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RESEARCH ARTICLE

Distinct Roles of Transcription Factors KLF4, Krox20, and Peroxisome Proliferator-Activated Receptor γ in Adipogenesis

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ABSTRACT Much of our knowledge on adipogenesis comes from cell culture models of preadipocyte differentiation. Adipogenesis is induced by treating confluent preadipocytes with the adipogenic cocktail, which activates transcription factors (TFs) glucocorticoid receptor (GR) and CREB within minutes and increases expression of TFs C/EBPβ, C/EBPδ, KLF4, and Krox20 within hours. All of these TFs have been shown to be capable of promoting adipogenesis in culture when they are overexpressed. However, it has remained unclear whether endogenous KLF4 and Krox20 are required for adipogenesis in culture and *in vivo*. Using conditional knockout mice and derived white and brown preadipocytes, we show that endogenous KLF4 and Krox20 are dispensable for adipogenesis in culture and for brown adipose tissue development in mice. In contrast, the master adipogenic TF peroxisome proliferator-activated receptor γ (PPARγ) is essential. These results challenge the existing model on transcriptional regulation in the early phase of adipogenesis and highlight the need of studying adipogenesis *in vivo*.

KEYWORDS KLF4, Krox20, PPARγ, adipogenesis

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Adipose tissues, including white adipose tissue (WAT) and brown adipose tissue (BAT), play critical roles in energy metabolism and homeostasis. Understanding molecular mechanisms underlying the development of adipose tissues is of potential importance for treating obesity and type II diabetes. Much of our knowledge on adipose tissue development comes from studies using cell culture model systems such as the mouse 3T3-L1 preadipocytes (1, 2). Adipogenesis of 3T3-L1 is induced by stimulating confluent cells with the adipogenic cocktail, which consists of synthetic glucocorticoid dexamethasone, insulin, the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), and fetal bovine serum (FBS) (3–5).

Studies on transcriptional regulation of adipogenesis have revealed a cascade of sequentially induced transcription factors (TFs) that promotes adipogenesis in culture (6, 7). The adipogenic cocktail activates TFs GR (also known as NR3C1) and CREB within minutes and induces expression of TFs C/EBPβ, C/EBPδ, KLF4, and Krox20 (also known as Egr2) within hours (8). C/EBPβ and C/EBPδ directly activate the expression of two principal adipogenic TFs, peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPα, which cooperate to induce expression of thousands of adipocyte genes, resulting in differentiation toward mature adipocytes (9). C/EBPα, -β, and -δ and PPARγ have been shown to be essential for adipogenesis in mice (10, 11).

Besides C/EBPβ and C/EBPδ, KLF4 and Krox20 are also induced in the early phase of 3T3-L1 adipogenesis (12, 13). Krox20 and Klf4 are specifically induced by FBS and IBMX, respectively. *Krox20* is induced within 1 h, while the *Klf4* mRNA level peaks at 2 h after the addition of an adipogenic cocktail to confluent 3T3-L1 cells. Ectopic expression of

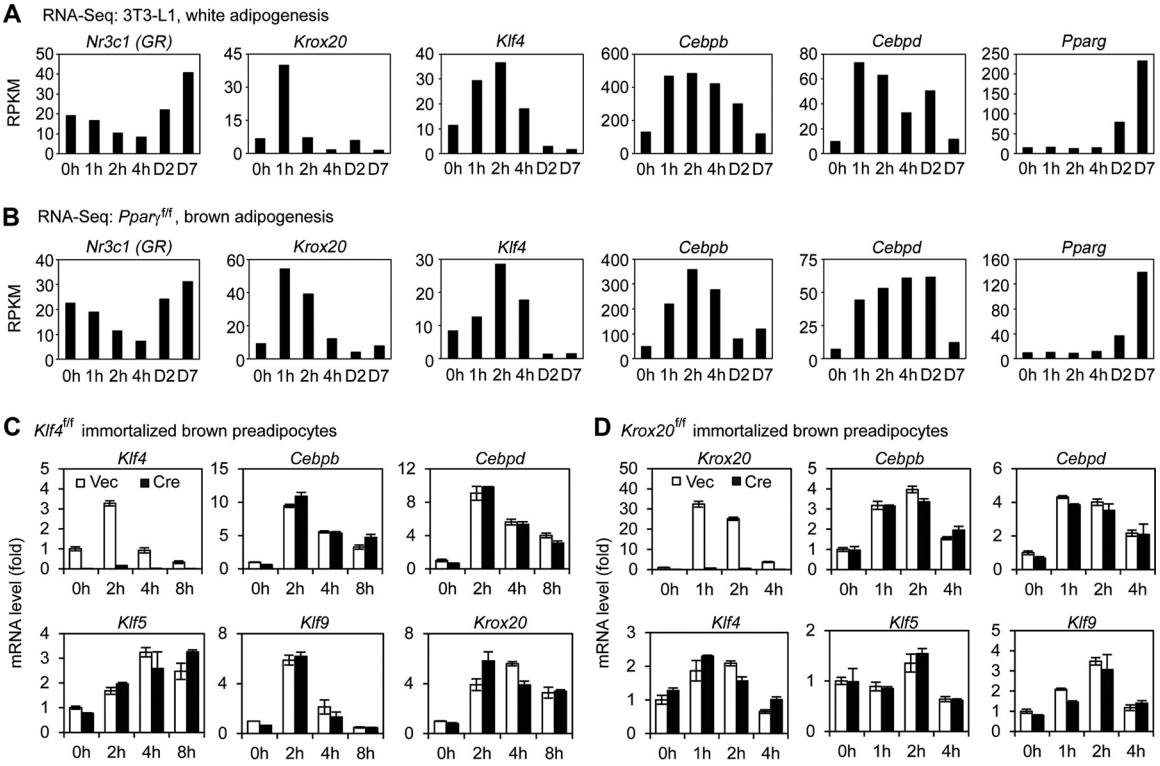

FIG 1 KLF4 and Krox20 are dispensable for induction of early adipogenic TFs. (A, B) 3T3-L1 white preadipocytes (A) and *Pparγ*$^{f/f}$ immortalized brown preadipocytes (B) were induced to undergo adipogenesis. Cells were collected at the indicated time points for RNA-Seq. RPKM values from RNA-Seq indicate gene expression levels. D2, day 2; D7, day 7. (C, D) *Klf4*$^{f/f}$ (C) and *Krox20*$^{f/f}$ (D) immortalized brown preadipocytes were infected with retroviral vector MSCVhygro expressing Cre. Adipogenesis was induced with the adipogenic cocktail, and cells were collected at indicated time points for qRT-PCR analysis of *Klf4*, *Krox20*, *Cebpβ*, *Cebpδ*, *Klf5*, and *Klf9*.

Krox20 promotes adipogenesis in both 3T3-L1 preadipocytes and NIH 3T3 fibroblasts. Conversely, RNA interference (RNAi)-mediated knockdown of *Krox20* or *Klf4* has been shown to inhibit 3T3-L1 adipogenesis (12, 13). Ectopic KLF4 and Krox20 cooperate to promote C/EBPβ expression, thus providing a possible mechanism by which these two TFs positively regulate adipogenesis in culture (13). However, the roles of endogenous Krox20 and KLF4 in adipogenesis have remained unclear.

We have investigated the roles of adipogenic TFs in adipogenesis by employing conditional knockout (cKO) mice and derived white and brown preadipocytes. By deleting *Klf4*, *Krox20*, and *Pparγ* genes individually, we found that surprisingly, endogenous Krox20 and KLF4 are dispensable for adipogenesis of preadipocytes in culture and for BAT development in mice. In contrast, PPARγ is essential for adipogenesis both in culture and in mice.

RESULTS

KLF4 and Krox20 are dispensable for the induction of early adipogenic TFs. To profile gene expression changes during adipogenesis, confluent 3T3-L1 white preadipocytes and *Pparγ*$^{f/f}$ brown preadipocytes were induced to undergo differentiation. RNAs were extracted in the early phase (0 h, 1 h, 2 h, and 4 h) and later phase (day 2 and day 7) of adipogenesis for RNA sequencing (RNA-Seq) analysis. The results from both cell lines confirmed previous observations that *Krox20* and *Klf4* were induced transiently in the early phase of adipogenesis (Fig. 1A and B) (12, 13). *Krox20* was induced to the maximum level at 1 h but dropped quickly at 4 h. *Klf4* was induced to the maximum level at 2 h but started to drop at 4 h. Both *Krox20* and *Klf4* levels were very low at day 2 and day 7 of adipogenesis. *Cebpβ* and *Cebpδ* were also induced in the early phase but were expressed throughout the differentiation. In contrast, the master adipogenic TF *Pparγ* was induced at day 2 and reached the maximal level at day 7.

To investigate whether endogenous KLF4 and Krox20 are required for the expression of early adipogenesis factors, primary brown preadipocytes were isolated from *Klf4*$^{f/f}$ or *Krox20*$^{f/f}$ BATs and were immortalized with simian virus 40 large T antigen (SV40T). The immortalized brown preadipocytes were infected with retroviral Cre to stably delete *Klf4* or *Krox20* gene (Fig. 1C and D). Consistent with RNA-Seq data, *Klf4* and *Krox20* were induced at early time points after induction of differentiation in control cells but not in KO cells. However, deletion of *Klf4* or *Krox20* from brown preadipocytes had little effect on the induction of early adipogenic TFs, including *Cebpβ*, *Cebpδ*, *Klf5*, *Klf9*, *Klf4*, and *Krox20* by the adipogenic cocktail (Fig. 1C and D). These data indicate that KLF4 and Krox20 are dispensable for the induction of early adipogenic TFs.

KLF4 is dispensable for adipogenesis in culture. Next, we investigated whether KLF4 is required for adipogenesis. We induced adipogenesis in retroviral Cre-infected *Klf4*$^{f/f}$ immortalized brown preadipocytes. To our surprise, deletion of *Klf4* had little effect on brown adipogenesis and induction of adipogenesis markers *Pparγ*, *Cebpα*, and *Fabp4* and brown adipocyte markers *Prdm16* and *Ucp1* (Fig. 2A to C). To eliminate the possible involvement of any compensation mechanism during the 2-week process required for retroviral Cre-mediated stable gene deletion, we acutely infected *Klf4*$^{f/f}$ brown preadipocytes with adenoviral Cre. Acute deletion of *Klf4* also had little effect on adipogenesis (Fig. 2D and E).

Deletion of *Klf4* also had little effect on adipogenesis of primary brown preadipocytes (Fig. 2F and G). After primary preadipocytes differentiated to mature brown adipocytes, cells were treated by CL316,243, a selective adrenergic-β3 receptor agonist. Deletion of *Klf4* had little effect on CL316,243-induced expression of *Ucp1*, a critical thermogenic gene, in brown adipocytes (Fig. 2H). Consistent with our observations in brown adipogenesis, deletion of *Klf4* in primary white preadipocytes or knockdown of *Klf4* in immortalized 3T3-L1 cells had little effect on adipogenesis (Fig. 3). Together, these data indicate that KLF4 is dispensable for adipogenesis in culture.

Krox20 is dispensable for adipogenesis in culture. To investigate whether endogenous Krox20 is required for adipogenesis, primary white preadipocytes were isolated from *Krox20*$^{f/f}$ mice and were infected with adenoviral Cre. Deletion of *Krox20* in primary white preadipocytes led to a growth defect (Fig. 4A and B). After cells reached confluence, adipogenesis was induced. Although Cre-infected cells showed reduced lipid droplet accumulation after 10 days of differentiation (Fig. 4C), reverse transcription-quantitative PCR (qRT-PCR) revealed that deletion of *Krox20* in preadipocytes had little effect on the induction of adipogenesis markers (Fig. 4D). To distinguish the roles of *Krox20* in cell differentiation versus proliferation, we turned to *Krox20*$^{f/f}$ immortalized brown preadipocytes. Deletion of *Krox20* had little effect on the growth of immortalized brown preadipocytes (Fig. 4E). We found that either stable deletion by retroviral Cre or acute deletion by adenoviral Cre of *Krox20* in preadipocytes did not affect brown adipogenesis and induction of adipogenesis markers (Fig. 4F to I), suggesting that Krox20 is dispensable for adipogenesis in culture.

KLF4 is dispensable for BAT development. To find out whether KLF4 is required for BAT development, we generated *Klf4*$^{f/f}$; *Myf5-Cre* mice. The *Myf5-Cre* transgene specifically deletes floxed genes in precursor cells of both BAT and muscles in the back (14). Littermate *Klf4*$^{f/f}$ mice were used as the control. *Klf4*$^{f/f}$; *Myf5-Cre* pups survived well beyond 6 weeks after birth and showed body weight similar to that of control mice, although the *Klf4* gene level was reduced by over 70% in the BAT of KO mice (Fig. 5A to C). Consistent with the adipogenesis data in cell culture, deletion of *Klf4* by *Myf5-Cre* in *Klf4*$^{f/f}$ mice did not affect the development of BAT and associated expression of adipogenesis and BAT markers (Fig. 5D and E). Consistently, *Klf4*$^{f/f}$; *Myf5-Cre* mice maintained normal body temperatures, were cold tolerant, and displayed behavior similar to that of control mice in the cold tolerance test (Fig. 5F and G). Similar results obtained from E18.5 embryos confirmed that KLF4 is dispensable for BAT development (Fig. 5H to K). While a full physiological characterization of *Klf4*$^{f/f}$; *Myf5-Cre* mice needs

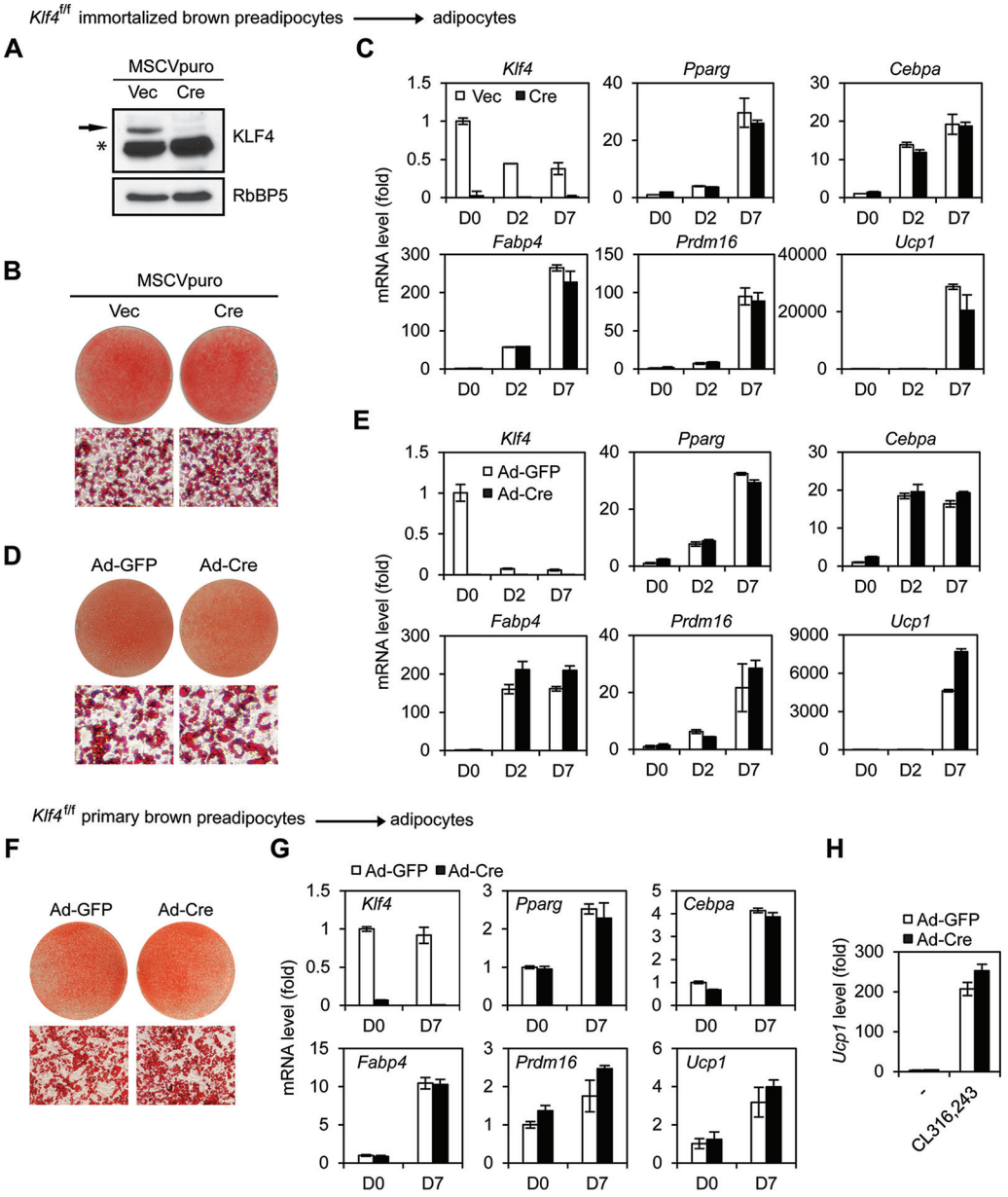

FIG 2 KLF4 is dispensable for brown adipogenesis in culture. (A to C) *Klf4*$^{f/f}$ immortalized brown preadipocytes were infected with retroviral vector MSCVpuro expressing Cre. (A) Western blot analysis of KLF4 before differentiation. The asterisk indicates a nonspecific band. RbBP5 was used as a loading control. (B) Oil Red O staining at day 7 of adipogenesis. Upper panels, stained dishes; lower panels, representative fields under the microscope. (C) qRT-PCR analysis of *Klf4*, adipogenesis markers (*Pparg*, *Cebpa*, and *Fabp4*) and brown adipocyte markers (*Prdm16* and *Ucp1*) at indicated time points of adipogenesis. D0, day 0. (D, E) *Klf4*$^{f/f}$ immortalized brown preadipocytes were infected with adenoviruses expressing GFP (Ad-GFP) or Cre (Ad-Cre). Two days later, cells were replated, followed by adipogenesis assays. (D) Oil Red O staining. (E) qRT-PCR analysis of gene expression. (F to H) *Klf4*$^{f/f}$ primary brown preadipocytes were infected with Ad-GFP or Ad-Cre, followed by adipogenesis assays. (F) Oil Red O staining. (G) qRT-PCR analysis of gene expression. (H) D7 mature brown adipocytes were treated with 100 nM CL-316,243 for 4 h, followed by qRT-PCR analysis of *Ucp1* expression.

to be done in the future, these data indicate that KLF4 is dispensable for BAT development in mice.

Krox20 is dispensable for BAT development. To investigate whether Krox20 is required for BAT development, we tried to generate *Krox20*$^{f/f}$; *Myf5-Cre* mice. However, since the *Krox20* gene (chromosome 10 [Chr10], bp 67534453 to 67547493) is located on the same chromosome as the *Myf5-Cre* transgene (Chr10, bp 107482908 to 107486927), the recombination frequency is 1/20 according to the distance between

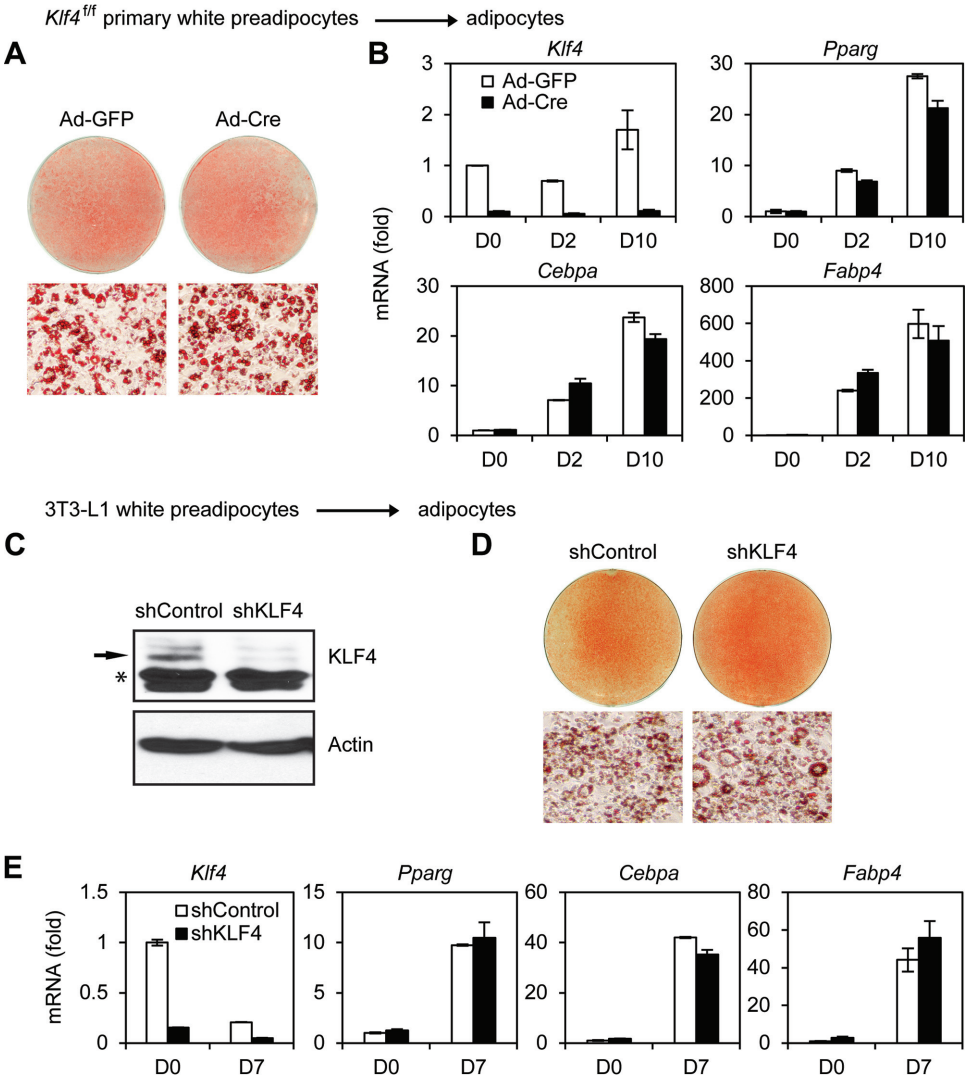

FIG 3 KLF4 is dispensable for white adipogenesis in culture. (A, B) *Klf4*$^{f/f}$ primary white preadipocytes were infected with Ad-GFP or Ad-Cre, followed by adipogenesis assays. (A) Oil Red O staining of differentiated cells at D10 (day 10). (B) qRT-PCR analysis of gene expression. (C to E) 3T3-L1 white preadipocytes were infected with lentiviral vector expressing control (shControl) or KLF4 knockdown shRNA (shKLF4), followed by adipogenesis assays. (C) Western blot analysis of KLF4 in preadipocytes. The asterisk indicates a nonspecific band. (D) Oil Red O staining of differentiated cells at D7. (E) qRT-PCR analysis of gene expression.

two loci (40 Mb) (15). Among 80 mice, we obtained 9 *Krox20*$^{f/f}$; *Myf5-Cre* adult mice (Fig. 6A). *Krox20*$^{f/f}$; *Myf5-Cre* pups survived well beyond 8 weeks after birth without any obvious developmental problems. Consistent with the adipogenesis data in cell culture, deletion of *Krox20* did not affect BAT development and expression of adipogenesis and BAT markers, although the *Krox20* gene level was reduced by over 90% in the BAT of KO mice (Fig. 6B to D). At the E18.5 stage, we obtained only 2 *Krox20*$^{f/f}$; *Myf5-Cre* embryos of 37, which appeared grossly normal compared to the littermate *Krox20*$^{f/f}$ embryos (Fig. 6E). One embryo was used for extracting RNA to confirm *Krox20* deletion (Fig. 6F). The other was subjected to histological analysis. The results revealed similar morphology of BATs and muscles for *Krox20*$^{f/f}$; *Myf5-Cre* and *Krox20*$^{f/f}$ embryos (Fig. 6G). Furthermore, RNA-Seq analysis of interscapular BAT from wild-type adult (8-week-old) mice and embryos (E18.5) showed that compared with other early adipogenic TFs (*GR*, *Cebpβ*, *Cebpδ*, and *Klf4*) or adipogenesis markers (*Pparγ*, *Cebpα*, *Fabp4*, *Prdm16*, and *Ucp1*), the expression level of *Krox20* was very low (RPKM $<$ 0.1) in BAT (Fig. 6H), suggesting that Krox20 is unlikely to play a major role in BAT function. Together, while

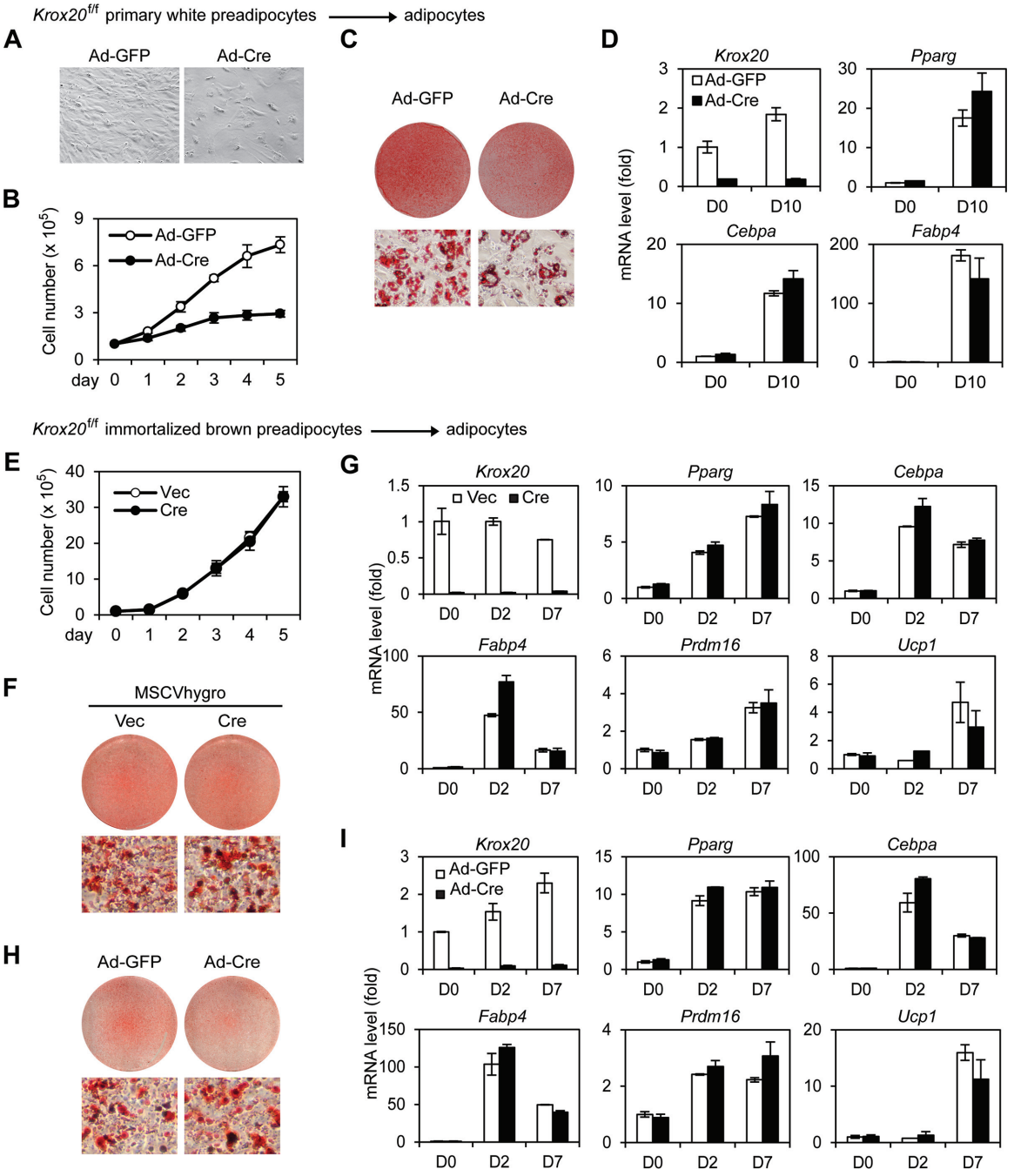

FIG 4 Krox20 is dispensable for adipogenesis in culture. (A, B) *Krox20* knockout (KO) reduces growth of primary white preadipocytes. *Krox20*$^{f/f}$ primary white preadipocytes were infected with Ad-GFP or Ad-Cre. (A) Cell morphology of *Krox20* KO cells under the microscope. (B) Cell growth rates: 1×10^5 cells were plated, and the cumulative cell numbers were determined every day for 5 days. (C, D) Ad-GFP or Ad-Cre infected *Krox20*$^{f/f}$ primary white preadipocytes were cultured until confluence. Two days later, cells were treated with the adipogenic cocktail to induce adipogenesis. (C) Oil Red O staining at D10. (D) qRT-PCR analysis of gene expression. (E to G) *Krox20*$^{f/f}$ immortalized brown preadipocytes were infected with MSCVhygro expressing Cre. (E) Cell growth rates. (F) Oil Red O staining at day 7 of adipogenesis. (G) qRT-PCR analysis of *Krox20* and adipogenesis markers at indicated time points of adipogenesis. (H, I) *Krox20*$^{f/f}$ immortalized brown preadipocytes were infected with Ad-GFP or Ad-Cre. Two days later, cells were replated, followed by adipogenesis assays. (H) Oil Red O staining at day 7 of adipogenesis. (I) qRT-PCR analysis of gene expression.

a full physiological characterization of *Krox20*$^{f/f}$; *Myf5-Cre* mice may need to be done, these data indicate that Krox20 is dispensable for BAT development in mice.

PPARγ is essential for adipogenesis and BAT development. As a positive control, we infected *Pparγ*$^{f/f}$ immortalized brown preadipocytes with retroviral Cre. Deletion of *Pparγ* gene in preadipocytes prevented adipogenesis and induction of adipogenesis

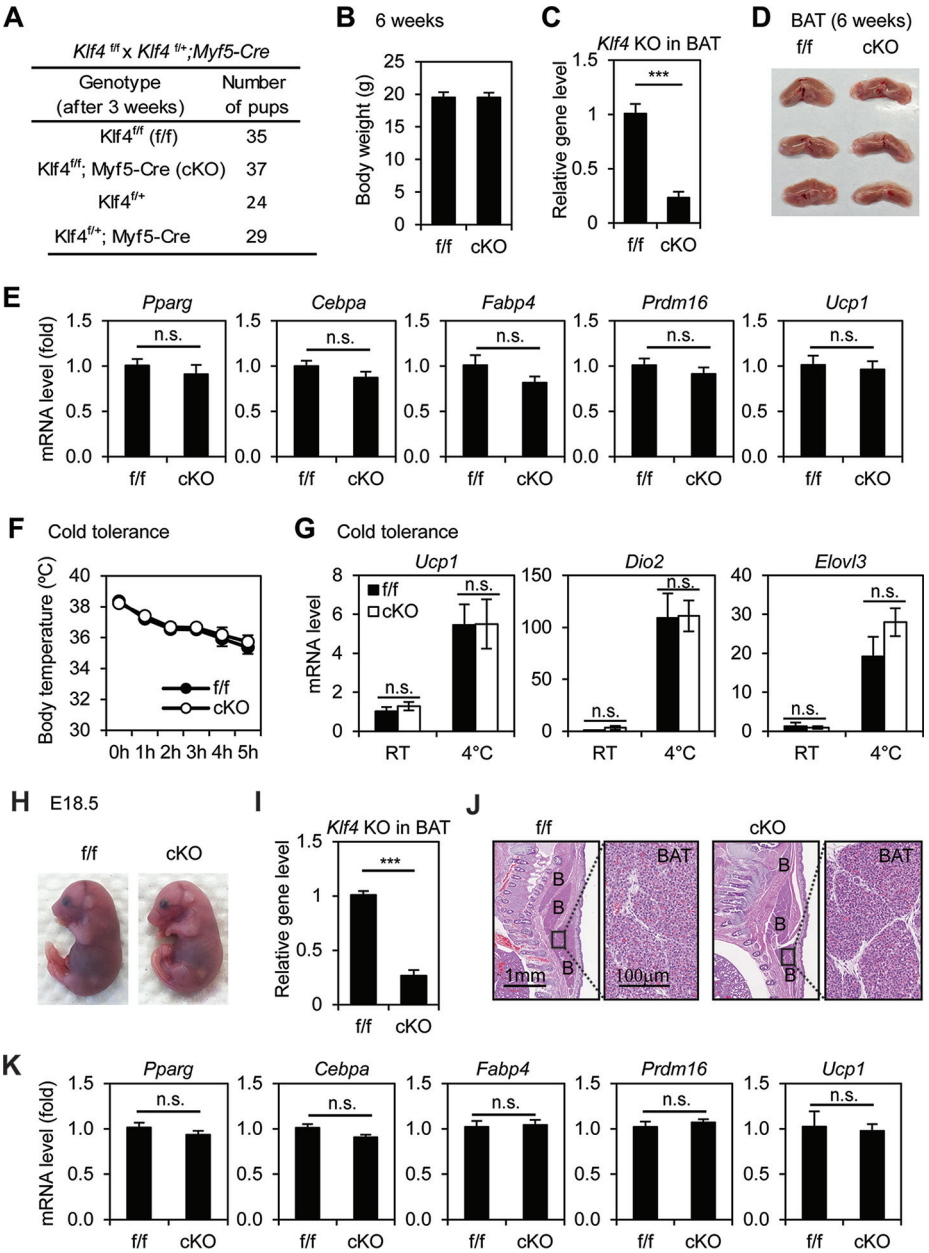

FIG 5 KLF4 is dispensable for BAT development and function. *Klf4*$^{f/f}$ mice were crossed with *Klf4*$^{f/+}$; *Myf5-Cre* mice to obtain *Klf4*$^{f/f}$; *Myf5-Cre* (conditional KO [cKO]) mice and littermate control (*Klf4*$^{f/f}$ [f/f]) mice. (A to E) KLF4 is dispensable for brown adipose tissue (BAT) development. Six-week-old cKO and f/f mice were characterized. (A) Genotyping results. The expected ratios of the four genotypes are 1:1:1:1. (B) Body weight of f/f ($n = 8$) or cKO ($n = 13$) male mice. (C) Confirmation of *Klf4* deletion in BAT by qPCR analysis of genomic DNA. ***, $P < 0.001$. (D) Pictures of BATs isolated from f/f and cKO mice ($n = 3$ per group). (E) RNA was extracted from BAT of f/f ($n = 8$) or cKO ($n = 13$) mice for qRT-PCR analysis of adipogenesis markers *Pparγ*, *Cebpα*, and *Fabp4* and BAT markers *Prdm16* and *Ucp1*. Data are presented as means ± SEM. n.s., no significance. (F, G) Cold tolerance test. cKO mice and their littermate controls ($n = 5$ per group) were acutely exposed to 4°C for 5 h. (F) Body temperatures were measured every hour. (G) qRT-PCR analysis of genes involved in thermogenesis in BAT. RT, room temperature. Data are presented as means ± SEM. (H to K) Characterization of E18.5 embryos. (H) Representative pictures of E18.5 embryos. (I) Confirmation of *Klf4* deletion in E18.5 BAT by qPCR analysis of genomic DNA. (J) E18.5 embryos were sagittally sectioned along the midline. The sections of the interscapular area were stained with hematoxylin and eosin (H&E). B, BAT. (K) RNAs were extracted from E18.5 BAT of f/f ($n = 7$) or cKO ($n = 9$) embryos for qRT-PCR analysis.

markers such as *Cebpα* and *Fabp4* (Fig. 7A to C). This result is consistent with the known essential role of PPARγ for adipogenesis.

We also generated *Pparγ*$^{f/f}$; *Myf5-Cre* mice by crossing *Pparγ*$^{f/f}$ with *Myf5-Cre* mice to define the role of PPARγ in BAT development in mice. *Pparγ*$^{f/f}$; *Myf5-Cre* mice were

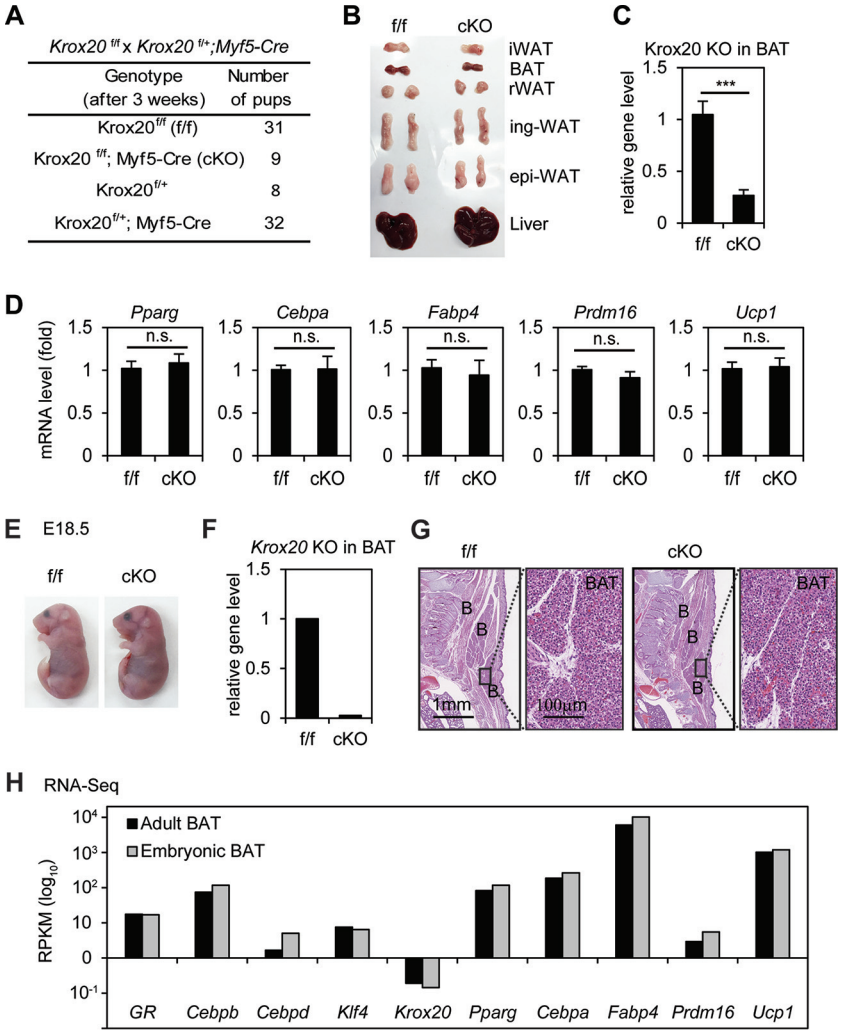

FIG 6 Krox20 is dispensable for BAT development. *Krox20*$^{f/f}$ were crossed with *Krox20*$^{f/+}$; *Myf5-Cre* to obtain *Krox20*$^{f/f}$; *Myf5-Cre* (conditional KO [cKO]) and littermate control (*Krox20*$^{f/f}$ [f/f]) mice. (A to D) *Krox20* is dispensable for BAT development. Eight-week-old cKO and f/f mice were characterized. (A) Genotyping results. The expected ratios of the four genotypes are 4:1:1:4. (B) Isolated adipose tissues. iWAT, interscapular WAT; epi-WAT, epididymal WAT; ing-WAT, inguinal WAT; rWAT, retroperitoneal WAT. (C) Confirmation of *Krox20* deletion in BAT by qPCR analysis of genomic DNA. (D) RNA was extracted from BAT of f/f and cKO mice for qRT-PCR analysis of adipogenesis markers *Pparγ*, *Cebpα*, and *Fabp4* and BAT markers *Prdm16* and *Ucp1*. (E to G) Characterization of E18.5 embryos. (E) Representative pictures of E18.5 embryos. (F) *Krox20* deletion in BAT was confirmed by qRT-PCR of genomic DNA. (G) H&E staining of the interscapular area. B, BAT. (H) *Krox20* mRNA is largely absent in BAT. The mRNA levels of early (*GR*, *Cebpβ*, *Cebpδ*, *Klf4*, and *Krox20*) or late (*Pparγ*, *Cebpα*, *Fabp4*, *Prdm16*, and *Ucp1*) adipogenesis genes in BATs isolated from adult (8-week-old) mice or embryos (E18.5) were determined by RNA-Seq. RPKM values from RNA-Seq indicate gene expression levels ($\log_{10}$ scale). ***, $P < 0.001$; n.s., no significance.

obtained close to the expected Mendelian ratio, and all survived well beyond 6 weeks after birth without any obvious developmental defects (Fig. 7D). Compared to littermate controls, *Pparγ*$^{f/f}$; *Myf5-Cre* mice showed near-complete absence of BAT and marked decreases of interscapular WAT mass (Fig. 7E and F). Consistent with the lack of BAT, *Pparγ*$^{f/f}$; *Myf5-Cre* mice were unable to maintain body temperatures and were cold intolerant when exposed to environmental cold (Fig. 7G). Immunohistochemistry analysis of E18.5 embryos using antibodies against the BAT marker UCP1 and the muscle marker myosin confirmed the severe defect in BAT but not in muscle development in *Pparγ*$^{f/f}$; *Myf5-Cre* embryos (Fig. 7H). Since *Myf5-Cre* is selectively expressed in precursor cells giving rise to BAT and muscles in the back (14), these results indicate that PPARγ is required for BAT but not muscle development and that BAT is dispensable for the survival of mice in the laboratory setting.

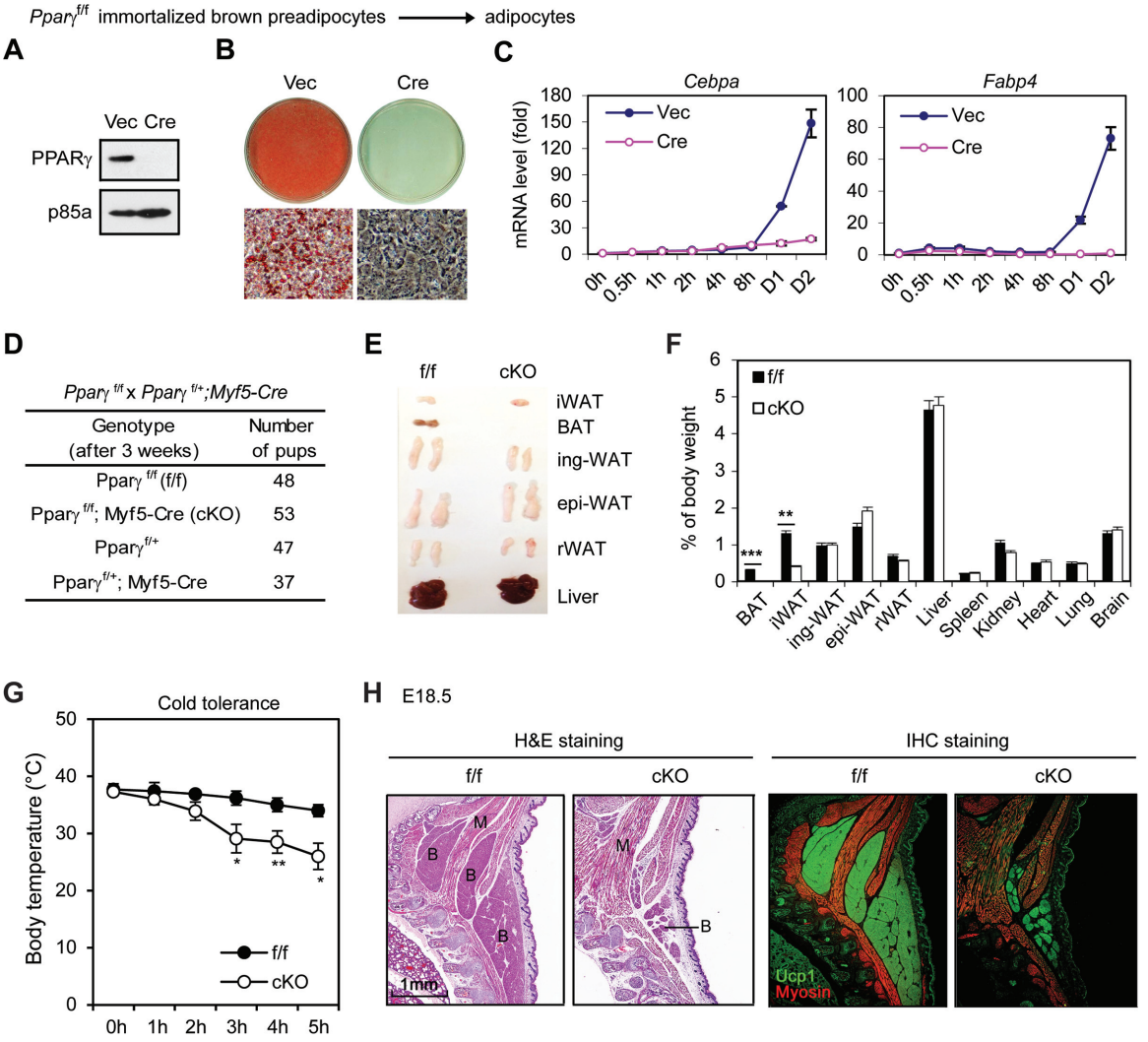

FIG 7 PPARγ is essential for adipogenesis and BAT development. (A to C) *Pparγ*$^{f/f}$ immortalized brown preadipocytes were infected with MSCVhygro expressing Cre. (A) Western blot analysis of PPARγ. p85a was used as a loading control. (B) Seven days after induction of adipogenesis, cells were stained with Oil Red O. (C) Time course qRT-PCR analysis of adipogenic markers *Cebpα* and *Fabp4*. (D to G) Characterization of 6-week-old *Pparγ*$^{f/f}$; *Myf5-Cre* mice. (D) Genotyping results. (E) Representative pictures of isolated adipose tissues and livers. (F) The relative weight of each tissue and organ is shown as a percentage of body weight. (G) *Pparγ*$^{f/f}$; *Myf5-Cre* mice are cold intolerant. Six-week-old mice were housed at 4°C, and their body temperatures were measured every hour ($n = 6$). *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$. (H) E18.5 embryos were sagittally sectioned along the midline. The sections of the interscapular area were stained with H&E (left panels) or with antibodies recognizing BAT marker UCP1 (green) and skeletal muscle marker myosin (red) (right panels). IHC, immunohistochemistry.

DISCUSSION

We show that endogenous KLF4 and Krox20 are dispensable for white and brown adipogenesis in culture. Consistent with these observations, KLF4 and Krox20 are dispensable, while PPARγ is essential, for BAT development in mice. These results indicate that while KLF4 and Krox20 are transiently induced by the adipogenic cocktail in the early phase of preadipocyte differentiation in culture, they do not play major roles in adipogenesis. Among components of the adipogenic cocktail, IBMX and FBS induce the expression of *Klf4* and *Krox20*, respectively (12, 13). The unexpected findings presented here suggest that the adipogenic cocktail accelerates adipogenesis independent of KLF4 and Krox20.

Using 3T3-L1 white preadipocytes and immortalized brown preadipocytes, we confirmed the previous observations that *Klf4* and *Krox20* are transiently induced in the early phase of adipogenesis in culture (12, 13). Surprisingly, deletion of *Klf4* or *Krox20* did not affect the induction of other early adipogenic TFs, including C/EBPβ and

C/EBPδ, which is consistent with our data that KLF4 and Krox20 are dispensable for adipogenesis. Our results do not exclude the possibility that overexpression of KLF4 and Krox20 stimulates adipogenesis in culture.

It was shown previously that small interfering RNA (siRNA)-mediated knockdown of *Klf4* inhibited 3T3-L1 adipogenesis (13). In our hands, however, *Klf4* knockdown by short hairpin RNA (shRNA) did not affect adipogenesis in 3T3-L1 cells (Fig. 3). Further, we demonstrated that KLF4 is dispensable for adipogenesis by deleting *Klf4* gene in white and brown preadipocytes as well as in mice. It was also shown that siRNA-mediated knockdown of *Krox20* inhibited 3T3-L1 adipogenesis (12). However, by deleting *Krox20* gene in white and brown preadipocytes as well as in mice, we demonstrated that Krox20 is dispensable for adipogenesis.

Studies using cell culture model systems, especially 3T3-L1 adipogenesis, have enabled the discoveries of major adipogenic TFs, including C/EBPα, C/EBPβ, C/EBPδ, and PPARγ (6, 7). Our data showing that endogenous KLF4 and Krox20 are dispensable for adipogenesis in culture and BAT development in mice suggest that it is also important to study adipogenesis *in vivo*, a process that is poorly understood (16).

MATERIALS AND METHODS

Plasmids and antibodies. Retroviral plasmids MSCVhygro-Cre and MSCVpuro-Cre have been described previously (17–19). The lentiviral shRNA plasmid pLKO.1 targeting *Klf4* (clone ID TRCN0000238251) and shRNA control plasmid were from Sigma. Anti-KLF4 antibody (catalog number 4038) was from Cell Signaling. Anti-PPARγ (sc-7196X) and anti-p85α (sc-1637) antibodies were from Santa Cruz. Anti-RbBP5 (A300-109A) antibody was from Bethyl Laboratories.

Mouse experiments. *Klf4*$^{f/f}$ mice (20), *Krox20*$^{f/f}$ mice (21), and *Pparγ*$^{f/f}$ mice (22) (Jackson no. 004584) were crossed with *Myf5-Cre* mice (Jackson no. 007893). Histology and immunohistochemistry analyses of E18.5 embryos were done as described previously (18). For the cold tolerance test, individual mice were housed at 4°C as described previously (17). Body temperature was measured every hour for 5 h using a mouse rectal probe (Thermalert TH-5). At the end of experiments, mice were euthanized and BATs were collected. Data were presented as means ± standard errors of the means (SEM). Differences were analyzed with Student's *t* test. All mouse work was approved by the Animal Care and Use Committee of NIDDK, NIH.

Isolation of primary preadipocytes, immortalization, virus infection, and adipogenesis assay. Primary brown preadipocytes were isolated from interscapular BAT of newborn *Klf4*$^{f/f}$ or *Krox20*$^{f/f}$ pups. Primary white adipocytes were isolated from inguinal WAT of *Klf4*$^{f/f}$ or *Krox20*$^{f/f}$ adult mice. Immortalization of primary brown preadipocytes by retroviral vectors expressing SV40T was done as described previously (23). Adipogenesis of primary white preadipocytes and the 3T3-L1 cell line was carried out as described previously (19). For brown adipogenesis assays, preadipocytes were plated at a density of 1×10^5 in each well of 6-well plates in growth medium (Dulbecco's modified Eagle medium [DMEM] plus 10% FBS) 4 days before induction of adipogenesis. At day 0, cells were fully confluent and were treated with differentiation medium (DMEM plus 10% FBS, 0.1 μM insulin, and 1 nM T3) supplemented with 0.5 mM IBMX, 1 μM dexamethasone, and 0.125 mM indomethacin. Two days later, cells were changed to the differentiation medium. The medium was replenished at 2-day intervals. Fully differentiated cells were either stained with Oil Red O or subjected to gene expression analysis by qRT-PCR.

qRT-PCR. Total RNA was extracted using TRIzol (Invitrogen) and reverse transcribed using ProtoScript II first-strand cDNA synthesis kit (NEB), following the manufacturers' instructions. qRT-PCR was done using the following SYBR green primers: *Klf4*, forward, 5′-GTGCCCCGACTAACCGTTG-3′, and reverse, 5′-GTCGTTGAACTCCTCGGTCT-3′; *Krox20*, forward, 5′-TGACTATTGTGGCCGCAAGTT-3′, and reverse, 5′-TTCTGCCGAAGGTGGATCTT-3′. SYBR green primers for other genes were described previously (24).

RNA-Seq. RNA-Seq experiments were performed during adipogenesis of 3T3-L1 white preadipocytes and *Pparγ*$^{f/f}$ immortalized brown preadipocytes. For RNA-Seq analysis of BAT, interscapular BATs were isolated from wild-type adult (8-week-old) mice or E18.5 embryos. RNA-Seq library preparation was done following the protocol described previously (18). The expression levels of each gene were indicated by units of reads per kilobase per million (RPKM).

Statistical analyses. Quantitative data in all figures are expressed as means ± standard errors. To compare between two groups, the statistical significance was calculated using the two-tailed unpaired *t* test under two experimental conditions. A *P* value of less than 0.05 was considered statistically significant.

Accession number. All data sets described in this paper have been deposited in NCBI Gene Expression Omnibus under accession number GSE87113.

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