Compressive force induces the expression of osteoblast differentiation-related transcription factors via TGF-ßs signaling pathway in MC3T3-E1 cells.

Remi Sano

Nihon University Graduate School of Dentistry,

Major in Orthodontics

（Directors：Prof. Noriyoshi Shimizu and Assist. Prof. Akira Nakajima）

Contents

This doctoral thesis are consisted the following article and new unpublished data (Fig. 5C, Fig. 6C, Fig. 8C, and Fig. 9C): Remi Sano, Akira Nakajima, Takayuki Kawato, Masao Maeno and Noriyoshi Shimizu Effect of Compressive Force on TGF-β1/2 Signaling Pathway in MC3T3-E1 Cells, Journal of Hard Tissue Biology (in press)

Abstract

Transforming growth factor (TGF)-β is a 25-kD homodimer with three main isoforms, namely TGF-β1, TGF-β2 and TGF-β3, and TGF-β1 and -β2 have a well-documented role in bone formation wherein they induce the differentiation of mesenchymal cells toward an osteoblastic lineage and enhance the differentiated function of osteoblasts. The key transcription factors that drive the mesenchymal precursor cell toward an osteoblast lineage, and thus support bone formation, are Runx2 and Osterix. In addition, several other transcription factors, including Msx2, also affect osteoblast differentiation. These transcription factors have been associated with the bone formation process induced by mechanical stress such as compressive force (CF). However, the effects of different magnitudes of CF on osteoblasts differentiation and bone formation, and optimal force for osteogenesis via TGF-β have not been fully understood.

TGF-β transmits signals across the plasma membrane by forming a heteromeric complex with TGF-β type 1 and 2 receptors (Tβr1 and Tβr2). TGF-β binds the Tβr1/Tβr2 conglomerate, and then Tβr2 phosphorylates Tβr1, thereby enabling Tβr1 to bind a Smad2 or Smad3 proteins. TGF-βs also activate Smad-independent signaling cascades

such as p38 mitogen-activated protein kinase (MAPK). However, it has not been elucidated how Smad-dependent and/or -independent pathways are specified and how they function in osteoblasts under conditions of mechanical stress, including CF stimulation. In the present study, with the aim of gaining insight into the role of TGF-β signaling in mechanical stress-induced bone formation, the effects of CF on the expression of TGF-β1, TGF-β2, Tβr1, Tβr2, Runx2, Osterix and Msx2, and the phosphorylation of Smad2, Smad3 and p38 were investigated using osteoblast-like MC3T3-E1 cells. Moreover, the effects of Tβr inhibitor (LY2109761) on the phosphorylation of Smad2, Smad3 and p38 and the expression of Runx2, Osterix and Msx2 in cells stimulated with CF were additionally examined.

MC3T3-E1 cells were seeded in 100-mm cell culture dishes at a density of 2.0×10^4 cells/cm^2 and incubated overnight until confluent. They were then compressed continuously by application of a uniform compression method. Briefly, thin round glass plates were placed over the layer of confluent cells, and CF was adjusted by placing a lead weight on the glass plates. Confluent cells in a culture dish were covered with the glass plate. The cells were subjected to 1.0 g/cm^2 (98 Pa) or 2.0 g/cm^2 (196 Pa) CF. Control cells were covered with the same type of glass plates, but without lead weights,

2

which produced a CF of 0.035 g/cm^2. Exogenous 25 nM LY2109761 as Tβr1/2 inhibitor was added immediately before 30 min of CF stimulation in order to prevent TGF-β from binding Tβr1 and Tβr2. The expression at mRNA and protein levels of TGF-β1, TGF-β2, Tβr1, Tβr2, Runx2, Osterix and Msx2 were measured by real-time polymerase chain reaction and Western blot analysis, respectively. Phosphorylation levels of Smad2, Smad3 and p38 were determined by Western blot analysis. These expressions or phosphorylation levels were statistically compared between cells stimulated with different magnitude of CF (the 1.0 g/cm^2 CF group and the 2.0 g/cm^2 CF group) and unstimulated control cells (the control group).

TGF-β1 and TGF-β2 mRNA expressions were significantly increased in the 1.0 g/cm^2 CF group compared with both the control and the 2.0 g/cm^2 CF groups. The 1.0 g/cm^2 CF group, but not the 2.0 g/cm^2 CF group also had significantly higher TGF-β1 and TGF-β2 protein levels than the control group. Tβr1 and Tβr2 expressions were unaffected by either magnitude of CF. The 1.0 g/cm^2 CF group had significantly higher levels of phosphorylated Smad2, Smad3 and p38 than did the control and the 2.0 g/cm^2 CF groups. These results suggest that the 1.0 g/cm^2 CF induce the expression of TGF-β1 and TGF-β2 and the phosphorylation of Smad2, Smad3 and p38, whereas, 2.0 g/cm^2

CF is a excess magnitude for these up-regulations.

The 1.0 g/cm^2 CF group also significantly increased mRNA and protein expressions of Runx2, Osterix and Msx2 compared with the control and the 2.0 g/cm^2 CF groups. Moreover, elevation of phosphorylated Smad2, Smad3 and p38 levels observed in the 1.0 g/cm^2 CF group were blocked by pretreatment with the Tβr1/2 inhibitor LY2109761. LY2109761 pretreatment also blocked the stimulatory effects of CF on Runx2, Osterix and Msx2 mRNA and protein expressions in the 1.0 g/cm^2 CF group. These results suggest that 1.0 g/cm^2 CF-induced TGF-β increases the expression of osteogenesis-related transcription factors including Runx2, Osterix and Msx2 via autocrine action of CF-induced TGF-β in osteoblasts.

In conclusion, the 1.0 g/cm^2 CF is within an optimal mechanical stress range for promoting osteoblast differentiation via upregulation of Runx2, Osterix and Msx2 expressions by autocrine action of CF-induced TGF-β. Moreover, these upregulations were reflected in both Smad-dependent and -independent signaling pathway molecules, suggesting that optimal force has an osteogenic bone formation, at least under the present experimental conditions.

Introduction

Transforming growth factor (TGF)-β is a potent cell regulatory polypeptide[1]. It is a multifunctional signaling molecule that plays a role in a wide array of cellular processes, including embryonic development as well as cell growth, differentiation, motility, and apoptosis[1,2]. TGF-β is a 25-kD homodimer with three main isoforms, namely TGF-β1, TGF-β2 and TGF-β3, and 40 related family members[3,4]. TGF-β1 and -β2 have a well-documented role in bone formation wherein they induce the differentiation of mesenchymal cells toward an osteoblastic lineage and enhance the differentiated function of osteoblasts[1-7].

Bone contains large amounts of TGF-β1/2 and target cells of TGF-β activity expressing TGF-β type 1 and 2 receptors (Tβr1 and Tβr2). The key transcription factor that drives the mesenchymal precursor cell toward an osteoblast lineage, and thus supports bone formation, is Runx2 and Osterix, which regulates the expression of all known marker genes expressed by the osteoblast[8-11]. In addition, several other transcription factors, including Msx2, also affect osteoblast differentiation[12]. These transcription factors have been associated with the bone formation process induced by mechanical stress such as compressive force (CF)[13-18]. However, the effects of different magnitudes of CF on osteoblasts reactions and bone formation, and optimal force for osteogenesis via TGF-β signaling interacts have not been fully understood.

TGF-β transmits signals across the plasma membrane by forming a heteromeric complex with Tβr1 and Tβr2, which are serine/threonine kinase receptors[3,4]. Activated Tβrs initiate intracellular signaling through phosphorylation of linked Smad proteins,

5

and then translocate to the nucleus to direct a transcriptional response[1,3-7]. More specifically, when TGF-β binds the Tβr1/Tβr2 conglomerate, Tβr2 phosphorylates Tβr1, thereby enabling Tβr1 to bind a Smad2 or Smad3 proteins (so-called receptor-activated Smad proteins)[19]. TGF-βs also activate Smad-independent signaling cascades, including mitogen-activated protein kinase (MAPK) pathways[19-22]. Notably, activation of p38 signaling by TGF-β is accompanied by Tβr-kinase activity-independent TNF receptor associated factor-6/TGF-β activation kinase1 (TAK1) phosphorylation[22-26]. However, it has not been elucidated how Smad-dependent and/or -independent pathways are specified and how they function in osteoblasts under conditions of mechanical stress, including CF stimulation.

In the present study, with the aim of gaining insight into the role of TGF-β signaling in mechanical stress-induced bone formation, the author investigated the effects of CF (two magnitudes) on the expression of TGF-β1, TGF-β2 and their receptors, and the expression of bone-related transcription factors, namely Runx2, Osterix and Msx2. The author also investigated effect of CF on the phosphorylation of Smad2, Smad3 and p38 in cultured osteoblasts.

Materials and Methods

Cell culture

The osteoblast-like MC3T3-E1 cells, which were originated from newborn mouse calvaria, were obtained from the RIKEN Bio Resource Center (Ibaraki, Japan)[27]. The cells were maintained in α-minimal essential medium (α-MEM; Gibco BRL, Rockville, MD, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Application of CF

MC3T3-E1 cells were seeded in 100-mm cell culture dishes at a density of 2.0 × 10^4 cells/cm^2 and incubated overnight until confluent. They were then compressed continuously by application of a uniform compression method similar to those described previously[14-18] (Fig. 1). Briefly, thin round glass plates were placed over the layer of confluent cells without FBS, and CF was adjusted by placing a lead weight on the glass plates. Confluent cells in a culture dish (inner diameter 83 mm, thickness 1.0 mm) were covered with the glass plate (inner diameter 78 mm, thickness 0.85 mm)[14-18]. The weight was positioned so that the force was evenly distributed across the cell monolayer. The stainless steel wire bridges with tripod(angle 120°) were placed on the glass plates such that the weight did not touch the culture medium[14-18]. The cells were subjected to 1.0 g/cm^2 (98 Pa) or 2.0 g/cm^2 (196 Pa) CF for 1 h, 3 h, 6 h and 9 h[14-18] (Fig. 1). Control cells were covered with the same type of glass plates, but without lead weights, which produced a CF of 0.035 g/cm^2. The possibility that the removal of the glass plates might

cause static electricity, which could affect the cells, was controlled for using glass plates on the CF cells and the control cells.

Cell culture for exogenous Tβr1/2 inhibitor (LY2109761)

A series of enzymatic and cellular assays were carried out to characterize the ability of orally active Tβr1/2 kinase dual inhibitor LY2109761 (I, K_i = 38 nmol/L; II, K_i = 300 nmol/L *in vitro* kinase assay) to inhibit TGF-β signaling *in vitro* by preventing TGF-β from binding Tβr1 and Tβr2 receptors[28,29]. MC3T3-E1 cells were plated in 100-mm cell culture dishes at a density of 2.0×10^4 cells/cm^2. After incubating the cells overnight, exogenous 25 nM LY2109761 (G-T, Minneapolis, MN, USA) as Tβr1/2 inhibitor was added immediately before 30 min of CF stimulation[28]. The cells were then incubated for up to 6 h with 1.0 g/cm^2 CF or without CF in preparation for measurement of total and phosphorylated levels of Smad2, Smad3 and p38, and the expression of Runx2, Osterix and Msx2.

Real-time polymerase chain reaction (PCR)

Total RNA was isolated from cultured MC3T3-E1 cells using a commercially available kit (RNeasy Mini Kit; Qiagen, Valencia, CA, USA). Aliquots containing equal amounts of mRNA were subjected to real-time PCR. First-strand cDNA synthesis was carried out with 1 μg of DNase-treated total mRNA in a 20-μl volume of a solution containing first-strand buffer, random primers (50 ng), 10 mM dNTP mixture, 1 mM DTT, and 0.5 U reverse transcriptase at 42°C for 60 min. The cDNA mixtures were diluted five-fold in sterile distilled water, and 2-μl aliquots were subjected to real-time

PCR with SYBR Green I dye. Each real-time PCR was performed in a 25-μl volume of a solution containing 1X R-PR buffer, 1.5 mM dNTP mixture, 1X SYBR Green I, 15 mM $MgCl_2$, 0.25 U Ex Taq polymerase real-time PCR version (TaKaRa, Tokyo, Japan), and 20 mM specific primers (sense and antisense), as shown in Table 1. The primers were designed in Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). The PCRs were carried out in a thermal cycler (Smart Cycler, Cepheid, Sunnyvale, CA, USA), and the data were analyzed in Smart Cycler software (ver. 1.2d). The real-time PCR conditions were 40 cycles of 95°C for 3 s and 68°C for 20 s. Measurements were taken at the end of the annealing step at 68°C in each cycle. The specificity of the PCR products was verified by adding melting curve analysis (68-94°C). All real-time PCRs were performed in triplicate, and the levels of mRNA expression were calculated and normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA detected at each time point.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

To obtain whole-cell extracts, MC3T3-E1 cells cultured with or without CF were rinsed with phosphate buffered saline, and then exposed to a lysis buffer that consisted of 50 mM Tris-HCl, 0.1% Triton X-100, 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, in which they were sonicated for 10 s three times. Aliquots that contained equal amounts of protein were resolved by 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes with a semidry transfer unit. The membranes were probed with anti-TGF-β1, anti-TGF-β2,

anti-Runx2, anti-Osterix, anti-Msx2 (these five antibodies: Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:500, polyclonal rabbit antibodies), anti-Smad2, anti-p-Smad2, anti-Smad3, anti-p-Smad3, anti-p38, anti-p-p38 (these six antibodies: Cell Signaling Technology, Boston, MA; 1:1000, polyclonal rabbit anti-bodies) or anti-GAPDH (Millipore, Billerica, MA, USA; 1:500, internal standard, monoclonal mouse antibody), followed by a biotin-conjugated secondary antibody (dilution 1:10,000). Then, membranes were treated with horseradish peroxidase-conjugated streptavidin. Immunoreactive proteins were visualized with a chemiluminesence kit (Amersham Life Science, Buckinghamshire, UK). The intensities of the blots were quantified by computer using scanner (Epson PX-603F, Seiko Epson, Tokyo, Japan) and digital image analysis software (Scion image, version Beta 4.0.3, Scion corporation, NIH, Bethesda, ML, USA). The protein expressions of TGF-β1, TGF-β2, Runx2, Osterix and Msx2 were performed in triplicate, and the levels of protein expression was calculated and normalized to GAPDH detected at each time point. And the expressions of p-Smad2, p-Smad3 and p-p38 were also examined triplicate, and normalized to the expressions of total-Smad2, total-Smad3 and total-p38, respectively.

Statistical analysis

All data are presented as means ± standard deviations (SDs). Statistical significance was determined using Turkey's HSD test after analyses of variance (ANOVAs). Values of $p < 0.05$ were considered to be statistically significant.

Results

Effects of CF on expression of TGF-β1, TGF-β2 and their receptors

As shown in Figure 2A and B, real-time PCR of MC3T3-E1 cells stimulated, or not, with continuous CF (1.0 and 2.0 g/cm^2) indicated that expression of TGF-β1 and TGF-β2 mRNA increased gradually up to the 3-h or 6-h time point, and decreased gradually thereafter. TGF-β1 and TGF-β2 mRNA expressions were increased in the 1.0 g/cm^2 CF group compared with both the control and 2.0 g/cm^2 CF groups at the 3-h and 6-h time points (Fig. 2A and B). Western blot analysis confirmed that the cells stimulated with 1.0 g/cm^2, but not 2.0 g/cm^2, continuous CF also had higher TGF-β1 and TGF-β2 protein levels than control cells (Fig. 3A and B).

Tβr1 and Tβr2 transcript levels in unstimulated control cells and CF-stimulated cells peaked at 1 h and 3 h, respectively, and thereafter decreased gradually (Fig. 2C and D). Thus, TGF-β receptor mRNA expression preceded TGF-β ligand mRNA expression. Tβr1 and Tβr2 transcript levels were statistically similar between the groups at all time points.

Effects of CF on Smad2, Smad3 and p38 phosphorylation

Western blot analysis showed that total Smad2 and Smad3 protein levels were similar between control and CF-treated cells at 6 h. Conversely, the 1.0 g/cm^2 CF group had higher levels of phosphorylated Smad2 and Smad3 than did the control and 2.0 g/cm^2 CF groups at the 6-h time point (Fig. 4A and B). To determine the effects of CF on the Smad-independent signaling pathway, the phosphorylation of p38 was also

investigated by Western blot analysis. As a result, CF did not alter p38 expression level, whereas phosphorylated-p38 level significantly increased in the 1.0 g/cm^2 CF group compared with those observed for the control and 2.0 g/cm^2 CF groups (Fig. 4C).

Effects of CF on expression of Runx2, Osterix and Msx2

Real-time PCR analysis indicated that the 1.0 g/cm^2 CF group significantly increased mRNA expression of Runx2, Osterix and Msx2 (Fig. 5A, B and C), compared with the control and 2.0 g/cm^2 CF groups, at 3 h and 6 h, the same time points when CF effects on TGF-β1/2 expressions. Western blots run with samples taken at the 6-h time point showed significantly increased Runx2, Osterix and Msx2 protein levels in the 1.0 g/cm^2 CF group compared with the control and 2.0 g/cm^2 CF groups (Fig. 6A, B and C).

Effect of Tβr-1/2 inhibition on CF-induced phosphorylation of Smad2, Smad3 and p38, and expression of Runx2, Osterix and Msx2

Elevation of phosphorylated Smad2, Smad3 and p38 levels observed in 1.0 g/cm^2 CF-stimulated cells were blocked by pretreatment with the Tβr1/2 inhibitor LY2109761 (Fig. 7A, B and C). The Tβr1/2 inhibitor pretreatment blocked the effects of the CF condition on Runx2, Osterix and Msx2 mRNA expression (Fig. 8A, B and C) and attenuated CF-induced increases in Runx2, Osterix and Msx2 protein expression at the 6-h time point (Fig. 9A, B and C).

Discussion

The central findings of the present study were that 1.0 g/cm^2 CF stimulated TGF-β1 and TGF-β2 production in osteoblast-like MC3T3-E1 cells, and also stimulated the phosphorylation of Smad2/3 in the Smad-dependent signaling pathway, and p38, which is linked to the Smad-independent pathway. Expression levels of the transcription factors Runx2, Osterix and Msx2 were also increased by 1.0 g/cm^2 CF.

The aforementioned stimulative effects of 1.0 g/cm^2 CF were not observed for cells loaded with 2.0 g/cm^2 CF. Hence, these findings are consistent with prior studies showing that 1.0 g/cm^2 CF is within an optimal mechanical stress range for facilitating osteogenesis in osteoblasts by increasing type I collagen, bone sialoprotein and bone morphogenetic protein expression levels, whereas excessive mechanical stress (e.g. 3.0 g/cm^2) attenuates these processes[14,15].

The findings showed that Tβr1/2 inhibition blocked CF-induced phosphorylation of Smad2/3 and CF-induced expression of Runx2, Osterix and Msx2. They meant TGF-β signaling pathway included by 1.0 g/cm^2 CF was most to occur via an autocrine action of CF-induced TGF-βs binding their receptors. Given that TGF-β can have positive or negative regulatory effects on osteoblast differentiation[30], 1.0 g/cm^2 CF condition could be presumed to promote osteoblast differentiation, whereas the 2.0 g/cm^2 condition was not.

Osteoblast differentiation and bone formation appear to be influenced by Smad-independent TGF-β signaling pathways as well as Smad-dependent TGF-β signaling[3-7,19-24]. For example, the association of TAK1 and TAK1 binding protein 1 that is induced

by TGF-β1 results in activation of the MAPK kinase 3-p38 MAPK signaling cascade that leads to the induction of type I collagen expression[30], and TAK1 regulates the steady-state protein levels of these two kinases[23-26]. Interestingly, a recent study demonstrated that following TGF-β induction, both the Smad and p38 pathways converged at Runx2 in their control of mesenchymal precursor cell differentiation[31]. Indeed, here, I found that TGF-β inhibition prevented CF-induced phosphorylation of p38 and expression of Runx2, Osterix and Msx2, data which support the notion that Smad-independent signaling can also promote osteoblast differentiation via expression of osteogenesis-related transcription factors.

Bone morphogenetic proteins (BMPs), which are members of the TGF-β superfamily and share 32-37% sequence homology with TGF-βs, have profound effects on osteoblast activity[31-37]. The net effect of TGF-βs and BMPs on gene expression depends on the intricate balance of these two signal transduction pathways[37]. Following BMP induction, Smad1/5 and p38 cascades converge at Runx2 to control mesenchymal precursor cell differentiation[36,37]. Previous studies reported that CF increased the expression of BMPs and their receptors, increased levels of phosphorylated-Smad1, the expression of Runx2 and Osterix, and augment *in vitro* mineralization; these were attenuated by BMP receptor antagonism[14,18]. These previous findings and the present results indicate that both the BMP and TGF-β signaling pathways might be stimulated by CF, and osteoblast differentiation was induced via Smad-dependent/-independent cascades, and cross-talk of BMP and TGF-β signaling pathways might be related to these phenomena.

Conclusion

The 1.0 g/cm^2 CF is within an optimal mechanical stress range for promoting osteoblast differentiation via upregulation of Runx2, Osterix and Msx2 expressions by autocrine action of CF-induced TGF-β. In addition, these increases were attenuated by pretreatment with LY2109761. The present findings indicate that 1.0 g/cm^2 CF can induce bone-specific transcription factors via autocrine action of CF-induced TGF-β signaling in osteoblasts.

These upregulation were reflected in both Smad-dependent and -independent signaling pathway molecules, suggesting that optimal force has an osteogenic bone formation, at least under the present experimental conditions.

Acknowledgements

References

1. Massague J, Blain SW and Lo RS. TGFβ signaling in growth control, cancer, and heritable disorders. Cell 103: 295-309, 2000

2. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM and Wang EA. Novel regulators of bone formation: molecular clones and activities. Science 242: 1528-1534, 1998

3. Derynck R, Zhang Y and Feng XH. Smads: transcriptional activators of TGF-β responses. Cell 95: 737-740, 1998

4. Kretzschmar M and Massagué J. SMADS: Mediators and regulatios of TGF-β signaling. Curr Opin Gen Develop 8: 103-111, 1998

5. Centrella M, McCarthy TL and Canalis E. Skeletal tissue and transforming growth factor β. FASEB J 2: 3066-3073, 1988

6. Janssens K, ten Dijke P, Janssens S and Van Hul W. Transforming growth factor-β1 to the bone. Endocr Rev 26: 743-774, 2005

7. Shi Y and Massague J. Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell 113: 685-700, 2003

8. Ducy P, Zhang R, Geoffroy V, Ridall AL and Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89: 747-754, 1997

9. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR and de Crombrugghe B. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108: 17-29, 2002

10. Fujita T, Izumo N, Fukuyama R, Meguro T, Nakamuta H, Kohno T and Koida M.

Phosphate provides an extracellular signal that drives nuclear export of Runx2/Cbfa1 in bone cells. Biochem Biophys Res Commun 280: 348-352, 2001

11. Gilbert L, He X, Farmer P, Rubin J, Drissi H, van Wijnen AJ, Lian JB, Stein GS and Nanes MS. Expression of the osteoblast differentiation factor RUNX2 (Cbfa1/AML3/Pebp2αA) is inhibited by tumor necrosis factor-α. J Biol Chem 277: 2695-2701, 2002

12. Ducy P, Schinke T and Karsenty G. The osteoblast: a sophisticated fibroblast under central surveillance. Science 289: 1501-1504, 2000

13. Kanai K, Nohara H and Hanada K. Initial effects of continuously applied compressive stress to human periodontal ligament fibroblasts. J Jpn Orthod Soc 51: 153-163, 1992

14. Mitsui N, Suzuki N, Maeno M, Mayahara K, Yanagisawa M, Otsuka K and Shimizu N. Optimal compressive force induces bone formation via increasing bone sialoprotein and prostaglandin E_2 production appropriately. Life Sci 77: 3168-3182, 2005

15. Mitsui N, Suzuki N, Maeno M, Yanagisawa M, Koyama Y, Otsuka K and Shimizu N. Optimal compressive force induces bone formation via increasing bone morphogenetic proteins production and decreasing their antagonists production by Saos-2 cells. Life Sci 78: 2697-2706, 2006

16. Yanagisawa M, Suzuki N, Mitsui N, Koyama Y, Otsuka K and Shimizu N. Compressive force stimulates the expression of osteogenesis-related transcription factors in ROS 17/2.8 cells. Arch Oral Biol 53: 214-219, 2007

17. Zhang F, Wang CL, Koyama Y, Mitsui N, Shionome C, Sanuki R, Suzuki N,

Mayahara K, Shimizu N and Maeno M. Compressive force stimulates the gene expression of IL-17s and their receptors in MC3T3-E1 cells. Connect Tissue Res 51: 359-369, 2010

18. Shionome C, Kawato T, Tanabe N, Kariya T, Suzuki R, Koyama Y, Suzuki N, Shimizu N and Maeno M. Compressive force induces the expression of bone remodeling-related proteins via inter leukin-11 production in MC3T3-E1. J Hard Tissue Biol 21: 65-74, 2012

19. Massague J, Seoane J and Wotton D. Smad transcription factors. Genes Dev 19: 2783-2810, 2005

20. Kang JS, Liu C and Derynck R. New regulatory mechanisms of TGF-β receptor function. Trends Cell Biol 19: 385-394, 2009

21. Zhang YE. Non-Smad pathways in TGF-β signaling. Cell Res 19: 128-139, 2009

22. Iwata J, Parada C and Chai Y. The mechanism of TGF-β signaling during palate development. Oral Dis 17: 733-744, 2011

23. Kimura N, Matsuo R, Shibuya H, Nakashima K and Taga T. BMP2-induced apoptosis is mediated by activation of the TAK1-p38 kinase pathway that is negatively regulated by Smad6. J Biol Chem 275: 17647-17652, 2000

24. Sorrentino A, Thakur N, Grimsby S, Marcusson A, von Bulow V, Schuster N, Zhang S, Heldin CH and Landström M. The type I TGF-β receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. Nat Cell Biol 10: 1199-1207, 2008

25. Yamashita M, Fatyol K, Jin C, Wang X, Liu Z and Zhang YE. TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-β. Mol Cell 31: 918-924,

2008

26. Jung SM, Lee JH, Park J, Oh YS, Lee SK, Park JS, Lee YS, Kim JH, Lee JY, Bae YS, Koo SH, Kim SJ and Park SH. Smad6 inhibits non-canonical TGF-β1 signaling by recruiting the deubiquitinase A20 to TRAF6. Nat Commun 4: 2562, 2013

27. Sudo H, Kodama HA, Amagai Y, Yamamoto S and Kasai S. *In vitro* differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 96: 191-198, 1983

28. Melisi D, Ishiyama S, Sclabas GM, Fleming JB, Xia Q, Tortora G, Abbruzzese JL and Chiao PJ. LY2109761, a novel transforming growth factor β receptor type I and type II dual inhibitor, as a therapeutic approach to suppressing pancreatic cancer metastasis. Mol Cancer Ther 7: 829-840, 2008

29. Fransvea E, Angelotti U, Antonaci S and Giannelli G. Blocking transforming growth factor-beta up-regulates E-cadherin and reduces migration and invasion of hepatocellular carcinoma cells. Hepatology 47: 1557-1566, 2008

30. Kim SI, Kwak JH, Zachariah M, He Y, Wang L and Choi ME. TGF-β-activated kinase 1 and TAK1-binding protein 1 cooperate to mediate TGF-β1-induced MKK3-p38 MAPK activation and stimulation of type I collagen. Am J Physiol Renal Physiol 292: 1471-1478, 2007

31. Lee KS, Hong SH and Bae SC. Both the Smad and p38 MAPK pathways play a crucial role in Runx2 expression following induction by transforming growth factor-β and bone morphogenetic protein. Oncogene 21: 7156-7163, 2002

32. Rosen V and Wozney JM. Bone morphogenetic proteins. In: Bilezikian JP, Raiz

LA and Rodan GA (eds) Principles bone biology, 2nd ed. Academic Press, Cambridge, 2001, pp 919-928

33. Lai CF and Cheng SL. Signal transductions induced by bone morphogenetic protein-2 and transforming growth factor-β in normal human osteoblastic cells. J Biol Chem 277: 15514-15522, 2002

34. Miyazono K, Maeda S and Imamura T. BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. Cytokine Growth Factor Rev 16: 251-263, 2005

35. Derynck R and Akhurst RJ. Differentiation plasticity regulated by TGF-β family proteins in development and disease. Nat Cell Biol 9: 1000-1004, 2007

36. Chen G, Deng C and Li YP. TGF-β and BMP signaling in osteoblast differentiation and bone formation. Int J Bio Sci 8: 272-288, 2012

37. Rahman MS, Akhtar N, Jamil HM, Banik RS and Asaduzzaman SM. TGF-β/BMP signaling and other molecular events: regulation of osteoblastogenesis and bone formation. Bone Res 3: 15005, 2015

Table 1.

PCR primers used in the experiments

| Gene | Primer sequence | | GenBank |
	Forward	Reverse	Accession No.
TGF-β1	5'-GTGTGGAGCAACATGTGGAACTCTA-3'	5'-CGCTGAATCGAAAGCCCTGTA-3'	NM_011577
TGF-β2	5'-TCGACATGGATCAGTTTATGCG-3'	5'-CCCTGGTACTGTTGTAGATGGA-3'	NM_009367
Tβr1	5'-CAG AGG GCA CCA CCT TAA AA-3'	5'-AATGGTCCTGGCAATTGTTC-3'	NM_009370
Tβr2	5'-ATGAGCAACTGCAGCATCAC-3'	5'-GGCAAACCGTCTCCAGAGTA-3'	NM_029575
Runx2	5'-TCCTGCAAGTCCCCTGATAC-3'	5'-AACCTGGAGGGAAAATGCTT-3'	NM_009820
Msx2	5'-TGCAAGCGGCATCCATATACA-3'	5'-GCGTGGCATAGAGTCCCACA-3'	NM_013601
Osterix	5'-GCATCACCCAGAAGAAGAGC-3'	5-GTCCATCCAGAGGCACTCAT-3'	NM_152860
GAPDH	5'-CAATGACCCCTTCATTGACC-3'	5'-GACAAGCTTCCCGTTCTCAG-3'	XM_001473623
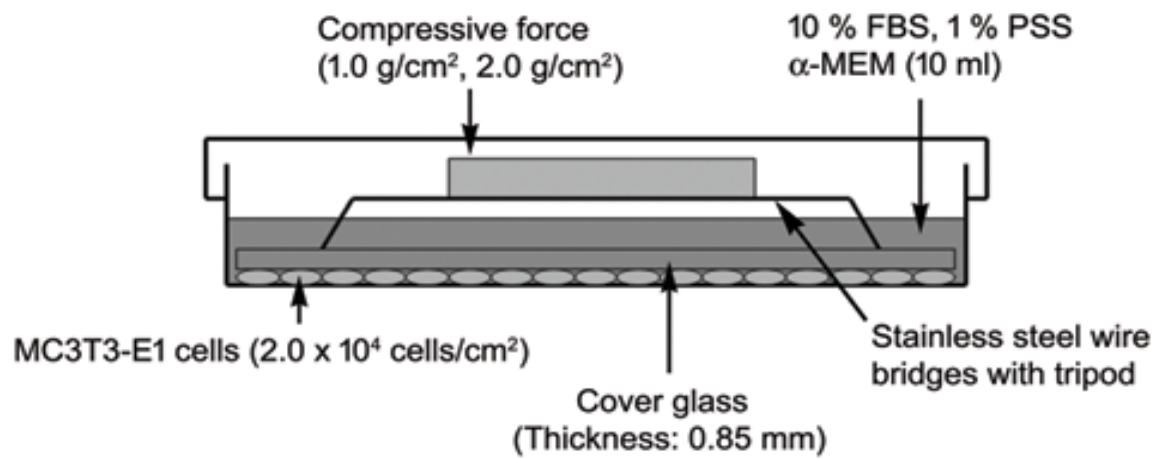
Figure 1. Diagram of compressive force application paradigm (image modified from the previous report[16])
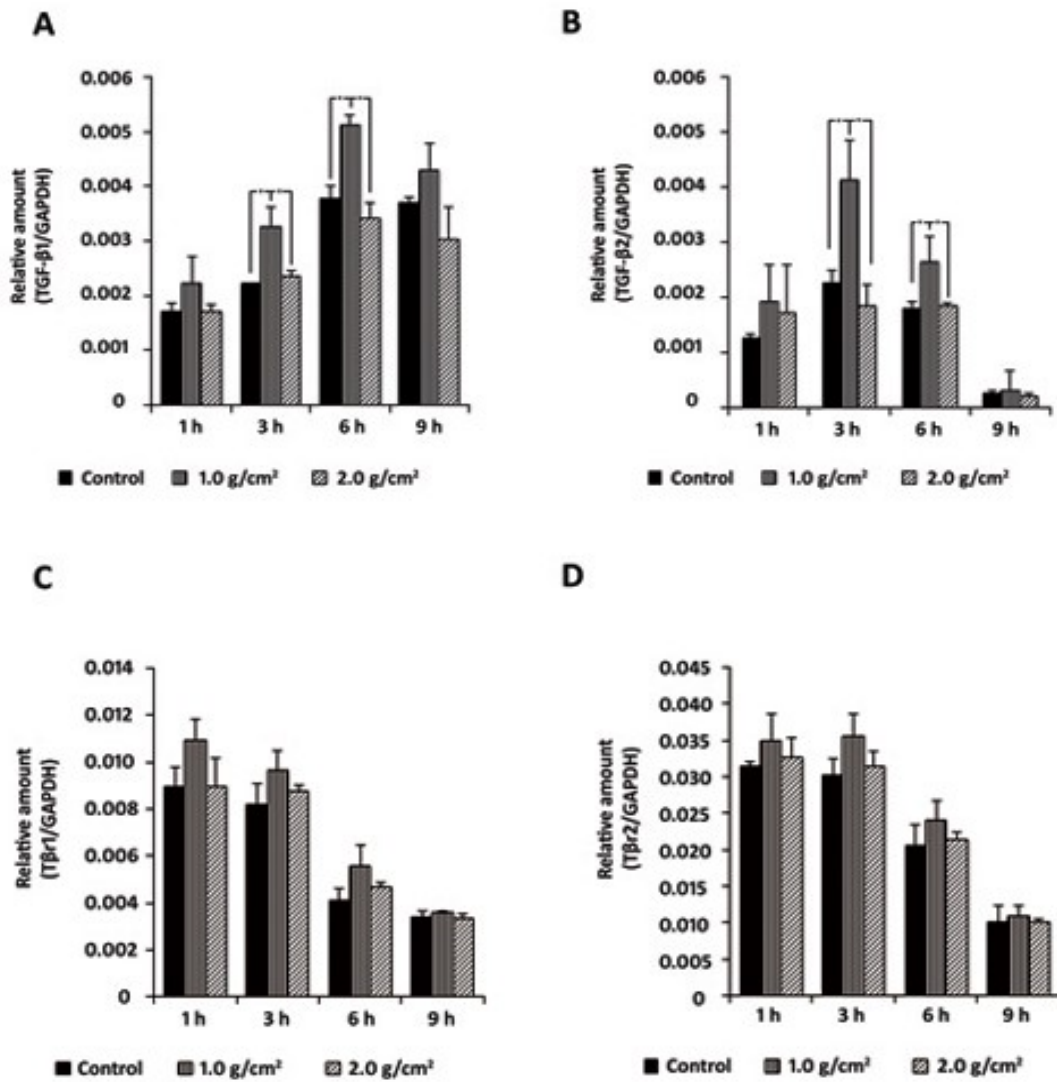
Figure 2. Effects of CF on the mRNA expression of TGF-β1, TGF-β2 and their receptors

The mRNA expressions of TGF-β1 (A) and TGF-β2 (B) increased gradually up to the 3-h and

6-h time point, respectively, and then decreased gradually thereafter. The mRNA expressions of

Tβr1 (C) and Tβr2 (D) peaked at the 1-h time point, and these expressions were slightly increase

in 1.0 g/cm^2 CF compared with the control and 2.0 g/cm^2 CF (n = 3, *p < 0.05).
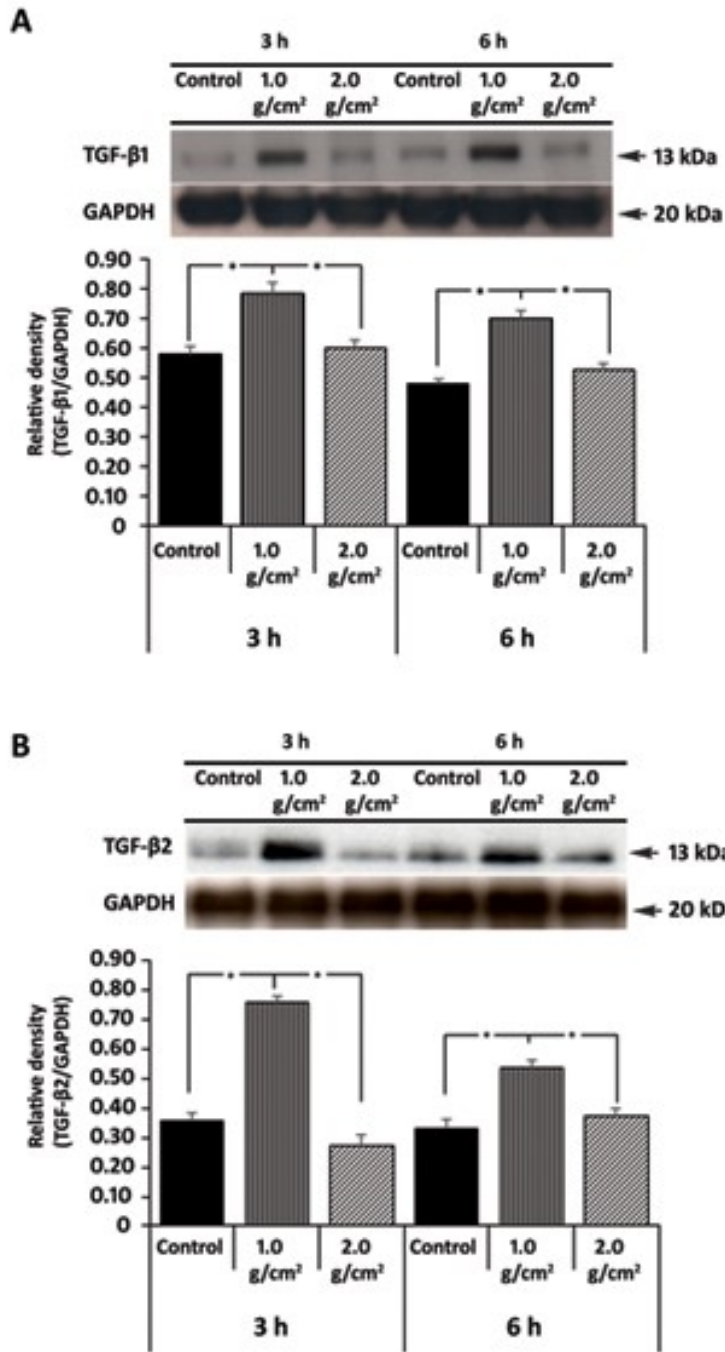
Figure 3. Effects of CF on the protein expression of TGF-β1 and TGF-β2

Western blots of TGF-β1 (A) and TGF-β2 (B) protein expression in MC3T3-E1 cells stimulated,

with or without continuous CF (1.0 g/cm^2 or 2.0 g/cm^2). Both proteins were significantly

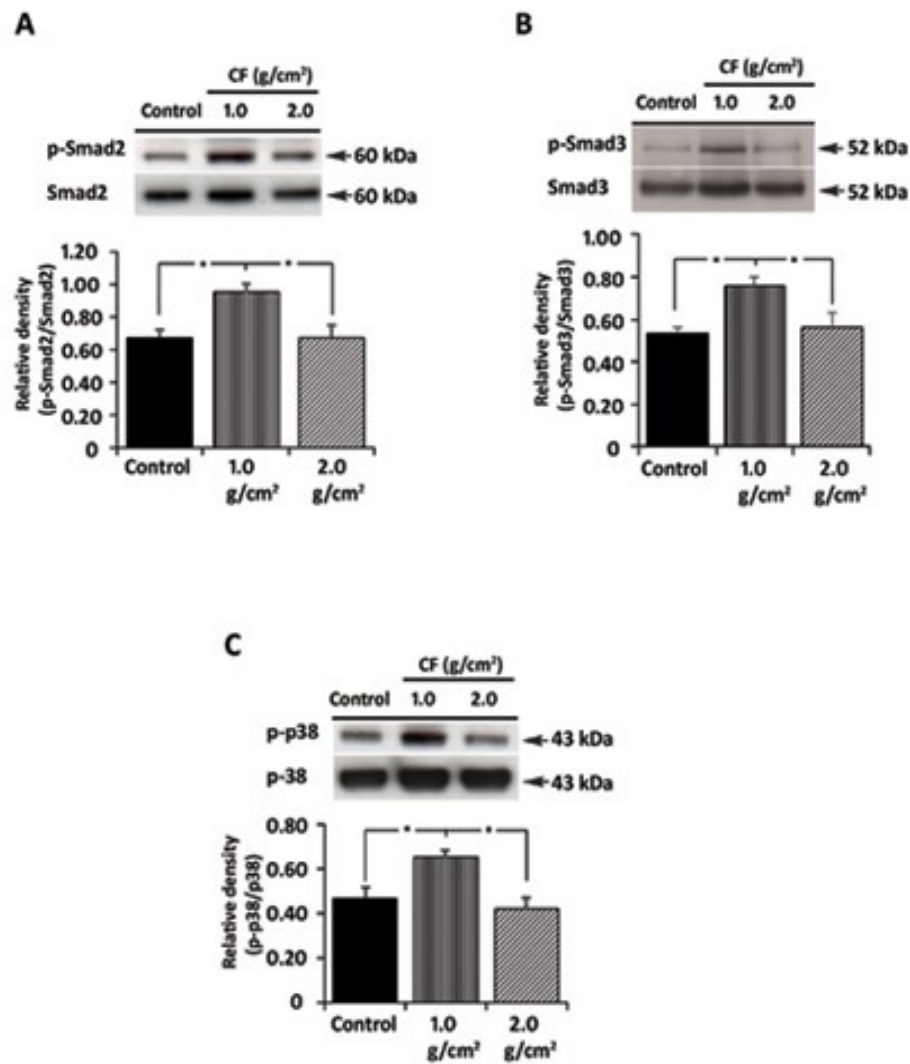increased in the 1.0 g/cm^2 CF compared with control and the 2.0 g/cm^2 CF (n = 3, *p < 0.05).

Figure 4. Effects of CF on Smad2, Smad3 and p38 phosphorylation

Phosphorylated levels of Smad2 (A), Smad3 (B) and p38 (C) were significantly increased at the

6-h time point in the 1.0 g/cm^2 CF, but not the 2.0 g/cm^2 CF. However, there were no significantly

different the total expressions of Smad2, Smad3 and p38 between control and CF-treatment
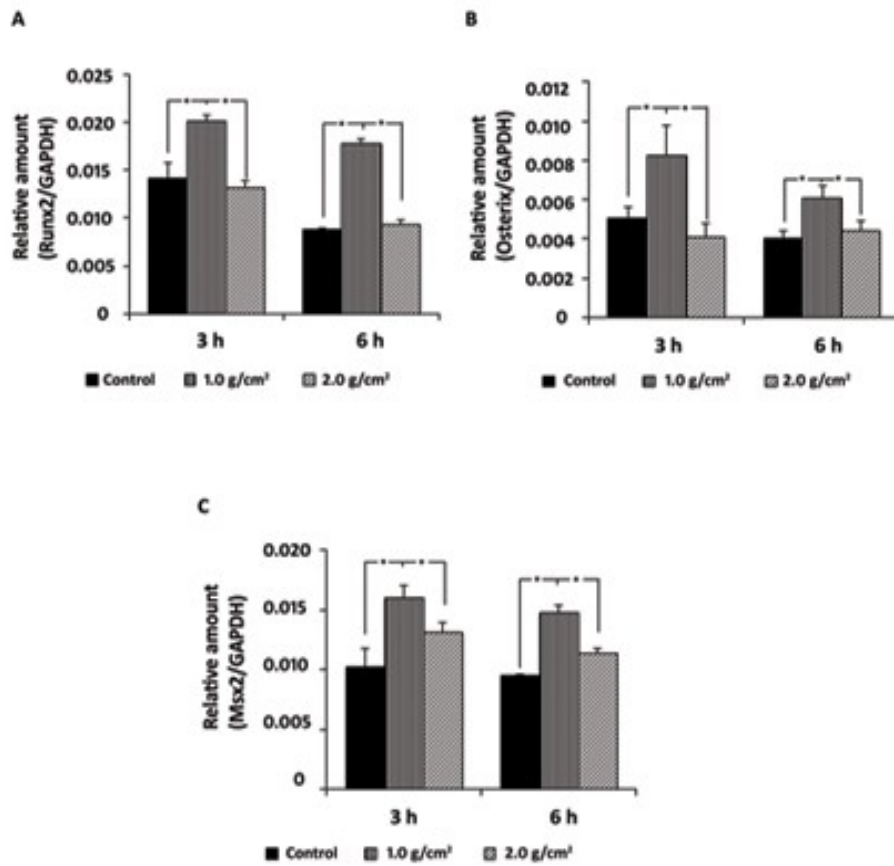
groups (n = 3, *p < 0.05).

Figure 5. Effects of CF on Runx2, Osterix and Msx2 mRNA levels

The mRNA expressions of Runx2 (A), Osterix (B) and Msx2 (C) were increased in cells stimulated with 1.0 g/cm^2 CF compared with control and 2.0 g/cm^2 CF at the 3-h and 6-h time points (n = 3, *p < 0.05).
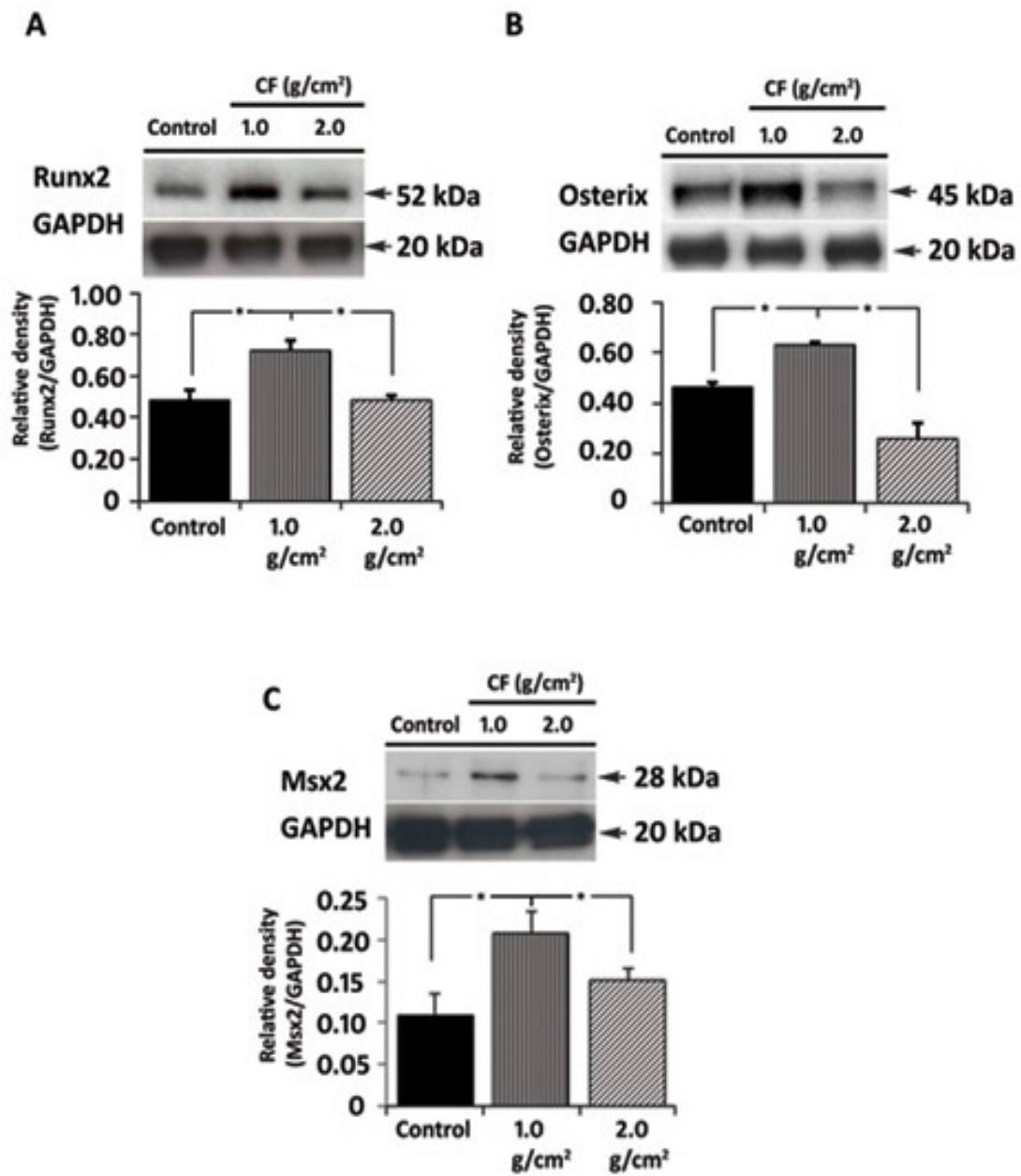
Figure 6. Effects of CF on Runx2, Osterix and Msx2 protein levels

The protein expressions of Runx2 (A), Osterix (B) and Msx2 (C) were increased in cells stimulated with 1.0 g/cm^2 compared with control and 2.0 g/cm^2 CF at the 6-h time point (n = 3, *p < 0.05).
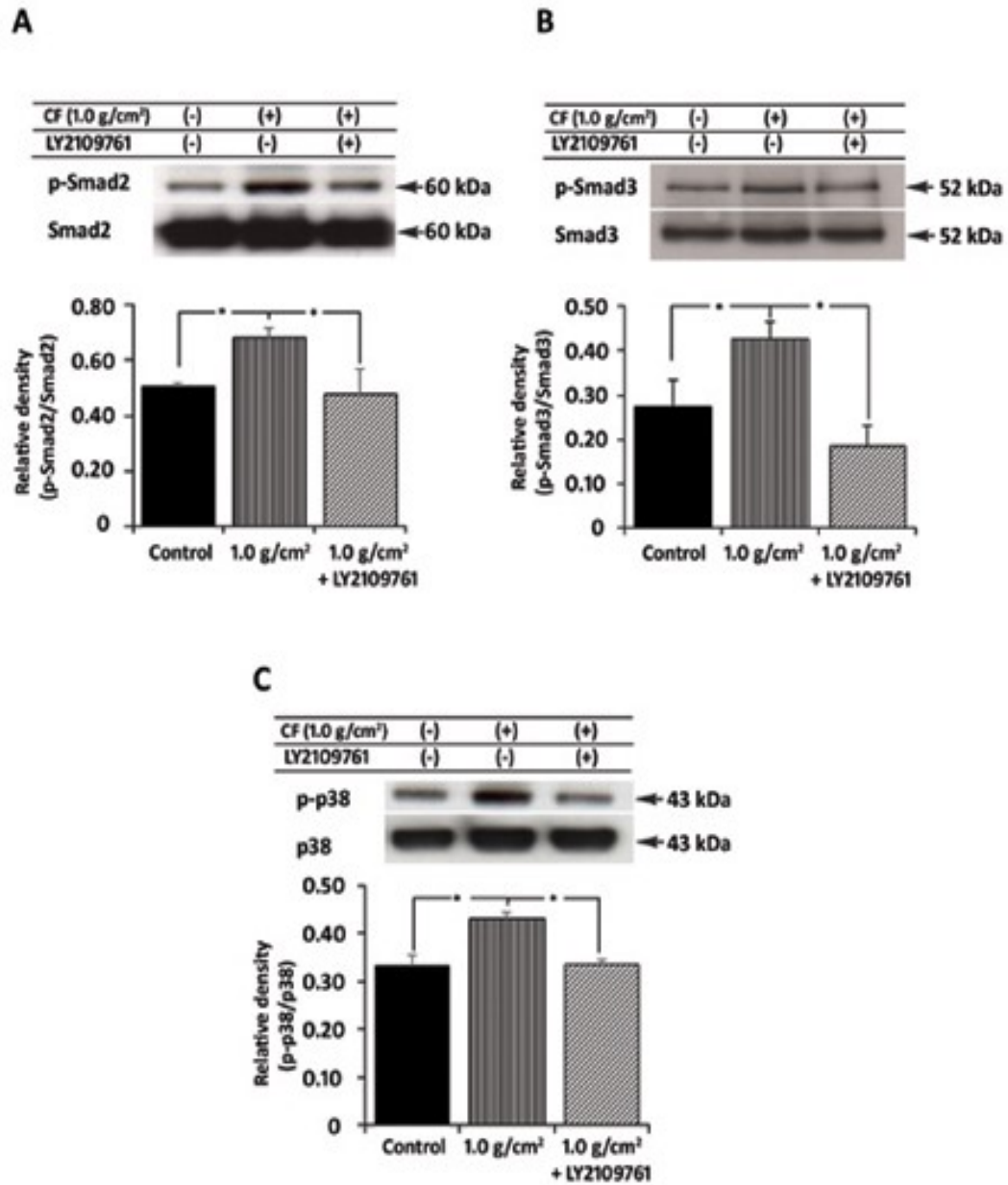
Figure 7. Effects of Tβr1/2 inhibition on the phosphorylation of Smad2, Smad3 and p38

The increased phosphorylation of Smad2 (A), Smad3 (B) and p38 (C) observed with 1.0 g/cm^2

CF cells was blocked by the Tβr1/2 inhibitor LY2709761 at the 6-h time point. The expression

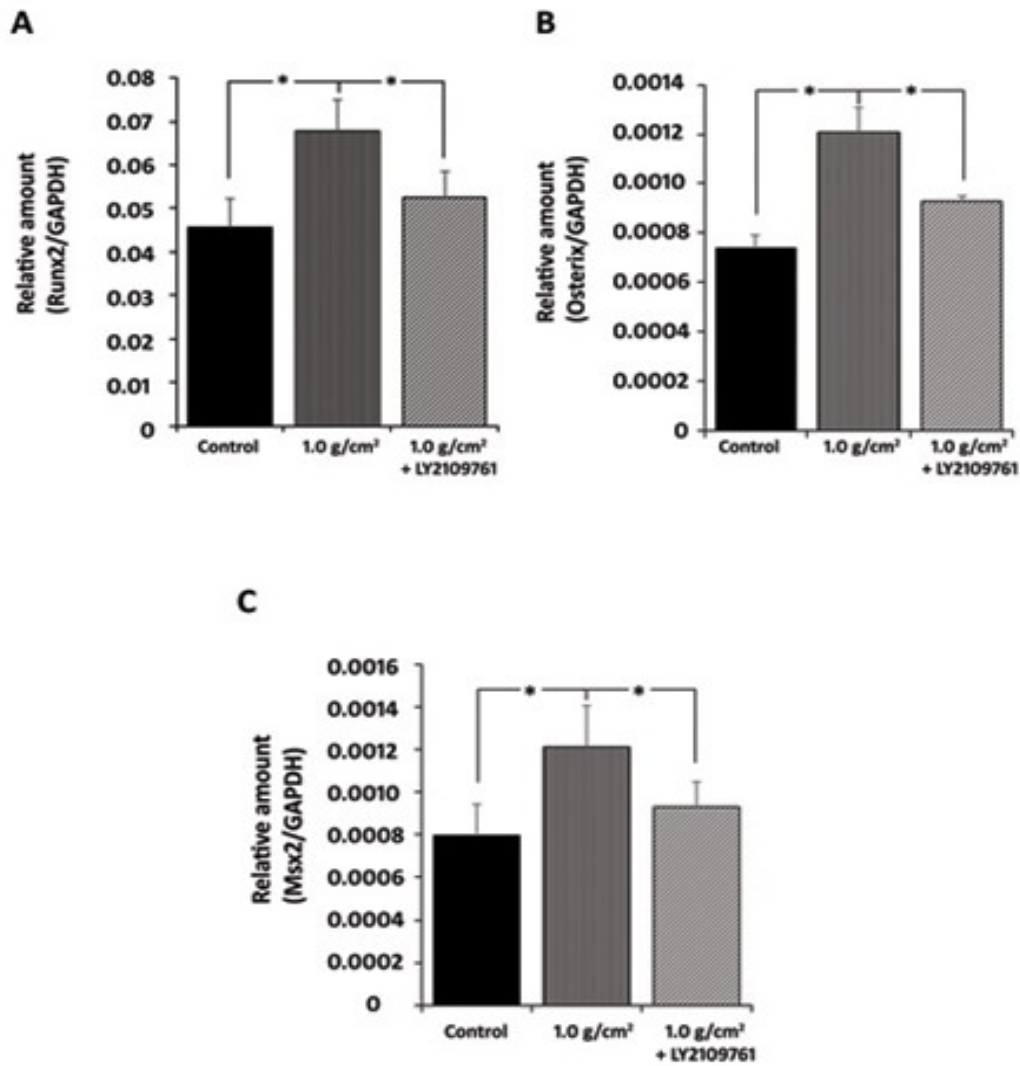levels of Smad2, Smad3 and p38 did not differ among the three groups (n = 3, *p < 0.05).

Figure 8. Effects of Tβr1/2 inhibition on the mRNA expressions of Runx2, Osterix and Msx2

Real-time PCR results showed that 1.0 g/cm^2 CF-induced increases in the transcription of Runx2 (A), Osterix (B) and Msx2 (C) were significantly attenuated by the Tβr1/2 inhibitor (LY2109761) compared with levels observed in the group at the 6-h time point after loading CF (n = 3, *p < 0.05).
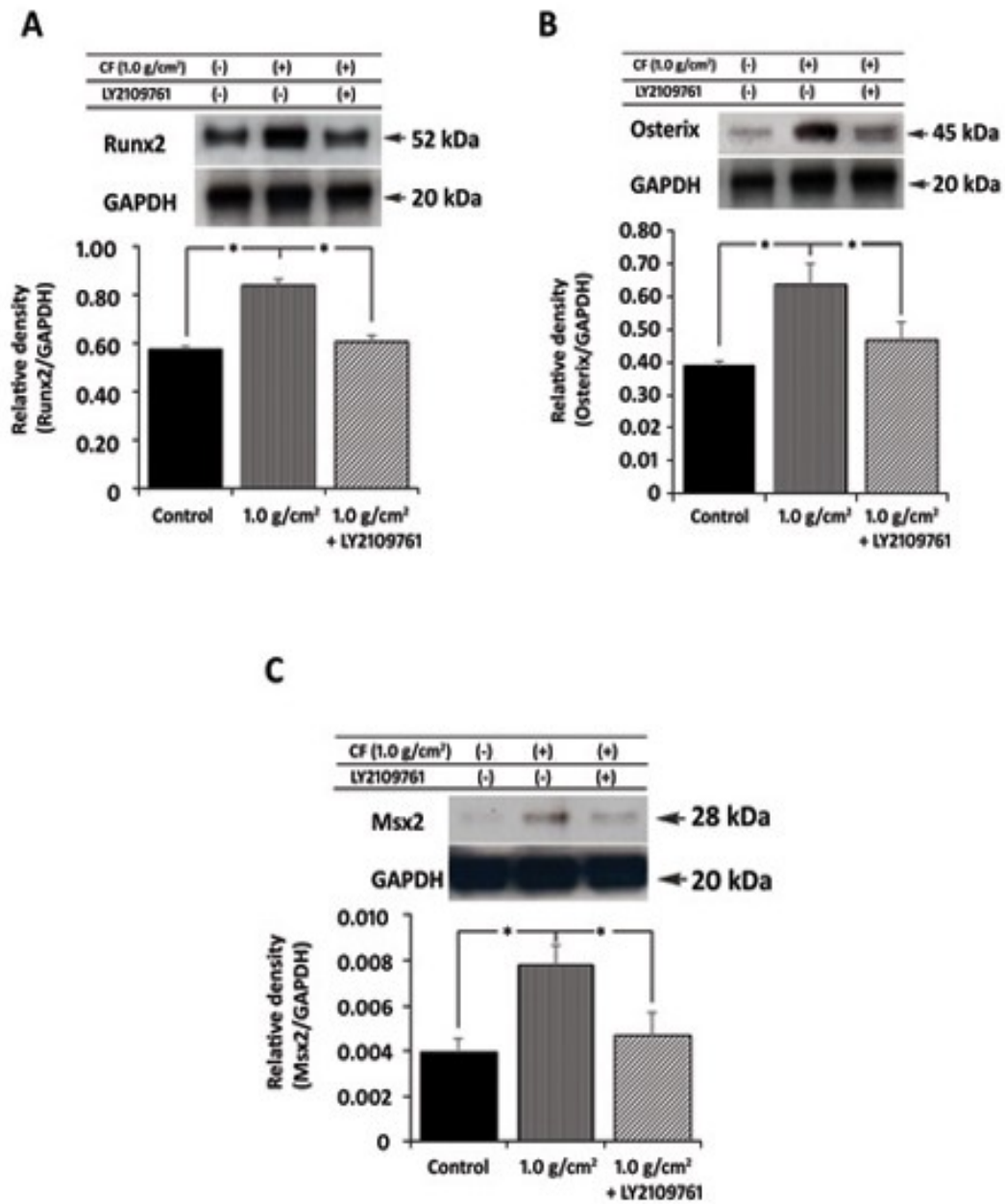
Figure 9. Effects of Tβr1/2 inhibition on the protein expressions of Runx2, Osterix and Msx2

The increased protein expression levels of Runx2 (A), Osterix (B) and Msx2 (C) observed the 6-h time point after loading with 1.0 g/cm^2 CF were also decreased significantly by Tβr1/2 inhibitor (LY2109761) (n = 3, *p < 0.05).