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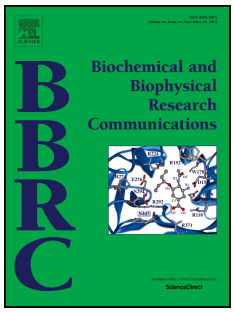

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Suppression of CD36 attenuates adipogenesis with a reduction of P2X7 expression in 3T3-L1 cells

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Abstract

Adipogenesis is a process of differentiation from preadipocyte into adipocyte, and is regulated by several transcription factors, including the peroxisome proliferator-activated receptor gamma (PPARγ) and the CCAAT-enhancer-binding protein alpha (C/EBPα). CD36 is a membrane protein which contributes to the metabolic disorders such as obesity. Although the previous study demonstrated CD36 participated in the progression of adipogenesis, the mechanism is still unclear. We report here that knockdown of CD36 expression by CD36

small interfering RNA (siRNA) resulted in a reduction of adipocyte differentiation and adipogenic protein expression. In addition, purinergic receptor P2X, ligand-gated ion channel 7 (P2X7) was downregulated in CD36-knockdown 3T3-L1 cells, suggesting that the suppression of CD36 attenuates adipogenesis via the P2X7 pathway in 3T3-L1 cells.

Keywords: CD36, adipogenesis, ATP, P2X7

1. Introduction

Obesity is a risk factor for several diseases, including type II diabetes, cancer, and cardiovascular disease[1]. In humans, white adipose tissue (WAT) is the main fat mass, therefore, figuring out the molecular mechanisms of the development of WAT is important. Adipogenesis is the primary process of conversion of preadipocyte into adipocyte[2], which is still not well understood. It is well-described that the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors and Peroxisome proliferator-activated receptor-γ (PPARγ) act as key regulators in the transcriptional process of adipogenesis[3, 4]. Mitochondrial biogenesis was also involved in the progress of adipogenesis[5, 6]. At the early stage of adipogenesis, mitochondrial biogenesis and metabolism are crucial for the initiation and promotion of adipocyte differentiation[7]. The generation of adenosine 5'-triphosphate (ATP) by mitochondria is to ensure the normal metabolism of lipid synthesis during the progress of adipogenesis[8].

CD36 is a receptor for long-chain free fatty acids (FFAs) that has been shown to facilitate FFA uptake into the adipose tissue and muscle of rodents and humans. CD36 also has been recently implicated in several other aspects related to inflammation, immune responses, atherogenesis and thrombosis[9, 10]. Several groups have found that adipocyte bind oxLDL in a CD36-dependent manner that results in insulin resistance in adipose tissue[11, 12]. The previous study has demonstrated that CD36 plays a critical role in adipose tissue biology[13]. However, the molecule mechanism of CD36 in adipogenesis remains unclear.

P2X7 receptor is a ligand-gated cation channel encoded by P2RX7 gene (purinergic receptor P2X, ligand-gated ion channel 7), belonging to the P2X receptor family. Activation of P2X7 by extracellular ATP causes the movement of Ca^{2+}, Na^{+} and K^{+} across the plasma membrane. P2X7 is predominately expressed on cells of the hematopoietic, epithelial, mesenchymal and neural lineages[14]. Subsequently, P2X7 plays important roles in inflammation, immunity, bone homoeostasis, neurological function and neoplasia. P2X7 receptors have also been reported to connect with lipid metabolism. For instance, male mice

lacking functional P2X7 receptors develop ectopic lipid accumulations as they age, and P2X7R plays a generalised role in regulating lipid storage and metabolism in vivo[15]. The progression of adipogenesis is associated with the generation and consumption of ATP. Activation of P2X7 by extracellular ATP causes the downstream events, including pro-inflammatory mediators. However, the effect of P2X7 on adipogenesis is unknown.

In this study, we demonstrated that CD36 was increased after induction of differentiation in 3T3-L1 cells, while knocking down CD36 suppressed adipogenesis by both decreasing the delivery of fatty acids to mitochondria and inhibiting mitochondrial biogenesis. This may suppress adipogenesis by inhibiting P2X7.

2. Material and Methods

2.1. Cell culture and adipocyte differentiation

3T3-L1 preadipocytes were provided by Prof Fiona M. Watt (Kings College London) and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum (Sigma, UK). The cells were cultured for 2 days after confluence (day 0), differentiation was induced in DMEM containing 10% fetal bovine serum (Labtech, UK), 5µg/ml insulin (Sigma), 1µM dexamethasone, and 0.5mM 3-isobutyl-1-methylxanthine (Sigma) for 2 days. After 2 days, the medium was changed to growth medium containing 5µg/ml insulin for another 2 days. Then the medium was replaced with growth medium and replaced every other day until day 8.

2.2. Oil Red O staining

In brief, following fixation of the mature adipocyte in 10% (v/v) formaldehyde solution for 30 minutes, the cells were stained with 0.5% Oil Red O for 30 minutes at room temperature and were washed three times with PBS. Cells were visualised by a microscopy. Oil red O was extracted with 100% isopropanol and concentration were determined by measuring absorbance at 510 nm.

2.3. Small interfering RNA

Small interfering RNA (siRNA) transfections were performed with Lipofectamine RNAiMAX (Invitrogen, UK), using siRNA targeting murine CD36 (catalogue number AM16708 160083; Invitrogen), or a nontargeting control (catalogue number 12935300; Invitrogen) at a final concentration of 50 nM. At the day 4, we used another transfection for persistent inhibition of CD36 protein synthesis.

2.4. Protein isolation and Western blot analysis

3T3-L1 cells were lysed in RIPA buffer supplied with protease inhibitor cocktail (Sigma).

20-30 µg protein was separated by SDS-PAGE and then transferred to the PVDF membrane, blocked with 5% BSA 1hour at room temperature and incubated the primary antibody overnight at the cold room. Antibodies against PPARγ, FABP4, cebpα, were purchased from Cell Signaling Technology (USA). The antibody for β-actin was obtained from Sigma. The antibody against CD36 (MAB1955) was purchased from R & D company (UK). The p2x7 antibody was purchased from Abcam (UK). After incubated with second antibody 1h at room temperature, washed the membrane with TBST three times, then, the membrane was exposed to ECL. The membrane was stripped by stripping buffer (Sigma) and re-probed for actin as loading control.

2.5. Real-Time RT-PCR Analysis

RNA was extracted using TRIzol Reagent (Invitrogen) according to manufacturers' instructions. The total RNA (2 µg) per sample was made into cDNA using reverse transcriptase (Applied Biosystems, UK). Prepared cDNA was amplified using the LightCycler 96 system (Roche) and analysed using the SYBR Green PCR Master Mix (Lab science, UK). Cycle threshold (Ct) values were normalised for amplification using b actin, and the data were analysed using the Ct method. The primer sequences used in this study are shown in Table S1.

2.6. Statistical analysis

Data is presented as the mean±SEM of three independent experiments. Statistical significance was analysed by an unpaired t test. Statistical significance was set at $P<0.05$.

3. Results

3.1. CD36 is increased during the adipogenic differentiation

To clarify the precise function of CD36 during adipogenesis, we first observed the different time course of CD36 expression during the 3T3-L1 adipocyte differentiation into mature adipocyte. As shown in Figure 1A and B, the CD36 protein level was increased after adipogenic induction and maintained a high-level of expression in the process of adipocyte differentiation. We also detected the expression of several key transcription factors in adipogenesis, such as PPARγ , cebpα and FABP4, increased appreciably in the progress of adipogenesis. The mRNA levels of CD36 and adipogenic factors exhibited a similar expression with protein levels (Fig 1C). The gene, such as SREBP1 which is involved in lipogenesis were also increased during the adipogenic differentiation (Fig 1C).

3.2. Knockdown of CD36 decreased lipid accumulation

To figure out whether CD36 participates in the adipogenic process, we knockdown CD36

by transfecting CD36 siRNA into 3T3-L1 preadipocyte during the differentiation induction. CD36 knockdown resulted in the markedly decreased numbers of mature adipocyte by Oil Red O staining (Fig 2).

3.3. Suppression of CD36 reduced the adipogenic gene expression

The suppression of CD36 significantly suppresses the expression of adipogenic marker genes and proteins, such as FABP4, cebpα, PPARγ (Fig 3A, B). Moreover, mitochondrial development is associated with adipocyte differentiation; we tested the mRNA expression of the mitochondrial gene including peroxisome proliferator activated receptor (PPAR) coactivator-1α (PGC-1α) and mitochondrial transcription factor A (mtTFA) which were significantly decreased along with knockdown of CD36 (Fig 3C).

3.4. P2X7 was downregulated in CD36-knockdown 3T3-L1 cells

we demonstrated that suppression of CD36 decreased the expression of P2X7 (Fig 4A, B, C). To assess whether the effect of CD36 is mediated by PPARγ, we added rosiglitazone into the differentiation progress. We found that rosiglitazone could reverse the inhibition of adipogenesis and P2X7 downregulation which were caused by knockdown of CD36 (Fig 4D, E). This outcome suggests that suppression of CD36 in preadipocyte decreases the generation of ATP or increases the consumption of ATP during the progress of adipogenesis.

4. Discussion

In this study, we have demonstrated that CD36 regulates the progression of adipogenesis by showing that the suppression of CD36 reduced lipid accumulation accompanying reductions of the adipogenic markers, such as PPARγ, Interestingly, P2X7 was also down regulated by CD36 knockdown. To our knowledge, this is the first report linking the role of CD36 with P2X7 on adipogenesis process.

CD36 has been shown to regulate fatty acid uptake in several cell types, including adipocyte, macrophage, microvascular endothelial cells and platelets[16]. It has been demonstrated that CD36 is located on the outer mitochondrial membrane, thereby contributing to the regulation of mitochondria fatty acid transport[17]. In this context, suppression of CD36 in preadipocyte may reduce the supply of fatty acids to mitochondrial for beta oxidation, decreasing the generation of ATP.

Numerous mitochondrial proteins can be detected in 3T3-L1 preadipocyte after induction of the differentiation, which means mitochondrial biogenesis mediated by the mitochondrial proteins in adipogenesis has a crucial function[18]. In human mesenchymal stem cells, mitochondria respiration regulated adipogenic differentiation[6]. In our present study,

suppression of CD36 using siRNA transiently inhibit the expression of PGC1a which is one important mitochondrial biogenesis gene. The mitochondrial transcription factor was also reduced after knockdown of CD36. These results demonstrated that knockdown of CD36 suppressed the formation of mitochondrial by which ATP production may be reduced.

The generation of ATP is mainly from mitochondrial. So far, the effect of ATP on adipogenesis is controversial. One side, there had been several pieces of evidence suggesting that the relationship between mitochondrial ATP production and adipogenesis of preadipocyte was strong[19]. Several ATP synthase were increased during the adipogenesis of 3T3-L1 preadipocyte[20]. However, on the other hand, the previous study demonstrated that the consumption of ATP during the adipogenesis resulted in down regulation of the generation of ATP during adipocyte differentiation[8]. Although after addition of exogenous ATP alone failed to stimulate adipogenesis of 3T3-L1 preadipocyte[21]. Suppression of mitochondrial ATP production by depletion of mitoferrin 1/2 repressed the adipogenesis of preadipocyte[22]. These results suggest that absolute levels of ATP may not be a decisive factor for adipogenesis, but P2X7, which is a receptor for ATP could be an important factor in regulating adipogenesis. The previous study has demonstrated that P2X7 was involved in the regulation of bone cell formation, survival and function. Recent studies have suggested the possibility that P2X7 facilitate the invasion and survival of tumor cell[23, 24]. However, the mechanisms of P2X7 modulates lipid metabolism like adipogenesis was unknown. In this study, we showed for the first time that P2X7 was downregulated after knockdown of CD36 which suppress the adipogenesis.

In conclusion, the present study showed that suppression of CD36 decreased the expression of adipogenic genes and mitochondrial biogenesis gene during adipogenesis in 3T3-L1 cells. Furthermore, the suppression of mitochondrial formation resulted in a reduction of ATP generation by which the downregulation of P2X7. These results indicate that CD36 and P2X7 are vital factors in the regulation of adipocyte differentiation.

Author's contributions

HQ G and XZ R conceived and designed the study. HQ G and DY L conducted the experiments. YX C, L Z, P Y, L W and HQ G analyzed the data. HQ G and XZ R wrote the manuscript. All authors reviewed the manuscript.

Conflicts of interest

The authors have no conflicts of interest exist.

Acknowledgments

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Fig 1, Expression of CD36 during adipogenesis in 3T3-L1 cells.

A, Adipogenesis of 3T3-L1 cells was induced with MDI. The protein levels of CD36, cebpβ, cebpα, and PPARγ were compared at different time-points by immunoblotting. Actin was used as loading controls. B, Quantification of immunoblots. C, mRNA levels at time points of differentiation of 3T3-L1 preadipocyte (*p<0.05 and **p<0.01 compared with 0D)

Fig 2, knockdown of CD36 attenuate lipid accumulation in 3T3-L1 preadipocyte.

After 8 days of differentiation, lipid droplets were stained with Oil Red O. Lipid accumulation was quantified by extracting Oil Red O. (** P<0.01 compared with sinc)

Fig 3, suppression of CD36 decrease the expression of adipogenic genes in 3T3-L1 preadipocyets

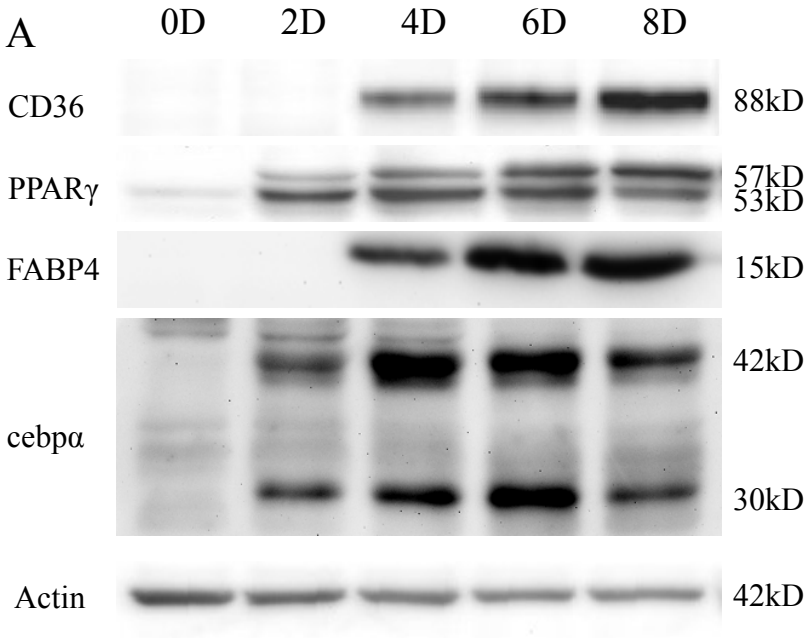
A, after induction of differentiation, the cells were collected at indicated time from nontarget and CD36-knockdown cells for Western blot analysis of CD36, PPARγ, cebpα, FABP4 and β-actin. β-actin was used as a loading control. B, Quantification of immunoblots. C, mRNA levels at time points of differentiation of 3T3-L1 preadipocyte transfected with siNC and siCD36. (*p<0.05 and **p<0.01 compared with siNC)

Fig 4, Expression of P2X7 in CD36-knockdown 3T3-L1 cells.

The control and CD36-knockdown 3T3-L1 cells were differentiated under the addition of differentiation medium. cells were harvested at indicated time points after induction. A, Protein expression of P2X7 was analyzed by Western blot. B, Quantification of immunoblots.

C, The mRNA expression of P2X7 was analyzed by RT-PCR and normalized relative to β-actin. D, 3T3-L1 cells were induced to differentiate into adipocytes for 8 d with (+Rosi) 1 μM rosiglitazone and protein was extracted at day 8 and determined using WB. E, Quantification of immunoblots. (*$p<0.05$ and **$p<0.01$ compared with siNC)

Fig1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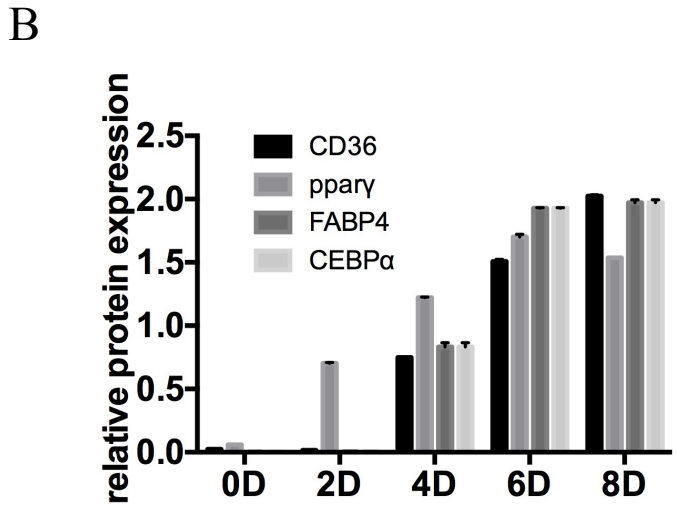
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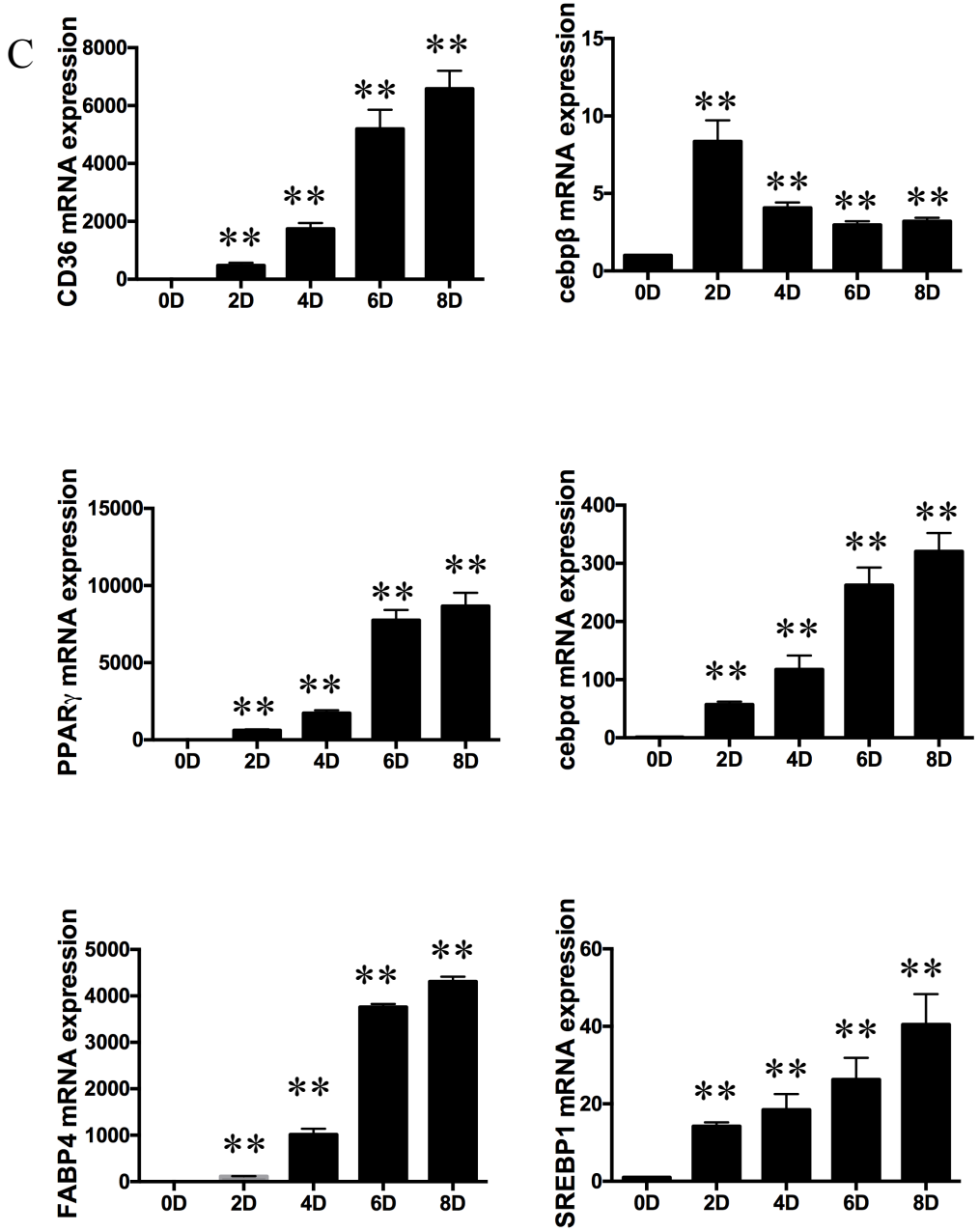
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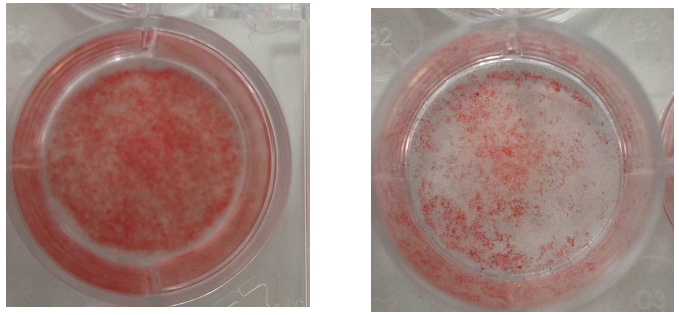
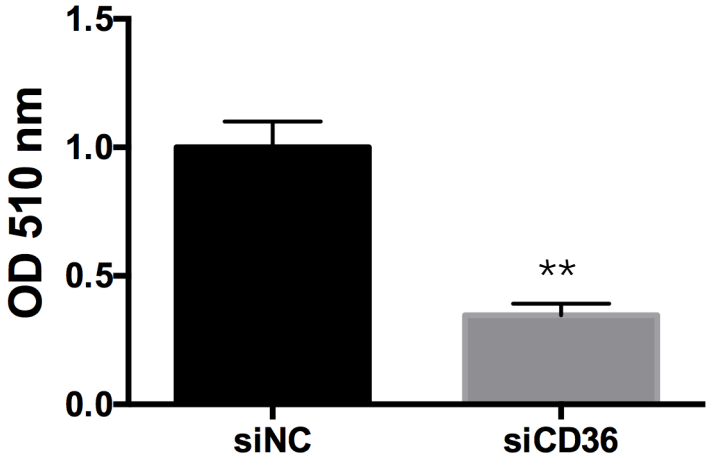
Fig2





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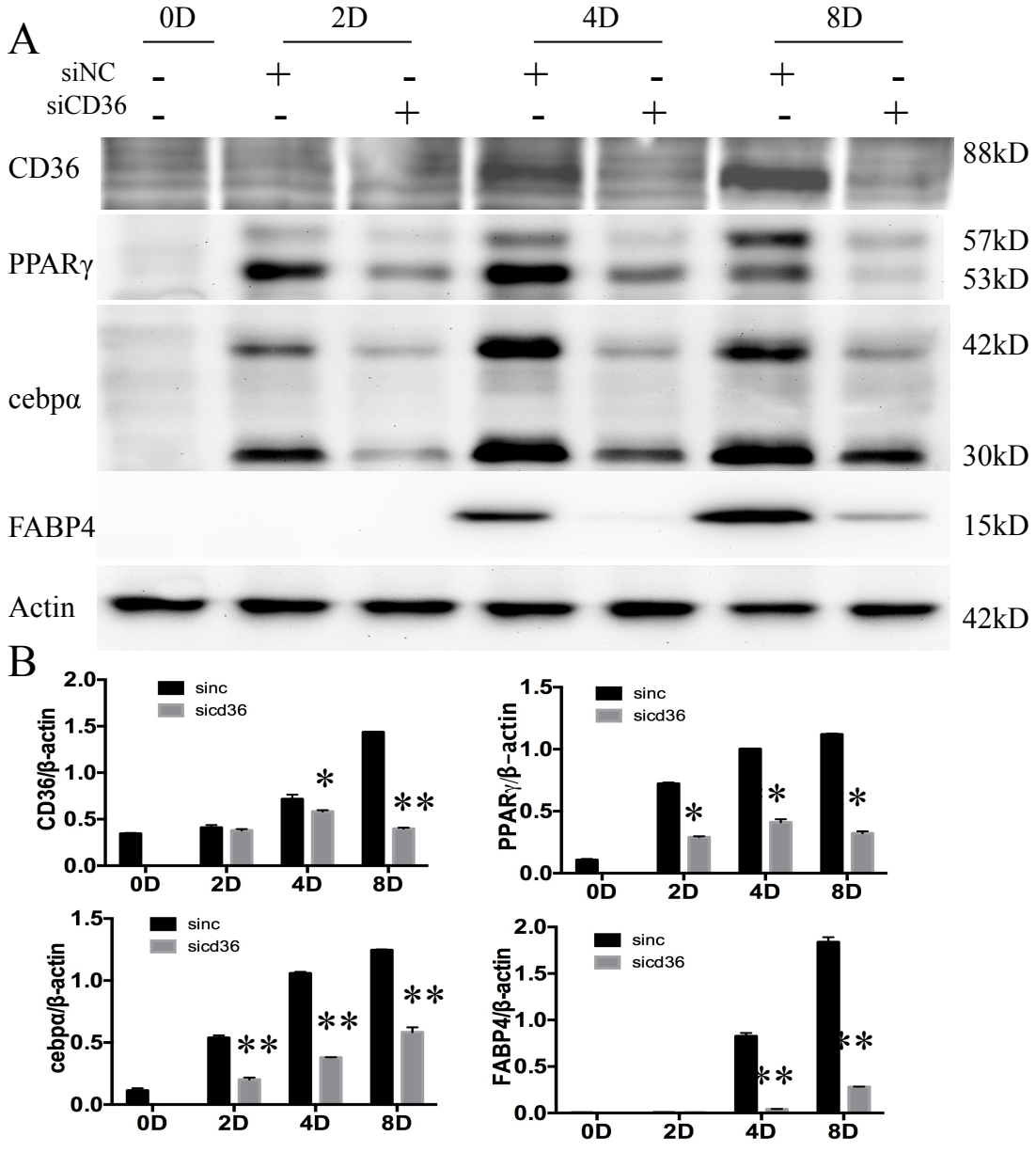
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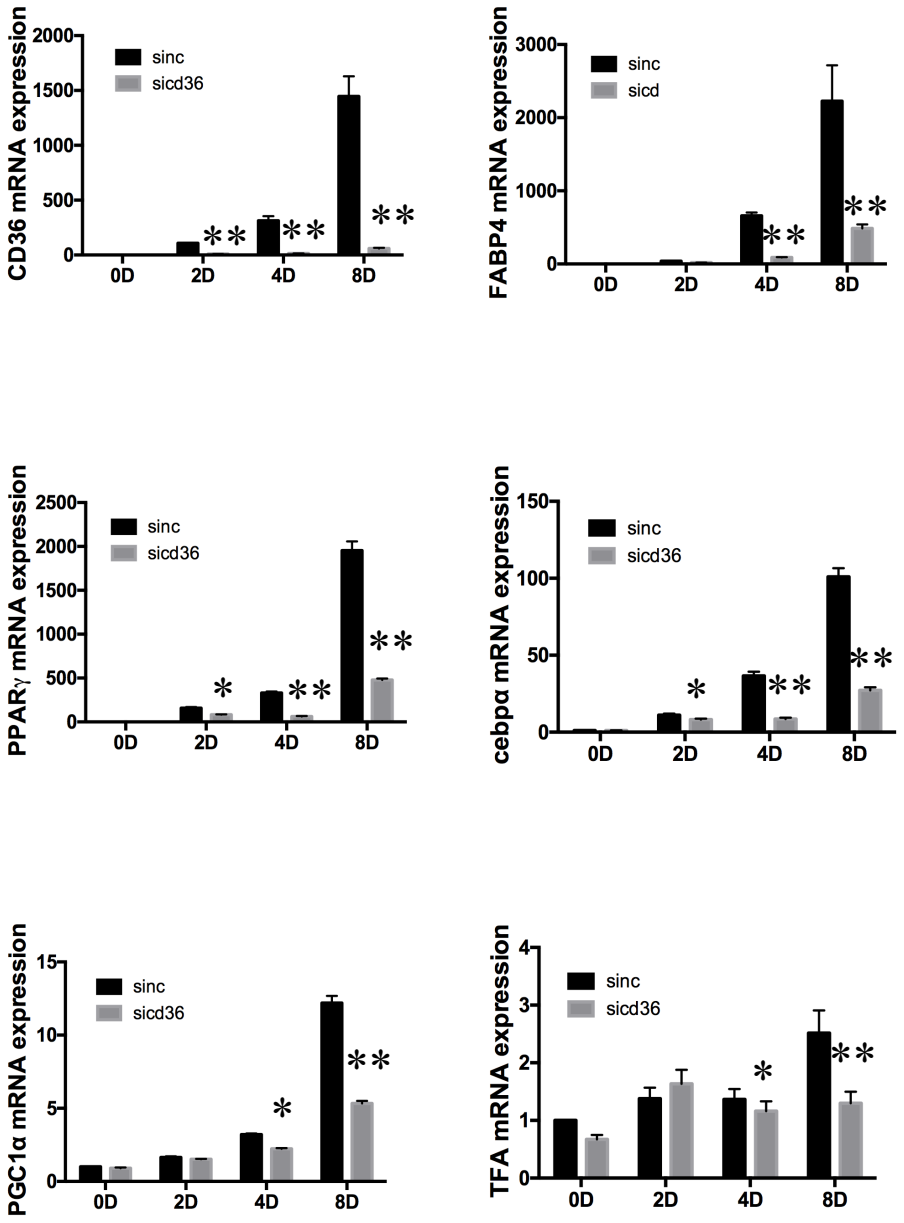
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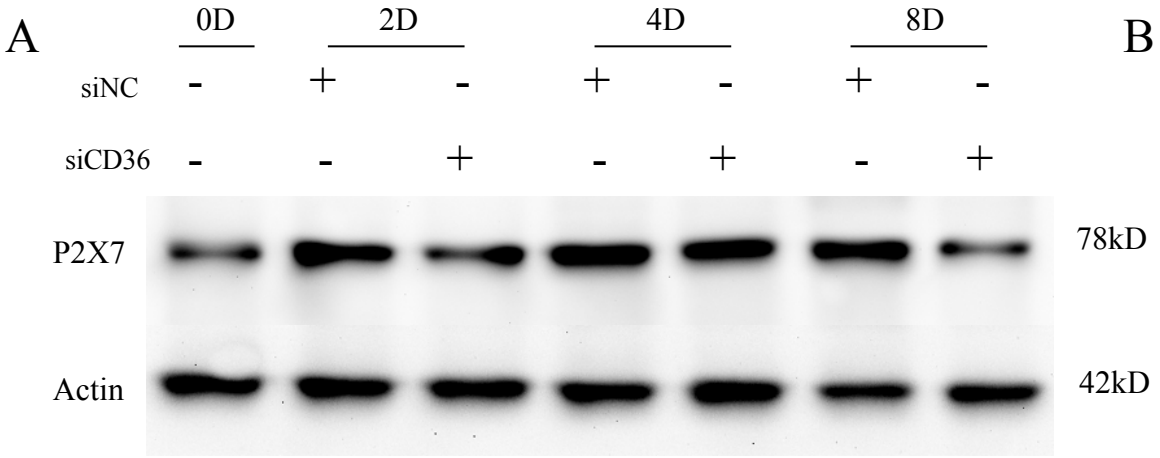
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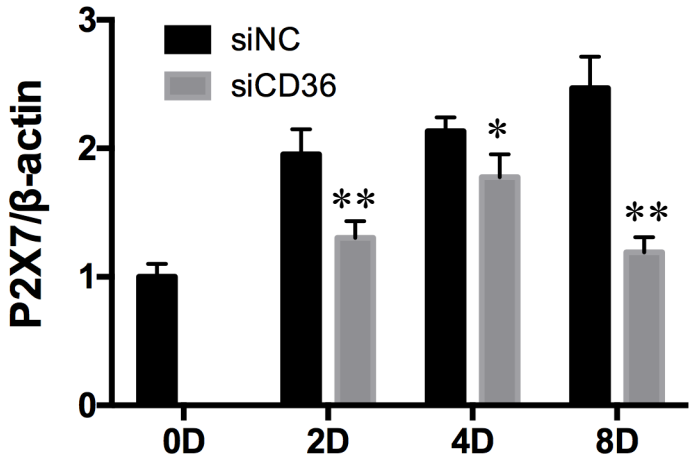
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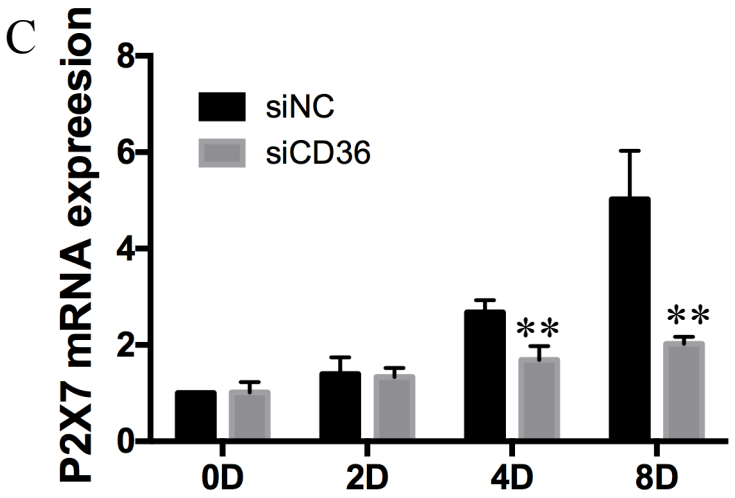
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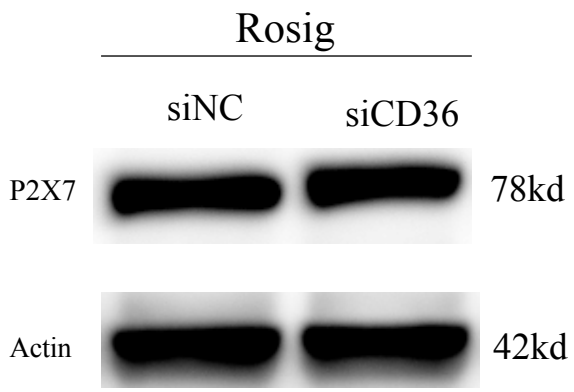
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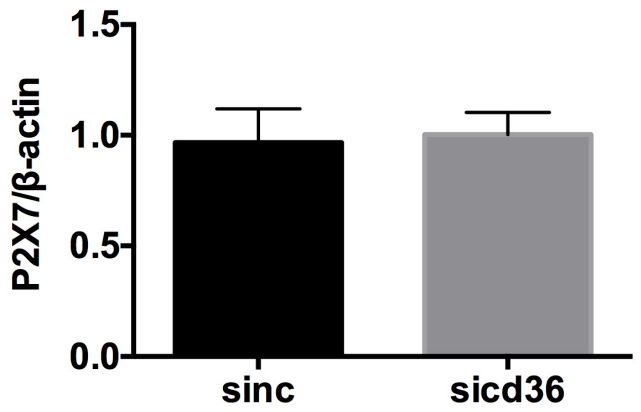
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Highlights

1. CD36 regulated adipogenesis in 3T3-L1 cells.
2. Suppression of CD36 downregulated the expression of adipogenic gene.
3. P2X7 was downregulated in CD36-knockdown 3T3-L1 cells.