

Activation of G Protein-coupled Receptor 30 Promotes Proliferation of Goat Mammary Epithelial Cells via MEK/Erk&PI3K/Akt Signaling Pathway

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Research

Keywords: Estrogen, G protein-coupled receptor 30, Goat mammary epithelial cells, Cell cycle, Cell proliferation

Posted Date: October 2nd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-84329/v1>

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Abstract

Background

Goat is an important dairy animal. During lactation, maintaining a high proliferative activity in goat mammary epithelial cells (GMECs) is significant to improve the yield and composition of goat milk. Estrogen is an essential hormone in epithelial cell proliferation and ductal morphogenesis of mammary gland. G protein-coupled receptor 30 (GPR30) is a novel membrane receptor of estrogen. However, the relationship between estrogen/GPR30 signaling and proliferation of goat mammary epithelial cells has not been reported. And the molecular mechanisms underlying the proliferative effect of estrogen via GPR30 on GMECs remain unclear.

Results

To investigate the effect of estrogen/GPR30 signaling on GMECs proliferation, goat mammary epithelial cells, which expressed cytokeratin 18 and β -casein, were isolated and identified, defining their mammary alveolar epithelium origination. Estrogen and GPR30 agonist G1 obviously promoted the proliferation of GMECs, in contrast, GPR30 antagonist G15 partly abolished estrogen-induced cell proliferation. Remarkably, the stimulatory effect of estrogen and G1 on GMECs growth was suppressed by GPR30 knockdown detected by cell counting assay, CCK-8 assay, and BrdU assay, suggesting that estrogen/GPR30 signaling was involved in GMECs proliferation. Additionally, G15 decreased cyclin D1, cyclin B1, CDK1, and p-CDK1 expression, resulting in cell cycle arrest in the G2/M phase via a down-regulated phosphorylation of Erk1/2 and Akt compared with estrogen alone. What's more, knock-down GPR30 led to an accumulation in the G2/M phase and inhibition of cyclin D1, cyclin B1, CDK1, and p-CDK1 expression via a down-regulation of phosphorylated Erk1/2 and Akt despite the presence of estrogen and G1. Furthermore, MEK inhibitor and PI3K inhibitor decreased the expression of cyclin D1, cyclin B1, CDK1, and p-CDK1, and repressed estrogen-induced and G1-driven promotion of cell growth. It indicated that estrogen/GPR30 signaling played an important role in GMECs proliferation by affecting cell cycle progression via MEK/Erk&PI3K/Akt signaling pathway.

Conclusion

This study may provide a new insight into the effect of estrogen/GPR30 signaling on the regulatory action of goat mammary gland development.

Background

Goat is an important dairy animal. The synthetic capacity of milk from the mammary gland relies largely on the quantity and efficiency of functional mammary epithelium [1]. During a pregnancy/lactation cycle, the mammary epithelial cells proliferate rapidly to form ducts and secretory alveoli through hormonal regulation, resulting in remodeling of the glandular tissue architecture into a milk-secretory organ [2, 3]. Estrogen (mainly 17β -estradiol) is a key regulator of mammary gland development, especially for its essential in the epithelial cell proliferation and ductal morphogenesis of the mammary gland [4, 5].

As a membrane estrogen receptor, G protein-coupled receptor 30 (GPR30) is widely expressed in reproductive tissues, such as mammary glands [5], ovary [6], bone [7], uterus [8], and others [9, 10]. After binding with estrogen, GPR30 mediates the rapid non-genomic actions of estrogen by stimulating the intracellular second messenger signals [11]. In normal human breast epithelial cells, estrogenic GPR30 signaling promoted cell proliferation via the MEK/Erk pathway, but GPR30 knockdown caused a reverse [12]. Similarly, this phenomenon was also found in endometrial carcinoma cells [13] and Sertoli TM4 cells [9]. On the other hand, estrogen-stimulated and GPR30-mediated promotion of uterine epithelial cell growth was regulated via PI3K/Akt signaling, but GPR30 antagonist G15 inhibited the effect induced by estrogen [14, 15]. Moreover, depletion of GPR30 abolished estrogen-induced PI3K/Akt signaling activation and led to the suppression of endometrial cancer cell proliferation [8]. These findings indicate that GPR30-mediated non-genomic signaling could play a significant role in cell proliferation.

Normal cell-cycle progression is a crucial event for every multicellular organism, due to it deciding body size and shape, tissue renewal and senescence, and is also important for reproduction [16], which is closely related to cell proliferation. In bovine mammary epithelial cells, an abundance of S and G2 phases [17, 18] and up-regulated expression of cyclin D1 mRNA [17] were accompanied in the promotion of cell proliferation. In addition, G1 promoted the proliferation of GPR30-positive human thyroid cancer cells and upregulated the expression of cyclin A and D1 [19]. Interestingly, G1 repressed the survival ability via cell arrest in the G2 phase of prostate cancer cells [20], which was verified in the mouse melanoma cells [21]. Parallely, the expression of cyclin A2, cyclin B1, cdc25c, and cdc2 was down-regulated after G1 treatment [20], suggesting that estrogen/GPR30 signaling played a vital role in the initiation and progression of cell proliferation. Although estrogen/GPR30 signaling has been demonstrated involving in cell proliferation regulation of breast cancer cells [12, 19], endometrial carcinoma cells [13], prostate cancer cells [20], ovarian cancer cells [6], and others [9, 10], the relationship between estrogen/GPR30 signaling and proliferation of goat mammary epithelial cells has not been reported. And the molecular mechanisms underlying the proliferative effect of estrogen via GPR30 on GMECs remain unclear.

In this study, we explored whether estrogen/GPR30 signaling affected the proliferation of GMECs. The results presented here may provide a new perspective on the effect of estrogen/GPR30 signaling on the regulation of goat mammary gland development.

Methods

Goat mammary epithelial cell isolation and culture

Goat mammary gland tissues were surgically isolated from a 2-year-old lactating Guanzhong dairy goat at 36 d of lactation as described previously [22], which were reared in Shaanxi Province (P. R. China). The mammary parenchyma was obtained under sterile conditions, placed in sterilized PBS with penicillin, streptomycin, and amphotericin B at 100 U/mL, 100 µg/mL, and 25 ng/mL (Sigma, St. Louis, MO, USA), and immediately transported to the laboratory. Tissue pieces were finely minced and put into a 24-well culture plates, one piece for each hole, and cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; BasalMedia, Shanghai, China) containing 10% KnockOut Serum Replacement (SR; Gibco,

Waltham, MA, USA) and 10 ng/mL epidermal growth factor (EGF; Sigma). The medium was replaced every 3 d, until cells had spread across the bottom of the plate. All cells were cultured in a 37°C incubator with 5% CO₂.

Cell lines and cell culture

The HEK-293T cells was purchased from the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China), and were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) prior to further treatment. All cells were cultured in a 37°C incubator with 5% CO₂.

Construction and identification of shGPR30 lentiviral vectors

To knockdown the expression of GPR30 gene (GenBank: XM_018039918), short hairpin RNAs against GPR30 (shGPR30) and negative control (shRNA) were designed by online software (<https://rnaidesigner.invitrogen.com/rnaiexpress/>), and synthesized by Sangon Biotech (Shanghai) Co., Ltd. All sequences are as described in Table 1. The method of constructing short hairpin RNA lentivirus vectors was performed as previous description [23]. After detecting by ampicillin (Solarbio, Beijing, China), drug-resistant colonies were delivered to be analyzed by Sangon Biotech (Shanghai) Co., Ltd. For knockdown of the target GPR30, synthesized vectors and packaging vectors were transfected into HEK-293T cells using TurboFect Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA) to form lentivirus. GMECs were incubated with lentivirus of shGPR30 and shRNA for 18 h before removing. After transfection, cells were allowed to grow, and then gained for protein extraction. Western blot analysis was used to evaluate the knock-down efficiency of GPR30.

Table 1 Hairpin short interfering RNA (shRNA) inserts.	
shRNA	Sequence (loop in bold letters) (5'-3')
shGPR30-1	GATCCGCTCATCCTGGTGGTGAACAT TTCAAGAGA AATGTTCCACCACCAGGATGAGCTTTTTTG
	AATTCAAAAAAGCTCATCCTGGTGGTGAACAT TCTCTTGAA AATGTTCCACCACCAGGATGAGCG
shGPR30-2	GATCCGCTCATCGAGGTGTTCAACCT TTCAAGAGA AAGGTTGAACACCTCGATGAGCTTTTTTG
	AATTCAAAAAAGCTCATCGAGGTGTTCAACCT TCTCTTGAA AAGGTTGAACACCTCGATGAGCG
shRNA	GATCCTTCTCCGAACGTGTCACGT TTCAAGAGA ACGTGACACGTTCCGGAGAATTTTTTG
	AATTCAAAAAATTCTCCGAACGTGTCACGT TCTCTTGAA ACGTGACACGTTCCGGAGAAG
Abbreviations: shGPR30, short hairpin G protein-coupled receptor 30; shRNA, short hairpin negative	

Immunofluorescence

The GMECs were seeded in 24-well culture plates (1×10^4 cells/well) and allowed to grow for 6 d. After this, cells were washed 3 times with PBS for 5 min each time, and fixed for 30 min using 4% paraformaldehyde (Solarbio) at room temperature. All fixed cells were washed 3 times with PBS and incubated with a blocking buffer (1% FBS in PBS) for 2 h at room temperature. Subsequently, cells were incubated with primary antibody against cytokeratin 18 (CK18; ab668, abcam, Cambridge, MA, USA) and GPR30 (ab39742, abcam) overnight at 4°C. The nuclei were stained with Hoechst 33342 (Sigma) in dark for 5 min at room temperature. Images were captured using a fluorescence microscope (Olympus, Tokyo, Japan).

Cell cycle analysis

Flow cytometry analysis was used to measure cell cycle distribution. GMECs were cultured in 6-well culture plates at a density of 1.5×10^5 cells/well and allowed to grow for 4 d. Then, cells were cultured with estrogen (Sigma), G1 (Cayman Chemical Co, Ann Arbor, MI, USA), and estrogen + G15 (Cayman Chemical Co) for 48 h. Subsequently, cells were harvested with 0.25% (w/v) trypsin, fixed in 70% ice-cold ethanol overnight at 4°C, incubated with RNase A (20 µg/mL; CWbio, Jiangsu, China) for 30 min, and stained with 50 µg/mL of propidium iodide (Sigma) in dark for 20 min at room temperature. The cell cycle data were assayed using the flow cytometer (BD Biosciences, San Jose, CA), and analyzed by the Modfit software (Tree Star, Inc., Ashland, OR).

Cell counting assay

Cell proliferation was tested by cell counting. Briefly, 1×10^4 cells/well were seeded in 24-well plates for 24 h, and then cultured with estrogen (Sigma), G1 (Cayman Chemical Co), and estrogen + G15 (Cayman Chemical Co) for 5 d. Next, cells were harvested with 0.25% (w/v) trypsin and resuspended in fresh medium. Cell numbers were determined using a hemocytometer, and proliferation was expressed as population doubling time (T_D). $T_D = t \times \log 2 / (\log N_t - \log N_0)$, where t is the culturing time; N_0 is the initial cell numbers; N_t is the cell numbers after culturing time.

Cell viability assay

Cell viability was evaluated using the Cell Counting Kit-8 kit (BOSTER, Wuhan, China). In detail, 6×10^3 cells/well were seeded in 96-well plates for 24 h and then cultured with estrogen (Sigma), G1 (Cayman Chemical Co), and estrogen + G15 (Cayman Chemical Co) for 5 d. Next, CCK-8 solution (10 µL/well) was added and incubated for 2 h, and the absorbance value was measured at 450 nm.

Bromodeoxyuridine labeling and immunofluorescence assay

Cell proliferation was detected using the BrdU assay. Briefly, GMECs were seeded in 24-well culture plates at a density of 1×10^4 cells/well and allowed to grow for 4 d. Next, cells were cultured with estrogen (Sigma), G1 (Cayman Chemical Co), and estrogen + G15 (Cayman Chemical Co) for 48 h and were then cocultured with bromodeoxyuridine (BrdU; 10 µM; Sigma,) for 2 h in a 37°C incubator with 5% CO₂. Next, cells were fixed in 4% paraformaldehyde (Solarbio) for 30 min, permeabilized with 0.2% TritonX-100 (Sigma) for 15 min, treated with 1 M HCl for 15 min, incubated with an anti-BrdU antibody (SAB4700630, Sigma) overnight at

4°C. The nuclei were counterstained by Hoechst 33342 (Sigma) in dark for 5 min at room temperature. BrdU positive cells were observed and counted through an inverted fluorescence microscope (Olympus)

Western blot

Western blot was implemented to test the phosphorylation and total levels of Erk1/2 and Akt as well as the expression of cell cycle checkpoint regulators including cyclin D1, cyclin B1, CDK1, and p-CDK1 in GMECs. Total cellular and cytoplasmic proteins were lysed with High-efficiency RIPA buffer (Solarbio) including protease and phosphatase inhibitors and applied to SDS-PAGE with equal amounts of protein. After separation, protein was transferred to a polyvinylidene difluoride membrane (PVDF; Millipore, MA, USA). Following transfer, the membranes were blocked with 3% BSA (Solarbio) diluted in Tris buffer saline containing 0.1% tween (TBST) for 2 h at room temperature. Then, membranes were incubated overnight at 4°C with the specific primary antibodies against GPR30 (ab39742, abcom), β -casein (sc-166530, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Erk1/2 (4695, Cell Signaling Technology, Beverly, MA, USA), p-Erk1/2 (4370, Cell Signaling Technology), Akt (BM4390, BOSTER), p-Akt (BM4390, BOSTER), cyclin D1 (D160236, Sangon Biotech), cyclin B1 (4135, Cell Signaling Technology), CDK1 (77055, Cell Signaling Technology), p-CDK1 (4539, Cell Signaling Technology), and β -actin (4970, Cell Signaling Technology). Membranes were washed 3 times and incubated with the corresponding horseradish peroxidase conjugated secondary antibodies overnight at 4°C. Finally, the protein bands were detected with a chemiluminescence kit (Biotanon, Shanghai, China).

Statistical analysis

The data were presented as average values \pm standard error of the means from 3 independent experiments of cell counting assay, cell viability assay, BrdU assay, and Western blot analysis. Statistical analyses were performed with SPSS (version 20.0; SPSS Inc., Chicago, IL, USA), which were checked using Tukey's test. Probability (p) value < 0.05 was considered statistically significant.

Results

Estrogen promotes GMECs proliferation via GPR30

The isolated cells expressed cytokeratin 18 and β -casein tested by immunofluorescence and Western blot respectively (Fig. 1a and b), defining their mammary alveolar epithelium origination. Furthermore, the expression of GPR30 was identified by immunofluorescence and Western blot. As the results showed, GMECs continuously express GPR30 during the experiments (P3-P9) (Fig. 1c and d). Since estrogen is the ligand of GPR30, GMECs were treated with different concentrations of estrogen. After treatment with 0.1 μ M and 1 μ M estrogen, population doubling time was obviously decreased compared with control (0 μ M) (Fig. 1e). Meanwhile, the CCK-8 assay and BrdU assay showed the similar results that 0.1 μ M and 1 μ M estrogen evidently promoted the survival and mitosis of GMECs compared to control (0 μ M) (Fig. 1f, g, and h). These results suggested that estrogen promoted the proliferation of GMECs. As the specific agonist of GPR30, G1 was used to treat GMECs. Similar to estrogen, G1 group presented a decrease of population doubling time (Fig. 1e) as well as an improvement of cell survival (Fig. 1f) and mitosis of GMECs (Fig. 1g and h) compared

with control group (0 μM), and there was no significance between 0.1 μM and 1 μM estrogen group. G15, the specific antagonist of GPR30, was added to determine whether the effect of estrogen could be inhibited. As predictably, in the presence of G15, estrogen-induced GMECs proliferation was obviously repressed compared with estrogen alone (Fig. 1e, f, g, and h). These results indicated that estrogen promoted the proliferation of GMECs via GPR30.

Fig. 1 Estrogen promotes GMECs proliferation via GPR30. GMECs expressed (a) cytokeratin 18, (b) β -casein, and (c, d) GPR30 identified by immunofluorescence and Western blot. The nuclei were stained with Hoechst 33342. The effect of estrogen (0.01 μM , 0.1 μM , and 1 μM), G1 (0.1 μM), and estrogen (0.1 μM) + G15 (1 μM) on cell growth was detected by (e) cell counting assay, (f) CCK-8 assay, and (g, h) BrdU assay. β -actin serves as a loading control. Data are shown as mean \pm SEM, $n = 3$. Statistical significance was determined using Tukey's test. **, $p < 0.01$; ***, $p < 0.001$; ns, not significant. GMF, goat mammary fibroblast (negative control). Scale bar = 50 μm .

RNAi-mediated silence of GPR30 suppresses estrogen-induced GMECs proliferation

To further explore the role of GPR30 activation induced by estrogen in GMECs proliferation, GPR30 expression was silenced by RNAi. As showed in Fig. 2a, shGPR30-2 treatment had the greatest down-regulated effect on GPR30 expression (Fig. 2a). Therefore, shGPR30-2 was chosen to use in followed experiments. When GPR30 was knocked down, in contrast to negative control, population doubling time was markedly increased in spite of the presence of estrogen and G1 (Fig. 2b). Similarly, cell viability was visibly inhibited as measured by the CCK-8 assay (Fig. 2c), and the proportion of BrdU-positive cells was also clearly downregulated (Fig. 2d and e) after GPR30 knockdown. These data revealed that RNAi-mediated silence of GPR30 suppressed estrogen-induced and G1-driven promotion of cell proliferation, suggesting a proliferative role of GPR30 activation in GMECs.

Fig. 2 GPR30 Knockdown suppresses the estrogen-induced and G1-driven promotion of cell proliferation. (a) Western blot was used to determine the protein levels of GPR30 in GMECs. Cell growth was assessed by (b) cell counting assay, (c) CCK-8 assay, and (d, e) BrdU assay after GPR30 knockdown despite the presence of estrogen (0.1 μM) and G1 (0.1 μM). The nuclei were stained with Hoechst 33342. β -actin serves as a loading control. Data are shown as mean \pm SEM, $n = 3$. Statistical significance was determined using Tukey's test. *, $p < 0.05$. Scale bar = 50 μm .

GPR30 activation and inactivation altered cell cycle distribution

Because the proliferation of GMECs was obviously promoted in response to estrogen exposure, we then explored whether the cell cycle was also altered following estrogen treatment. Thus, flow cytometry was performed to analyze the cell cycle distribution. Here, in contrast to control, estrogen treatment upregulated the S phase proportion ($26.01 \pm 3.47\%$ vs. $16.97 \pm 3.97\%$), but did not change the G2/M phase proportion ($9.41 \pm 0.64\%$ vs. $9.59 \pm 0.30\%$). However, G15 treatment increased the proportion of G2/M phase contrasted with estrogen alone ($16.87 \pm 0.61\%$ vs. $9.41 \pm 0.64\%$). On the other hand, G1 treatment increased the ratio of S and G2/M phase ($30.22 \pm 2.65\%$ vs. $16.97 \pm 3.97\%$; $11.64 \pm 0.51\%$ vs. $9.59 \pm 0.30\%$) contrasted to control (Fig. 3a). In particular, GPR30 knockdown altered cell cycle distribution, accumulating in the G2/M phase

compared to negative control in spite of the presence of estrogen and G1 ($10.93 \pm 0.62\%$ vs. $8.12 \pm 0.15\%$; $9.87 \pm 0.57\%$ vs. $7.75 \pm 0.43\%$) (Fig. 3b).

To further elucidate the underlying mechanisms, the expression of cell cycle checkpoint regulators was examined. Estrogen treatment improved the level of cyclin D1, cyclin B1, CDK1, and p-CDK1 contrasted with control. Similar to estrogen, G1 treatment presented a positive effect on the expression of cyclin D1, cyclin B1, CDK1, and p-CDK1. Nevertheless, G15 treatment cause a reverse, suppressing estrogen-induced expression of cyclin D1, cyclin B1, CDK1, and p-CDK1 (Fig. 3c). When GPR30 was knocked down, the levels of cyclin D1, cyclin B1, CDK1, and p-CDK1 were downregulated than negative control (Fig. 3d). These results demonstrated that GPR30 activation driven by estrogen upregulated S phase proportion, and GPR30 inactivation induced cell cycle arrest in the G2/M phase.

Fig. 3 Inactivation of GPR30 induces cell cycle arrest in the G2/M phase. (a, b) Flow cytometry was used to analyze the distribution of cell cycle after treatment with estrogen (0.1 μ M), G1 (0.1 μ M), estrogen (0.1 μ M) + G15 (1 μ M), and GPR30 knockdown. (c, d) The expression of cell cycle checkpoint regulators of GMECs was detected by immunoblot analysis after treatment with estrogen (0.1 μ M), G1 (0.1 μ M), estrogen (0.1 μ M) + G15 (1 μ M), and GPR30 knockdown. The level of protein was shown by densitometric analysis. β -actin serves as a loading control. Data are shown as mean \pm SEM, $n = 3$. Statistical significance was determined using Tukey's test. *, $p < 0.05$; **, $p < 0.01$; ns, not significant.

MEK/Erk and PI3K/Akt signaling pathway are involved in GMECs proliferation

For in-depth investigation of the molecular regulation mechanisms of GPR30 activation induced by estrogen involved in cell proliferation, small molecular inhibitors were applied. Western blot results revealed that estrogen treatment obviously promoted the phosphorylation of Erk1/2 and Akt compared with control. After G1 treatment, an abundance of phosphorylated Erk1/2 and Akt were detected by Western blot. Conversely, G15 treatment visibly inhibited the estrogen-induced phosphorylation of Erk1/2 and Akt (Fig. 4a). When GPR30 was silenced, in comparison to negative control, a decrease in p-Erk1/2 and p-Akt was determined (Fig. 4b). Additionally, with the treatment of MEK inhibitor U0126, the rise in protein levels of Cyclin D1, Cyclin B1, CDK1, and p-CDK1 induced by estrogen and G1 was abolished. Similarly, LY294002, the PI3K inhibitor, markedly decreased the expression of cyclin D1, cyclin B1, CDK1, and p-CDK1 driven by estrogen and G1 (Fig. 4c).

To investigate the relationship between MEK/Erk&PI3K/Akt signaling pathway and GMECs proliferation further, we detected the growth of GMECs after treatment with U0126 and LY294002. In the presence of estrogen and G1, U0126 treatment upregulated the population doubling time (Fig. 5a). In addition, the CCK-8 assay discovered that U0126 treatment obviously impaired the viability of GMECs elicited by estrogen and G1, respectively (Fig. 5b). BrdU assay further confirmed the result that U0126 treatment inhibited the estrogen-induced and G1-driven promotion of mitosis (Fig. 5c and d). In parallel, LY294002 treatment evidently suppressed the growth of GMECs originated from estrogen and G1 (Fig. 5). These data suggested that GPR30 activation induced by estrogen promoted GMECs proliferation via the MEK/Erk and PI3K/Akt signaling pathway.

Fig. 4 MEK/Erk&PI3K/Akt signaling pathway is involved in the regulation of GMECs proliferation. (a, b) Western blot was performed to detect the phosphorylation and total protein levels of Erk1/2 and Akt of GMECs cultured with estrogen (0.1 μ M) and G1 (0.1 μ M) for 1 h with or without G15 (0.1 μ M) pretreatment for 3 h. (c) The protein expression of cell cycle checkpoint regulators was detected by Western blot after treatment with MEK inhibitor U0126 (10 μ M) and PI3K inhibitor LY294002 (10 μ M) for 48 h in the presence of estrogen (0.1 μ M) and G1 (0.1 μ M). The level of protein was shown by densitometric analysis. β -actin serves as a loading control. Data are shown as mean \pm SEM, $n = 3$. Statistical significance was determined using Tukey's test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 5 Estrogen-induced and GPR30-mediated cell proliferation via MEK/Erk&PI3K/Akt signaling pathway. (a) Cell counting assay, (b) CCK-8 assay, and (c, d) BrdU assay were implemented to evaluate GMECs proliferation after treatment with MEK inhibitor U0126 (10 μ M) and PI3K inhibitor LY294002 (10 μ M) in the presence of estrogen (0.1 μ M) and G1 (0.1 μ M). Data are shown as mean \pm SEM, $n = 3$. Statistical significance was determined using Tukey's test. *, $p < 0.05$; **, $p < 0.01$. Scale bar = 50 μ m.

Discussion

In this study, we discovered that estrogen promoted GPR30-mediated proliferation of mammary epithelial cells by altering cell cycle distribution via MEK/Erk&PI3K/Akt signaling pathway, providing a new perspective on the effect of estrogen/GPR30 signaling on the regulatory action of goat mammary gland development.

Estrogen is a key regulator of mammary gland development, especially for its essential role in the proliferation of epithelial cells and ductal morphogenesis of mammary gland [4, 5]. As a novel estrogen membrane receptor, GPR30 plays an important role in the regulation of estrogen mediated signaling pathways [11]. In mammary epithelial cells, GPR30 agonist G1 promoted cell growth, nevertheless, GPR30 antagonist G36 partly abolished G1-mediated cell proliferation [12]. In addition, estrogen significantly enhanced the proliferation of cervical adenocarcinoma cells [24] and endometrial carcinoma cells [8, 25] by stimulating GPR30. Intriguingly, GPR30 activation by G1 inhibited the growth of mouse-derived neural stem/progenitor cells, what's more, GPR30 siRNA reversed the inhibitory effect of G1 on cell proliferation [26]. Here, this study found that estrogen/GPR30 signaling was involved in GMECs proliferation. Activation of GPR30 induced by estrogen could promote GMECs proliferation, as predictably, G15 suppressed estrogen-stimulated and GPR30-mediated GMECs proliferation (Fig. 1). Importantly, RNAi-mediated silence of GPR30 inhibited estrogen-induced and G1-driven promotion of cell proliferation (Fig. 2), suggesting a proliferative role of GPR30 activation in GMECs.

Generally, cell proliferation is closely associated with cell cycle [27, 28]. In breast cancer cells, estrogen promoted cell progression by significant accumulation in the S phase and a high expression of cyclin D1 [29]. In addition, a high proportion in the S and G2/M phases was also observed in the promotion of mouse osteoblast cell growth [30, 31], and up-regulated expression of cyclin B, cyclin D, and cyclin E at the transcription and translation levels were accompanied in the positive proliferation of porcine granulosa cells [32]. Particularly, GPR30 activation by G1 inhibited prostate cancer cell growth and downregulated the expression of cyclin A2, cyclin B1, cdc25c, and cdc2, resulting in the cell cycle arrest in the G2 phase [20]. In

this study, GPR30 stimulation caused obvious differences in the cell cycle distribution and checkpoint regulator expression. GPR30 activation driven by estrogen upregulated S phase proportion, and GPR30 inactivation induced cell cycle arrest in the G2/M phase via down-regulation of cyclin D1, cyclin B1, CDK1, and p-CDK1 expression (Fig. 3).

After stimulation, GPR30 regulates a series of cellular downstream effectors, including EGFR [33, 34], adenylyl cyclase [35], cAMP [35], Erk1/2 [36, 37], PI3K/Akt [38, 39], and others [40, 41]. Estrogenic induction of cell proliferation was mediated by GPR30 via Erk/Akt signaling in cervical adenocarcinomas cells [24], on the other hand, an activation in PI3K/Akt and MAPK signaling pathways were also in response to the promotion of bovine mammary epithelial cell survival [39, 42]. In addition, in GPR30-positive breast cancer cells, GPR30 activation has been observed to induce rapid activation of Erk1/2 [37], in turn, G15 and knock-down GPR30 caused a reverse [36]. In GMECs, GPR30 knockdown obviously inhibited the estrogen-induced and G1-driven phosphorylation of Erk1/2 and Akt (Fig. 4). Furthermore, MEK inhibitor and PI3K inhibitor visibly abolished estrogen-induced and G1-driven promotion of cell growth (Fig. 5), suggesting that MEK/Erk&PI3K/Akt signaling pathway was required for estrogen-induced and GPR30-mediated proliferation of GMECs.

Conclusions

In summary, this work determines that estrogen acting on GPR30 contributes to the proliferation of mammary epithelial cells by affecting cell cycle progression via MEK/Erk&PI3K/Akt signaling pathway, which is helpful to improve milk yield. This work may provide a new insight into the effect of estrogen/GPR30 signaling on the regulation of goat mammary gland development.

Abbreviations

GMECs: goat mammary epithelial cells; GPR30: G protein-coupled receptor 30; E2: 17 β -estradiol; CK18: cytokeratin 18; GMF: goat mammary fibroblast; P3, P6, P9: the 3, 6, and 9 generation of GMECs; shGPR30: short hairpin G protein-coupled receptor 30; shRNA: short hairpin negative; cyclin A2: cell cycle protein A2; cyclin B1: cell cycle protein B1; cyclin D1: cell cycle protein D1; cyclin E1: cell cycle protein E1; cdc25c: cell division cyclin 25C; CDK1(cdc2): cyclin-dependent kinase 1; p-CDK1: phospho-cyclin-dependent kinase 1; PI3K/Akt: phosphatidylinositol 3-kinase/proteinkinase B; MAPK: mitogen-activated protein kinase; MEK/Erk: MAK kinase kinase/extracellular signal related kinases.

Declarations

Ethics approval and consent to participate

In the present study, all procedures were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee of College of Veterinary Medicine, Northwest A&F University.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Natural Science Foundation of China (31772818) and Key Research and Development Program of Shaanxi (Program No. 2018NY-023).

Authors' contributions

Conceived and designed the experiments: YZ, BHM, QW and XEZ. Performed the experiments: YZ, HKL, and MZF. Analyzed the data: YZ and YYM. Writing-original draft: YZ and HKL. Writing-review and editing: YZ. All read and approved the final manuscript.

Acknowledgments

We are grateful to Professor Yulin Chen (College of Animal Science and Technology, Northwest A&F University, China) for the vectors of synthesized lentivirus and their technical assistance.

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Figures

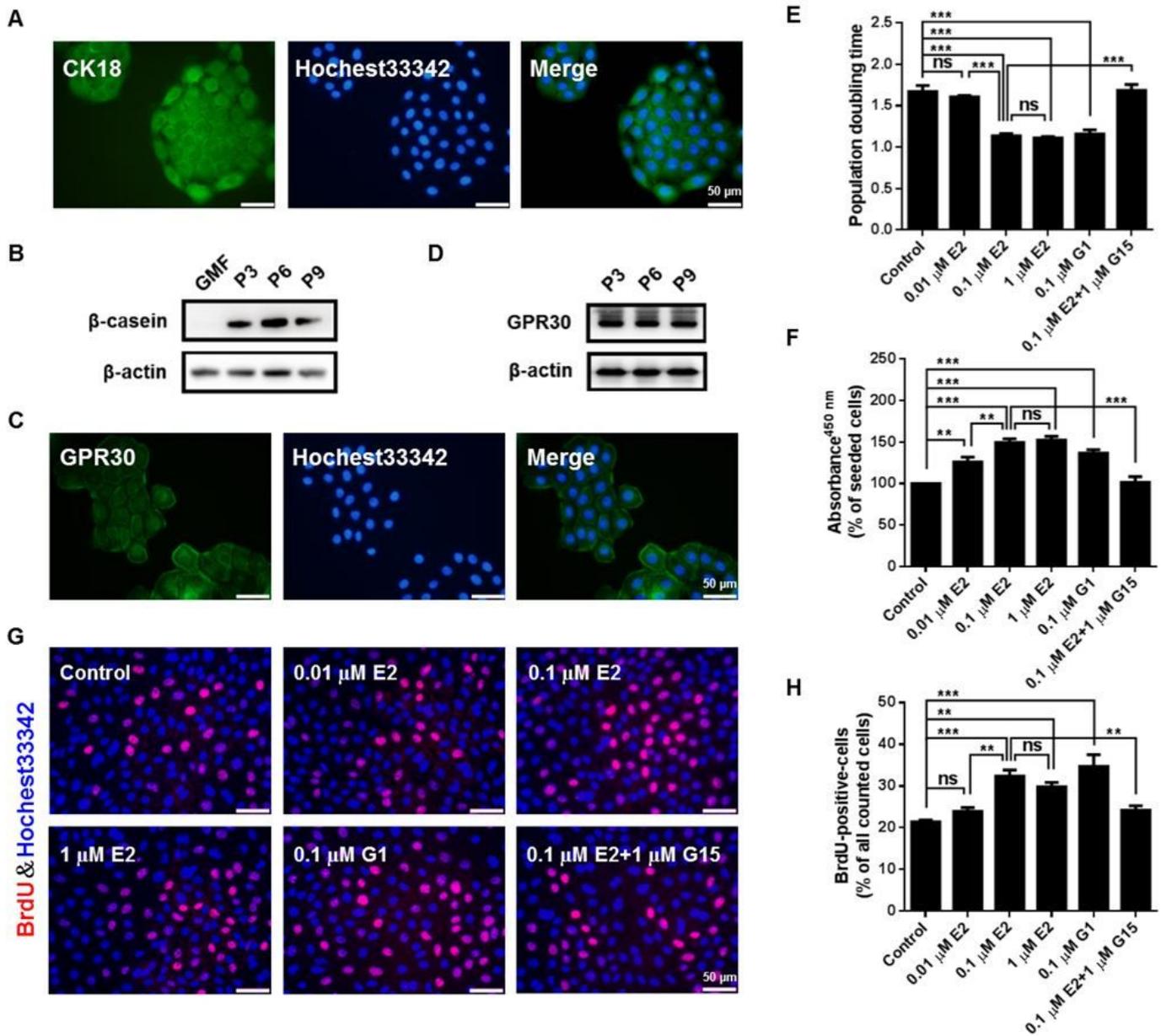


Figure 1

Estrogen promotes GMECs proliferation via GPR30. GMECs expressed (a) cytokeratin 18, (b) β -casein, and (c, d) GPR30 identified by immunofluorescence and Western blot. The nuclei were stained with Hoechst 33342. The effect of estrogen (0.01 μ M, 0.1 μ M, and 1 μ M), G1 (0.1 μ M), and estrogen (0.1 μ M) + G15 (1 μ M) on cell growth was detected by (e) cell counting assay, (f) CCK-8 assay, and (g, h) BrdU assay. β -actin serves as a loading control. Data are shown as mean \pm SEM, n = 3. Statistical significance was determined using Tukey's test. **, p < 0.01; ***, p < 0.001; ns, not significant. GMF, goat mammary fibroblast (negative control). Scale bar = 50 μ m.

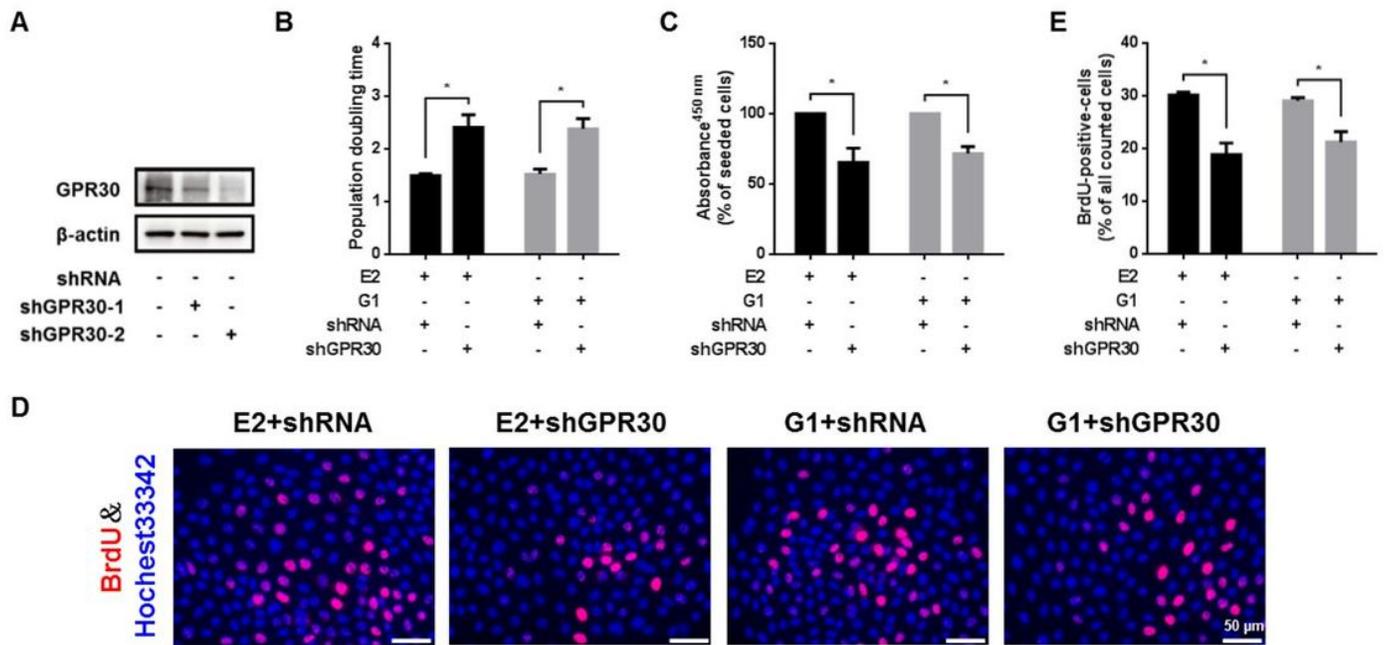


Figure 2

GPR30 Knockdown suppresses the estrogen-induced and G1-driven promotion of cell proliferation. (a) Western blot was used to determine the protein levels of GPR30 in GMECs. Cell growth was assessed by (b) cell counting assay, (c) CCK-8 assay, and (d, e) BrdU assay after GPR30 knockdown despite the presence of estrogen (0.1 μ M) and G1 (0.1 μ M). The nuclei were stained with Hoechst 33342. β -actin serves as a loading control. Data are shown as mean \pm SEM, $n = 3$. Statistical significance was determined using Tukey's test. *, $p < 0.05$. Scale bar = 50 μ m.

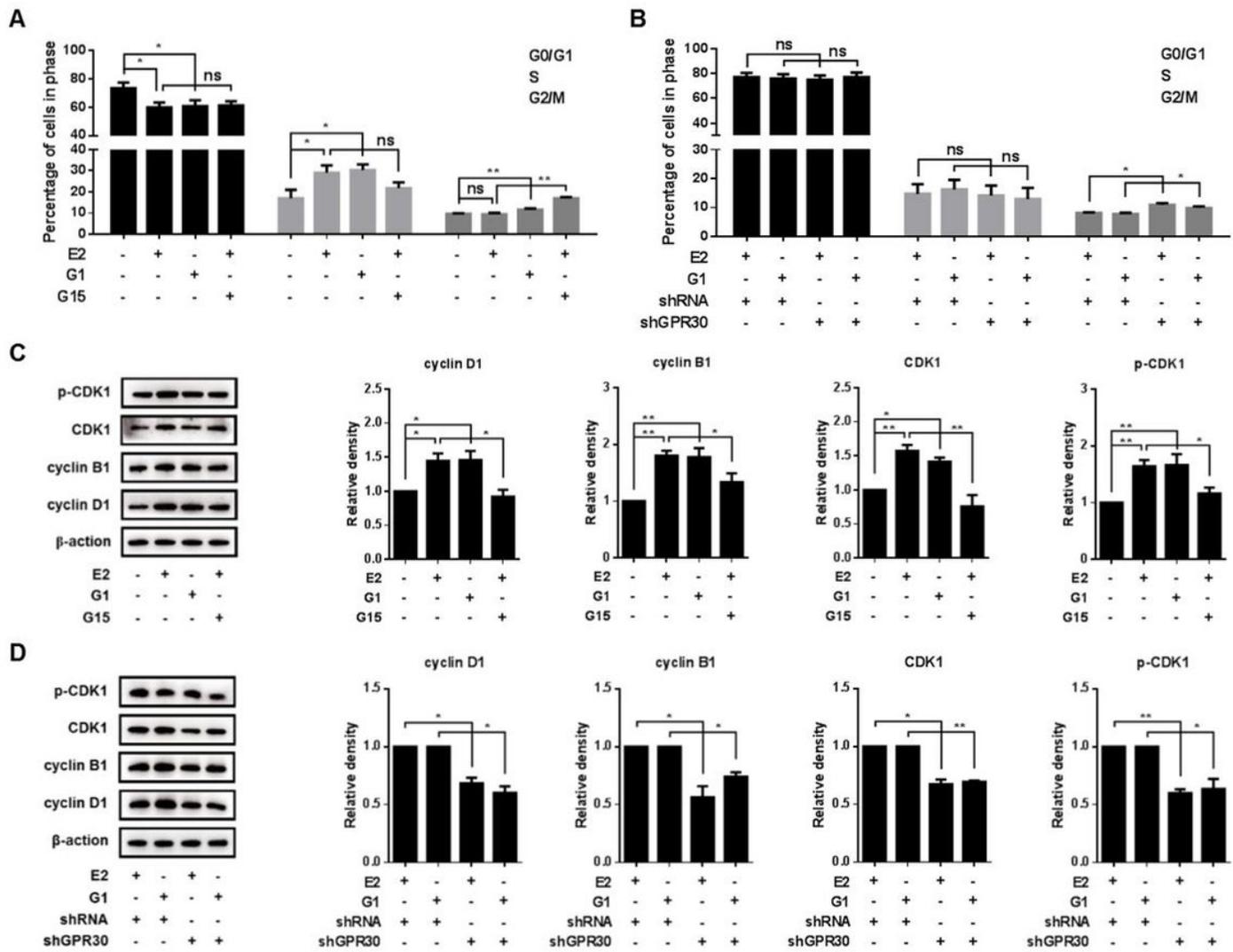


Figure 3

Inactivation of GPR30 induces cell cycle arrest in the G2/M phase. (a, b) Flow cytometry was used to analyze the distribution of cell cycle after treatment with estrogen (0.1 μ M), G1 (0.1 μ M), estrogen (0.1 μ M) + G15 (1 μ M), and GPR30 knockdown. (c, d) The expression of cell cycle checkpoint regulators of GMECs was detected by immunoblot analysis after treatment with estrogen (0.1 μ M), G1 (0.1 μ M), estrogen (0.1 μ M) + G15 (1 μ M), and GPR30 knockdown. The level of protein was shown by densitometric analysis. β -actin serves as a loading control. Data are shown as mean \pm SEM, $n = 3$. Statistical significance was determined using Tukey's test. *, $p < 0.05$; **, $p < 0.01$; ns, not significant.

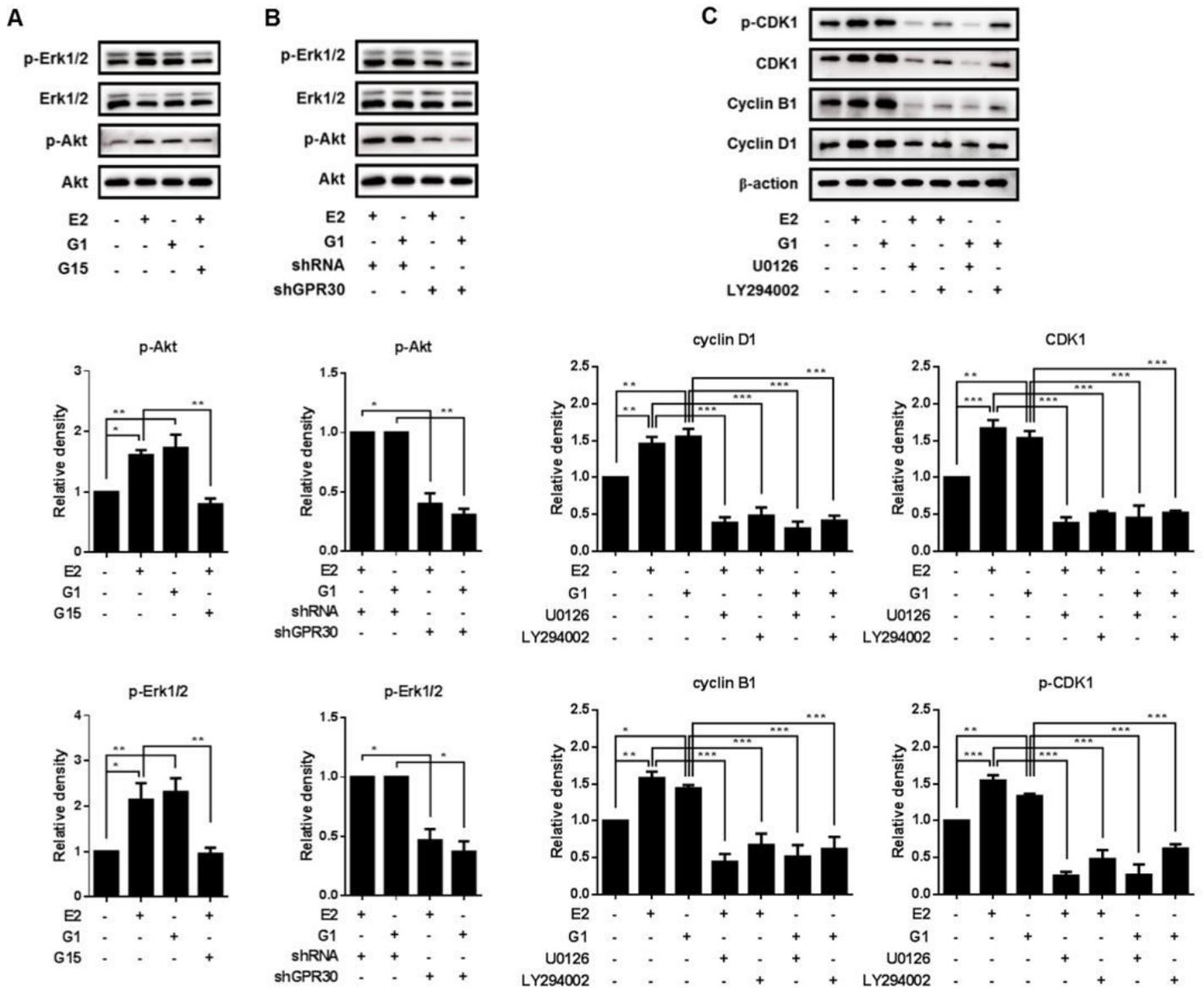


Figure 4

MEK/Erk&PI3K/Akt signaling pathway is involved in the regulation of GMECs proliferation. (a, b) Western blot was performed to detect the phosphorylation and total protein levels of Erk1/2 and Akt of GMECs cultured with estrogen (0.1 μ M) and G1 (0.1 μ M) for 1 h with or without G15 (0.1 μ M) pretreatment for 3 h. (c) The protein expression of cell cycle checkpoint regulators was detected by Western blot after treatment with MEK inhibitor U0126 (10 μ M) and PI3K inhibitor LY294002 (10 μ M) for 48 h in the presence of estrogen (0.1 μ M) and G1 (0.1 μ M). The level of protein was shown by densitometric analysis. β -actin serves as a loading control. Data are shown as mean \pm SEM, n = 3. Statistical significance was determined using Tukey's test. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

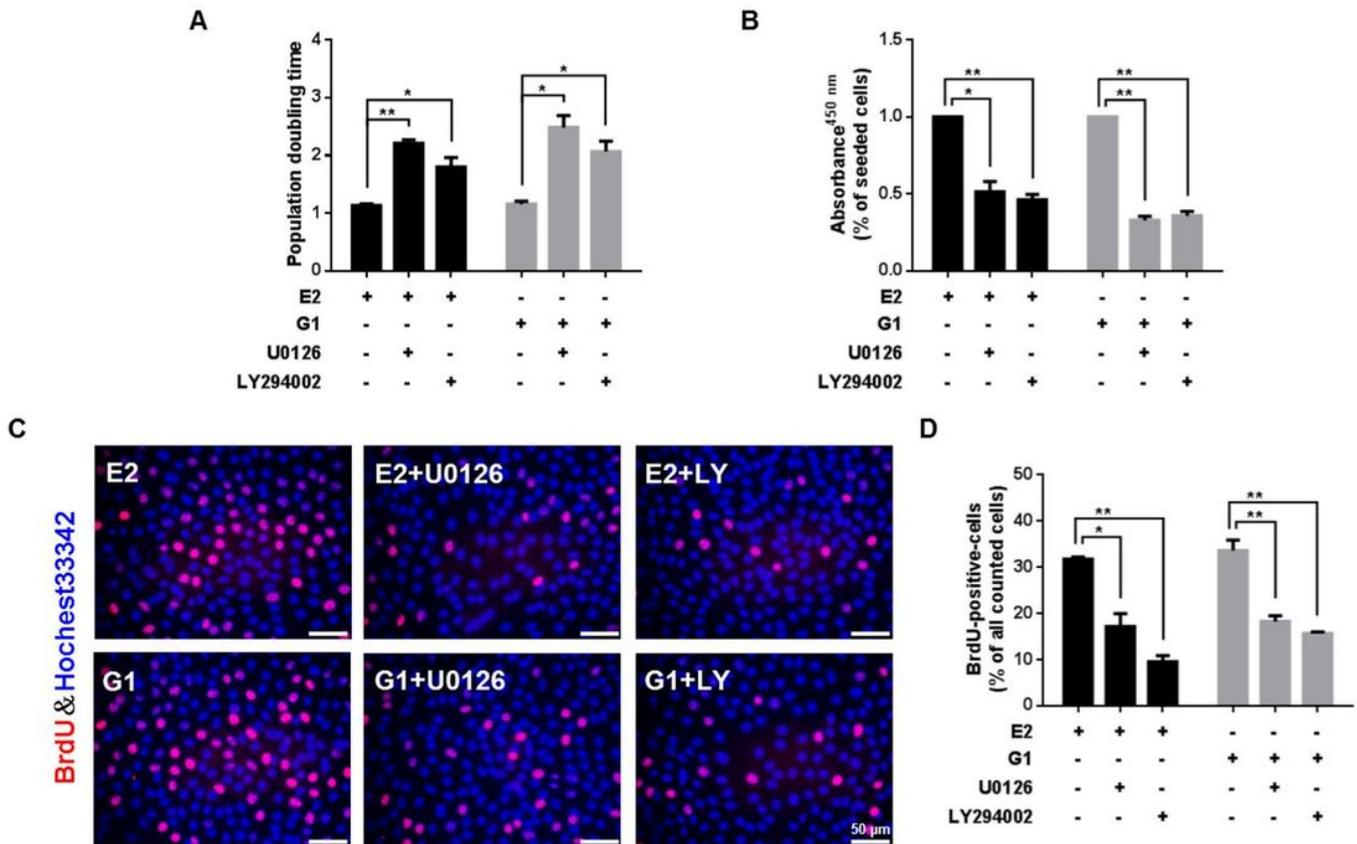


Figure 5

Estrogen-induced and GPR30-mediated cell proliferation via MEK/Erk&PI3K/Akt signaling pathway. (a) Cell counting assay, (b) CCK-8 assay, and (c, d) BrdU assay were implemented to evaluate GMECs proliferation after treatment with MEK inhibitor U0126 (10 μ M) and PI3K inhibitor LY294002 (10 μ M) in the presence of estrogen (0.1 μ M) and G1 (0.1 μ M). Data are shown as mean \pm SEM, n = 3. Statistical significance was determined using Tukey's test. *, p < 0.05; **, p < 0.01. Scale bar = 50 μ m.