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C/EBPβ converts bovine fibroblasts to adipocytes without hormone cocktail induction

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ABSTRACT

Background: Adipogenesis and fibrogenesis can be considered as a competitive process in muscle, which may affect the intramuscular fat deposition. The CCAAT/enhancer-binding protein beta (C/EBPβ) plays an important role in adipogenesis, which is well-characterized in mice, but little known in bovine so far.
Results: In this study, real-time qPCR revealed that the level of C/EBPβ was increased during the developmental stages of bovine and adipogenesis process of preadipocytes. Overexpression of C/EBPβ promoted bovine fibroblast proliferation through mitotic clonal expansion (MCE), a necessary process for initiating adipogenesis, by significantly downregulating levels of *p21* and *p27* ($p < 0.01$). Also, the *PPARγ* expression was inhibited during the MCE stage ($p < 0.01$). 31.28% of transfected fibroblasts adopted lipid-laden adipocyte morphology after 8 d. Real-time qPCR showed that C/EBPβ activated the transcription of early stage adipogenesis markers *C/EBPα* and *PPARγ*. Expression of *ACCα*, *FASN*, *FABP4* and LPL was also significantly upregulated, while the expression of *LEPR* was weakened.
Conclusions: It was concluded C/EBPβ can convert bovine fibroblasts into adipocytes without hormone induction by initiating the MCE process and promoting adipogenic genes expression, which may provide new insights into the potential functions of C/EBPβ in regulating intramuscular fat deposition in beef cattle.
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1. Introduction

Intramuscular fat (IMF) content is a vital contributor to beef quality, which influences taste, juiciness, and tenderness [1,2,3]. The accumulation of IMF is affected by genetic background, as well as age and nutrition [4,5,6]. Deposition of IMF involves adipocyte proliferation or an increase in cell numbers, as well as differentiation, wherein cell size increases with lipid accumulation. These processes are regulated by a complex network of adipogenic factors [7]. The CCAAT enhancer-binding protein family (C/EBPs) includes six members (α, β, δ, γ, ε, and ζ), which contain a leucine zipper domain and a DNA-binding domain that binds to promoter regions of target genes. These pivotal regulators of cellular differentiation are expressed in a variety of tissues [8]. Adipogenesis is a complex process involving a cascade of transcriptional, factors and proteins that induce gene expression and adipocyte differentiation. C/EBPβ is a vital transcriptional factor expressed immediately after cocktail hormone induction in 3T3-L1 cells. However, DNA-binding activity of C/EBPβ is gained only after a long lag, as the cells synchronously begin mitotic clonal expansion (MCE), which is a process necessary to adipogenesis [9]. After MCE, C/EBPβ activates the expression of the adipogenesis markers such as C/EBPα and peroxisome proliferator-activated receptor γ (*PPARγ*). These genes play a profound role in regulating a wide array of downstream genes important in the regulation of adipogenesis [10].

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These underlying mechanisms are likely best understood in rodents. C/EBPβ overexpression has been shown to induce adipocyte phenotype in both mouse embryo fibroblasts (MEFs) and human mesenchymal stem cells [11]. In mice lacking C/EBPβ, adipogenesis is impaired [12]. As an important early factor of adipogenesis, C/EBPβ promotes terminal adipocyte differentiation. However, there are apparent species variations in the temporal pattern of appearance of these transcription factors. Meat animal species have species-specific differentiation processes, as well as different gene and protein markers from the mouse model [7,13]. Moreover, specific adipose deposits in ruminant animals do not respond to regulatory compounds in the same way as monogastric animals [14]. Thus, identifying the function of C/EBPβ and the regulatory expression patterns in cattle cells undergoing adipocyte differentiation, especially in intramuscular adipogenesis, may be useful for improving carcass fat traits.

Recent research has shown that intramuscular adipocytes and fibroblasts share common mesenchymal progenitor cells located in the extracellular matrix of primordial muscle fibers, in which the fibroblasts but not myogenic cells are the major cellular source of intramuscular adipose tissue. Conversion of fibroblasts to adipocytes may reduce intramuscular connective tissue and increase intramuscular fat deposition [15,16]. In addition, adipogenic ability and gene expression patterns of stem cells and preadipocytes derived from adipose tissue are influenced by isolation methods, reducing their modeling value [17,18,19,20]. Therefore, fibroblasts may provide a useful cell model for research on intramuscular adipocyte differentiation. We investigated C/EBPβ function and regulation in cattle fibroblasts to provide insights into the precise mechanism of intramuscular adipose formation in beef cattle.

2. Material and methods

2.1. Experimental animal and tissues collection

Tissue samples (muscle, heart, omasum, kidney, rumen, liver, lung, spleen, perinephric fat, large intestine and intramuscular fat) were obtained at a local slaughterhouse (Yangling, China) from three adults (24 months of age) and three newborn Chinese indigenous Qinchuan cattle. All animal procedures were approved by the Institutional Animal Care and Use Committee (Northwest A&F University, No. 2013-23).

2.2. Isolation of preadipocytes and induction of differentiation

Preadipocytes were isolated from longissimus dorsi muscle of calves as described before with minor alterations [21]. Briefly, samples were separated from the longissimus dorsi of cattle, and approximately 1 mm^3 sections were obtained using scissors under sterile conditions. Tissue samples was digested with type I collagenase for 45 min in a 37°C shaking water bath. Digested materials were sequentially filtered through 100 and 40 μm cell strainers (BD Biosciences, USA). The filtrate was centrifuged at 800 × *g* for 10 min and the supernatant was removed. Following this, the pellet was resuspended in DMEM/F12 and centrifuged again. The pellet was then re-suspended in DMEM/F12 + 10% FBS + 1% PS (penicillin and streptomycin) media and incubated at 37°C, under a humidified air containing 5% CO_2 for 1.5 h. Afterwards, the non-adherent cells were removed by PBS, and the remained cells cultured with fresh growth medium at 37°C in a 5% CO_2 incubator.

The cells were cultured in 12-well plates for differentiation induction. Briefly, Medium was changed to differentiation medium (DMEM/F12 + 10%FBS + 5 μg/mL insulin + 0.5 mM IBMX + 1 μM DEX) after achieving 90% cell confluence. This point in time was considered day 0. Two days later, medium was changed to DMEM/F12 supplemented with 10% FBS with 5 μg/mL insulin and cultured until adipocytes were completely differentiated, with medium changed every two days. Total RNA of differentiated adipocytes was isolated on days 0, 2, 4, 6 and 8.

2.3. Isolation of fibroblasts and troglitazone treatment

Primary fibroblasts from newborn Qinchuan cattle ear epidermis were isolated using explant culture as described before with minor modification [22]. In brief, after removal of the cartilage tissue using scissors, ear skin tissue was soaked in 75% ethanol for 30 s, then rinsed three times with PBS containing 5% PS. Following this, approximately 1 mm^3 tissue sections were obtained using scissors under sterile condition. These were seeded at the bottom of a T-25 flask with DMEM/F12 + 10% FBS + 1% PS culture media for ceiling culture at 37°C under humidified air containing 5% CO2. After 3–4 h, the flask was turned over when the pieces had attached, and culture continued until the primary outgrowing cells reached confluence around the explants. Fibroblasts were expanded and purified by short trypsinization with 0.1% trypsin/EDTA method as described previously [23].

Fibroblasts were cultured in the complete medium (DMEM/F12 + 10% FBS + 1% PS) until 90% confluence. Then, cells were treated with 10 μM troglitazone (Sigma, USA), a PPARγ agonist, to test for purity and whether the PPARγ activation could initiate adipogenesis of fibroblasts. Cattle preadipocytes were used as control. Fibroblasts and preadipocytes were treated with troglitazone for 8 d, with medium changed every 2 d. Total RNA was isolated on days 0, 2, 4, 6, and 8. Lipid accumulation was detected using oil red O staining at day 8 and quantified using Image J 1.47 [24].

2.4. Adenovirus package and transfection

The CDS region of the cattle *C/EBPβ* gene (GenBank No. NM_176788.1) was cloned and added to the *kozak* sequence to improve translation efficiency [25]. C/EBPβ overexpression adenoviruses (Ad-C/EBPβ) and negative control (Ad-GFP) were packaged in HEK293A cells (DMEM containing 5% FBS) using Adenoviral Vector System (Invitrogen, USA). Following this, 80%-confluent fibroblasts were transfected with Ad-C/EBPβ and Ad-GFP at a multiplicity of infection (MOI) of 20.

2.5. RNA isolation and real-time qPCR

Total RNA was extracted using TRIzol (Invitrogen, USA), then reverse-transcribed using a PrimeScript™ RT reagent kit with gDNA Eraser to eliminate genomic DNA contamination (Takara, Japan). Real-time qPCR was carried out using an ABI 7500 real-time qPCR system (Applied Biosystems, USA) with SYBR Premix Ex Taq kit (Takara, Japan). A melting curve analysis and electrophoresis of amplification products were performed to confirm that only one product was amplified. PCR products were electrophoresed on 2% agarose gels to confirm size. mRNA expression levels were quantified as $2^{-\text{delta Ct}}$ [26]. All experiments were performed in triplicate. All the data were normalized to the mRNA level of endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers are shown in Table 1.

2.6. Cell proliferation assay

To determine the effect of C/EBPβ on cell proliferation, 80%-confluent fibroblasts cultured in 12- or 6-well plates were infected by Ad-C/EBPβ for 5-ethynyl-2′-deoxyuridine (EdU) (RiboBio, China) assay or flow cytometry analysis. At 48 h post-infection, 50 μM of EdU was added into each well and incubated at 37°C for 2 h. Cells were then fixed with 4% formaldehyde for 30 min and treated with

Table 1
Primers used for real-time qPCR.

Gene	Accession number	Primer sequence (5′–3′)	Product size, bp
C/EBPβ	NM_176788.1	F-TTCCTCTCCGACCTCTTCTC R-CCAGACTCACGTAGCCGTACT	79 bp
CCND2	NM_001076372.1	F-GGGCAAGTTGAAATGGAA R-TCATCGACGGCGGGTAC	173 bp
p21	NM_001098958.2	F-GACCAGCATGACAGATTTCTACCA R-TGAAGGCCCAAGGCAAAAG	144 bp
p27	NM_001100346.1	F-AGATGTCAAACGTGCGAGTG R-GCCAAAGAGGTTTCTGCAAG	104 bp
C/EBPα	NM_176784.2	F-ATCTGCGAACACGAGACG R-CCAGGAACTCGTCGTTGAA	73 bp
PPARγ	NM_181024.2	F-GAGATCACAGAGTACGCCAAG R-GGGCTCCATAAAGTCACCAA	216 bp
SREBP1	NM_001113302.1	F-CAATGTGTGAGAAGGCCAGT R-ACAAGGAGCAGGTCACACAG	109 bp
LEPR	NM_001012285.2	F-GAACGTGGTTGGAAGATGTG R-GCCAGCACCATAACAGAATG	82 bp
LPL	NM_001075120.1	F-ACGATTATTGCTCAGCATGG R-ACTTTGTACAGGCACAACCG	130 bp
FABP4	NM_174314.2	F-TGAGATTTCCTTCAAATTGGG R-CTTGTACCAGAGCACCTTCATC	101 bp
FASN	NM_001012669.1	F-TAAGGTTCAAATTGCTGCGT R-TCCAGAGCGAAGGAGAGATT	138 bp
ACCα	NM_174224.2	F-CTCCAACCTCAACCACTACGG R-GGGGAATCACAGAAGCAGCC	171 bp
GAPDH	NM_001034034.2	F-CCAACGTGTCTGTTGTGGAT R-CTGCTTCACCACCTTCTTGA	80 bp

PBS, containing 0.5% TritonX-100, for 10 min on a shaking plate. 500 μL of 1 × Apollo reaction buffer was added in each well and allowed to react for 30 min. Then, cells were washed twice with PBS containing 0.5% Triton X-100 and stained in a 1 × Hoechst 33342 solution for 30 min. Cells were detected and analyzed using a fluorescent microscope at 100×. The cell proliferation activity was evaluated by the ratio of EdU-stained cells (with red fluorescence) to Hoechst-stained cells (with blue fluorescence).

Flow cytometry was performed as described previously [27]. Briefly, cells were collected by trypsin digestion and resuspended in 1 ml PBS. This was then mixed with 3 ml ice-cold ethanol and left overnight at −20°C. For cell cycle analysis, cells were centrifuged at 800 × g for 5 min, then resuspended in 4 ml PBS and hydrated for 15 min at room temperature. After that, re-centrifuged, washed with PBS a second time, and stained with 500 μl at 2 μg/mL DAPI (Sigma, USA) for 30 min. Finally, cells were filtered through 57-μm nylon mesh and subjected to flow cytometry using BD FACSAria III (BD Biosciences, USA).

2.7. Oil red O stain and adipogenesis analysis

For oil red O staining, fibroblasts on days 0, 2, 4, 6, and 8 after viral infection were washed twice with PBS and fixed with 10% formalin for 1 h at room temperature. After rinsing with 60% (v/v) isopropanol, cells were stained with filtered oil red O (Sigma, USA) for 30 min. Excess dye was quickly removed with 30% isopropanol. Cells were washed twice with distilled water and visualized using light microscopy. Ad-GFP treated fibroblasts were also stained with oil red O on days 0, 2, 4, 6, and 8 as negative controls. Lipid accumulation in cells was quantified using image J as mentioned above. Area fractions were collected for each treatment and normalized to control based on day 0 measures.

2.8. Statistical analysis

Statistical analyses were performed with SPSS 17.0 software. All data were presented as the mean ± SEM. Statistical analysis conducted using ANOVA, followed by t-test for multiple comparisons, and statistical significance was defined as "*"and "**" for at $p < 0.05$ and $p < 0.01$.

3. Results

3.1. Temporal and spatial expression pattern of C/EBPβ in cattle

In adult cattle, C/EBPβ expression was high in the intramuscular fat, large intestine, and perinephric fat; moderate in the spleen, lung, liver, rumen, kidney, and omasum; and low in the heart and muscle. Among newborn cattle, C/EBPβ expression was high in the liver and perinephric fat; moderate in the omasum, spleen, intramuscular fat, and rumen; and lowest in the lung, large intestine, muscle, kidney, and heart. Taken as a whole, the expression of C/EBPβ was greater in adults, especially in the intramuscular fat, large intestine, perinephric fat, spleen, lung, kidney, and heart of adult cattle ($p < 0.01$) (Fig. 1A).

The temporal expression pattern of *C/EBPβ* in cattle preadipocyte result showed that *C/EBPβ* expression gradually increased during the adipogenesis process to reach a peak at day 6, then decreased slightly (Fig. 1B). This result was consistent with the expression pattern of which in fat tissue between newborn and adult cattle. The expression level of *C/EBPβ* increased in perirenal fat and subcutaneous fat tissues in association with the development process of cattle, due to the gradual deposition. These results indicate that C/EBPβ may play a role in the fat deposition of cattle.

3.2. Troglitazone stimulated PPARγ expression but does not initiate adipogenesis in cattle fibroblasts

Cattle fibroblasts were isolated by explant culture method. Ear epidermis explants were cultured for about 6–8 d; it can be seen that the primary fibroblasts outgrow in pieces around (Fig. 2A). Then, the relatively homogeneous cattle fibroblasts were purified by 2 round short trypsinization method (Fig. 2B).

The PPARγ agonist troglitazone was used to test whether the troglitazone treatment could induce adipogenesis of primary fibroblasts. Meanwhile, the cattle preadipocytes were used as a control. As a result, cattle preadipocytes could differentiated spontaneously without cocktail induction (Fig. 3B) and the adipogenesis and lipid accumulation ability were improved by troglitazone treatment (44% vs. 25%, $p < 0.01$) (Fig. 3C). However, troglitazone treatment alone was not capable of inducing cattle fibroblast convert into adipocyte-like cells or to form lipid droplets (Fig. 3A, C), though the mRNA level of PPARγ was significantly increased after day 4 ($p < 0.05$, $p < 0.01$) (Fig. 3D). Moreover, C/EBPβ expression was not considerably affected by troglitazone (Fig. 3E). These results indicate that activation of PPARγ alone by troglitazone could not initiate adipogenesis of cattle fibroblasts.

3.3. C/EBPβ initiated the MCE process necessary for subsequent differentiation

As mitotic clonal expansion is a key process required for initiating adipogenesis, the effect of C/EBPβ expression on arrested fibroblasts was assessed using EdU labeling and flow cytometry analysis. C/EBPβ expression promoted fibroblast proliferation, with greater EdU incorporation than the control group ($p < 0.05$) (Fig. 4A, B). Flow cytometry showed that S-phase fibroblasts increased under C/EBPβ treatment, indicating that C/EBPβ promoted DNA replication and initiated the MCE process of arrested fibroblasts (Fig. 4C, D). In addition, real-time qPCR indicated that C/EBPβ down-regulated the expression of *p21* and *p27* ($p < 0.05$, $p < 0.01$) (Fig. 4E), the two cyclin-dependent kinase inhibitors,

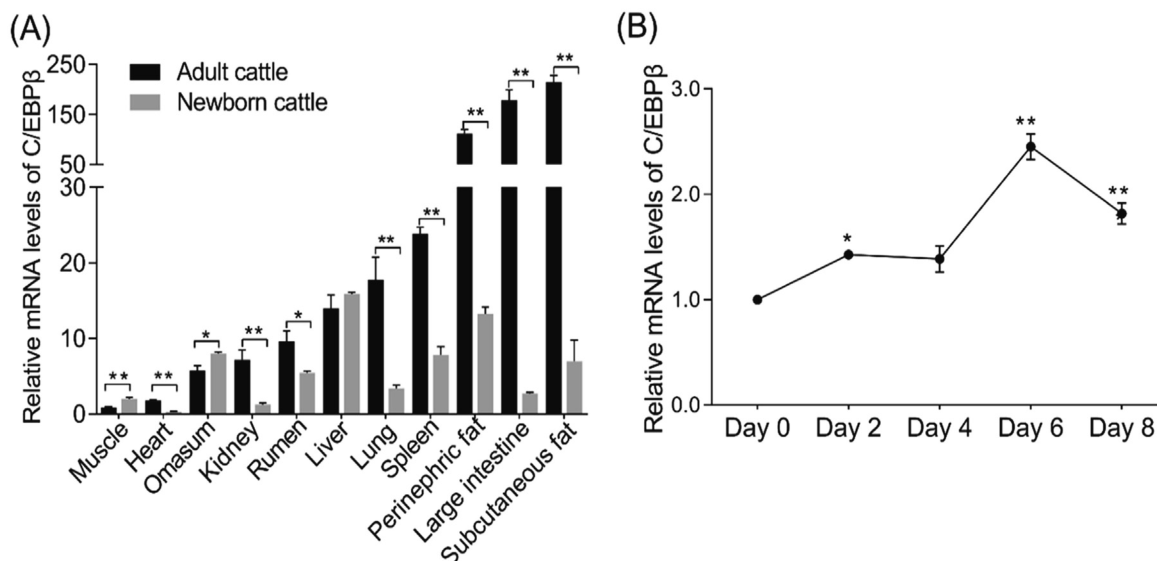

Fig. 1. Temporal and spatial expression pattern of *C/EBPβ* in cattle. (A) Tissue expression profile of *C/EBPβ* between newborn and adult cattle. The *C/EBPβ* mRNA expression level in various tissue between adults (24 months old, $n = 3$) and newborn ($n = 3$) was detected using real-time qPCR. (B) Relative mRNA levels of cattle *C/EBPβ* during preadipocyte differentiation on days 0, 2, 4, 6, and 8 after cocktail stimulus induction. Bars indicate means ± SEM ($n = 3$). Asterisks indicate significant difference between adult and newborn cattle or preadipocytes differentiated on day 0 and day 2, 4, 6, 8, where $^{*}p < 0.05$ or $^{**}p < 0.01$. *GAPDH* expression was used as a reference.

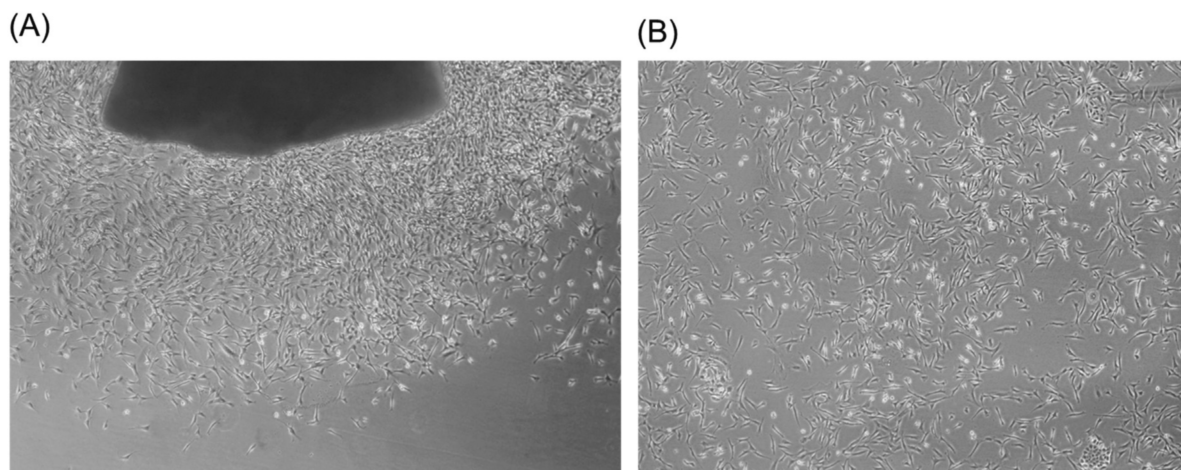

Fig. 2. Cattle primary fibroblasts isolated by explants culture method. (A) Primary fibroblasts outgrown near the explants inoculated to a petri dish at day 6 (40×). (B) Fibroblasts cultured and purified by differential digestion method (40×).

promoting G1-S phase transition. No significant changes were found in cyclin D2 (CCND2) (Fig. 4E).

3.4. C/EBPβ promoted adipogenic conversion of cattle fibroblasts by stimulating CEBPα and PPARγ expression

Oil Red O staining showed that Ad-C/EBPβ treatment initiated the adipogenesis of cattle fibroblasts, and lipid droplets accumulated gradually (Fig. 5A). Nearly a third (31.28%) of fibroblasts showed cytoplasmic triglyceride accumulation at day 8 (Fig. 5B), suggesting that C/EBPβ alone could promote adipogenic conversion of cattle fibroblasts. Real-time qPCR showed that the mRNA level of *C/EBPβ* was significant up-regulated by Ad-C/EBPβ infection after day 2 ($p < 0.01$) (Fig. 5C). Meanwhile, the *C/EBPα* and *PPARγ* were significantly activated by C/EBPβ at day 4 ($p < 0.01$) and reached a peak at day 8 ($p < 0.01$) (Fig. 5D, E). Interestingly, the *PPARγ* gene was initially down-regulated by exogenous C/EBPβ on day 2 prior to its increase on day 4 ($p < 0.01$) (Fig. 5D). Measures of *PPARγ* expression at 0, 24, 48, 72, and 96 h showed that it was inhibited by Ad-C/EBPβ at the early differentiation stages within the first 72 h (data not shown). No significant changes were found in *SREBP1* in the Ad-C/EBPβ group (Fig. 5F).

3.5. Effects of Ad-C/EBPβ on genes related to lipid metabolism

The mRNA levels of several key lipid metabolism genes such as *ACCα*, *FASN*, *FABP4* and *LPL* were significantly increased ($p < 0.05$, $p < 0.01$) after day 4 or day 8 during the adipogenesis process of cattle fibroblasts induced by C/EBPβ overexpression (Fig. 6A–D), while *LEPR* was inhibited by Ad-C/EBPβ after day 2 ($p < 0.05$, $p < 0.01$) (Fig. 6E).

4. Discussion

Intramuscular fat is visible as marbling, and contributes critically to the flavor and tenderness of beef cattle. Adipocytes and

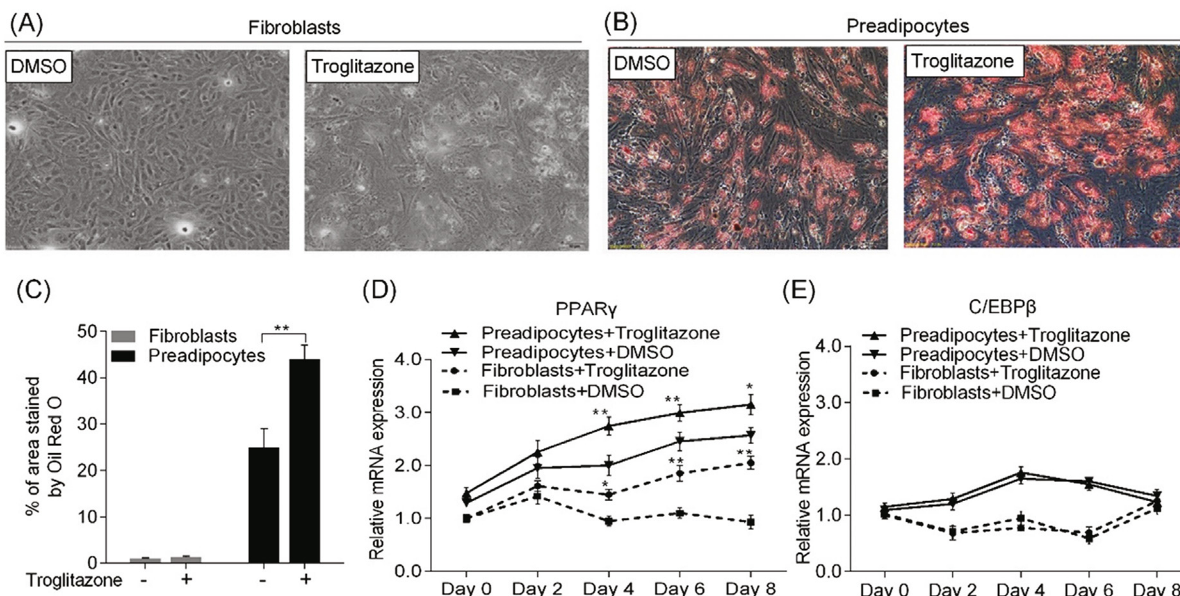

Fig. 3. Troglitazone stimulated PPARγ expression but did not initiate adipogenesis in cattle fibroblasts. (A) Oil red O dye after 90%-confluent cattle fibroblasts were treated with 10 μM troglitazone (using DMSO as a control) for 8 days, with medium changed every 2 days (100×). (B) Control preadipocytes treated with 10 μM troglitazone for 8 days (see above). (C) Lipid accumulation in fibroblasts and preadipocytes treated with troglitazone for 8 days, quantified using Image J software. Area fractions were collected for each treatment and normalized to controls ($n = 3$). (D) Relative PPARγ mRNA levels in fibroblasts and preadipocytes treated with troglitazone on days 0, 2, 4, 6, and 8. Bars indicate means ± SEM ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ versus day 0, respectively. (E) Relative C/EBPβ mRNA in fibroblasts and preadipocytes treated with troglitazone on days 0, 2, 4, 6, and 8. Data shown represent mean ± SEM ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ versus day 0, respectively.

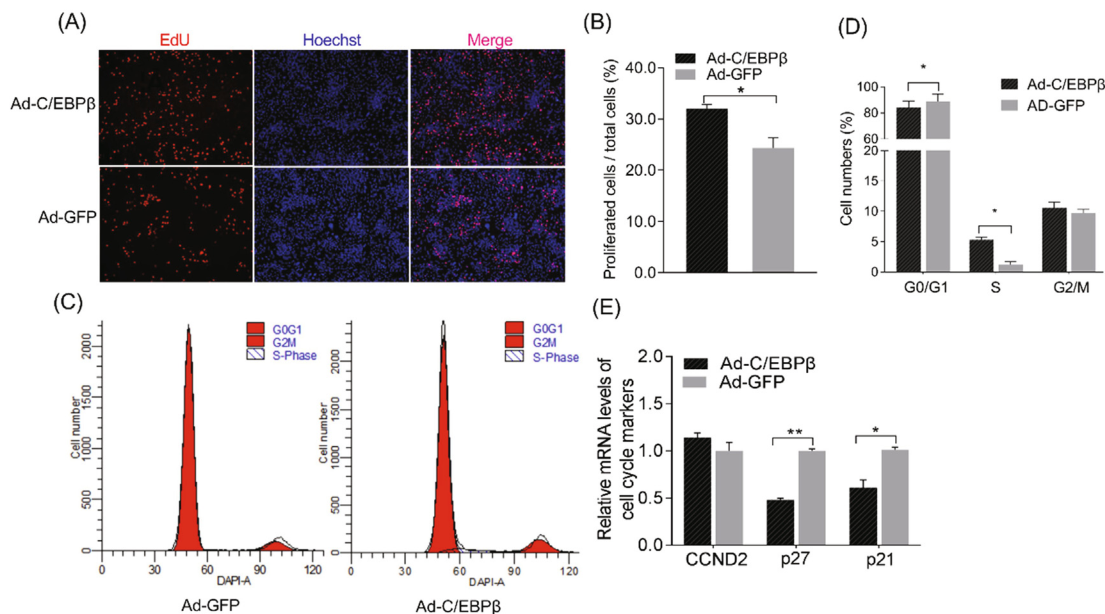

Fig. 4. C/EBPβ initiated fibroblasts' MCE process by downregulating the expression of two cyclin-dependent kinase inhibitors (p21 and p27). (A) Cell proliferation was analyzed using EdU dyeing at 48 h after Ad-C/EBPβ transfection. Cells in the process of DNA replication were stained by EdU (red) and cell nuclei were stained with Hoechst (blue). Magnification was 100×. (B). The percentage of EdU positive cells/total cells was quantified using Image J. (C) Cell cycle analysis was performed using flow cytometry at 48 h after Ad-C/EBPβ infection. (D) Statistical results of cell cycle analysis. (E) Relative mRNA levels of cell cycle markers were measured at 48 h after Ad-C/EBPβ infection. Data shown represent means ± SEM ($n = 3$). $^{*}p < 0.05$ or $^{**}p < 0.01$ versus control.

fibroblasts share immediate progenitor cells located in the extracellular matrix of primordial muscle fibers [28,29]. Early adipogenesis and fibrogenesis can be considered a competitive process, where the conversion of fibroblasts to adipocytes may reduce intramuscular connective tissue and increase intramuscular fat deposition to improve beef flavor and juiciness [30,31,32,33]. C/EBPβ is expressed immediately after cocktail hormone induction in 3T3-L1 cells and is critically important in adipogenesis. However, little is known about its mechanisms in cattle.

In the present study, *C/EBPβ* was found to be highly expressed in the intramuscular fat, large intestine, and perinephric fat of adult cattle, as well as in the liver and perinephric fat of newborn cattle.

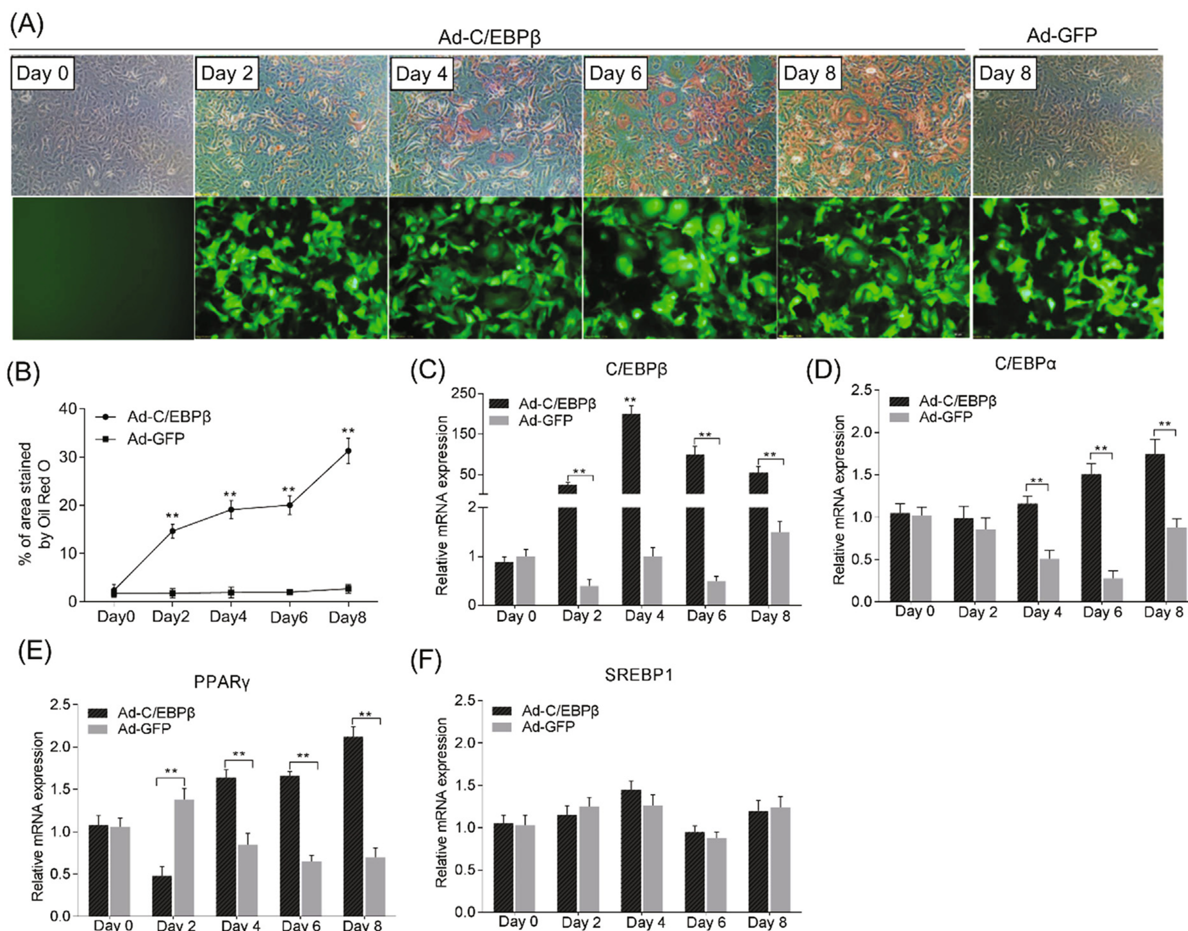

Fig. 5. C/EBPβ promoted adipogenic conversion of cattle fibroblasts by stimulating CEBPα and PPARγ expression. (A) Lipid accumulation in fibroblasts transfected with Ad-C/EBPβ was detected by Oil red O staining on days 0, 2, 4, 6, and 8 after transfection. The Ad-GFP group on day 8 was used as a control. Transfection efficiency was observed using dark field microscopy at 100×. (B) Lipid accumulation in fibroblasts treated with Ad-C/EBPβ and Ad-GFP was quantified using Image J. Area fractions were collected for each treatment and normalized to the control at day 0. (C) Relative mRNA levels of *C/EBPβ* after Ad-C/EBPβ infection. (D, E, and F) Relative mRNA levels of *C/EBPα*, *PPARγ* and *SREBP1* during the adipogenic conversion process induced by C/EBPβ. Data represents mean ± SEM (n = 3). *p < 0.05 and **p < 0.01 versus control.

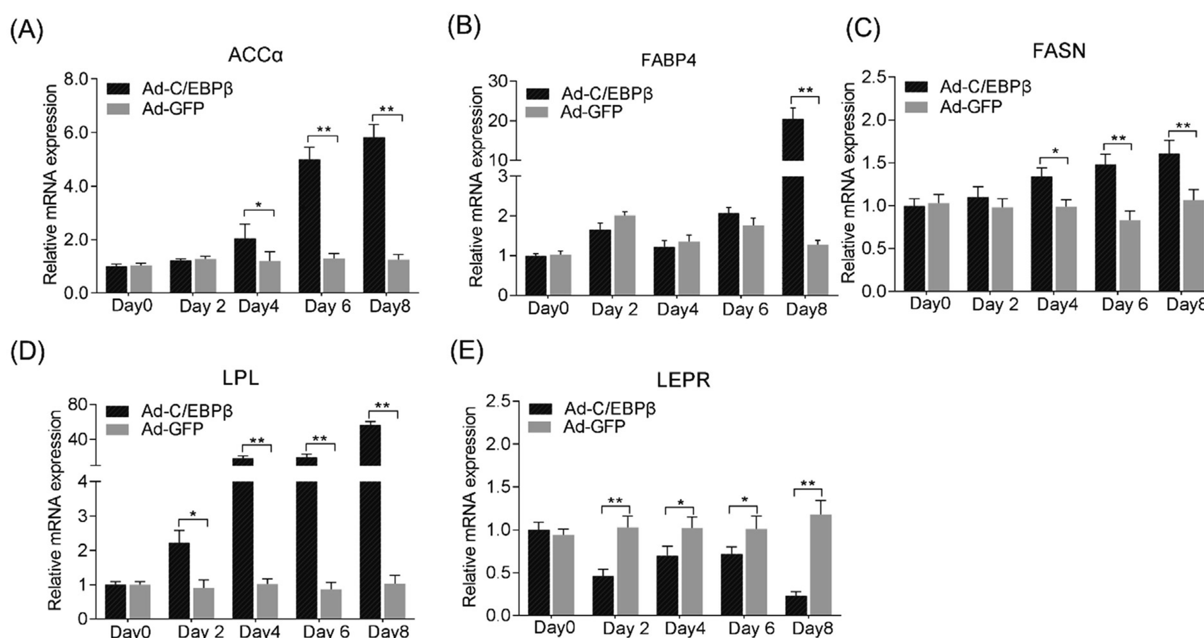

Fig. 6. Effects of C/EBPβ on the mRNA levels of adipogenic genes. After the cattle fibroblasts were infected by Ad-C/EBPβ and Ad-GFP, the relative mRNA levels of *ACCα* (A), *FABP4* (B), *FASN* (C), *LPL* (D), and *LEPR* (E) on days 0, 2, 4, 6, and 8 by real-time qPCR. Data shown indicate mean ± SEM (n = 3). *p < 0.05 and **p < 0.01 versus control.

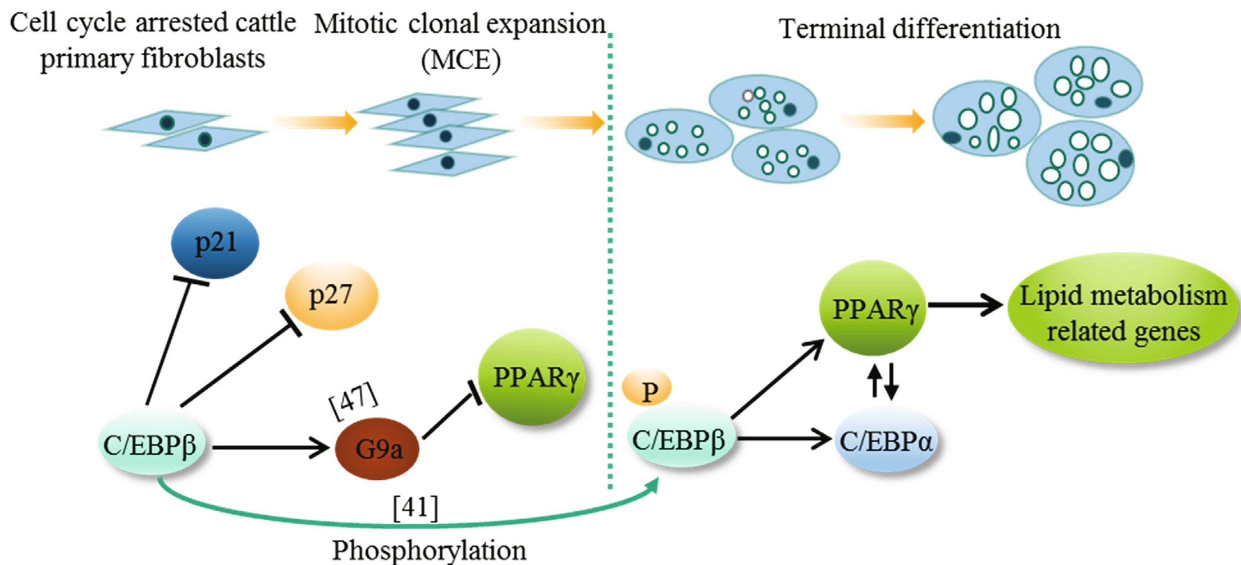

Fig. 7. A proposed schematic summary for the role of C/EBPβ in adipogenic conversion of cattle fibroblasts. The expression of C/EBPβ promotes cell cycle-arrested fibroblasts' G1-S transition by downregulating p27 and p21. The expression of PPARγ is also attenuated by C/EBPβ through the G9a dimethylation in order to guarantee the MCE process. Binding activity of C/EBPβ is activated by subsequent phosphorylation. Finally, PPARγ and C/EBPα, two key factors that regulate adipogenesis, are induced promoting terminal adipocyte differentiation and lipid metabolism gene expression.

In general, when comparing adults to newborn cattle, *C/EBPβ* levels were up-regulated and associated with the growth, the process accompanied with the intramuscular fat deposition. Meanwhile, during adipogenesis, *C/EBPβ* expression was gradually increased, then decreased toward the end of the process. This indicates that C/EBPβ might contribute to fat deposition overall.

Because adipogenesis and fibrogenesis can be considered competitive processes, cattle fibroblasts were isolated and used as a model to elucidate the role of C/EBPβ in the adipogenesis of cattle. We found that transient expression of C/EBPβ gene alone is sufficient to convert cattle fibroblasts into adipocyte-like cells, without the hormonal inducers normally required. These results were consistent with previous studies in non-adipogenic NIH-3T3 cells [34]. Interestingly, 3T3-L1 cells can also be induced to accumulate lipids in the absence of the hormone cocktail, when physiological levels of fatty acids are provided in the medium [35]. Thus, either transcription factors or nutritional factors alone can induce lipid accumulation in different cell types.

The initiation of adipogenesis occurs through a cascade of events. Mitotic clonal expansion is an early event which is indispensable for adipogenesis in 3T3-L1, and its inhibition can prevent adipogenesis entirely. C/EBPβ is a prerequisite for MCE in the adipogenesis process, and its overexpression can promote proliferation of cycle-arrested cattle fibroblasts by reentering the cell cycle and facilitating G1-S transition [36]. Real-time qPCR result showed that C/EBPβ initiated the MCE process by inhibiting expression of *p27* and *p21*, which are cyclin-dependent kinase inhibitors, in turn promoting the cell cycle-arrested fibroblasts to pass through the G1-S checkpoint. However, the role of C/EBPβ in cell proliferation is still somewhat unclear. C/EBPβ has been found to inhibit proliferation in malignant cells through a p21 dependent mechanism [37]. Nevertheless, C/EBPβ alone had little effect on p21 activation [38]. By contrast, blocking C/EBPβ binding activity has been shown to promote p27 expression and inhibit cell proliferation during the MCE process, as well as impede adipogenesis of 3T3-L1 [39]. As C/EBPα can bind to the promoter region and promote p21 expression, its function of C/EBPα seems to be purely antiproliferative [40]. C/EBPβ expressed immediately after adipogenic stimuli induction; however, its binding activity was obtained only after a long lag period until it is phosphorylated. After that, the C/EBPα and PPARγ were coordinately activated by phosphorylated C/EBPβ. This delay seems to guarantee the MCE process which is required for subsequent adipogenesis differentiation [41,42]. As such, C/EBPβ might have dual effects on cell proliferation depending on both the cell and cellular context (e.g., quiescent or dividing cells) [43].

Our data showed that C/EBPβ can activate and upregulate *C/EBPα* expression, which is consistent with other cell lines [44]. Unexpectedly, the expression of *PPARγ* was inhibited by exogenous C/EBPβ at early stage, but then rebounded steadily after day 4. As reported previously, C/EBPβ can activate expression of PPARγ during the adipogenesis process [45]. Nevertheless, we found that overexpression of C/EBPβ alone enhanced PPARγ expression at latter phases of cells conversion process. The initial downregulation observed in the present study provides a novel insight into the interaction between C/EBPβ and PPARγ in cattle fibroblasts.

C/EBPβ is able to bind to relatively 'closed' or only partially open chromatin, about one third of the transcription factors, during the adipogenesis process, can be bound by C/EBPβ which precedes chromatin remodeling [46]. Earlier studies have reported that, C/EBPβ can activate histone H3K9 methyltransferase G9a expression, which inhibits PPARγ and C/EBPα expression through H3K9 dimethylation of their promoters. Hence, C/EBPβ upregulates G9a and delays activation of PPARγ and C/EBPα so as to guarantee MCE [47,48]. Therefore, at the early stage of adipogenesis transdifferentiation, the downregulation of PPARγ by C/EBPβ may be due to the dimethylation on its promoter by G9a. However, no inhibition effect was observed on C/EBPα in cattle fibroblasts.

LPL, FASN, and ACCα, and FABP4 play central roles in controlling lipid accumulation and mobilization [49]. We found that overexpression of C/EBPβ enhanced *LPL*, *FASN*, *ACCα*, and *FABP4* gene expressions in cattle fibroblasts. These changes in gene expression also closely reflected lipid accumulation as assessed by Oil Red O staining. Exogenous C/EBPβ was observed to consistently down-regulate *LEPR* gene expression, suggesting a possible feedback regulatory mechanism between the leptin signaling pathway

and C/EBPβ [50]. At least one study in goose adipocytes reports that knockdown of LEPR represses several adipocyte differentiation related genes, implying a link between these pathways [51].

In conclusion, we have shown that *C/EBPβ* levels are upregulated during cattle growth, with the process accompanying intramuscular fat deposition. C/EBPβ expression can convert cattle fibroblasts into adipocyte-like cells without hormone induction by initiating the MCE process that inhibits early-stage *p27*, *p21*, and *PPARγ* expression. This then induces cellular adipogenesis by upregulating the expression of *C/EBPα*, *PPARγ*, *LPL*, *FASN*, *ACCα* and *FABP4* (as illustrated in Fig. 7). The present study provides novel insights into the role of C/EBPβ in cattle and may accelerate the elucidation of the C/EBPβ gene-signaling pathway, with potential for use in selection for favorable meat quality traits of cattle.

5. Conclusions

We can conclude that C/EBPβ converts bovine fibroblasts into adipocyte-like cells without hormone induction by initiating the MCE process that inhibits early-stage p27, p21, and PPARγ expression, which in turn induce cellular adipogenesis by upregulating the expression of C/EBPα, PPARγ, LPL, FASN, ACCα, and FABP4. The present study provides an insight into the regulatory role of C/EBPβ in bovine adipogenesis and may elucidate the mechanism of C/EBPβ gene-signaling pathway in the context of its potential role as a molecular marker for intramuscular fat in bovine species.

Conflict of Interest

The Authors declare no conflict of interest.

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