ORIGINAL ARTICLE

Knockdown of ubiquitin D inhibits adipogenesis during the differentiation of porcine intramuscular and subcutaneous preadipocytes

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Abstract

Objectives: Intramuscular fat (IMF) has a significant influence on porcine meat quality. Ubiquitin D (UBD) is involved in the management of diverse intracellular processes. However, its physiological functions in adipose cell differentiation and proliferation are still poorly defined.

Materials and methods: Intramuscular and subcutaneous preadipocytes were isolated from the longissimus dorsi and neck subcutaneous deposits of Chinese native Guanzhong Black piglets (3-5 days old), respectively. Lentivirus with short hairpin RNA (shRNA) for UBD was applied to knockdown UBD expression. We used real-time PCR and Western blot analysis to detect gene expression. Lipid droplets were dyed with Oil Red O, and cell proliferation was assessed using flow cytometry, 5-ethynyl-2'-deoxyuridine incorporation and cell counting assays.

Results: Lipogenesis through the Akt/mTOR pathway was inhibited when preadipocytes were transfected with UBD shRNA. The expression of adipogenic genes and the number of lipid droplets were obviously diminished. Moreover, repression of UBD attenuated cell proliferation. UBD downregulation resulted in cell cycle arrest because of a decreased proportion of S-phase cells, and the expression of positive cell proliferation markers was significantly decreased.

Conclusion: These observations illustrated that knockdown of UBD partially suppressed porcine intramuscular and subcutaneous preadipocyte adipogenesis through the Akt/mTOR signalling and inhibited cell proliferation, suggesting the essential role of UBD in the differentiation of preadipocytes.

1 | INTRODUCTION

Obesity contributes to some of the most serious health problems around the world. It is a vital risk factor for many diseases including type-2 diabetes and hyperlipidaemia.^{1,2} It is crucial to comprehend the mechanisms regulating adipogenesis in order to combat the rapidly climbing occurrence rate of obesity. In addition, intramuscular fat (IMF) is closely connected with the quality of meat, affecting properties such as flavour, water-holding capacity and tenderness.³ For the

past few years, studies about IMF have become increasingly frequent in the field of porcine fat deposition. Thus, it is essential to study candidate genes that can control IMF deposition.⁴

Ubiquitin D (UBD) is an 18 kDa protein consisting of two ubiquitin-like (UBL) domains.⁵ It is a member of UBL protein family that is encoded by the major histocompatibility class I locus and possesses 29% and 36% identity with the N- and C-terminus of ubiquitin, respectively.⁶⁻⁸ UBD expression is highly induced by its agonists tumour necrosis factor- α (TNF α) and interferon- γ (IFN γ).^{9,10} In numerous cancer

studies, its expression is considered to be cell-cycle regulated, and downregulated by p53, and it has been shown to interact with mitotic arrest deficient-2 (MAD2), all of which are key regulators in apoptotic or mitotic process.^{11,12} These studies powerfully demonstrate that UBD has relevant impacts on cell proliferation and apoptosis.

Systemic UBD-deficient mice showed hypersensitivity to lipopolysaccharide.^{8,13} In addition, ageing knockout mice exhibited a distinct phenotype of delayed senility, accompanied by a considerable reduction in adipose mass, indicating that UBD is key regulator of the inflammatory response and fat metabolism.¹⁴ These data highlight the potential role of UBD in regulating lipogenesis in mice adipose tissue, which is a vital energy storage tissue. Although UBD plays a critical role in various intracellular activities, such as inflammation, apoptosis and tumorigenesis, whether it can modulate adipogenesis is still unclear.^{5,9,14-17} Therefore, this study tried to elucidate the effect of UBD on lipogenesis in porcine preadipocytes.

There is a series of adipogenic marker genes that are pivotal for the conversion of preadipocytes to adipocytes in IMF, such as peroxisome proliferator-activated receptor γ (PPAR γ) and fatty acid-binding protein (ap2).¹⁸ Thus, intramuscular preadipocytes may be a perfect cell model to investigate the entire differentiation process. Furthermore, Akt/mTOR signalling has been indispensable for adipogenesis.¹⁹ For this reason, we detected the phosphorylation of Akt/mTOR after knocking down UBD expression.

This report is the first to illustrate that UBD can induce adipogenesis in intramuscular preadipocytes through Akt/mTOR signalling. Moreover, the decreased level of UBD was accompanied by the suppression of cell proliferation. These findings strongly point to the essential impact of UBD in the development of intramuscular preadipocytes, and indicate its important significance to IMF deposition. Also, it could be a potential therapeutic target against metabolic diseases induced by obesity.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

The male Chinese native Guanzhong Black piglets (3-5 days old) and adult pigs (180 days old) derived from the Guanzhong area of Shaanxi province were purchased from the experimental animal station of Northwest A&F University. These littermate pigs lived in similar rearing conditions. All experimental operations were consistent with the Guide for the Care and Use of Experimental Animals.

2.2 | The isolation of intramuscular and subcutaneous preadipocytes

Intramuscular preadipocytes were isolated from the longissimus dorsi muscle (LDM), and subcutaneous adipocytes were stripped from the subcutaneous deposits in the neck and back of 3-day-old piglets. First, subcutaneous tissues were cut into pieces and digested with 1 mg/mL collagenase type I (Invitrogen, Carlsbad, CA, USA) at 37°C for 60 minutes in a reciprocating shaker bath, followed by filtration. LDM samples were digested for 1.5-2 hours with 2 mg/mL collagenase type I. Preadipocytes were cultured in growth medium (GM) containing Dulbecco's modified eagle medium (DMEM)/F12, 10% foetal bovine serum (Science Cell, Carlsbad, CA, USA), 100 mg/mL streptomycin and 100 U/mL penicillin in a humidified 5% CO₂ atmosphere at 37°C. Because of differential adhesion capacities, intramuscular preadipocytes adhered to the dish after approximately 1 hour, while most other cells could not. Therefore, cells were washed with PBS 1 hour later and cultured in GM. In this way, we acquired relatively pure samples of intramuscular preadipocytes.^{1,20}

2.3 | Cell culture and induction of adipogenic differentiation

Cells were cultured in 12-well dishes at 5×10^5 cells/well (Corning, New York, NY, USA). We added 1 mL GM per well. The cell culture medium was changed every 2 days. When the primary cells reached 100% confluence, adipogenesis was induced with differentiation medium (DM), consisted of GM, 5 μ g/mL insulin, 500 μ mol/L 3-isobutyl-1-methylxanthine and 1 μ mol/L dexamethasone, for 2 days. Subsequently, cells were cultured in maintenance medium, which only consisted of 5 μ g/mL insulin in GM. The maintenance medium was also replaced every 2 days.

2.4 | Lentiviral plasmid construction and lentivirus production

We designed the UBD shRNAs described in Table 1. Through annealing treatment at 95°C for 10 minutes, shRNA nucleotides were inserted into the BamHI/XhoI site of a pLenti-H1 lentiviral vector (GenePharma, Shanghai, China). Using Lipofectamine-mediated transfection, HEK293T cells were co-transfected with 10 μ g of either pLentiHI-UBD shRNA or scrambled shRNA, 5 μ g of vesicular stomatitis virus G protein plasmid and 6 μ g of Δ 8.9 packaging plasmid (GenePharma).²¹ After 48 hours, cells generated a mature lentivirus-containing supernatant.

TABLE 1 The shRNA oligo DNA targeting porcine UBD CDS

UBD shRNAs	Sense strands	Antisense strands
shRNA1	5'-GATCCGGAAGATAGAGTGAAGAAGATCTC GA GATCTTCTTCACTCTATCTTCCTTTTC-3'	5'-TCGAGAAAAAGGAAGATAGAGTGAAGAA GATCTCGAGATCTTCTTCACTCTATCTTCG-3'
shRNA2	5'-GATCCGCATTGACAAGGAGACCACCACTCG AGT GGTGGTCTCCTTGCAATGCTTTTC-3'	5'-TCGAGAAAAAGCATTGACAAGGAGACCACCA TCGAGTGGTGGTCTCCTTGCAATGCG-3'

The target UBD sequence of shRNA1 was 5'-GGAAGATAGAGTGAAGAAGAT-3'; And the target UBD sequence of shRNA2 was 5'-GCATTGACAAGGAGACCACCA-3'. The underlined sequences represented the sense and anti-sense sequences of UBD shRNA1 or shRNA2.

2.5 | Lentivirus infection of porcine intramuscular and subcutaneous preadipocytes

Green fluorescent protein (GFP) labelling existed inside the pLenti-H1 lentiviral vector and was used to detect lentivirus. When the density of preadipocytes reached 70%-80%, they were infected with 200 μ L of lentivirus (encoding UBD shRNAs or scrambled shRNA) and cultured in 200 μ L of GM for 24 hours. After this period, the medium was exchanged with normal GM. The successfully infected preadipocytes revealed green fluorescence. When cells reached 100% confluence, they were cultured in DM.

2.6 | Oil Red O staining and dye extraction analysis

After adding DM, intramuscular preadipocytes were cultured for 8 days. Cells were fixed with 4% paraformaldehyde for 45 minutes at room temperature. Afterwards, the fixed cells were washed and stained with 1% filtered Oil Red O in isopropyl alcohol for 30 minutes. Then, they were washed and observed with phase-contrast microscopy (IS-Elements software, Nikon Eclipse, Tokyo, Japan). Finally, we extracted Oil Red O dye from the stained cells with isopropanol for 20 minutes, and performed a quantitative analysis using a colorimetric spectrophotometer. The only difference in the procedure for subcutaneous preadipocytes was that staining was performed 6 days after induction.

2.7 | RNA extraction and real-time qPCR

Total RNA was extracted with TRIzol reagent (TaKaRa, Otsu, Japan). RNA reverse transcription PCR was carried out using reverse transcription kits (TaKaRa). The specific primer sequences were shown in Table 2, and we used a SYBR green kit (Vazyme, Nanjing, China)

with a Bio-Rad iQ5 system (BioRad, Hercules, CA, USA) to complete the qPCR reactions. Relative gene expression was analysed using the $2^{-\Delta\Delta ct}$ method.

2.8 | Western blot analysis

The major Western blotting analysis was performed as reported previously reported.²² Using lysis buffer (Beyotime, Shanghai, China), we collected cells and boiled the mixed lysates with SDS loading buffer (Beyotime). After samples were electrophoresed in 12% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), all membranes were blocked in 5% non-fat milk for 2 hours and allowed to react with specific primary antibodies at 4°C overnight. Membranes were incubated at 4°C for 1.5 hours with homologous HRP-conjugated secondary antibodies. Protein bands were examined with chemiluminescence reagents (Millipore) using a ChemiDoc XRS imaging system (BioRad), and quantitative statistical analysis was performed with Image J. The primary antibodies were specific for the following proteins. PPAR γ , cyclin D and cyclin B (Abcam, Cambridge, UK), adipose triglyceride lipase (ATGL), proliferating cell nuclear antigen (PCNA), mammalian target of rapamycin (mTOR), phospho-mTOR (Ser2448), protein kinase B (AKT) and phospho-AKT (Thr308) (Cell Signalling Technology, Danvers, MA, USA), UBD (R&D, MN, USA), ap2, fatty acid synthase (FAS), β -tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Dallas, TX, USA).

2.9 | Flow cytometry detection

Intramuscular preadipocytes were cultured in 60-mm dishes at 4×10^3 cells/dish and we added 3 mL GM per dish (Corning). First, cells were

TABLE 2 Specific primers for qPCR

Genes	Accession No.	Primer sequence	Length	Tm/°C
UBD	NM_001160088.1	F: GCGTCTGTGTGATGGTCTCTT R: TCTGTGGGGCTTTAGGGTCT	164	60
PPAR γ	NM_214379.1	F: AGGACTACCAAAGTGCCATCAAA R: GAGGCTTTATCCCCACAGACAC	142	60
ap2	HM453202	F: GAGCACCATAACCTTAGATGGA R: AAATTCTGGTAGCCGTGACA	121	60
SREBP1c	NM_214157	F: GAGCACCATAACCTTAGATGGA R: AAATTCTGGTAGCCGTGACA	201	60
ATGL	NM_001098605.1	F: CCTCATTCCACCTGCTCTCC R: GTGATGGTGCTCTTGAGTTCGT	90	62
CyclinB	NM_001170768.1	F: AATCCCTTCTTGTTGGTTA R: CTTAGATGTGGCATACTTG	104	60
CDK4	NM_001123097.1	F: ATCAGCACGTTCTGTAAGT R: GCTCAAACACACGGGTCACT	132	60
CDKN2B	NM_214157	F: AGTGGCGGCGGTGGAGAT R: GGGTGAGGGTGGCAGGGT	217	60
p27	NM_214316.1	F: CCAGGCGGTGCCTTTAATTG R: GTGGCAGGTCGCTTCCTAT	132	60
β -actin	NM_007393	F: GGACTTCGAGCAGGAGATGG R: AGGAAGGAGGGCTGGAAGAG	138	60

FIGURE 1 Ubiquitin D (UBD) expression in porcine preadipocyte differentiation period and in various tissues. A, UBD mRNA expression in various tissues. B, The levels of UBD in subcutaneous adipose tissue (SAT) and intramuscular fat (IMF) at 3-day-old and 180-day-old pigs. C, Intramuscular preadipocytes were cultured in GM before induction and viewed by phase-contrast microscopy (left panel). Intramuscular preadipocytes were induced adipogenesis for 8 days after addition of DM and observed by phase-contrast microscopy (middle panel) or stained by Oil Red O (right panel). D, UBD expression during subcutaneous and intramuscular preadipocytes adipogenesis. β -actin as internal control. E, UBD protein levels during differentiation period of intramuscular preadipocytes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was interior control. The results were analysed as means $\pm$ SEM, $n = 3$; * $P < .05$

digested with trypsin and washed with PBS after 48 hours of infection with lentivirus. Second, cells were fixed in cold 70% ethanol for 30 minutes and treated with 1 mg/mL RNaseA for 40 minutes at 37°C. Last, cells were stained with 50 μ g/mL propidium iodide (PI) and analysed using a FACScan argon laser cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

2.10 | EdU incorporation assay

Cell proliferation was examined with a Click-iT EdU Alexa Fluor 594 Imaging Kit (Invitrogen). Cells were cultured with 10 μ mol/L 5-ethynyl-2'-deoxyuridine (EdU) for 1 hour to stain proliferative cell

nuclei. Total cell nuclei were stained with 5 μ g/mL Hoechst 33342 (Beyotime) for 15 minutes. Finally, we detected the diverse fluorescence signals using a fluorescence microscope. For statistical results, the ratio of EdU-positive cells/nuclei was evaluated with Photoshop software.

2.11 | Cell Counting assay

In order to examine the proliferation of preadipocytes, we used CCK-8 kit (Beyotime) to detect the number of viable cells. Preadipocytes were seeded in 96-well plates at 2.5×10^3 cells/well and cultured in 100 μ L GM per well. After 24 hours of lentivirus infection, we added 10 μ L of CCK-8 kit reagent and 90 μ L of GM to each well and incubated the

FIGURE 2 Interference of Ubiquitin D (UBD) inhibited adipogenic marker genes expression. A, UBD mRNA levels at 72 hours after intramuscular preadipocytes infected by UBD shRNAs. B, The infected intramuscular preadipocytes by lentivirus. Top: Transfected HEK293T cells by scrambled or UBD shRNA1 vector and generated mature packaged lentivirus after 48 hours. Bottom: The infected intramuscular preadipocytes by lentivirus after 72 hours. C, UBD expression in infected intramuscular preadipocytes with UBD shRNA1 after 72 hours. D, The mRNA expression of adipogenic marker genes peroxisome proliferator-activated receptor γ (PPAR γ), fatty acid-binding protein (ap2), adipose triglyceride lipase (ATGL), sterol regulatory element binding protein-1c (SREBP-1c) and CCAAT/Enhancer-Binding Protein beta (C/EBP β) at 4, 6, 8 days after induction. β -actin acted as control. E-F, The protein profiles of adipogenic genes at 8 days after induction and UBD expression during the period of differentiation (E) and quantitative analysis by ImageJ (F). GAPDH acted as loading control. The data were analysed as means $\pm$ SEM, $n = 3$; * $P < .05$, ** $P < .01$

cells at 37°C for 4 hours. Then, a VICTOR™ X5 multilabel plate reader (PerkinElmer, Waltham, MA, USA) was used to detect absorbance values to determine the cell proliferation index. Specific steps were in accordance with the manufacturer's instructions.

2.12 | Statistical analysis

We used SPSS 19.0 statistical software (Chicago, Illinois, USA) to perform statistical analyses with Student's t test. Experimental data

are shown as the mean $\pm$ SEM. Statistical significance is represented as follows: * $P < .05$; ** $P < .01$.

3 | RESULTS

3.1 | Expression of UBD in the porcine preadipocytes differentiation period and in various tissues

To clarify the tissue distribution of UBD in various porcine tissues, we extracted RNA from seven different tissues of 3-day-old piglets. Moderate UBD expression was observed in subcutaneous adipose tissue (SAT) and in IMF (Figure 1A). In adipose tissue, mRNA expression was more elevated in 180-day-old pigs compared with 3-day-old piglets (Figure 1B). These results suggest that UBD may be involved in the adipocyte differentiation process.

Lipogenesis is associated with dynamic regulation of gene expression. Therefore, we detected the UBD expression pattern in differentiated intramuscular and subcutaneous preadipocytes. First, intramuscular preadipocytes were induced to generate lipid droplets, which were visualized with Oil Red O staining (Figure 1C). In intramuscular and subcutaneous preadipocytes, UBD mRNA expression increased gradually during the differentiation period (Figure 1D). Protein levels showed a tendency similar to that of mRNA expression (Figure 1E). Thus, these results indicate that UBD is likely to play a crucial role in porcine adipogenesis development of SAT and IMF.

3.2 | Knockdown of UBD inhibits intramuscular preadipocytes differentiation

Preadipocytes were infected with lentivirus (encoding UBD shRNA1 or shRNA2). The resulting interference efficiency of shRNA1 reached 80% after 72 hours of infection, which was higher than that of shRNA2 (Figure 2A). Hence, we selected the more efficient shRNA1 for use in the following experiments.

The GFP in HEK293T cells indicated the presence of mature lentivirus, and intramuscular preadipocytes were infected with virus when reaching 70%-80% confluence, as evidenced by green fluorescence (Figure 2B). The qPCR results showed that UBD expression dramatically declined at 72 hours after induction (Figure 2C). Meanwhile, during the adipogenesis period, unsurprisingly, the mRNA levels of the lipogenic genes PPAR γ , ap2, ATGL and sterol regulatory element binding transcription factor 1 (SREBP-1c) all significantly dropped in response to UBD shRNA1 treatment (Figure 2D). Consistently, knockdown of UBD resulted in a notable decrease in protein levels of adipocyte genes, including PPAR γ , FAS, ap2 and the lipolytic gene ATGL, during terminal differentiation (Figure 2E,F). Moreover, consistent with the above outcomes, Oil Red O staining indicated that UBD repression strongly decreased the number of lipid droplets. The OD value of stained adipocytes was also significantly reduced after Oil Red O extraction (Figure 3A,B). In addition, to investigate downstream UBD signalling, we examined the

phosphorylation (activation) levels of Akt and mTOR (p-Akt/mTOR). The phosphorylation of them was suppressed in terminal differentiation (Figure 3C).

Taken together, these results suggest that UBD inhibition down-regulated intramuscular preadipocyte differentiation, indicating that UBD may be a positive adipogenic regulator.

FIGURE 3 Knockdown of Ubiquitin D (UBD) repressed Akt/mTOR signalling protein levels and lipid droplets synthesis. A-B, Oil Red O staining of intramuscular preadipocytes (A) and quantitative assay (B) at 8 days after adipogenic induction. C, The protein expression of Akt and mammalian target of rapamycin (mTOR) phosphorylation levels during adipogenesis. The data were analysed as means $\pm$ SEM, $n = 3$; * $P < .05$

FIGURE 4 Ubiquitin D (UBD) promoted intramuscular preadipocyte proliferation. A, The mRNA expression of CyclinB, cyclin-dependent kinase 4 (CDK4), cyclin-dependent kinase inhibitor 1B (p27) and cyclin-dependent kinase inhibitor 2B (CDKN2B) after 24 and 48 hours infection, and the interference efficiency of UBD. β -actin as the internal control. B, The protein expression of proliferative marker genes PCNA, CyclinB and CyclinD and their relevant quantitative analysis after 24 hours treatment. GAPDH was internal control. C-D, The proliferation was examined by EdU immunofluorescent staining after 24 hours infection. Red represented EdU staining, and blue was cell nuclei (C). The percentage of EdU-positive cells (D). E, After 24 hours treatment, the cell proliferation index was examined by CCK-8 kit with absorbance value. F-G, Cell cycle phase analysis with flow cytometry after 24 hours infection (F) and its statistical results (G). The data were analysed as means $\pm$ SEM, $n = 3$; * $P < .05$, ** $P < .01$

FIGURE 5 Inhibition of UBD suppressed subcutaneous adipocyte differentiation and proliferation. A, The infected subcutaneous preadipocytes by scramble or UBD shRNA1 lentivirus after 24 hours of infection. B, The mRNA levels of adipogenic marker genes at 2, 4 and 6 days after induction (left to right). β -actin as internal control. C, The protein expression of adipogenic genes at 6 days after adipogenic induction. GAPDH was interior control. D, Oil Red O staining at day 6 after adipogenic differentiation. E, Expression of proliferation genes CDK4, CyclinB and CDKN2B after 24 hours of infection. F, Protein expression of proliferation genes after 24 hours of infection during the period of propagation stage. G-H, The EdU immunofluorescent staining after 24 hours of infection (G). And the percentage of EdU-positive cells (H). I, The cell proliferation index with CCK-8 kit after 24 hours of infection. The data were analysed as means $\pm$ SEM, $n = 3$; * $P < .05$, ** $P < .01$

3.3 | UBD promotes porcine intramuscular preadipocytes proliferation

Previous studies have confirmed that UBD can regulate cell apoptosis in hepatoma carcinoma cells.^{8,23} However, the influence of UBD on cell proliferation has not been clearly determined. To this end, we analysed the proliferation of intramuscular preadipocyte after UBD suppression.

Preadipocytes were infected with UBD shRNA1 at 40% density. First, we found that the UBD mRNA level had achieved 70% inhibition 24 hours later (Figure 4A). The mRNA levels of the representative proliferation markers cyclin-dependent kinase 4 (CDK4) and cyclin B were decreased upon knockdown of UBD (Figure 4A). Meanwhile, the repressor genes cyclin-dependent kinase inhibitor 1B (p27) and cyclin-dependent kinase inhibitor 2B (CDKN2B) were markedly increased (Figure 4A). The Western blot analysis results were coincident with the above described results. Cyclin B, cyclin D and PCNA protein levels were observably decreased after UBD shRNA1 treatment (Figure 4B). These suggest that UBD plays a pivotal role in positively modulating cell proliferation of preadipocytes.

After 24 hours of infection, the proportion of EdU-positive cells was notably reduced (Figure 4C,D). In addition, the CCK-8 assays showed that the cell proliferation index significantly decreased (Figure 4E). The flow cytometry analysis of the cell cycle distribution also indicated that UBD downregulation resulted in cell cycle arrest as demonstrated by the declining number of S-phase cells and the rising proportion of G0/G1 phase cells after 24 hours of infection (Figure 4F,G), which further supports the inhibitory effect.

3.4 | Inhibition of UBD suppresses subcutaneous adipocytes differentiation and proliferation

Owing to the dramatic phenomenon induced by UBD shRNA1 in intramuscular preadipocytes, we also performed relevant experiments in subcutaneous adipocytes to further confirm the role of UBD via two-way authentication.

Subcutaneous preadipocytes were infected with UBD lentivirus when they reached 70%-80% confluence and exhibited green fluorescence (Figure 5A). Throughout adipogenesis period, Western blot analysis and qPCR results suggested that the expression of PPAR γ , ap2, ATGL and SREBP1c were markedly decreased in UBD shRNA1-treated cells (Figure 5B,C). As expected, consistent with the gene detection results, lipid accumulation determined with Oil Red O staining was notably reduced (Figure 5D). Taken together, these results further demonstrate that UBD has an active effect on porcine adipogenesis.

In examining the influence of UBD on cell cycle regulation of subcutaneous adipocytes, we found that the percentage of EdU-positive cells and the cell proliferation index were dramatically decreased under UBD knockdown treatment (Figure 5G-I). Moreover, further quantitative assessment indicated that positive marker genes were significantly decreased, while CDKN2B was increased (Figure 5E,F). On the whole, these findings indicate that UBD can alter the proliferation of porcine subcutaneous preadipocytes and has identical effects regardless of the type of porcine cell.

4 | DISCUSSION

This work is the first to report that UBD plays a positive role in the differentiation of porcine intramuscular and subcutaneous preadipocytes. These findings predict critical function of UBD in porcine adipogenesis and proliferation, help us to understand whether UBD has a potential impact on improving IMF deposition, and provide a therapeutic approach to human metabolic disorders induced by obesity.

UBD has been studied as a potential modulator of cell development and growth. UBD downregulation accelerated unnatural transformations in the inflammatory response, cell mitosis and apoptosis.^{12,24,25} Overexpression of UBD strongly stimulated tumour growth.²⁶ Currently, the role of UBD in apoptosis and proliferation of different cell types is uncertain. UBD overexpression attenuated cardiac myocyte apoptosis.²⁷ However, in mouse fibroblasts and HCC cells, it induced apoptosis in a caspase-dependent manner.¹⁰ In our investigation, we found that UBD could promote porcine preadipocytes proliferation. Owing to different species and cell types, these discrepant results indicate the numerous functions of UBD in diverse cell types. Cell proliferation and adipogenesis are closely related, and blocking the cell cycle and mitotic clonal expansion can prevent adipocytes differentiation.²⁸ Therefore, our investigation strongly confirms the influence of UBD on adipocytes development.

Ubiquitin D levels were highly elevated in response to the proinflammatory cytokines TNF α and IFN γ .^{24,29} These cytokines play important roles in chronic inflammation-associated tumourigenesis.³⁰ They are closely related to obesity-associated insulin resistance and type-2 diabetes.^{31,32} Systemic UBD-deficient mice exhibited a distinct phenotype of delayed senility accompanied by global alterations in energy metabolism, adiposity, insulin sensitivity and inflammation.¹⁴ In recent years, evidence has indicated that a variety of UBD target proteins and interacting partners that are involved in insulin sensitivity and energy metabolism can be modulated by cAMP-dependent signalling, PI3K/Akt/mTOR signalling and the NF- κ B pathway.³³⁻³⁵ UBD

acts as an oncogene that regulates tumourigenesis and tumour metastasis through Akt/GSK3 β signalling in hepatocarcinoma.^{15,26} In addition, novel post-transcriptional control of Akt activation has verified that ubiquitination may have a significant function in this pathway, and UBD happened to be involved in the ubiquitination process.³⁶ In general, UBD may have a close relationship with Akt signalling as a member of the ubiquitination family. Therefore, we had paid attention to Akt signalling firstly in our experiments. Fortunately, with regard to our differentiation results, there was a certain degree of reduction in the phosphorylation levels of Akt and its downstreams signalling mTOR under UBD inhibition treatment, suggesting that UBD may exert its critical regulation in adipogenesis in part through Akt/mTOR signalling.

In addition, one study demonstrated that UBD-KO mice exhibited increased β -oxidation and upregulated expression of PGC1 α , UCP1 and PPAR α , accompanied by intensive glucose-insulin homeostasis.¹⁴ On the basis of these reports, we are inspired to analyse the potential impact of UBD on white adipocyte browning in the future.

We made use of intramuscular and subcutaneous preadipocytes to confirm UBD function. As expected, both verification tests led to the same results. The only difference between the results was that the extent of adipogenesis in IMF cells was slightly poorer than that in subcutaneous cells. Recently, some laboratories have successfully isolated intramuscular adipocytes that can form obvious lipid droplets, and compared gene expression patterns of various adipocytes.^{37,38} Deposition of IMF is mainly determined by hyperplasia.³⁹⁻⁴¹ Preadipocytes are likely to exhibit multifarious adipogenic potential in various adipose deposits.⁴² There are also benefits for meat quality promotion in studying the regulatory mechanisms of candidate genes in IMF.⁴³

In conclusion, we have confirmed that UBD may be a positive modulator in porcine intramuscular preadipocytes differentiation. The present study provides evidence that a decrease in UBD levels leads to a significant reduction in mRNA and protein levels of key adipogenic markers through inhibition of the Akt/mTOR signalling pathway. Taken together, although our investigation is still the tip of the iceberg, these findings reveal that UBD is an essential regulator of the deposition of IMF and SAT and a novel therapeutic target against metabolic diseases induced by obesity.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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