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Original Article

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G protein-coupled estrogen receptor 1 mediates proliferation and adipogenic differentiation of goat adipose-derived stem cells through ERK1/2-NF-κB signaling pathway

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Abstract

Adipose tissue formation and moderate fat deposition are important for the production performance and eating quality of livestock meats. The self-renewal and adipogenic differentiation of adipose-derived stem cells are responsible for the formation and development of adipose tissue. In addition, estrogen targeting G protein-coupled estrogen receptor 1 (GPER1) has been reported to modulate cell proliferation and differentiation during tissue and organ development. However, the potential correlation among estrogen, GPER1, proliferation, and adipogenic differentiation in goat adipose-derived stem cells (gADSCs) is still unclear. Herein, we demonstrated that 17β-estradiol enhances the proliferative ability of gADSCs, indicated by the increased cell number and cell viability, accompanied by up-regulated expressions of cyclin D1 and PCNA. Meanwhile, the adipogenic differentiation is promoted by 17β-estradiol, supported by higher ccumulation of intracellular lipids and increased expressions of PPARγ, ACC, and FABP4. Notably, these activities are all obviously reduced by administration with GPER1 antagonist G15, but GPER1 agonist G1 enhances cell proliferation and adipogenic differentiation. Moreover, GPER1 silencing diminishes cell proliferation and adipogenic differentiation. In parallel, 17β-estradiol elevates the protein level of nuclear p-p65. Furthermore, the phosphorylation of p65 is enhanced by G1 but inhibited by G15 and GPER1 silencing. In addition, the phosphorylation of p65 is mediated by ERK1/2, suggesting that estrogen targeting GPER1 regulates cell proliferation and adipogenic differentiation of gADSCs through the ERK1/2-NF-κB signaling pathway. This study may provide a strong theoretical basis for improving meat quality, flavor, and cold resistance of livestock.

Key words 17β-estradiol, G protein-coupled estrogen receptor 1, goat adipose-derived stem cell, proliferation, adipogenic differentiation

Introduction

Goats play a pivotal role in meat and milk consumption of the diet for people. They are good candidates for producing exogenous nutrition due to its high protein production, abundant amino acids, and microelements for the human body [1,2]. Therefore, it is necessary to improve the production and quality of mutton [3]. Adipose tissue formation and moderate fat deposition are important for improving the tenderness and flavor of livestock meats [4,5]. Adipose tissue, originating from the mesoderm during embryonic development, presents in every mammalian species and locates throughout the body [6]. Adipose-derived stem cells (ADSCs) are widely distributed in adipose tissue, whose proliferation and differentiation are fundamental for the formation and growth of adipose tissue [7].

Estrogen (mainly 17β-estradiol) is an important hormone which plays multifunctional roles in tissue and organ development through influencing cell proliferation, differentiation, and metabolism. Studies have found that estrogen stimulates cell proliferation [8] and promotes adipogenic differentiation, increasing the number of adipocytes and the abundance of lipid droplets [9]. Furthermore, most estrogenic effects are mediated by the estrogen membrane receptor G protein-coupled estrogen receptor 1 (GPER1) which is

expressed in numerous tissues, performing a number of significant cellular and physiological functions. GPER1 activation increases cell viability and proliferation [10] and enhances the number of adipocytes and lipid accumulation [11], while GPER1 silencing is associated with the opposite effects; moreover, decreases of body weight, body fat depot mass, and adipocyte size were detected in *GPER1*-knockout female mice [12]. In addition, GPER1 exhibits beneficial effects on osteogenic [13,14] and neuronal [15] differentiation, suggesting that GPER1 participates in the regulation of cell proliferation and multiple differentiation potentials.

Nuclear transcription factor-kappa B (NF-κB) signaling regulates cell proliferation and multi-differentiation ability, including adipogenic, osteogenic, and chondrogenic differentiation [16]. NF-κB signaling activity promotes cell proliferation [17] and adipogenic differentiation [18,19]. In turn, inhibition of adipogenic differentiation and adipocytokines secretion are mediated by antagonizing NF-κB transactivation and p65 nuclear translocation [20]. Additionally, NF-κB activity is elevated in adipocytes derived from myoblasts cultured in adipocyte differentiation medium, but NF-κB inhibition in myoblast-derived adipocytes restores them into fusion-competent myoblasts [21]. Based on previous studies, we hypothesized that estrogen may bind to GPER1 to regulate cell proliferation and adipogenic differentiation of goat adipose-derived stem cells (gADSCs).

The aim of the present study is to explore whether estrogen targeting GPER1 effects the proliferation and adipogenic differentiation of gADSCs. We found that GPER1 activation increases cell proliferation and adipogenic differentiation of gADSCs, whereas GPER1 silencing is associated with the opposite effects. Furthermore, GPER1 functions in gADSCs are mediated by the ERK1/2-NF-κB signaling pathway.

Materials and Methods

Ethics statement

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Northwest A&F University. All animal experiments were performed under the control of the Guidelines for Animal Experiments by the Institutional Animal Care and Use Committee (IACUC) of Northwest A&F University.

gADSCs isolation and identification

Adipose tissue samples were obtained from the inguinal areas of female goats aged 2 to 3 months. gADSCs were isolated by collagenase digestion as previously described [22] with the following modifications. Briefly, the samples were minced into pieces and digested in 0.1% Collagenase Type I (Gibco, Carlsbad, USA) at 37°C for 40 min with shaking every 10 min. Then, the digested samples were centrifuged at 2000 rpm for 5 min to obtain a bottom pellet containing gADSCs. The pellet was resuspended in complete basal medium of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (phenol red-free, DMEM/F-12; BasalMedia, Shanghai, China), supplemented with 10% KnockOut Serum Replacement (SR; Gibco) and 1 × antibiotic antimycotic solution (Sigma, St Louis, USA), and centrifuged again at 1500 rpm for 10 min. Then, cells were collected and cultured in complete basal medium at 37°C with 5% CO_2 in culture dishes. The medium was refreshed every 3 days thereafter.

For the flow cytometric analysis, gADSCs in 100 μL PBS were mixed with fluorescein-labelled monoclonal antibody anti-CD29 (Abcam, Cambridge, USA), anti-CD34 (BD Pharmingen, San Diego, USA), anti-CD44 (Abcam), anti-CD45 (BD Pharmingen), anti-CD90 (BD Pharmingen), or anti-CD105 (BD Pharmingen), and incubated in the dark for 2 h at room temperature. After washing, cells were resuspended in 200 μL PBS, and analyzed using a flow cytometer (BD Biosciences, San Diego, USA).

For the multidirectional differentiation assay, gADSCs were induced with specific adipogenic, osteogenic, or chondrogenic differentiation medium for 14 days. Subsequently, cells were identified by Oil Red O staining, Alizarin Red staining, and Alcian Blue staining according to manufacturer's instructions, respectively.

Cell counting assay

gADSCs were seeded into 24-well plates (1×10^4 cells/well) and cultured in the complete basal mediumwith different treatments at 37°C for 4 days. Cell number was determined in triplicate wells.

Cell viability assay

gADSCs were seeded into 96-well plates (3×10^3 cells/well) and cultured in complete basal medium with different treatments at 37°C for 4 days. CCK-8 kit (BOSTER, Shanghai, China) was used to evaluate the viability of cells according to the instructions of the manufacturer.

Adipogenic differentiation

gADSCs were seeded into 6-well plates and grown until confluent. The differentiation was initiated with the adipogenic differentiation medium containing DMEM/F-12, 10% SR, 10 ng/mL insulin-like growth factor-I (Peprotech, Rocky Hill, USA), 1 μM dexamethasone (MP Biomedicals, Santa Ana, USA), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), and 0.2 mM indomethacin (Sigma). Cells were continuously cultured in the medium for 14 days, and the medium was refreshed every 3 days. During adipogenic differentiation, gADSCs were treated with 17β-estradiol (E2, 0.1 μM; Sigma), GPER1 agonist G1 (0.1 μM; Cayman, Ann Arbor, USA), GPER1 antagonist G15 (1 μM; Cayman), or mitogen-activated protein kinase kinase (MEK) inhibitor U0126 (10 μM; Selleck, Houston, USA) alone or in combination as specified.

Oil Red O staining

The accumulation of intracellular lipid was measured by Oil red O staining. Cells were washed twice with PBS, fixed in 4% paraformaldehyde for 30 min, washed with 60% isopropanol, and dried at room temperature. Then, cells were incubated with working Oil red O solution (Solaibio, Beijing, China) for 1 h at room temperature and washed with 60% isopropanol to remove excessive stain. To quantify the intracellular lipid, stained cells were incubated with 100% isopropanol for 20 min to extract the dye, and the absorbance value was determined at 492 nm.

Transfection of shRNA targeting GPER1

The short hairpin GPER1 (GenBank: XM_018039918, shGPER1) and short hairpin negative control (shRNA) were synthesized by Sangon Biotech (Shanghai, China) and the sequences are shown in Table 1. The procedure of constructing short hairpin RNA lentivirus vectors and packaging virus were performed as previously described [23]. Thereafter, gADSCs were infected with shRNA or shGPER1 lentivirus for 18 h, and expanded until they achieved about80% confluence, respectively. The protein level of GPER1 was detected by western blot analysis.

Table 1. Short hairpin interfering RNAs used in this study

shRNA	Sequence (loop in bold letters) (5′→3′)
shGPER1-1	GATCCGCTCATCCTGGTGGTGAACAT**TTCAAGAGA** ATGTTCACCACCAGGATGAGCTTTTTTG
	AATTCAAAAAAGCTCATCCTGGTGGTGAACAT **TCTCTTGAA**ATGTTCACCACCAGGATGAGCG
shGPER1-2	GATCCGCTCATCGAGGTGTTCAACCT**TTCAAGAGA** AGGTTGAACACCTCGATGAGCTTTTTTG
	AATTCAAAAAAGCTCATCGAGGTGTTCAACCT **TCTCTTGAA**AGGTTGAACACCTCGATGAGCG
Sh-NC	GATCCTTCTCCGAACGTGTCACGT**TTCAAGAGA** ACGTGACACGTTCGGAGAATTTTTTG
	AATTCAAAAAATTCTCCGAACGTGTCACGT**TCTCTT GAA**ACGTGACACGTTCGGAGAAG

Immunofluorescence staining

According to manufacturer's instructions, gADSCs were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton-X 100 for 15 min, and blocked with 1% BSA for 2 h at room temperature. Then, the cells were incubated with the anti-GPER1 primary antibody (Abcam) overnight at 4°C, followed by incubation with fluorescein-labelled secondary antibody (Invitrogen, Carlsbad, USA). DAPI (Beyotime, Shanghai, China) was used to stain the nuclei in the dark for 5 min at room temperature. All images were collected using a fluorescence microscope (Olympus, Tokyo, Japan).

RNA preparation and gene expression analysis

Total RNA was extracted using RNAiso Plus kit (TaKaRa, Dalian, China), and cDNA was synthesized from the total RNA using the Evo M-MLV RT kit with gDNA Clean for qPCRII(Agbio, Changsha, China). The levels of mRNAs were measured using the SYBR Green Premix Pro Taq HS qPCR kit (Agbio) according to the manufacturer's protocol. GAPDH is used as the internal control. All primers were synthesized by Sangon Biotech (Shanghai), and the sequences are shown in Table 2.

Western blot analysis

Cells were lysed with high-efficiency RIPA buffer (Solarbio) containing protease and phosphatase inhibitors, or with Nuclear Protein Extraction kit (BestBio, Shanghai, China) to obtain total protein or nuclear protein. Protein concentration was determined using a BCA Assay kit (Genstar, Beijing, China). Equal amounts of proteins were separated by SDS-PAGE and subsequently transferred to PVDF membranes (Millipore, Billerica, USA). The membranes were blocked with 3% BSA, and incubated with specific primary antibodies overnight at 4°C, followed by incubation with the corresponding HRP-conjugated secondary antibody (Proteintech, Chicago, USA). Finally, the protein bands were visualized using the Chemiluminescence kit (Biotanon, Shanghai, China), and quantified using Gel-Pro analyzer (Media Cybernetics, Maryland, USA). GAPDH is used as the internal control. The following primary antibodies were used: anti-GAPDH antibody (Cell Signaling Technology, Beverly, USA), anti-Histone H3 antibody (Abcam), anti-GPER1 antibody (Abcam), anti-ERK1/2 antibody (Cell Signaling Technology), anti-phospho-ERK1/2 antibody (Cell Signaling Technology), and anti-phospho-NF-κB antibody (Cell Signaling Technology).

Table 2. Sequence of primers used in this study

Gene	Primer sequence (5′→3′)	GenBank
Cyclin D1	Forward: AGACCCTCGCTTGTGCTTAC	XM_018043271
	Reverse: AACGTGCCGGTTACATGTCT	
PCNA	Forward: ATCAGCTCAAGTGGCGTGAA	XM_005688167
	Reverse: TGCCAAGGTGTCCGCATTAT	
PPARγ	Forward: ACTTTGGGATCAGCTCCGTG	NM_001285658
	Reverse: GTCAGCTCTTGGGAACGGAA	
ACC	Forward: AGCCTGCGGAATAGCATCTC	XM_018064168
	Reverse: GCACGTCAACTTCCACACAC	
FABP4	Forward: TGGGCCAGGAATTTGATGAA GTC	NM_001285623
	Reverse: CTCTGGTGGTAGTGACACCGT	
GAPDH	Forward: TTATGACCACTGTCCACGCC	XM_005680968
	Reverse: TCAGATCCACAACGGACACG	

Statistical analysis

Data were obtained from three independent experiments and expressed as the mean ± SD. Statistical analysis was conducted using SPSS (version 20.0; SPSS, Chicago, USA), which was checked by Tukey's test. Statistical significance was set at $P < 0.05$.

Results

Characterization of gADSCs

The isolated cells were adherent and displayed the typical fibroblastic morphology (Figure 1A). Furthermore, they were capable of self-renewal and multilineage differentiation. When being exposed to appropriate induction medium, they could differentiate into adipogenic, osteogenic, and chondrogenic cells, which were identified by Oil red O staining (Figure 1B), Alizarin Red staining (Figure 1C), and Alcian Blue staining (Figure 1D), respectively. Flow cytometric analysis showed that these cells expressed the surface markers that were characteristic of mesenchymal stem cells (positive for CD29, CD44, CD90, and CD105; negative for CD34 and CD45) (Figure 1E), as previously described [22,24,25]. Based on these results, gADSCs possessed the common characteristics of mesenchymal stem cells.

17β-estradiol promotes the proliferation of gADSCs through GPER1

We firstly investigated the roles of 17β-estradiol in the proliferation of gADSCs. gADSCs were treated with different concentrations of 17β-estradiol. As shown in Figure 2, 0.1 μM and 1 μM 17β-estradiol treatment increased the cell number and cell viability of gADSCs compared to the control (0 μM), and there were no statistical significances between cells treated with 0.1 μM and 1 μM 17-β estradiol (Figure 2), suggesting that 17-β estradiol promotes the proliferation of gADSCs, and 0.1 μM 17β-estradiol can be used to perform the subsequent experiments.

To investigate whether estrogen membrane receptor GPER1 mediated the pro-proliferative effect of 17β-estradiol on gADSCs, we firstly examined the distribution and expression of GPER1. Immunofluorescence staining showed that GPER1 mainly located at the membrane and cytoplasm(Figure 3A), and gADSCs stably expressed GPER1 protein during the experiments (P3–P12) (Figure 3B). Thereafter, we evaluated the changes of cell proliferation of gADSCs exposed to GPER1 agonist and GPER1 antagonist. GPER1

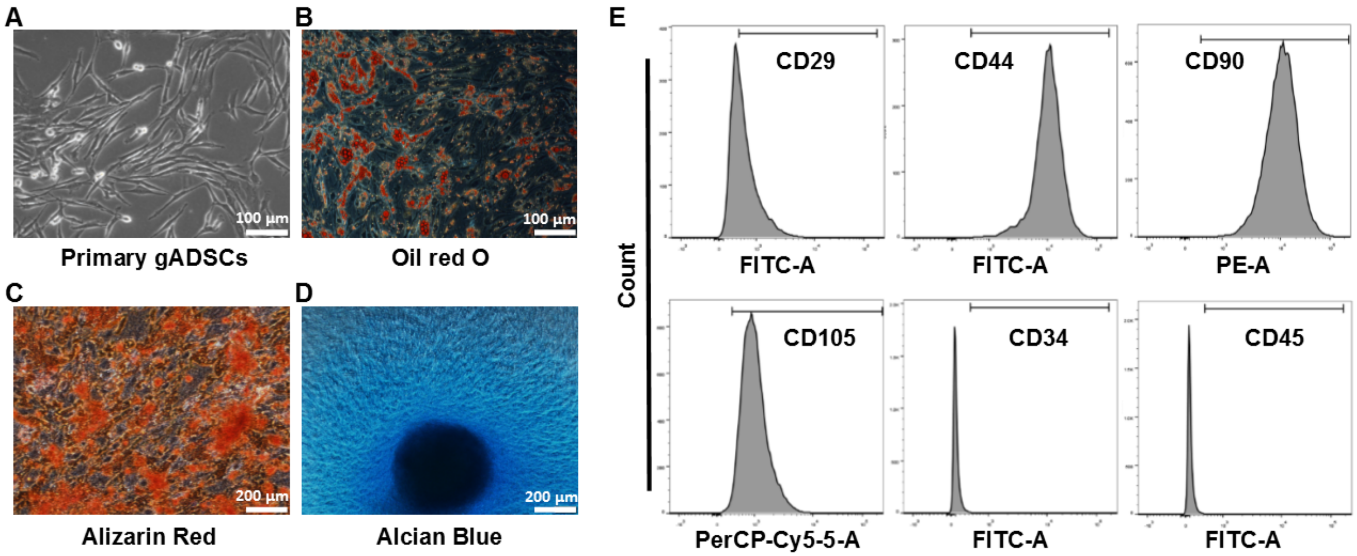

Figure 1. Characterization of gADSCs (A) Representative image of the morphology of gADSCs. The adipogenic, osteogenic, and chondrogenic ability of gADSCs were determined by Oil red O staining (B), Alizarin Red staining (C), and Alcian Blue staining (D), respectively. (E) Flow cytometric analysis identified the surface marker CD29, CD44, CD90, CD105, CD34, and CD45. Scale bar = 100 μM for (A,B) and scale bare = 200 μM for (C,D).

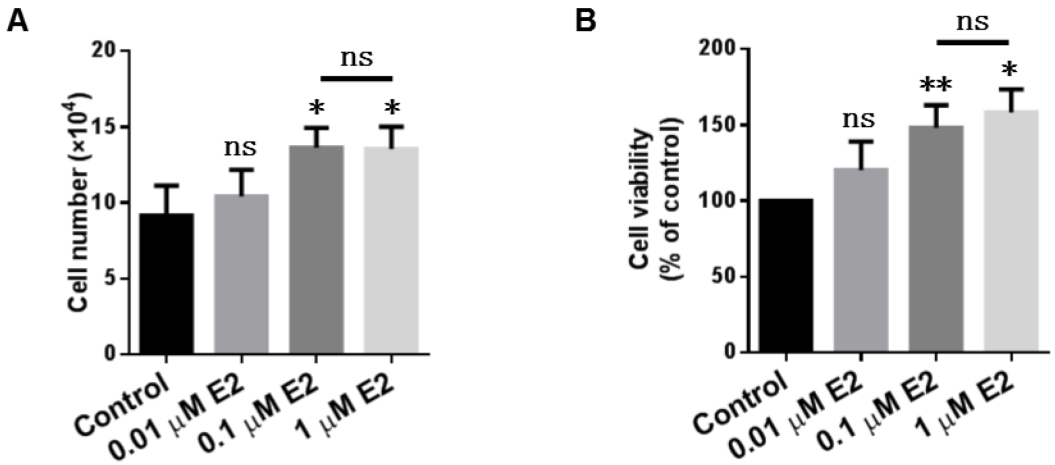

Figure 2. 17β-estradiol promotes the proliferation of gADSCs The effect of 17β-estradiol (E2; 0.01 μM, 0.1 μM, and 1 μM) on cell proliferation was detected by cell counting (A) and CCK-8 assay (B). ns, not statistically significant. *$P < 0.05$, **$P < 0.01$ (vs control).

agonist G1 treatment improved cell number and cell viability of gADSCs compared to the control, whereas GPER1 antagonist G15 obviously inhibited the pro-proliferative effect of 17β-estradiol on gADSCs (Figure 3C,D).

To further investigate the potential effects of GPER1 on cell proliferation, GPER1 silencing was performed in gADSCs using two specific shRNAs, *i.e.*, shGPER1-1 and shGPER1-2. The results showed that shGPER1-2 treatment had better knockdown efficiency on GPER1 expression (Figure 3E), therefore shGPER1-2 was used in the subsequent GPER1 silencing experiments. As predicted, GPER1 silencing obviously decreased cell number and cell viability compared to the negative control (Figure 3F,G).

Meanwhile, the mRNA levels of proliferation marker cyclin D1 and proliferating cell nuclear antigen (PCNA) [26] were found to be upregulated by 17β-estradiol, which was reversed by simultaneous exposure to G15. G1 treatment increased the mRNA levels of cyclin D1 and PCNA (Figure 3H), while GPER1 silencing decreased the mRNA levels of cyclin D1 and PCNA (Figure 3I). These data show that 17β-estradiol may bind with GPER1 to regulate cell proliferation of gADSCs.

GPER1 activation promotes adipogenic differentiation of gADSCs

To evaluate whether 17β-estradiol regulated adipogenic differentiation of gADSCs, lipid accumulation was measured by Oil red O staining. As shown in Figure 4A, 17β-estradiol treatment induced lipid accumulation compared to the control, indicating its promotive effect on adipogenic differentiation of gADSCs. Moreover, G1 treatment increased the accumulation of intracellular lipids compared to the control, while G15 treatment decreased the lipid accumulation compared to 17β-estradiol alone (Figure 4A). After GPER1 silencing, gADSCs showed a lower amount of intracellular

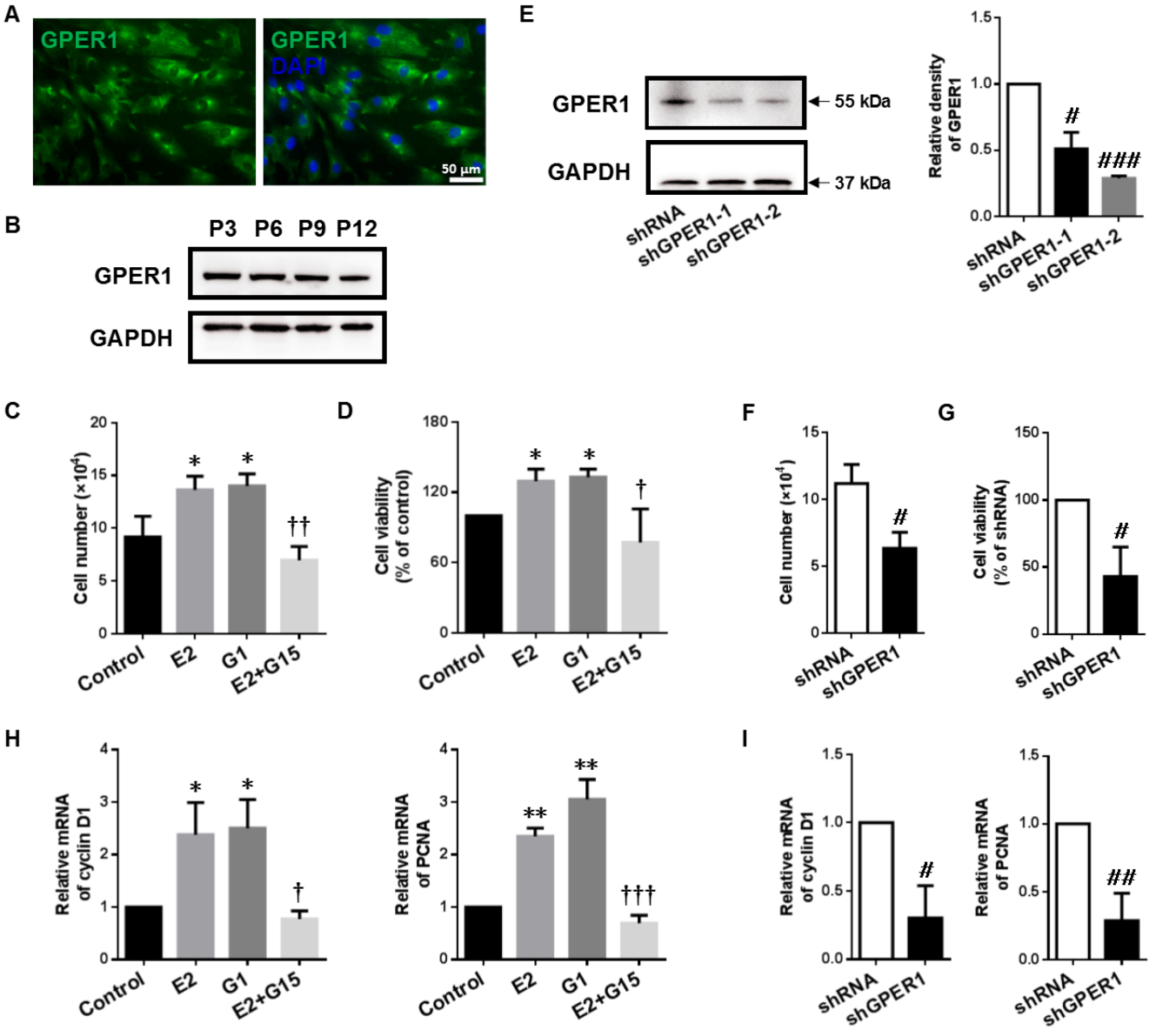

Figure 3. GPER1 mediates 17β-estradiol-induced proliferation of gADSCs Expression of GPER1 was determined by immunofluorescence staining (A) and western blot analysis (B). Cell counting (C) and CCK-8 assay (D) were performed to detect cell proliferation of gADSCs treated with 17β-estradiol (E2; 0.1 μM), GPER1 agonist G1 (0.1 μM), or 17β-estradiol + GPER1 antagonist G15 (1 μM) for 4 days. (E) The protein level of GPER1 was determined by western blot analysis. After GPER1 silencing, cell counting (F) and CCK-8 assay (G) were performed to detect cell proliferation of gADSCs. (H,I) The mRNA levels of cyclin D1 and PCNA in gADSCs subjected to various treatments were measured by RT-qPCR. P3, P6, P9, and P12 represent the 3, 6, 9, and 12 generation of gADSCs respectively. The nuclei were stained with DAPI. GAPDH serves as the internal control. Scale bar = 50 μm. $^{*}P<0.05$, $^{**}P<0.01$ (vs control); $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ (vs E2); and $^{\#}P<0.05$, $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$ (vs shRNA).

lipid accumulation compared to the negative control (Figure 4B).

To further confirm the effects of GPER1 on adipogenic differentiation of gADSCs, the expressions of adipogenic marker genes [27,28] were detected. As shown in Figure 4C, 17β-estradiol treatment upregulated the mRNA levels of proliferator-activated receptor gamma (PPARγ), acetyl CoA carboxylase (ACC), and fatty acid binding protein 4 (FABP4) compared to the control. Likewise, the mRNA levels of PPARγ, ACC, and FABP4 showed evident upregulation after treatment with G1, whereas their levels were decreased by G15 treatment compared to 17β-estradiol alone (Figure 4C). Furthermore, GPER1 silencing downregulated the mRNA levels of PPARγ, ACC, and FABP4 (Figure 4D). These data indicate that 17β-estradiol targets GPER1 to regulate adipogenic differentiation of gADSCs.

Effect of 17β-estradiol targeting GPER1 on NF-κB signaling

NF-κB signaling regulates cell proliferation and multi-differentiation ability, such as adipogenic differentiation [16]. Herein, we examined the changes of elements involved in the NF-κB signaling. The results showed that 17β-estradiol treatment elevated the nuclear protein level of phosphorylated NF-κB p65 subunit (p-p65). Likewise, the protein level of nuclear p-p65 was upregulated upon G1 stimulation. In turn, G15 treatment reversed these effects in-

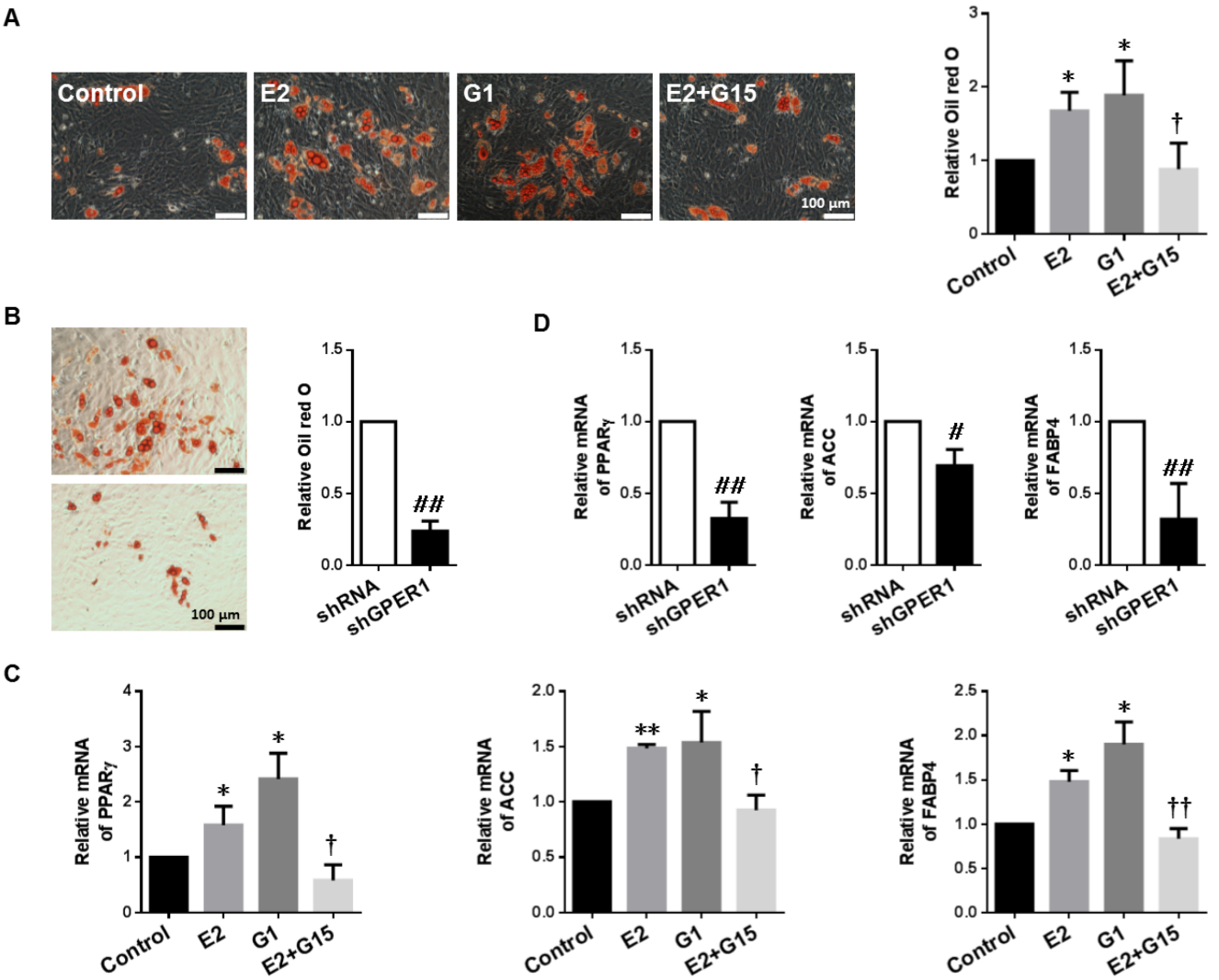

Figure 4. Activation of GPER1 promotes adipogenic differentiation of gADSCs (A) Representative images of intracellular lipid accumulation of gADSCs incubated with 17β-estradiol (E2; 0.1 μM), G1 (0.1 μM), or 17β-estradiol + G15 (1 μM) for 14 days. Lipid accumulation was detected by Oil red O staining. (B) Representative images of intracellular lipid accumulation of gADSCs with GPER1 silencing. Lipid accumulation was determined by Oil red O staining. (C,D) The mRNA levels of PPARγ, ACC, and FABP4 in gADSCs subjected to various treatments were measured by RT-qPCR. GAPDH serves as the internal control. Scale bar = 100 μm. $^{*}P < 0.05$, $^{**}P < 0.01$ (vs control); $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ (vs E2); and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (vs shRNA).

duced by 17β-estradiol alone (Figure 5A). What's more, GPER1 silencing inhibited the phosphorylation of p65 compared to the negative control (Figure 5B). These results demonstrated the involvement of NF-κB signaling in the promotion of proliferative and adipogenic effect of GPER1 activation.

Cross-talk between GPER1 and NF-κB signaling

Based on above results of GPER1 agonist and antagonist, we hypothesized that there was a cross-talk between GPER1 and NF-κB signaling participating in the regulation of cell proliferation and adipogenic differentiation of gADSCs. Our results showed that ERK1/2 was phosphorylated upon 17β-estradiol stimulation. Furthermore, G1 treatment upregulated the protein level of phospho-ERK1/2 (p-ERK1/2) compared to the control. On the contrary, G15 treatment inhibited the phosphorylation of ERK1/2 compared to 17β-estradiol alone (Figure 5C). Moreover, GPER1 silencing decreased the phosphorylation of ERK1/2 compared to the negative control (Figure 5D).

Notably, U0126, an inhibitor of MEK, decreased the nuclear protein level of p-p65 compared to 17β-estradiol or G1 alone (Figure 5E), suggesting that the phosphorylation of p65 is mediated by ERK1/2 in gADSCs.

Effect of 17β-estradiol on cell proliferation and adipogenic differentiation through ERK1/2-NF-κB signaling pathway

We further investigated the effect of MEK/ERK signaling on cell proliferation and adipogenic differentiation of gADSCs, and found that MEK inhibitor U0126 decreased the cell number and cell viability of gADSCs (Figure 6A,B), as evidenced by down-regulated expressions of cyclin D1 and PCNA mRNA compared to 17β-estradiol or G1 alone (Figure 6C). Moreover, U0126 treatment inhibited

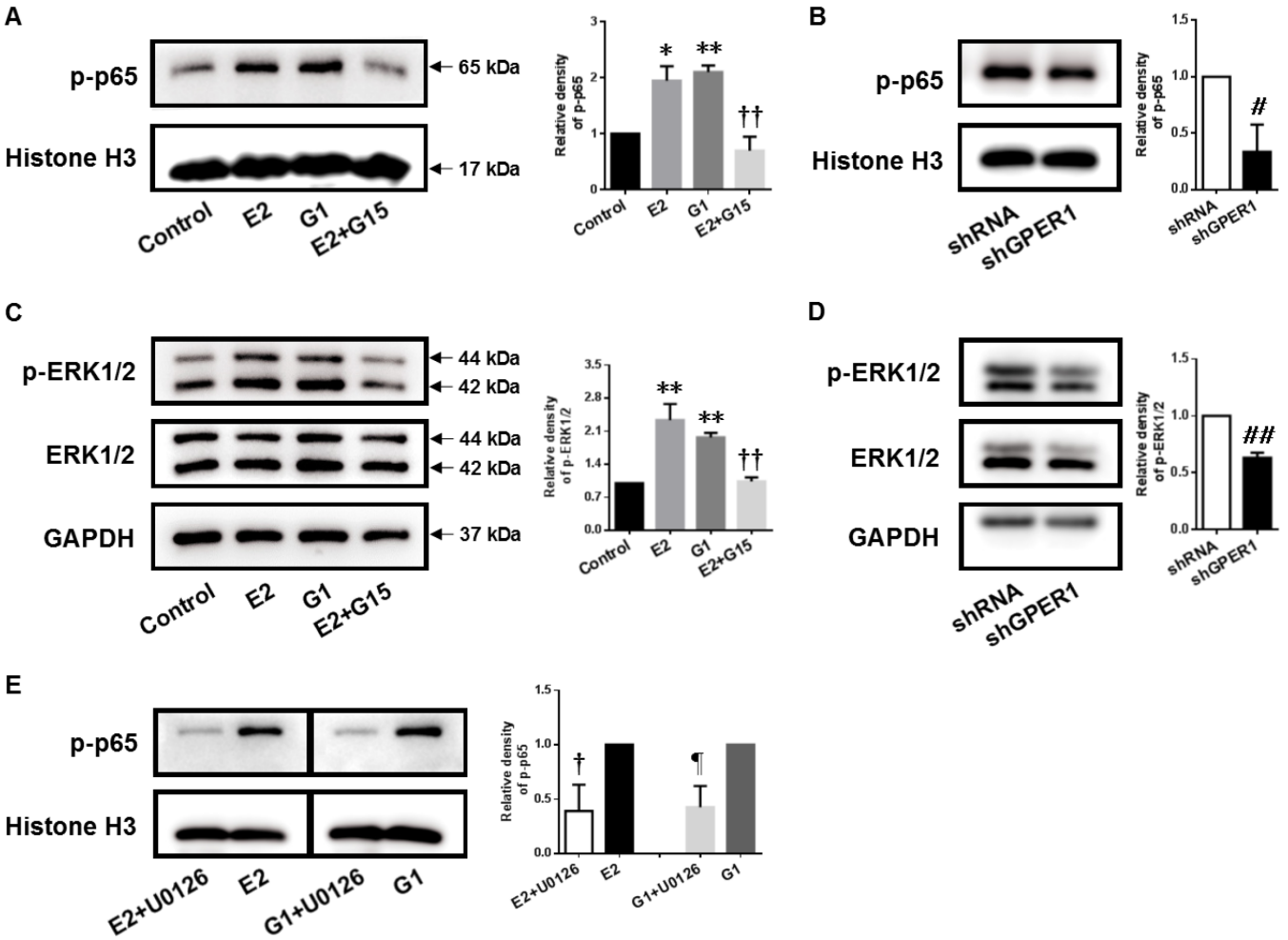

Figure 5. The cross-talk between GPER1 and NF-κB signaling (A) gADSCs were incubated with 17β-estradiol (E2; 0.1 μM), G1 (0.1 μM), or 17β-estradiol + G15 (1 μM) for 4 days. The expressions of nuclear p-p65 (A) and the total and phosphorylated protein level of ERK1/2 (C) were tested by western blot analysis. After GPER1 silencing, the nuclear protein level of p-p65 (B) and the total and phosphorylated protein level of ERK1/2 (D) were detected by western blot analysis. (E) gADSCs were incubated with 17β-estradiol (0.1 μM) or G1 (0.1 μM) with or without MEK inhibitor U0126 (10 μM) for 4 days, and the nuclear protein level of p-p65 was detected by western blot analysis. GAPDH serves as the internal control. $^{*}P<0.05$, $^{**}P<0.01$ (vs control); $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ (vs E2); $^{\#}P<0.05$, $^{\#\#}P<0.01$ (vs shRNA); and $^{\P}P<0.05$ (vs G1).

intracellular lipid accumulation (Figure 6D), and decreased the mRNA levels of PPARγ, ACC, andFABP4 in gADSCs, compared to 17β-estradiol or G1 alone (Figure 6E). These data suggest that ERK1/2-NF-κB signaling pathway is responsible for 17β-estradiol-induced and GPER1-mediated cell proliferation and adipogenic differentiation of gADSCs.

Discussion

Adipose tissue formation and moderate fat deposition are important for the quality of livestock meats [4,5]. And the self-renewal and adipocyte differentiation of ADSCs are responsible for the formation and growth of adipose tissue [7]. Notably, estrogen targeting GPER1 is discovered to participate in the regulation of cell proliferation and multi-differentiation potentials, positively or negatively. Previous studies reported that estrogen stimulated cell viability and proliferation [8], and GPER1 activation presented a promotive effect on cell viability and proliferation [10]. Our data showed that estrogen binds to GPER1, which increases cell number and cell viability, producing a pro-proliferative effect. Interestingly, GPER1 was also reported to negatively regulate cell viability and cell proliferation [29], indicating that GPER1 plays critical roles in cell fate determination.

In the present study, we found that GPER1 mediated estrogen-induced adipogenic differentiation. Estrogen increased the formation of lipid vacuoles and the expressions of adipogenic genes, producing a pro-adipogenic effect [30,31]. GPER1 is responsible for the promotion of adipogenic differentiation [11,12]. In turn, GPER1 deletion inhibits adipogenic differentiation. Moreover, decreases in body weight, body fat depot mass, and adipocyte size were detected in *GPER1*-knockout female mice [12]. On the other hand, estrogen has a strong inhibitory effect on body weight, abdominal adipose tissue, total visceral fat pad mass, and adipocyte size after ovariectomy, with down-regulated expressions of adipogenic genes [32,33]. During osteo-adipogenic trans-differentiation, estrogen decreased the number and size of lipid droplets [34]. This difference may be related to culture conditions, tissues, gender, or species. Nevertheless, all these studies show that estrogen has moderate effects on cell proliferation and adipogenesis through GPER1.

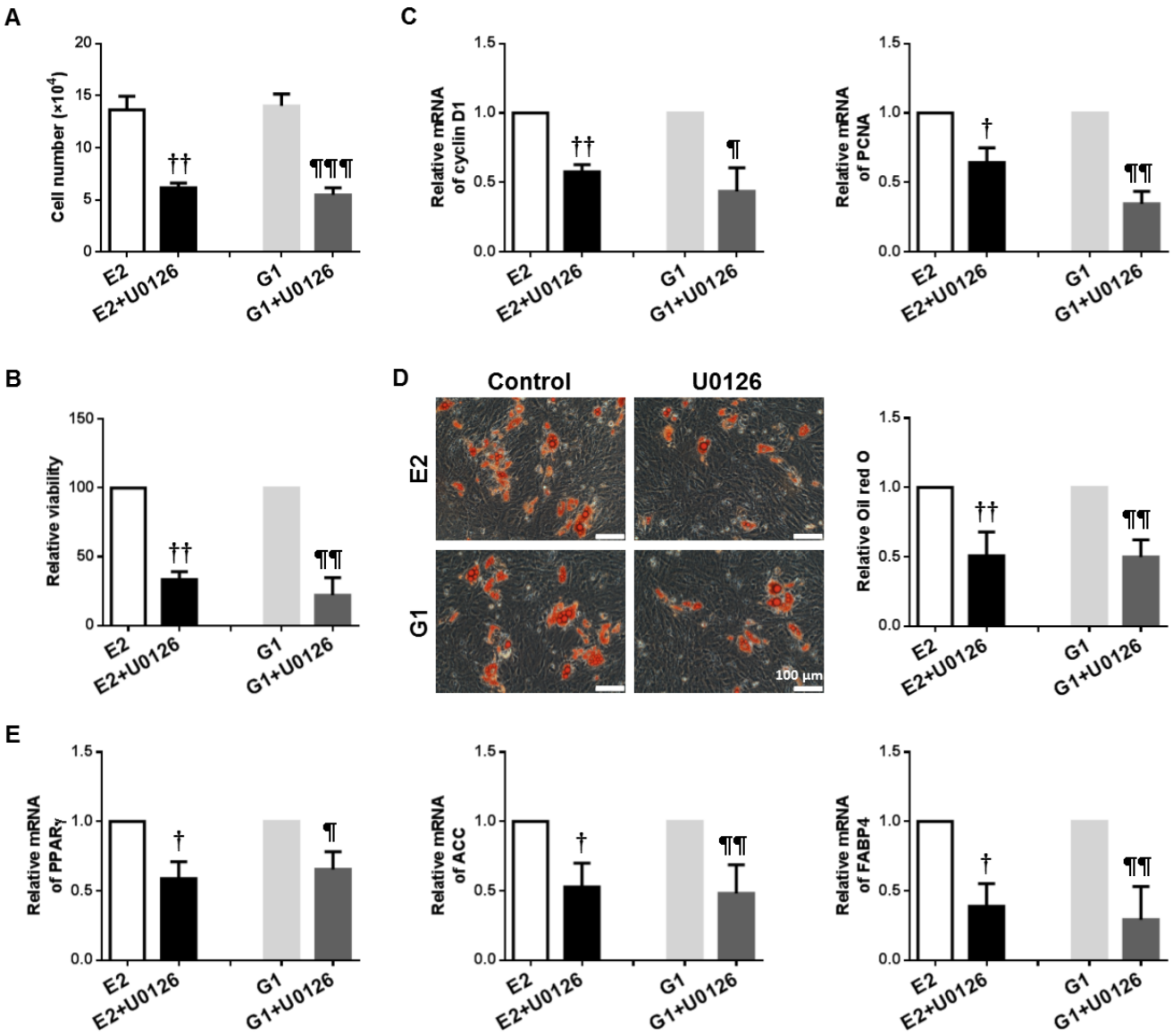

Figure 6. Effect of 17β-estradiol targeting GPER1 on cell proliferation and adipogenic differentiation is through ERK1/2-NF-κB signaling pathway
gADSCs were cultured with 17β-estradiol (E2; 0.1 μM) or G1 (0.1 μM) with or without U0126 (10 μM) for 4 days. Cell counting (A) and CCK-8 assay (B) were performed to detect cell proliferation. (C) The mRNA levels of cyclin D1 and PCNA of gADSCs were measured by RT-qPCR. gADSCs were cultured with 17β-estradiol (0.1 μM) or G1 (0.1 μM) with or without U0126 (10 μM) for 14 days. (D) Representative images of intracellular lipid accumulation of gADSCs detected by Oil red O staining. (E) The mRNA levels of PPARγ, ACC, and FABP4 of gADSCs were measured by RT-qPCR. GAPDH serves as the internal control. Scale bar = 100 μm. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ (vs E2); and$^{¶}P < 0.05$, $^{¶¶}P < 0.01$, $^{¶¶¶}P < 0.001$ (vs G1).

NF-κB signaling is involved in the regulation of cell proliferation and multi-differentiation ability [16,35]. Activation of NF-κB signaling enhances cell proliferation [36] and adipogenic differentiation [18,19]. In turn, inhibition of adipogenic differentiation and adipocytokine secretion is mediated by antagonizing NF-κB transactivation and p65 nuclear translocation [20]. Our data showed that GPER1 activation increased the level of nuclear p-p65, whereas GPER1 silencing decreased the nuclear protein level of p-p65, confirming that the proliferative and adipogenic effect of GPER1 is mediated through the NF-κB signaling.

In addition, NF-κB signaling is activated by estrogen, and its pro-proliferative and -adipogenic effects are diminished by GPER1 antagonist, implying the involvement of a cross-talk between estrogen receptor signaling and NF-κB signaling. ERK1/2 serves as a link between these two pathways [37,38]. Activation of ERK1/2 upregulates the phosphorylation of NF-κB p65 subunit and promotes its activity [25]. Here we revealed that GPER1 activation upregulates the phosphorylation of ERK1/2, whereas GPER1 silencing reverses the phosphorylation of ERK1/2. Of note, ERK1/2 inactivity inhibits the phosphorylation of p65, which contributes to the suppression of cell proliferation and adipogenic differentiation. Activation of ERK1/2 signaling was found to promote cell proliferation [39,40] and adipogenic differentiation [41–43]. However, another study showed that inhibition of proliferation and adipogenic differentiation was related to the activation of ERK1/2 and NF-κB signaling pathways [25], suggesting that the activity of NF-κB needs to be tightly regulated.

In summary, this study revealed the relationship among estrogen,

GPER1, proliferation, and adipogenic differentiation of goat adipose-derived stem cells and illuminated its regulatory mechanism (Figure 7), providing a strong theoretical basis for improving meat quality, flavor, and cold resistance of livestock.

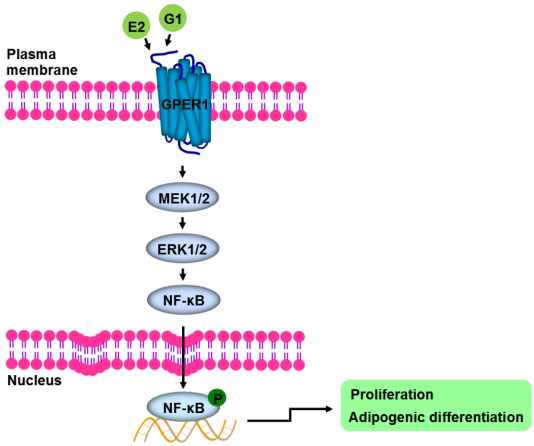

Figure 7. Working model for the effect of estrogen binding to GPER1 on cell proliferation and adipogenic differentiation of goat adipose-derived stem cells Estrogenic GPER1 promotes cell proliferation and adipogenic differentiation of goat adipose-derived stem cells through activating the ERK1/2-NF-κB signaling pathway.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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