Single Bout Short Duration Fluid Shear Stress Induces Osteogenic Differentiation of MC3T3-E1 Cells via Integrin α1 and BMP2 Signaling Cross-Talk

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Abstract

Fluid shear stress plays an important role in bone osteogenic differentiation. It is traditionally believed that pulsed and continuous stress load is more favorable for fracture recovery and bone homeostasis. However, according to our clinical practice, we notice that single short duration stress load is sufficient to trigger osteogenic differentiation. In the present study, we subject osteoblast MC3T3-E1 cells to single bouts of short duration fluid shear stress by using a parallel plate flow system. The results show that 1 hour of fluid shear stress at 12 dyn/cm² promotes osteogenic differentiation, including rearrangement of F-actin stress fiber, up-regulation of osteogenic genes expression, elevation of alkaline phosphatase activity, secretion of type I collagen and osteoid nodule formation. Moreover, collaboration of BMP2 and integrin α1 pathways plays a significant role in such differentiation processes. Our findings provide further experimental evidence to support the notion that single bout short duration fluid shear stress can promote osteogenic differentiation.

Introduction

Mechanical stress is crucial for bone development, homeostasis and repair [1]. Mechanical stress-induced deformation in the mineralized matrix causes a heterogeneous pressure gradient that drives interstitial fluid flow in the lacunar-canalicular network and haversian systems, creating fluid shear stress (FSS) across the surface of bone cells [2]. It has been demonstrated that predicted level of FSS is a potent regulator of bone cell behavior, which enhances cell proliferation and induces osteogenic differentiation [3]. Conventional researches have found that long-term or pulse FSS is capable of stimulating osteoblast differentiation in vitro [4,5]. M. Patel et al. reported that capillary shear stress can trigger osteogenic differentiation in muscle-derived precursor cells [6,7]. By contrast, Lanyon et al. have demonstrated that a single, short exposure to an exogenous loading is sufficient to increase metabolic activity of osteocytes and activate quiescent cells on bone surface [8,9]. Indeed, in clinical practice, a short-term and appropriate mechanical stimulus, e.g. osteoplastic distraction forces (rapid palatal expansion) [10] and orthodontic forces (lacedback) [11] can rapidly activate the function of osteoblasts, resulting in new bone formation on the tension side. Thus we raise a hypothesis that single bout short duration FSS could also promote osteogenic differentiation.

Bone morphogenetic protein 2 (BMP2) is a prominent factor to regulate osteoblast differentiation [12]. BMP2 binds and activates transmembrane serine/threonine kinase receptors, namely type II BMP receptors. Activated BMP type II receptors further activates type I receptors and triggers phosphorylation of Smad 1, 5, and 8 proteins. Phosphorylated Smad 1, 5, and 8 oligomerize with Smad 4 and then translocate into the nucleus to regulate the transcription of osteogenic-related genes including RUNX2 and SP7, eventually leading to extracellular matrix (ECM) mineralization to create osteoid [13]. BMP2 has been demonstrated as a responsive factor in chemical stimulation-induced osteoblast differentiation [14]. Accumulated evidences indicate that BMP2 positively regulates expressions of integrins to further promote osteogenic differentiation [15,16]. Until now, experimental evidences have shown that mechanical force including FSS and uniaxial cyclic tensile strain can promote the gene transcription of BMP2 [17,18]. However, whether BMP2 directly mediates FSS-directed osteogenic differentiation in preosteoblast/osteoblast has not yet been carefully examined.

Integrins have been identified as mechanoreceptors in a wide range of cell types including osteoblasts. In particular, integrin β1 subunit, coding by ITGB1 gene, plays predominant functional role in osteoblasts, which dimerizes with α subunits including α1 through α5 and αV. Ligands of integrin β1 contain type I and III...
collagen and fibronectin. The cytoplasmic tail of the β1 subunit is responsible for integrin signaling [19]. Abundant evidences suggest that integrin signaling is required for osteoblast cell proliferation and differentiation [19–21].

Based on the literature review and our clinical observations, we try to find out whether single bout short duration FSS could also promote osteogenic differentiation in this study. Our results show that a single load of FSS for 1 h at 12 dyn/cm² was capable of inducing osteogenesis-related processes, including rearrangement of F-actin stress fiber, up-regulation of osteogenic genes, elevation of alkaline phosphatase (ALP) activity, secretion of type I collagen and mineralized nodule formation in murine pre-osteoblastic cell line MC3T3-E1. Moreover, up-regulation of BMP2 and integrin β1 may form positive feedback signaling pathway to promote FSS-induced osteogenic differentiation of MC3T3-E1. Our research provides mechanism of osteogenic differentiation induced by single bout short duration fluid shear stress in osteoblasts, and potentially offer an experimental basis for study of orthodontic bone remodeling and bone tissue engineering mechanisms.

Results

FSS promotes osteogenic differentiation of MC3T3-E1 cells

To begin with, we optimized the strength of FSS treatment. We found that one single load of 12 dyn/cm² FSS for 1 h exhibited most potent expression of osteogenic differentiation markers (Fig. S1). As a result, we chose this condition for further experiments.

We found that FSS treatment induced an early morphological change of MC3T3-E1 cells. As is shown in Fig. 1A, polymerization of actin cytoskeleton was triggered immediately after FSS treatment, with F-actin fibers paralleling to the long axis of the cell along the orientation of the fluid flow, as compared with the random distribution in the control cells. Mean fluorescence intensity of F-actin was slightly but significantly higher after FSS treatment, which indicates that FSS induced actin stress fiber formation.

Subsequently, mRNA levels of osteogenic differentiation markers including alkaline phosphatase (ALP), runt related transcription factor 2 (RUNX2) and Sp7 transcription factor 7 (SP7) were elevated in 3–12 h post-FSS treatment (pf.). (Fig. 1B, 1G and 1D) ALP activity was up-regulated as soon as 24 h pf. (Fig. 1E).

Type I collagen is the major component of extracellular matrix (ECM) whose mineralization is required for osteoid construction [22]. Through FSS treatment, the secretion of type I collagen notably increased at 24 h pf. (Fig. 1F). Correspondingly, enhanced mineralization of ECM examined by Alizarin Red S staining was observed at 12 day pf., showing a terminal differentiation phenotype of osteoblast (Fig. 1G). To further confirm this phenomenon, we isolated primary mesenchymal stem cells from mouse bone marrow. Similarly, transcription of molecular differentiation markers ALP, RUNX2 and SP7 were consistently up-regulated in 7 days pf. and dropped close to basal line in 14 days pf. (Fig. 1H, 1I and 1J). ALP activity was also significantly increased at 7 day pf. (Fig. 1K). Alizarin Red S staining revealed that dramatic ECM mineralization was observed at 14 day pf. (Fig. 1I).

These results indicated that FSS is capable of inducing a systematic and sequential differentiation of MC3T3-E1 osteoblasts.

FSS promotes synthesis and secretion of BMP2

BMP2 is the most competent factor to induce osteogenic differentiation [23]. As is shown in Fig. 2A, we found that mRNA level of BMP2 was significantly up-regulated at 3 h pf. and peaked at 12 h pf.. Accordingly, secretion of BMP2 was also promoted at 3 h pf. and peaked at 24 h pf. (Fig. 2B).

The data suggested that single bout short duration FSS may stimulate transcription and protein processing to induce osteogenic differentiation.

BMP2 induces osteogenic differentiation of MC3T3-E1 cells

BMP2 has been proved to be a potent factor induced differentiation of MC3T3-E1 cells [24,25]. To further confirm whether BMP2 could simulate differentiation pattern as FSS model, we exogenously supplied recombinant BMP2 and examined differentiation indicators of various periods.

After BMP2 incubation lasted for 12 h, transcriptional levels of ALP, RUNX2 and SP7 were consistently up-regulated. In addition, additive dorsomorphin which is a competitive inhibitor of smad1/5/8 known to suppress BMP2 signaling abrogated the up-regulation of these molecular markers (Fig. 3A): Accordingly, subsequent ALP activity (Fig. 3B) and terminal mineralization of ECM (Fig. 3C and 3D) were also elicited by recombinant BMP2, which could be canceled by dorsomorphin.

These results showed that BMP2 functions as bona fide factor of osteogenic differentiation in MC3T3-E1 cells.

Blocking BMP2 signaling abolishes single bout short duration FSS-induced osteogenic differentiation of MC3T3-E1 cells

To further address single bout short duration FSS that indeed utilizes BMP2 signaling to promote osteogenic differentiation, we tested whether dorsomorphin and silencing BMP2 expression could suppress differentiation process after FSS treatment.

After introducing dorsomorphin at the end of FSS treatment, transcription and secretion of BMP2 were remarkably attenuated at each time point (Fig. 4A and 4C). In agreement with this finding, silencing BMP2 with RNAi prior to FSS loading also reversed the transcriptional and secretory induction by FSS. (Fig. 4B and 4D). Correspondingly, transcription of ALP, RUNX2 and SP7 were persistently repressed over the expected peak time point both by dorsomorphin treatment (Fig. 4E, 4G and 4I) and RNAi (Fig. 4F, 4H and 4J). Subsequent elevated ALP activity induced by FSS was also abrogated by additional dorsomorphin treatment (Fig. 4K) and BMP2 silencing (Fig. 4L). Finally, terminal mineralization of ECM was canceled likewise by inhibiting BMP2 signaling (Fig. 4M and 4N).

Taken together, these results above indicated that single bout short duration FSS is capable of facilitating BMP2 transcription and synthesis to further induce osteogenic differentiation of MC3T3-E1 cells.

Interaction between integrin β1 and BMP2 pathway plays an important role in FSS promoted-osteogenic differentiation of MC3T3-E1 cells

FSS is a type of hydromechanical signal. How such physical signal is transduced into a biological signal to promote BMP2 synthesis and secretion is still unclear. Integrin β1 is an essential cell adhesion molecule that connects with ECM and senses extracellular mechanical signals [26]. To explore the possibility that integrin β1 is involved in our model, we measured the mRNA level of ITGB1 (coding integrin β1) after FSS. Fig. 5A shows that...
the transcriptional level of ITGB1 was significantly up-regulated just after FSS and peaked at 12 h pf. Furthermore, cells pre-incubated with integrin β1 inhibitor RGD peptide showed no positive modulation of ITGB1 expression after FSS, indicating RGD peptide successfully blocks integrin β1 signaling in our model. In consistency with this finding, silencing ITGB1 was also capable of abolishing FSS-induced expression of ITGB1 (Fig. 5B). As shown in Fig. 5C and 5E, blocking integrin β1 signaling with RGD abolished FSS-induced BMP2 transcription and secretion. Likewise, silencing ITGB1 achieved similar inhibitory effect on BMP2 production (Fig. 5D and 5F). Correspondingly, complement of recombinant BMP2 rescued the differentiation phenotypes that were blocked by RGD (Fig. S3). In agreement with these results, all positive modulated osteogenic differentiation indicators triggered by FSS, including molecular makers ALP (Fig. 5G & 5H), RUNX2 (Fig. 5I & 5J) and SP7 (Fig. 5K & 5L), ALP activities (Fig. 5M &5N), and final ECM mineralization (Fig. 5O & 5P) were all countered both through RGD pre-treatment and ITGB1 RNAi.

In this way, blocking integrin β1 is capable of abrogating FSS-induced BMP2 processing and subsequent differentiation mechanism, indicating that integrin β1 functions as a physical-biological transducer under single bout short duration FSS. To our surprise, by dorsomorphin treatment and BMP2 silencing after FSS, transcription of ITGB1 gene was notably suppressed (Fig. 5Q & 5R). This result suggests that there is a complicated interaction
between integrin β1 and BMP2 signaling to collaboratively promote osteogenic differentiation of MC3T3-E1 cells.

**Discussion**

Osteoblast differentiation induced by mechanical force is a tightly regulated process. Changes of extracellular mechanical force environment lead to rearrangement of cytoskeleton. Meanwhile, transmembrane signal transducer proteins convert physical signal into intracellular biological signal, triggering transcription of osteogenic-related genes, promoting generation of ECM and secretion of ALP, and finally resulting in mineralization of ECM to construct osteoid.

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**Figure 2. FSS promotes BMP2 synthesis and secretion.** (A) FSS up-regulated transcriptional level of BMP2 gene. Relative level of BMP2 was determined by qRT-PCR at a series of time points after FSS load. (B) Extracellular level of BMP2 protein was examined using ELISA. (Data were shown as mean ± SD, n = 3. *, P < 0.05; **, P < 0.01; ***, P < 0.001.) doi:10.1371/journal.pone.0061600.g002

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**Figure 3. BMP2 governs osteogenic differentiation of MC3T3-E1 cells.** (A) mRNA Levels of ALP, RUNX2 and SP7 were measured after recombinant BMP2 or/and dorsomorphin (DM) treatment for 12 h. *, BMP2 group versus control group; #, BMP2 + DM group versus BMP2 group. (B) ALP activity was detected as indicated at 24 h pf. (C) Microscopic examination and (D) quantification of ARS stain were carried out as above to assess the terminal differentiation at day 6 pf. (Scale bar: 50 μm. Data were shown as mean ± SD. n = 3. ***, P < 0.001; ####, P < 0.001.) doi:10.1371/journal.pone.0061600.g003
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A: BMP2 mRNA level (relative)

B: BMP2 mRNA level (relative)

C: ALP mRNA level (relative)

D: ALP mRNA level (relative)

E: ALP mRNA level (relative)

F: ALP mRNA level (relative)

G: RUNX2 mRNA level (relative)

H: SP7 mRNA level (relative)

I: SP7 mRNA level (relative)

J: SP7 mRNA level (relative)

K: ALP activity (pg/mg protein)

L: ALP activity (pg/mg protein)

M: Osteogenic differentiation assay

N: Osteogenic differentiation assay
RUNX2 is essential for differentiation of osteoblast lineage cells and bone formation in vitro and in vivo by binding osteoblast-specific cis-acting element 2 (OSE2) which is commonly found in the promoter regions of various osteogenic differentiation genes (e.g. osteocalcin, type I collagen, bone sialoprotein, osteopontin, alkaline phosphatase and collagenase-3) to promote osteogenic differentiation [27]. SP7 (ostexis) is a zinc finger-containing transcriptional factor which runs downstream of RUNX2 to induce maturation of osteoblasts [28].

Synthesis and secretion of collagen proteins occur during the proliferation stage of osteoblasts for ECM formation, which are necessary for osteogenesis [29]. In bones, collagens are the major type of ECM proteins with ~95% type I and ~5% type V collagens assembling into hetero-fibrils [30]. As for ALP that hydrolyzes various substrates including nucleic acid, protein and alkaloid, it catalyzes formation of phosphate anion that reacts with extracellular calcium ion to produce calcium phosphate. Deposition of calcium phosphate into collagen fiber network forms osteoid, which is the functional phenotype of the terminal step of osteogenic differentiation [31,32].

Previous studies have demonstrated that mechanical force is capable of triggering osteogenic differentiation signal pathway and biochemical process. Donahue/Jacobs’ group has found the effects of oscillatory fluid flow on osteoblastic (MC3T3-E1, hFOB) and osteocytic (MLO-Y4) cells, triggering intracellular Ca2+ mobilization [33], prostaglandin E2 release [34], osteopontin gene expression, mitogen-activated protein kinase activation [35], and NF-κB DNA binding inactivation [36]. In our present study it was found that a single load of steady FSS for 1 h at 12 dyn/cm² immediately increased F-actin stress fibers formation and rearrangement. Subsequently, osteogenic differentiation genes including ALP, RUNX2 and SP7 were remarkably up-regulated. In the later time point, secretion of type I collagen and elevation of ALP activity were observed. These molecular and biochemical changes induced by a steady and short duration FSS load ultimately facilitated terminal differentiation of osteoblasts.

BMP2 is a well-characterized regulatory factor that stimulates osteoblast differentiation. BMP2 promotes differentiation of mesenchymal cells into osteoblasts in vitro and induce bone formation in vivo [37]. Conversely, mice lacking limb-specific expression of BMP2 have a significant delay in formation of secondary ossification centers in each endochondral bone of limbs, showing obvious micro-fractures two weeks after birth and finally causing seriously damage under high loading stresses and strains in adult bones [38]. Our data reveals that a single load of short duration FSS dramatically induced transcription and secretion of BMP2. Blocking BMP2 signaling by dorsomorphin and RNAi abolished both FSS-induced terminal differentiation of osteoblasts, confirming that BMP2 signaling also contributes to osteoblast differentiation governed by single bout short duration FSS.

Previous reports have demonstrated that integrin β1 directs BMP2-induced osteogenic differentiation. Blocking integrin signal can significantly inhibit osteoblast differentiation induced by BMP2 [19,39]. In our model, FSS is the direct stimulus rather than chemical signal. We speculated that there should be a physical-biological transducer possibly located on cell surface. Our data supports that integrin β1 was up-regulated by single bout short duration FSS. Blocking integrin β1 by RGD peptide and RNAi abrogated osteogenic differentiation. In addition, inhibiting integrin β1 attenuated BMP2 synthesis and secretion, vice versa. These results suggest an interaction between integrin β1 and BMP2 signaling. Lai and Cheng have demonstrated a colocalization pattern of integrin β1 and BMP2 in human osteoblasts [19]. Further investigations are required to fully understand the collaboration of these proteins.

Traditional opinions prefer that pulsed and continuous mechanical stimulation is more favorable for fracture recovery and bone homeostasis rather than single stimulation. Dimitrios Pavlidis et al. found that only intermittent forces can induce phosphorylation of osteogenic related factor ERK1/2 in the pressure side of the rats’ molars, as compared with different strength of steady force for 2 and 4 hours [40]. Yu Ban et al. reported that continuous flow perfusion is a more favorable environment for the initiation of osteoblast activity compared with intermittent flow perfusion [41]. Kreke MR et al. found that repeated application of shear stress stimulates late phenotypic markers of osteoblastic differentiation of bone marrow stromal cells in a manner that depends on the duration of stimulus [42]. However, Lanyon and colleagues demonstrated that a single, short exposure to an exogenous load is sufficient to elevate metabolic activity of osteocytes and activate quiescent cells on the bone surface [8,9]. In our clinical practice, we found that a single stress loading on teeth was capable of inducing alveolar resorption on the tension side and new bone formation on the traction side within 4–5 weeks. By contrast, repeating load on teeth led to significant alveolar resorption but reduced new bone formation, ultimately resulting in complication including root resorption and teeth loosening. Our study confirms that one single load of short duration FSS was capable of triggering a long-term potentiated differentiation pathway and inducing terminal differentiation of osteoblast, which indicated that optimized strength and time interval could achieve better clinical effects.

In summary, our results demonstrate that single bout short duration and appropriate FSS is one of the effective approaches to promote terminal differentiation of osteoblast MC3T3-E1 cells, and interaction between BMP2 and integrin β1 signaling contributes to this cellular process. Our study provides further interpretation for mechanism of osteogenic differentiation triggered by single bout short duration fluid shear stress and may potentially offer experimental basis for further study of orthodontic bone remodeling and bone tissue engineering mechanisms.

Materials and Methods

Cell culture and drug treatment

MC3T3-E1 cells, murine pre-osteoblastic cell line from ATCC (Manassas, VA, USA), were cultured in α-MEM media (Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (Life Technologies), 1% penicillin-streptomycin (Life Technologies) and maintained in a 5% CO2 humidified environment at 37°C. Medium was changed every three days. BMP2 and dorsomorphin (Sigma-aldrich, St. Louis, MO, USA) were added to the culture medium with 50 μg/ml ascorbic acid and 10 mM β-
glycerophosphate at a concentration of 200 ng/ml and 10 μM respectively. Dorsomorphin was added after FSS load to block BMP2 signaling. 500 μg/ml RGD peptide (Sigma-aldrich) was added with before FSS load to block integrin β1 signaling. Mouse primary bone mesenchymal stem cells were isolated as described previously [43] and maintained in DMEM with 10% fetal bovine serum. Multipotent differentiation capacity was identified (Fig. S2).

Fluid flow stress application

When reaching 85–90% confluence on glass slides coated with 10 μg/ml polylysine (Sigma-aldrich), cells were starved for 12 hours in serum-free medium and then subjected to FSS (12 dyn/cm² for 1 hour) using a parallel plate flow system. The system, which consists of a parallel-plate flow chamber (PPFC), a multichannel pump (BT00-100/YZ1515, Baoding Longer Precision Pump Co., Ltd, Baoding, China) and a medium reservoir, generated a laminar unidirectional flow across the cells as described previously [44] The PPFC generated a laminar unidirectional flow across the cells consisted of two Polymethyl methacrylate organic glass plates(Guangzhou Suiming Artwork Co., Ltd, Guangzhou, China), a silicone gasket (Wanhe Plastic MaterialsCo., Ltd, Guangzhou, China), and a 76×26 mm cell-seeded glass slide. Here, the gasket maintained a watertight seal and a uniform channel of height, h = 0.03 cm. The flow system maintained at 37 ˚C and was filled with 1% serum-containing medium aerated with 5% CO₂. Control experiments were performed separately by placing the cells in the same condition as the corresponding FSS experiments without applying FSS stimulus. After FSS treatment, cells were grown in the same osteogenic conditions (50 μg/ml ascorbic acid and 10 mM β-glycerophosphate).

Confocal microscopy, immunostaining and quantitative analysis

F-actin was stained with Rhodamine-phalloidin mixed solution (Life Technologies, Eugene, OR, USA) overnight at 4 ˚C and nucleus was labeled with Hoechst 33258 (Life Technologies) for 10 min. Images were visualized using confocal microscopy (LSM 710, Zeiss, Germany). For type I collagen immunostaining, cells were fixed and permeabilized followed by 5% BSA (Sigma-aldrich) blocking. Primary antibody rabbit anti-mouse collagen type I (ab34170, 1:150; Abcam, Cambridge, MA,USA) and secondary antibody goat anti-rabbit (ZSGB-BIO, Beijing, China) was used. Five images were randomly taken in each section under light microscope (200×, Olympus, Japan). Relative integrated optical density (IOD) of immunostaining was calculated with Imaging-Pro Plus 6.0 software. Each group was assessed by estimating the median staining intensity per cell.

RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA was extracted by using TRIzol reagent (Life Technologies). 3 μg of total RNA from each sample was subjected to first-strand cdNA synthesis by using M-MLV reverse transcriptase (Life Technologies). Transcriptional levels of tested genes were quantified by quantitative Realtime-PCR (qRT-PCR) by LightCycler 480 (Roche, Switzerland) by using Platinum SYBR Green qPCR SuperMix-UDG (Life Technologies). Relative quantity of mRNA level was performed by using comparative CT method (ΔΔCT) with GAPDH as internal reference. The qRT-PCR consisted of 40 cycles (94 ˚C for 15 s, 60.5 ˚C for 15 s, 72 ˚C for 15 s) after an initial denaturation step (94 ˚C for 2 min). Primers’ information was provided in Table S1.

ELISA

The concentration of BMP2 in culture supernatants was measured using BMP2 Mouse ELISA kit (ab119582, abcam) according to the manufacturer’s instructions. Data were normalized by viable cell numbers which were calculated by trypan blue staining.

Alkaline phosphatase activity assay

ALP activity was determined by PNPP method with p-Nitrophenylphosphate as substrate(LabAssay TM ALP, Wako, Japanese) according to the manufacturers’ instructions. The enzyme activity (units/mg protein) is equal to concentration of p-Nitrophenol (μmol/μl) released by sample within 17 minutes after excluding background. ALP activity of each sample was normalized by protein concentration detected by BCA protein assay kit (Thermo Scientific Pierce, USA).

Alizarin Red S staining

To detect mineralization of ECM as a marker of terminal differentiation, cells were washed and fixed with 4% paraformaldehyde. Fixed cells were stained with 1% Alizarin Red S solution (Sigma-aldrich). Images were randomly taken under light microscope (400×, Olympus). Quantification of Alizarin Red S stain was assessed via extraction with cetyl-pyridinium chloride monohydrate (TCl, Japanese). Absorbance was read at 560 nm.

RNAi

Double strand siRNAs were designed and synthesized by Ribobio (Guangzhou, China). 50 nM of siRNAs were transfected by Lipofectamine RNAiMAX (Life Technologies) according to the manual. Double strand scramble RNA was used as the negative control (NC). Cells seeded on glass slides were transfected for 12 h and subjected to further treatment.

Statistical analysis

All experiments were carried out three times independently and presented as mean ± SD. Statistical analysis was performed by using one way ANOVA. Differences were considered to be statistically significant at P<0.05 (*) and #, P<0.01; ** and ##, P<0.001; *** and ###, P<0.001.).
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Supporting Information

Figure S1 FSS promoted ALP and Runx2 gene expression in MC3T3-E1 cells. mRNA levels of ALP and Runx2 were determined by qRT-PCR at 12 h post FSS. Data are shown as fold change relative to control. Data were shown as means ± SD. N ≥ 3; *** p < 0.001. (TIF)

Figure S2 Multipotent differentiation capacity of mouse bone marrow stromal cells (BMSCs). (A) BMSCs were cultured in DMEM media containing 10% fetal bovine serum, 1% penicillin-streptomycin and Osteogenesis induced fluid (50 μg/ml ascorbic acid 10 mM β-glycerophosphate and 0.1 μM dexamethasone) and stained with Alizarin Red S at day 21; (B) BMSCs were induced by Adipogenic liquid (0.1 μM dexamethasone, 10 μg/ml insulin and 0.45 mM 3-isobutyl-1-methyl-xanthin) and stained with Oil Red at day 21. (Scale-bar: 50 μm) (TIF)

Figure S3 BMP2 rescued RGD blocked differentiation phenotype. RGD blocked FSS-induced ECM mineralization, while supplemental of BMP2 rescued the differentiation phenotype. (TIF)

Table S1 Primers for quantitative RT-PCR. (DOC)

Author Contributions
Conceived and designed the experiments: ZM SW HA. Performed the experiments: ZM ZP JZ LG. Analyzed the data: SW HL DB GY. Contributed reagents/materials/analysis tools: DB GY. Wrote the paper: ZM SW HA.

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