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Research Article

TGFβ3 regulates adipogenesis of bovine subcutaneous preadipocytes via typical Smad and atypical MAPK signaling pathways

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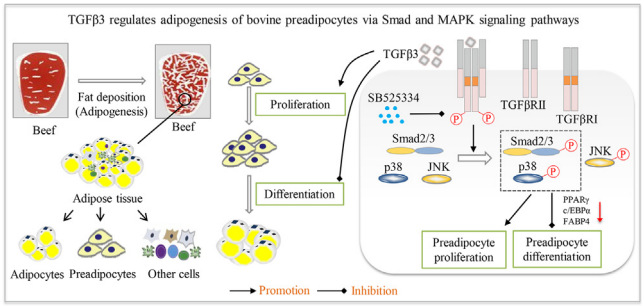
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Transforming growth factor β3 (TGFβ3) is a member of TGFβ superfamily. Studies have shown that TGFβ is involved in adipose tissue biology. Whether TGFβ3 is a crucial regulator in adipogenic differentiation of bovine preadipocytes remains unclear. The main objective of this study was to investigate the effect of TGFβ3 on the adipogenic differentiation of bovine preadipocytes as well as the underlying mechanisms.

Results: Primary subcutaneous preadipocytes derived from bovine subcutaneous adipose tissue were isolated and used as a cellular model. The results showed that TGFβ3 significantly promoted the proliferation of bovine preadipocytes and markedly decreased the lipid accumulation and expression levels of key adipogenic marker genes (PPARγ, c/EBPα, and FABP4) during adipocyte differentiation. In addition, TGFβ3 significantly increased the phosphorylation levels of Smad2, Smad3, JNK, and p38 in bovine preadipocytes, which could be eliminated by SB525334 (a TGFβRI inhibitor). Further studies showed that Smad3 and p38 mediated the inhibitory effect of TGFβ3 on differentiation, whereas Smad2 attenuated the anti-adipogenic effect of TGFβ3.

Conclusions: To sum up, our findings indicate that TGFβ3 is an important regulator of adipogenesis in bovine. TGFβ3 promotes the proliferation and regulates the differentiation of bovine preadipocytes through Smad2/3 and p38 signaling pathways.

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1. Introduction

Adipocytes comprise most of the volume of adipose tissues, and adipose tissues expand through the proliferation (number increase) and differentiation (size increase) of preadipocytes [1]. Excessive expansion of adipose tissue results in obesity. Plenty of diseases are induced by obesity, including hypertension, cardiovascular disease, type II diabetes (T2D), and cancer [2,3], which poses a big challenge to human health. On the other side, adipose tissue plays an irreplaceable role in thermoregulation, mechanical organ protection, and storing triglycerides in the presence of excessive calories and releasing them during fasting [4,5]. Adipose tissue has also been recognized as an endocrine organ, exerting specific effects on a variety of biological processes involved in maintaining whole-body homeostasis [6]. In livestock production, intramuscular fat accumulation makes beef appear marbling and contributes to beef flavor and juiciness [7], enhanced adipogenesis in intramuscular fat is often pursued. Therefore, further exploration of the underlying mechanisms in adipogenesis would be vital to both human health and animal production.

The transforming growth factor β (TGFβ) superfamily contains TGFβ1, 2, and 3, growth differentiation factors (GDFs), bone morphogenetic proteins (BMPs), nodal-related proteins, and activins. TGFβ is highly expressed in 10T1/2 cells, 3T3-L1 cells, and ASCs isolated from mouse and human adipose tissues [8,9,10], regulating pivotal cellular processes such as cell growth, cell fate specification, and differentiation [11]. Studies have shown that many members of TGFβ superfamily exert pleiotropic actions in adipogenesis [9,12,13,14,15]. Generally, TGFβ initiates cellular signaling through binding to specific receptor complexes referred as type I and type II receptors on the cell surface. And then, the activated type I receptor phosphorylates and thereby activates specific Smad proteins in the canonical signaling pathway, which translocate into the nucleus to control target gene transcription [16]. TGFβ also functions through atypical signaling pathways which involve in activation of several kinase cascades, including the p38, JNK, and ERK mitogen-activated protein kinase (MAPK) pathways [17]. Both typical Smad signaling pathway and atypical MAPK signaling pathways are closely related to the regulation of adipogenesis [18,19].

Fat deposition is controlled by a complicated interaction network, which involves hundreds of adipogenesis-related genes [20,21,22,23]. Additionally, it has been reported that TGFβ3, a member of the TGFβ superfamily, was also involved in adipose tissue biology. TGFβ3 overexpressing mice display smaller fat cells and improved glucose tolerance upon a high-fat diet [24]. In human, mutation of TGFβ3 gene can markedly reduce subcutaneous fat deposition [25]. Moreover, TGFβ3 stimulates adipocyte progenitor proliferation and results in a higher number of cells undergoing differentiation *in vitro* [26]. *In vivo*, TGFβ3$^{+/-}$ mice exhibit lower subcutaneous adipocyte progenitor proliferation, white adipose tissue hypertrophy, and glucose intolerance [26]. Above findings indicate that TGFβ3 is a potential regulator of adipogenesis. However, whether and how TGFβ3 directly regulates adipogenesis in bovine subcutaneous preadipocytes remains unclear. Here, this study attempts to explore the fundamental functions as well as molecular mechanism of TGFβ3 in adipogenesis regulation.

2. Materials and methods

2.1. Experimental animals and reagents

Samples of dorsal subcutaneous adipose tissue and other tissues from four 24-month-old fattening finished Simmental steers were collected at a local slaughterhouse (Nanjing, China). This study was approved by the Animal Ethics Committee of Nanjing Agricultural University (SYXK 2017-0027).

DMEM/F12 medium, phosphate-buffered saline (PBS), penicillin, and streptomycin used in this study were obtained from Hyclone (Logan, UT). Fetal bovine serum (FBS) was from Sciencell (San Diego, CA). Recombinant TGFβ3 was purchased from Peprotech (Rocky Hill, NJ). SB525334 was obtained from Selleck (Shanghai, China).

2.2. Cell culture

Dorsal subcutaneous fat tissues were isolated from the experimental animals under sterile conditions. Samples were rinsed with warm PBS several times and then cut with a scissor into approximately 1 mm^3 pieces. The tissue was digested with type I collagenase (Invitrogen, Carlsbad, CA) at 37°C for 2 h in a shaking water bath and stopped by adding two times the volume of growth medium (DMEM/F12 medium + 10% FBS + 100 IU/mL penicillin + 100 μg/mL streptomycin). Then, the digested mixture was filtrated through a 200 μm cell strainer followed by centrifugation at 1,200 *rpm* for 10 min. The supernatant containing mature adipocytes was collected for RNA extraction. The pellet containing preadipocytes was re-suspended in fresh growth medium and maintained in a humidified atmosphere containing 5% CO_2 at 37°C. The medium was changed every 2 d.

2.3. Cell proliferation assay

Bovine preadipocytes were seeded in 96-well culture plates and treated with siRNAs or recombinant protein for 48 h. Then, the cell viability was assessed with CCK-8 Cell Counting Kit (Vazyme, Nanjing, China) according to the manufacturer's protocol. Briefly, the cells were incubated with growth medium containing 10% CCK-8 solution for 4 h at 37°C and then quantified by absorbance values at 450 nm using a microplate reader (Tecan, Salzburg, Austria).

2.4. Adipogenic differentiation and Oil Red O staining

Two days after confluence (d 0), a standard inductive differentiation medium (DMI) cocktail (growth medium + 1 μg/mL insulin + 1 μM dexamethasone + 0.5 mM isobutylmethylxanthine + 2 μM rosiglitazone) was used to induce adipogenic differentiation. Then 6 d later, the DMI cocktail was replaced by maintenance medium (growth medium + 1 μg/mL insulin). At d 8 of induction, adipocytes were washed with cold PBS three times and fixed with 4% paraformaldehyde for 30 min at room temperature. Then, cells were washed again and stained with 60% saturated Oil Red O (Sigma-Aldrich, St. Louis, MO) for 30 min. The results were visualized on a microscope (CKX41, Olympus, Tokyo, Japan). Then, the lipid of cells was extracted by 100% isopropanol and quantified by absorbance values at 510 nm using a microplate reader (Tecan, Salzburg, Austria).

2.5. Immunohistochemistry

The dorsal subcutaneous adipose tissue specimens were fixed with formalin, embedded in paraffin, and then sectioned at 4 μm thicknesses. Sections of the paraffin-embedded were kept at 60°C for 24 h in the oven and then followed by deparaffinizing with xylene and hydrating with an ethanol gradient (100–70%) and incubating with antigen retrieval solution (Shanghai Shunbai Biotechnology Company, Shanghai, China) and 3% H_2O_2 for 30 min. Then, the sections were washed three times with water and incubated with the primary antibody against TGFβ3 (1:50, diluted with PBS) or PBS (negative control) overnight at 4°C. Next, the sections were rinsed and incubated with the biotinylated sec-

ondary antibody for 60 min followed by 3,3′-diaminobenzidine. After hematoxylin staining, the sections were mounted with gum and observed by a microscope and photographed.

2.6. RNA isolation and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted by Trizol Reagent (Invitrogen, Carlsbad, CA). The RNA concentrations were detected by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA). Isolated RNA was reverse transcribed by Prime Script RT reagent Kit (Takara, Kusatsu, Japan). The RT-qPCR was performed on the QuantStudio 7 Flex System (ABI, Foster City, CA) and used the SYBR Premix Ex Taq™ (Takara, Kusatsu, Japan). Gene-specific primers were obtained from Shanghai Sangon Biological Engineering Technology (Shanghai, China), as shown in Table 1. The relative expression level of mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and analyzed by the $2^{-\Delta\Delta CT}$ method.

2.7. Small interfering RNA (siRNA) transfection

A negative control siRNA (si-NC) and siRNAs specific to TGFβ3, Smad2, Smad3, JNK, and p38 were purchased from Shanghai GenePharma Biological Engineering Technology (Shanghai, China). The sequences of siRNAs were shown in Table 2. SiRNAs were transfected into subcutaneous preadipocytes using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. In brief, cells were washed three times with warm DMEM/F12 medium before the transfection, then transected with 50 nM siRNA (diluted with antibiotic-free DMEM/F12 medium) using Lipofectamine 2000 for 6 h and changed medium with fresh growth medium for further downstream treatment. The knockdown efficiency was examined at 48 h after transfection.

2.8. Western blot analysis

After specified treatments, cells were lysed in ice-cold RIPA buffer (Beyotime, Shanghai, China) containing 1 mM protease inhibitor PMSF (Beyotime, Shanghai, China). The concentration of protein was measured using the BCA method (Beyotime, Shanghai, China). Equal amounts of protein (25 μg) were separated via 4–12% GenScript ExpressPlus™ PAGE Gels (GenScript, Piscataway, NJ) and then transferred onto PVDF membranes (Millipore, Billerica, MA). The membranes were blocked with 5% nonfat dry milk in 1XTBST (Solarbio, Beijing, China) for 2 h and incubated overnight at 4°C with the following primary antibodies: TGFβ3 (1:1000, Novus, Littleton, CO), ERK1/2, phospho-ERK1/2, p38, phospho-p38 (1:1000, Cell Signaling Technology, Danvers, MA), PPARγ, JNK, phospho-JNK (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Smad2/3, GAPDH (1:500, Bioss, Beijing, China), and Smad2/3 (1:500, Boster, Wuhan, China). Followed by incubation with anti-rabbit IgG secondary antibody (1:4000, Cell Signaling Technology, Danvers, MA) for 1 h at room temperature, corresponding protein bands were detected using the ECL Chemiluminescence Detection Kit (Vazyme, Nanjing, China) and photographed using the Versa-Doc 4000 MP system (Bio-Rad, Hercules, CA). GAPDH was regarded as a housekeeping gene. Protein band density was quantified by Image-J Software.

2.9. Statistical analysis

The experimental data were obtained from at least three independent experiments, each containing three replicates. Results were expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using SPSS software (version 20.0, IBM, USA). Average difference was determined using a two-tailed *t*-test. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. TGFβ3 is expressed abundantly in bovine subcutaneous fat and changed regularly during adipocyte differentiation

To analyze TGFβ3 expression across different tissues, RNA was isolated from heart, liver, spleen, lung, kidney, intestine, muscle, intramuscular and subcutaneous adipose tissues (IAT and SAT) of Simmental bulls, and analyzed by quantitative real-time PCR. Among these tissues, subcutaneous adipose tissue showed high levels of TGFβ3 and ranked second only to lung tissue (Fig. 1a). Meanwhile, Immunohistochemical staining showed that TGFβ3 protein was predominantly localized in the subcutaneous adipose tissue (Fig. 1e). Above results suggested that TGFβ3 could play an important role in adipose tissue.

In order to unveil the function of TGFβ3 in adipocytes, Preadipocytes and mature adipocytes directly isolated from bovine subcuta-

Table 1
Primer sequences for RT-qPCR.

Gene	Gene accession number	mRNA accession number	Primer sequence (5′ to 3′)		Product size
TGFβ3	NC_037337.1	NM_001101183.1	Forward:	CCGCTTCAACGTGTCCTCAGTG	157 bp
			Reverse:	CCGCTGCTTGGCTATGTGCTC	
TGFβR I	NC_037335.1	NM_174621.2	Forward:	ACATATTGCTGCAACCAGGACCAC	91 bp
			Reverse:	CTGCCAGTTCAACAGGACCAAGG	
TGFβRII	NC_037349.1	NM_001159566.2	Forward:	CCGACTTGCTGCTGGTTATCTTCC	81 bp
			Reverse:	TGATGATGACTGCGATGGCGATG	
PPARγ	NC_037349.1	NM_181024.2	Forward:	TGGAGACCGCCCAGGTTTGC	111 bp
			Reverse:	AGCTGGGAGGACTCGGGGTG	
c/EBPα	NC_037340.1	NM_176784.2	Forward:	TGGGCAAGAGCCGGGACAAG	166 bp
			Reverse:	ACCAGGGAGCTCTCGGGCAG	
FABP4	NC_037341.1	NM_174314.2	Forward:	TCCTTCAAATTGGGCCAGGAA	218 bp
			Reverse:	CCCTTGGCTTATGCTCTCTCA	
Smad2	NC_037351.1	NM_001046218.1	Forward:	GCTGGCTCAGTCCGTTAATC	121 bp
			Reverse:	GCTTGTTACCGTCTGCCTTC	
Smad3	NC_037337.1	NM_001205805.1	Forward:	AGCTGACACGGAGGCATATC	109 bp
			Reverse:	CAGTTGGGAGACTGCACAAA	
JNK	NC_037355.1	NM_001192974.2	Forward:	ATGAGCAGAAGCAAGCGTGACAG	124 bp
			Reverse:	CACATACTATTCCTTGGGCTCCTGAAC	
p38	NC_037350.1	NM_001102174.1	Forward:	GGCTCCTGAGATCATGCTGAACTG	137 bp
			Reverse:	TCTGCTGAAGCTGGTTAATATGGTCTG	
GAPDH	NC_037332.1	NM_001034034.2	Forward:	TGCCCGTTCGACAGATAGCC	148 bp
			Reverse:	GCGACGATGTCCACTTTGCC	

Table 2
Sequences of siRNAs.

Item	Sense (5′ to 3′)	Antisense (5′ to 3′)
siRNA1	GGAGUAUUAUGCCAAAGAATT	UUCUUUGGCAUAAUACUCCTT
siRNA2	CCAACUUAGGUCUGGAAAUTT	AUUUCCAGACCUAAGUUGGTT
siRNA3	GCAGCUGUCUAACAUGGUGTT	CACCAUGUUAGACAGCUGCTT
si-Smad2	GCCAGUUACUUACUCAGAGTT	CUCUGAGUAAGUAACUGGCTT
si-Smad3	GCAUGUGCACCAUUCGCAUGATT	UCAUGCGAAUGGUGCACAUGCTT
si-JNK	GCCCAGUAAUAUAGUAGUATT	UACUACUAUAUUACUGGGCTT
si-p38	CATAATTCACAGGGACCTATT	UAGGUCCCUGUGAAUUAUGTT
si-NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

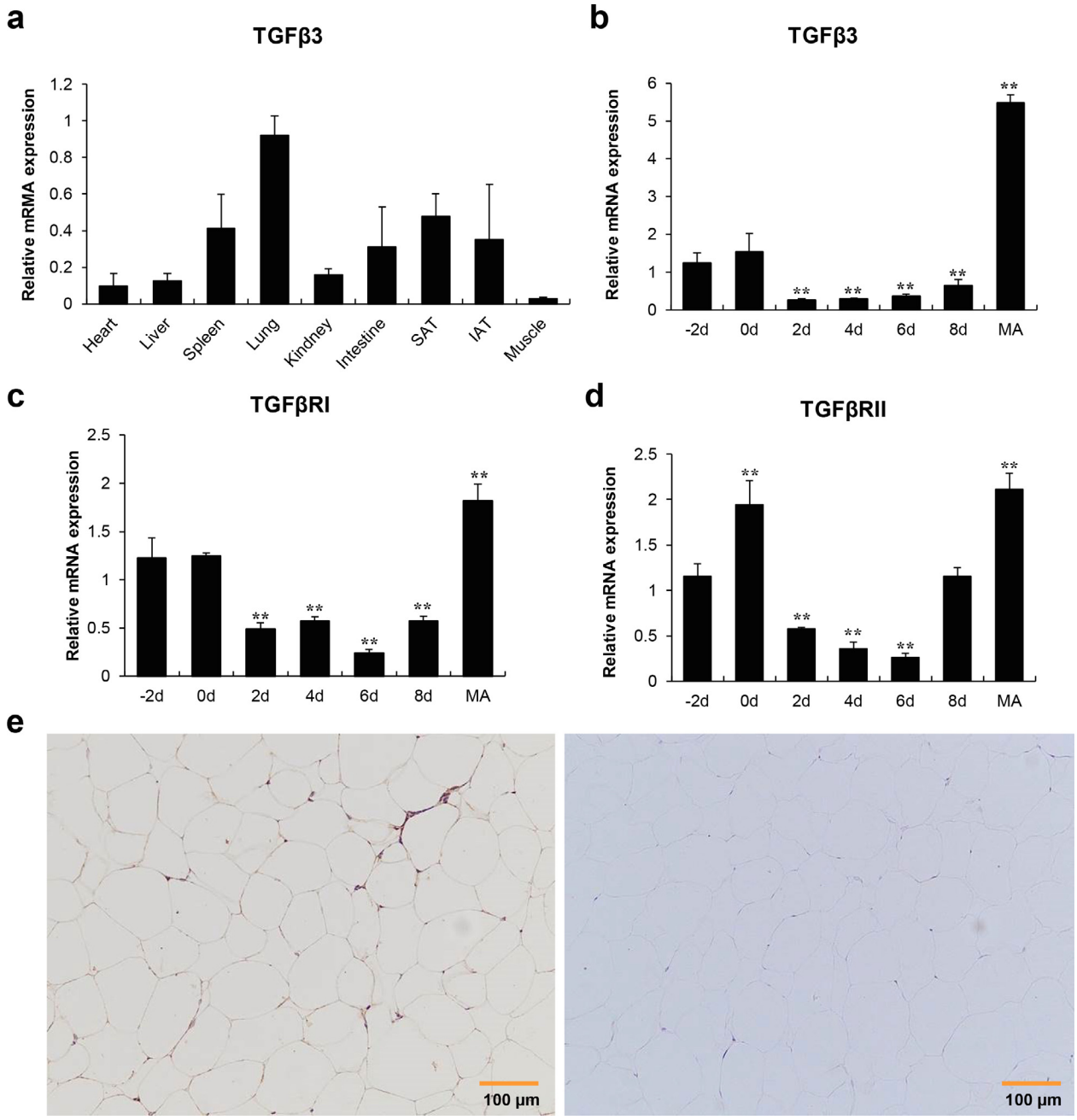


Fig. 1. Expression patterns of TGFβ3 and related receptor genes. (a) Relative expression of TGFβ3 mRNA in various tissues (n = 4). (b) The time-spatial mRNA expressions of TGFβ3 during differentiation of preadipocytes and in mature adipocytes. (c–d) The time-spatial mRNA expressions of TGFβRI (c) and TGFβRII (d) during differentiation of preadipocytes and in mature adipocytes. Data were compared with day −2. (e) Immunohistochemistry with anti-TGFβ3 antibody (left) and PBS (right) in bovine subcutaneous adipose tissue. SAT: subcutaneous adipose tissue, IAT: intramuscular adipose tissue, MA: mature adipocytes. n = 3, $^{*}p < 0.05$, $^{**}p < 0.01$.

neous adipose tissue were used to analyze TGFβ3 and related receptor gene expression during adipocyte maturation. RNA was isolated at different time points during the adipogenic differentiation. And as shown in Fig. 1b–d), mature adipocytes had very significantly higher expressions of TGFβ3, TGFβRI, and TGFβRII in mRNA level compared with preadipocytes ($p < 0.01$). Moreover, these genes were highly expressed at day −2, 0 of induction and peaked at day 0. After adipogenic treatment, their expression decreased significantly and then remained a low expression level. These results showed that TGFβ3 might be a crucial regulator in the adipogenic differentiation of bovine preadipocytes.

3.2. Recombinant TGFβ3 protein promotes proliferation and inhibits differentiation of bovine adipocytes

First, to examine the proliferative effects of recombinant TGFβ3. Bovine preadipocytes were incubated with various concentrations (1-100 ng/mL) of recombinant TGFβ3 for 48 h, and the cell viability was determined by CCK-8 assay. We observed that the cell viability was significantly increased following recombinant TGFβ3 treatment, and the optimal treatment concentration was 10 ng/mL (Fig. 2a).

To investigate the effects of TGFβ3 on adipocyte differentiation, we treated the bovine preadipocytes with 10 ng/mL recombinant TGFβ3 at day −2, 0, 3 of induction for 48 h, respectively. At day 8, the formation of lipid droplets was observed by staining with Oil Red O (Fig. 2b). TGFβ3 treatment obviously decreased the number of lipid droplets in bovine adipocytes relative to blank group. The lipid content was quantified based on absorbance values. Compared with the blank group, TGFβ3 treatment could significantly decrease lipid accumulation in adipocytes ($p < 0.01$; Fig. 2c). Furthermore, we observed the best inhibitory effect when we treated cells at day −2 of induction. Similarly, bovine preadipocytes were treated with 10 ng/mL TGFβ3 at the day −2 of induction for 48 h, and mRNA expressions of key adipogenic genes, including PPARγ, c/EBPα, and FABP4, were detected at the 24 h and day 8 of induction during adipocyte differentiation. As shown in Fig. 2d–e, TGFβ3 treatment dramatically decreased expressions of PPARγ, c/EBPα, and FABP4 through the differentiation process ($p < 0.01$) compared with the blank control group. These results indicated that TGFβ3 could promote proliferation while inhibiting differentiation of bovine adipocytes.

3.3. Knockdown of TGFβ3 inhibits proliferation and promotes differentiation of bovine adipocytes

To further validate our findings, three small interfering RNAs (siRNA1, 2, and 3) were synthetized to knock down the expression of TGFβ3 and transfected into proliferating bovine preadipocytes. TGFβ3 expression level was detected by quantitative real-time PCR and western blot analysis at 48 h post-transfection. The results showed that the interference efficiency of siRNA3 was the greatest (Fig. 3a–b) and the siRNA3 was suitable for further study.

CCK-8 assay showed that knockdown of TGFβ3 significantly downregulated the cell viability ($p < 0.05$; Fig. 3c). Interestingly, attenuated cell viability caused by siRNA3 could be rescued by incubation with recombinant TGFβ3 (Fig. 3c). TGFβ3 knockdown also obviously increased the number of lipid droplets (Fig. 3d) and the lipid content ($p < 0.01$; Fig. 3e) in bovine adipocytes which were transfected with siRNA3 at day −2, 0 of induction compared with negative control group. Transfecting at d 3 of induction had no significant promoting action. Consistent with the above outcomes, the levels of FABP4 were markedly upregulated at 24 h of induction in the knockdown-treated cells relative to the negative control group ($p < 0.05$; Fig. 3f). These results showed that knockdown of TGFβ3 inhibited proliferation and promoted differentiation of bovine adipocytes.

3.4. TGFβ3 activates the smad2/3 and MAPK signaling pathways in bovine adipocytes

To understand the potential mechanism of TGFβ3 on adipocyte maturation, bovine preadipocytes were treated with 10 ng/mL recombinant TGFβ3 for 15 min, 30 min, and 1 h, respectively. The phosphorylated protein levels of Smad2/3, JNK, p38, and ERK1/2 were analyzed by western blot analysis. The results showed that TGFβ3 treatment markedly increased the phosphorylation of Smad3 and JNK at both time points examined and significantly increased the phosphorylation of p38 and Smad2 at time points of 30 min and 1 h ($p < 0.01$; Fig. 4a–f). However, the phosphorylated protein level of ERK1/2 had no significant change (Fig. 4g). These results suggested that TGFβ3 could activate the Smad2/3, JNK, and p38 signaling pathways in bovine adipocytes.

3.5. Inhibition of TGFβRI by inhibitor SB525334 blocks TGFβ3-induced signaling pathways and attenuates the effect of TGFβ3 on adipogenic differentiation

SB525334, a TGFβRI specific inhibitor, was used to further confirm our findings. Bovine preadipocytes were pretreated with SB525334 (10 μM) for 2 h and then incubated with or without 10 ng/mL recombinant TGFβ3 for 1 h. Western blotting results showed that SB525334 dramatically decreased the phosphorylation levels of Smad2/3, JNK, and p38 ($p < 0.01$; Fig. 5a–c). Furthermore, we determined the effect of SB525334 on adipogenic differentiation of bovine adipocytes. As shown in Fig. 5d–f, TGFβ3 treatment obviously upregulated the cell viability of bovine preadipocytes ($p < 0.01$), and this effect was completely eliminated after incubation with 10 μM SB525334. During adipocyte differentiation, 10 μM SB525334 obviously attenuated the inhibitory effect of TGFβ3 on PPARγ mRNA expression level at d 8 of induction. These results showed that inhibition of TGFβRI by inhibitor SB525334 blocked TGFβ3-induced signaling pathways and attenuated the effect of TGFβ3 on adipogenic differentiation.

3.6. TGFβ3 regulates adipogenesis by smad2/3 and p38 signaling pathways in bovine adipocytes

Knockdown experiment was carried out to further examine the involvement of the signaling pathways in regulating adipogenic differentiation of bovine preadipocytes in response to TGFβ3. We first confirmed the effectiveness of siRNAs by quantitative real-time PCR and western blot analysis. As depicted in Fig. 6a, transfection with si-Smad2, si-Smad3, si-JNK, or si-p38 for 48 h specifically decreased their targeted gene mRNA levels in bovine adipocytes when compared to negative control group. Consistent with the above findings, significant decrease in the protein levels of phosphorylated Smad2, Smad3, JNK, and p38 was observed upon specific siRNAs treatment in the absence or presence of recombinant TGFβ3 ($p < 0.01$; Fig. 6b–e). In the subsequent experiment, we transfected specific siRNAs into bovine preadipocytes followed by TGFβ3 challenge. The mRNA expression of key adipogenic genes were analyzed at the 24 h of induction. The results show that TGFβ3 treatment notably decreased PPARγ, c/EBPα, and FABP4 mRNA levels ($p < 0.01$; Fig. 6f–h). Smad2 knockdown markedly decreased c/EBPα mRNA levels, whereas p38 knockdown obviously increased c/EBPα mRNA levels in the presence of recombinant TGFβ3 ($p < 0.01$; Fig. 6h). Moreover, Smad3 knockdown significantly increased PPARγ mRNA levels in the presence of recombinant TGFβ3 ($p < 0.01$; Fig. 6f).Taken together, it was

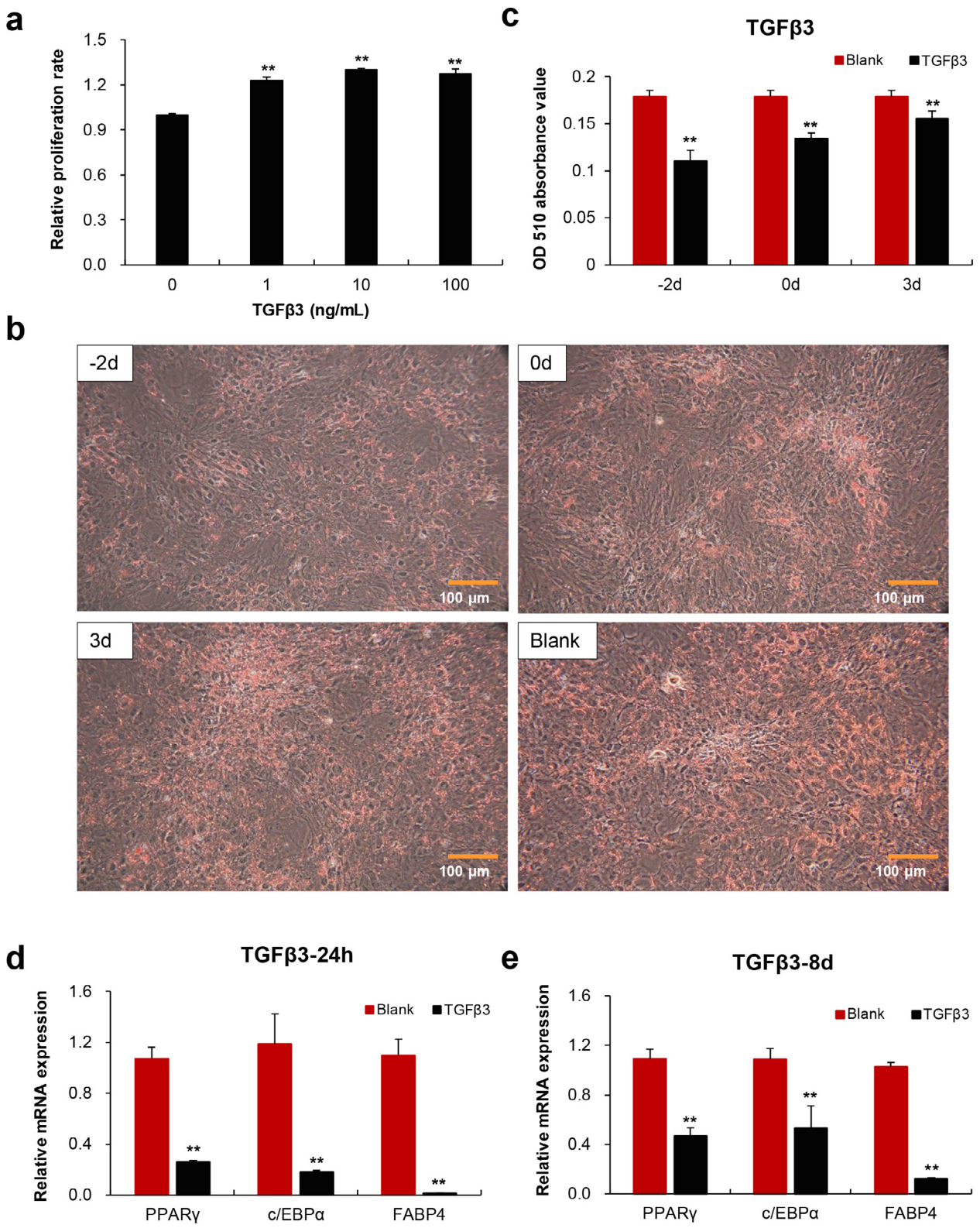
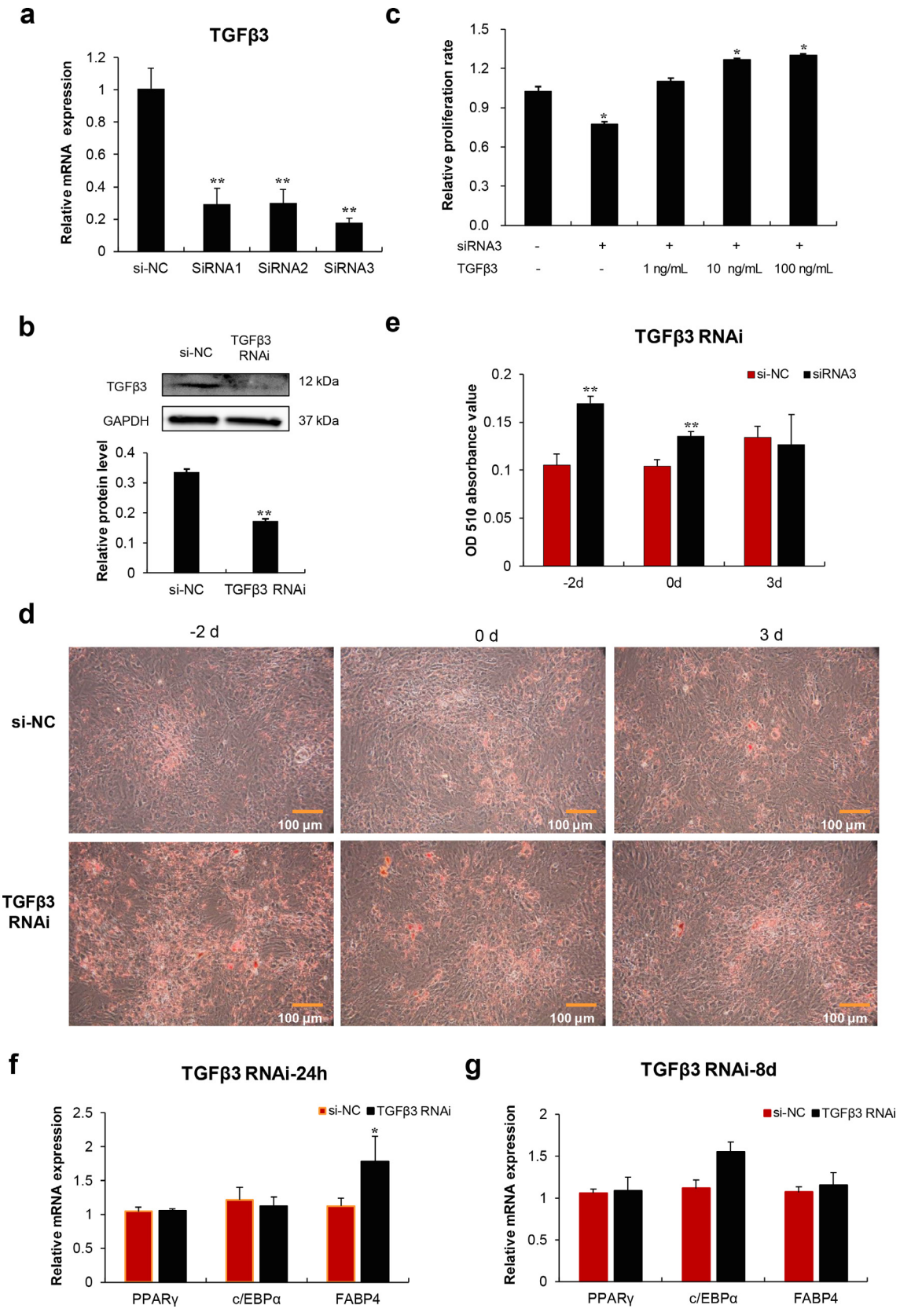


Fig. 2. Recombinant TGFβ3 protein regulates bovine preadipocyte proliferation and differentiation. (a) Bovine preadipocytes were incubated with 1–100 ng/mL recombinant TGFβ3 for 48 h and cell viability was assessed by CCK-8 assay ($n = 6$). (b) Bovine preadipocytes were treated with 10 ng/mL recombinant TGFβ3 at day −2, 0, 3 of induction for 48 h, and lipid accumulation was monitored with Oil Red O staining under a microscope (100 × magnification) at day 8. (c) Quantification of lipid content by measuring the absorbance at 510 nm. Bovine preadipocytes were treated with 10 ng/mL TGFβ3 at day −2 of induction for 48 h, and relative mRNA expressions of key adipogenic genes were detected at the 24 h (d) and day 8 (e) of induction during adipocyte differentiation. $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$.


a
TGFβ3
Relative mRNA expression
si-NC
SiRNA1
SiRNA2
SiRNA3
c
Relative proliferation rate
siRNA3
TGFβ3
1 ng/mL
10 ng/mL
100 ng/mL
b
si-NC
TGFβ3 RNAi
TGFβ3
GAPDH
12 kDa
37 kDa
Relative protein level
e
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siRNA3
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-2d
0d
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-2 d
0 d
3 d
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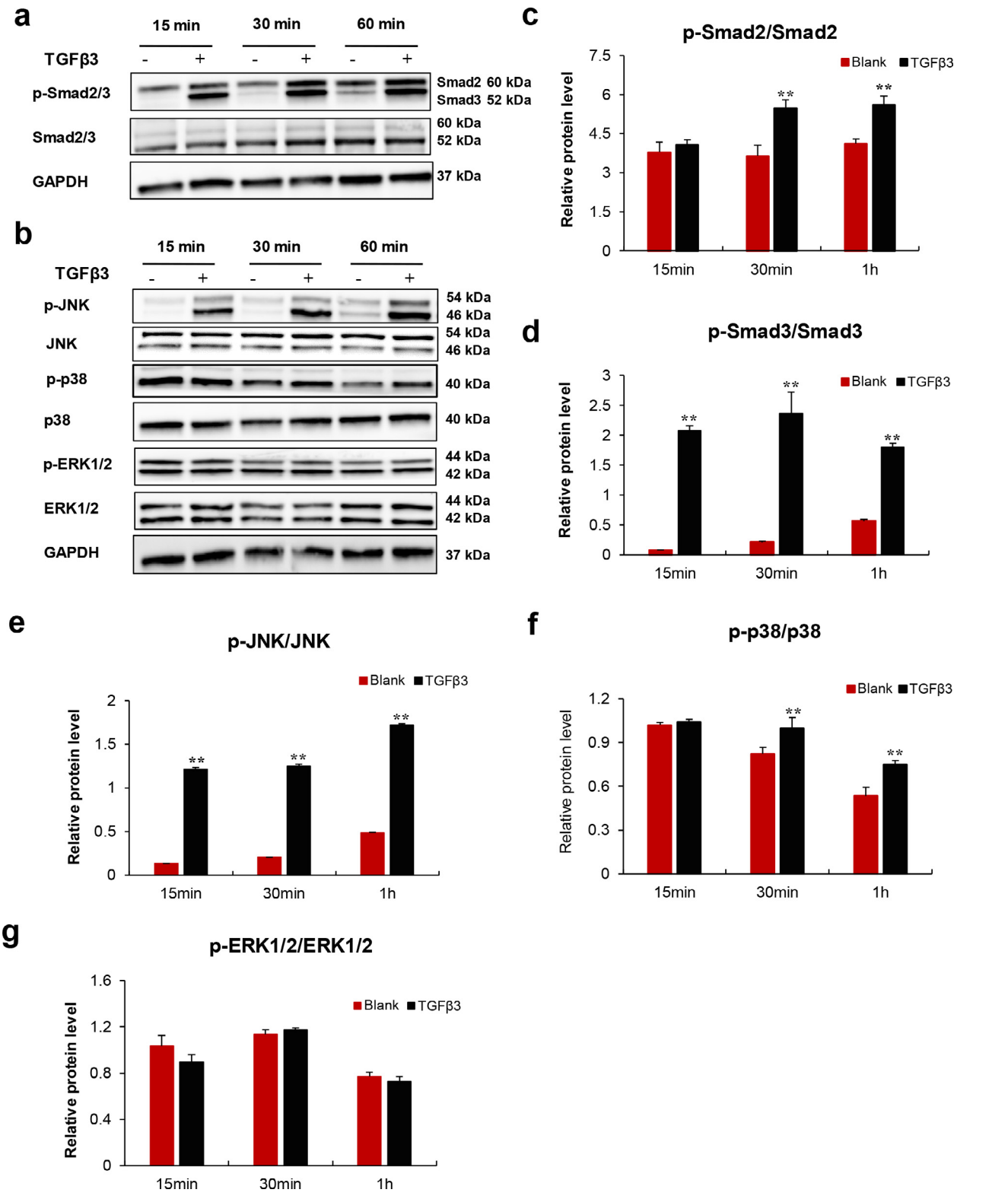

Fig. 4. Signaling pathways induced by TGFβ3. Bovine preadipocytes were treated with 10 ng/mL recombinant TGFβ3 for 15 min, 30 min, and 1 h, respectively. The phosphorylated protein levels of Smad2, Smad3 (a), JNK, p38, and ERK1/2 (b) were analyzed by western blot analysis. (c-g) Relative density values of Smad2 (c), Smad3 (d), JNK (e), p38 (f), and ERK1/2 (g). $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$.

Fig. 3. Knockdown of TGFβ3 promotes the proliferation and inhibits differentiation of bovine adipocytes. (a) Three siRNAs were transfected into proliferating bovine preadipocytes, respectively, for 48 h and TGFβ3 expression levels were detected by RT-qPCR. (b) SiRNA3 was transfected into proliferating bovine preadipocytes for 48 h and TGFβ3 protein expression levels were detected by western blot analysis. (c) Bovine preadipocytes were transfected with or without siRNA3 and incubated with various concentrations of recombinant TGFβ3 for 48 h. Cell viability was assessed by CCK-8 assay ($n = 6$). (d) Bovine preadipocytes were transfected with siRNA3 at the days −2, 0, and 3 of induction. Lipid accumulation was monitored with Oil Red O staining under a microscope (100 × magnification) on day 8. (e) Quantification of lipid content by measuring the absorbance at 510 nm. Bovine preadipocytes were transfected with siRNA3 at day −2 of induction for 48 h, and relative mRNA expressions of key adipogenic genes were detected at the 24 h (f) and day 8 (g) of induction during adipocyte differentiation. $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$.

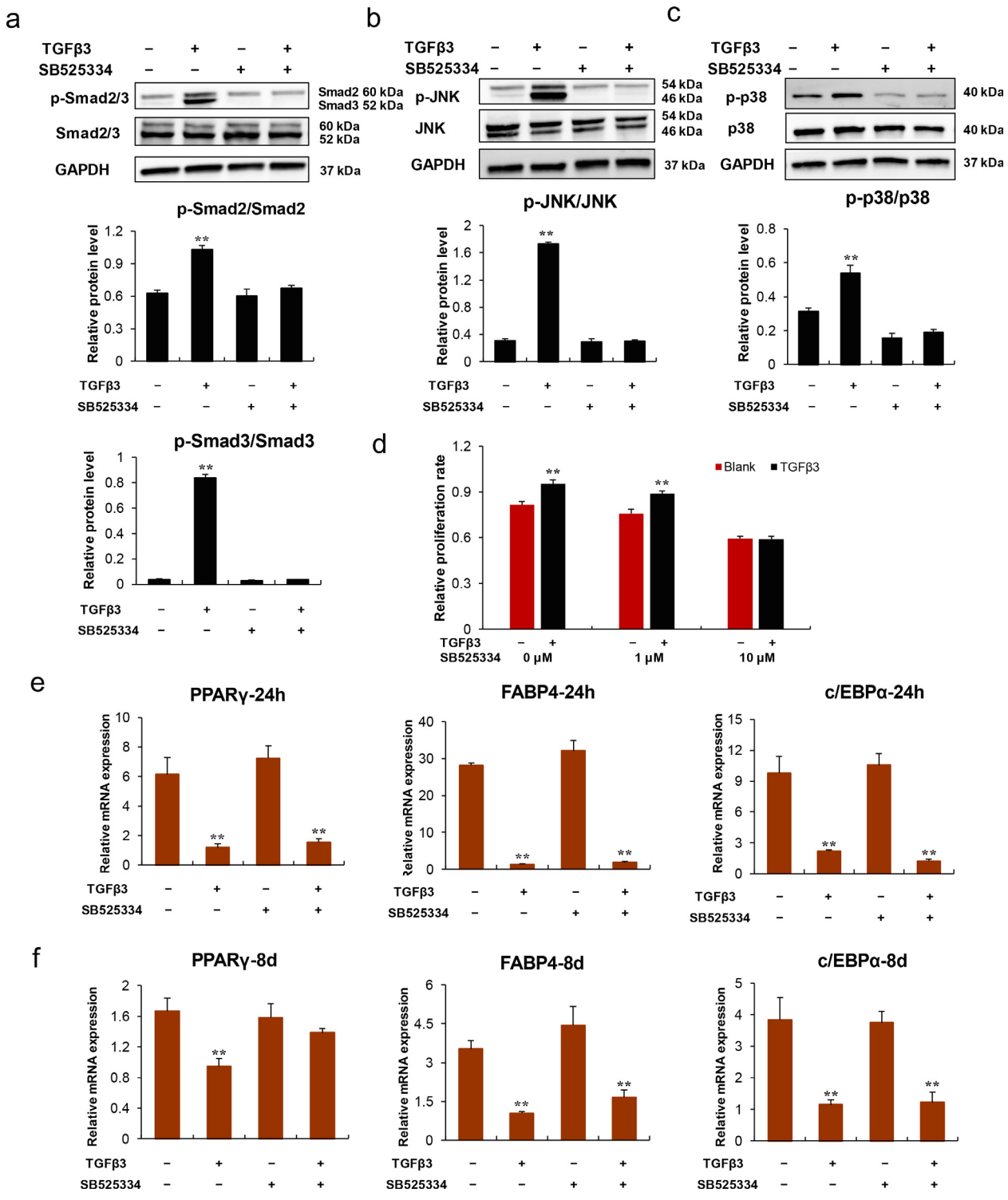

Fig. 5. SB525334 blocks TGFβ3-induced signaling pathways and attenuates the effect of TGFβ3 on adipogenic differentiation. (a-c) Bovine preadipocytes were pretreated with SB525334 (10 μM) for 2 h and then incubated with or without 10 ng/mL recombinant TGFβ3 for 1 h. The phosphorylated protein levels of Smad2/3 (a), JNK (b) and p38 (c) were analyzed by western blot analysis. Data were compared with the first group. (d) Bovine preadipocytes were treated with SB525334 (10 μM) in the absence or presence of recombinant TGFβ3 for 48 h. Cell viability was assessed by CCK-8 assay ($n = 6$). (e–f) Bovine preadipocytes were treated with SB525334 (10 μM) in the absence or presence of recombinant TGFβ3 at day −2 of induction for 48 h. Relative mRNA expressions of key adipogenic genes were detected at the 24 h (e) and day 8 (f) of induction during adipocyte differentiation. Data were compared with the first group. $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$.

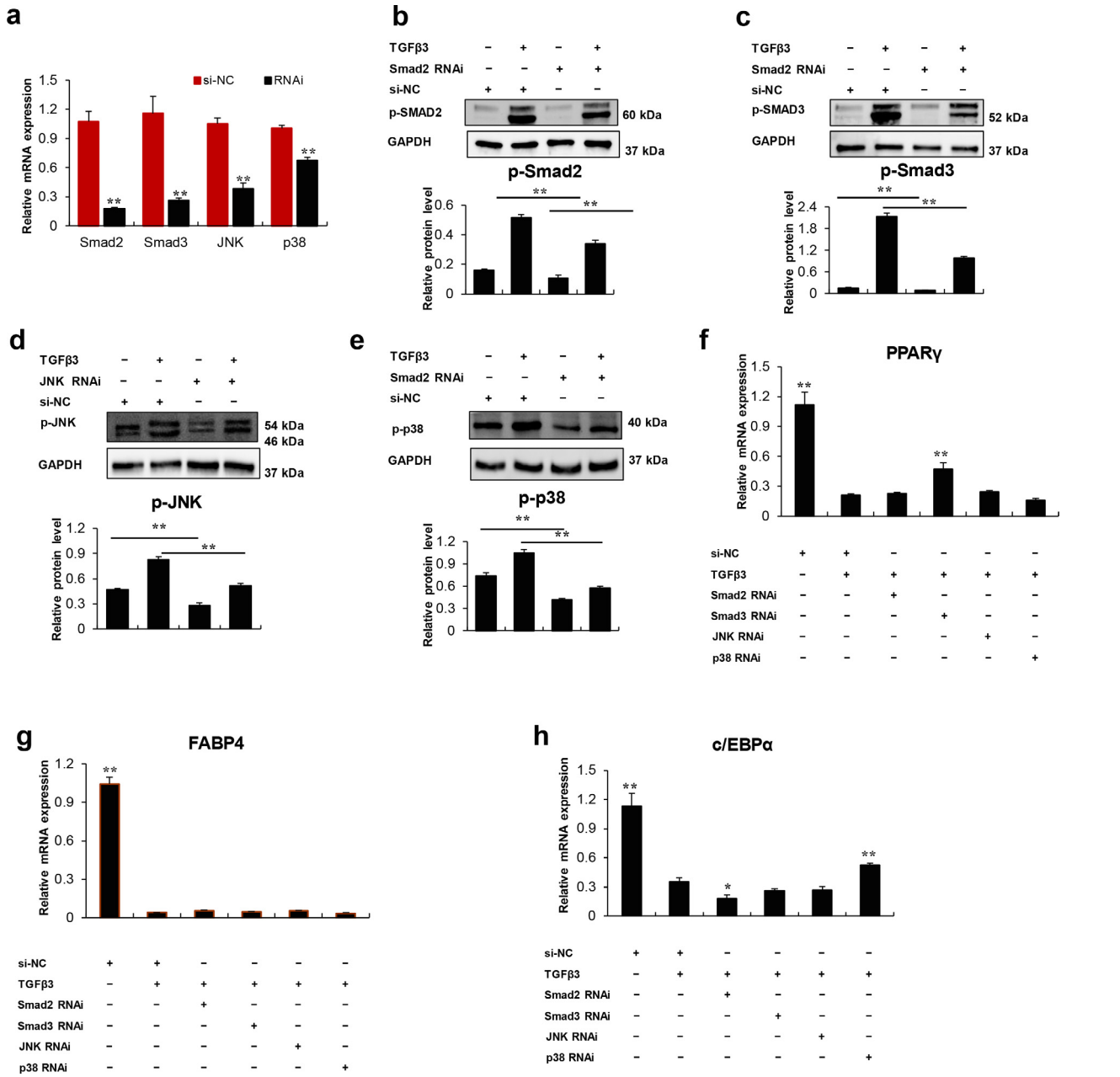

Fig. 6. TGFβ3 regulates adipogenesis by Smad2/3 and p38 Signaling Pathways. (a) Specific siRNAs were transfected into proliferating bovine preadipocytes for 48 h and mRNA levels of their target genes were detected by RT-qPCR. (b-e) Bovine preadipocytes were transfected with si-Smad2, si-Smad3, si-JNK, or si-p38 in the absence or presence of recombinant TGFβ3 for 48 h. The protein levels of phosphorylated Smad2 (b), Smad3 (c), JNK (d), and p38 (e) were analyzed by western blot analysis. (f-h) Bovine preadipocytes were transfected with si-Smad2, si-Smad3, si-JNK, or si-p38 in the absence or presence of recombinant TGFβ3 at day −2 of induction for 48 h. Relative mRNA expressions of key adipogenic genes were detected at the 24 h of induction during adipocyte differentiation. Data were compared with the group NC in the presence of recombinant TGFβ3. $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$.

unequivocally demonstrated that TGFβ3 regulated adipogenesis by Smad2, 3, and p38 signaling pathways in bovine adipocytes.

4. Discussion

Adipogenesis involves a complex network of gene-for-gene interactions which leads to the formation of mature adipocyte [27]. This process can be divided into three main phases: growth arrest, clonal expansion, and terminal differentiation [28]. Interrupting any of these stages can be used as a potential therapeutic strategy to inhibit or promote adipogenesis. Hence, identifying key genes that control adipogenesis would provide a feasible solution for obesity and meat quality improvement. We herein report that TGFβ3 is a novel regulator of adipogenesis in bovine, which promote the proliferation and inhibit the differentiation of subcutaneous preadipocytes via the activation of Smad2/3 and p38 signaling cascades.

The mammalian genome contains three related isoforms of the TGFβ subfamily including TGFβ1, TGFβ2, and TGFβ3. These isoforms are widely expressed in a variety of tissues and cell types [24,26,29]. Miller et al. [30] demonstrated that TGFβ3 mRNA expression is prominent in the placenta, adipose tissue, lung, brain, heart, and male submaxillary gland of adult mouse. An *in vitro* study showed that TGFb3 was secreted by undifferentiated human subcutaneous adipocyte precursor cells and reduced during

adipogenesis [26]. The expression pattern of TGFβ3 gene in bovine was determined in this study. Similarly, we observed that TGFβ3 expressed in different tissues, notably subcutaneous adipose tissue, showed high levels of TGFβ3. During adipogenesis, high TGFβ3 expression levels in mature adipocytes and un-induced preadipocytes were observed, as well as a significant reduction after adipogenic treatment. In addition, the expression patterns of TGFβ3 receptor genes (TGFβRI and TGFβRII) showed a similar trend to TGFβ3. These results imply that TGFβ3 may engage in the process of adipogenesis.

TGFβ1 was known to promote the proliferation of preadipocytes while inhibiting their differentiation into adipocytes [13,14]. Although the TGFβ1 and TGFβ3 share 60–80% identity at the amino acid level, the heterology between them is also evolutionarily well conserved [24,31]. The isoform-specific properties are likely to exist. Whether TGFβ3 regulates adipogenic differentiation of bovine preadipocytes is still unknown. To explore the role of TGFβ3 in adipogenic differentiation, bovine primary subcutaneous preadipocytes were used as the cellular model. We found that cell proliferation was promoted, while lipid accumulation and expression levels of key adipogenic marker genes (including PPARγ, c/EBPα, FABP4) were significantly inhibited upon recombinant TGFβ3 treatment. Consistently, TGFβ3 knockdown by RNAi resulted in suppression of cell proliferation and enhanced adipogenic differentiation. These results indicated that TGFβ1 and TGFβ3 appear to play similar roles in adipogenic differentiation. Interestingly, our results were inconsistent with the research of Petrus et al. [26], in which TGFβ3 increased the proliferation of human and murine adipocyte precursors but no direct effects on adipogenesis. It might be caused by different species or cell type-specific differences. For instance, ZBTB16 enhanced the adipogenesis of bovine intramuscular preadipocytes while significantly repressed differentiation of 3T3-L1 preadipocytes [32,33]. Further research is needed to find out the TGFβ3 functions on preadipocyte differentiation.

TGFβ3 typically initiates cell signaling through a heterogeneous combination of two type I receptors (TGFβRI) and two type II receptors (TGFβRII), followed by phosphorylation and activation of Smad2 and Smad3 [34]. It should be noted that TGFβ receptors were shown to activate other pathways like ERK, p38, JNK, and Akt/PKB [35]. In present study, we found that TGFβ3 observably increased the phosphorylated levels of Smad2, Smad3, p38, and JNK in bovine preadipocytes. It has been shown that TGF3 affected a sustained activation of JNK in breast cancer cells [36]. TGFβ3 (500 ng/mL) induction significantly increased the phosphorylated p38 levels during osteogenic differentiation of primary human periodontal ligament stem cells [37]. These researches could support our results. However, TGFβ3 could activate both ERK and JNK signaling pathways in junctional adhesion molecule-B mRNA destabilization [38]. A study conducted in rats revealed that TGFβ3 cooperates with different adapters (TAB1 and CD2AP) to activate p38 and ERK signaling pathways, reversibly disrupting blood-testicular barrier integrity and support-germ cell adhesion [39]. There was a dispute over the activation of ERK pathway in response to TGFβ3. In view of TGFβ family, receptor complexes activate Smad signaling as well as non-Smad signaling pathways depending on cell/tissue type and physiological context [40]. This difference may exist, but it remains to be verified. Moreover, the drug inhibition test by using SB525334, a TGFβRI specific inhibitor, markedly blocked Smad2/3, p38, and JNK signaling pathways, and confirmed our conclusions.

Further research found that SB525334 obviously downregulated the cell proliferation and attenuated the anti-adipogenic effects of TGFβ3 in bovine preadipocytes. This result indicated that Smad2, Smad3, p38, and/or JNK signaling pathway may be involved in the regulation of adipogenic differentiation in response to TGFβ3. To investigate which pathways participate in the process of adipogenesis, knockdown experiments were carried out. Our results showed that Smad2 knockdown markedly decreased c/EBPα mRNA levels, whereas Smad3 knockdown significantly increased PPARγ mRNA levels in the presence of TGFβ3. Consistent with our findings, previous studies reported that Smad3 mutant could significantly increase the expression levels of PPARγ, ADD-1, c/EBPα, FABP4, and adipsin, and promote lipid accumulation in the presence of TGFβ in 3T3-F442A cells. In contrast to Smad3, Smad2 mutant enhanced the TGFβ-mediated suppression of these differentiation markers and inhibited the differentiation of 3T3-F442A cells [14]. In addition to the Smad signaling pathway, previous studies demonstrated that p38 played a negative regulatory role during adipogenesis. For example, p38α knockout increased the adipogenesis of embryonic stem cells *in vitro* and *in vivo* [41]. In mouse, inhibition of p38 activity increased c/EBPβ phosphorylation at Threonine^{188} and augmented adipocyte differentiation [42]. Consistent with these previous studies, our findings indicated that p38 knockdown obviously increased c/EBPα mRNA levels in the presence of TGFβ3. However, JNK knockdown did not significantly alter the expression levels of PPARγ, c/EBPα, and FABP4, implying that the JNK pathway was not involved in TGFβ3-mediated differentiation-inhibiting. The mechanisms by which Smad2, 3 and MAPKs involve in adipogenic differentiation may be related to their well-known ability to interact with and modify the activity of other regulators. Whether these downstream factors of TGFβ3 target the regulators that drive adipose differentiation is worth exploring.

In summary, the present study provided clear evidence that TGFβ3 promoted the proliferation and inhibited the differentiation of bovine preadipocytes. TGFβ3 significantly reduced lipid accumulation and key adipogenic marker gene expression during adipogenic differentiation. Mechanically, TGFβ3 activated Smad2/3 and p38 signaling pathways and engaged in adipogenesis regulation of TGFβ3. These findings help to elucidate the mechanisms by which TGFβ3 affects adipogenesis and further provide a new approach for fat management by controlling adipogenesis through TGFβ3.

Author contributions

- Study conception and design: S Wei, P Yan.
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- Analysis and interpretation of results: S Wei, T Li, H Fang, H Bai.
- Draft manuscript preparation: L Yang.
- Revision of the results and approved the final version of the manuscript: S Wei, P Yan, L Yang, H Wang, W Hao, T Li, H Fang, H Bai.

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Conflicts of interest

The authors declare no conflicts of interest.

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