Krüppel-Like Factor KLF8 Plays a Critical Role in Adipocyte Differentiation

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Abstract

KLF8 (Krüppel-like factor 8) is a zinc-finger transcription factor known to play an essential role in the regulation of the cell cycle, apoptosis, and differentiation. However, its physiological roles and functions in adipogenesis remain unclear. In the present study, we show that KLF8 acts as a key regulator controlling adipocyte differentiation. In 3T3-L1 preadipocytes, we found that KLF8 expression was induced during differentiation, which was followed by expression of peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPa). Adipocyte differentiation was significantly attenuated by the addition of siRNA against KLF8, whereas overexpression of KLF8 resulted in enhanced differentiation. Furthermore, luciferase reporter assays demonstrated that overexpression of KLF8 induced PPARγ2 and C/EBPz promoter activity, suggesting that KLF8 is an upstream regulator of PPARγ and C/EBPz. The KLF8 binding sites were localized by site mutation analysis to −191 region in C/EBPz promoter and −303 region in PPARγ promoter, respectively. Taken together, these data reveal that KLF8 is a key component of the transcription factor network that controls terminal differentiation during adipogenesis.

Introduction

Obesity is the main cause of metabolic syndrome and leads to various complications, including an increased risk of diabetes and cardiovascular diseases [1]. Obesity is characterized by increased adipose tissue mass due to increased adipocyte number (hypertrophy) and increased adipocyte size (hypertrophy) [2]. The number of adipocytes is determined to a large degree by the adipocyte differentiation process, which generates mature adipocytes from fibroblast-like preadipocytes. Therefore, understanding the regulatory processes involved in adipocyte differentiation may help to limit obesity and its pathological consequences.

Adipocyte differentiation is influenced by endocrine and autocrine factors that promote or constrain adipogenesis by intracellular mechanisms that induce the synthesis and activation of adipogenic transcription factors [3]. Murine 3T3-L1 cells differentiate into mature adipocytes when treated with serum-containing medium supplemented with 1-methyl 3-isobutylxanthine, dexamethasone, and insulin. After hormonal induction, growth-arrested 3T3-L1 preadipocytes reenter the cell cycle for additional two rounds of division, known as mitotic clonal expansion, and then express genes required for the adipocyte phenotype [4,5,6]. Several transcription factors are involved in adipocyte differentiation. These include CCAAT/enhancer-binding proteins (C/EBPz) [7,8] and peroxisome proliferator-activated receptor γ (PPARγ) [9,10]. Both C/EBPz and C/EBPa are induced immediately [11] and stimulate cell proliferation and expression of PPARγ and C/EBPz [12]. PPARγ and C/EBPz serve as pleiotropic transcriptional activators that coordinately induce the expression of adipocyte-specific genes that lead to formation of a mature adipocyte.

Members of the Krüppel-like factor (KLF) family of transcription factors are important regulators of development, growth, differentiation, and a number of other physiological cellular processes [13]. The KLF family is composed of at least 17 transcription factors that share homology in their tandem three C2H2 zinc-fingers near their C-terminus [14]. In adipogenesis, KLF family transcription factors act as both activators and repressors in the transcriptional cascade. KLF5, for example, is induced at an early stage of differentiation and activates the PPARγ2 promoter in concert with C/EBPz [15]. Moreover, KLF4 acts as a regulator at an earlier stage of differentiation by binding to the C/EBPz promoter together with Krox20, thereby inducing adipocyte differentiation [16]. In contrast, KLF3 represses adipocyte differentiation by recruiting C-terminal binding protein (CtBP) corepressors [17]. Other KLF family proteins have also been proven to promote or inhibit adipocyte differentiation [18].
In the present study, we analyzed the expression patterns of KLF family proteins during 3T3-L1 differentiation using microarray and found that KLF8 was significantly induced after the mitotic clonal expansion period. Adipocyte differentiation was significantly attenuated by KLF8 knockdown, whereas overexpression of KLF8 resulted in enhanced differentiation. Furthermore, overexpression of KLF8 induced PPARγ2 and C/EBPα transcriptional activities, as shown by luciferase assay, suggesting that KLF8 is an upstream regulator of PPARγ and C/EBPα. These results indicate that KLF8 plays an important role in early adipocyte differentiation.

Materials and Methods

Cell Isolation from Mice and Ethic Statement

The stromal vascular fraction (SVF) and fat fraction were isolated from mouse epididymal fat pads of 12-week-old mice by digestion with type I collagenase as described [19]. All procedures were approved by the Committee on Animal Investigations of the Yonsei University.

Cell Culture and in vitro Differentiation

Methods for maintenance and induction of differentiation of 3T3-L1 preadipocytes have been described previously [20]. Briefly, 3T3-L1 preadipocytes were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 8 μg/ml biotin, supplemented with 10% heat-inactivated fetal calf serum at 37°C, in an atmosphere of 90% air and 10% CO2. To induce differentiation, 2-day postconfluent 3T3-L1 cells (designated day 0) were incubated in DMEM containing 10% FBS for 2 days. Cells were then cultured in DMEM containing 10% FBS and insulin for another 2 days, after which they were grown in DMEM containing 10% FBS. Cell numbers were determined on day 2, and oil red-O staining was performed on day 8.

Western Blot Analysis

At each time indicated, cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed in a buffer containing 1% SDS and 60 mM Tris-Cl, pH 6.8. Lysates were briefly vortexed, boiled for 10 min, and cleared by centrifugation at 12,000 g for 10 min at 4°C. The supernatants were collected, and protein concentrations were assayed using a BCA assay kit (Pierce). Protein samples of equal amount were separated by SDS-PAGE and transferred to nitrocellulose membranes. Anti-KLF8 rabbit polyclonal antibody was generated against the murine KLF8 peptide (residues 5–16, IDNMDVRIKSES) by Atigen (Korea). Immunoblot analyses were performed using the following antibodies: polyclonal antibody against KLF8, C/EBPα [21], C/EBPβ [19], mouse monoclonal antibodies against PPARγ (Santa Cruz Biotechnology), FLAG antibody (Sigma), and β-actin antibody (Santa Cruz Biotechnology). The immunoreactive bands were detected using an enhanced chemiluminescence detection system (Amersham) following the manufacturer’s instructions.

RNA Isolation and Real-time RT-PCR

Total RNA was isolated from cultured cells using TRIzol (Invitrogen) according to the manufacturer’s instructions. For quantitative RT-PCR, cDNA was synthesized from 5 μg of total RNA using random hexamer primers and SuperScript reverse transcriptase II (Invitrogen), following the manufacturer’s instructions. An aliquot (1/40) of the reaction was used for quantitative PCR using the SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers. RT-PCR products were quantified using the ABI PRISM 7300 RT-PCR System (Applied Biosystems). RT-PCR was performed using the following primers: KLF8-sense, 5’-CGTGAT CATT A TGCGT GCCACG-3’; KLF8-antisense, 5’-CTAGA GGCCG GAGTG AGAAG-3’. All reactions were performed in triplicate. The relative amounts of the mRNA were calculated using the comparative cycle-time method (Applied Biosystems). GAPDH mRNA was also measured as an invariant control.

Small Interfering RNA (siRNA)

Preadipocytes (3T3-L1) were plated into 60-mm-diameter dishes 18–24 h prior to transfection. Cells were transfected with control or gene-specific siRNA at 50 nM (Dharmacon) in OPTI-MEM medium using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer’s protocol. The next day, the medium was replaced with fresh DMEM containing 10% calf serum and the cells were incubated for 24 h before the induction of differentiation. Total RNA and protein extracts were prepared from the cells at the indicated time points, and RT-PCR and immunoblot analyses were performed. Oil red-O staining of KLF8 knockdown was performed on day 8. The siRNA sequences are as follows: si-C/EBPβ, 5’-AGUAG AAGUU GGCA CUCUU AUGGGG-3’; si-KLF8a, 5’-UCAAG UAGGC ACCAU AUUGG CUUGA-3’; and si-KLF8b, 5’-UCAG CCAUU AUUGG GCCUA CUUCA-3’. We used the Stealth RNA siRNA negative control (Invitrogen) as siRNA control.

Transient Transfection Assay

KLF8 overexpressing vector (pcDNA3.0-KLF8-FLAG) was generated by inserting the whole open reading frame of mouse KLF8 with a C-terminal FLAG tag into pcDNA3.0 (Invitrogen). To maximize the transfection efficiency, microoliter volume electroporation of 3T3-L1 preadipocytes was performed with OneDrop MicroPorator MP-100 (Digital Bio). The cells were trypsinized, washed with 1× PBS, and finally resuspended in 10 μl of resuspension buffer R and 0.5 μg of plasmid at a concentration of 200,000 cells per pipette. The cells were then microporated at 1,300 V, with a 20-ms pulse width, 2 pulses. Following microporation, the cells were seeded in 35-mm cell culture dishes and placed at 37°C in a 10% CO2-humidified atmosphere. For luciferase assays of the promoter constructs, Lipofectamin and Plus Reagent (Invitrogen) was used. Briefly, NIH3T3 cells were cultured at a density of 2.5×105 cells/well in DMEM. The next day, cells were transfected with the indicated luciferase reporter plasmids using Lipofectamin and Plus Reagent following the manufacturer’s instructions. After 3 h of incubation, the cell medium was replaced with fresh complete medium. After 48 h of incubation, the cells were washed with PBS and harvested in 200 μl of passive lysis buffer (Promega). The cells were mixed vigorously for 15 s and centrifuged at 12,000 g for 10 min at 4°C. The supernatants were transferred into a fresh tube, and 5-μl aliquots of the cleared whole-cell lysate were assayed for luciferase activity using a Dual-Luciferase Reporter Assay System (Promega). Each transfection experiment was performed in triplicate.

Site-directed Mutagenesis

PCR amplification of the wild-type luciferase reporter plasmid was performed using site-directed mutation primers (C/EBPβmt-C/EBP-sense 5’-AGCCG AGGAG TCGAT TTCGGG CGGCG GTTGA tACAG ATCCTC-3’; C/EBPβmt-C/EBP-antisense 5’-GAAGAT CGTGGt atCAAT GCAGCC ACTGA CTCCT GCGGT-3’; C/EBPβmt-KLF-sense 5’-AGCCG AGGAG TCGAT GTGGG TTGGG GCCAG ATCCTC-3’; C/EBPβmt-KLF-antisense 5’-
GAGAT CGTG CGCAA acacCC ACTGA CTCCT GCGCT-3', PPARγ2mtKLF-sense 5'-AICTA CTGTA CAGTT acacGC CCCCTC ACAGA-3', PPARγ2mt-KLF-antisense 5'-TCTGT GAGGG GCGTG AACTG TACAG-3'. The substituted bases are indicated in small case letter. PCR amplification was performed using 50 ng template DNA and 15 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 7 min. PCR products were digested with DpnI for 2 h at 37°C, prior to transformation into Escherichia coli DH5α competent cells. Colonies were screened by DNA sequencing.

Electrophoretic Mobility Shift Assays (EMSA)

Briefly, pcDNA3.0-C/EBPβ-FLAG and KLF8-FLAG were used for in vitro-transfection reaction using TNT T7 quick master mix (Promega). EMSAs were performed using in vitro-translated protein as previously described [21]. Double-stranded probes were labeled with [γ-32P]ATP, using T4 polynucleotide kinase. Protein-DNA complexes were resolved from the free probe by electrophoresis on a 4%(wt/vol) polyacrylamide gel in 0.25 M TBE buffer.

Chromatin Immunoprecipitation (ChIP) Assays

ChIP analysis was performed following the protocol of the ChIP assay kit (Upstate). DNA-protein complexes were immunoprecipitated with antibodies against C/EBPβ and KLF8 for 4 h and then collected with protein A-agarose for 3 h at 4°C with rotation. The beads were washed, and chromatin complexes were eluted from the beads. After reversal of the cross-links, the DNA was purified. Input control and ChIP samples were used as PCR templates to amplify the PPARγ and C/EBPα promoters containing the C/EBP and KLF8 binding sites using the following primers: C/EBPα, the region from −335 to −63 was amplified, sense 5'-TCCCT AGTGT TGGCT GGAAG-3', antisense 5'-CTGTA CAGTT CACGC CCCTC ACAGA-3'; C/EBPβ, sense 5'-AGCGC AGGAG TTTAC CAGGG-3', antisense 5'-CAGTA GGATG GTGCC TGCTG-3'.

Statistical Analysis

All results are expressed as mean ± SD. Statistical comparisons of groups were made using an unpaired Student's t test and two-way ANOVA.

Results

KLF8 is Induced After Mitotic Clonal Expansion in 3T3-L1 Cell Differentiation

In order to analyze the roles of KLF family proteins in adipogenesis, we conducted microarray during 3T3-L1 cell differentiation induced by the standard hormone cocktail (MDI) (Table 1). In this analysis, it is remarkable that KLF8, KLF9, KLF12, and KLF17 were induced during adipocyte differentiation. Interestingly, the expression pattern of KLF8, KLF12, and KLF17 were similar, as they reached peak expression at day 2 after differentiation induction, suggesting that these KLFs may play roles in terminal differentiation during adipogenesis.

To confirm the microarray data, the expression levels of these KLFs were analyzed by RT-PCR at different time points. As shown in Figure 1A, the amount of KLF8 mRNA was increased during 3T3-L1 differentiation, reaching its maximum at 36–48 h after induction. In contrast, the mRNA level of KLF5 was elevated at an earlier point during differentiation. Similar to KLF8, KLF17 also was upregulated at the mRNA level during 3T3-L1 differentiation. Contrary to the microarray data, however, KLF12 was not found to be induced during differentiation by RT-PCR. Therefore, we focused on the potential roles of KLF8 and KLF17 in the differentiation program of adipogenesis. We generated polyclonal anti-KLF8 antibody (Figure 1B) and verified the increase of KLF8 protein during adipocyte differentiation, which is also expressed in the SVF, is not required for differentiation of adipocytes. It is interesting to note that KLF8 was mainly expressed in the stromal vascular fraction (SVF) compared to the fat fraction (Figure 1D). The SVF is known to have many adipocyte progenitor cells [22], whereas the fat fraction contains mature adipocytes. This suggests that KLF8, like KLF5, which is also expressed in the SVF, is not required for maintenance of the mature adipocyte phenotype, but instead plays a role in the differentiation of adipocytes.

KLF8, but not KLF17, is Necessary for Adipocyte Differentiation in 3T3-L1 Preadipocytes

To confirm the function of KLF8 directly, we observed the effect of KLF8 knockdown in 3T3-L1 preadipocytes. Two KLF8-specific siRNAs (designated as si-KLF8a and b) were used in 3T3-L1 cell transfection prior to hormonal induction of differentiation.

Table 1. Gene expression change of KLF family members during 3T3-L1 differentiation.

| Gene  | Alternative Names | GenBank No. | D0 | D2 | D4 | D7 |
|-------|------------------|-------------|----|----|----|----|
| Klf1  | Elk8             | NM_010635   | 1  | 0.916 | 1.020 | 1.233 |
| Klf2  | Lklf             | NM_008452   | 1  | 1.195 | 1.166 | 1.187 |
| Klf3  | Bklf; Tef-2      | NM_008453   | 1  | 0.800 | 1.291 | 0.852 |
| Klf4  | Ezf; Gikf        | NM_010637   | 1  | 0.404 | 0.352 | 0.341 |
| Klf5  | CKLF; Iklf; Beb2 | NM_009769   | 1  | 1.544 | 0.858 | 0.584 |
| Klf6  | FM2; FM6; Zf9    | NM_011803   | 1  | 0.271 | 0.547 | 0.546 |
| Klf7  | –                | NM_033563   | 1  | 1.642 | 0.643 | 0.621 |
| Klf8  | BkLF3; ZNF74     | NM_173780   | 1  | 2.147 | 1.928 | 1.830 |
| Klf9  | Bteb1; BTEB-1    | NM_010638   | 1  | 2.160 | 4.008 | 3.286 |
| Klf10 | Tieg; mgIF; Egral; Tieg1NM_013692 | 1  | 1.088 | 0.670 | 0.621 |
| Klf11 | Tieg2; Tieg3; Tieg2b | NM_178357 | 1  | 0.288 | 0.348 | 0.686 |
| Klf12 | AP-2reg         | NM_010636   | 1  | 3.108 | 1.765 | 1.167 |
| Klf13 | Bteb3; FKL2; NSLP1 | NM_021366 | 1  | 1.796 | 1.702 | 3.855 |
| Klf16 | DRRF; BTEB4      | NM_078477   | 1  | 1.516 | 0.736 | 1.142 |
| Klf17 | Gzf; C5123; Zfp393 | NM_029416 | 1  | 2.489 | 1.631 | 0.858 |

*Microarray was performed using total RNA samples from 3T3-L1 cells after 0, 2, 4, and 7 days of differentiation. Gene expression was analyzed by Agilent Mouse Genome 4×44 k oligo chip. The preparation for hybridization and the scanning of mouse chips were performed according to the manufacturer’s protocols (Genocheck). More than 2-fold changes of expression are indicated in bold.

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* The gene expression change of KLF family members during 3T3-L1 differentiation is shown in Table 1. The fold change of expression is compared to the control (D0) group.
The mRNA levels of KLF8 decreased approximately to 75% and 40% by si-KLF8a and b, respectively, compared to control siRNA (Figure 2A). Importantly, oil red-O staining on day 8 showed that KLF8 siRNA significantly diminished the accumulation of lipid droplets (Figure 2B). Moreover, the expression levels of PPARγ and C/EBPα were effectively suppressed by KLF8 siRNA during differentiation compared to expression levels in the control siRNA group, whereas C/EBPβ expression was relatively unaffected (Figure 2C). This suggests that KLF8 acts as an upstream regulator of C/EBPα and PPARγ, independent of C/EBPβ. Meanwhile, although the expression of KLF17 was induced during differentiation, knockdown of KLF17 did not affect 3T3-L1 cell differentiation (data not shown).

While expression of C/EBPβ increases after induction of differentiation, it has been reported that the upregulation of C/EBPβ is delayed [23], suggesting the presence of another regulator between the two transcription factors. Because C/EBPβ is also required for mitotic clonal expansion [24], knockdown of C/EBPβ resulted in the inhibition of cell proliferation (Figure 2D). Meanwhile, cell numbers at day 2 indicate that knockdown of KLF8 did not inhibit mitotic clonal expansion to the level observed in si-C/EBPβ (Figure 2D), suggesting that the role of KLF8 is downstream of C/EBPβ and mitotic clonal expansion. This is consistent with the result that expression of KLF8 increases at 36–48 h after the first round of division during clonal expansion (Figure 1A). Thus, KLF8 might not affect cell cycle or the expression of C/EBPβ but play a critical role in terminal differentiation during adipogenesis.

We next examined whether overexpression of KLF8 might induce adipogenesis in 3T3-L1 cells. KLF8 was overexpressed in 3T3-L1 preadipocytes by microporation prior to hormonal induction of differentiation. As expected, KLF8 overexpression increased the accumulation of lipid droplets (Figure 3A). In addition, PPARγ and C/EBPα were induced earlier in KLF8-overexpressing cells than in control cells, whereas no difference was observed in the induction of C/EBPβ (Figure 3B). Thus, it appears that KLF8 is able to induce the expression of PPARγ and C/EBPβ.

KLF8 Directly Controls and Binds to the PPARγ2 and C/EBPα Promoters

Because we found that KLF8 plays an important role in PPARγ and C/EBPα expression, we next examined whether KLF8 directly controls transcription of these genes. To clarify which region is responsible for KLF8 transactivation, serial deletion constructs of a −450 fragment of the C/EBPα promoter were made [25]. KLF8, similar to C/EBPα, significantly stimulated C/EBPα promoter activity in reporter assays (Figure 4A). This activation was almost completely abolished when the minimal proximal promoter (C/EBPα-89) was used (Figure 4A), indicating that KLF8 plays a critical role in C/EBPα promoter activity and the direct binding site exists between −205 and −89 bp in the C/EBPα promoter. Also, we found that KLF8 stimulated PPARγ promoter activity directly (Figure 4B). Sequence analysis of the C/EBPα promoter revealed two potential binding sites for KLF family members, the consensus sequence for which has been identified as CNCCCG [26]. Of these, site-specific mutation of the −191 KLF site abolished luciferase activity driven by KLF8 overexpression (Figure 4C). It should be noted that the KLF8 binding site is a GC-rich region right next to the C/EBP regulatory element [7]. The overexpression of both C/EBPβ and KLF8 resulted in the activation of the C/EBPβ-205-luciferase construct in a partially additive manner, not synergistically. Consistently, the mutation study revealed that C/EBPβ and
KLF8 bind to the C/EBP\(\alpha\) promoter separately, without affecting the action of the other (Figure 4C). Similarly, KLF8 site was localized to \(-303\) region of the PPAR\(c\) promoter, which has been identified as a KLF5 binding site previously \([15]\) (Figure 4D). Mutation of this sequence also abolished KLF8-driven transactivation of the promoter, without affecting C/EBP\(\beta\) action.

The direct binding of KLF8 to the promoters was also confirmed by EMSA. As shown in Figure 5A, in vitro translated KLF8 bound to \(-191\) region of the C/EBP\(\alpha\) promoter and \(-303\) region of the PPAR\(c\) promoter. These findings suggest that KLF8 plays a critical role in regulating adipocyte differentiation through direct binding to specific promoters.
region of the PPARc promoter, respectively. A known sequence of the b-globin promoter was shown as a positive control. As mentioned above, 2191 region of the C/EBPa promoter has an interesting feature that KLF8 and C/EBPb binding sites exist in a short sequence. Thus, we tested whether KLF8 and C/EBPb bind separately using mutated probes. The Figure 5B and 5C show that KLF8 or C/EBPb binding was not basically affected by each other factor. This is consistent with the luciferase result: KLF8 and C/EBPb transactivate the promoter in an additive manner (Figure 4C).

To further confirm the role of KLF8 on these promoters, we next performed ChIP to analyze the binding of KLF8 to these target genes. Chromatin samples were prepared from 3T3-L1 cells before (day 0) and after the induction of adipocyte differentiation (days 1 and 2) and were then immunoprecipitated with a KLF8-specific antibody. We found that KLF8 bound to these promoters (day 1) and the strength of binding increased further after 2 days of MDI treatment (Figure 5D). Anti-C/EBPb and anti-IgG were used as positive and negative control, respectively. Taken together, the results of this study implicate KLF8 as a key component of the transcription factor network that controls the regulation of C/EBPb and PPARγ during adipogenesis.

Discussion

In this study, we showed that KLF8 expression increased at both the mRNA and protein level during adipocyte differentiation in 3T3-L1 cells. In addition, KLF8 controlled the expression of PPARγ and C/EBPb by directly binding to the promoter regions but had no effect on C/EBPc. These results suggest that KLF8 is a key intermediate component of the transcription factor network regulating adipogenesis.

Previous studies identified that differentiation of the 3T3-L1 preadipocyte cell line into adipocytes requires sequential activation of various transcription factors, including C/EBPb, C/EBPc, PPARγ, and C/EBPb [4]. C/EBPb and C/EBPc are expressed in the early stage of adipogenesis (at ~4 h), whereas the activation of the C/EBPc or PPARγ gene is initiated ~36 h after differentiation is induced. Therefore, it is suggested that additional events are required for the terminal differentiation. The phosphorylation-dependent activation process of C/EBPb by MAPK and GSK3β [21,27], as well as redox control of DNA-binding [19] were suggested for those events. In addition, we also reported that upstream stimulatory factors are required for the full activation of C/EBPb promoter [25].
Role of KLF8 in Adipocyte Differentiation

Meanwhile, the involvement of KLF family proteins provides another example for the completion of transcriptional cascade in adipogenesis. KLF family proteins are known to play diverse roles in cell differentiation and development in mammals. Although KLF proteins exhibit homology in their carboxyl-terminal zinc finger domains, different amino-terminal sequences provide unique regions for interaction with specific binding partners. It is well established that KLF8 acts in critical cellular processes, such as differentiation, cell cycle progression, transformation, epithelial-to-mesenchymal transition, migration, and invasion [14]. In the present study, we first report the involvement of KLF8 in the transcriptional cascade of adipocyte differentiation. Importantly, KLF8 was expressed at ~36 h just prior to the activation of C/EBPα and PPARγ promoter. In addition, KLF8 overexpression in 3T3-L1 cells strongly upregulated PPARγ and C/EBPα expression by direct binding to these gene promoters. Furthermore, our analysis showed that the role of KLF8 is initiated after mitotic clonal expansion, suggesting that KLF8 is a critical regulator of terminal differentiation in adipogenesis.

We found that KLF8 binding site on the C/EBPα promoter is a GC-rich region right next to the C/EBP regulatory element. This GC-rich region has been analyzed thoroughly, demonstrating that Sp1 occupies the GC-box, which prevents access of the C/EBP protein in preadipocytes [28]. Upon differentiation stimuli, Sp1 level is decreased and C/EBP then binds to the regulatory region, thereby activating the C/EBPα promoter activity [28]. In our data, KLF8 and C/EBPα bind to and activate the C/EBPα promoter, in an additive manner. We also tested whether these two transcription factors interact with each other by protein-protein interaction; however, we could not observe any detectible interaction by immunoprecipitation (data not shown). Therefore, we suggest a possible mechanism involved in an activation process of the C/EBPα promoter as follows; in preadipocytes, Sp1 expression is high enough to repress the C/EBPα promoter, by competing the regulatory element with C/EBPα. When the differentiation is induced, the cells down-regulate Sp1 and begin to express C/EBPβ and KLF8. These two transcription factors occupy the critical regulatory elements of the C/EBPα promoter, thereby leading to a steady expression of C/EBPα protein. It is also possible that this combination of KLF8 and C/EBPα recruits specific co-activators to the promoter region. In previous studies, KLF8 has been found to recruit p300/CBP in order to activate expression of the cyclin D1 gene and promote acetylation of nearby histones [29]. Post-translational modification of KLF8 via SUMOylation attenuates this ability [30]. Whether recruitment of p300/CBP or post-translational modification of KLF8 are involved in adipogenesis needs to be further investigated.

Recently, it was reported that the closely related family member KLF3 is highly expressed in undifferentiated preadipocytes and reduced upon differentiation into adipocytes [17]. Other study demonstrated that KLF3 repressed expression of KLF8 in other cell types [31], raising a possibility of network between KLF family of proteins during adipocyte differentiation. In this regard, it is possible that the activities of KLF3 and KLF8 are reciprocally regulated during adipogenesis. Thus, it would be interesting to investigate the KLF family protein network in adipogenesis, including regulation of the KLF3-KLF8 axis.

In summary, the present study provides interesting evidence for the pivotal role played by KLF8 in adipocyte differentiation. Further studies of the mechanism by which KLF8 expression and function are regulated should provide additional insight into adipocyte differentiation.

Author Contributions
Conceived and designed the experiments: Haemi Lee HJK MYL YJL JwK. Performed the experiments: Haemi Lee HJK YJL MYL HC Hyemin Lee. Analyzed the data: Haemi Lee HJK MYL YJL JwK. Wrote the paper: Haemi Lee HJK MYL JwK.
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