Krüppel-like factor 10 (KLF10) is transactivated by the transcription factor C/EBPβ and involved in early 3T3-L1 preadipocyte differentiation

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Yuan Liu, Wan-Qiu Peng, Ying-Ying Guo, Yang Liu, Qi-Qun Tang1, and Liang Guo2

From the Key Laboratory of Metabolism and Molecular Medicine of the Ministry of Education, Institute of Stem Cell Research and Regenerative Medicine of Institutes of Biomedical Sciences, Department of Biochemistry and Molecular Biology of School of Basic Medical Sciences and Department of Endocrinology and Metabolism of Zhongshan Hospital, Fudan University, Shanghai 200032, China

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Adipose tissue stores energy and plays an important role in energy homeostasis. CCAAT/enhancer-binding protein β (C/EBPβ) is an important early transcription factor for 3T3-L1 preadipocyte differentiation, facilitating mitotic clonal expansion (MCE) and transactivating C/EBPα and peroxisome proliferator–activated receptor–γ (PPARγ) to promote adipogenesis. C/EBPβ is induced early, but the expression of antimitotic C/EBPα and PPARγ is not induced until ~48 h. The delayed expression of C/EBPα and PPARγ is thought to ensure MCE progression, but the molecular mechanism for this delay remains elusive. Here, we show that the zinc-finger transcription factor Krüppel-like factor 10 (KLF10) is induced after adipogenic induction and that its expression positively correlates with that of C/EBPβ but inversely correlates with expression of C/EBPα and PPARγ. C/EBPβ bound to the KLF10 promoter and transactivated its expression during MCE. KLF10 overexpression in 3T3-L1 preadipocyte repressed adipogenesis and decreased C/EBPα and PPARγ expression, whereas siRNA-mediated down-regulation of KLF10 enhanced adipogenesis and increased C/EBPα and PPARγ expression. Luciferase assays revealed an inhibitory effect of KLF10 on C/EBPα promoter activity. Using promoter deletion and mutation analysis, we identified a KLF10-binding site within the proximal promoter region of C/EBPα. Furthermore, KLF10 interacted with and recruited histone deacetylase 1 (HDAC1) to the C/EBPα promoter, decreasing acetylated histone H4 on the C/EBPα promoter and inactivating C/EBPα transcription. Because C/EBPα can transactivate PPARγ, our results suggest a mechanism by which expression of C/EBPα and PPARγ is delayed via KLF10 expression and shed light on the negative feedback loop for C/EBPβ-regulated adipogenesis in 3T3-L1 preadipocyte.

Adipose tissue, the energy storage organ, plays an important role in energy homeostasis (1). The change of adipose tissue amount is due to a variety of physiological or pathological factors and is closely related to many metabolic disorder diseases, including obesity and type 2 diabetes (2–5). Enlarged adipose tissue mass can rise through the increase in cell number (hyperplasia), cell size (hypertrophy), or both (6–8). Understanding the mechanisms regulating adipocyte differentiation may provide valuable information in limiting obesity and its pathological consequences (5, 9).

3T3-L1, a murine preadipose cell line, has been extensively used to investigate adipocyte differentiation (10). After exposure to hormonal adipogenic stimulus, preadipocytes trigger a sequential expression of transcription factors, in which CCAAT/enhancer-binding proteins (C/EBP) β and δ are induced rapidly within 4 h postinduction, and then C/EBPβ induces C/EBPα and PPARγ expression after a certain time lag (~48 h). During this time lag, adipocytes reenter the cell cycle and undergo approximately two rounds of mitosis, known as mitotic clonal expansion (MCE). When MCE is completed, the cells express adipocyte-specific proteins and obtain mature adipocytes biochemical and morphological characteristics (11, 12). Because C/EBPα and PPARγ are antimitotic factors, their delayed expression is thought to ensure the successful progression of MCE. However, the mechanism for the delayed expression of C/EBPα and PPARγ is not fully understood.

By using 3T3-L1 cell model, it has been identified that a variety of transcription factors and signaling pathways are involved in adipocyte differentiation (7). In recent years, the Krüppel-like factor (KLF) family proteins are reported to play important roles in this process (13). KLFs are a large family of transcription factors that regulate cell proliferation, differentiation, and apoptosis. All members of the family share a common sequence, three C2H2 zinc finger motifs, in their C terminus. The domain can recognize and bind to the GC-rich elements including CACCC boxes and GC boxes, making their functions in regulating target genes expression. Recently, a number of

1 To whom correspondence may be addressed. Tel.: 86-21-54237198; E-mail: qqtang@shmu.edu.cn.
2 To whom correspondence may be addressed. Tel.: 86-2--54237546; E-mail: liangguo@fudan.edu.cn.

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studies have identified several KLF proteins involved in adipocyte differentiation. KLF15 promotes adipocyte differentiation by inducing the expression of PPARγ and GLUT4 (14). KLF4 is expressed in the early stage of the differentiation and promotes adipogenesis by inducing C/EBPβ expression (15). KLF5, which is induced by C/EBPβ/δ, activates PPARγ expression and promotes adipocyte differentiation (16). However, not all of the KLF members promote adipocyte differentiation. For example, KLF3 represses the expression of C/EBPα and blocks adipogenesis by recruiting CtBP to C/EBPα promoter (17). So far, studies have confirmed seven of the other KLF proteins participating in the regulation of adipocyte differentiation (13, 18–22). Here, we investigated the role of KLF10, another member of the KLFs family, in 3T3-L1 preadipocyte differentiation.

KLF10, also named as TGF-β–inducible early gene, was first identified in human osteoblasts (23). Early studies show that KLF10 plays a role in osteogenic differentiation through enhancing TGF-β signaling pathway or directly acting on osteogenic differentiation genes such as Runx2 and Osterix (24), and epidemiological studies show that the expression of KLF10 between patients with osteoporosis and normal individuals is significantly different (23). In recent years, more studies have found that KLF10 plays a regulatory role in tumor formation and metastasis (25). KLF10 controls the formation of tumor by inhibiting cell proliferation and promoting cell apoptosis, and it inhibits the tumor metastasis by directly acting on the promoters of SLUG/SNAI2 and other tumor metastasis–related genes (26). According to the database online, KLF10 is widely expressed in many tissues and organs, especially in bone marrow, lung, and skin. It is also expressed in adipose tissue with its expression increased in obese mice. However, the role of KLF10 in adipocyte differentiation and adipose tissue function is still unclear.

Previously, our laboratory has identified a series of potential target genes of C/EBPβ by using ChIP-on-chip analysis at the early stage of 3T3-L1 preadipocyte differentiation (20 h after induction) (27). Among these candidate genes is KLF10. In this study, it is confirmed that C/EBPβ transactivates KLF10, whereas KLF10 negatively regulates C/EBPα transcription by recruiting and interacting with HDAC1. Thus, KLF10 functions as a downstream effector of C/EBPβ and may contribute to the delayed expression of C/EBPα and PPARγ. KLF10 may also be involved in the self-restricted pathway of C/EBPβ, which could help to maintain the adipogenesis at an appropriate level.

Results

The expression of KLF10 correlates with those of C/EBPβ, C/EBPα, and PPARγ during 3T3-L1 preadipocyte differentiation

C/EBPβ has been shown to play a pivotal role in the initiation of 3T3-L1 preadipocyte differentiation (12). To better understand the mechanisms of adipogenesis and to identify potential C/EBPβ target genes during MCE, we performed a promoter-wide ChIP-on-chip analysis using 3T3-L1 cells at 20 h after hormonal induction, when the cells were in the 5 phase during the first round of MCE (27). This identified a putative binding site on the proximal promoter (−749 to −200 bp from the transcription start site) of KLF10, a member of Krüppel-like factors family of zinc-finger transcription factors.

The expression profile of KLF10 during 3T3-L1 preadipocyte differentiation was investigated first. After adipogenic induction, KLF10 mRNA expression level is increased, reaches the highest level at ~24 h postinduction, and then gradually declined to its basal level at 72 h (Fig. 1A). This expression profile positively correlates with that of C/EBPβ (Fig. 1A). In contrast, the mRNA expression levels of C/EBPα and PPARγ, the two key pro-adipogenic factors, are not induced until ~48 h postinduction, when the MCE is coming to the end, and are dramatically increased after that (Fig. 1A). Similar trends for these expression profiles are also detected at the protein level (Fig. 1B), and the quantitative results for KLF10 and C/EBPβ proteins are shown in Fig. 1C. Thus, during the adipogenesis of 3T3-L1 preadipocyte, the expression profile of KLF10 positively correlates with that of C/EBPβ but inversely correlates with those of C/EBPα and PPARγ.

C/EBPβ transactivates KLF10 during MCE in the adipogenesis of 3T3-L1 preadipocyte

Our ChIP-on-chip data and the expression profile analyses suggest that KLF10 could be a transcriptional target of C/EBPβ.

Figure 1. The expression profile of Klf10 positively correlates with that of C/EBPβ but inversely correlates with those of C/EBPα and PPARγ during 3T3-L1 preadipocyte differentiation. A, the mRNA expression of KLF10, C/EBPβ, C/EBPα, and PPARγ in 3T3-L1 cells during adipocyte differentiation. The mRNA level was determined by real-time PCR and normalized to 18S rRNA. The numbers indicate the time points (hour) of differentiation induction. The results are expressed as means ± S.D. (n = 3). B, the protein expression of KLF10, C/EBPβ, C/EBPα, and PPARγ in 3T3-L1 cells during adipocyte differentiation. The targeted proteins were detected by Western blotting. Hsp90 is the loading control. C, Western blotting results in B were quantified against Hsp90. All values are represented as means with error bars representing S.D.*, p < 0.05; **, p < 0.01; ***, p < 0.001.
ChIP–qPCR confirmed the significant binding of C/EBPβ to the proximal promoter of KLF10 in differentiating cells after induction (24 h) but not in quiescent cells before induction (0 h), as shown in Fig. 2A. On the basis of the results from the ChIP-on-chip analysis, we analyzed the KLF10 proximal promoter and found a C/EBP-binding site (at approximately -591 to -582 bp) from the transcription start site of KLF10 (Fig. 2B). Using a luciferase assay, we found that C/EBPβ could transac-
tivate KLF10 in a dose-dependent manner in 3T3-L1 cells (Fig. 2C). When the predicted C/EBP-binding site was mutated, this transactivation was significantly blunted (Fig. 2D), suggesting a critical role of this C/EBP-binding site in the action of C/EBPβ. To further validate the role of C/EBPβ in KLF10 induction, C/EBPβ was overexpressed or knocked down in 3T3-L1 preadipocyte, and then the cells were induced to differentiation. Overexpression of C/EBPβ enhanced (Fig. 2, E and F), while knockdown of C/EBPβ impaired (Fig. 2, G and H), the expression of KLF10 at 24 h postinduction. These results demonstrate that KLF10 is a bona fide target of C/EBPβ during the MCE of 3T3-L1 preadipocyte differentiation.

**Modulation of KLF10 expression regulates adipogenesis**

Because C/EBPβ is an important transcription factor for adipogenic differentiation, the strong correlation between KLF10 and C/EBPβ expression and the transactivation of KLF10 by C/EBPβ suggests that KLF10 may also influence the adipogenic differentiation of 3T3-L1 preadipocyte. Gain-of-function and loss-of-function of KLF10 experiments were then performed to examine the role of KLF10 in adipogenesis. KLF10 was overexpressed or knocked down in 3T3-L1 preadipocyte, and then the cells were induced to differentiation. Overexpression of KLF10 led to a significant decrease in lipid accumulation in 3T3-L1 cells at 8 days postinduction, as evidenced by Oil Red O staining (Fig. 3, A and B). Moreover, the expression of C/EBPα and PPARγ, two key pro-adipogenic transcription factors, was inhibited by the overexpression of KLF10 (Fig. 3, C and D). On the contrary, siRNA-mediated knockdown of KLF10 resulted in a significant increase in lipid accumulation in 3T3-L1 cells at 5 days postinduction (Fig. 3, E and F), with enhanced expression of C/EBPα and PPARγ (Fig. 3, G and H). Collectively, the data above illustrate an inhibitory role of KLF10 in the adipogenesis of 3T3-L1 cells, suppressing the expression of C/EBPα and PPARγ.

**KLF10 inhibits C/EBPα transcription through binding to its promoter**

Because the gain of function and loss of function of KLF10 influenced the expression of C/EBPα and PPARγ, we ask whether KLF10 represses adipogenesis through the transcriptional inhibition of C/EBPα and PPARγ. 3T3-L1 preadipocyte was co-transfected with a plasmid expressing KLF10 and a firefly luciferase reporter plasmid containing C/EBPα or PPARγ proximal promoters (−1500 to +1 bp from the transcription start site), respectively. The results showed that KLF10 had no effects on the activity of PPARγ promoter (Fig. 4A) but inhibited C/EBPα promoter activity in a dose-dependent manner (Fig. 4B). Bioinformatics analysis predicted a potential binding site for KLF10 on the C/EBPα proximal promoter (−974 to −964 bp from the transcription start site) as shown in Fig. 4C. After this binding site was deleted (WT-short) or mutated (KLF10-Mut), the inhibitory role of KLF10 in C/EBPα promoter activity was blunted (Fig. 4D), suggesting a critical role for this binding site in the action of KLF10. ChIP–qPCR was performed to confirm the binding of KLF10 to C/EBPα proximal promoter. Because the commercially available anti-mouse KLF10 antibodies are not suitable for ChIP assay, FLAG-tagged KLF10 was overexpressed in 3T3-L1 preadipocyte, and then the cells were induced to differentiation. ChIP was performed by using the anti-FLAG antibody, and the qPCR was performed by using a pair of primers flanking the predicted KLF10-binding site as described above. The result indicated a significant binding of FLAG-KLF10 to the proximal promoter of C/EBPα at 24 h postinduction (Fig. 4E). As a negative control, no binding of FLAG-KLF10 to the promoter of 36B4 was detected (Fig. 4E). Taken together, these data indicate that KLF10 could inhibit the transcription of C/EBPα through binding to a KLF10 consensus sequence on its promoter.
KLF10 interacts with HDAC1 to cooperatively inhibit C/EBPα transcription

KLF10 contains a repressor domain on the N terminus, which is shown to interact with Sin3-histone deacetylase (HDAC) complex in T regulatory cells (28). Moreover, HDAC1 is reported to repress C/EBPα expression at the transcription level (29). Therefore, the role of HDAC1 in KLF10-mediated inhibition of C/EBPα transcription was investigated. Inactivation of HDACs by trichostatin A (TSA) treatment blunted the inhibitory role of KLF10 in C/EBPα promoter activity in 3T3-L1 cells (Fig. 5A), suggesting an important role of HDACs activity in the suppression of C/EBPα expression by KLF10.

The interaction between KLF10 and HDAC1 was also investigated in 3T3-L1 cells at both the overexpression level and the endogenous level. As shown in Fig. 5(B and C), 3T3-L1 preadipocyte was transiently transfected with the plasmids encoding HA-KLF10 and/or FLAG-HDAC1 and then induced to differentiation. At 24 h postinduction, the cells were harvested, and co-immunoprecipitation experiments were performed. Immunoprecipitation with anti-HA antibody pulled down not only FLAG-HDAC1 itself, but also HA-KLF10 (Fig. 5B). Similarly, immunoprecipitation with anti-FLAG antibody pulled down not only FLAG-HDAC1 itself, but also HA-KLF10 (Fig. 5C). To further confirm the interaction between KLF10 and HDAC1, co-immunoprecipitation at the endogenous level was also performed. At 24 h postinduction, 3T3-L1 cells were harvested and subjected to immunoprecipitation with anti-HDAC1 antibody, which resulted in the pulldown of both endogenous HDAC1 and endogenous KLF10 (Fig. 5D). Moreover, luciferase assays demonstrated that HDAC1 enhanced the ability of KLF10 to inhibit the promoter of C/EBPα in 3T3-L1 cells (Fig. 5E). Taken together, these data indicate that HDAC1 could function as a co-factor of KLF10 to inhibit the transcription and expression of C/EBPα.

Klf10 promotes the recruitment of HDAC1 to C/EBPα promoter, resulting in decreased enrichment of acetylated histone H4 on C/EBPα promoter

The interaction between KLF10 and HDAC1 in 3T3-L1 cells prompts us to investigate the recruitment of HDAC1 to C/EBPα promoter by KLF10. KLF10 was overexpressed or knocked down in 3T3-L1 preadipocyte, and then the cells were induced to differentiation. The cells were collected at 24 h postinduction for Western blotting and ChIP–qPCR analyses.
Overexpression of KLF10 did not affect the expression level of HDAC1 (Fig. 6A) but enhanced the binding of HDAC1 to C/EBPα promoter (Fig. 6B). The enrichment of acetylated histone H4, which is a marker for genes transcriptional activation and could be removed by HDACs (30), was decreased on C/EBPα promoter (Fig. 6C), suggesting the inhibition of C/EBPα transcription. The siRNA-mediated knockdown of KLF10 in 3T3-L1 cells also did not affect the expression level of HDAC1 (Fig. 6D). In contrast to the overexpression of KLF10, knockdown of KLF10 attenuated the binding of HDAC1 to C/EBPα promoter and increased the enrichment of acetylated histone H4 on C/EBPα promoter (Fig. 6E and F), suggesting the enhancement of C/EBPα transcription. Taken together, these data indicate that KLF10 interacts with and recruits HDAC1 to C/EBPα promoter, which leads to the inactivation of the C/EBPα transcription during the early phase of 3T3-L1 preadipocyte differentiation.

Discussion

C/EBPβ is considered as an important regulatory factor in the initiation of adipogenesis. It is expressed shortly after adipogenic induction and then activates the expression of a cascade of genes, including C/EBPα and PPARγ (12). Although some C/EBPβ-regulated genes have been found, there are still a lot unknown. Our laboratory used ChIP-on-chip experiments to identify a series of C/EBPβ target genes at the early stage of 3T3-L1 preadipocyte differentiation (27, 31–34), among which is KLF10.

In the current study, we demonstrated that the expression profile of KLF10 positively correlated with that of C/EBPβ during 3T3-L1 preadipocyte differentiation (Fig. 1). We used both ChIP–qPCR and luciferase assays to confirm that C/EBPβ can bind to the promoter of KLF10 and transactivates it. Moreover, overexpression of C/EBPβ increased, while knockdown of C/EBPβ decreased, KLF10 expression (Fig. 2–4). Then we used gain-of-function and loss-of-function experiments to examine the role of KLF10 in adipocyte differentiation. Overexpression of KLF10 in 3T3-L1 preadipocyte inhibited lipid accumulation and down-regulated C/EBPα and PPARγ expression. Consistently, knockdown of KLF10 promoted adipogenesis and up-regulated C/EBPα and PPARγ expression (Fig. 3). Thus, KLF10, which is a transcriptional target of C/EBPβ, may function as an anti-adipogenic factor.

Our data showed that KLF10 was a transcriptional target of C/EBPβ. However, based on the expression profile study, there was not a lag time between C/EBPβ expression and KLF10 expression (Fig. 1A). A possible explanation is that C/EBPβ could be not the only factor regulating KLF10 expression. The
KLF10 is involved in the early phase of adipogenesis

Potential involvement of other factors may make the regulation of KLF10 expression more complicated, which could lead to no obvious lag time between C/EBPβ expression and KLF10 expression. In addition, although C/EBPα and PPARγ expression started to increase from \( \sim 48 \) h postinduction, C/EBPβ expression gradually dropped to preinduction level during this period. At \( 48 \) h postinduction, C/EBPβ expression level was still \( \sim 1.5 \)-fold higher than the preinduction level (Fig. 1), which may facilitate the activation of C/EBPα and PPARγ expression. Once C/EBPα and PPARγ expression is induced and reaches a certain threshold, a cycle of positive cross-regulation between C/EBPα and PPARγ will be started, further enhancing the expression of C/EBPα and PPARγ themselves, achieving and maintaining a differentiated state of adipose cells (35). Moreover, C/EBPβ is not the only factor activating C/EBPα and PPARγ expression. For example, the long noncoding RNA ADINR can regulate adipogenesis by transcriptionally activating C/EBPα (36). KLF9 can promote PPARγ transactivation at the middle stage of adipogenesis (19). Taken together, the notion above may explain why C/EBPα and PPARγ expression is increased and maintained at a high level despite the down-regulation of C/EBPβ from the middle stage of adipogenesis.

C/EBPβ is induced shortly after induction in 3T3-L1 preadipocyte, although it does not transactivate C/EBPα and PPARγ until \( \sim 48 \) h postinduction. C/EBPα and PPARγ have been defined as anti-proliferation genes, and MCE is an important process of 3T3-L1 adipocyte differentiation, which may contribute to the increase in adipocytes number (11). The delayed C/EBPα and PPARγ expression is thought to ensure MCE, but the mechanism underlying this delay is poorly understood. Here our results indicate that KLF10 may play a role in this process. During 3T3-L1 preadipocyte differentiation, the expression profile of KLF10 negatively correlated with those of C/EBPα and PPARγ (Fig. 1, A and B). KLF10 was able to repress a luciferase reporter driven by the −1 to −1.5kb C/EBPα promoter in a dose-dependent manner (Fig. 4B), and ChIP−qPCR assays confirmed the binding of KLF10 to C/EBPα promoter (Fig. 4E). Further deletion and mutation experiments localized the critical KLF10-binding region to between −974 and −964 bp on C/EBPα promoter (Fig. 4, C and D). Although our data showed that KLF10 repressed both C/EBPα and PPARγ expression (Fig. 3, C and D), it seems to have no effects on the proximal promoter of PPARγ (Fig. 4A). Because C/EBPα participates in a cross-regulatory loop with PPARγ (19), it is possible that KLF10 regulates PPARγ indirectly through C/EBPα. KLF10 protein contains three zinc-finger DNA-binding domains, several SH3-binding domains, and three unique repression domains. Studies have shown that KLF10 represses genes expression by interacting with other regulatory factors, like HDAC1, in some cell types (28). Moreover, HDAC1 is recruited to repress the expression of C/EBPα and PPARγ. Our data demonstrate that KLF10 promotes the recruitment of HDAC1 to the promoter of C/EBPα, which leads to the decreased enrichment of acetylated histone H4 on C/EBPα promoter and inactivation of C/EBPα transcription (Figs. 5 and 6).

Based on the results in this paper, we consider that C/EBPβ-regulated KLF10 could contribute to the delayed expression of C/EBPα and PPARγ, which may help to facilitate the successful progression of MCE (Fig. 7). To investigate whether KLF10 affected MCE, MSCV retrovirus-mediated overexpression of KLF10 in 3T3-L1 preadipocytes was performed, and then the 2-day postconfluent cells were induced to differentiation. Cell number was determined at 0 and 48 h after induction, and the relative cell number was plotted. As shown in Fig. S1A, no significant difference in the increase of cell number (48 h versus 0 h) was detected among the mock, MSCV−GFP, and MSCV−KLF10 groups, suggesting that overexpression of KLF10 in 3T3-L1 preadipocyte did not affect the subsequent MCE. A possible explanation could be that induction of KLF10 expression at the appropriate time frame may be important for the role of KLF10 in MCE. As shown in Fig. 1, during 3T3-L1 preadipocyte differentiation, KLF10 expression increased shortly after adipogenic induction, reached the highest level at \( \sim 24 \) h, and then gradually declined to its basal level at 72 h. MCE, which is a special cell proliferation process, occurs during the above period. In our study, KLF10 was overexpressed in 3T3-L1 preadipocytes. This led to a high expression of KLF10 at 0 h (Fig. S1B), which is before MCE and KLF10 expression and therefore should have been at its basal level. It is possible that overexpression of KLF10 at an inappropriate time could cause some other effects, influencing the role of KLF10 in MCE. Further studies are needed to manipulate the expression level of KLF10 at the appropriate time frame so that we can better dissect the role of KLF10 in MCE.

C/EBPβ is known to be a pro-adipogenic transcriptional factor. Overexpression of C/EBPβ in 3T3-L1 preadipocytes promotes adipogenesis with enhanced expression of C/EBPα and PPARγ (27), the two key adipogenic factors. Interestingly, our study identified KLF10 as a C/EBPβ target gene, which could act as an anti-adipogenic factor by inhibiting C/EBPα and PPARγ expression. This may reveal a self-restricted mecha-
nism by C/EBPβ in the regulation of adipocyte differentiation. Actually, the self-restricted mechanism widely exists in biological processes. For example, Wnt5a can be induced by TNFα, but it antagonizes TNFα-induced up-regulation of matrix metalloproteinase through the inhibition of NF-kB signaling (37). SMAD7 is both a transcriptional target and a negative regulator of TGFβ signaling and potentially restrains TGFβ responses in cancer cells (38). Thus, in 3T3-L1 preadipocyte, KLF10 may mediate a negative feedback loop with C/EBPβ that may help to maintain the adipogenesis at an appropriate level. C/EBPβ is an important early transcription factor for adipogenesis, but its expression level decreases from the middle stage of adipogenesis. The C/EBPβ/KLF10-mediated negative feedback mechanism may provide a rationale for the decrease of C/EBPβ as adipogenesis proceeds, because this decrease could facilitate the expression of C/EBPα and PPARγ and promote the terminal adipocyte differentiation.

In conclusion, we have explored KLF10, another member of KLFs, involved in adipocyte differentiation. On one hand, our findings may give a new clue to explain the delayed expression of C/EBPα and PPARγ. On the other hand, our data show a possible self-restricted mechanism for C/EBPβ to ensure the adipogenesis at an appropriate level (Fig. 7). KLF10 could be added to the growing list of KLF family members that function in the regulation of adipogenesis. The role of KLF10 in metabolism had been defined in the liver (39, 40), but it is still unknown whether KLF10 also plays a role in the metabolism of other tissues. Our finding that KLF10 could inhibit adipocyte differentiation in vitro suggests a role for KLF10 in the process of obesity and obesity-related metabolic consequences, which merits further investigation.

**Experimental procedures**

**Cell culture and induction of differentiation**

3T3-L1 preadipocyte was propagated and maintained in DMEM (Invitrogen) containing 10% calf serum (Gibco), 2-day postconfluent 3T3-L1 preadipocytes (designated 0 day) were induced to differentiation with DMEM containing 10% FBS (Gibco) and a cocktail of inducers, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 1 mM dexamethasone (Sigma), and 1 μg/ml insulin (Sigma), until day 2. The cells were then cultured in DMEM supplemented with 10% FBS and 1 μg/ml insulin for 2 days, after which they were fed every other day with DMEM containing 10% FBS. The cells expressed adipocyte-specific proteins from day 3 and obtained mature adipocytes biochemical and morphological characteristics by day 8. KLF10 overexpression or knockdown assays were performed before induction, and the cells were harvested at the indicated time points.

**Oil Red O staining**

At the indicated time points, to determine lipid accumulation, the medium was discarded, and the cells were washed with PBS. Then the cells were fixed with 3.7% formaldehyde for 10 min and incubated with Oil Red O for 2 h at room temperature. Oil Red O stock solution (0.5% in isopropanol) was diluted with water (3:2) before using. The cells were washed with water, and the stained fat droplets in the cells were visualized by light microscopy and photographed. Finally, all of the water was removed, 1 ml of isopropanol was added into the cells for 10 min, and optical density was measured at 510 nm.

**Plasmid constructs and siRNAs**

C/EBPβ, KLF10, and FLAG-KLF10 were cloned into MSCV vector, which is managed to generate stably transfected cell lines. MSCV retroviruses were prepared as described before (41). FLAG-HDAC1 and HA-KLF10 were cloned into PCDNA3.1 vector for transient transfection in 3T3-L1 cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The siRNAs were designed and synthesized by GenePharma. The target sequences for successful siRNAs were as following: siC/EBPβ, GCCCTGAGTAAATCCTTAAAG; siKLF10-1, GGAAGTCTCATTCAAGAA; siKLF10-2, GGAAGCACCATTAAACCA; and siNC, TTCTCCGACGTTCAGTCTACGT. 3T3-L1 cells were transfected at ~50% confluence with siRNAs using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. 36 h after cells reached confluence, they were subjected to the standard differentiation protocol as described earlier, and at various times thereafter, cells were prepared for the test. siNC was used as a negative control.

**ChIP**

ChIP was performed as described before (27). The cells were fixed with 1% formaldehyde for 10 min at room temperature with swirling. Glycine was added to a final concentration of 0.125 M, and the incubation was continued for an additional 5 min. The cells were washed twice with ice-cold PBS, harvested by scraping, pelleted, and resuspended in 1 ml of SDS lysis buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 10 mM EDTA, and protease inhibitors). Samples were sonicated eight times for 30 s each with an interval of 30 s with a Bioruptor sonicator (Diagenode, Denville, NJ). Samples were centrifuged at 14,000 × g at 4 °C for 10 min. After removal of an input aliquot (whole-cell extract), supernatants were diluted 10-fold in ChIP dilution buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and complete protease inhibitor tablets). The protein–DNA complexes were immunoprecipitated using the indicated antibodies: anti-C/EBPβ (Santa Cruz, sc-150), anti-FLAG (Sigma, # F1904), anti-HDAC1 (GeneTex, GTX100513), anti-acetylated histone H4 (active motif, #39925), and control IgG (Abcam, ab46540). Immunoprecipitated samples were eluted and reverse cross-linked by incubation overnight at 65 °C in elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS). Genomic DNA was then extracted with a PCR purification kit (Qiagen). Purified DNA was subjected to qPCR using primers specific to the promoters of the indicated genes. The primers used for ChIP–qPCR were: 5'-CGGTGAGTGC-GAGCAGGTTG-3' (KLF10-forward); 5'-CCGGTGAAGCCA-AGGGATGG-3' (KLF10-reverse); 5'-CCGAGCTACCAGA-TTATG-3' (C/EBPα-forward); 5'-AGAGGCGAGGTTG-TGGA-3' (C/EBPα-reverse); 5'-CTGGGACGATGAATGAG-GAT-3' (36b4-forward); and 5'-AGCAGCTGGCACCTAACA-CG-3' (36b4-reverse).

**KLF10 is involved in the early phase of adipogenesis**

ChIP-qPCR revealed that KLF10 is involved in the early phase of adipogenesis with three oligonucleotide sequences, which opens a new window to the study of adipogenesis. The findings may give a new clue to explain the delayed expression of C/EBPα.
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Immunoprecipitation assay

Immunoprecipitation experiments were performed as described before (42). The cells were washed with PBS, scraped off, and collected by centrifugation. Then cells were suspended in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) in the presence of protease inhibitors (Roche) for 3 h at 4°C. After centrifugation, the supernates were incubated with the indicated antibodies at 4°C overnight. The next day, protein A–agarose beads (Invitrogen) were added. After 3 h of incubation, the beads were washed with TBS-T (TBS + 0.05% Tween 20) in the presence of protease inhibitors (Roche). The immunoprecipitates were separated by SDS-PAGE and subjected to Western blotting.

RNA isolation and qPCR

Total RNAs were extracted with TRIzol (Invitrogen) and transcribed to cDNA using the Superscript III kit (Invitrogen) according to the manufacturer’s instructions. The cDNA were analyzed using the Power SYBR Green PCR kit on the ABI PRISM 7300 qPCR machine (Applied Biosystems). All qPCR assays were normalized to 18S rRNA. Primers used for RT–qPCR were: 5’-GCAGGCTTAGATGGGAAACTCT-3’ (18S rRNA-forward); 5’-CATTCTTGCCAATGCTTTTCG-3’ (18S rRNA-reverse); 5’-ACAGGCTCTTCTGGCACCTTTGT-3’ (C/EBPβ-forward); 5’-CGAGGCTTAGATGGGAAACTCT-3’ (C/EBPβ-reverse); 5’-CAAGAACGACGAGTAAGTACGG-3’ (C/EBPα-forward); 5’-GTCACTGTCTGACTTCCACGC-3’ (C/EBPα-reverse); 5’-TGCTGTATGGTGTTAACAAGCTC-3’ (PPARγ-forward); 5’-CGTCTGATTGGTTAGGAATATGC-3’ (PPARγ-reverse); 5’-AGAAGAACCCAGGCAATA-3’ (KLF10-forward); and 5’-GAGGAAGCGACAGCA-3’ (KLF10-reverse).

Western blotting analyses

The cells were lysed with lysis buffer containing 2% SDS, 10 mM DTT, 50 mM Tris-HCl, pH 6.8, 10% glycerol, 0.002% bromphenol blue, and 1× protease inhibitor mixture (Roche). Equal amounts of protein were separated by SDS-PAGE, transferred to polyvinylidene fluoride membranes (Millipore); immunoblotted with the indicated antibodies: anti-KLF10 (Abcam, ab73537), anti-C/EBPβ (Santa Cruz, sc-150), anti-C/EBPα (Cell Signaling, #2295), anti-PPARγ (Cell Signaling, #2430), anti-HDAC1 (GeneTex, GTX100513), anti-Hsp90 (Santa Cruz, sc-7947), anti-FLAG (Sigma, # F1904), anti-HA (Santa Cruz, sc-805); and then visualized with horseradish peroxidase–coupled secondary antibodies.

Luciferase reporter assays

The proximal promoter regions of mouse KLF10, C/EBPα, PPARγ, and their artificial mutants were amplified via PCR and subcloned into the firefly luciferase reporter construct PGL3-basic (Promega). 3T3-L1 cells (2 × 10⁵ cells/well) were transfected with 300 ng/well firefly luciferase reporter constructs, 2 ng/well Renilla luciferase reporter plasmids, in combination with pcDNA3.1(−) vector, C/EBPβ plasmid or KLF10 plasmid, by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Where indicated, TSA, which was from Sigma, was added into the cell culture medium to inhibit HDACs activity. After 48 h, luciferase activity was measured using dual luciferase reporter assay (Promega), normalizing firefly luciferase to Renilla activity.

Statistical analysis

The results are expressed as the means ± S.D. Comparisons between groups and were made using unpaired two-tailed Student’s t tests. For comparison of more than two groups with comparable variances, one-way analysis of variance and Bonferroni’s post hoc tests were carried out. p < 0.05 was considered statistically significant. All experiments were repeated a minimum of three times with triplicate samples, and representative data are shown.

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