) α: sense GGG TGA GTT CAT GGA GAA TGG, anti-sense CAG TTT GGC AAG AAT CAG AGC A; peroxisome proliferator-activated receptor (PPAR) γ: sense AGG CCG AGA AGG AGA AGC TGT TG, anti-sense TGG CCA CCT CTT TGC TCT GCT G; adipocyte protein 2 (aP2): sense TCT CAC CTG GAA GAC AGC TCC TCG, anti-sense TTC CAT CCA GGC CTC TTC CTT TGG CTC; GAPDH: sense CAT GTT CCA GTA TGA CTC CAC TC, anti-sense GGC CTC ACC CCA TTT GAT GT. For lipid staining, cells were fixed with formalin, stained with 0.5% oil red O solution, and imaged with optical microscope. Stained oil red O was extracted by isopropanol and quantified by measuring with a Bio-Tek spectrophotometer at 570 nm.

2.4. Immunoblotting of FAK, Smad, and p38 phosphorylation

Phosphorylation of FAK and two major BMP signaling cascades (Smad and p38) in response to BMP4 exposure was tested. Cells were treated with BMP4 at 50 ng/ml for various time periods (up to 60 min) and the phosphorylation of FAK, Smad, and p38 was quantified by western blotting. Immunoblotting followed previous protocols [5,6]. Primary antibodies for FAK (Cell Signaling, 3285), phosphorylated (p-) FAK (pY397, BD, 611806), Smad1 (Santa Cruz, sc-7965), p-Smad1/5/8 (Cell Signaling, 9511), p38 (Cell Signaling, 9212), p-p38 (Cell Signaling, 9216) were used. Phosphorylation was measured by dividing the immunoblot band intensity of the phosphorylated protein by that of the total protein. The loading control (GAPDH) is also shown in immunoblot images.

2.5. FAK inhibition by FAK inhibitor 14

Assays were repeated with using known pharmacological FAK blocker, FAK inhibitor 14 (Santa Cruz, sc-203950). Cells were treated for 20 min with 1 μM FAK inhibitor 14 before exposure to BMP4, and adipogenesis and phosphorylation assays were repeated. Note that FAK inhibitor 14 was included in the medium throughout the culture protocol, and cells under FAK inhibitor 14 also reached confluence after 4 days of growth.

2.6. Statistics

Three assays each in triplicate were completed. Statistics was assessed by one-way analysis of variance followed by post hoc tests. See figure legends for detailed comparisons.

3. Results

3.1. BMP4 induces FAK phosphorylation in C3H10T1/2 MSCs

In our previous studies [5,6], it was confirmed that BMP4 has the potential to induce MSC adipogenesis. To test the role of FAK in BMP4 induction of MSC adipogenesis, we first examined whether FAK is activated by BMP4 treatment. Parent C3H10T1/2 MSCs were exposed to BMP4 for 5, 10, 15, 30, and 60 min, and FAK phosphorylation at pY397 auto-phosphorylation site was quantified by normalizing its immunoblot band intensity with that of the corresponding total FAK protein expression (Fig. 1). FAK was phosphorylated in MSCs for 5–30 min of BMP4 treatments (∗, ∗∗) relative to untreated control (Un). FAK phosphorylation was decreased by 60 min of BMP4 exposure. This indicates that BMP4 has the potential to activate FAK pathway.

3.2. BMP4 induction of MSC adipogenesis is suppressed by shRNA-FAK

We next examined the effects of FAK silencing on the adipocytic differentiation of MSCs under BMP4. To test this, we transfected

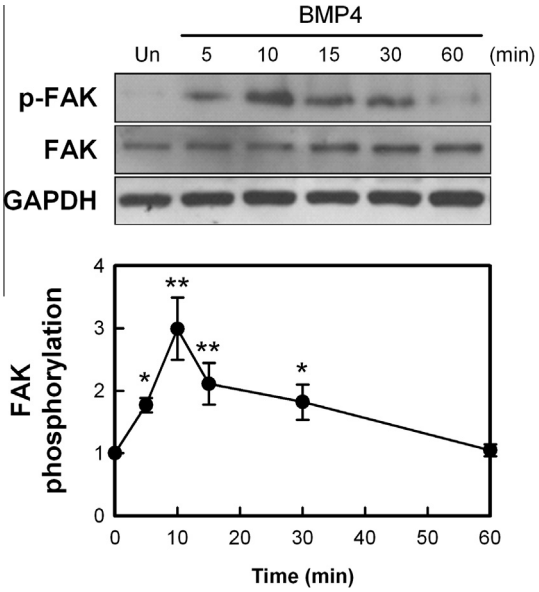

Fig. 1. BMP4 induces the phosphorylation of FAK at pY397 in C3H10T1/2 MSCs. Cells were exposed to BMP4 (50 ng/ml) for 5, 10, 15, 30, and 60 min, and FAK phosphorylation at pY397 auto-phosphorylation site was measured. Relative phosphorylation level was quantified by dividing the western immunoblot band intensity of phosphorylated (p-) FAK by that of total FAK protein expression. GAPDH loading control is shown. Comparison with BMP4-untreated control (Un) is shown at $^{*}p < 0.05$ and $^{**}p < 0.01$.

shRNA-FAK and control shRNA plasmids into C3H10T1/2 MSCs. As seen in Fig. 2A, FAK expression was significantly reduced for MSCs displaying shRNA-FAK relative to vector control (**). There was no significant difference in FAK expression between parent cells and shRNA vector control. Upon adipogenesis induction, shRNA vector control showed distinct characteristics of BMP4-triggered adipogenic activities, e.g., well-developed lipid staining (Fig. 2B) and upregulations in key adipogenic transcription factors (Fig. 2C). In the presence of FAK knock-down by shRNA, interestingly, such BMP4-induced MSC adipogenic commitment and differentiation were significantly suppressed. Lipid accumulation quantified by spectrophotometer was significantly decreased (Fig. 2B, **) and key adipogenic genes (C/EBPα, PPARγ, aP2) were significantly downregulated (Fig. 2C, **), both for cells displaying shRNA-FAK relative to vector control. These strongly suggest FAK is involved in BMP4 functioning to trigger adipogenic transcription and differentiation.

3.3. shRNA-FAK downregulates BMP4-Smad/p38 signaling

To investigate the role of FAK in BMP4 functioning, two established BMP4 signaling pathways (Smad, p38) were screened without or with FAK silencing. We first retested BMP4 induction of FAK activation. BMP4 treatment clearly induced FAK phosphorylation in vector control cells (Fig. 3A), as is the same as FAK activation by BMP4 in parent cells (Fig. 1). In the presence of shRNA-FAK, BMP4 induction of FAK phosphorylation was significantly reduced (Fig. 3B, ## for comparisons at the same time points). Next, BMP4 signaling through Smad and p38 was confirmed, i.e., phosphorylation of Smad1/5/8 and p38 after 15 min of BMP4 exposure for shRNA vector control. Interestingly, FAK silencing by shRNA significantly abrogated BMP4 induction of Smad and p38 phosphorylation relative to vector control (Fig. 3C,D, #, ## compared at the same time points). All data (Figs. 1–3) taken together, our results suggest FAK is required for BMP4 direction of MSC adipogenesis and this may be governed by FAK mediation of BMP4 signaling through Smad/p38.

3.4. FAK inhibitor 14 reduces MSC adipogenesis and Smad/p38 phosphorylation

Data from shRNA were further validated with pharmacological FAK inhibitor. Parent MSCs were treated with FAK inhibitor 14 and adipogenesis assays were repeated using the same BMP4 exposure and adipogenic induction (Fig. S1). FAK inhibitor 14 induced significant reductions in lipid accumulation and adipogenic

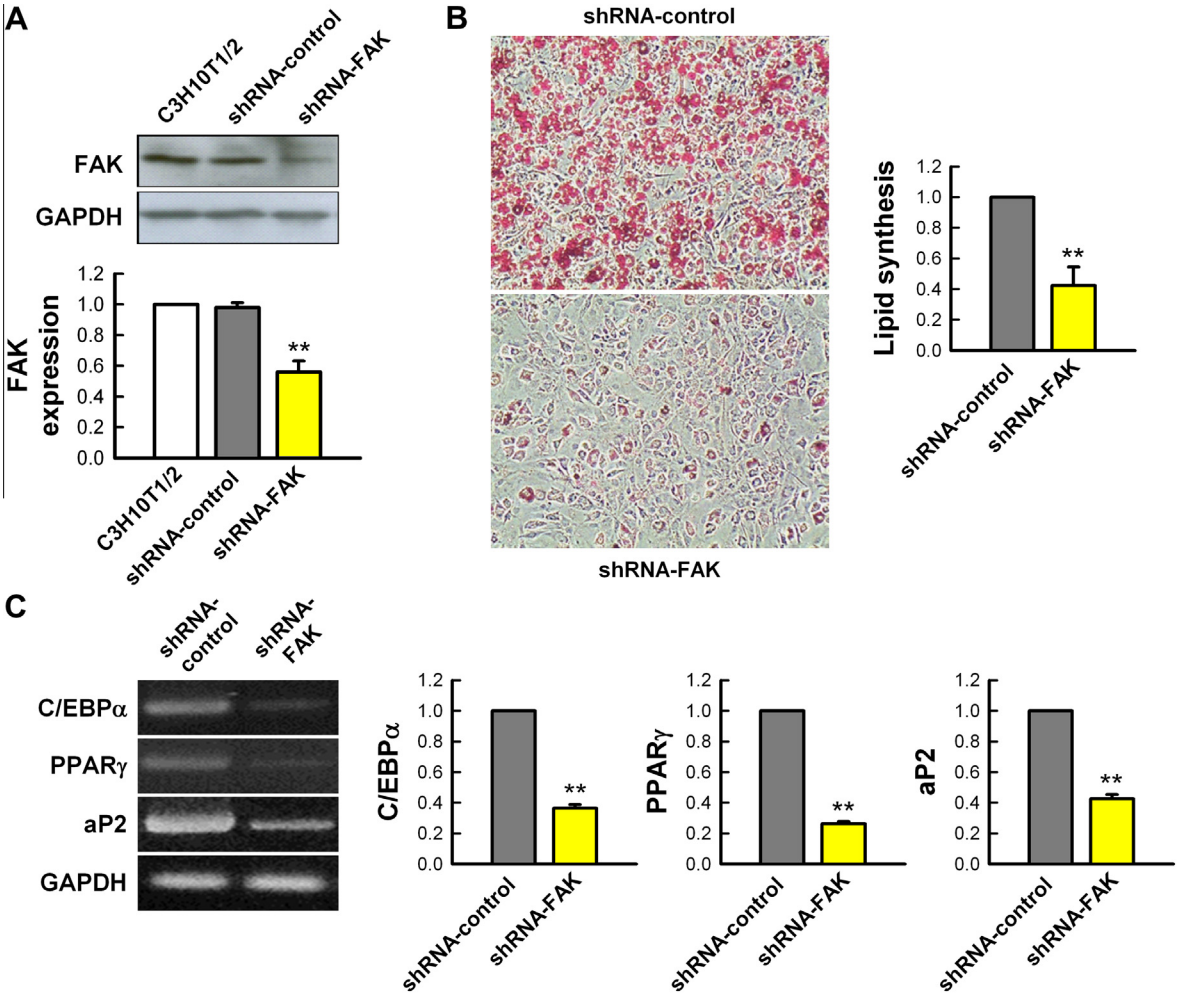

Fig. 2. BMP4 induction of MSC adipogenesis is suppressed by FAK interference through shRNA. (A) Establishment of FAK-silenced cells via shRNA was confirmed by western blotting. FAK expression in cells with shRNA-FAK was significantly lower than that of shRNA vector control (**$p < 0.01$). (B,C) FAK silencing suppressed BMP4-induced MSC adipogenesis. Cells were treated with BMP4 at 50 ng/ml for 4 days and then induced to differentiate to adipogenesis for 8 days (2 days of induction and 6 days of maintenance). MSCs with shRNA-FAK displayed significantly reduced lipid synthesis relative to vector control, as assessed by oil red O staining and spectrophotometer quantification (**$p < 0.01$). Also, shRNA-FAK markedly downregulated key adipogenic gene (C/EBPα, PPARγ, aP2) expression relative to vector control (**$p < 0.01$).

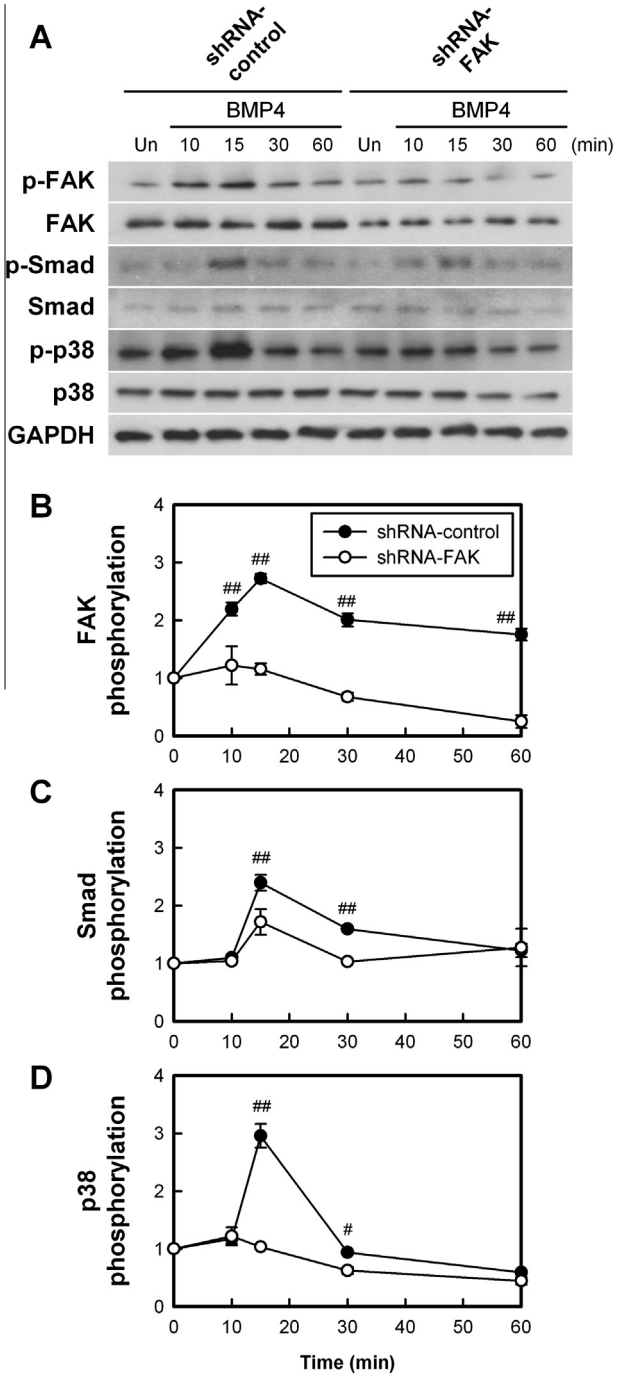

Fig. 3. FAK silencing by shRNA downregulates BMP4 signaling through Smad/p38. (A) Western blotting of FAK, Smad, p38, and their phosphorylation (-p) in cells with shRNA-FAK and shRNA vector control in response to BMP4 exposure at 50 ng/ml. (B) BMP4 induced FAK pY397 phosphorylation in vector control, which was abrogated by shRNA-FAK. Comparisons between shRNA-FAK and vector control at the same time points are shown with $^{\#\#}p < 0.01$. (C,D) Major BMP4 signaling pathways were confirmed by the phosphorylation of Smad1/5/8 and p38 under 15–30 min of BMP4 exposure. These were significantly downregulated in the presence of shRNA-FAK ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$ at the same time points).

gene (C/EBPα, PPARγ, aP2) expression when compared with vector control. Effects from inhibitor appeared less strong relative to shRNA, which may be due to relatively mild inhibitor concentration. BMP4 signaling was also tested with FAK inhibitor (Fig. 4). Parent cells displayed similar trends as those of shRNA-FAK with respect to BMP4 and FAK interference, e.g., BMP4-induced Smad and p38 phosphorylation was downregulated in the presence of FAK inhibitor 14 (FAK 14, $^{\#}$, $^{\#\#}$ compared at the same time points). Thus, both shRNA and inhibitor studies indicate the regulatory role of FAK in BMP4 induction of MSC adipogenesis potentially through mediating Smad/p38.

4. Discussion

We demonstrated that the interference of FAK, the forceful focal adhesion signaling cascade, suppresses the BMP4 induction of MSC adipogenesis. BMP4 induction of adipogenic transcription factor (C/EBPα, PPARγ, aP2) expression and lipid accumulation in MSCs were significantly decreased in the presence of shRNA-FAK or FAK inhibitor 14. BMP4 signaling through Smad and p38 was significantly impaired by FAK interference, suggesting a potential regulatory mechanism.

FAK belongs to focal adhesion anchoring proteins that connect transmembrane integrins to actin cytoskeletons. FAK colocalizes with integrins at the C-terminal focal adhesion targeting domain via associations with other anchoring proteins such as paxillin and talin [14]. FAK is a tyrosine kinase that can be phosphorylated at various sites including tyrosine-397, -576, -577, -861, and -925. Among these, tyrosine-397 (pY397) is auto-phosphorylated when integrin and extracellular ligand binding is activated. pY397 can provide binding sites for Src homology 2 (SH2) domains of a variety of structural and cytosolic signaling molecules such as

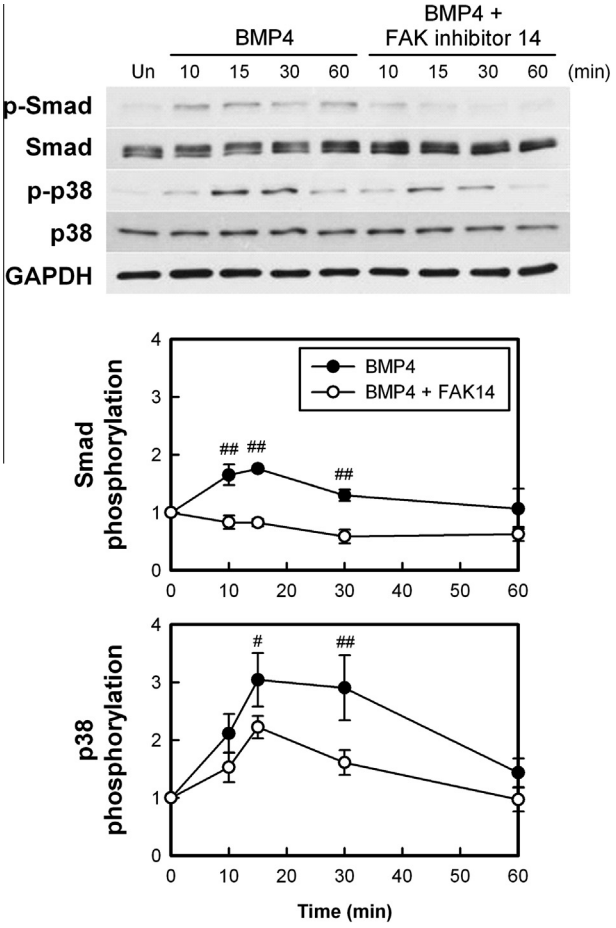

Fig. 4. FAK inhibitor 14 reduces BMP4-mediated Smad/p38 phosphorylation. Parent MSCs were treated with BMP4 (50 ng/ml) without or with FAK inhibitor 14 (FAK 14), and Smad and p38 phosphorylation was assessed. FAK inhibitor 14 downregulated BMP4-mediated Smad1/5/8 and p38 phosphorylation ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$ at the same time points).

Src-family tyrosine kinases, and has been proposed to play an essential role in cell adhesion, growth, and differentiation [15]. We previously demonstrated that FAK phosphorylation at pY397 can play a regulatory role in differential human fetal osteoblastic cell adhesion on varying nanotopographic substrates [16]. On the other hand, FAK phosphorylation at other sites may play a major role for cells under mechanical stimuli, e.g., pY576 was a predominant mechanosensor for pulmonary epithelial cells under stretching [17] and for artery endothelial cells under fluid shear [18]. In the current study using static culture, we tested pY397. It was observed that FAK phosphorylation at pY397 is stimulated by BMP4 soluble signal (Fig. 1) and such activation is abolished in the presence of shRNA-FAK (Fig. 3A,B).

The regulatory role of FAK in lineage commitment and differentiation is relatively well-established for cardiogenesis [19], myogenesis [20], and osteogenesis [21]. However, very little is known on its role in adipogenesis. Adipocytic differentiation accompanies cell morphological change from a fibroblastic shape to a relatively spherical shape, cytoskeletal reorganization and remodeling, and change in extracellular matrix composition and organization [12]. Considering such changes in general are mediated by focal adhesion activities [13], it is intuitive to speculate that adipogenesis may also be governed by FAK, one of the dominant focal adhesion signaling molecules. We demonstrated that FAK is required for BMP4 induction of MSC adipogenesis, as supported by decreases in adipogenic gene expression and lipid synthesis with FAK interference. Positive transcriptional feedback loop between C/EBPα and PPARγ coordinates downstream adipocyte biology [22]. Thus, our data (Fig. 2C, S1B) specifically indicate that interfering FAK activity could impair adipocytic developmental biology triggered by BMP4.

To reveal how FAK is involved in BMP4 functioning, we tested two of the established BMP4 signaling pathways (Smad, p38) in the absence or presence of FAK interference. BMPs bind to membrane receptors (type II and type I), which induce the phosphorylation of Smad1/5/8 [7]. Activated Smad1/5/8 constitutes heteromer with Smad4, which is then translocated into the nucleus to trigger transcriptional activities. Other than Smad, it was documented that BMPs can signal through non-Smad pathways such as p38 mitogen-activated protein kinase (MAPK)[10]. In our previous study, retinoic acid inhibited BMP4 induction of MSC adipogenesis via directly disabling BMP4-Smad and BMP4-p38 signaling [5]. The current data may suggest a similar mechanism that interfering FAK may destroy BMP4 functioning by downregulating two BMP4 pathways, Smad and p38. This was evidenced for molecularly manipulated cells (shRNA, Fig. 3C,D) and parent cells (with FAK inhibitor 14, Fig. 4).

Our study on FAK and BMP interactive control of MSC adipogenesis is, as far as we know, the first of a kind. Interestingly, potential mirror mechanism may be traced for BMP-induced osteogenesis, as BMPs were initially recognized by the potent effect to accelerate osteoblast maturation [7]. In stimulating trabecular bone-derived osteoblastic cell osteogenesis by BMP2, it was observed that FAK activation by BMP2 occurred with the same kinetics as BMP2-induced Smad1 phosphorylation [23]. This result may be mirrored with ours (Fig. 3B,C), e.g., FAK and Smad activation displaying similar phosphorylation kinetics under BMP4. Though the type of BMP (2 and 4), cells (osteoblasts and MSCs), and terminal fate (osteogenesis and adipogenesis) are different, the conclusion of the study on osteogenesis [23] may be shared such that BMPs may trigger crosstalk between BMP signaling (Smad) pathway and focal adhesion signaling (FAK) pathway. Similarly, in a study using osteoblastic MC3T3-E1 cells [24], cells manipulated to have FAK antisense failed to exhibit BMP2-induced osteogenic transcription through Smad6. This result, again, suggests FAK involvement in BMP functionality, though not for adipogenesis.

There is one study that pursued a similar theme as ours, examining the role of focal adhesion in adipogenesis, but targeting integrin of preadipocytes from rat adipose tissue [25]. When preadipocytes were treated with rhodostomin (snake venom-derived arginine-glycine-aspartic acid containing disintegrin), cells showed detachment, decreases in FAK expression and phosphorylation, and increase in apoptosis. Also, rhodostomin treatment during the initial 3 days of growth produced less mature adipocytes after 10 days of differentiation [25]. These data on integrin control of preadipocytic cell adipogenesis suggest a consistent mechanism as ours in that integrin-mediated focal adhesion and related signaling would be required for adipogenic activities (regardless of different adipogenesis model, i.e., preadipocytic cell adipogenesis vs. BMP4-induced MSC fate decision into adipocyte).

Additionally, though not directly relevant to this study, it may be notable that another aspect of FAK in adipogenesis was reported [26]. For 3T3-L1 and primary rat preadipocytes, it was proposed that FAK may participate in adipogenesis by displaying gradual cleavage during the differentiation. Inhibition of calpain, a mediator of FAK cleavage, resulted in the disturbance of terminal adipocytic maturation [26]. Note, we focused on the FAK involvement in BMP4-induced MSC adipogenic direction potentially via regulating BMP4-Smad/p38 signaling, but did not assess FAK cleavage along the differentiation time span.

In conclusion, it was demonstrated that FAK, a vital focal adhesion signaling molecule, would be required for BMP4 induction of MSC adipogenesis. BMP4-induced MSC adipogenic commitment and differentiation, assessed by expression of key adipogenic factors (C/EBPα, PPARγ, aP2) and lipid synthesis, were significantly suppressed in the presence of shRNA-FAK or with FAK inhibitor 14. As a potential molecular mechanism, BMP4-induced phosphorylation in Smad and p38 was markedly inactivated by molecular or pharmacological interference of FAK. Our results suggest a link between focal adhesion signaling, such as FAK, and BMP4-regulated MSC adipogenic activities. The data indicating FAK is required for MSC fate decision toward adipogenesis may further suggest a potential of therapeutic attempt that targets FAK for treating or preventing obesity.

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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.2013.05.045.

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