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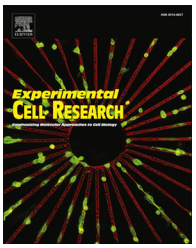

Research Article

Insulin suppresses distal-less homeobox 5 expression through the up-regulation of microRNA-124 in 3T3-L1 cells

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ABSTRACT

Distal-less homeobox 5 (Dlx5) is a pro-osteogenic but anti-adipogenic transcription factor that regulates lineage commitment in mesenchymal stem cells. Although the expression of Dlx5 is known to be decreased by adipogenic stimuli, the mechanism of Dlx5 down-regulation has not yet been clarified. MicroRNAs (miRNAs) are small regulatory RNAs that post-transcriptionally regulate many biological functions, including cell differentiation. In this study, we examined whether miRNAs are involved in down-regulation of Dlx5 following adipogenic stimuli. We screened candidate miRNAs that have a direct target site in the Dlx5 3′UTR using computational prediction programs, selected seven miRNA candidates with the highest binding score and observed their expression levels in 3T3-L1 murine pre-adipocytes. Among the miRNAs examined, only miR-124 was significantly up-regulated by 24-h incubation in adipogenic medium. Among the four components of adipogenic stimuli (1-methy-3-isobutyl xanthine, insulin, indomethacin and dexamethasone), insulin exhibited the highest stimulatory effect on miR-124 expression. Insulin significantly increased the expression of miR-124 precursors including pri-miR-124-1, pri-miR124-2 and pri-miR-124-3. LY294002, an inhibitor of phosphatidylinositol-3-kinase, prevented the regulatory effect of insulin on the expression levels of miR-124 and Dlx5. Over-expression of a miR-124 mimic decreased the expression of Dlx5 while increasing adipogenic differentiation in 3T3-L1 cells. Blocking miR-124 with anti-miR-124, a hairpin inhibitor of miR-124, increased the expression level of Dlx5 and suppressed adipogenic differentiation. When reporter assays were performed with a reporter construct containing the Dlx5 3′UTR sequence downstream of a luciferase gene, miR-124 mimic suppressed, but anti-miR-124 enhanced, luciferase activity in an miR-124 binding site-dependent manner. These results suggest that insulin-induced miR-124 plays a pivotal role in post-transcriptional regulation of Dlx5 during adipogenic differentiation and that miR-124 exerts pro-adipogenic effects by targeting Dlx5, at least in part.

Abbreviations: anti-miR-124, a hairpin inhibitor for miR-124; anti-miR-Control, a non-targeting control hairpin inhibitor; IBMX, 1-methy-3-isobutyl xanthine; LPL, lipoprotein lipase; miRNA, microRNA; miR-124-mimic, a small RNA mimic of mature miR-124; control-mimic, miRNA mimics negative control.

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Introduction

Adipocytes are derived from mesenchymal stem cells or progenitor cells via a lineage-specific differentiation process called adipogenesis. Adipogenic transcription factors regulate the expression of many adipogenic genes, leading to the differentiation of adipocytes [1,2]. Peroxisome proliferator-activated receptor-γ (PPAR-γ), CCAAT/enhancer binding protein α and β, glucocorticoid receptor and Kruppel-like factor 5 (KLF5) have been identified as essential regulators of adipogenesis [3]. PPAR-γ is commonly referred to as the master regulator of adipogenesis because ectopic expression or activation of PPAR-γ in other cell types such as fibroblasts and pre-myoblasts can trigger differentiation into adipocytes [4,5]. Agents such as dexamethasone, 1-methy-3-isobutyl xanthine (IBMX), indomethacin, prostaglandin $F_{2\alpha}$ and prostacylin accelerate the expression of an adipose phenotype [6–8]. Insulin and insulin-like growth factor I also stimulate adipogenesis [9,10].

Many transcription factors function as negative regulators of adipogenesis, including several members of the GATA-binding protein family, CHOP10, ETO/MTG8, Delta-interacting protein A, KLF2 and KLF7 [11,12]. Recently, we have shown that Distal-less homeobox 5 (Dlx5) exerts an anti-adipogenic effect through the down-regulation of PPARγ [13]. Dlx5, a member of the Distal-less homeobox domain protein family, is a well-known transcription factor for osteogenic differentiation [14]. Dlx5 is suggested to play a role as an osteoblast lineage determinant in bone marrow mesenchymal stem cells through the up-regulation of Runx2 and the down-regulation of PPARγ [13,15]. Dlx5 expression is enhanced by osteogenic signals such as bone morphogenetic protein 2 while it is suppressed by adipogenic stimuli in bone marrow mesenchymal stem cells [13]. The mechanism of Dlx5 down-regulation by adipogenic signals has not yet been clarified.

microRNAs (miRNAs) are endogenous non-coding RNAs, 22–25 nucleotides in length. miRNAs regulate various biological processes by down-regulating target gene expression via controlling mRNA stability and/or translation [16]. Many studies have profiled miRNA expression during adipogenic differentiation, often using murine 3T3-L1 pre-adipocytes [4,17,18]. Some miRNAs have been shown to inhibit adipogenesis: miR-27a and miR-27b suppress adipogenesis by targeting PPARγ [19,20] and miR-448 suppresses KLF5 expression [21]. Many miRNAs have also been reported to accelerate adipocyte differentiation. miR-143 is up-regulated by induction of adipocyte differentiation in mouse 3T3-L1 and human pre-adipocyte cells [22,23]. miR-375 was reported to promote 3T3-L1 preadipocyte differentiation [24]. miR-103 and the miRNA cluster containing miRs 17–92 enhance adipogenesis [4,17,18]. miR-8 family members also regulate adipogenesis, possibly by inhibiting Wnt signaling in ST2 marrow stromal cells [25]. Recently, it was shown that miR-124 expression is up-regulated during adipogenesis in human mesenchymal stem cells [26].

In this study, we examined whether miRNAs are involved in inhibition of Dlx5 expression caused by adipogenic stimuli in 3T3-L1 cells. Among the several candidate miRNAs that have a seed sequence targeting the Dlx5 3′UTR, the expression of miR-124 was significantly up-regulated by adipogenic medium and especially by insulin. Over-expression of a miR-124 mimic decreased Dlx5 expression, whereas a hairpin inhibitor of miR-124 suppressed insulin inhibition of Dlx5 expression. A miR-124 mimic enhanced adipogenic differentiation, whereas inhibition of miR-124 suppressed adipogenic differentiation in 3T3-L1 cells. These results indicate that miR-124 is potentially an important negative regulator of Dlx5 expression during adipogenesis. To the best of our knowledge, this is the first report to demonstrate the regulatory role of miR-124 on Dlx5 expression in adipocytes.

Materials and methods

Materials

Insulin, IBMX, indomethacin, dexamethasone, LY294002 and Oil red O stain were purchased from Sigma (St Louis, MO, USA). Anti-Dlx5 antibody was purchased from Millipore (Billerica, MA, USA). Anti-lamin antibody and HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Supex reagent for Western blot analysis was ordered from Dyne-Bio (Sungnam, Korea). The NE-PER Nuclear and Cytoplasmic Extraction Reagent was obtained from PIERCE Biotechnology (Rockford, IL, USA). The easy-BLUE™ and *Star*Taq™ reagents were ordered from iNtRON Biotechnology (Sungnam, Korea) and the AccuPower RT-PreMix was purchased from Bioneer (Daejeon, Korea). A microRNA isolation kit was purchased from Ambion (Austin, TX, USA). The Mir-X™ miRNA First-Strand Synthesis Kit and SYBR Advantage miRNA qRT-PCR Kit were obtained from Clonetech (Mountain View, CA, USA). PCR primers were synthesized by TaKaRa Korea (Seoul, Korea). The SYBR Premix Ex Taq™ was purchased from TaKaRa (Otsu, Japan). Tissue culture sera were purchased from HyClone (Logan, UT, USA). A Dual-Glo luciferase assay kit was obtained from Promega (Madison, WI, USA).

Cell culture and Oil red O staining

3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. After 2 days of confluence, the cells were treated with 50 μM indomethacin, 0.5 mM IBMX, 0.1 μM dexamethasone and 10 μg/ml insulin for the indicated time periods to induce adipogenic differentiation. When indicated, a single component of these adipogenic stimuli was added and the expression levels of Dlx5 and miR-124 were examined.

Oil red O staining was performed to identify the accumulation of lipid droplets in 3T3-L1 cells. In brief, the 3T3-L1 cells were seeded at a density of 1×10^5 cells per well in a 24-well plate, transiently transfected with miRNA mimics or anti-miRNAs and incubated for 6 days in adipogenic medium. The cells were then washed with phosphate-buffered saline (PBS), fixed in 10% formalin for 1 h and stained with freshly diluted Oil red O solution for 30 min. The cellular morphology was examined under a light microscope ($\times 100$).

Bioinformatics method

miRNA candidates that have predicted binding sites within the Dlx5 3′UTR were screened by computer-aided algorithms from PicTar (http://pictar.org/), miRDB (http://mirdb.org/miRDB/), miR Gen (http://www.diana.pcbi.upenn.edu/miRGen.html), TargetScan