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Lactoferrin promotes osteogenesis through TGF- β receptor II binding in osteoblasts and activation of canonical TGF- β signaling in MC3T3-E1 cells and C57BL/6J mice. --Manuscript Draft--

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Abstract:	Background: Lactoferrin (LF), as a major functional protein in dairy products, is known to modulate bone anabolic effects. However, the underlying molecular mechanisms remain unclear; the receptor of LF in osteoblast differentiation has not been identified. Objective: The aims of the study were to 1) illuminate whether the receptor of LF in osteoblast differentiation is TGF-β Receptor II (TβRII) ; and 2) determine whether the TGF-β signaling pathway is activated by LF in promoting osteogenesis in vitro and in vivo, in addition to P38 and ERK pathways. Methods: We utilized co-immunoprecipitation (Co-IP) to detect any binding of LF to TβRII. Subsequently, the role of the TGF-β signaling pathway involved in LF-induced osteoblast proliferation and differentiation was determined by inhibition of TGF-β Receptor I (TβRI) activity by inhibition and knockout of TβRII expression by sgRNAs in MC3T3-E1 cells. In addition, 4-week-old male C57BL/6J mice were orally administered 100 mg/kg BW LF for 16 weeks, after which any activation of the TGF-β signaling pathway in vivo was measured by western blots. Results: LF was shown to directly interact with the TβRII protein and activate the TGF-β signaling pathway in MC3T3-E1 cells. Inhibition of TβRI activity and knockout TβRII expression both attenuated the stimulation of LF in osteoblast proliferation and differentiation by 30-50%. LF-induced activation of TGF-β canonical signaling resulted in upregulation of osteogenic factors. Moreover, the expression of p-SMAD2 was increased by 1 fold after LF treatment in femoral tissue of mice. Conclusions: This study provide evidence identifying TβRII as a LF receptor in LF-induced osteoblast differentiation. In addition, the TβRII-dependent TGF-β canonical signaling pathways was proved to play an important role in mediating LF-induced osteogenesis both in MC3T3-E1 cells and in C57BL/6J mice.

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Lactoferrin promotes osteogenesis through TGF- β receptor II binding in osteoblasts and activation of canonical TGF- β signaling in MC3T3-E1 cells and C57BL/6J mice.

Running title: Lactoferrin promotes osteogenesis via TGF- β pathway

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Abbreviation: ALP, Alkaline phosphatase; Co-IP, Co-Immunoprecipitation; *Col2a1*, Collagen-2a1; *Fgf2*, Fibroblast growth factor 2; IgG, immunoglobulin G; LF, Lactoferrin; LRP1, Low-density lipoprotein receptor-related protein 1; MOI, multiplicity of infection; MAPK, Mitogen-activated protein kinase; *Ocn*, Osteocalcin; *Opn*, Osteopontin; TGF- β , Transforming Growth Factor- β ; T β R, TGF- β receptor;

LF Group: Experimental group with lactoferrin treatment; LF+SB Group, experimental group with lactoferrin and SB431542; NC, negative control; LF+NC, lactoferrin and negative sgRNA treatment group; T1-3, sgRNA 1-3 treatment group; LF+T3, lactoferrin and sgRNA 3 treatment group; si, T β RII siRNA 2 treatment group; LF+si, lactoferrin and T β RII siRNA 2 treatment group;

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1 Abstract

2 *Background:* Lactoferrin (LF), as a major functional protein in dairy products, is known
3 to modulate bone anabolic effects. However, the underlying molecular mechanisms
4 remain unclear; the receptor of LF in osteoblast differentiation has not been identified.

5 *Objective:* The aims of the study were to 1) illuminate whether the receptor of LF in
6 osteoblast differentiation is TGF- β Receptor II (T β RII); and 2) determine whether the
7 TGF- β signaling pathway is activated by LF in promoting osteogenesis in vitro and in
8 vivo, in addition to P38 and ERK pathways.

9 *Methods:* We utilized co-immunoprecipitation (Co-IP) to detect any binding of LF to
10 T β RII. Subsequently, the role of the TGF- β signaling pathway involved in LF-induced
11 osteoblast proliferation and differentiation was determined by inhibition of TGF- β
12 Receptor I (T β RI) activity by inhibition and knockout of T β RII expression by sgRNAs
13 in MC3T3-E1 cells. In addition, 4-week-old male C57BL/6J mice were orally
14 administered 100 mg/kg BW LF for 16 weeks, after which any activation of the TGF-
15 β signaling pathway in vivo was measured by western blots.

16 *Results:* LF was shown to directly interact with the T β RII protein and activate the TGF-
17 β signaling pathway in MC3T3-E1 cells. Inhibition of T β RI activity and knockout
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19 differentiation by 30-50%. LF-induced activation of TGF- β canonical signaling
20 resulted in upregulation of osteogenic factors. Moreover, the expression of p-SMAD2
21 was increased by 1 fold after LF treatment in femoral tissue of mice.

22 *Conclusions:* This study provide evidence identifying T β RII as a LF receptor in LF-

induced osteoblast differentiation. In addition, the T β RII-dependent TGF- β canonical signaling pathways was proved to play an important role in mediating LF-induced osteogenesis both in MC3T3-E1 cells and in C57BL/6J mice.

Key words: Lactoferrin, T β RII, SMAD2, TGF- β signaling pathway, osteogenesis

Introduction

Lactoferrin (LF), an 80-kDa iron-binding glycoprotein, is present at high concentrations in colostrum and milk (1), and has been demonstrated to be responsible for the resistance of infants to infections (2,3). In addition, LF also regulates many other biological activities that are essential for adequate infant health, such as antimicrobial, immunomodulatory and anti-inflammatory activities, and even has anti-carcinogenic potential (3), and is thereby widely used as a functional ingredient in infant formulas (4-7). Recently, there has been growing interest in the use of LF for improving bone health as LF appears to be a potent bone growth factor (8). Bone health depends on the balance between osteoblast and osteoclast activities (9) and LF acts as a bone anabolic factor to induce osteoblast-controlled bone formation and to inhibit osteoclast resorption both in vitro and in vivo (10,11). Administration of LF increased the calvaria formation in adult male mice (12) and improved bone density in rats (13). Furthermore, LF potently stimulated osteoblast proliferation and differentiation and also inhibited the activities of osteoclasts in vitro (12).

Several studies attempting to explore the molecular mechanisms underlying the

osteotropic activity of LF revealed that different signaling pathways may be involved in different activities of LF in osteoblasts. Cornish and colleagues reported that LF induces osteoblast growth by binding to the low-density lipoprotein receptor related protein 1 (LRP1) to activate the mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase 1/2 (ERK1/2, also called P42/44 MAPK) pathway (14), whereas it inhibits osteoblast apoptosis through LRP1-independent mechanisms, with no involvement of the PI3 kinase-AKT and MAPK-ERK pathways (10). In our previous study, LF was found to stimulate MC3T3-E1 preosteoblast differentiation mainly through the LRP1-independent PKA and P38 MAPK signaling pathways (15). Notwithstanding the above studies, two important issues remain to be resolved: (1) Blocking ERK1/2 and P38 with inhibitors U0126 and SB203580, respectively, did not completely degrade LF-induced enhancement in proliferation and differentiation in osteoblasts (14,15), indicating that some other signaling pathways might be involved in mediating these effects. (2) To date, LRP1 is the only identified LF receptor that mediates its action in osteoblasts (16), yet LRP1 is not involved in osteoblast differentiation. Thus, the receptor of LF in this process remains to be identified.

Transforming growth factor-beta (TGF- β) signaling plays an important role during bone formation (17,18). Previous studies demonstrated that TGF- β signaling promotes osteoprogenitor proliferation (19) and early differentiation (20,21) and that the commitment of osteoprogenitors to the osteoblastic lineage is regulated through both TGF- β canonical and non-canonical pathways (22,23). LF was shown to stimulate

proliferation of chondrocytes similar to that of TGF- β signaling (24). Furthermore, it was reported that LF could directly interact with β -glycan in mouse B cells, through which it could stimulate canonical TGF- β signaling pathways to promote immunoglobulin production (25). However, whether TGF- β signaling is involved in LF-induced osteoblast activity is not clear.

In this study, we hypothesized that the mechanism for LF-induced osteogenesis might be through its interaction with T β RII, which subsequently stimulates receptor I/II-mediated SMAD2 signaling and up-regulates the expression of growth factors that are important for osteoblast proliferation and differentiation. To verify our hypothesis, we utilized Co-IP to observe any binding of LF to T β RII, and to demonstrate whether the TGF- β signaling pathway is activated by LF, we utilized MC3T3-E1 cells and mice, *in vivo*.

Materials and Methods

Experimental reagents

The following experimental reagents were used in the experimental methods: bovine LF with 95% purity (SDS-PAGE) (26) (Australian Yosica Holding, Melbourne, Australia); Rabbit antibody against SMAD2, SMAD4, phospho-SMAD2 (p-SMAD2), and anti-mouse and rabbit immunoglobulin G (IgG) peroxidase conjugate antibodies (Cell Signaling Technology, Beverly, MA, USA); SB431542 (Calbiochem, Cambridge, MA, USA); Mouse anti-T β RII antibody for immunoprecipitation and goat anti-rat IgG-

HRP antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and Rabbit anti-LF antibody and Co-IP assay kits (Thermo Fisher Scientific, Waltham, MA, USA).

Cell culture and treatment

The mouse preosteoblastic cell line MC3T3-E1 subclone 4 (ATCC, Rockville, MD, USA). was cultured every 2 to 3 days until reaching confluency (15). Upon confluency, cells entered the differentiating phase even in the growth medium, but achieved more differentiation when cultured in the differentiation medium (15). To examine the downstream signaling pathways in response to LF treatment, cells were seeded into 60-mm dishes at a density of 2×10^4 cells/cm² and cultured for 24 h. Cells were then starved in α -MEM without serum for 24 h before the addition of LF (100 μ g/mL). In experiments designed to assess the effects of kinase inhibitor on LF-induced cell activity, the inhibitor SB431542 (20 μ M) was added 1 h before LF, and an inhibitor-only treatment was included.

Cell Proliferation Assay

The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) assay was used to assess the effect of LF on proliferation of osteoblasts. Cells were seeded into 96-well tissue culture plates at a density of 2×10^4 cells/cm² and cultured with growth medium. After 24 h, when cells were still in the log growth phase, cells were starved for 24 h in a serum-free medium, after which SB431542 and LF (or different concentrations of LF) were added. Cells were treated for 48 h before subjected into the MTT assay. MTT (5 mg/mL in PBS) was added to each well. After 4 h dimethylsulfoxide was added to solubilize MTT for another 4 h

after removal of the supernatant. After extraction with dimethylsulfoxide, the optical density was measured at 495 nm (plate reader, Bio-Rad Laboratories, Hercules, CA, USA). The data are presented as fold increases over the values obtained from cells exposed to vehicle or SB431542 alone.

ALP activity assay

Cells were seeded into 24-well plates at a density of 2×10^4 cells/cm² in the growth medium and cultured for 7 days. At day 7, cells were treated with SB431542 and LF (or different concentrations of LF) stimulation for 24 h. Cells were then scraped and sonicated in 0.1M Tris buffer (pH 7.4) containing 1% Triton X-100 after PBS washing. Activity was quantitated in cell lysate using an ALP activity diagnostic kit (Roche Diagnostics GmbH, Basle, Swiss) with an automated clinical chemistry analyzer (Hitachi, Tokyo, Japan) and normalized to the total protein measured using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The ALP activities were presented as a fold increase over the values obtained from vehicle or SB431542 alone treatment, respectively.

Mineralization assay

Confluent cells were incubated in the differentiation medium containing LF of indicated concentrations with or without SB431542 in six-well plates for 35 days (15). The media were refreshed every 2 days. For bone nodule detection, the cells were rinsed with ice-cooled PBS and fixed with 70% ethyl alcohol. They were then stained for 10 minutes with 40 mM Alizarin red S (pH 4.2) before observation under light microscope.

Animals and experimental design

A total of 12, 4-week-old male C57BL/6J mice (Beijing Vital River Laboratory Animal Technology Co. Ltd., Beijing, China) were housed 3 per cage in a controlled environment (12:12 h light–dark cycle, temperature 24 ± 1 °C) with free access to food and water. Following a 1-week acclimatisation period, all mice were continually fed a standard diet (M02-F-20110831007, SLAC Laboratory Animal, Shanghai, China), the standard diet provided containing 20% protein, 7% fat, 5% fiber, 3.5% mineral mixtures, 1% vitamin mixtures, 1.3% calcium (Ca), and 0.9% phosphorus. The 100 mg/kg BW of LF was suspended in sterile 0.85% NaCl and administered into the mice (n=6) via oral gavage once daily for 16 weeks (LF group), while the same amount of saline was orally administered to the control group (n=6) over the same period. The dose of LF was chosen based on the extended dose-response study of LF on osteogenic function performed by Guo and colleagues (13). To assess pathway activation, at age 20 weeks all mice were euthanized and a femoral bone tissue sample was collected for protein analyses. All mice were treated in accordance with the guidelines in the Care and Use of Animals and all experiments were approved by the Animal Experimentation Committee of the China Agricultural University (Beijing, China).

Western Blot

MC3T3-E1 cells were lysed for 30 minutes on ice. Nuclear extracts were prepared with a nuclear and cytoplasmic protein extraction kit. The concentration of protein from cell lysate and tissue was determined using a BCA protein assay kit . Proteins were separated in SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Temecula, CA, USA). The membrane was incubated with the

primary antibody after blocking with 5% milk, followed by incubation with the horseradish peroxidase-conjugated secondary antibody. The washed blot was developed using enhanced chemiluminescence reagent (Millipore).

Co-immunoprecipitation

MC3T3-E1 cells were lysed in a buffer containing 20 mM Tris (pH 7.4), 50 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.5% SDS, 0.5% deoxycholate, and protease inhibitors. An appropriate amount of rabbit anti-LF (or mouse anti-T β RII) antibody as well as control IgG were precleared with 50 μ L protein A-sepharose beads for 2 h at 4°C, and then incubated with a mixture of 500 μ g lysate and 500 μ g LF overnight at 4°C. After the beads were washed with a high salt lysis buffer, immunoprecipitated proteins were eluted into an elution solution and then subjected to immunoblot analysis with appropriate antibodies.

Knockout of T β RII with Lenti-Cas9-sgRNA-Puro vectors

T β RII-sgRNA expression vectors as well as lentivirus vector generations were produced by GeneChem using their Lenti-CRISPR dual-vector system (GeneChem, Shanghai, China). MC3T3-E1 cells were seeded at 1×10^4 cells/cm² in six-well plates the day before transfection. Lentivirus at 100 multiplicity of infection (MOI) and 10mg/mL polybrene were added to α -MEM with 10% FBS in 1mL total volume. After 24 h, the medium was replaced for expansion of transduced cells. Forty-eight h later the medium was removed and cells were selected by puromycin treatment (3 μ g/mL) for 7 days. Deficiency of T β RII protein was confirmed by western blotting and the cells were used for subsequent experiments.

siRNA knockdown of T β RII expression

MC3T3-E1 cells were seeded into six-well plates and grown for 24 h to reach 50% confluence before transfection. The medium was then changed to an opti-MEM reduced serum medium and the MC3T3-E1 cells were transfected with T β RII siRNA (Supplemental Table 1) and control scramble siRNA using Lipofectamine 2000. Forty-eight h after siRNA transfection, cells were prepared or treated with LF for 1h for western blotting analysis (15).

Quantitative Real-time PCR analysis

MC3T3-E1 cells were treated with 20 μ M SB431542 for 1 h and 100 μ g /mL LF for 4 h before total RNAs were isolated with TRIzol (Invitrogen, Carlsbad, CA, USA). cDNAs were generated using a first-strand cDNA synthesis kit (Takara Biotechnology, Dalian, China). The expression of desired genes (Supplemental Table 1) were analyzed by quantitative RT-PCR with SYBER Premix Ex Taq according to the company's instructions (Takara Biotechnology, Dalian, China) with *GAPDH* as a control. The experiments were performed in duplicate and with three biological repeats for each experiment.

Statistical analysis

All data were expressed as means $\pm$ standard deviations. Data analysis was carried out using SPSS software (version 17.0 SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) followed by Duncan's post hoc test was used to evaluate differences between groups in vitro. The main effects and interactions of two factors (LF $\times$ SB, LF $\times$ T3, and LF $\times$ si) were analyzed by univariate 2-way ANOVA. Mean comparisons

were conducted by using a Tukey's post hoc test when the main effect was significant. If an interaction between LF and SB was found, a mean comparison were done conditionally. Differences between two groups in vivo were analyzed by t-test. In all tests, statistical significance was defined at a level of $P < 0.05$.

Results

TGF- β pathway is required for LF-induced osteoblast proliferation and differentiation in MC3T3-E1 cells

To further address whether LF has an effect on the proliferation of osteoblasts, we found that the MC3T3-E1 cells exhibited a dose-dependent increase in proliferation, resulting in a significant increase in the proliferation rate when an LF dose over 10 $\mu\text{g/mL}$ was used. The treatment also led to a similar pattern of increase in alkaline phosphatase (ALP) activity, a bone differentiation marker, when the cells entered the differentiation phase after reaching confluence (**Fig. 1A**). These results indicate that LF promotes both MC3T3-E1 cell proliferation and bone differentiation. Since 100 $\mu\text{g/mL}$ is an achievable physiological concentration and is also the concentration of LF in human milk, we focused on this concentration for all subsequent experiments. Given that the effects of LF on these cells were similar to that of TGF- β 1 treatment (Fig. 1B) and that TGF- β signaling plays an important role in bone formation (16,17), we questioned whether LF performs its function through TGF- β pathways. To address this, we treated cells with LF in the presence or absence of T β RI-specific inhibitor SB431542. In the log-growth phase, pretreatment of MC3T3-E1 cells with SB431542 reduced the LF-

induced proliferation up to 50-70%. Similarly, SB431542 pretreatment also significantly reduced LF-induced ALP activity (Fig. 1C). Furthermore, SB431542 considerably diminished the LF-induced mineralization in MC3T3-E1 cells (Fig. 1D). These results suggest that TGF- β signaling is likely required for LF-induced proliferation and differentiation in osteoblast cells.

LF induces anabolic effect in MC3T3-E1 cells through interaction with T β RII

Canonical TGF- β signaling is activated by ligand binding to T β RII, which subsequently recruits and phosphorylates T β RI to activate the transducers and effectors. Therefore, we examined whether LF interacts with T β RII to activate this signaling pathway. Co-IP experiments with LF as a bait protein showed that the T β RII protein was only detected in the eluate of LF immunoprecipitation, indicating an interaction between LF and T β RII. This interaction was further confirmed by using T β RII as a bait protein for Co-IP, in which LF was clearly shown in T β RII immunoprecipitants but was undetectable in IgG immunoprecipitation (**Fig. 2A**). These data demonstrate that LF is able to interact with T β RII, which may account for its function to activate TGF- β signaling.

To verify whether T β RII plays a role in LF-induced anabolic effect in MC3T3-E1 cells, we proceeded to abolish T β RII in these cells to investigate the effect of LF treatment. We tested three T β RII sgRNAs in the lentiviral-CRISPR system for efficient knockout of T β RII expression in MC3T3-E1 cells. After puromycin selection, the deficiency of T β RII expression was confirmed by western blotting, which revealed that only sgRNA

T3 showed a loss of T β RII expression, which, as expected, led to a dramatic reduction in the activation of SMAD2 (Fig. 2B). We then evaluated the osteoinductive effect of LF in these T β RII-deficient MC3T3-E1 cells. The results showed that a deficiency in T β RII significantly attenuated LF's effect on both proliferation in the early growth stage and ALP activity in the differentiation phase by up to 50%-60% in MC3T3-E1 cells (Fig. 2C), suggesting that T β RII could be involved in LF-induced activities in MC3T3-E1 cells.

LF activates the canonical TGF- β signaling pathway in MC3T3-E1 cells

Since LF was shown to interact with T β RII and the LF-induced effect in MC3T3-E1 cells was impeded by inhibition of T β RI activity and T β RII-deficiency, we found it plausible that the LF-induced anabolic effect in MC3T3-E1 cells could be through activation of the TGF- β signaling pathway. Our results showed that LF indeed stimulated phosphorylation and nuclear translocation of SMAD2 in these cells and that this activation exhibited a dosage-dependent manner (**Fig. 3A**), similar to that shown in LF-induced proliferation and ALP activity (Fig. 1A). Time-course analysis of LF-induced activation at 100 μ g/mL revealed that LF-induced SMAD2 phosphorylation and nuclear translocation most likely occurred less than 15 minutes from administration of LF, peaked around 30 minutes post-treatment, and then gradually declined (Fig. 3B). In addition, we found that LF only significantly activated SMAD2, but not SMAD3. Unlike LF, SMAD2 and SMAD3 were both activated by TGF- β 1 (**Fig. 4A-B**). Furthermore, inhibition of T β RI with SB431542 dramatically attenuated LF-induced SMAD2 phosphorylation and nuclear translocation (Fig. 3C), implying that LF-induced

SMAD2 activation is T β RI dependent.

In our previous study, P38 activation was observed in LF-treated osteoblasts (15), therefore it is known that the P38 pathway is a TGF- β non-canonical pathway. Thus, in this study, we sought to determine whether LF activated the P38 pathway via the TGF- β receptor. First, we used an siRNA knockdown approach to examine the effect of T β RII knockdown. As shown in **Fig. 5A**, MC3T3-E1 cells were transfected with T β RII siRNA 1, 2, 3, and control siRNA, and T β RII siRNA 2 transfection effectively decreased the basal level of T β RII protein expression. However, T β RII knockdown did not suppress any LF-induced enhancement of P38 activation (Fig. 5B). In addition, as shown in Fig. 5C, any attenuation on P38 activation was not observed to be from inhibition of T β RI activity. Together, these results demonstrate that LF can function as a potent ligand for the activation of canonical TGF- β signaling in MC3T3-E1 cells, but not non-canonical pathways.

LF activates the canonical TGF- β signaling pathway in femoral tissue from C57BL/6J mice

To demonstrate the activation of the TGF- β signaling pathway by LF in vivo, as shown in **Fig. 6**, we assessed the expression levels of p-SMAD2 and SMAD4 in femoral tissue from the control ($n=6$) and LF mice groups ($n=6$) after 4 months. Although p-SMAD2 expression levels were low, it was markedly increased by LF (100 mg/kg BW) treatment. Moreover, there was no significant difference in SMAD4 expression between the LF and the control groups. These findings indicate that oral LF activates the canonical

TGF- β signaling pathway in vivo, in accordance with the results seen in MC3T3-E1 cells.

The TGF- β signaling pathway is involved in the LF-induced expression of Ocn, Opn, Col2a1 and Fgf2

During osteogenesis, many growth factors are actively expressed and produced to facilitate bone formation, including *Ocn*, *Opn*, *Col2a1*, and *Fgf2* (27,28). To validate the role of LF in promoting osteogenesis at the molecular level, we compared the relative mRNA expression of these factors in MC3T3-E1 cells with or without LF treatment. As expected, all factors exhibited a significant upregulation in their expression after LF treatment (**Fig. 7**), which was consistent with the phenotypic changes associated with LF treatment. More importantly, this increase in mRNA expression was significantly hindered when the cells were pre-incubated with SB431542 inhibitor prior to LF treatment (Fig. 7). These data demonstrate that the TGF- β signaling pathway plays an essential role in the LF-induced osteotropic factors as expressed in the MC3T3-E1 cells.

Discussion

LF has been established a potent anabolic factor that promotes bone formation and inhibits bone resorption (12). Although previous studies have explored the molecular mechanisms underlying LF's osteotropic effect, key information regarding the functional receptor that mediates LF's effect on osteoblast differentiation remains largely unknown. In this study, we provided direct evidence that LF, similar to TGF- β 1,

is able to bind to T β RII and activate the TGF- β signaling pathway, thereby promoting the proliferation and differentiation of osteoblasts. To the best of our knowledge, this study is the first to provide evidence that LF interacts with T β RII to activate TGF- β signaling for the osteotropic effect in osteoblasts.

It has been previously shown that LRP-1 mediates LF-induced activation of SMAD2, resulting in SMAD2 nuclear translocation in chondrocytes (24). However, we revealed that p-SMAD2 was almost completely abolished upon knockout of T β RII by sgRNA in MC3T3-E1 cells (Fig. 2B), suggesting that SMAD2 activation in these cells is primarily mediated by T β RII. This is in agreement with a previous report that clearly showed LF-induced osteoblast differentiation independent of LRP1 (15). Jang et al. showed that LF also modulated the immune response in B cells through activation of the TGF β -SMAD3 pathway (25). Moreover, in the study by Jang et al., pretreating LF with soluble T β RII did not reduce a LF-induced immune response. In addition, LF activated TGF- β signaling by interacting with T β RIII (also called betaglycan), which in turn induced the phosphorylation of T β RI and SMAD3 through the formation of a T β RIII/T β RII/T β RI complex (25). However, our current results clearly show that LF interacts with T β RII in MC3T3-E1 cells (Fig. 2A) and disables T β RII to eliminating LF-induced SMAD2 activity and function in these cells (Fig. 2B). The discrepancy between our study and that of Jang et al. might be due to the fact that LF binds to different receptors in different cell types, or that LF does not stably interact with soluble T β RII.

Our lab previously reported that LF-stimulated MC3T3-E1 cells differentiation is through LRP1-independent PKA and P38 signaling pathways (15). In our current study we demonstrate that LF can function by interacting with T β RII to stimulate TGF- β canonical signaling. Thus, it might be possible that LF also activates the P38 pathway via T β RII by way of a TGF- β non-canonical pathway. However, T β RII knockdown by siRNA in MC3T3-E1 cells did not reveal any reduction in LF-induced P38 activation. Nor was there any attenuation observed on P38 activation by inhibition of T β RI activity (Fig. 5). These results demonstrate that TGF- β receptors may not participate in the P38 pathway induced by LF and suggest that the existence of other potential receptors exploited by LF are responsible for the LF-stimulated activation of the P38 pathway. Therefore, we conclude that multiple signaling pathways might be involved in the LF-induced osteotropic effect in pre-osteoblasts. Further work is required to define the cross-talk mechanisms underlying the anabolic effect of LF on bone.

The TGF- β signal often involves two receptor-regulated SMADs (R-SMADs), SMAD2 and 3, as the signal effectors upon activation of the T β RI transducer. These activated R-SMADs form complexes with the common mediator SMAD (co-SMAD; SMAD4) and accumulate in the nucleus, where they directly activate target genes in conjunction with other transcription factors (30,31). However, the SMAD2 and SMAD3 proteins can execute different functions in cells. For example, in fibroblasts, SMAD3 functions to mediate the induction of collagenase-3 expression by TGF- β , whereas SMAD2 is not involved in this context (32); however in osteoblasts, only SMAD2 (not SMAD3)

mediates its effect for collagenase-3 promoter activation (33). Similarly, in MC3T3-E1 cells, we found that LF significantly activated SMAD2, inducing its nuclear translocation (Fig. 3), while the expression of SMAD3 was low in these cells and was not activated by LF (Fig. 4). This is consistent with previous findings that LF stimulated proliferation of chondrocytes by inducing nuclear SMAD2 translocation (24). In addition, the expression and localization of SMAD4 showed no change in our study regardless of LF treatment, with or without inhibition of T β RI (Fig. 3). It is likely that nuclear translocation of SMAD2 does not require SMAD4. Analogously, a functional study found that SMAD2 can translocate into the nucleus independently of SMAD4 in SMAD4-deficient tumor cell lines (34).

In our study, we showed that LF increased the expression of *Ocn*, *Opn*, *Col2a1* and *Fgf2* in MC3T3-E1 cells, while blocking the TGF- β pathway by SB431542 which significantly decreased this effect (Fig. 7). This suggests that the TGF- β pathway is involved in the upregulation of *Ocn*, *Opn*, *Col2a1* and *Fgf2* by LF, which might confer osteoblast differentiation (35-38).

In conclusion, we demonstrated that LF stimulates osteoblast proliferation and differentiation through binding to T β RII, further activating SMADs signaling to induce bone health (**Fig. 8**). Our work contributes new understanding regarding the molecular mechanisms underlying the anabolic effect of LF on bone and supports the continued use of LF as a natural infant formula reagent. Further adequately powered studies are

needed to investigate the effects on clinically relevant endpoints in healthy term infants.

Acknowledgments

Authors' contributions

YL conducted all of the experimental work and contributed to the drafting of the manuscript; JW contributed to the experimental work; FR, WZ and WC helped to prepare the manuscript and interpret the results; HZ, LZ, and MZ conducted the data analysis; XW helped to prepare and edit the manuscript; HG contributed to the research design and takes primary responsibility for the final content. All authors read and approved the final manuscript.

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Figure legends

Fig. 1. LF-induced MC3T3-E1 osteoblastic cells proliferation and differentiation is inhibited by TGF- β inhibitor.

MC3T3-E1 cells were treated with LF and/or SB as described in Materials and Methods before they were subjected to proliferation and differentiation analysis. The effect of LF (A) or TGF- β 1 (B) treatment for indicated concentrations on osteoblast proliferation and differentiation. (C) 100 μ g/mL LF treatment in the presence of SB on osteoblast proliferation and differentiation. (D) The enhancement of their mineralization by LF, which is inhibited by SB. Values are mean $\pm$ SDs, $n=3$ (means of 5 replicates). (Means without a common letter differ, $P<0.05$; * $P<0.05$ versus control group; # $P<0.05$ LF-treated group versus LF+SB treated group).

Fig. 2. T β RII-LF interaction is important for LF-induced osteogenesis effect in MC3T3-E1 osteoblastic cells.

(A) Co-IP experiments showing interaction between LF and T β RII proteins. LF or T β RII were used as bait proteins to detect the combination, respectively (B) Western blot and the gray analysis showing efficient elimination of T β RII expression by sgRNA T3. (C) MTT and ALP assay showing attenuation of LF-induced proliferation and differentiation by T β RII deficiency. Values are mean $\pm$ SDs, $n=3$ (means of 5 replicates). (Means without a common letter differ, $P<0.05$; * $P<0.05$ versus control group; # $P<0.05$ versus LF+T3 treated group).

Fig. 3. LF stimulates TGF- β /SMAD2 signaling pathway in MC3T3-E1 osteoblastic cells.

(A) Western blot and the gray analysis showing dose-dependent activation of SMAD2 phosphorylation and nuclear translocation by LF treatment for 30 minutes. (B) Time-course analysis of SMAD2 phosphorylation and nuclear translocation after treatment with 100 μ g/mL LF. (C) Pre-treating cells with SB attenuates LF (100 μ g/mL)-induced SMAD2 activation. Values are mean $\pm$ SDs, $n=3$. (Means without a common letter differ, $P<0.05$; * $P<0.05$ versus control group; # $P<0.05$ versus LF+SB treated group).

Fig. 4. LF activates SMAD2, not SMAD3 in MC3T3-E1 osteoblastic cells.

Time-course analysis of p-SMAD2/SMAD3 in MC3T3-E1 cells after treatment with (A) 2ng/mL TGF- β 1 and (B) 100 μ g/mL LF.

Fig. 5. Inhibition of either T β RI activity or T β RII expression cannot attenuate LF-induced P38 activation in MC3T3-E1 osteoblastic cells.

(A) Knockdown effect of T β RII siRNA 1, 2, 3 on T β RII expression and (B) the phosphorylation level of LF-induced P38 was determined. (C) Pre-treating cells with

SB, LF (100 μ g/mL)-induced P38 activation was detected. Values are mean $\pm$ SDs, $n=3$.
(Means without a common letter differ, $P < 0.05$; * $P < 0.05$ versus control group; # $P < 0.05$ versus LF+si or SB treated group).

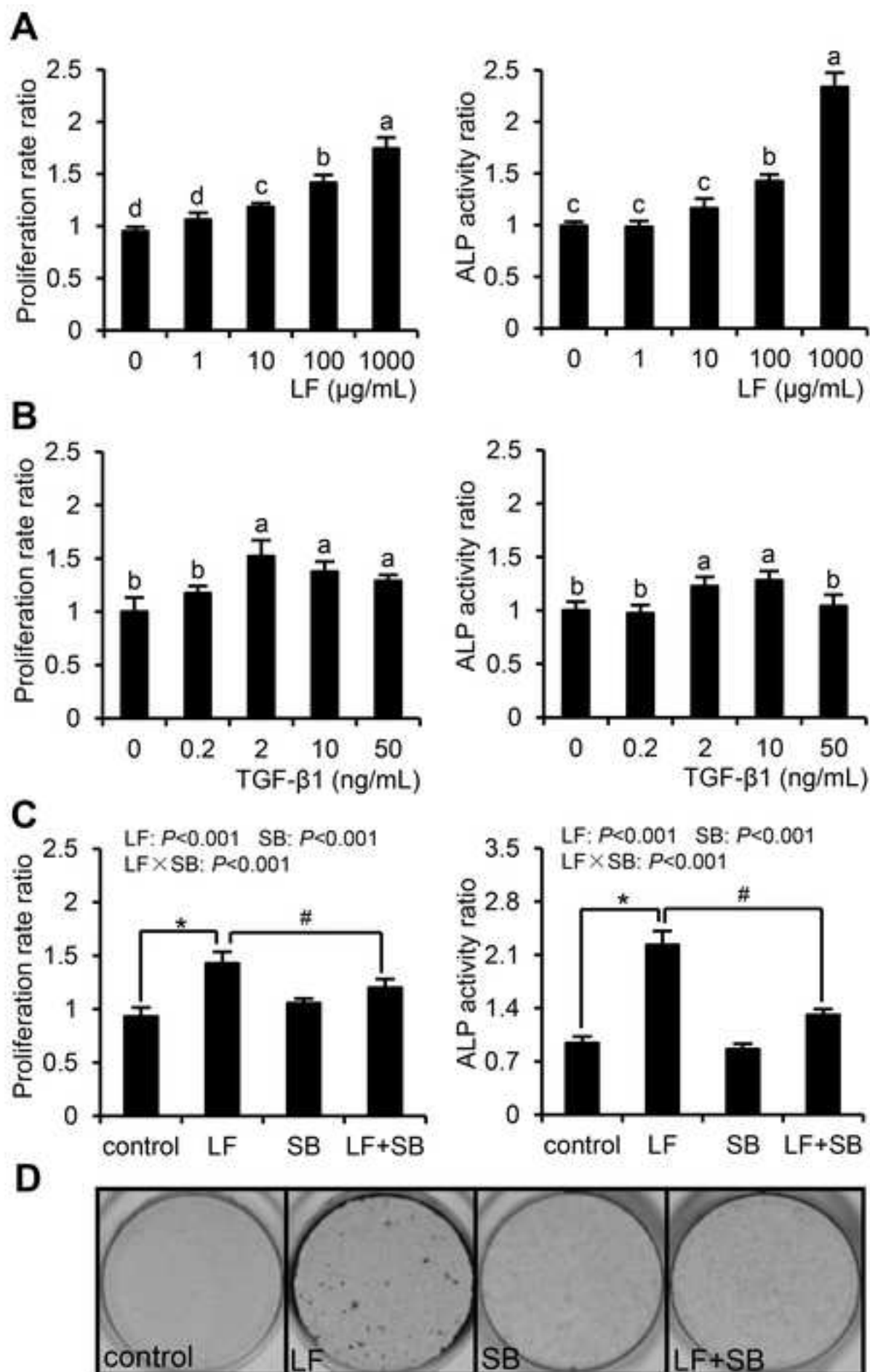
Fig. 6. LF activates TGF- β / SMADs pathway in femoral tissue from C57BL/6J mice.

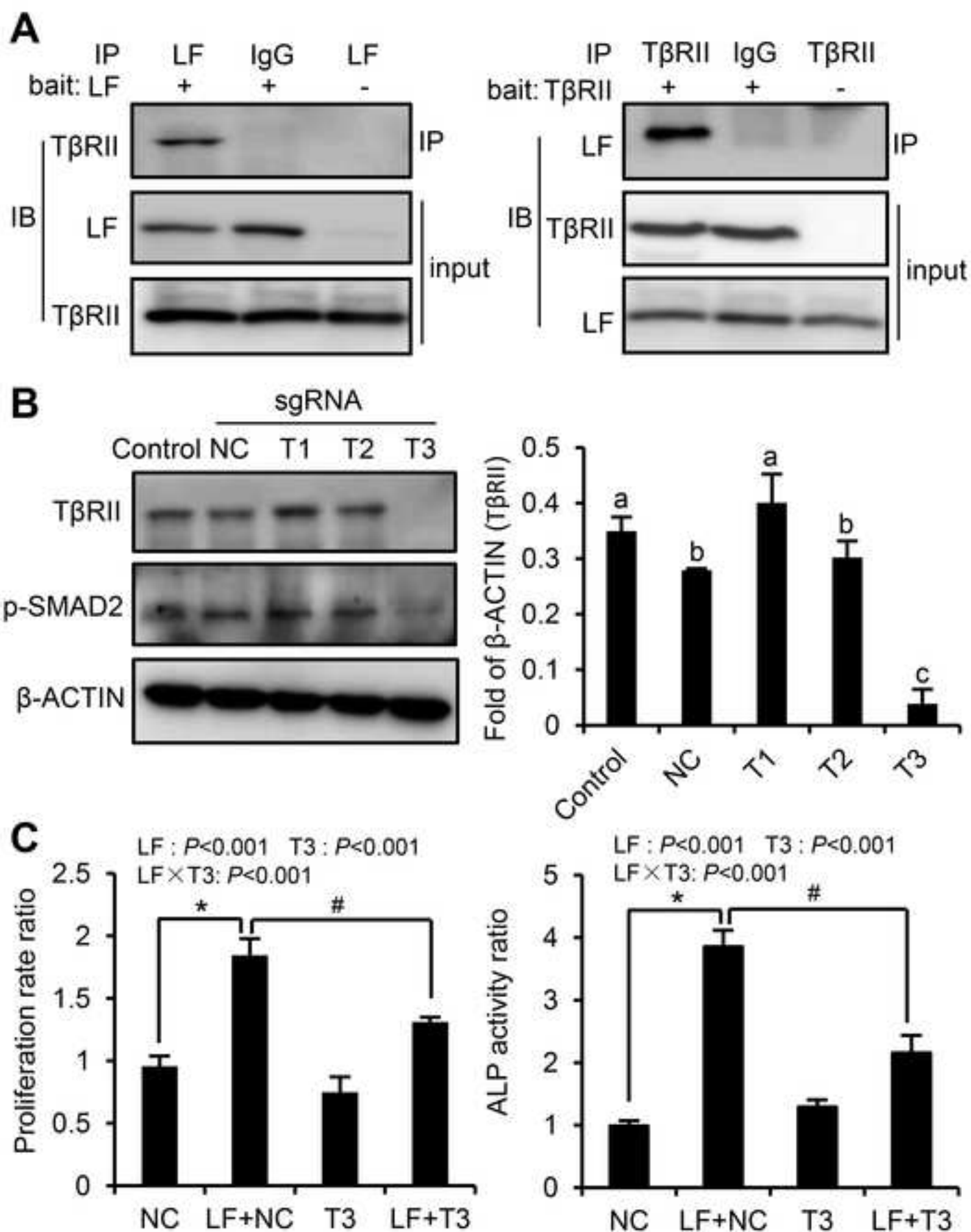
The expression of p-SMAD2 and SMAD4 in mouse femoral tissue for the control group ($n=6$) and the 100mg/kg LF-treated group ($n=6$) are shown. Values are mean $\pm$ SDs, $n=6$. (Means without a common letter differ, $P < 0.05$).

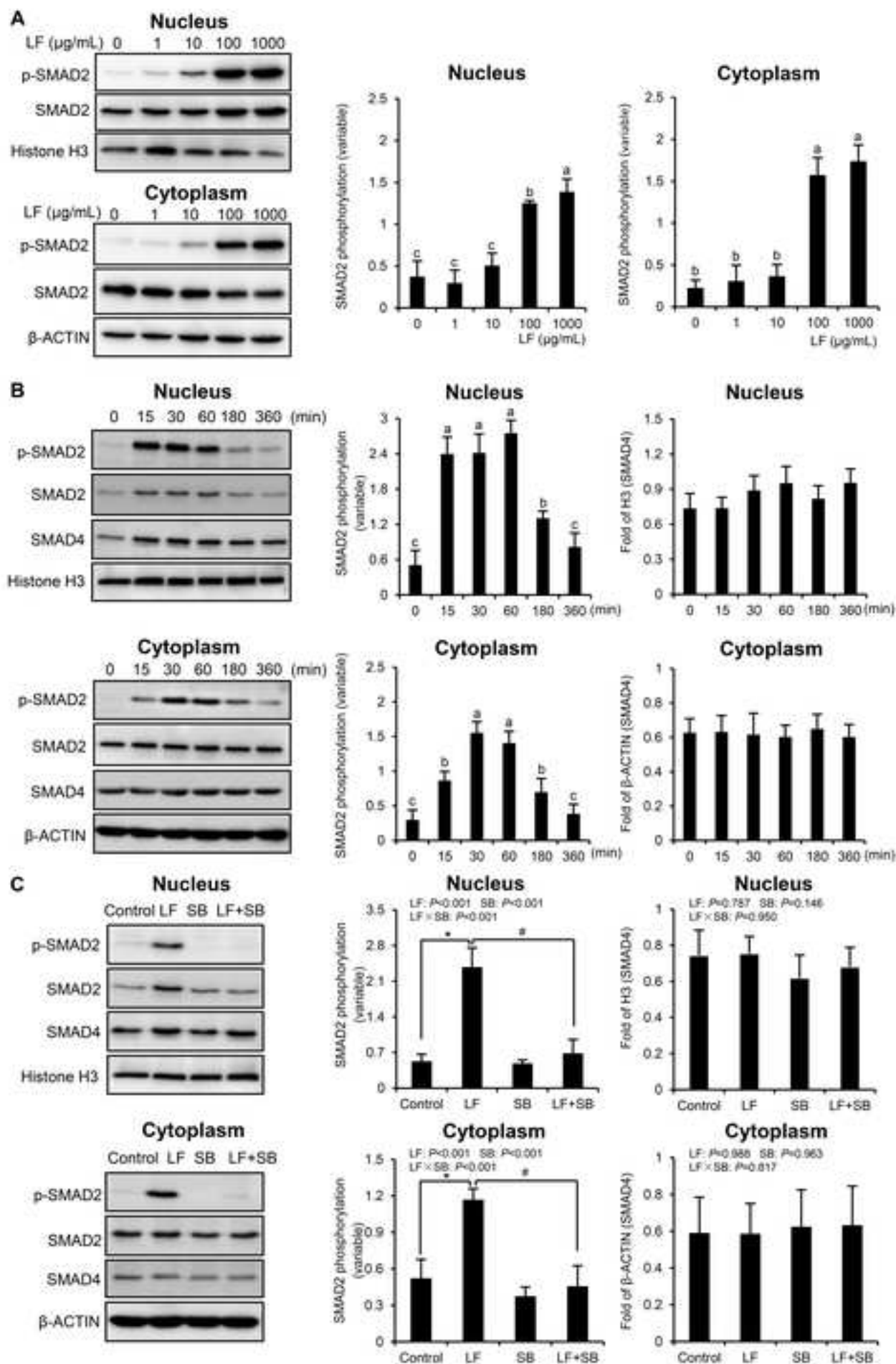
Fig. 7. TGF- β pathway is involved in LF-induced upregulation of osteogenic factors in MC3T3-E1 osteoblastic cells.

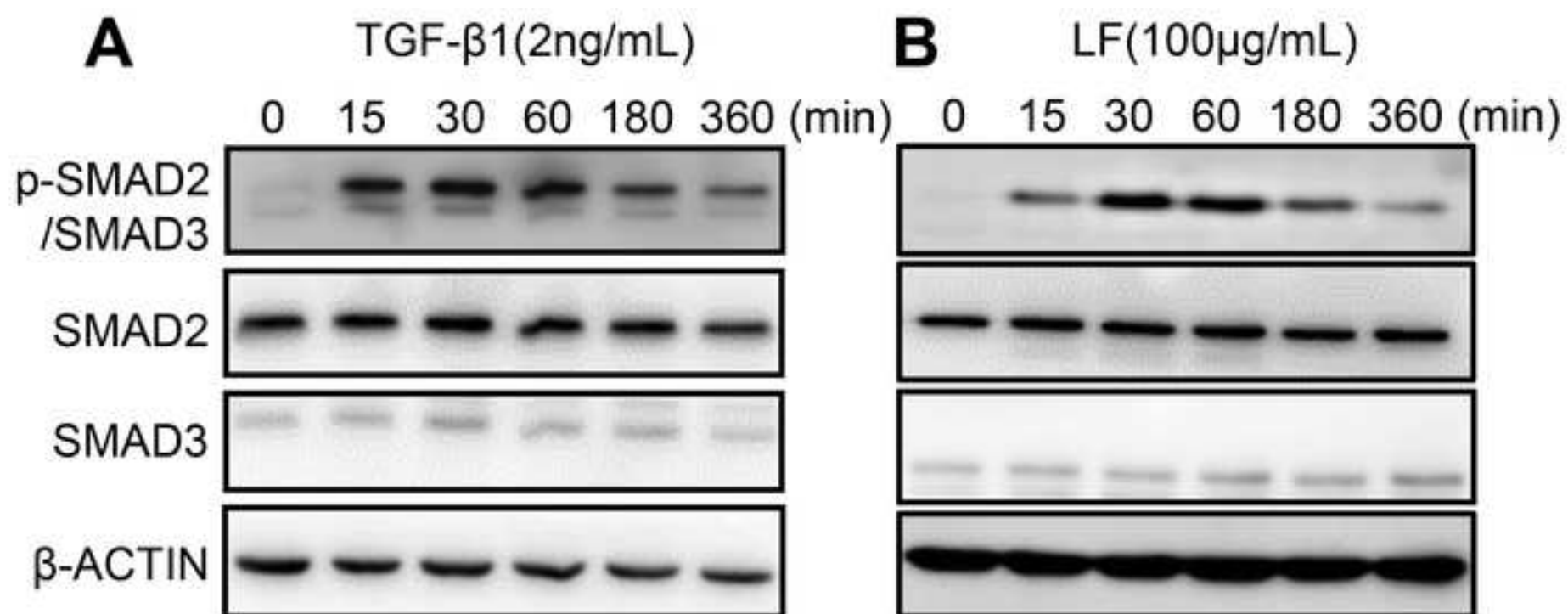
MC3T3-E1 cells were pretreated with SB and followed by treatment with LF. Expression of indicated factors were measured by q-RT-PCR. Values are mean $\pm$ SDs, $n=3$ (means of 5 replicates). (* $P < 0.05$ versus control group; # $P < 0.05$ versus LF+SB treated group).

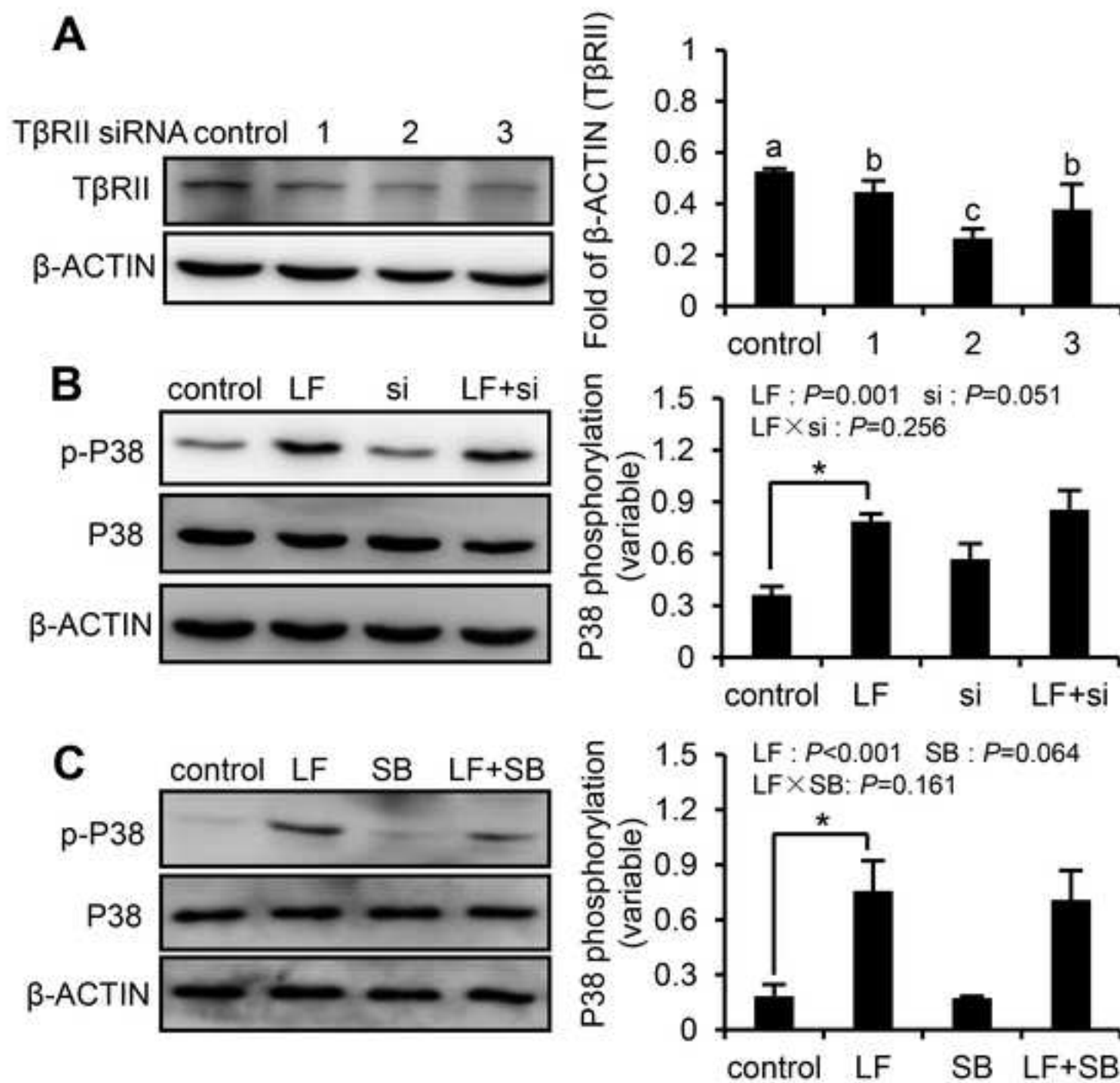
Fig. 8. Illustration of LF-induced osteogenic proliferation and differentiation through TGF- β /SMAD2 pathway in MC3T3-E1 osteoblastic cells.



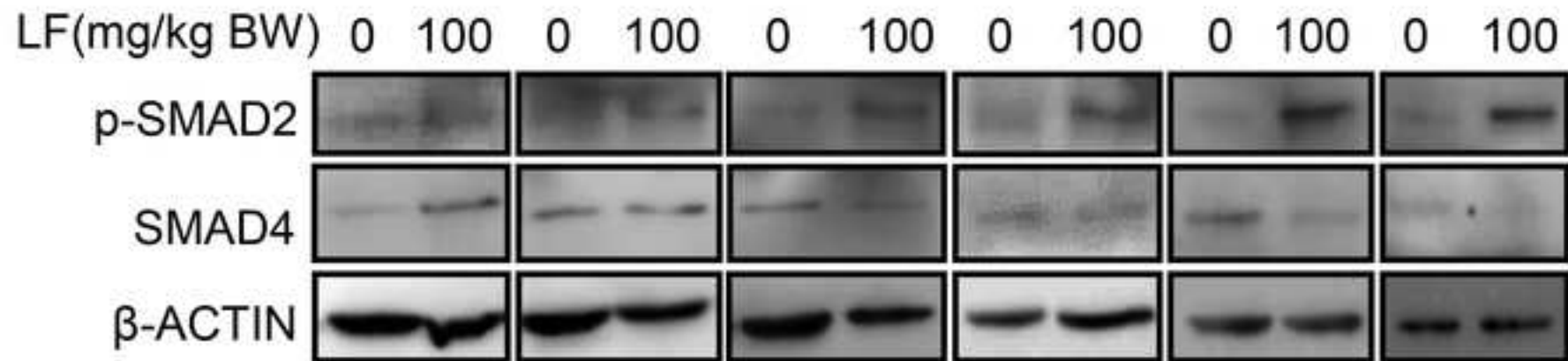




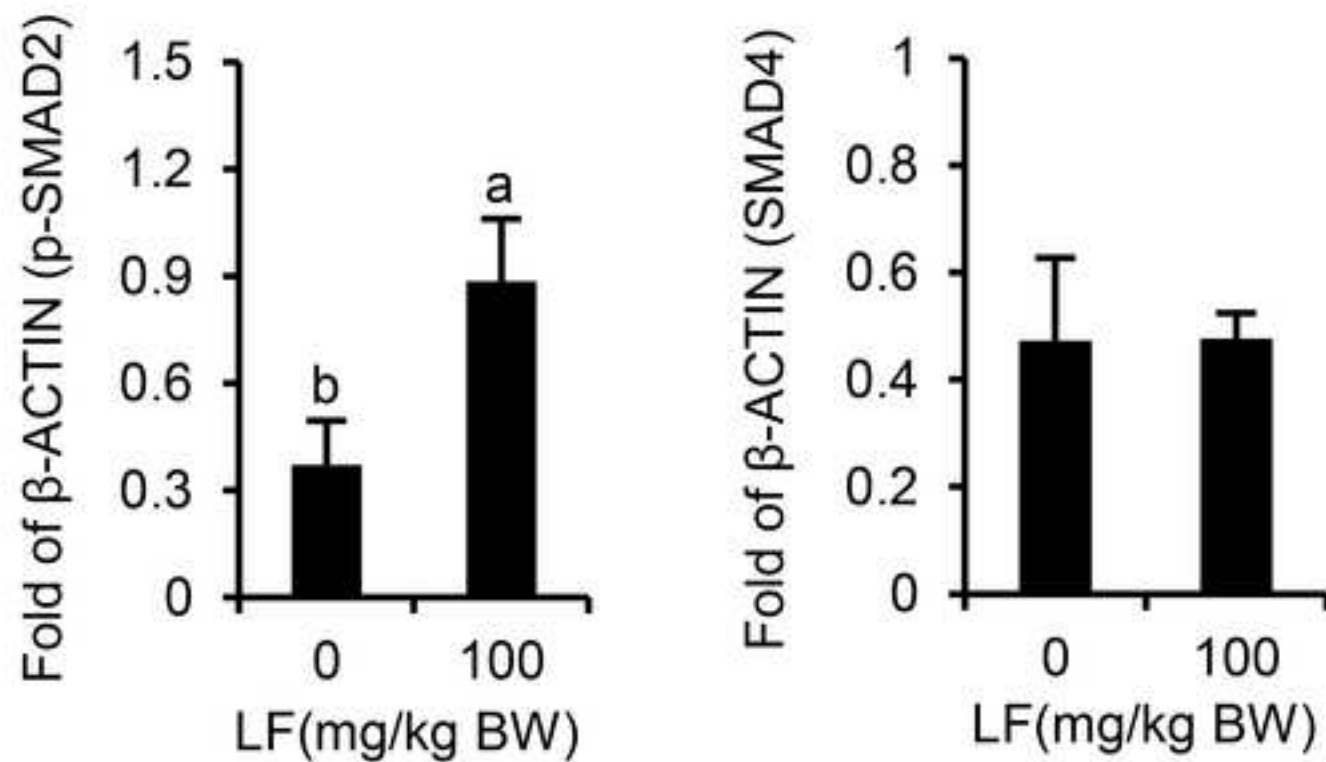


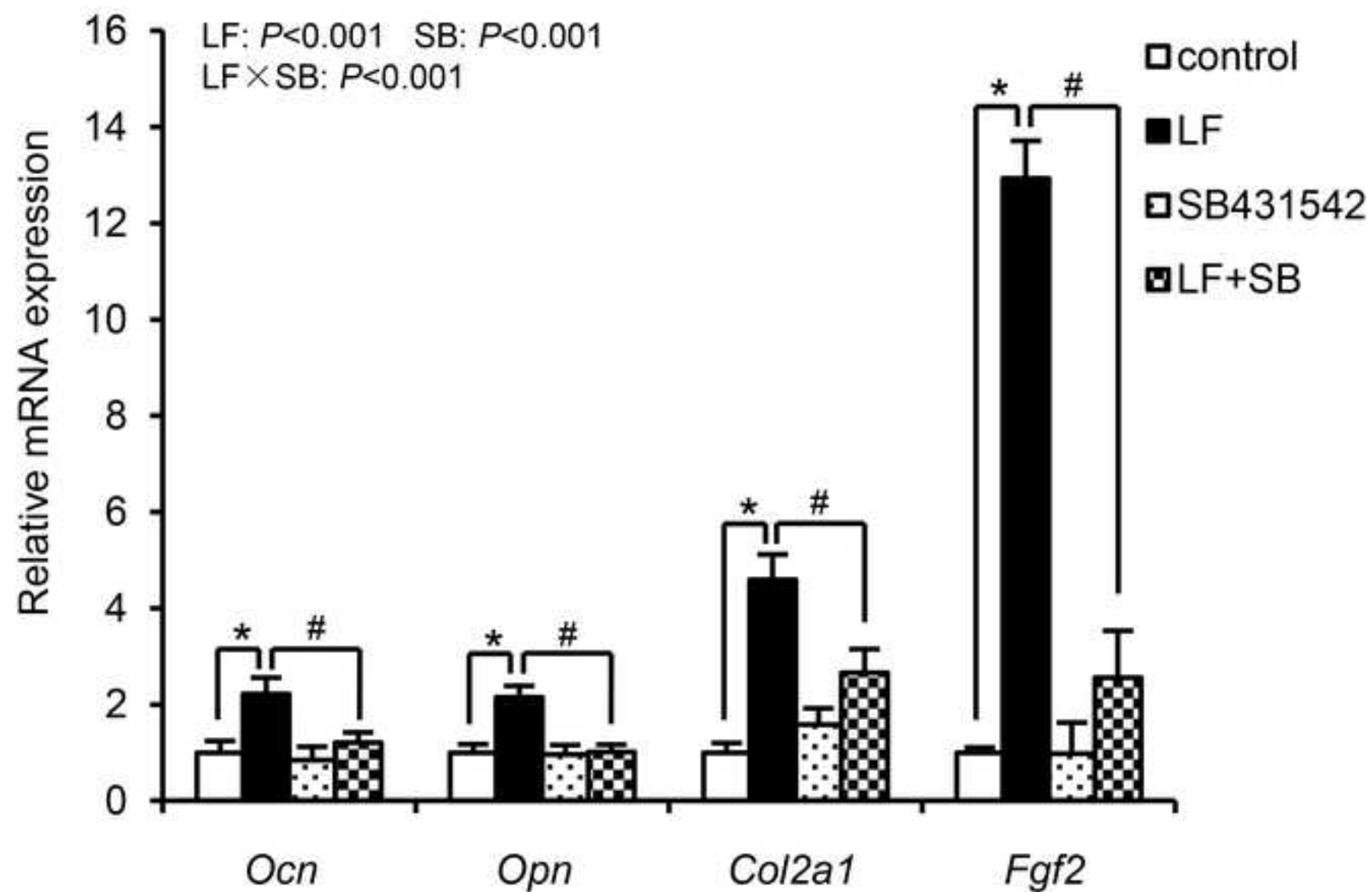


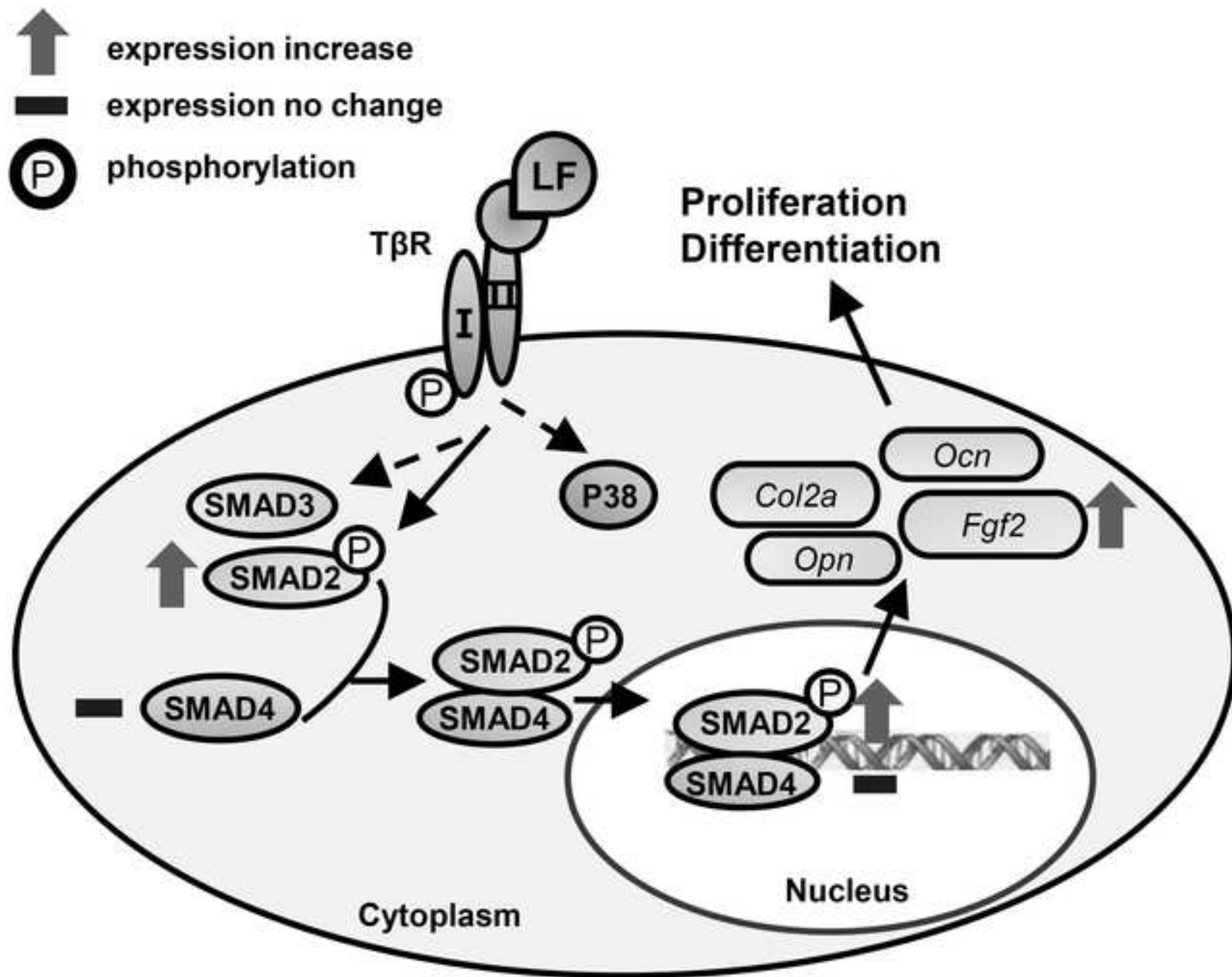
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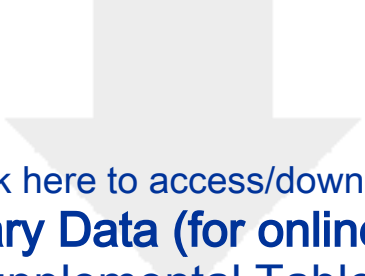


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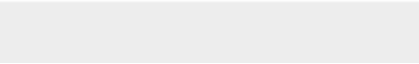






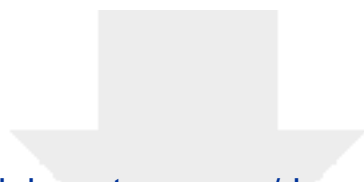
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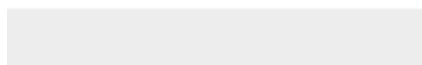
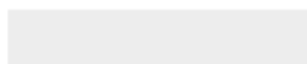


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