The KLF2 Transcription Factor Does Not Affect the Formation of Preadipocytes but Inhibits Their Differentiation into Adipocytes[†]

Jinghai Wu,[‡] Seetha V. Srinivasan,[‡] Jon C. Neumann, and Jerry B. Lingrel*

Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati Medical Center, 231 Albert Sabin Way, Cincinnati, Ohio 45267

Received January 27, 2005; Revised Manuscript Received June 3, 2005

ABSTRACT: Kruppel-like transcription factor 2 (KLF2), a critical gene for mouse embryogenesis, was recently identified as an inhibitor of adipogenesis. However, it is still unknown whether KLF2 is a natural repressor of adipocyte differentiation and if KLF2 affects the formation of preadipocytes. It may also be important for preadipocyte formation, as KLF2 is crucial for lung development and blood vessel formation. In this study, we show that differentiation of preadipocytes not only results in a concomitant decrease in the levels of KLF2 protein but also significantly reduces KLF2 promoter activity. We have generated tet-responsive lines of 3T3L1 that express physiological levels of KLF2 and show that reexpression of KLF2 prevents preadipocyte differentiation, thereby confirming the inhibition of adipogenesis by KLF2, partially via the restoration of Pref-1. In addition, we studied the contribution of KLF2-negative cells to the formation and subsequent differentiation of preadipocytes. We demonstrate that embryoid bodies derived from KLF2$^{-/-}$ ES cells can differentiate into adipocytes as evidenced by the accumulation of lipids and expression of several biochemical markers. Moreover, mouse embryonic fibroblasts (MEFs) derived from KLF2$^{-/-}$ mouse embryos differentiate efficiently into adipocytes. Interestingly, quantification of lipid accumulation in MEFs indicated that KLF2$^{-/-}$ cells are more prone to differentiate at the early stage of the process, suggesting that KLF2 is a natural repressor of differentiation in vivo. Taken together, these studies demonstrate that KLF2 does not affect the commitment of multipotent stem cells into the preadipocytic lineage but rather maintains their preadipocyte state and thereby negatively regulates their transition into adipocytes.

The global emergence of obesity as a disease of epidemic proportions necessitates the understanding of molecular switches regulating adipose development. Adipogenesis involves the formation of preadipocytes from mesenchymal precursors and their differentiation into adipocytes, a complex event controlled by multiple factors including transcriptional control and neural regulation (*1*, *2*). The understanding of this process has been greatly facilitated by the use of model systems such as the 3T3L1 preadipocyte cells or freshly isolated primary cultures that are subjected to adipogenic stimuli.

The differentiation of preadipocytes is defined by specific stages, each associated with the expression of several transcription factors that regulate expression of adipose-specific genes (*1*) and coordinate tissue-specific adipogenesis. At the molecular level, the accepted sequence of events is that C/EBP-β[1] and C/EBP-δ are induced early in response to adipogenic stimuli such as insulin and these two CAAT-element binding protein isoforms then induce the transcription of PPAR-γ. In addition, the basic helix−loop−helix protein ADD-1/SREBP-1c (adipocyte differentiation and determination factor 1/sterol regulatory element binding protein 1) is also induced and thought to accelerate differentiation by upregulating PPAR-γ and providing the required ligands. Finally, the maintenance of this state requires the combined action and coregulation of PPAR-γ and CEBP-α. While several studies characterize C/EBPs and PPARs as primary players in adipogenesis, a more intricate regulatory pattern is emerging especially as the process is also regulated by GATA-2, GATA-3, Pref-1, retinoic acid receptor α, and Wnts (*3*−*7*). In addition, there are reports of Kruppel-like family members associated with the adipogenic pathway (*8*−*10*). Recently, the retroviral transfected KLF2 was found to inhibit adipogenesis (*11*).

KLF2 is emerging as a common component in seemingly diverse cellular functions such as lung development, T-cell quiescence, endothelial activation, and adipogenesis. Early studies showed that KLF2-null mice are embryonically lethal (*12*, *13*). The use of chimeric mice has revealed that KLF2 is critical for lung development (*14*). KLF2-deficient T-cells show a spontaneously activated phenotype and high rates of cell death, thereby identifying a role for KLF2 in maintaining a pool of quiescent peripheral T-cells in peripheral lymphoid organs and blood (*15*). KLF2 influences blood vessel

[†] This work was supported by NIH Grant RO1 HL57281 to J.B.L.

* Corresponding author. E-mail: jerry.lingrel@uc.edu. Phone: 513-558-5324. Fax: 513-558-1190.

[‡] These two authors contributed equally to this work.

[1] Abbreviations: AP2, adipose fatty acid binding protein; β3-AR, β3 adrenergic receptor; C/EBP, CAAT element binding protein; Dox, doxycycline; EBs, embryoid bodies; ES cells, embryonic stem cells; GPI, glucose-phosphate isomerase; IBMX, 3-isobutyl-1-methylxanthine; KLF2, Kruppel-like factor 2; MEFs, mouse embryonic fibroblasts; PPAR-γ, peroxisome proliferator-activated receptor γ; Pref-1, preadipocyte factor 1.

10.1021/bi050166i CCC: $30.25
Published on Web 08/02/2005

formation, and KLF2-null mice display endothelial cell necrosis and defective tunica media formation (*13*). More recent studies show that KLF2 is induced by sheer stress and is therefore likely to maintain a physiologically healthy and functional endothelium (*16*−*18*).

A recent study showed KLF2 to be a modulator of PPAR-γ, a ligand-induced nuclear receptor considered to be a primary player in adipogenesis (*11*). This inactivation of PPAR-γ could also reduce the response of cells to other fat-determining factors, such as C/EBPs. In fact, CEBP-α levels are reduced when KLF2 is retrovirally transfected in 3T3L1 cells. Additionally, mutation of the KLF2 binding site on the PPAR-γ promoter is not sufficient to completely abrogate KLF2 function, indicating that KLF2 is probably involved in other functions that culminate in the inhibition of adipocyte differentiation. Since stem cell determination and preadipocyte proliferation as well as differentiation are other potential sites where adipogenesis could be targeted (*2*), and KLF2 is crucial for the lung development and formation of the blood vessel (*12*, *13*), it is also quite interesting to explore whether KLF2 regulates other aspects of adipogenesis such as the formation of adipocyte precursors, their commitment into the adipocytic lineage, and thereafter the transition of these cells from their preadipocytic phenotype to their differentiated counterparts. In addition, the molecular mechanisms leading to the commitment of precursors into cells of the adipocytic lineage are poorly understood (*19*), although glucocorticoids and their analogues are known to enhance preadipocyte recruitment and differentiation in serum and insulin-containing medium (*20*). Taken together, it is necessary to determine whether KLF2 is a physiological inhibitor of differentiation and if KLF2 also regulates other crucial aspects during adipogenesis such as preadipocyte formation. In the present study, reexpression of physiological levels of KLF2 in differentiation-induced cells via a KLF2-inducible system exhibits a marked inhibition of adipogenic differentiation, partially through its regulation on Pref-1. We also use a combination of in vitro and in vivo approaches to demonstrate that KLF2 deficiency does not interfere with formation of adipocyte precursors that can differentiate into adipocytes when appropriately induced. More interestingly, KLF2$^{-/-}$ MEFs are more prone to differentiation at the early stage of the process, suggesting KLF2 as a natural repressor of adipocyte differentiation.

MATERIALS AND METHODS

3T3L1 Preadipocyte Cell Culture and Differentiation. The 3T3L1 preadipocytic cell line was purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) containing Hepes buffer (Sigma Aldrich), gentamycin (Sigma Aldrich), and 10% calf serum (Invitrogen). Cells were grown to confluence in 10 cm dishes and treated to the differentiation medium 2 days post-confluence. For differentiation studies, the basal DMEM medium was supplemented with 10% fetal calf serum and a differentiation cocktail composed of 1.7 μM insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 1 μM dexamethasone (Sigma Aldrich) (*11*). Two days post-induction of differentiation, the cells were maintained in medium supplemented with insulin. Experiments were done in duplicate and repeated three times. Simultaneously, an alternate set of cells were cultured as described above and harvested in Laemmli buffer for Western blot analysis.

Transfection Assays. Cells (2×10^5) were plated in 10 cm dishes in DMEM supplemented with 5% fetal calf serum. Forty-eight hours later, cells were transfected with 1.0 μg of pGL3-basic (Promega) alone or with 342 bp of the KLF2 promoter cloned into the pGL3-basic luciferase plasmid. TK-Renilla (0.05 μg/10 cm dish) was cotransfected to correct for differences in transfection efficiency. The total DNA in each plate was kept constant by addition of vector DNA. Forty-eight hours after transfection, cells were harvested in luciferase assay buffer, and the dual luciferase kit (Promega) was used to measure the luciferase activity.

Generation of Stable Transformants of KLF2 and 3T3L1-KLF2 Cell Lines. The reverse tetracycline repressor system was used to produce inducible expression of KLF2 in 3T3L1 preadipocytes. Cells were first transfected with the regulatory plasmid pUHD172-1 neo, selected by growth in G418 sulfate (800 μg/mL, Invitrogen), and single-cell clones were generated by limiting dilution. Clones showing the highest expression of rtTA were subsequently transfected with the response plasmid pTRE2hyg-KLF2 as previously described (*21*). Doubly transfected clones were selected for growth in hygromycin B (200 μg/mL, Clontech) and G418 (200 μg/mL, Invitrogen), and single-cell clones were generated by limiting dilution. The clones were called 3T3L1-KLF2 cells.

For differentiation of 3T3L1-KLF2 cells, the method employed was identical to that described for 3T3L1 cells except that the growth medium had maintenance doses of hygromycin (100 μg/mL) and G418 (200 μg/mL).

Mouse Embryonic Fibroblast (MEF) Culture and Differentiation. Since KLF2$^{-/-}$ mouse embryos die around 12.5 days, we used KLF2$^{-/-}$ and KLF2$^{+/+}$embryos that were harvested at day 11.5. The preparation of MEFs was established by a modified protocol (*22*). In brief, fibroblasts were obtained by overnight trypsin digestion of eviscerated embryos and plating of cells in DMEM with 15% FBS. Cells used for adipocyte differentiation were from passages 3−6. For the differentiation, MEFs were plated on 60 mm dishes and six-well plates at 5×10^4 per plate/well in DMEM medium plus 10% FBS. Two days post-confluence, the medium was supplemented with 5 μg/mL insulin, 0.5 mM IBMX, and 1 μM dexamethasone for 2 days followed by supplementation with insulin alone from day 3. Differentiation into adipocytes was observed from day 7, and cells were stained for lipid accumulation by Oil Red O stain on day 12. The second set of plates was used to prepare whole cell lysates for Western blot analysis of expressed proteins.

Generation and Differentiation of Embryoid Bodies (EBs). KLF2$^{-/-}$ ES cells were created using a two-step targeting procedure as described previously (*14*). The established protocol for EB culture and differentiation into adipocytes is described previously (*23*). In summary, KLF2$^{-/-}$ and KLF2$^{+/+}$ ES cells were grown in DMEM supplemented with 15% fetal calf serum on mitomycin-treated fibroblast cell layers. ES cells were harvested and resuspended in medium and diluted to 5×10^5 cells/mL. Microdrops comprising 25 μL were positioned for the hanging drop cultures and allowed to grow for 2 days and differentiate into EBs. EBs were then harvested, washed once in PBS, resuspended in DMEM supplemented with 15% FBS and 10^{-7} M retinoic acid (Sigma Aldrich), and transferred to nontissue culture treated

60 mm Petri dishes and later used for differentiation studies. For the differentiation, EBs were seeded in 60 mm dishes and treated with retinoic acid-supplemented medium as described above. After 3 days of RA treatment with a daily media change, an adipocyte differentiation cocktail comprising 1.7 μM insulin, 1 μM dexamethasone, 0.5 mM IBMX, and 2 nM triiodothyronine was added to the embryoid bodies. The EBs were cultured in differentiation medium for 14 days and then stained for differentiation using Oil Red O. One set of plates was used for preparation of cell lysates for Western blot analysis of endogenous proteins.

Oil Red O Staining and Quantification of Lipid Accumulation in the Kinetics of MEF Differentiation. The accumulation of lipids signifying the formation of adipocytes was observed by staining the differentiated cells or EBs with Oil Red O. Oil Red O stock solution (0.5%) was prepared in 60% triethyl phosphate and filtered in cellulose nitrate filters as described previously (*24*). The stock solution was diluted 6:4 in water and double filtered before use. Cells were washed before staining 2 h with the Oil Red O working solution and then washed with tap water. To measure the lipid accumulation of MEFs during differentiation, the Oil Red O dye was extracted with isopropyl alcohol by gentle shaking, and its absorbance was measured immediately at 510 nm (*25*, *26*).

Microscopy and Digital Image Generation. A D1X digital camera (Nikon) attached to a Microphot FXA upright microscope (Nikon) with a 63× dippable Zeiss objective and a 10× eyepiece was used to take pictures of Oil Red O-stained cells. NikonView5 software was used to transfer images. For pictures of Oil Red O-stained KLF2$^{+/+}$ and KLF2$^{-/-}$mouse embryonic fibroblasts, a lower magnification objective (40×) was used.

Western Blotting Assays. Cultured cells were washed with PBS twice. Total cell extracts were prepared by lysing cells in 1 mL of Laemmli buffer per 10 cm dish. The samples were boiled before determination of the protein concentration by using the BCA kit. The samples were stored frozen at −20 °C or electrophoresed as described. Forty micrograms of protein was loaded on each lane and electrophoresed using a 4−15% gradient gel (Bio-Rad). Proteins were then transferred to 0.45 μm Immobilon-P membrane (Millipore). A 5% solution of nonfat milk in TBS−0.1% Tween 20 was used as a blocking agent for 1 h. Membranes were incubated with a 1:1000 dilution of specific antibodies (described below) overnight. The next day, membranes were washed in TBS-T, incubated with the appropriate secondary antibody, and developed with the chemiluminescent ECL kit (Amersham). Kodak Biomax film was used for autoradiography. The goat anti-KLF2 antibody was a kind gift from Dr. Leiden, through Dr. Glimcher. AP2/422 and adiponectin antibodies were purchased from R&D Systems, Pref-1 antibody was from Chemicon International, and β3-adrenergic receptor and anti-HA antibodies were from Santa Cruz Biotechnology Inc.

RESULTS

Endogenous KLF2 Protein Decreases upon Differentiation of Preadipocytes. The levels of KLF2 mRNA are known to decrease in preadipocytic 3T3L1 cells upon induction of differentiation (*11*). To examine if this reduction of KLF2 mRNA is followed by a concomitant decrease in KLF2 protein, we cultured 3T3L1 cells and induced them to differentiate using the standard differentiation mixture of insulin, dexamethasone, and IBMX. The cells were stained with Oil Red O to ascertain differentiation and oil droplet accumulation (Figure 1A). Differentiation was further confirmed by the decrease of Pref-1, a preadipocyte marker, and also by the increased expression of adipogenic proteins such as the adipocyte lipid binding protein AP2 and adiponectin, an adipocyte-specific cytokine (Figure 1B). In agreement with the reduced mRNA levels, our results show that KLF2 protein expression markedly decreases upon differentiation (Figure 1B), indicating that the differentiation of preadipocytes is accompanied by a consequent decrease in KLF2 protein.

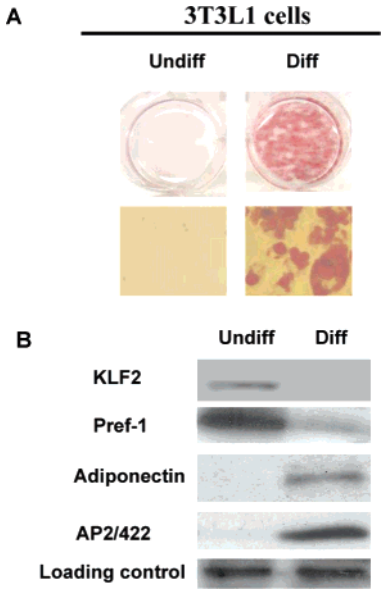

FIGURE 1: Differentiation of 3T3L1 preadipocytes results in decreased levels of KLF2. (A) 3T3L1 preadipocytes were plated at 2 × 10^6 per 10 cm dish in basal medium supplemented with calf serum and grown to confluence. Two days post-confluence, the cells were treated to a DMEM-based differentiation medium that included fetal calf serum, insulin, dexamethasone, and IBMX. Forty-eight hours later, the differentiation-induced cells were maintained in medium containing 10% fetal calf serum and insulin. Medium supplemented with 10% calf serum was used to culture undifferentiated cells. Oil Red O staining was used to stain plates 8 days post-differentiation. (B) An identical set of plates was harvested in Laemmli buffer for Western blot analysis using the indicated antibodies.

Differentiation of 3T3L1 Downregulates KLF2 Promoter Activity. Given this reduction of KLF2 mRNA levels and our own studies demonstrating reduction of KLF2 protein upon differentiation, we hypothesized that the observed downregulation could be explained, at least in part, by the regulation of the KLF2 gene at the transcriptional level. We therefore assessed the activity of the KLF2 promoter in undifferentiated or freshly differentiated and replated cultures of 3T3L1 by transfection of a KLF2 promoter−luciferase reporter plasmid. We found that the initiation of differentiation significantly reduced KLF2 promoter activity 5-fold (Figure 2). We ascertained that these differences were not the result of ineffective transfection by cotransfection of the TK-renilla plasmid (Figure 2) and by the use of a control PGL3-SV40 plasmid which showed that both undifferentiated and differentiation-initiated 3T3L1 cells have the ability to transactivate a reporter plasmid (data not shown). These

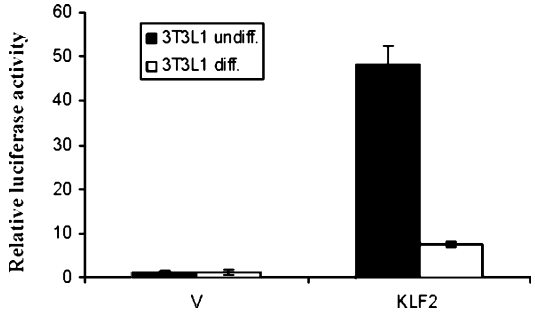

FIGURE 2: The activity of a luciferase reporter under the control of the KLF2 gene promoter is significantly reduced in differentiation-induced 3T3L1 cells. 3T3L1 cells were grown and differentiated as described in Materials and Methods. After 2 days of differentiation, cells were harvested and replated at 2×10^5 cells per 10 cm dish to facilitate transfection studies. Two days after plating, vector alone (V) or the murine KLF2 promoter cloned in pGL3-basic vector (KLF2) was transfected into cells using Fugene6. TK renilla was cotransfected in all plates to assess transfection efficiency. Forty-eight hours after transfection, cells were harvested in lysis buffer. Dual luciferase assays were carried out using a dual luciferase kit. Experiments were replicated three times and performed in duplicates each time.

results clearly indicate that KLF2 gene expression is controlled to a major extent at the transcriptional level.

Reexpression of Physiological Levels of KLF2 in Preadipocytes Prevents Their Differentiation and Maintains Their Preadipocyte Characteristics. Forced expression of KLF2 using a retroviral system has been shown to inhibit adipogenesis (*11*). However, the effect of reexpressing physiological levels of KLF2 upon differentiation is unknown. We employed the reverse tetracycline repressor system to generate a 3T3L1 preadipocytic cell line that ectopically expresses KLF2 upon induction with doxycycline (Figure 3A,B) to confirm the negative regulation of adipogenesis by KLF2. This system makes it possible to express physiological levels of KLF2 by optimizing the dose of doxycycline used. KLF2 expressing clones were selected by growth in hygromycin B and G418 and also confirmed by an anti-HA Western blot of the Dox-treated cells (Figure 3B). The positive clones were designated as 3T3L1-KLF2. The amounts of Dox used were optimized to finally obtain the dose necessary for physiological levels of KLF2 expression. To differentiate these 3T3L1-KLF2 cells, we used medium supplemented with fetal calf serum and the standard differentiation cocktail containing insulin, dexamethasone, and IBMX (Figure 3C, panels 3 and 4), while undifferentiated controls were maintained in medium supplemented with calf serum alone (Figure 3C, panels 1 and 2). Upon stimulation of differentiation, the cells underwent the expected transition into adipocytes as seen by enlargement of cells, acquisition of rounded morphology, and, most strikingly, an increased accumulation of oil droplets after day 6 (Figure 3C, panel 3). However, cells induced to express KLF2 did not exhibit adipogenic characteristics and retained, instead, their fibroblastic morphology (Figure 3C, panel 4). We assayed for HA-tagged KLF2 expression in these cells and found that KLF2 was indeed induced (Figure 3D). KLF2 is expressed in these cells at a level comparable to that present in preadipocytes as shown using the KLF2 antibody (Figure 3C). Importantly, this indicates that the expression system resulted in inducing KLF2 at physiologically relevant levels and that the KLF2 gene was not grossly overexpressed (compare lanes 1 and 4). As expected, lane 2 showed a more prominent KLF2 band since the antibody detected both endogenous and ectopically expressed KLF2. Furthermore, in KLF2-expressing cells that were induced to differentiate, the preadipocytic marker Pref-1 was restored to the levels seen prior to induction of differentiation (Figure 3D). To further ascertain that KLF2 maintained the preadipocytic state of cells, we determined the levels of adipogenic marker AP2/422 and found it to be decreased in KLF2-expressing cells (Figure 3D). Upon extended differentiation past day 8, we observed further accumulation of oil drops and their coalescing to form larger droplets in differentiated cells without doxycycline. However, cells induced for KLF2 expression failed to demonstrate this effect and continued to retain features of preadipocytes (Figure 3C, compare panels 3 and 4). Identical results were obtained from all of the 3T3L1-KLF2 clones analyzed. In summary, KLF2-expressing cells did not accumulate fat; they maintained high levels of Pref-1 and showed decreased levels of adipogenic markers, all of which demonstrate that the reexpression of KLF2 in differentiation-induced preadipocytes inhibits their conversion into adipocytes. The inability of 3T3L1 cells to differentiate appears to be a consequence of the revived expression of KLF2 in these induced cells.

KLF2$^{-/-}$ MEFs Are Not Defective in Adipocyte Precursor Cell Formation and, When Induced, Exhibit the Adipocytic Phenotype. We investigated the role of KLF2 in the formation of adipocyte precursors and their subsequent differentiation into mature adipocytes using two in vitro approaches in our study. The first was the analysis of embryonic fibroblasts derived from KLF2$^{+/+}$ and KLF2 $^{-/-}$ embryos; the second involved the differentiation of embryoid bodies from KLF2-null ES cells.

We generated embryonic fibroblasts from wild-type KLF2 and KLF2-deficient E11.5 mice embryos, since preadipocytes are derived from fibroblast-like precursors (*27*). The cells were obtained from trypsin-digested wild-type and null E11.5 mouse embryos and passaged 1−5 times to generate homozygous KLF2$^{+/+}$ and KLF2$^{-/-}$ primary cell cultures composed of morphologically similar fibroblasts. For induction of adipocyte differentiation, insulin, dexamethasone, and IBMX were used. After the first week of treatment, the fibroblasts acquired the characteristic rounded morphology of adipocyte cells and showed accumulation of oil droplets (Figure 4A). To corroborate our Oil Red O data, we also examined the biochemical markers associated with adipogenesis and found the expected correlation as indicated by decreased Pref-1 levels and increased AP2/422, adiponectin, and β3-AR (Figure 4B). Since differentiation is known to induce decreases in actin and tubulin levels (*28, 29*), we used Ponceau S stained bands to indicate equal loading of protein. Taken together, the results show that KLF2-null fibroblasts are capable of producing preadipocytes that can subsequently differentiate, when induced, into fat-accumulating adipocytic cells.

Embryoid bodies derived from KLF2$^{-/-}$ mouse ES cells retain the ability to form functional adipocyte precursors that differentiate upon adipogenic stimuli. The generation of KLF2$^{-/-}$ ES cells and their subsequent use in the in vitro culture were also necessary to bypass the embryonic lethality problem of KLF2-null mice. Embryoid bodies derived from embryonic stem cells with or without the KLF2 gene were

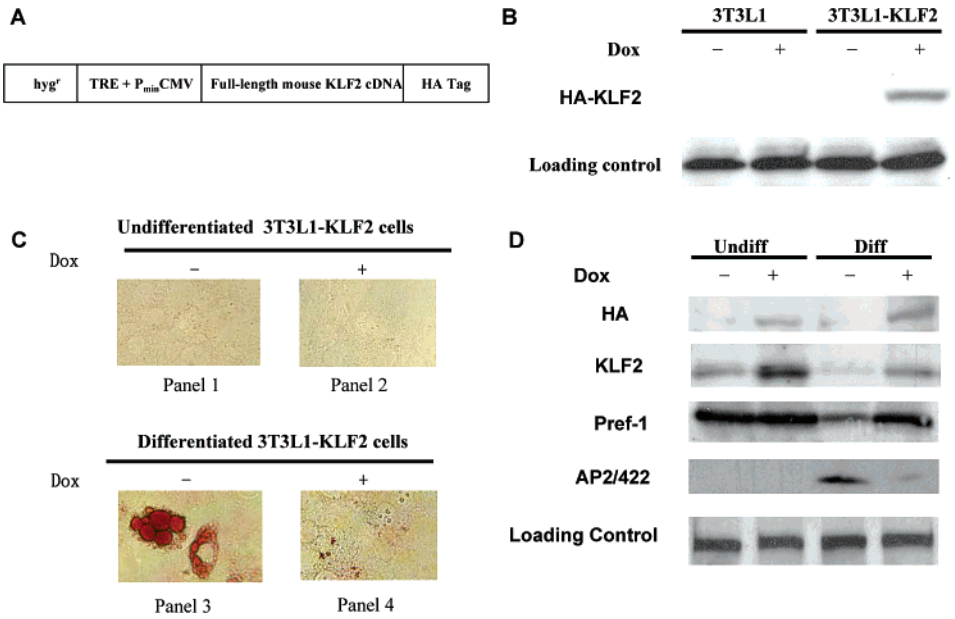

FIGURE 3: Ectopic expression of KLF2 blocks adipogenesis, decreases endogenous levels of adipogenic genes, and simultaneously restores Pref-1 expression to preadipocytic levels. The reverse tetracycline repressor system was used to produce inducible expression of KLF2 in 3T3L1 preadipocytes as described in Materials and Methods. (A) Schematic diagram of the KLF2 tet-inducible construct using full-length KLF2. The construct carried a C-terminal HA tag. (B) 3T3L1 cells were cultured as described. Cells were either mock-treated with PBS or treated with 1 μg/mL tetracycline as indicated and harvested 48 h later for Western blot analysis. Anti-HA antibody was used to detect KLF2 tagged by the HA epitope. (C) 3T3L1-KLF2 cells were cultured in six-well plates (1×10^4) as described. When confluent, cells were treated with doxycycline to induce KLF2 and then differentiated 2 days post-induction. Panels 2 and 4 show cells that were induced for KLF2 expression by the addition of doxycycline. Cells were photographed on day 10, after staining for lipid accumulation. (D) 3T3L1-KLF2 cells were cultured in 10 cm dishes and, upon confluence, induced with 1 μg/mL doxycycline. Cells were then differentiated into adipocytes as described in Materials and Methods. Cell lysates were prepared 6 days post-differentiation and subjected to Western blotting with the indicated antibodies.

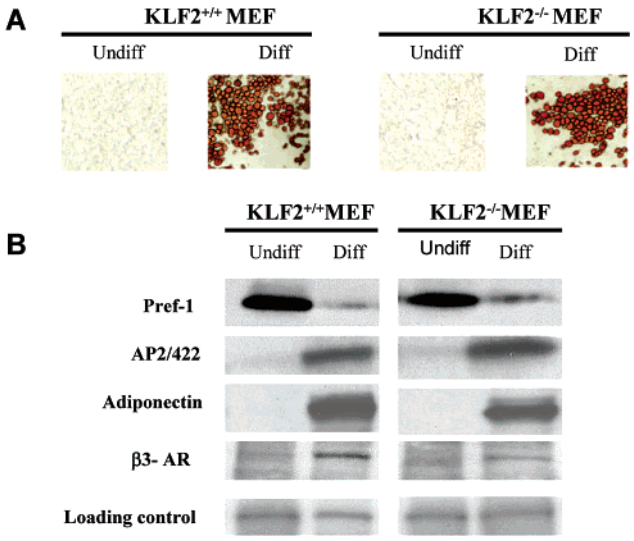

FIGURE 4: Fibroblasts derived from E11.5 KLF2$^{-/-}$ mouse embryos are capable of differentiating into adipocyte cells that accumulate oil droplets and show decreased Pref-1. Fibroblasts were derived from mouse embryos as described in Materials and Methods. Both KLF2$^{-/-}$ and KLF2$^{+/+}$ fibroblasts were plated in six-well plates and differentiated as described. Plates were stained with Oil Red O to assess lipid accumulation on day 10 (A) or were harvested for protein analysis of preadipocytic and adipogenic markers (B).

analyzed for their ability to differentiate into adipocytes. KLF2$^{-/-}$ and KLF2$^{+/+}$ cells were grown on mitomycin-treated fibroblast feeder layers initially and later suspended as microdrops on inverted plates to form embryoid bodies (EBs). Following the administration of the standard differentiation protocol to these cultures (*23*), microscopic examination revealed oil-accumulating cells in embryoid bodies that were stimulated for adipocyte differentiation in both wild-type and KLF2$^{-/-}$ cultures. Cell enlargement was also apparent by day 5. The results of the embryoid bodies stained with Oil Red O are shown in Figure 5A. An alternate set of KLF2$^{-/-}$ and KLF2$^{+/+}$ EBs were maintained under uninduced control conditions (Figure 5A). These cells expressed the preadipocytic marker Pref-1 and did not stain with Oil Red O (Figure 5A). This experiment was extended for 2 weeks and resulted in enlargement of cells, rounded morphology, and accumulation of coalescing oil droplets (Figure 5A). Pref-1 showed a significant decrease in expression in response to differentiation in both KLF2$^{+/+}$ and KLF2$^{-/-}$ MEFs (Figure 5B). To further characterize the biochemical basis for the observed differentiation of EBs, we examined AP2/422, adiponectin, and β3-AR in the embryoid bodies and found that these known markers of adipogenesis increased significantly in the differentiated cultures, regardless of whether they were derived from KLF2-containing or KLF2-deficient ES cells (Figure 5B). Thus the lack of KLF2 in embryoid bodies does not render them incapable of differentiating into adipocytes when appropriately stimulated.

KLF2$^{-/-}$ MEFs Are Prone To Differentiate at the Early Stage of Differentiation. As described above, on the basis of the data of Oil Red O staining and biochemical markers, the differentiation of KLF2-null cells and EBs is not impaired or accelerated during the process. To further ascertain if KLF2 is a natural repressor of differentiation, we carried out the kinetic quantification of lipid accumulation during KLF2$^{-/-}$ differentiation. As shown in Figure 6, after a full

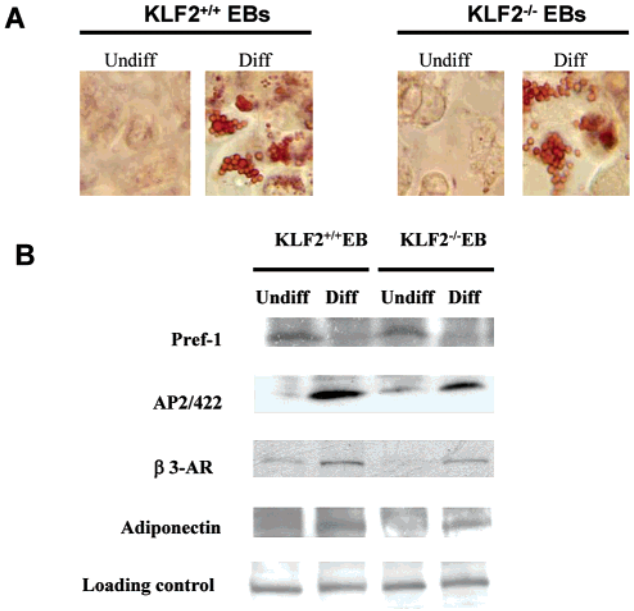

FIGURE 5: Embryoid bodies derived from KLF2-null mouse ES cells are not defective in adipocyte precursor cell formation and upon differentiation form lipid-laden cells with the adipocytic phenotype. Embryoid bodies from either wild-type or null KLF2 cells were derived by the hanging drop culture of ES cells and differentiated as described in the text. Oil Red O staining was done on day 14 (A), and a matched set of EBs were harvested for analysis of preadipogenic and adipogenic (B) markers.

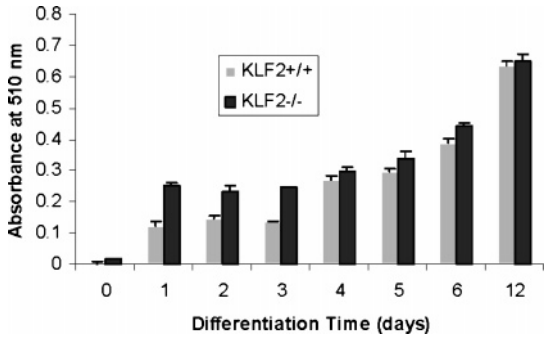

FIGURE 6: Kinetic quantification of lipid accumulation in MEF differentiation. $KLF2^{+/+}$ MEFs and $KLF2^{-/-}$ MEFs are subject to differentiation in six-well plates. At the different time points, cells were washed and stained with Oil Red O. After extraction by isopropyl alcohol, the lipid quantification was measured at 510 nm.

12 days of differentiation, $KLF2^{-/-}$ cells do not exhibit a significant difference of lipid accumulation compared to $KLF2^{+/+}$. However, upon 2 days after confluence (day 0 of differentiation), $KLF2^{-/-}$ cells showed autonomously more lipid deposit (~10-fold) compared to wild-type cells. At the initial stage of differentiation, i.e., day 1−3, $KLF2^{-/-}$ cells still exhibited about 100% more lipid accumulation than $KLF2^{+/+}$ cells. These data indicate that $KLF2^{-/-}$ cells are more prone to differentiate at the early stage of adipocyte differentiation, suggesting that KLF2 is a natural repressor of differentiation.

DISCUSSION

Our demonstration of a significant decrease of KLF2 protein (over 90%) upon preadipocyte differentiation relates well with the published report of reduced KLF2 mRNA upon the induction of differentiation (*11*). It therefore appears that, similar to the downregulation of Pref-1, Wnts, and GATA-3, the decrease of KLF2 protein is a prerequisite for the onset of adipogenic events. It is interesting to note that a KLF2 promoter-driven luciferase reporter showed a significant and reproducible loss of activity upon differentiation, thereby indicating that one possible mechanism of KLF2 downregulation occurs through the reduction of its promoter activity and is therefore at the transcriptional level. This also suggests that the absence of positive acting factors and/or the recruitment of negative regulators (*31*) of the KLF2 promoter may be responsible for the downregulation of KLF2. We have previously shown that KLF2 protein binds directly to WWP1, an E3 ubiquitin ligase (*32*), and that this interaction leads to the ubiquitination and degradation of KLF2 (*33*). The present results suggest that transcriptional regulation of KLF2 is an additional mechanism by which KLF2 is regulated. It will be interesting to extensively characterize the KLF2 promoter to identify the specific cis elements involved in this inhibition and the factors that bind to them.

This is the first report of physiological levels of KLF2 inhibiting 3T3L1 cell adipogenesis, thereby suggesting that KLF2 is a natural repressor of differentiation. Moreover, the reexpression of KLF2 sustains the levels of Pref-1, an inhibitor of differentiation that is normally downregulated upon the induction of adipogenesis. Pref-1 is an epidermal growth factor-like, transmembrane protein (*4*), and its downregulation is a necessary step for adipose development (*34*). Cells stably expressing Pref-1 fail to differentiate (*5*), and Pref-1 knockout mice show increased adiposity (*35*). Therefore, our finding that physiological reexpression of KLF2 is able to restore Pref-1 expression even in differentiating cultures suggests that one mechanism by which KLF2 helps to maintain preadipocytic characteristics is by circumventing the glucocorticoid-induced transcriptional repression of Pref-1. Additionally, it is interesting to note that physiological expression of KLF2 downregulates AP2 in 3T3L1 cells that are induced to differentiate. AP2, a fatty acid binding protein, is a marker of terminal adipocyte differentiation and is regulated by PPAR-γ and C/EBPs (*36*, *37*). The observation that AP2 is downregulated in 3T3L1-KLF2 cells raises the intriguing possibility that, in addition to being upregulated by PPAR-γ/CEBP, AP2 could also be subjected to negative regulation by KLF2. Alternatively, it is possible that the observed decrease in AP2 is simply the consequence of the downregulation of PPAR-γ. Nevertheless, the reduction of AP2 provides biochemical evidence that KLF2 negatively regulates the preadipocyte to adipocyte transition as it either directly or indirectly decreases adipose-specific gene expression.

Although the commitment phase of pluripotent cells is not well understood, it is thought to be triggered by factors that activate genes responsible for initiating a lineage-specific developmental program. Recent studies have begun to identify proteins involved in the early lineage-determining events of pluripotent cells. For instance, bone morphogenic protein 4 (BMP4) can induce the commitment of pluripotent C3H10T1/2 cells into differentiation-competent preadipocytes (*38*). Testosterone is thought to promote commitment toward the myogenic lineage and inhibit adipocyte formation (*39*). The commitment of ES cells toward adipocytes is also influenced by cell shape, cytoskeletal tension, and RhoA signaling (*40*). Also, protein kinase C ϵ (PKC ϵ) has a stimulatory role in both adipogenic commitment of multi-

potent fibroblasts and the diffferentiation of preadipocytes (*41*). Another report showed that overexpression of Pref-1 inhibits the conversion of mesenchymal stem cells into adipocytes and is also associated with reduced levels of late stage adipogenic markers (*42*). Considering that KLF2 is involved in the development of the lung and blood vessel, it was possible that KLF2 is required not just for the maintenance of preadipocytic characteristics but also for their formation. In such a scenario, the lack of KLF2 would prevent the formation of or lead to a severely reduced pool of preadipocytes. We therefore hypothesized that if KLF2 is required to generate preadipocytes, then KLF2-null cells would have a reduced preadipocytic pool and such cells could not form adipocytes because they would lack adipocyte precursors. Since the 3T3L1 cell system represents a cell model where the cells have already committed to the preadipocyte lineage, this alternate approach of using embryoid bodies was necessary as it enabled us to study the initial stages of adipose cell formation including the "commitment" of mesenchymal precursors into preadipocytes. Embryonic stem (ES) cells can be induced to differentiate along the adipogenic pathway, and this is known to be an effective model system to study the regulation of the first steps of adipose cell development (*27*). Embryonic stem cells give rise to mesenchymal cells that are capable of developing into cells of different lineages such as chondrocytes, osteocytes, myocytes, and adipocytes (*43*). When appropriately induced, embryonic stem cells (ES), which are normally pluripotent, can be channeled to form adipocytes (*44*). This allows the study of the initial steps of adipogenesis in addition to identifying genes involved in the commitment of the pluripotent mesenchymal stem cells into cells of adipocyte lineage. In the present study, both embryonic fibroblast data and embryoid body differentiation studies show that KLF2-negative cells differentiate efficiently; by extension these studies also indicate that KLF2-deficient cells can form a differentiation-competent pool of preadipocytes. It therefore appears that KLF2 does not have a pivotal role in converting pluripotent ES cells into preadipocytes. However, this does not rule out the possibility of an indirect role for KLF2 in the establishment of fat cell lineage, since expression of KLF2 can sustain Pref-1, a factor that is now known to be involved in mesenchymal cell fate determination (*42*).

In the present study, KLF2$^{-/-}$ ES cells were used to generate chimeric mice, and their ability to form fat pads was tested. If KLF2 is required for the formation of fat pads, then KLF2$^{-/-}$ cells would be incapable of forming fat. However, our present data demonstrate that KLF2-null cells can and do contribute to the formation of epididymal fat pads in adult mice equally when compared to wild-type ES cells (data not shown). This corroborates with the two in vitro approaches used in our study. It is therefore logical to infer, from the analysis of fat pad formation in chimeric mice and in vitro differentiation studies of embryonic fibroblasts and embryoid bodies, that the competency to contribute toward adipocyte precursor formation is in a KLF2-independent manner. Taken together, our studies show that KLF2 is a negative regulator of adipogenesis but is dispensable for the formation of differentiation-competent precursors of fat cells.

Our present study shows that KLF2$^{-/-}$ MEF cells differentiate faster than wild-type cells and indicates that loss of KLF2 promotes adipocyte differentiation. This is consistent with the role of KLF2 in T-cell quiescence.

There are several other transcription factors that repress adipogenic genes, some of which are members of the zinc-finger family. For instance, AEBP2, a protein containing three Gli-Kruppel (Cys2-His2) type zinc fingers, binds to a regulatory element located in the proximal promoter of AP2 and represses its expression (*45*). Sirt1 represses PPAR-γ by docking with its cofactors NCoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptors) (*46*). Interestingly, parathyroid hormone-related protein (PTHrP), which affects the developing lung by regulating branching morphogenesis and type II cell maturation, is also known to downregulate PPAR-γ via a MAPK-dependent pathway and thereby inhibit differentiation. Furthermore, PTHrP is also thought to alter the commitment and differentiation of pluripotent cells (*47*). There are reports of the GATA family of transcription factors negatively regulating adipogenesis by downregulating PPAR-γ (*6*) and interacting with the C/EBP family (*48*). Collectively, these studies suggest that multiple families of transcription factors are involved in negative regulation of adipogenesis and they act by downregulating primary players of adipocyte differentiation. Along with the upregulation of positively acting factors, the onset of adipogenesis therefore requires the sequential suppression of several negative regulators for effectively establishing the adipocyte phenotype. Given that loss of cyclin-dependent kinase inhibitors such as p27 and p21 markedly stimulate adipogenesis, p27/p21 double knockout mice show an obese phenotype (*49*), and our studies demonstrate that KLF2 upregulates p21 (*21*), future studies should address the possibility that positive regulation of p21 by KLF2 is an additional mechanism by which KLF2 could regulate adipogenesis.

ACKNOWLEDGMENT

We thank Dr. Maqsood Wani for generating the ES cells, Dr. Nancy Kleene for expert help in photographing Oil Red O images, and Dr. Leiden through Dr. Laurie Glimcher for kindly providing the KLF2 antibody.

REFERENCES

1. Hausman, D. B., DiGirolamo, M., Bartness, T. J., Hausman, G. J., and Martin, R. J. (2001) The biology of white adipocyte proliferation, *Obes. Rev. 2*, 239−254.
2. Harp, J. B. (2004) New insights into inhibitors of adipogenesis, *Curr. Opin. Lipidol. 15*, 303−307.
3. Tong, Q., Dalgin, G., Xu, H., Ting, C. N., Leiden, J. M., and Hotamisligil, G. S. (2000) Function of GATA transcription factors in preadipocyte-adipocyte transition, *Science 290*, 134−138.
4. Smas, C. M., and Sul, H. S. (1993) Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation, *Cell 73*, 725−734.
5. Smas, C. M., and Sul, H. S. (1996) Characterization of Pref-1 and its inhibitory role in adipocyte differentiation, *Int. J. Obes. Relat. Metab. Disord. 20* (Suppl. 3), S65−S72.
6. Schwarz, E. J., Reginato, M. J., Shao, D., Krakow, S. L., and Lazar, M. A. (1997) Retinoic acid blocks adipogenesis by inhibiting C/EBPbeta-mediated transcription, *Mol. Cell. Biol. 17*, 1552−1561.
7. Ross, S. E., Hemati, N., Longo, K. A., Bennett, C. N., Lucas, P. C., Erickson, R. L., and MacDougald, O. A. (2000) Inhibition of adipogenesis by Wnt signaling, *Science 289*, 950−953.
8. Inuzuka, H., Wakao, H., Masuho, Y., Muramatsu, M., Tojo, H., and Nanbu-Wakao, R. (1999) cDNA cloning and expression analysis of mouse zf9, a Kruppel-like transcription factor gene

that is induced by adipogenic hormonal stimulation in 3T3-L1 cells, *Biochim. Biophys. Acta 1447*, 199−207.
9. Gray, S., Feinberg, M. W., Hull, S., Kuo, C. T., Watanabe, M., Sen-Banerjee, S., DePina, A., Haspel, R., and Jain, M. K. (2002) The Kruppel-like factor KLF15 regulates the insulin-sensitive glucose transporter GLUT4, *J. Biol. Chem. 277*, 34322−34328.
10. Besnar, N., Persuy, M. A., Stinnakre, M. G., Lepourry, L., Da Silva, J. C., Goubin, G., and Vilotte, J. L. (2002) Targeted expression of the only zinc finger gene in transgenic mice is associated with impaired mammary development, *Transgenic Res. 11*, 505−513.
11. Banerjee, S. S., Feinberg, M. W., Watanabe, M., Gray, S., Haspel, R. L., Denkinger, D. J., Kawahara, R., Hauner, H., and Jain, M. K. (2003) The Kruppel-like factor KLF2 inhibits peroxisome proliferator-activated receptor-γ expression and adipogenesis, *J. Biol. Chem. 278*, 2581−2584.
12. Wani, M. A., Means, R. T., Jr., and Lingrel, J. B. (1998) Loss of LKLF function results in embryonic lethality in mice, *Transgenic Res. 7*, 229−238.
13. Kuo, C. T., Veselits, M. L., Barton, K. P., Lu, M. M., Clendenin, C., and Leiden, J. M. (1997) The LKLF transcription factor is required for normal tunica media formation and blood vessel stabilization during murine embryogenesis, *Genes Dev. 11*, 2996−3006.
14. Wani, M. A., Wert, S. E., and Lingrel, J. B. (1999) Lung Kruppel-like factor, a zinc finger transcription factor, is essential for normal lung development, *J. Biol. Chem. 274*, 21180−21185.
15. Kuo, C. T., Veselits, M. L., and Leiden, J. M. (1997) LKLF: A transcriptional regulator of single-positive T cell quiescence and survival, *Science 277*, 1986−1990.
16. Dekker, R. J., van Soest, S., Fontijn, R. D., Salamanca, S., de Groot, P. G., VanBavel, E., Pannekoek, H., and Horrevoets, A. J. (2002) Prolonged fluid shear stress induces a distinct set of endothelial cell genes, most specifically lung Kruppel-like factor (KLF2), *Blood 100*, 1689−1698.
17. Huddleson, J. P., Srinivasan, S., Ahmad, N., and Lingrel, J. B. (2004) Fluid shear stress induces endothelial KLF2 gene expression through a defined promoter region, *Biol. Chem. 385*, 723−729.
18. SenBanerjee, S., Lin, Z., Atkins, G. B., Greif, D. M., Rao, R. M., Kumar, A., Feinberg, M. W., Chen, Z., Simon, D. I., Luscinskas, F. W., Michel, T. M., Gimbrone, M. A., Jr., Garcia-Cardena, G., and Jain, M. K. (2004) KLF2 is a novel transcriptional regulator of endothelial proinflammatory activation, *J. Exp. Med. 199*, 1305−1315.
19. Farmer, S. R., and Auwerx, J. (2004) Adipose tissue: new therapeutic targets from molecular and genetic studies—IASO Stock Conference 2003 Report, *Obes. Rev. 5*, 189−196.
20. Kras, K. M., Hausman, D. B., Hausman, G. J., and Martin, R. J. (1999) Adipocyte development is dependent upon stem cell recruitment and proliferation of preadipocytes, *Obes. Res. 7*, 491−497.
21. Wu, J., and Lingrel, J. B. (2004) KLF2 inhibits Jurkat T leukemia cell growth via upregulation of cyclin-dependent kinase inhibitor p21 (WAF1/CIP1), *Oncogene 21*, 8088−8096.
22. Wu, Z., Rosen, E. D., Brun, R., Hauser, S., Adelmant, G., Troy, A. E., McKeon, C., Darlington, G. J., and Spiegelman, B. M. (1999) Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity, *Mol. Cell 3*, 151−158.
23. Dani, C., Smith, A. G., Dessolin, S., Leroy, P., Staccini, L., Villageois, P., Darimont, C., and Ailhaud, G. (1997) Differentiation of embryonic stem cells into adipocytes in vitro, *J. Cell. Sci. 110*, 1279−1285.
24. Koopman, R., Schaart, G., and Hesselink, M. K. (2001) Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids, *Histochem. Cell. Biol. 116*, 63−68.
25. Selvarajan, S., Lund, L. R., Takeuchi, T., Craik, C. S., and Werb, Z. (2001) A plasma kallikrein-dependent plasminogen cascade required for adipocyte differentiation, *Nat. Cell Biol. 3*, 267−275.
26. Viravaidya, K., and Shuler, M. L. (2002) The effect of various substrates on cell attachment and differentiation of 3T3-F442A preadipocytes, *Biotechnol. Bioeng. 78*, 454−458.
27. Phillips, B. W., Vernochet, C., and Dani, C. (2003) Differentiation of embryonic stem cells for pharmacological studies on adipose cells, *Pharmacol. Res. 47*, 263−268.
28. Spiegelman, B. M., and Farmer, S. R. (1982) Decreases in tubulin and actin gene expression prior to morphological differentiation of 3T3 adipocytes, *Cell 29*, 53−60.
29. Teichert-Kuliszewska, K., Hamilton, B. S., Roncari, D. A., Kirkland, J. L., Gillon, W. S., Deitel, M., and Hollenberg, C. H. (1996) Increasing vimentin expression associated with differentiation of human and rat preadipocytes, *Int. J. Obes. Relat. Metab. Disord. 20* (Suppl. 3), S108−S113.
30. Lyon, M, F., and Searle, A. G. (1990) *Genetic Variants and Strains of the Laboratory Mouse*, Oxford University Press, Oxford, U.K.
31. Levine, M., and Tjian, R. (2003) Transcription regulation and animal diversity, *Nature 424*, 147−151.
32. Conkright, M. D., Wani, M. A., and Lingrel, J. B. (2001) Lung Kruppel-like factor contains an autoinhibitory domain that regulates its transcriptional activation by binding WWP1, an E3 ubiquitin ligase, *J. Biol. Chem. 276*, 29299−29306.
33. Zhang, X., Srinivasan, S. V., and Lingrel, J. B. (2004) WWP1-dependent ubiquitination and degradation of the lung Kruppel-like factor, KLF2, *Biochem. Biophys. Res. Commun. 316*, 139−148.
34. Smas, C. M., Chen, L., Zhao, L., Latasa, M. J., and Sul, H. S. (1999) Transcriptional repression of pref-1 by glucocorticoids promotes 3T3-L1 adipocyte differentiation, *J. Biol. Chem. 274*, 12632−12641.
35. Moon, Y. S., Smas, C. M., Lee, K., Villena, J. A., Kim, K. H., Yun, E. J., and Sul, H. S. (2002) Mice lacking paternally expressed Pref-1/Dlk1 display growth retardation and accelerated adiposity, *Mol. Cell. Biol. 22*, 5585−5592.
36. Mandrup, S., and Lane, M. D. (1997) Regulating adipogenesis, *J. Biol. Chem. 272*, 5367−5370.
37. Gregoire, F. M., Smas, C. M., and Sul, H. S. (1998) Understanding adipocyte differentiation, *Physiol. Rev. 78*, 783−809.
38. Tang, Q. Q., Otto, T. C., and Lane, M. D. (2004) Commitment of C3H10T1/2 pluripotent stem cells to the adipocyte lineage, *Proc. Natl. Acad. Sci. U.S.A. 101*, 9607−9611.
39. Bhasin, S., Taylor, W. E., Singh, R., Artaza, J., Sinha-Hikim, I., Jasuja, R., Choi, H., and Gonzalez-Cadavid, N. F. (2003) The mechanisms of androgen effects on body composition: mesenchymal pluripotent cell as the target of androgen action, *J. Gerontol., Ser. A 58*, M1103−M1110.
40. McBeath, R., Pirone, D. M., Nelson, C. M., Bhadriraju, K., and Chen, C. S. (2004) Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment, *Dev. Cell 6*, 483−495.
41. Webb, P. R., Doyle, C., and Anderson, N. G. (2003) Protein kinase C-epsilon promotes adipogenic commitment and is essential for terminal differentiation of 3T3-F442A preadipocytes, *Cell. Mol. Life Sci. 60*, 1504−1512.
42. Abdallah, B. M., Jensen, C. H., Gutierrez, G., Leslie, R. G., Jensen, T. G., and Kassem, M. (2004) Regulation of human skeletal stem cells differentiation by Dlk1/Pref-1, *J. Bone Miner. Res. 19*, 841−852.
43. Doss, M. X., Koehler, C. I., Gissel, C., Hescheler, J., and Sachinidis, A. (2004) Embryonic stem cells: a promising tool for cell replacement therapy, *J. Cell. Mol. Med. 8*, 465−473.
44. Dani, C. (2002) Differentiation of embryonic stem cells as a model to study gene function during the development of adipose cells, *Methods Mol. Biol. 185*, 107−116.
45. He, G. P., Kim, S., and Ro, H. S. (1999) Cloning and characterization of a novel zinc finger transcriptional repressor. A direct role of the zinc finger motif in repression, *J. Biol. Chem. 274*, 14678−14684.
46. Picard, F., Kurtev, M., Chung, N., Topark-Ngarm, A., Senawong, T., Machado De Oliveira, R., Leid, M., McBurney, M. W., and Guarente, L. (2004) Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma, *Nature 429*, 771−776.
47. Chan, G. K., Deckelbaum, R. A., Bolivar, I., Goltzman, D., and Karaplis, A. C. (2001) PTHrP inhibits adipocyte differentiation by down-regulating PPAR gamma activity via a MAPK-dependent pathway, *Endocrinology 142*, 4900−4909.
48. Tong, Q., Tsai, J., Tan, G., Dalgin, G., and Hotamisligil, G. S. (2005) Interaction between GATA and the C/EBP family of transcription factors is critical in GATA-mediated suppression of adipocyte differentiation, *Mol. Cell. Biol. 25*, 706−715.
49. Naaz, A., Holsberger, D. R., Iwamoto, G. A., Nelson, A., Kiyokawa, H., and Cooke, P. S. (2004) Loss of cyclin-dependent kinase inhibitors produces adipocyte hyperplasia and obesity, *FASEB J., 18*, 1925−1927.

BI050166I