DNA binding-dependent glucocorticoid receptor activity promotes adipogenesis via Krüppel-like factor 15 gene expression

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Glucocorticoids, such as dexamethasone, have been used as *in vitro* inducers of adipogenesis. However, the roles of the glucocorticoid receptor (GR) in adipogenesis have not been well characterized yet. Here, we show that inhibition of GR activity using the GR antagonist RU486 prevents human mesenchymal stem cell and mouse embryonic fibroblast (MEF) differentiation into adipocytes. Moreover, in MEFs isolated from GR knockout (GR^null) and GR^dim mice deficient in GR DNA-binding activity, adipogenesis was blocked. We identified glucocorticoid response element sites in the first intron of KLF15 by bioinformatical promoter analysis and confirmed their functional relevance by demonstrating GR interaction by chromatin immunoprecipitation. Moreover, transfection of MEFs with siRNA for KLF15 significantly attenuated the expressions of adipogenic-marker genes and the lipid accumulation. Our results provide a new mechanism for understanding glucocorticoids-dependent adipogenesis and that GR promotes adipogenesis via KLF15 gene expression as a transcriptional direct target.

Adipose tissue has a key role in the regulation of energy balance and mobilization as well as the endocrine regulation of nutrient intake. Because of its role in obesity and the metabolic syndrome, there is increasing interest in adipocyte biology, including the differentiation of adipose tissue.

It is well established that fibroblastic cells including 3T3-L1, mesenchymal stem cells, and mouse embryonic fibroblasts (MEFs) can be differentiated into adipocytes under exposure to adipogenic inducers such as fetal bovine serum (FBS), dexamethasone (Dex), 3-isobutyl-1-methylxanthine (IBMX), and insulin.[1–4] This adipogenesis process is tightly regulated by the complicated temporal expression of transcription factors.[5–7] Among them, the peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα) have been shown to have critical roles in adipogenesis.[8,9] These two proteins act synergistically in the transcriptional activation of various adipocyte-specific genes.[10] In addition, two other members of the C/EBP family, C/EBPβ and C/EBPδ, are induced earlier than C/EBPα and PPARγ just after adipogenic stimulation. The ectopic expression of C/EBPβ and C/EBPδ promotes the expression of PPARγ and C/EBPα in fibroblasts and 3T3-L1,[11–13] suggesting that C/EBPβ and C/EBPδ are upstream transcriptional regulators for PPARγ and C/EBPα.

Glucocorticoids are potent regulators of adipose differentiation both *in vitro* and *in vivo*.[14–16] The synthetic glucocorticoid Dex is widely used as a component of the adipogenic-induction cocktail for *in vitro* adipogenesis systems. Cao *et al*[11] have shown that treatment of 3T3-L1 preadipocytes with Dex activates C/EBPδ, suggesting that the

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potential mechanism of the role of Dex in adipogenesis is to enhance PPARγ and C/EBPα expression via promoting C/EBPδ expression.[12] In a mesenchymal progenitor cell line, Dex induced C/EBPα, C/EBPβ, and C/EBPδ, which lead to an increase in PPARγ and aP2 to induce adipocyte differentiation.[17]

Glucocorticoids exert their effects through the glucocorticoid receptor (GR), a ligand-activated transcription factor that belongs to the nuclear receptor superfamily.[18,19] Molecular functions of the GR can be divided into two main actions; a transcriptional activator as a dimer and a transcriptional repressor as a monomer. As an activator, the ligand-bound GR binds to palindromic glucocorticoid response elements (GREs) as a dimer, thereby promoting target genes' transcription.[20–23] Dimerization depends on the D-loop in the DNA-binding domain. Transcriptional repression by GR appears to be mediated by GR monomers' protein–protein interaction with other transcription factors, and neither needs dimerization nor DNA binding. GR dimerizaion activity is abolished by an amino-acid substitution in the GR D-loop (A458T).[24] In mice carrying this point mutation (GR^dim mice), GRE binding-dependent gene expression is impaired, but the monomer GR function, that is, cross-talk with other transcription factors (protein–protein interaction), is maintained.[25] To get insight into the precise molecular mechanism of adipogenesis triggered by Dex, we used MEF from two GR-mutant mice; GR-deficient (GR^null) mice and GR^dim mice. These tools allow us to characterize direct GR-target genes in adipogenesis.

Among the set of GR targets during adipogenesis screened in this study, we focused on the role of KLF15, a member of the Krüppel-like family of transcription factors. Krüppel-like factors (KLFs) have diverse roles in cell proliferation, cell differentiation, and development. Some members of KLFs, such as KLF2,[26] KLF5,[27] and KLF15[28] have been implicated in adipogenesis. KLF15 has previously been shown to regulate adipogenesis by inducing GLUT4[29] and PPARγ[28] expression in 3T3-L1 preadipocytes; KLF15^−/− mice exhibit severe hypoglycemia after an overnight fast.[30] Previous studies revealed that KLF15 is induced by ectopic expression of C/EBPδ and C/EBPβ, Dex treatment, and cAMP hormonal stimulation in 3T3-L1 cells.[28] However, the molecular mechanism for the promotion of KLF15 gene expression is still unknown.

Here, we report that glucocorticoid stimulation was required for *in vitro* adipogenesis and GR activation with glucocorticoids induced KLF15 gene expression in a DNA-dependent manner.

MATERIALS AND METHODS
Reagents and Cells
Dex, IBMX, insulin, and RU486 were obtained from Sigma-Aldrich (St Louis, MO, USA). Pioglitazone was obtained from ALEXIS Biochemicals (Lausen, Switzerland). BMGM culture media for human mesenchymal stem cells (hMSCs) were obtained from Cambrex Bio Science Walkersville (Walkersville, MD, USA). Mouse embryonic fibroblast (MEF) cells from GR^null mice and GR^dim mice were isolated as described.[31] The 293T cell line was obtained from American Type Culture Collection. Anti-GR antibody (sc-1002) for chromatin immunoprecipitation (ChIP) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).[32]

Cell Culture and Differentiation
hMSCs were maintained in BMGM culture media, and cells from passages 3–6 were used for experiments. MEFs were isolated from 13.5-day-old embryos obtained from C57BL/6 mice as described previously.[33] Briefly, after dissection of head and visceral organs, embryos were minced and trypsinized for 30 min at 37°C. Embryonic fibroblasts were filtered, plated, and maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. All experiments with wild-type, GR^null and GR^dim MEFs were performed with cells in passages 2–10. For adipocyte differentiation, 2-day post-confluent cells were incubated for 3 days in adipogenesis-inducing medium (AIM), consisting of DMEM supplemented with 10% FBS, 500 μM IBMX, 1 μM Dex, 10 μM pioglitazone, 10 μg/ml insulin. Cells were then incubated in adipogenesis maintenance medium (DMEM supplemented with 10% FBS, 10 μg/ml insulin) for 4 days, with a fresh change of medium after 2 days (MEFs), or for 3 days (hMSCs); cells were then returned to AIM. Subsequently, lipid accumulation was visualized using Oil Red O stain as described previously.[34] Nile Red staining was performed to quantify lipid accumulation. PFA-fixed cells were incubated with 1:1000 of a Nile Red stock (0.5 mg/ml in acetone) in 80% glycerol 20% water and 1 mg/ml DAPI for nuclear counter staining. Lipid droplets were analyzed using Cellomics automated fluorescent microscopy. Cell numbers were counted according to DAPI staining and Nile red stained objects > 5.16 μm were considered as lipid droplets and their fluorescent intensity was determined.

Quantitative RT–PCR
Total RNA was extracted using Isogen (Wako, Osaka, Japan), and 2 μg RNA was used for cDNA synthesis with oligo(dT)18 primer by the Ready-to-Go You-First cDNA kit (GE Healthcare Life Sciences, Uppsala, Sweden), according to the manufacturer's instructions. Quantitative real-time RT–PCR was performed using TaqMan Gene Expression Assay probe for *Adiponectin* (Hs00605917_m1), *C/EBPα* (Hs00269972_s1), *C/EBPδ* (Hs00270931_s1), *KLF15* (Hs00362736_m1), *PPARγ* (Hs01115513_m1), *GAPDH* (Hs99999905_m1), *mAdiponectin* (Mm00456425_m1), *mC/EBPα* (Mm01265914_s1), *mC/EBPδ* (Mm00786711_s1), *mGR* (Mm00433832_m1), *mHIF3α* (Mm00469373_m1), *mKLF9* (Mm00495172_m1), *mKLF15* (Hs00164004_m1), *mPPARγ* (Mm01184322_m1), *mZbtb16* (Mm01176868_m1), *mZhx3* (Mm00770117_m1), and *mGAPDH* (Mm99999915_g1) (All Applied Biosystems,

Carlsbad, CA, USA). Relative expression was calculated using the $\Delta\Delta Ct$ values, and results were expressed as $2-\Delta\Delta Ct$. *GAPDH* was used as an internal control to normalize differences in each sample. The value of each control sample was set at 1 and was used to calculate the fold change in target genes. For the results in Figures 2c, 4b and d and Supplementary Figure 3, quantitative RT–PCR was performed as described.[35] Primer information can be provided upon request. Data were analyzed statistically using the Mann–Whitney *U* test or Steel–Dwass' test (multiple comparison). *P*-values < 0.05 were considered statistically significant.

Microarray Data Analysis

To generate first-strand cDNA, each reaction contained T7-linked oligo(dT) primers and $5\,\mu g$ total RNA (Affymetrix, Santa Clara, CA, USA). After second-strand synthesis using the SuperScript cDNA system (Invitrogen, Carlsbad, CA, USA), *in vitro* transcription was performed with biotinylated UTP and CTP (Enzo Life Sciences, New York, NY, USA), resulting in ~ 20-fold amplification of RNA. The biotinylated cRNA targets ($20\,\mu g$) were then cleaned up, fragmented, and hybridized to GeneChip expression arrays (Mouse Genome 430 2.0 Array, Affymetrix). Arrays were then washed and stained with streptavidin phycoerythrin before being scanned on an Affymetrix GeneChip scanner. Gene expression was analyzed using the GeneSpring Software 7 (Agilent Technologies, Santa Clara, CA, USA). Genes with signal intensities below 50 were eliminated and normalized, and then genes from cells incubated with AIM or Dex treatment that exhibited at least a twofold change relative to the control treatment were selected for analysis. In addition, genes from cells incubated with AIM and RU486 treatment (adipogenesis-RU486-regulated group) that exhibited at least a twofold change compared with cells incubated in AIM were also selected. Genes that belong to both the adipogenesis-RU486 treatment group and Dex-treated group were identified as GR-regulated adipogenic genes.

Location Analysis of the Promoter Sequences of KLF15 gene

Promoter regions (from $-7500\,\text{bp}$ to $+2000\,\text{bp}$) of GR-regulated adipogenic genes conserved between mouse and human genome were analyzed using rVista 2.0 and visualized using the ECR browser (http://ecrbrowser.dcode.org/). Mat Inspector software searched the highly conserved region for putative GRE sites (Genomatix, Munich, Germany). These GRE sites included androgen receptor (AR) response elements (AREs) and progesterone receptor (PR) response elements, because GR, AR, and PR bind to a consensus sequence for the superfamily of steroid hormone receptors.

Chromatin Immunoprecipitation

ChIP assays were performed as previously described.[36,37] MEFs were washed with PBS and then cross-linked using 1% formaldehyde for 10 min. The cross-linking was quenched with glycine (final concentration, 0.125 M). The samples were then washed twice in PBS and homogenized in ChIP cell lysis buffer (5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% Nonidet P-40, and protease inhibitor cocktail). Nuclei were sedimented and separated from the cellular debris, and then placed into nuclear lysis buffer (50 mM Tris HCl (pH 8.1), 10 mM EDTA, 1% SDS, $10\,\mu M$ aprotinin, $10\,\mu M$ leupeptin, and 1 mM phenylmethylsulfonyl fluoride). After 10 min on ice, the lysate was sonicated on ice (Digital Sonifier; BRANSON, Danbury, CT, USA) using three pulses of 2 s each at 4–6 W, and then centrifuged for 20 min at $21\,000\,g$ at $4^\circ C$. The supernatant was collected as chromatin samples. At this point, one aliquot was reserved as the input sample. When visualized on an agarose gel, the DNA produced a smear of fragments ~ 200–600 bp long. For each immunoprecipitation reaction, $2\,\mu g$ of chromatin was used. The chromatin was precleared by incubating with protein-G agarose at $4^\circ C$ for 1 h. Antiserum raised against the GR or preimmune IgG were added ($2\,\mu g$ each), and the samples were rotated at $4^\circ C$ overnight. Immunoprecipitates were isolated by incubating with blocked protein-G agarose and then washed extensively. Chromatin was eluted from the antibody by incubating for 10 min at room temperature with elution buffer (0.1 M NaHCO$_3$ and 1% SDS). Cross-linking was reversed with NaCl (0.2 M, final concentration) and incubating at $65^\circ C$ for at least 4 h. Samples were then digested with 40 ng of proteinase K, and DNA was isolated via phenol/chloroform extraction followed by ethanol precipitation. DNA concentrations were calculated by measuring absorbance at 260 nm. PCR primer pairs used were 5′-GCACGCTGACC CAATGGC-3′ and 5′-AGGCCGCTGGACTCCG-3′ for the GRE1 site ($+980/+998$), 5′-CGGAGTCCAGCGGCCT-3′, and 5′-GATCCAGGACTTCCTGGACCC-3′ for the GRE2 site ($+1085/+1103$) in the first intron of the KLF15 gene.

Luciferase Reporter Assay

The mouse KLF15 enhancer region ($+933/+1216$) was amplified by PCR using genomic DNA from C57BL/6 mice, with the sense primer 5′-GCACGCTGACCCAATGGC-3′ and the antisense primer 5′-GATCCAGGACTTCCTGGACCC-3′ and then inserted into the pTA vector (TOYOBO, Osaka, Japan). This plasmid was digested with *Nhe*I and *Xho*I restriction enzymes and ligated into the luciferase expression vector pGL3-tk, which had been constructed by insertion of the thymidine kinase (TK) promoter from the herpes simplex virus (HSV) into pGL3-Basic (Promega). The GR-binding site of the KLF15 enhancer region was mutated using the KOD mutagenesis kit according to the manufacturer's recommendations (TOYOBO). This mutation changed the wild-type sequence 5′-ATTACATGTTGTTCC-3′ to 5′-A**C AA**CATGTT**CA**TCC-3′ for GRE1 and the wild-type sequence 5′-AGAACAGGGTGCCCC-3′ to 5′-A**C**AACAGGGT**CA**CCC-3′ for GRE2. For the transfection experiment, 293T cells were plated into 96-well plates at 1×10^3 cells/well, and transient transfections were performed the following day using FuGENE

HD (Roche Applied Science, Indianapolis, IN, USA). The total amount of plasmid used for the transfection assay per well was 0.1 μg reporter vector/0.01 μg GR expression vector or pcDNA3.1 (Invitrogen)/phRL-TK vector (Promega). On the following day, 100 nM Dex or vehicle (DMSO) were added to cells. After 24 h, luciferase assays were carried out using Dual-Glo Luciferase Assay kit (Promega). The GR-dependent promoter activity of each construct was expressed as the ratio of activity in Dex/activity in DMSO. All luciferase experiments were performed in triplicate. Statistical significance of the difference between pGL3-intKLF15-tk and mutant reporters was determined by Steel's test.

Transfection of siRNA for *KLF15* into MEFs
siRNA for *KLF15* (siKLF15) (Silencer Select siRNAs-s82946, Applied Biosystems) and negative siRNA (Silencer Select siRNAs Silencer Negative Control #1, Applied Biosystems) were transfected into MEFs at 80–90% confluence (50 nM concentration) with Lipofectamine RNAi Max (Invitrogen) according to the manufacturer's instructions. After 48 h, transfected MEFs were stimulated with AIM or control medium.

RESULTS
Inhibition of Adipogenesis by GR Antagonist RU486 in hMSCs and MEFs
To investigate the role of GR in adipocyte differentiation, hMSCs were induced to differentiate into adipocytes by incubation in adipogenic-induction medium containing 500 μM IBMX, 1 μM Dex, 10 μM pioglitazone, 10 μg/ml insulin with or without the GR antagonist RU486 (10 μM), followed by insulin treatment every 3 days. As shown in Figure 1a, hMSCs differentiated into adipocytes and accumulated lipid droplets in the presence of AIM, but no obvious lipid accumulation was observed in hMSCs treated with RU486 during adipogenic stimulation. As the adipogenic process is characterized by expression of specific adipocyte markers such as PPARγ, C/EBPα, and adiponectin, we next examined the expression of these adipocyte-specific genes by quantitave RT–PCR (Figure 1b). The expression of PPARγ, C/EBPα, and adiponectin was increased in hMSCs incubated with adipogenic inducers for 2 days, and these gene expression levels were inhibited completely by RU486. These findings suggest that GR activity is necessary for the adipogenic differentiation of hMSCs.

We next examined whether the RU486 effect observed in hMSCs is similar in MEFs from wild-type mice. Wild-type MEFs were differentiated into adipocytes, and RU486 inhibited the accumulation of lipid droplets albeit not as efficient as in hMSCs (Figure 1c). Expression of adipocyte-specific marker genes in MEFs from wild-type mice were also inhibited by RU486 (Supplementary Figure 1). These data indicated that GR has a critical role in adipose differentiation from MEFs and hMSCs.

Inhibition of Adipogenic Differentiation in MEFs from GR^null and GR^dim Mice
To further verify the role of GR in adipogenesis at the genetic level, we obtained MEFs from GR^null mice and observed that adipogenesis (Figure 2a; Supplementary Figure 2) and induction of marker gene expression was blocked in GR^null MEFs, as well as RU486 treatment (Figure 2b and c). Furthermore, cells with a dimerization impaired GR (GR^dim) failed to accumulate lipids and enhancement of adipocyte-marker expression during adipogenic stimulation (Figure 2a–c). Taken together, these data clearly indicate that ligand-dependent GR transcriptional activity with its DNA binding to the target promoter is critical for adipogenesis.

Screening for GR Targets in Adipogenesis by Microarray Expression Analysis
To identify the genes regulated by GR during adipogenesis, total RNA was extracted from MEFs cultured with control medium, AIM, AIM with RU486, or Dex alone for 2 days; transcription was analyzed by microarray. As shown in Figure 3a, 1096 genes from AIM-treated cells that displayed at least a twofold expression change relative to control were selected as adipogenesis-specific genes; 203 genes from cells incubated with AIM and RU486 that displayed at least a twofold change compared with cells treated with AIM alone were selected as adipogenesis-RU486-regulated genes; and 1580 genes from Dex-treated cells that displayed at least a twofold change relative to control were selected as Dex-regulated genes. Furthermore, after eliminating overlap and expressed sequence tags, 51 genes that belong to both the adipogenesis-RU486-regulated group and the Dex-regulated group were identified as GR-regulated adipogenic genes; a list of these 51 genes is displayed in Table 1. All 51 genes demonstrated an increase or decrease in expression with Dex treatment—45 genes were upregulated and 6 genes were downregulated during adipogenesis—and RU486 treatment had the opposite effect on their expression.

Figure 1 Effect of GR antagonist RU486 on adipogenesis in hMSCs and MEFs. hMSCs were differentiated into adipocytes with AIM (containing 500 μM IBMX, 1 μM Dex, 5 μg/ml insulin, 10 μM pioglitazone) with or without 10 μM RU486 for 3 days, followed by treatment with 5 μg/ml insulin alone for 3 days. These treatments were repeated. Cells were stained with Oil Red O at 12 days post-induction (**a**). Quantitative real-time PCR for the determination of expression levels adipogenic genes on day 2 after AIM exposure in the presence or absence of RU486 (**b**). MEFs were differentiated into adipocytes with AIM for 3 days, followed by treatment with insulin alone for 4 days. These treatments were repeated. Cells were stained with Oil Red O at 14 days post-induction. Macroscopic (upper panel) and microscopic (lower panel) views are shown (**c**). Data from three independent experiments are mean ± s.e.m.; $n = 6$. P-values were determined by Steel–Dwass' test (*$P < 0.05$ vs control, #$P < 0.05$ vs AIM).

Candidates for GR Direct Target Genes in MEF During Adipogenesis

To reveal transcriptional network in adipogenesis, we focused on transcription factors, which were induced as GR direct target genes in adipogenesis. Among the 45 genes showing adipogenesis and GR-dependent upregulation in Table 1, 5 genes are transcriptional factors: Zbtb16 (zinc finger and BTB domain containing 16), HIF3α (hypoxia-inducible factor 3,
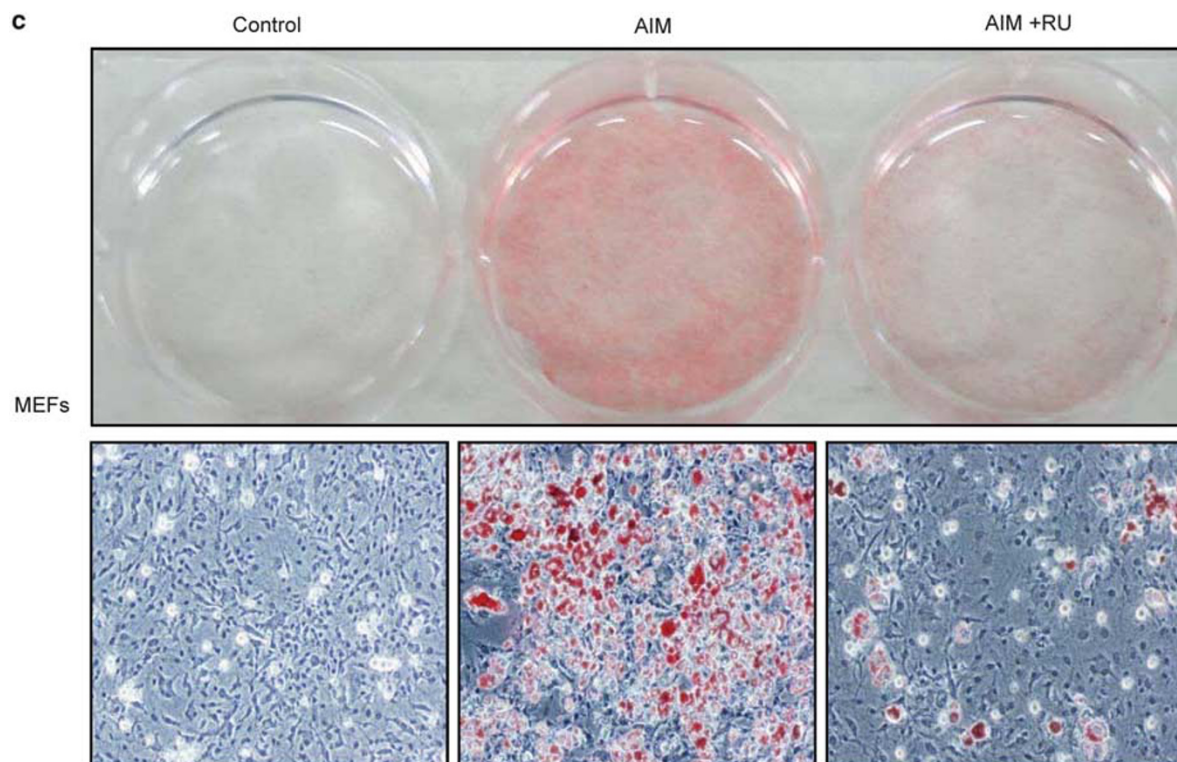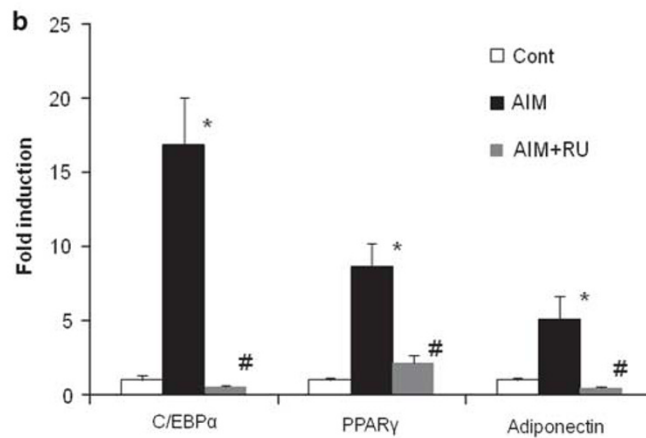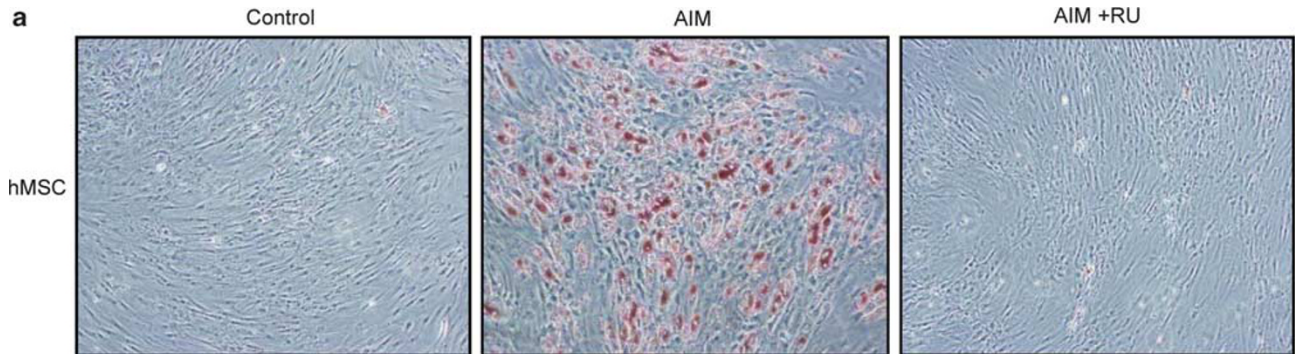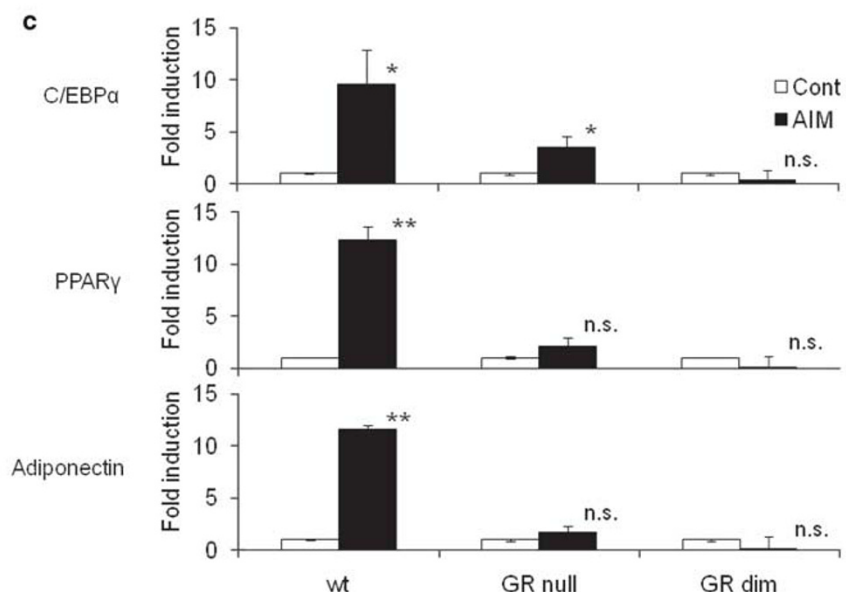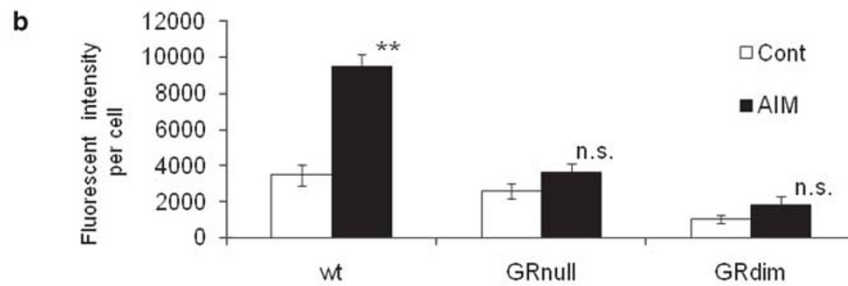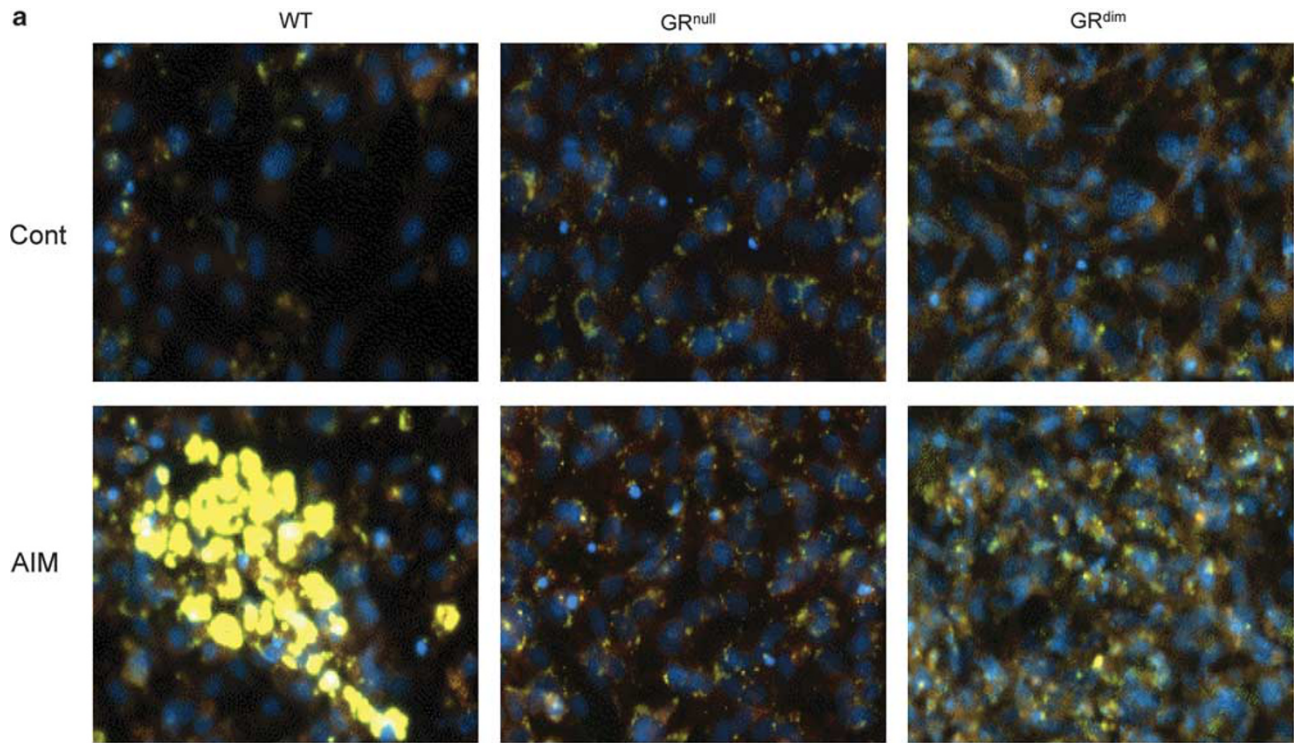
α subunit), Zhx3 (zinc fingers and homeoboxes 3), KLF9 (Krüppel-like factor 9), and KLF15.

Expression of these five genes was confirmed by real-time RT–PCR using cDNA from MEFs at day 2 after adipogenic induction ± RU486 or with Dex alone (Figure 3b). Expression of Zbtb16 (also known as ZFP145 or PLZF) was strongly

induced by Dex; consistent with a previous report showing that glucocorticoids regulate Zbtb16 gene expression via the promoter region of this gene,[38] and so far there is no report on the roles of Zbtb16, HIF3α, Zhx3, or KLF9 in adipogenesis. KLF15 has been reported to be induced by Dex and IBMX[28,39] and act as an adipogenic inducer via PPARγ
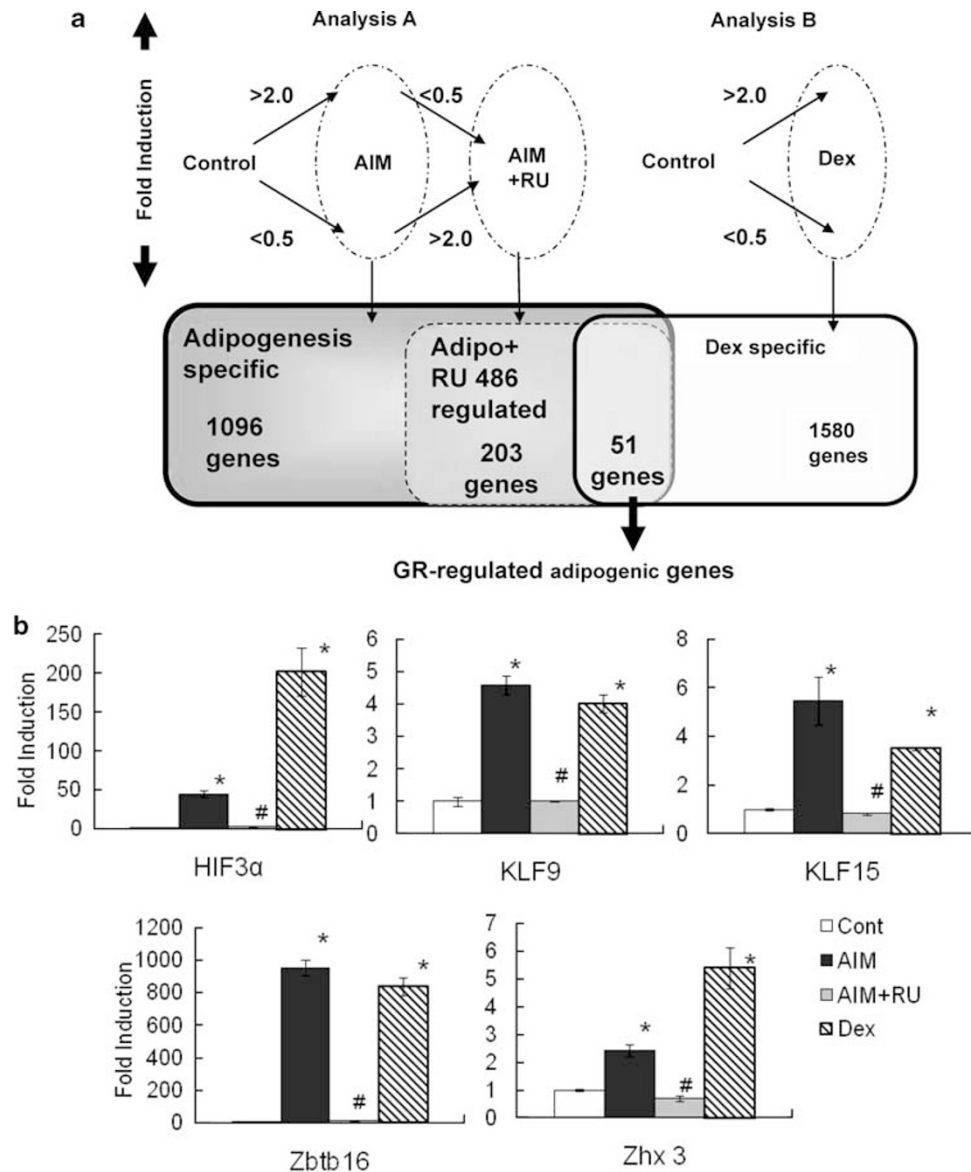

Figure 3 Screening for GR-regulated adipogenic-induced genes by microarray expression analysis. Scheme of microarray data analysis showing genes with increased or decreased expression in each treatment group (**a**). Genes with signal intensities below 50 were eliminated and normalized, and then genes from cells incubated with AIM or Dex treatment that exhibited at least a twofold change relative to the control treatment were selected for analysis. Expression profiles for GR-regulated adipogenic genes in MEF were confirmed with quantitative RT–PCR in Control, AIM, AIM + RU, and Dex treatment groups on day 2 (**b**). Data are mean ± s.e.m.; $n = 4$. P-values were determined by Steel–Dwass' test (*$P < 0.05$ vs control, #$P < 0.05$ vs AIM).

Figure 2 Induction of adipocyte differentiation in MEFs from GR[dim] and GR[null] mice. MEFs derived from wild type, GR[null] and GR[dim] mice were differentiated into adipocytes and stained with Nile Red for lipid droplets (yellow) and DAPI (blue) (**a**). Total fluorescent intensity of lipid droplets per cell were analyzed by Cellomics automated fluorescent microscopy (**b**). Data were mean ± s.e.m.; $n = 5$. P-values were determined by Mann–Whitney U test (**$P < 0.01$ vs control). Analysis of mRNA expression of adipogenesis-specific factors in wild type, GR[dim] and GR[null] MEFs during adipogenesis using quantitative RT–PCR 1 day after adipogenic differentiation (**c**). Data are mean ± s.e.m.; $n = 3$. P-values were determined by Mann–Whitney U test (**$P < 0.01$ vs control, *$P < 0.05$ vs control).

Table 1 Microarray analysis of GR-regulated adipogenic genes

Fold induction			Symbol	Description
AIM	AIM+RU	Dex		
367.2	19.0	249.9	**Zbtb16**	**Zinc finger and BTB domain containing 16**
144.1	55.2	39.8	Serpina3c	Serine (or cysteine) proteinase inhibitor, clade A, member 3C
78.0	15.8	39.2	Hp	Haptoglobin
70.5	16.0	14.8	Serpina3m	Serine (or cysteine) proteinase inhibitor, clade A, member 3M
36.2	15.5	3.8	Prg4	Proteoglycan 4 (megakaryocyte-stimulating factor, articular superficial zone protein)
23.6	0.9	11.3	**Hif3a**	**Hypoxia-inducible factor 3, α subunit**
23.4	1.6	22.6	BC055107	cDNA sequence BC055107
22.8	8.1	7.3	Fabp4	Fatty acid binding protein 4, adipocyte
20.1	2.3	8.6	Dpep1	Dipeptidase 1 (renal)
19.7	2.3	9.6	Fkbp5	FK506 binding protein 5
19.6	7.3	21.6	Cp	Ceruloplasmin
15.6	3.3	3.7	5430433G21Rik	RIKEN cDNA 5430433G21 gene
13.5	2.3	9.8	Aldh1a7	Aldehyde dehydrogenase family 1, subfamily A7
11.7	3.6	4.4	B230114P17Rik	RIKEN cDNA B230114P17 gene
11.2	4.1	10.0	AI607873	Expressed sequence AI607873
9.9	0.7	9.9	Fmo3	Flavin-containing monooxygenase 3
8.8	3.9	2.1	Cd24a	CD24a antigen
8.0	0.2	6.0	Fmo2	Flavin-containing monooxygenase 2
7.9	2.0	3.6	1300002F13Rik	RIKEN cDNA 1300002F13 gene
7.6	3.4	8.1	Ms4a4d	Membrane-spanning 4-domains, subfamily A, member 4D
7.5	2.4	2.9	Glul	Glutamate-ammonia ligase (glutamine synthase)
6.4	1.0	3.9	Tsc22d3	TSC22 domain family 3
5.6	0.9	6.4	Pdk4	Pyruvate dehydrogenase kinase, isoenzyme 4
4.6	1.5	2.1	Ms4a6b	Membrane-spanning 4-domains, subfamily A, member 6B
4.5	2.1	2.6	LOC553901	Hypothetical LOC553901
4.4	1.1	3.2	Ndrg2	*N*-myc downstream regulated gene 2
4.3	2.1	3.0	Rgs2	Regulator of G-protein signaling 2
4.3	1.1	6.4	Sult1a1	Sulfotransferase family 1A, phenol preferring, member 1
4.1	1.9	3.4	Gm368	Gene model 368 (NCBI)
3.8	1.8	2.1	**Zhx3**	**Zinc fingers and homeoboxes 3**
3.7	1.4	2.1	**Klf9**	**Kruppel-like factor 9**
3.5	1.7	4.1	Clca1///Clca2	Chloride channel calcium-activated 1///chloride channel calcium-activated 2
3.5	1.6	2.2	Admr	Adrenomedullin receptor
3.5	1.7	3.5	2310005P05Rik	RIKEN cDNA 2310005P05 gene
3.4	1.1	2.8	Crispld2	Cysteine-rich secretory protein LCCL domain containing 2
3.1	1.5	2.2	**Klf15**	**Kruppel-like factor 15**
3.1	0.9	4.3	Ctla2b	Cytotoxic T lymphocyte-associated protein 2 *β*
3.0	0.7	2.4	Stc2	Stanniocalcin 2
3.0	1.1	2.8	Aldh1a1	Aldehyde dehydrogenase family 1, subfamily A1
2.8	1.1	2.7	1810049K24Rik	Cysteine-rich secretory protein LCCL domain containing 2
2.4	1.1	2.3	Scn7a	Sodium channel, voltage-gated, type VII, α
2.4	1.1	2.4	Rab3d	RAB3D, member RAS oncogene family
2.2	0.8	2.3	Prkar2b	Protein kinase, cAMP-dependent regulatory, type II *β*
2.2	0.9	3.7	Clec1a	C-type lectin domain family 1, member a
2.1	1.0	2.3	Sgk	Serum/glucocorticoid-regulated kinase
0.4	0.9	0.4	4632408A20Rik	RIKEN cDNA 4632408A20 gene
0.3	1.0	0.4	Sec15l1	SEC15-like 1 (*S. cerevisiae*)
0.3	0.7	0.5	1810057P16Rik	RIKEN cDNA 1810057P16 gene
0.2	0.5	0.5	Slc7a5	Solute carrier family 7 (cationic amino-acid transporter, y+ system), member 5
0.2	0.4	0.5	E030004N02Rik	RIKEN cDNA E030004N02 gene
0.1	0.3	0.5	Gucy1a3	Guanylate cyclase 1, soluble, α 3

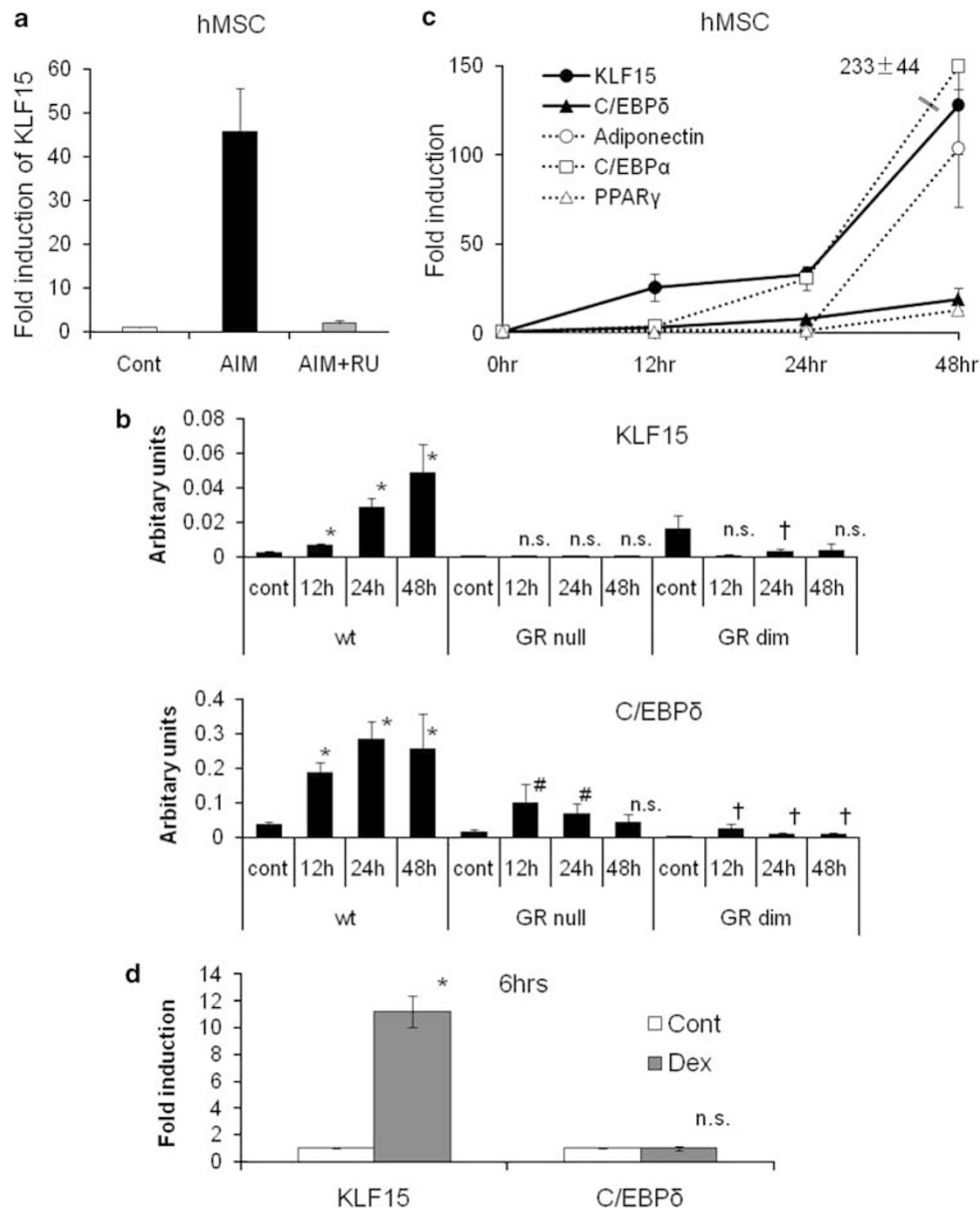**Figure 4** Expressions of KLF15 and C/EBPδ mRNA in MEFs from wild type, GR^dim and GR^null mice. Expression of KLF15 and effect of RU486 for KLF15 in hMSCs 2 days after induction (**a**) and expression of KLF15 and C/EBPδ in wild type, GR^dim and GR^null MEFs during adipogenesis (**b**). Data are mean ± s.e.m.; $n = 3$. Data were statistically analyzed with Steel's test (*$P < 0.05$ vs wild type 0 h, #$P < 0.05$ vs GR^null 0 h †$P < 0.05$ vs GR^dim 0 h). Time courses of expression of KLF15 and adipogenic-marker genes in hMSCs (**c**). Expressions of KLF15 and C/EBPδ in wild-type MEFs at 6 h after Dex alone stimulation (**d**). Total RNA was analyzed using quantitative RT–PCR. Data are mean ± s.e.m.; $n = 3–4$. P-values were determined by Mann–Whitney U test (*$P < 0.05$ vs control).

induction;[30,39] however, the molecular relationship between GR-dependent transcription and KLF15 expression is largely unrevealed yet.

KLF15 Expression Is Dependent on GR Dimerization

Consistent with the above observation, KLF15 expression was increased during adipose differentiation in both hMSCs and wild-type MEFs, and this induction was inhibited by RU486, suggesting the critical role of GR in KLF15 gene expression in adipogenesis (Figures 3b and 4a). To examine whether induction of KLF15 during adipogenesis is directly regulated

by GR or not, we tested the expression of KLF15 using GR^null and GR^dim MEFs and found that KLF15 gene expression during adipogenesis was inhibited (Figure 4b). These data suggest that KLF15 expression during adipogenesis needs ligand-dependent and DNA binding-dependent GR transcriptional activity.

Interestingly, C/EBPδ expression was induced with AIM although basic expression was decreased in both GR^null and GR^dim MEFs (fold induction: 4.97 ± 0.98 in wild type, 6.38 ± 3.33 in GR^null 7.91 ± 3.41 in GR^dim at 12 h) (Figure 4b). Kinetics analysis of gene expression showed
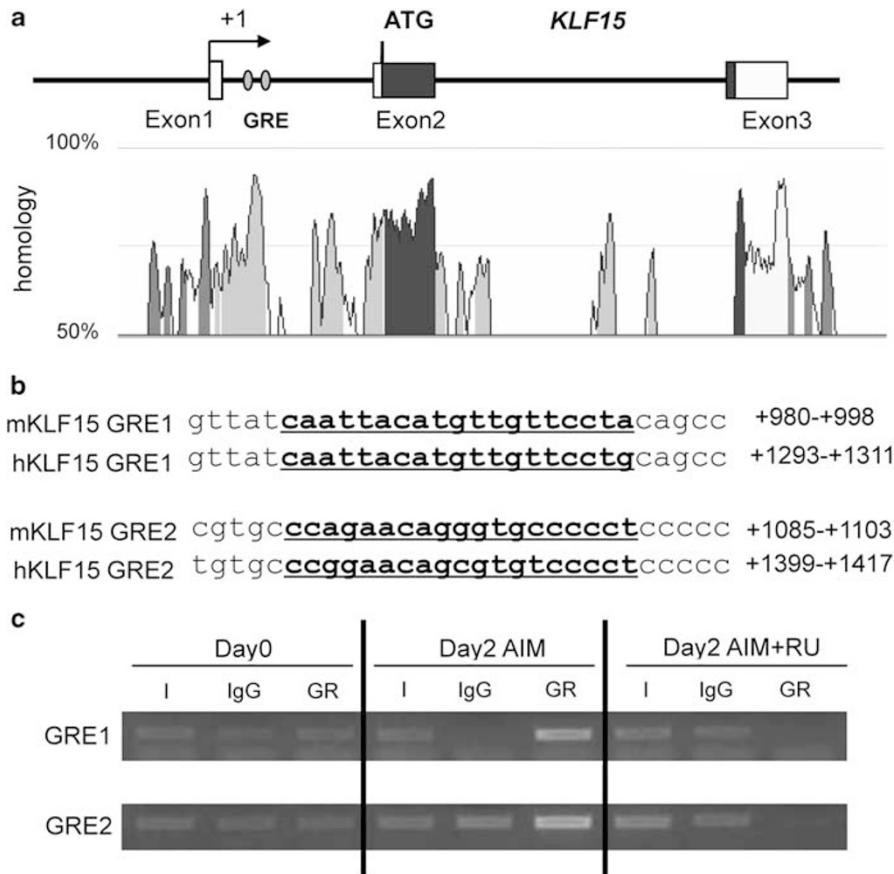
Figure 5 GRE sites in the KLF15 gene first intron. The highly conserved region between human and mouse in the KLF15 first intron (**a**). GRE site sequence in the first intron of KLF15 (**b**). The conserved region between human and mouse genomes was identified by rVista 2.0 and the homology graph from the ECR browser, and putative GRE sites were discovered with MatInspector. Chromatin immunoprecipitation was performed in MEFs differentiated with AIM for 2 days. DNA immunoprecipitated with anti-GR antibodies from treated cells was used to amplify the nucleotide sequences located between +933 to +1070 bp (GRE1), and +1055 to +1216 bp (GRE2) in the KLF15 first intron (**c**). I, input; IgG, normal rabbit serum IgG.

KLF15 expression is induced earlier than C/EBPδ expression in MSCs and MEFs (Figure 4c; Supplementary Figure 3). Consistent with these findings, Dex treatment strongly and promptly induced KLF15 gene expression, but not C/EBPδ gene expression (Figure 4d). These data suggest that the ligand-bound GR is critical for promoting KLF15 in adipogenesis and that C/EBPδ in adipogenesis can be alternatively induced by other factors (eg IBMX) without GR.

GR Directly Binds to GRE Sites in the First Intron of KLF15

Using bioinformatics, we analyzed the promoter regions—KLF15 (7500 to +2000 bp). A conserved region between human and mouse KLF15 promoters was identified in the first intron by rVista 2.0; two putative GRE sites (GRE1 and GRE2) were identified by analysis with MatInspector (Figure 5a and b).

To confirm these findings, we used the ChIP assay to determine whether GR binds to GRE sites in the first intron of KLF15 during adipocyte differentiation. Chromatin samples were prepared from MEFs undergoing adipogenic differentiation with or without RU486, then immunoprecipitated

with a specific antibody against GR. GR was found to be associated with both GRE1 and GRE2 in a conserved region of KLF15, and this association was inhibited by RU486 (Figure 5c). These results confirmed that GR directly binds to GRE sites in the first intron of KLF15 during adipogenesis.

GR Induces Promoter Activity via KLF15 First Intron

To define the mechanism by which GR induces KLF15 expression, we performed transient transfection studies in 293T cells using reporter constructs of the KLF15 intron (+933 to +1216), including two GRE sites under the HSV TK promoter (Figure 6a). Dex treatment resulted in an approximate 10-fold induction of expression of the KLF15 promoter, and this induction was inhibited by RU486 treatment (Figure 6b).

To determine which potential GR-binding site in the first intron of KLF15 was involved in transactivation, we introduced point mutations at the GRE 1 site (ATTAC ATGTTGTTCC→A**CA**ACATGTT**CA**TCC) and at the GRE2 site (AGAACAGGGTGCCCC→A**C**AACAGGGT**CA**CCC). Both mutations in the GRE1 or GRE2 resulted in a loss of transactivation (Figure 6c). Moreover, introduction of mutations at both GRE1 and GRE2 caused an even greater
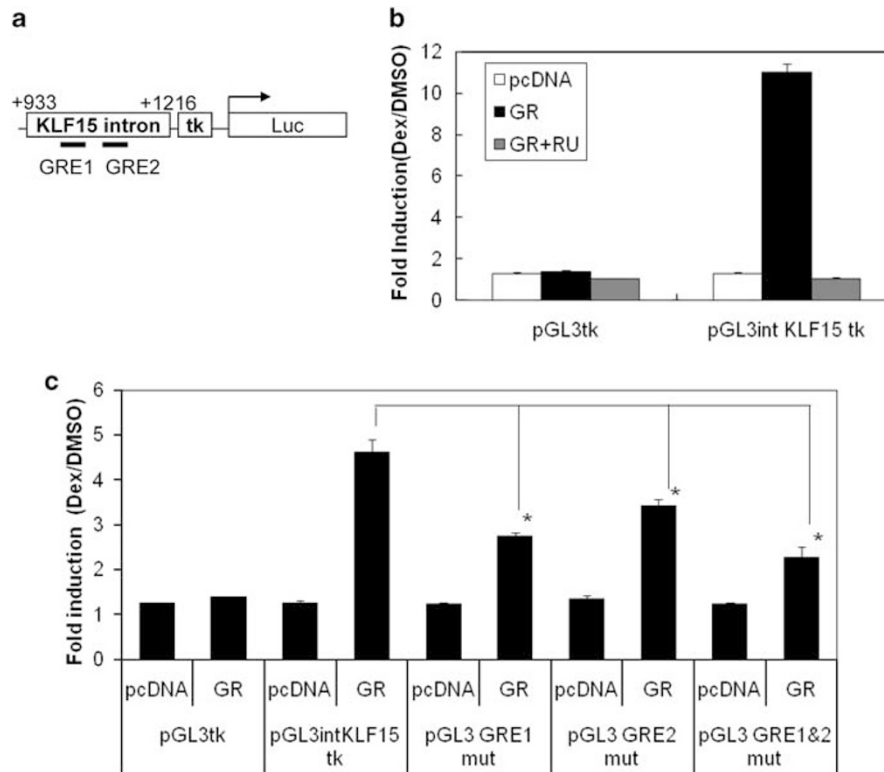
Figure 6 Promoter activity of reporter constructs containing $+933$ to $+1216$ bp of the KLF15 first intron. Map of pGL3 intKLF15 tk reporter vector (**a**). 293T cells were transiently transfected with the reporter construct pGL3-intKLF15-tk (**b**) or deletion constructs pGL3-GRE1mut (GRE1 mutation), pGL3-GRE2mut (GRE2 mutation), pGL3-GRE1&2mut (GRE1 and GRE2 mutations) (**c**), and empty vector (pcDNA) or GR expression vector. After 24 h, cells were incubated in Dex (100 nM) or DMSO (vehicle) with or without 10 μM RU486 for 24 h before the luciferase assay. Results were expressed as fold induction compared with cells treated with DMSO alone. Data are expressed as the mean $\pm$ s.d. of triplicates from representative experiments. Results were evaluated by Steel's test (*$P < 0.05$ *vs* pGL3-intKLF15-tk with Dex).

reduction in transactivation. These data suggest that GR can transactivate the KLF15 promoter activity by binding these GRE consensus sites.

Knockdown of KLF15 Inhibited Adipogenesis

Finally, to investigate the function of KLF15 on adipogenesis, we examined the effect of siKLF15 during adipogenic differentiation in MEFs. As shown in Figure 7a, MEFs transfected with nonspecific siRNA (siNega) differentiated into adipocytes and accumulated lipid droplets, but less lipid accumulation was observed in MEFs transfected with siKLF15 during adipogenic stimulation. We examined the expression of adipocyte-specific genes by quantitative RT–PCR 2 days after stimulation (Figure 7b). KLF15 gene expression was inhibited by siKLF15 and adipocyte-marker genes, C/EBPα and PPARγ were reduced whereas adiponectin was not affected. Thus, KLF15 induction contributes to adipocyte differentiation.

DISCUSSION

In this study, we used the GR antagonist RU486 and GR-mutant mice to provide evidence for an essential role for GR in adipogenesis. The GR and in particular its dimerization function are essential for *in vitro* adipogenesis, as cells with a GR impaired in dimerized DNA binding derived from GR[dim] mice fail to differentiate into adipocytes. Thus, genes under the control of GR–DNA interaction, which comprise a subset of GR-regulated genes are involved in adipogenesis.[40] The KLF15 gene was identified as a direct GR target in MEF during adipogenesis, and KLF15 has the critical role in adipogenic differentiation. Previous studies showed that KLF15 is induced by ectopic expression of C/EBPδ and C/EBPβ, and Dex and IBMX stimulation in 3T3-L1 cells.[28] Our data showed that KLF15 is more rapidly induced than C/EBPδ mRNA, suggesting a C/EBPδ- independent pathway for GR-mediated KLF15 induction (Figure 8).

KLF15 is expressed in major tissues such as liver, kidney, heart, skeletal muscle, and adipose tissue. KLF15^{−/−} mice display severe hypoglycemia, with a deficiency in amino-acid metabolic enzymes in the liver and muscle.[30] These findings raise the question of the function of adipose tissue in these mice. GR has an important role in glucose and amino-acid homeostasis in liver, muscle, and adipose tissue during gluconeogenesis under fasting conditions.[30] Fasting increases endogenous glucocorticoids, and induces gluconeogenesis-associated genes, such as PEPCK and G6Pase in the liver[41]; liver-specific GR-deficient mice displayed hypoglycemia while fasting, similar to KLF15^{−/−} mice. Expression of KLF15 is
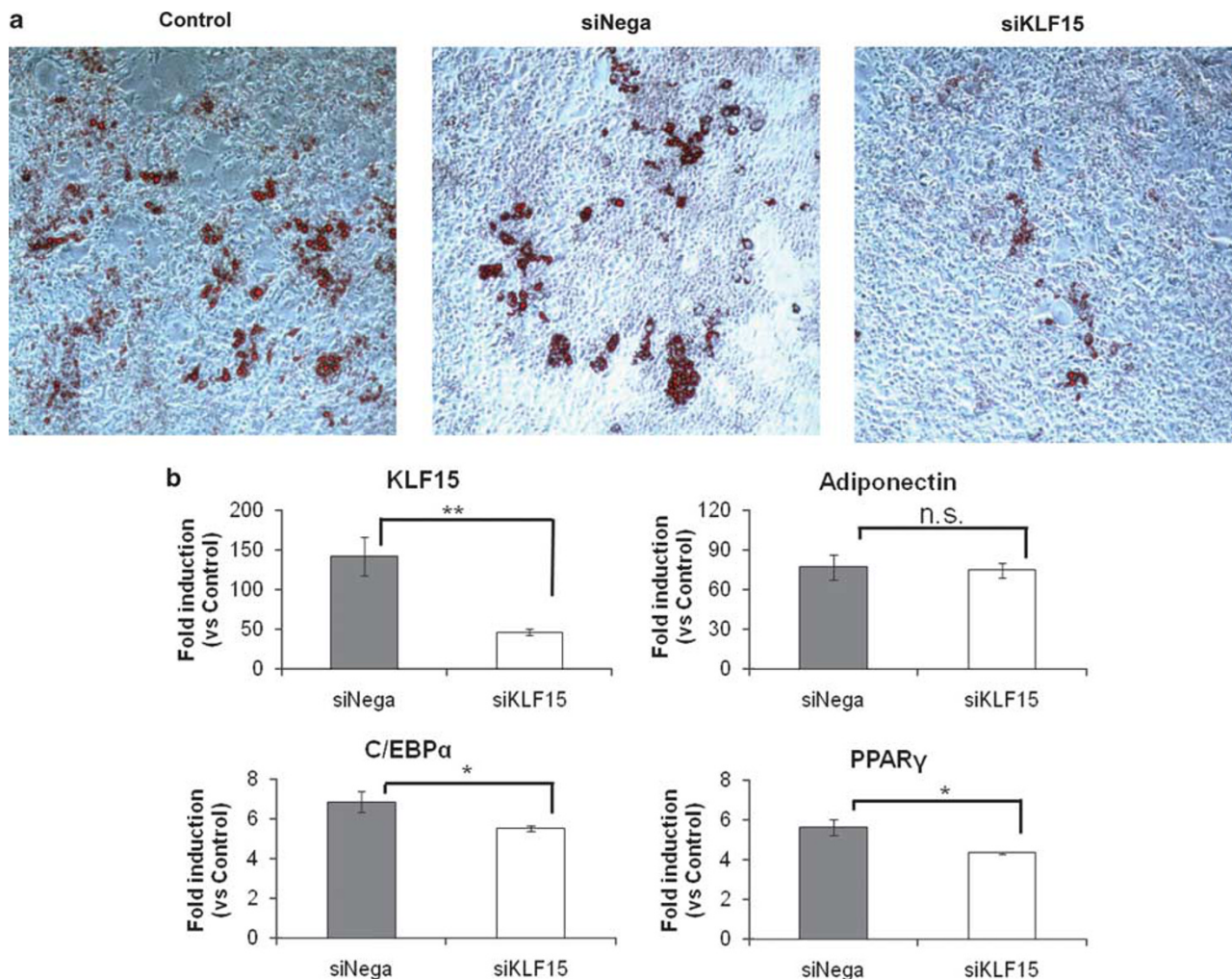
Figure 7 Effect of KLF15 knockdown on adipogenesis in MEFs. 50 nM siRNA for KLF15 (siKLF15) and 50 nM nonspecific siRNA (siNega) were transfected into MEFs. After 48 h, cells were differentiated into adipocytes in the same way as wild-type MEFs Figure 1 and were stained with Oil Red O at 14 days post-induction (**a**). Expression of KLF15 and adipogenic-specific marker genes in siRNA-transfected MEFs (**b**). Total RNA was extracted from cells 2 days after initiating adipose differentiation and analyzed using quantitative RT–PCR. Data are mean ± s.e.m.; $n = 6$–10. P-values were determined by Mann–Whitney U test (**$P < 0.01$ vs siNega, *$P < 0.05$ vs siNega).
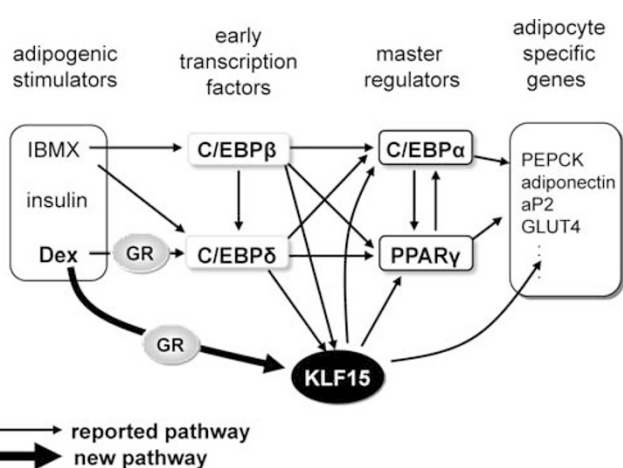

Figure 8 Schematic model of the pathways controlling differentiation. GR directly regulates KLF15 expression, which acts as an adipocyte differentiation activator at the transcriptional level in hMSCs and MEFs.

increased in the liver,[42] muscle,[43] and adipocytes in fasting conditions (data not shown). These findings suggest that the glucocorticoid effects may be partially mediated by KLF15 in vivo.

In GR[null] mice or GR[dim] mice, developments of white adipose tissues in embryos are normal comparative to wild type under normal conditions (data not shown); however, various stresses, such as a high-fat diet, may reveal the functions of GR in adipogenesis in vivo.

Further in vitro and in vivo studies to investigate the expression of the adipogenic regulators and GR-target genes (KLF15, etc) using GR[null] embryos and GR[dim] mice will generate a clearer picture of the roles of GR during adipocyte differentiation. Excessive glucocorticoids cause ectopic adipose distribution (central obesity, buffalo hump, etc) and contribute to metabolic disease through the facilitation of adipose tissue expansion. Our findings in this study will allow a more focused approach toward studying the

mechanisms underlying the effect of glucocorticoids on adipose tissue *in vivo*.

DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

1. Green H, Kehinde O. An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell 1975;5:19–27.
2. Green H, Kehinde O. Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. Cell 1976;7:105–113.
3. Rosen OM, Smith CJ, Fung C, et al. Development of hormone receptors and hormone responsiveness in vitro. Effect of prolonged insulin treatment on hexose uptake in 3T3-L1 adipocytes. J Biol Chem 1978;253:7579–7583.
4. Rubin CS, Hirsch A, Fung C, et al. Development of hormone receptors and hormonal responsiveness in vitro. Insulin receptors and insulin sensitivity in the preadipocyte and adipocyte forms of 3T3-L1 cells. J Biol Chem 1978;253:7570–7578.
5. Gregoire FM, Smas CM, Sul HS. Understanding adipocyte differentiation. Physiol Rev 1998;78:783–809.
6. Rosen ED, Walkey CJ, Puigserver P, et al. Transcriptional regulation of adipogenesis. Genes Dev 2000;14:1293–1307.
7. Farmer SR. Transcriptional control of adipocyte formation. Cell Metab 2006;4:263–273.
8. Rosen ED, Hsu CH, Wang X, et al. C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. Genes Dev 2002;16:22–26.
9. Rosen ED, Sarraf P, Troy AE, et al. PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. Mol Cell 1999;4:611–617.
10. Tontonoz P, Hu E, Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell 1994;79:1147–1156.
11. Cao Z, Umek RM, McKnight SL. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev 1991;5:1538–1552.
12. Wu Z, Bucher NL, Farmer SR. Induction of peroxisome proliferator-activated receptor gamma during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPbeta, C/EBPdelta, and glucocorticoids. Mol Cell Biol 1996;16:4128–4136.
13. Yeh WC, Cao Z, Classon M, et al. Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. Genes Dev 1995;9:168–181.
14. Masuzaki H, Paterson J, Shinyama H, et al. A transgenic model of visceral obesity and the metabolic syndrome. Science 2001;294:2166–2170.
15. Newell-Price J, Bertagna X, Grossman AB, et al. Cushing's syndrome. Lancet 2006;367:1605–1617.
16. Hauner H, Schmid P, Pfeiffer EF. Glucocorticoids and insulin promote the differentiation of human adipocyte precursor cells into fat cells. J Clin Endocrinol Metab 1987;64:832–835.
17. Ito S, Suzuki N, Kato S, et al. Glucocorticoids induce the differentiation of a mesenchymal progenitor cell line, ROB-C26 into adipocytes and osteoblasts, but fail to induce terminal osteoblast differentiation. Bone 2007;40:84–92.
18. Yamamoto KR. Steroid receptor regulated transcription of specific genes and gene networks. Annu Rev Genet 1985;19:209–252.
19. Beato M, Herrlich P, Schutz G. Steroid hormone receptors: many actors in search of a plot. Cell 1995;83:851–857.
20. Karin M. New twists in gene regulation by glucocorticoid receptor: is DNA binding dispensable? Cell 1998;93:487–490.
21. McKay LI, Cidlowski JA. Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. Endocr Rev 1999;20:435–459.
22. De Bosscher K, Vanden Berghe W, Haegeman G. The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. Endocr Rev 2003;24:488–522.
23. Barnes PJ. Corticosteroid effects on cell signalling. Eur Respir J 2006;27:413–426.
24. Heck S, Kullmann M, Gast A, et al. A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. EMBO J 1994;13:4087–4095.
25. Reichardt HM, Kaestner KH, Tuckermann J, et al. DNA binding of the glucocorticoid receptor is not essential for survival. Cell 1998;93:531–541.
26. Banerjee SS, Feinberg MW, Watanabe M, et al. The Kruppel-like factor KLF2 inhibits peroxisome proliferator-activated receptor-gamma expression and adipogenesis. J Biol Chem 2003;278:2581–2584.
27. Oishi Y, Manabe I, Tobe K, et al. Kruppel-like transcription factor KLF5 is a key regulator of adipocyte differentiation. Cell Metab 2005;1:27–39.
28. Mori T, Sakaue H, Iguchi H, et al. Role of Kruppel-like factor 15 (KLF15) in transcriptional regulation of adipogenesis. J Biol Chem 2005;280:12867–12875.
29. Gray S, Feinberg MW, Hull S, et al. The Kruppel-like factor KLF15 regulates the insulin-sensitive glucose transporter GLUT4. J Biol Chem 2002;277:34322–34328.
30. Gray S, Wang B, Orihuela Y, et al. Regulation of gluconeogenesis by Kruppel-like factor 15. Cell Metab 2007;5:305–312.
31. Reichardt HM, Kaestner KH, Tuckermann J, et al. DNA binding of the glucocorticoid receptor is not essential for survival. Cell 1998;93:531–541.
32. Pascussi JM, Busson-Le Coniat M, Maurel P, et al. Transcriptional analysis of the orphan nuclear receptor constitutive androstane receptor (NR1I3) gene promoter: identification of a distal glucocorticoid response element. Mol Endocrinol 2003;17:42–55.
33. Todaro GJ, Green H. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J Cell Biol 1963;17:299–313.
34. Ramirez-Zacarias JL, Castro-Munozledo F, Kuri-Harcuch W. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. Histochemistry 1992;97:493–497.
35. Tuckermann JP, Kleiman A, Moriggl R, et al. Macrophages and neutrophils are the targets for immune suppression by glucocorticoids in contact allergy. J Clin Invest 2007;117:1381–1390.
36. Phuc Le P, Friedman JR, Schug J, et al. Glucocorticoid-dependent gene regulatory networks. PLoS Genet 2005;1:e16.
37. Kassel O, Schneider S, Heilbock C, et al. A nuclear isoform of the focal adhesion LIM-domain protein Trip6 integrates activating and repressing signals at AP-1- and NF-kappaB-regulated promoters. Genes Dev 2004;18:2518–2528.
38. Fahnenstich J, Nandy A, Milde-Langosch K, et al. Promyelocytic leukaemia zinc finger protein (PLZF) is a glucocorticoid- and progesterone-induced transcription factor in human endometrial stromal cells and myometrial smooth muscle cells. Mol Hum Reprod 2003;9:611–623.
39. Baudry A, Yang ZZ, Hemmings BA. PKBalpha is required for adipose differentiation of mouse embryonic fibroblasts. J Cell Sci 2006;119:889–897.
40. Frijters R, Fleuren W, Toonen EJ, et al. Prednisolone-induced differential gene expression in mouse liver carrying wild type or a dimerization-defective glucocorticoid receptor. BMC Genomics 2010;11:359.
41. Opherk C, Tronche F, Kellendonk C, et al. Inactivation of the glucocorticoid receptor in hepatocytes leads to fasting hypoglycemia and ameliorates hyperglycemia in streptozotocin-induced diabetes mellitus. Mol Endocrinol 2004;18:1346–1353.
42. Teshigawara K, Ogawa W, Mori T, et al. Role of Kruppel-like factor 15 in PEPCK gene expression in the liver. Biochem Biophys Res Commun 2005;327:920–926.
43. Yamamoto J, Ikeda Y, Iguchi H, et al. A Kruppel-like factor KLF15 contributes fasting-induced transcriptional activation of mitochondrial acetyl-CoA synthetase gene AceCS2. J Biol Chem 2004;279:16954–16962.