Dlx5 Represses the Transcriptional Activity of PPARγ

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Peroxisome proliferator-activated receptor gamma (PPARγ) is a master transcription factor in adipocyte differentiation, while distal-less homeobox 5 (Dlx5) is essential for initiating osteoblast differentiation by driving Runx-related transcription factor 2 expression. Considering that adipocytes and osteoblasts share common progenitors, there is a reciprocal correlation between bone and fat formation. However, the mechanism by which Dlx5 controls PPARγ remains unclear. We elucidated that Dlx5 physically binds to PPARγ during immunoprecipitation; in particular, the ligand-binding and DNA-binding domains of PPARγ were involved in the interaction. Transcriptional activity of PPARγ was significantly decreased by Dlx5 overexpression, whereas the opposite results were detected with Dlx5 knockdown. Rosiglitazone, a PPARγ agonist, further enhanced the PPARγ-induced transcriptional activity; however, Dlx5 overexpression effectively repressed the rosiglitazone-mediated increase in activity. Finally, DNA-binding affinity assay revealed that Dlx5 interrupts the interaction of PPARγ with the PPARγ response element promoter. In conclusion, our findings indicate that Dlx5 impedes PPARγ-induced activity, and it may be useful for managing diabetes drug-mediated obesity.

Key words distal-less homeobox 5; peroxisome proliferator-activated receptor γ; transcription

INTRODUCTION

Obesity increases the risk of several lifestyle-related diseases, such as cardiovascular disease, hypertension, type 2 diabetes, and cancer because of serious health problems. The prevalence of obesity is increasing worldwide, resulting in diseases that increase healthcare costs. There are many causes of obesity, but from a physiological point of view, increasing numbers and rapid growth of adipocytes contributes to the occurrence of obesity; therefore, obesity is defined as an overabundance of white adipose tissue. Adipocytes play a crucial role in regulating consistent availability of energy by accumulating lipids; however, the overabundance of lipids in adipocytes, increase in adipocyte size, or generation of new adipocytes lead to obesity and related diseases. Adipocytes are differentiated from mesenchymal stem cells (MSCs) in many fatty tissues. This distinction occurs in two phases: lineage commitment and terminal maturation. MSCs are converted into committed whole adipocytes and then differentiated into mature adipocytes. Therefore, inhibiting adipocyte differentiation is an effective strategy for the prevention and treatment of obesity.

MSCs are postnatal adult stem cells identified from specific locations in the adult organism, including bone marrow, fat, skin, or skeletal muscle. MSCs have been elucidated to be differentiated into mature bone, adipose tissue, cartilage, and tendon. The lineage commitment of MSCs to adipocytes or other tissues is important in determining adipocyte fate. In particular, a reduced capacity for osteoblast differentiation by MSCs leads to increased adipocyte differentiation, subsequently resulting in an increase in fat accumulation. Adipocyte differentiation and fat formation are controlled by transcription factors such as CCAAT-induced lead protein (C/EBP) family members (C/EBPβ, C/EBPδ) and peroxisome proliferative activation receptor gamma (PPARγ), while osteoblast differentiation is regulated by Runx-related transcription factor 2 (Runx2). The relative activity of PPARγ versus Runx2 determines whether a mesenchymal progenitor undergoes osteoblast or adipocyte differentiation. Indeed, Runx2 deficiency in mice resulted in a loss of bone formation and spontaneously induced adipocyte differentiation, whereas PPARγ insufficiency in embryonic stem cells enhanced osteoblast differentiation and bone formation.

The Drosophila distal-less (Dll) gene, initially characterized in Drosophila, is required for the development and function of distal limbs, the forebrain, and branchial arches. The vertebrate distal-less (Dll) gene, which contains a homeodomain region that is highly conserved with Drosophila, encodes several types of homeodomain transcription factors and play an important role in early embryonic structures. Dlx5 enhances the development of mineralized bone nodules by regulating Runx2 activity and expression following the stimulation of bone morphogenetic protein signaling in almost every development stage. Considering that Dlx5 regulates Runx2 expression and that Runx2 is an important control factor determining the fate of MSCs for osteoblast differentiation, it is likely that Dlx5 adversely affects adipocyte differentiation.

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MATERIALS AND METHODS

Cell Culture  First, 3T3-L1 preadipocytes were maintained in Dulbecco’s modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY, U.S.A.) with 10% fetal bovine serum (FBS; Gibco BRL) and 1% penicillin–streptomycin antibiotics (Thermo Fisher Scientific, Waltham, MA, U.S.A.) at 37°C and 5% CO₂. To induce adipocyte differentiation, 0.5 mM 3-isobutyl-1-methylxanthine, 1mM dexamethasone, and 10mg/mL insulin (MDI) were used.

Plasmids and Reagents  For plasmids expressing N-terminal epitope-tagged mouse Dlx5 and PPARγ plasmids were subcloned with a Myc or HA tag into CMV promoter-derived mammalian expression vector (pCS4). Deletions of PPARγ (activation function 1 (AF1) domain, DNA-binding domain, and ligand-binding domain) were generated by PCR-based mutagenesis and confirmed by DNA sequencing. The constructs for adipose-specific fatty-acid-binding protein (aP2)–Luc and PPARγ response element (PPRE)-Luc have been described previously. 19) For Dlx5 knockdown experiments, oligonucleotides for short hairpin RNA (shRNA) were generated by targeting a 19-base sequence of the mouse Dlx5 gene: 5ʹ-CGA CGA CTA CAT TGA ACA A-3ʹ (shDlx5) to pSUPER vector (shCon; Oligo Engine, St. Madison, WA, U.S.A.).

Transient Transfection and Luciferase Reporter Assays  For transient transfection, polyethylenimine (PEI; Polysciences, Warrington, PA, U.S.A.) was used as previously described.20) The 3T3-L1 cells were plated in 24-well plates 1 d before transfection at a density of 3 × 10⁴ cells/well. The cells were co-transfected with a luciferase reporter gene (PPRE-Luc or aP2-Luc) and a plasmid cytomegalovirus (pCMV)-β-gal along with the indicated expression vectors (HA-PPARγ, Myc-Dlx5, shCon and shDlx5). The Luciferase Reporter Assay Kit (Promega, Madison, WI, U.S.A.) was used to measure the luciferase activity, and all experiments were performed in triplicate.

Immunoblotting and Immunoprecipitation  The 3T3-L1 cells were transfected for 48h and lysed in lysis buffer. Immunoblotting was performed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the proteins were transferred to a polyvinylidene fluoride mem-

![Fig. 1. Interaction between Dlx5 and PPARγ](image)

(A) 3T3-L1 cells were transfected with Myc-Dlx5 (0.5µg) and/or HA-PPARγ (0.5µg). Immunoprecipitation was performed with Myc or immunoglobulin G (IgG) antibody followed by immunoblotting with HA antibody. (B) 3T3-L1 cells were treated with 0.5mM 3-isobutyl-1-methylxanthine, 1mM dexamethasone, and 10mg/mL insulin (MDI) for 3d. Immunoprecipitation was performed with Dlx5 or IgG antibody followed by immunoblotting with PPARγ antibody. (C) Schematic representation and molecular weights of recombinant PPARγ domain constructs. (D) 3T3-L1 cells were transfected with Myc-Dlx5 (0.5µg) and/or HA-PPARγ (0.5µg) deletion constructs. Immunoprecipitation was performed with Myc antibody followed by immunoblotting with HA antibody. AF1, activation function 1; DBD, DNA-binding domain; LBD, ligand-binding domain.
brane and blocked with 5% skim milk. The membranes were probed with anti-Myc (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), HA (Santa Cruz Biotechnology), Dlx5 (Santa Cruz Biotechnology), PPARγ (Cell Signaling, Danvers, MA, U.S.A.), and α-tubulin (Cell Signaling) and then incubated with the appropriate secondary antibody. Immunoreactive bands were visualized using an enhanced chemiluminescence kit (GE Healthcare, Buckinghamshire, U.K.). The band strength was quantified using Multi Gauge V3.0 imaging software (FUJIFILM, Tokyo, Japan). Supernatants from centrifuged lysates were subjected to immunoprecipitation using appropriate antibodies and protein A or G-sepharose beads. The immunoprecipitated proteins were then subjected to SDS-PAGE and visualized by immunoblotting.

**DNA Affinity Precipitation Assay (DAPA)** The DNA-binding affinity was examined by nuclear extraction with a biotinylated DNA probe at the PPRE region located 1992–1968 bp upstream of the translational start site of the aP2 gene. The DNA-protein complexes were then precipitated using streptavidin-coated beads. The binding proteins were eluted by loading buffer and separated by 10% SDS-PAGE, followed by immunoblotting with anti-HA and Myc. The following biotinylated sequences were used for the binding and competition assay: PPRE promoter forward 5'-ATT TTA CTG CAA TTT TAA A A A G CA AT C A AT AT T G -3' and reverse 5'-TGA CGT TAA A AT T T T T C G T TA GT T ATA AC T T GT T-3'.
verify which part of Dlx5 interacts with PPARγ, we subcloned the activated domain of PPARγ, AF1 domain, DNA-binding domain, and ligand-binding domain (Fig. 1C). Similar to full-length PPARγ, the DNA-binding and ligand-binding domains co-immunoprecipitated with Myc-Dlx5, while AF1 was not detected by immunoblotting (Fig. 1D).

**Dlx5 Represses the Transcriptional Activity of PPARγ** PPARγ is a master ligand-activated transcription factor necessary and sufficient for adipocyte differentiation in mammals.21) To better understand the physiological influence of Dlx5 on PPARγ, we examined the effect of Dlx5 on the transcriptional activity of PPARγ in 3T3-L1 cells. We used two PPARγ-binding promoters: the aP2 promoter (aP2-Luc) contains nuclear receptor-type PPARγ response elements,22) and the PPRE promoter (PPRE-Luc) contains the PPAR-response elements of the aP2 gene.23) As expected, PPARγ alone markedly increased the transcriptional activity of the aP2-Luc and PPRE-Luc reporters. When Dlx5 and PPARγ were co-expressed, Dlx5 considerably suppressed the transcriptional activity of PPARγ in a dose-dependent manner (Figs. 2A, B). We further explored the effect of Dlx5 knockdown. Dlx5 abundance was suppressed by shRNA, which was confirmed Western blotting (Fig. 2C). In luciferase promoter assay, compared to the control, Dlx5 knockdown dose-dependently increased the transcriptional activity of PPARγ (Figs. 2D, E). These results indicate that Dlx5 has an inverse effect on the transcriptional activity of PPARγ.

**Dlx5 Diminishes Rosiglitazone-Mediated Transcriptional Activity of PPARγ** Rosiglitazone, a strong agonist for PPARγ that belongs to the class known as thiazolidinediones, has been shown to induce adipocyte differentiation in cell culture models and increase the tendency for weight gain clinically.24) We further assessed the inverse effects of Dlx5 on the transcriptional activity of PPARγ following stimulation with rosiglitazone. The results depicted in Figs. 3A and B demonstrate that rosiglitazone further increased the transcriptional activity of PPARγ, while Dlx5 overexpression significantly repressed the rosiglitazone-induced increase in the transcriptional activity of PPARγ on both aP2 and PPRE promoters.

**DISCUSSION**

PPARγ is particularly important for its unique and essential roles in adipogenesis, lipid metabolism, and insulin sensitivity.25) It is important to maintain PPARγ expression and activity for energy metabolism and insulin resistance; however, excessive PPARγ activity could result in obesity and...
differentiation. Runx2, a master transcription factor for osteogenesis, whereas knockdown of Dlx5 enhances adipogenic differentiation in 3T3-L1 cells and human bone marrow mesenchymal stem cells, whereas knockdown of Dlx5 enhances adipogenic differentiation. The reason for the difference between present study and the previous results is probably attributed to the difference in the sequence of promoters used in the experiments. However, taken together, Dlx5 is thought to lead the entire process of adipocyte differentiation because it regulates both the early differentiation markers CREB and the late differentiation markers C/EBPα and PPARγ. PPARγ is a nuclear transcription factor identified as a trans-acting factor that interacts with the gene encoding a fat-specific enhancer of aP2. The analysis of aP2 or PPRE promoter activity via the luciferase reporter assay revealed that PPARγ overexpression could significantly increase the promoter activity; however, co-overexpression of Dlx5 demonstrated weaker transcriptional activation (Figs. 2A, B). To further elucidate the negative regulatory effect of Dlx5, we adopted rosiglitazone as a PPARγ ligand. Rosiglitazone had been used as an antidiabetic drug to elevate cell sensitivity and responsiveness to insulin by directly interacting with PPARγ in fat cells. Rosiglitazone is currently off the market because of an increased risk of heart attacks, but when it was used as a diabetes treatment, the side effects of weight gain and obesity were an issue. The transcriptional activity of PPARγ was further induced by rosiglitazone treatment; however, this activity was lower in the Dlx5 overexpression group (Fig. 3). Although Dlx5 overexpression could not down-regulate the transcriptional activity to the basal level, considering the normal role and function of PPARγ, this proper effect can be considered more encouraging. In summary, Dlx5 has a function as an inhibitory gene that controls PPARγ activity, and it could be used to reduce the side effects of obesity caused by drugs used in diabetes treatment.

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Conflict of Interest The authors declare no conflict of interest.

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