

The anti-adipogenic effect of *PGRN* on porcine preadipocytes involves ERK_{1,2} mediated PPAR γ phosphorylation

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Abstract Recent researches indicate that PGRN is closely related to diabetes and is regarded as a novel adipokine associated with obesity development, affecting adipocyte biology. In the present study, we investigated the effects and mechanisms of PGRN on porcine preadipocytes differentiation. Porcine preadipocytes were induced to differentiation with the addition of lentivirus-expressed *PGRN* shRNA at the early or late stage of induction period, and in the presence or absence of recombinant PGRN protein. The effects of PGRN on adipogenic genes expression and ERK activation were investigated. At the early stage of induction, knockdown of *PGRN* promoted differentiation, evidenced by enhanced lipid accumulation, upregulation of adipocyte markers, as well as master adipogenic transcription factors, PPAR γ and C/EBP α . While, decreasing *PGRN* expression at the late stage of induction (day 3) had no effect on differentiation. These results suggested that PGRN functions in the early adipogenic events. Conversely, porcine preadipocytes differentiation was impaired by MDI and recombinant PGRN protein

induction, the expressions of adipocyte markers were decreased. Further studies revealed that PGRN can specifically facilitate ERK_{1,2} activation, and this activation can be abolished by U0126. Moreover, PPAR γ phosphorylation at serine 112 site was increased by PGRN treatment, which could reduce the transcriptional activity of PPAR γ . We conclude that PGRN inhibits adipogenesis in porcine preadipocytes partially through ERK activation mediated PPAR γ phosphorylation.

Keywords PGRN · Adipogenesis · ERK_{1,2} · PPAR γ · Phosphorylation · Porcine

Introduction

Obesity characterized by excess fat accumulation, associated with adipocytes hypertrophy and hyperplasia [1], is a major risk factor for the development of a broad range of diseases, including type II diabetes, cardiovascular diseases and cancer [2, 3]. Recent years, researches indicate that white adipose tissue (WAT) plays an important role in the generation of secreted adipokines rather than store excess energy [4]. These adipokines contain leptin, adiponectin, resistin, vaspin, tumor necrosis factor (TNF)- α , interleukins (ILs), which play important roles in regulating systemic processes, such as food intake and nutrient metabolism, insulin sensitivity, stress responses, reproduction, inflammation and so on [5–7].

Progranulin (PGRN), also known as proepithelin, granulin/epithelin precursor (GEP), acrogranin or PC cell-derived growth factor (PCDGF) [8]. Human PGRN is a 68.5 kDa secreted protein with a signal sequence and seven and one-half granulin domains connected by short linkers [9], these granulin domains are composed of highly

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conserved tandem repeats of a unique 12-cysteine motif [10]. PGRN is found in most eukaryotes, from yeast to arthropods, chordata and plants [11], but it is absent in drosophila [12]. It is widely expressed in tissues, abundantly expressed in rapidly cycling epithelial cells, immune system cells, and neurons [13]. PGRN has been shown to be a pluripotent growth factor that plays a role in many important biological processes, such as cell-cycle progression, wound healing and chondrogenesis [8]. PGRN is also implicated in various disease states, including breast and ovary cancers [14], neurodegenerative diseases [15], and rheumatoid arthritis [16].

In addition to this, recent researches indicate that PGRN is closely related to obesity development, involving in insulin resistance (IR), adipose tissue inflammation and food intake regulation. It was reported that PGRN is highly expressed in omental adipose tissue [17] and elevated serum PGRN is associated with visceral obesity, elevated plasma glucose and dyslipidemia [18]. Moreover, it's found that inhibition of hypothalamic *PGRN* expression or action increased food intake and promoted weight gain, suggesting that endogenous PGRN functions as an appetite suppressor in the hypothalamus [19]. These data revealed that PGRN functions as a key cytokine participating in regulation of whole body metabolic balance.

However, to the best of our knowledge, the pathophysiological significance of elevated PGRN level in these obesity-related diseases remains unknown; the function of PGRN in the development of obesity has not been clarified. Therefore, investigating the function of PGRN in preadipocytes growth and differentiation may provide pivotal information for understanding the mechanisms underlying the association of PGRN and obesity. In this study, we used pig preadipocytes as experimental model and aimed to find the effects and mechanisms of PGRN in the process of differentiation.

Materials and methods

Reagents

Dulbecco's Modified Eagle Medium (DMEM)/F12, fetal bovine serum (FBS), and type I collagenase were purchased from Gibco (Carlsbad, America). Oil red O was purchased from Sigma (Saint Louis, MO, America). Restriction enzyme BamHI and XhoI, Trizol, PrimeScript[®] RT reagent Kit (Perfect Real Time), and SYBR[®] Premix Ex Taq[™] II were purchased from TaKaRa Biotechnology (Dalian, China). Lipofectamine 2000 was purchased from Invitrogen (CA, America). Mouse monoclonal anti-C/EBP β and β -actin, rabbit polyclonal anti-C/EBP α , PPAR γ , Pref-1, FAS, SREBP-1c, ERK_{1,2}, phospho-ERK_{1,2} (Thr202/Tyr204),

SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185), p38 MAPK, and phospho-p38 MAPK (Thr180/Tyr182) antibodies were purchased from Cell Signaling Technology (Danvers, America). Goat polyclonal anti-aP2 and HRP-conjugated donkey anti-goat, goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology (CA, America).

Collection of animal tissues

Five healthy male crossbred (Duroc \times Yorkshire \times Landrace) pigs (180-day-old) were purchased from the experimental farm of Northwest A&F University (Yangling, China). All pigs were handled in accordance with the guidelines of Northwest A&F University Animal Care Committee. Heart, liver, spleen, lung, kidney, pancreas, skeletal muscle, subcutaneous fat adipose were isolated, collected, quickly frozen in liquid nitrogen, and stored at -80°C for later use.

Porcine primary preadipocytes culture and adipogenic differentiation

Preadipocytes were isolated under sterile conditions by the culture method with mild modification [20]. Briefly, subcutaneous adipose tissue was separated from the neck and back of piglets, and rinsed three times in phosphate-buffered saline (PBS) with 100 U/ml Penicillin and 100 $\mu\text{g/ml}$ Streptomycin (P/S). The tissue was minced into approximate 1 mm^3 sections and digested with 1 mg/ml type I collagenase at 37°C for 1 h in a shaking water bath, followed by adding DMEM/F12 medium containing 10 % FBS to stop digestion, then filtrated through 200 μm nylon mesh. The preadipocytes were collected by centrifugation at $1,000\times g$ for 5 min and resuspended with PBS for three times, and then treated with red cell lysis buffer (154 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA) for 5 min at room temperature. The preadipocytes were collected and seeded in culture plates at a density of 5×10^4 cells/ cm^2 and cultured at 37°C in humidified atmosphere with 5 % CO_2 . Culture medium was changed every 2 days. To induce differentiation, 2 days after confluence, cells were supplied with differentiation medium [DMEM/F12 containing 10 % FBS plus 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM DEX, 5 $\mu\text{g/ml}$ insulin (MDI)] for 2 days, and then cultured in maintain medium [DMEM/F12 containing 10 % FBS plus 5 $\mu\text{g/ml}$ insulin] for another 6 days, and medium was replaced every 2 days.

Lentivirus production and infection

PGRN shRNA oligonucleotides, corresponding to 208-228 (shRNA1), 686-706 (shRNA2), 1625-1643 (shRNA3) of

porcine *PGRN* mRNA (Accession Number: NM_001044578), were annealed and cloned into plenti-H1 plasmid (between BamHI and XhoI sites) respectively. A scrambled oligonucleotides expressing vector was constructed, serving as control. Plasmids (10 µg plenti-*PGRN* shRNAs, 6 µg Δ 8.9 and 9 µg VSVG) were co-transfected into 90 % confluent 293T cells in 100 mm plate with Lipofectamine 2000. 36 h and 72 h later, supernatant containing viral particles were collected, passed through 0.45 µm filters. For infection, cells were infected with lentivirus for 48 h, then the medium was replaced with MDI or maintain medium.

Real-time quantitative PCR analysis

Total RNA was extracted using Trizol reagent by standard procedure. 500 ng of total RNA was processed into single strand cDNA using reverse transcription kits with random primers. Table 1 lists the primers used for each gene studied. Real-time quantitative PCR reactions were performed in triplicate using the SYBR green kit with a Bio-Rad iQTM5 system. PCRs were performed with a 3 min pre-incubation at 95 °C, followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C. PCR products were verified by melting curve analysis. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expression level. Briefly, equivalent amount of cDNA template was used for transcripts amplification. Average Ct values were calculated and the ΔCt values were calculated by subtracting the *β-actin* average Ct value for each sample. Subsequently, $\Delta\Delta Ct$ values were calculated by subtracting the average ΔCt values of the control group. The final fold differences were calculated as $2^{-\Delta\Delta Ct}$ for each gene among treatments. These measurements were repeated 3 times.

Table 1 Specific primers for real-time quantitative PCR

Gene	Accession number	Primer sequences
<i>PGRN</i>	NM_001044578	F: CACCACCAATGAGACCTCCAAC R: CAGGAGCCGTCAAGCATAGTG
<i>β-actin</i>	NM_007393	F: GGACTTCGAGCAGGAGATGG R: AGGAAGGAGGGCTGGAAGAG
<i>PPARγ</i>	NM_214379	F: AGGACTACCAAAGTGCCATCAAA R: GAGGCTTTATCCCCACAGACAC
<i>aP2</i>	HM453202	F: GAGCACCATAACCTTAGATGGA R: AAATTCTGGTAGCCGTGACA
<i>LPL</i>	NM_214286	F: GGAGAGAGGAAGGGAAAACAGAG R: AGACCGACCAATAAACTGCAAAG
<i>SREBP-1c</i>	NM_214157	F: GCGACGGTGCCTCTGGTAGT R: CGCAAGACGGCGGATTTA

F forward primer, R reverse primer

Western blot analysis

The cellular protein was extracted with RIPA buffer (Ap-plygen, China) supplemented with protease inhibitor (Roche, USA). The protein content was measured by BCA method. The extracted proteins were electrophoresed in 12 % SDS–polyacrylamide gel under reducing conditions, and electrotransferred to nitrocellulose membranes (Milli-pore, USA). After blocking in 5 % skim milk, the mem-branes were incubated with primary antibodies overnight at 4 °C. After washing three times with TBST (Tris buffered saline plus 0.1 % Tween 20) at room temperature, the membranes were hybridized with secondary antibody for 1 h at room temperature, and then washed three times with TBST. The targeted proteins were detected using the Gel Doc XR System as per the instructions of the manufacturer.

Oil red O staining and extraction analysis

Differentiated porcine adipocytes were washed three times with PBS and fixed with 10 % paraformaldehyde for 30 min at room temperature. After washing three times with PBS, cells were stained with 1 % filtered Oil red O for 30 min at room temperature. Then cells were visualized by phase-contrast microscopy. To quantify intracellular lipid accumulation, the stained lipid droplets were dissolved with 100 % isopropanol for 10 min. The optical density was measured at 520 nm by spectrophotometer.

Statistical analysis

All the data were obtained from one independent experi-ment carried out in triplicate. The main and interactive effects were analyzed by one-way ANOVA using SPSS13.0 software. When justified by one-way ANOVA,

mean differences between individual groups were analyzed by one sample Student's *t* test. Differences were considered statistically significant if $P < 0.05$ and $P < 0.01$.

Results

PGRN was expressed in adipose and was developmentally regulated

To determine the tissue specificity of *PGRN* in normal porcine tissues, we extracted mRNA from tissues of 180-day-old pigs and subjected to real-time quantitative PCR analysis. As shown in Fig. 1a, *PGRN* is highly expressed in a number of tissues, including kidney, heart and spleen. Moreover, *PGRN* is moderately expressed in subcutaneous fat. In addition, we compared *PGRN* expression levels in preadipocytes and mature adipocytes, and we found preadipocytes are the dominant site of *PGRN* mRNA expression within the fat pad (Fig. 1b).

Next, primary porcine preadipocytes were used to assess *PGRN* expression over the course of adipogenesis. As differentiation proceeds, *PGRN* mRNA slightly declined on day 1, and then gradually increased to the maximum (about 4 fold of day 0's level) on day 6, and after that it decreased by approximately 25 % on day 10, about 2.5 fold over day 0's level (Fig. 1c).

Interference of *PGRN* promoted porcine preadipocytes differentiation and key adipogenic transcription factors expression

The developmental pattern of *PGRN* expression suggested that this gene might act as a regulator in adipogenesis. To test this hypothesis, three lentivirus-mediated shRNAs interfering of *PGRN* expression were constructed, and reduction of *PGRN* expression was performed during adipogenesis. As shown in Fig. 2A, shRNA1-3 can reduce *PGRN* mRNA expression, and the shRNA2 was the most effective one that can reduce 75 % of *PGRN* expression, then shRNA2 lentivirus was chosen to knockdown *PGRN* expression in the following experiments. Preadipocytes were infected with scrambled and shRNA2 lentivirus for 48 h, and then were induced to differentiation with MDI medium. Adipocyte formation, lipid accumulation, and adipocyte genes expression were evaluated on day 8. As shown in Fig. 2B, the shRNA2 treatment significantly promoted porcine preadipocytes differentiation. Lipid accumulation was also significantly enhanced in the shRNA2-treated cells (Fig. 2C). Moreover, the mRNA expression levels of adipocyte marker genes were robustly increased in shRNA2-treated cells (Fig. 2D), including *PPAR γ* and *aP2*, increased about 7-fold and 7.3-fold respectively, *SREBP-1c* and *LPL* were also increased about 4.5-fold and 2.2-fold respectively. These results were also confirmed by Western blot (Fig. 2E). The protein

Fig. 1 *PGRN* was expressed in adipose tissue in vivo and in vitro. **a** *PGRN* mRNA expression levels in different tissues of 180-day-old pigs were determined by real-time quantitative PCR. Liver serves as the control organ. **b** Subcutaneous fat tissue was further separated into preadipocytes and mature adipocytes. RNAs were extracted to perform real-time quantitative PCR for *PGRN* expression analysis. **c** The primary pig preadipocytes were induced to differentiation with MDI medium. RNAs were collected at the indicated time points (0, 1, 2, 4, 6, 8, 10 day) and were subjected to real-time quantitative PCR. Values represent the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$

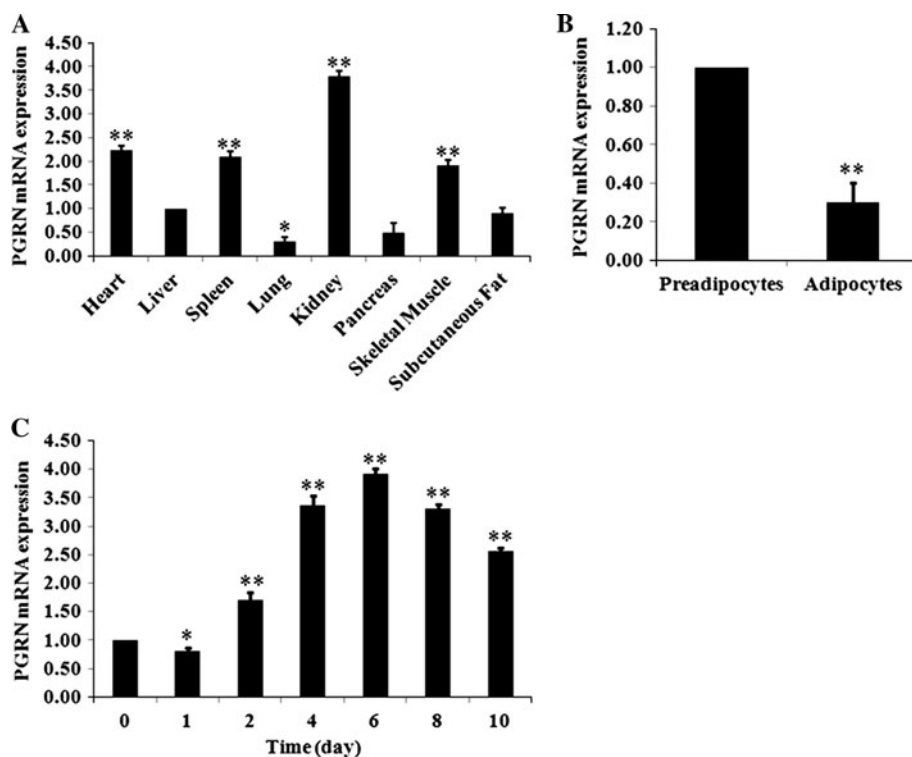
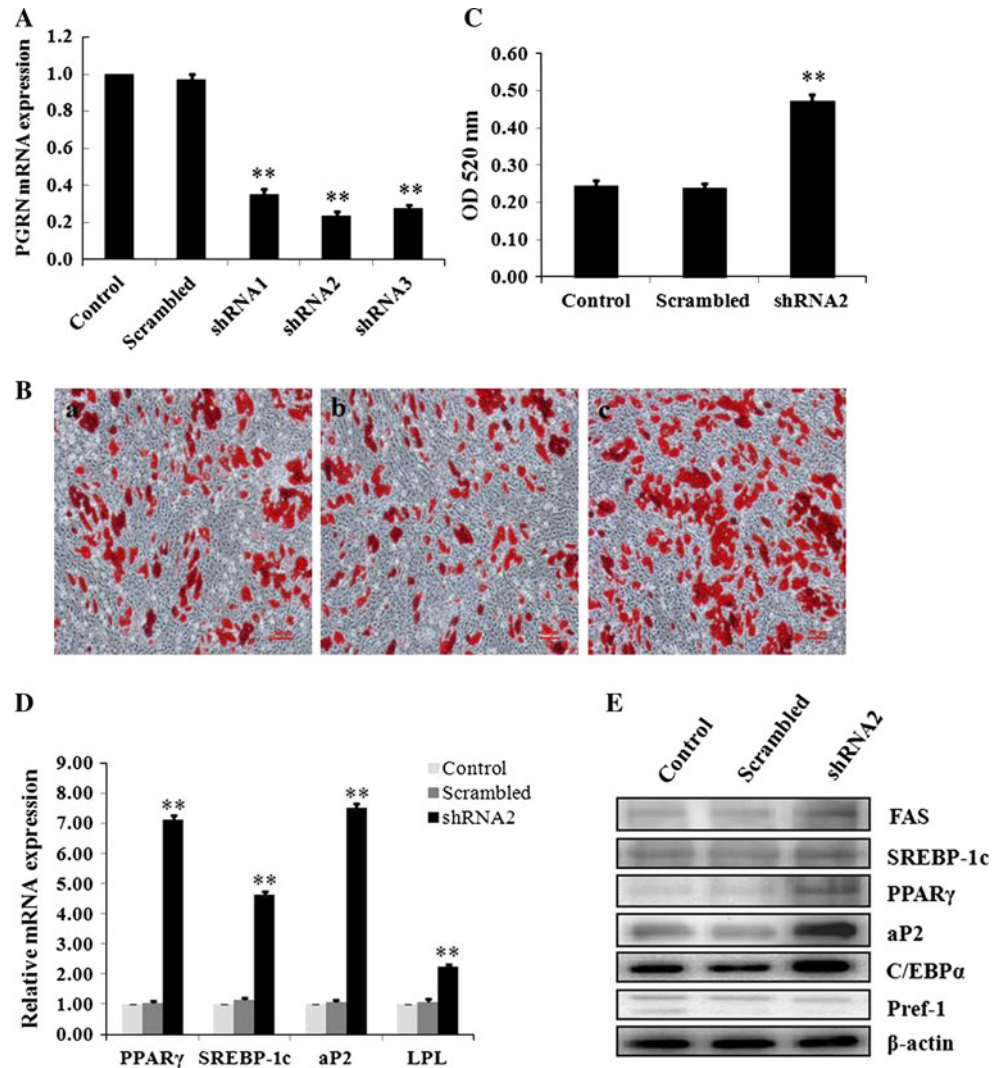


Fig. 2 Interference of *PGRN* expression at the early stage of MDI induction promoted the preadipocytes differentiation. **a** Interference efficiency of *PGRN* shRNAs was determined by real-time quantitative PCR. Preadipocytes were not infected or infected with scrambled, shRNA1, shRNA2, shRNA3 lentivirus, RNAs was collected for real-time quantitative PCR 48 h later. **b** The primary preadipocytes were not infected (a) or infected with scrambled (b), shRNA2 (c) lentivirus for 48 h, and then were induced to differentiation. Oil red O staining was performed on day 8. **c** Lipid content was quantified by spectrophotometer at the absorbance of OD 520 nm. **d** mRNA expression levels of adipocyte genes *PPAR γ* , *SREBP-1c*, *aP2* and *LPL* were analyzed by real-time quantitative PCR. **e** Protein expression levels of FAS, SREBP-1c, *PPAR γ* , *C/EBP α* , Pref-1 and *aP2* were detected by Western blot. No infected cells serve as Control. Values represent the mean \pm SEM. ****** $P < 0.01$



expression levels of FAS, SREBP-1c, *PPAR γ* and *C/EBP α* were enhanced in shRNA2 treated group, meanwhile, the expression of Pref-1 was reduced.

Interference of *PGRN* had no effect on late stage differentiation in porcine adipocytes

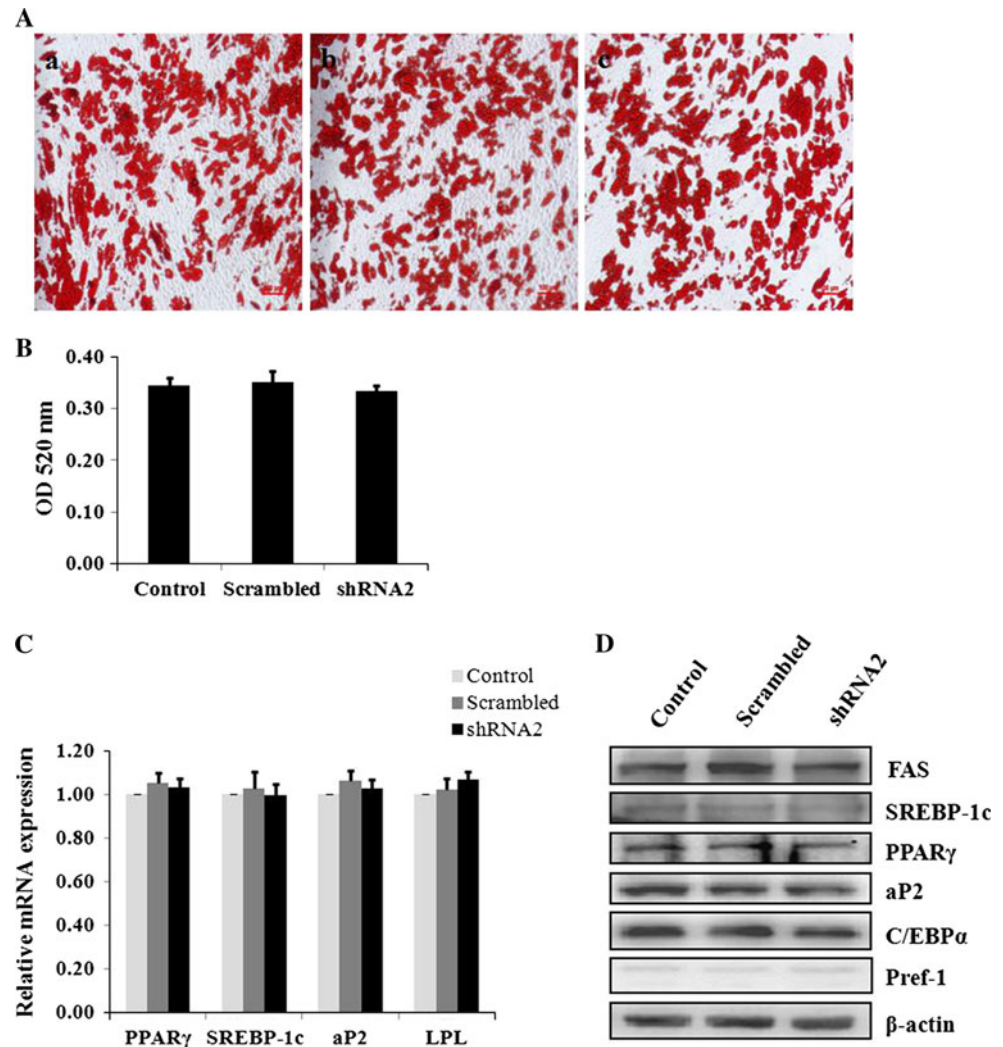
To determine whether *PGRN* had an effect on mature adipocyte differentiation, shRNA2 lentivirus was added to differentiating cells on day 3, and mature adipocyte formation, lipid accumulation and expression levels of key adipogenic factors were evaluated on day 8. The results showed that *PGRN* shRNA2 lentivirus did not affect mature adipocyte formation and lipid accumulation (Fig. 3A, B). In addition, the real-time quantitative PCR results indicated that shRNA2 lentivirus infection during the late stage of adipocyte differentiation did not alter the expression level of adipocyte markers, including *PPAR γ* , *SREBP-1c*, *aP2* and *LPL* (Fig. 3C). Consistent with this,

western blot results showed that no significant differences were detected between three groups (Fig. 3D). These results suggested that interference of *PGRN* expression had no effect on porcine adipocytes terminal differentiation.

Recombinant *PGRN* protein inhibited porcine preadipocytes differentiation, attenuated adipogenic transcription factors expression

To further confirm the effect of *PGRN* on porcine preadipocytes differentiation, bioactive recombinant *PGRN* protein was used in the experiments. Postconfluent porcine preadipocytes were exposed to differentiation medium in the presence or absence of *PGRN*. Adipocyte formation, lipid accumulation, and adipocyte genes expression were evaluated on day 8. The Oil red O staining results showed that preadipocytes differentiation was significantly promoted by MDI (Fig. 4A-c) and was remarkably inhibited by

Fig. 3 Reduction of the *PGRN* expression had no effect on late-stage differentiation in pig primary preadipocytes. **A** The differentiated preadipocytes were not infected (a) or infected with scrambled (b) and shRNA2 (c) lentivirus on day 3 for 48 h, and then were kept in maintain medium to day 8 till Oil red O staining was performed. **B** Lipid content was quantified by spectrophotometer at the absorbance of OD 520 nm. **C** mRNA levels of adipocyte genes *PPAR* γ , *SREBP-1c*, *aP2* and *LPL* were analyzed by real-time quantitative PCR. **D** Protein expression levels of FAS, SREBP-1c, *PPAR* γ , C/EBP α , Pref-1 and aP2. Values represent the mean \pm SEM



addition of PGRN protein (Fig. 4A-d), it seems that preadipocytes differentiation was also impaired by PGRN treatment alone, but didn't display significant effects (Fig. 4A-a, b). Compared with MDI induction, lipid accumulation was significantly reduced in the MDI+PGRN treated adipocytes (Fig. 4B). The real-time quantitative PCR results revealed that the mRNA expression levels of *PPAR* γ , *SREBP-1c*, *aP2* and *LPL* were decreased by MDI+PGRN treatment, comparing with MDI treatment alone (Fig. 4C). Western blot results showed that C/EBP α , Pref-1 and aP2 were decreased significantly in MDI+PGRN treated cells. (Fig. 4D).

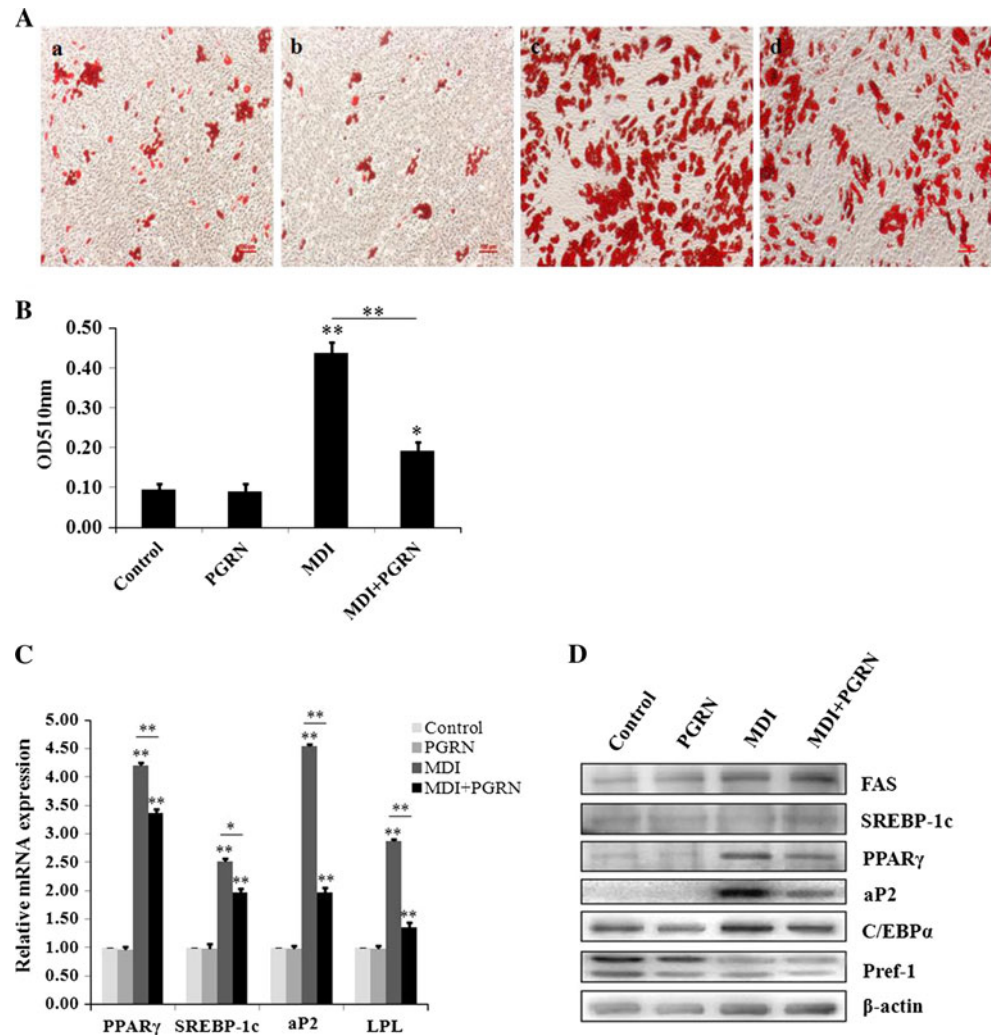
PGRN specifically activated the MDI-induced phosphorylation of ERK_{1,2}

It's reported that PGRN can activate ERK signaling pathway in different cell types, including tumor cells and epithelial cells [11]. However, its potential effect on adipocyte has not

been substantiated. Therefore we tested whether PGRN could activate ERK in the porcine preadipocytes. The porcine preadipocytes were treated with MDI medium plus PGRN, and the extent of phosphorylation of ERK_{1,2} (Thr202/Tyr204) was examined. The results showed that ERK_{1,2} phosphorylation was increased in a time-dependent manner in the cells which were incubated with 100 ng/ml PGRN, whereas the abundance of ERK_{1,2} was not affected (Fig. 5a). In control group, the ERK_{1,2} phosphorylation was peaked at 30 min, and then decreased to basal level at 60 min. Whereas in PGRN-treated group, the earliest increase in ERK_{1,2} phosphorylation was peaked at 15 min, and then maintained at a stable level. Meanwhile, phosphorylation of p38 MAPK and JNK was not affected by PGRN treatment.

To further confirm ERK_{1,2} activation in response to PGRN, MEK specific inhibitor U0126 was explored. Pre-exposure of preadipocytes to the U0126 prevented the PGRN-dependent increase in p-ERK_{1,2} (Fig. 5b). Relative abundance results showed that the ratio of p-ERK_{1,2}/

Fig. 4 PGRN treatment attenuated pig preadipocytes differentiation. Postconfluent preadipocytes were treated with PGRN (100 ng/ml) during differentiation. **A** Intracellular lipid was stained by Oil red O on day 8. (a) nondifferentiated control cells; (b) nondifferentiated cells treated with PGRN; (c) MDI treated cells; (d) MDI and PGRN treated cells. **B** Lipid content was quantified by spectrophotometer at the absorbance of OD 520 nm. **C** mRNA levels of adipocyte genes *PPAR γ* , *SREBP-1c*, *aP2* and *LPL* were analyzed by real-time quantitative PCR on day 8 after induction. **D** Protein expression levels of FAS, SREBP-1c, PPAR γ , C/EBP α , Pref-1 and aP2. Values represent the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$



ERK_{1,2} was 2.397 ± 0.18 in the cells which were maintained in PGRN containing medium. Whereas, in the presence of U0126, the value decreased, was 0.22 ± 0.011 , meanwhile, in PGRN and U0126 incubated cells, the ratio was decreased to a similar level as U0126-treated cells.

PGRN increased ERK-mediated phosphorylation of PPAR γ

PPAR γ , standing out as a master regulator in the development of adipocytes, can be phosphorylated by ERK_{1,2} at serine 112 site, and this phosphorylation leads to the reduction of transcriptional activity [21–25]. As shown in the previous results, PGRN can specifically activate ERK_{1,2}, we want to know whether PGRN have effects on PPAR γ expression as well as phosphorylation. Comparing with MDI treatment, MDI and PGRN treatment did not cause a significant change in the relative abundance of

PPAR γ (Fig. 6a). Whereas, phosphorylation of PPAR γ at serine 112 site was increased in the cells which were exposed to PGRN throughout the differentiation (Fig. 6a). Meanwhile, we also measured the phosphorylation of PPAR γ in the PGRN knockdown cells. In shRNA2-treated cells, the phosphorylation of PPAR γ was decreased (Fig. 6b). These results suggested that PGRN can increase PPAR γ phosphorylation at the serine 112 site.

Discussion

In the present study, we demonstrated for the first time that PGRN, a newly identified adipokine, inhibits adipogenesis in porcine preadipocytes. Furthermore, we show that one mechanism whereby PGRN inhibits adipogenesis is by activating ERK_{1,2} signaling and subsequently inducing PPAR γ phosphorylation. Interference of *PGRN* expression

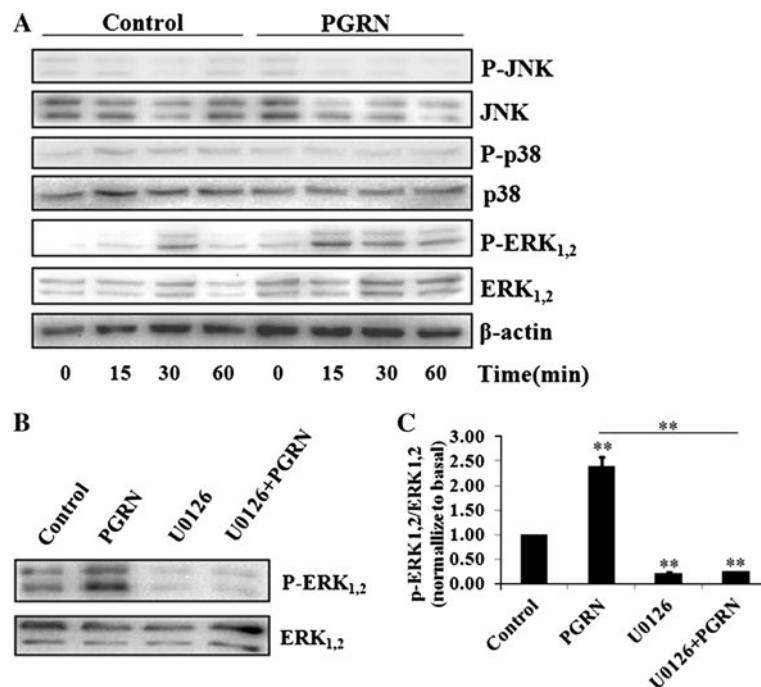


Fig. 5 Effects of PGRN on phosphorylation of ERK, p38 and JNK during early differentiation of porcine preadipocytes. **a** Postconfluent primary porcine preadipocytes were treated with MDI medium plus 100 ng/ml PGRN. Cell lysates were harvested at the indicated times and then subjected to western blot analysis detecting p-ERK_{1,2}, total ERK_{1,2}, p-p38 MAPK, total p38 MAPK, p-JNK, and total JNK expression levels. **b, c** Phosphorylated ERK_{1,2} levels were determined

in preadipocytes cultured in MDI medium and supplemented with PGRN. When indicated, 10 μ M U0126 was provided 1 h prior to MDI and PGRN treatment. **b** Western blot analysis of phosphorylated and total ERK_{1,2} under the experimental conditions. **c** The relative abundance of p-ERK_{1,2}/ERK_{1,2} ratio. The p-ERK_{1,2}/ERK_{1,2} ratio was normalized with respect to the adipogenic conditions (control). Values represent the mean \pm SEM. ** $P < 0.01$

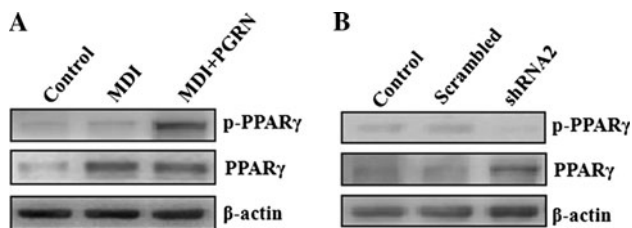


Fig. 6 PGRN activated PPAR γ phosphorylation at serine 112 site. **a** Postconfluent preadipocytes were treated with MDI, MDI plus 100 ng/ml PGRN for 8 days. The p-PPAR γ and PPAR γ were measured by western blot. **b** The primary preadipocytes were infected with scrambled, shRNA2 lentivirus for 48 h, and then were induced to differentiation. Western blot was performed on day 8 for p-PPAR γ and PPAR γ

at the early stage of adipogenesis in porcine preadipocytes promoted differentiation, whereas knockdown of PGRN at the late stage has no effect. The inhibitory effect of PGRN on adipogenesis was also confirmed by recombinant human PGRN protein treatment study. We found that the adipogenesis of porcine preadipocytes was significantly inhibited by 100 ng/ml PGRN treatment. These results collectively indicate that PGRN is a potent inhibitor on adipogenesis in porcine preadipocytes.

PGRN was first purified as a growth factor from condition media, which functions as a mitogen for 3T3 cells as

well as an autocrine growth factor for PC cells [26]. PGRN is ubiquitously expressed in various cell types and exhibits diverse functions [13]. In adult rat, *PGRN* is markedly expressed in spleen, lung, kidney and heart [27]. Consistent with the rat expression pattern, we observed that *PGRN* was highly expressed in kidney, heart and spleen in 180-day-old pigs. *PGRN* was also found to be predominantly expressed in omental adipose tissue of obese patients [17]. Our previous study showed that *PGRN* was highly expressed in subcutaneous adipose tissue of obesity-type and old pigs [28]. *PGRN* was predominantly detected in macrophages, as the development of obesity, macrophages infiltrate into adipose tissue, participating in inflammatory response and adipocyte biology regulation [29, 30]. In our study, mature adipocytes and preadipocytes were separated by digestion and low speed centrifugation, the macrophages were departed from adipocytes, but can mixed together with preadipocytes [31]. So under this circumstance, high expression of *PGRN* can be found in preadipocytes, which consists with the results obtained from mice [29]. It may suggest that macrophages is a source of PGRN. The serum concentration of PGRN is significantly higher in the type 2 diabetes patients, and is associated with omental obesity, elevated plasma glucose and dyslipidemia, and PGRN is regarded as a novel marker

of chronic inflammation [18]. In addition to this, it's found that alteration of PGRN production or action in hypothalamic may be linked to appetite dysregulation in obesity [19]. Recently, Matsubara demonstrated that PGRN is a key adipokine that mediates high fat diet induced IR and obesity [29]. These results collectively indicate that PGRN was involved in the regulation of energy homeostasis, and may play a key role in the development of obesity, IR and type 2 diabetes, but the expression and function of PGRN in adipocytes development has not been investigated. In current study, we found that PGRN was differentially expressed during the course of adipogenesis. It's highly expressed at the late stage maybe due to residual macrophages and dexamethasone induction, a potential explanation could be that PGRN may involve in IR in the mature adipocytes. This was proved by Matsubara [29]. Through knockdown and recombinant protein treatment studies, we found that PGRN inhibits porcine preadipocytes differentiation, suggesting a direct inhibitory effect of PGRN on adipocytes differentiation. Moreover, PGRN abolished preadipocytes differentiation at the early stage of MDI induction.

The activation of ERK_{1,2} in different stages plays different role in adipogenesis. Activation of ERK_{1,2} at the early stage promotes adipogenesis by enhancing PPAR γ and C/EBP α expression [32], while sustained activation of ERK_{1,2} signaling inhibits adipogenesis [33]. In the present study, we found that ERK_{1,2} phosphorylation was activated and maintained by PGRN treatment. As reported before, sustained activation of ERK_{1,2} impaired adipogenesis. Here, we found preadipocytes differentiation was attenuated by PGRN treatment. We also found that phosphorylation of PPAR γ at serine 112 site was increased in PGRN-treated cells. This phosphorylation would decrease ligand-binding affinity of PPAR γ , reduce the transcriptional activity, and eventually impair adipogenesis [21–25]. By the knockdown experiments, only interference of PGRN at the early stage can promote preadipocytes differentiation. This suggests that there may be an underlying pathway involving in accelerating adipogenesis by decreasing PGRN expression. Based on the existing results, we hypothesize that knockdown of PGRN may decrease the ERK_{1,2} phosphorylation after the initial adipogenic activation, and then maintain PPAR γ at a lower phosphorylation level, finally enhance adipogenic differentiation. Thus, we can say that MAP kinase, a central regulator of cell growth, modifies PPAR γ in a way that significantly reduces whose transcriptional activity, and impairs adipogenesis. In the previous reports, PGRN activates MAPK/ERK signaling pathway which has been widely illustrated [8, 18, 34]. However, the mechanism by which PGRN promotes ERK_{1,2} activation remains unresolved, which depends to a great extent on the biological

receptors have not been exactly identified. Gonzalez et al. [35] found that PGRN interacted with the extracellular six EGF-like repeats in Dlk1. Besides, Hu et al. [36], found GRN peptide can bind to SORT1 which mediated endocytosis determines level. It is also demonstrated that PGRN binds to TNFR and disturbs the TNF α –TNFR interaction [16]. All of these reports indicated that PGRN involves in adipocyte biology. But till to the recent time, no research demonstrates which molecule responses to PGRN's action involving in the regulation of adipocyte biology. Therefore, elucidating the potential interactions between PGRN and individual components in the adipogenic pathway may provide insight into the mechanism of PGRN in adipogenesis.

In summary, our studies provided evidence for inhibition effect of PGRN on porcine primary preadipocytes differentiation, and suggested that PGRN may function as a negative regulator in adipogenesis by regulating ERK-mediated PPAR γ phosphorylation. These results provide molecular information for further investigation of the mechanisms by which PGRN regulates adipocyte biology. Furthermore, these results could be important in devising mechanism-based and targeted therapeutic strategies for obesity.

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