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Characterization of Cre recombinase models for the study of adipose tissue

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The study of adipose tissue *in vivo* has been significantly advanced through the use of genetic mouse models. While the aP2-Cre^{BI} and aP2-Cre^{Salk} lines have been widely used to target adipose tissue, the specificity of these lines for adipocytes has recently been questioned. Here we characterize Cre recombinase activity in multiple cell populations of the major adipose tissue depots of these and other Cre lines using the membrane-Tomato/membrane-GFP (mT/mG) dual fluorescent reporter. We find that the aP2-Cre^{BI} and aP2-Cre^{Salk} lines lack specificity for adipocytes within adipose tissue, and that the aP2-Cre^{BI} line does not efficiently target adipocytes in white adipose depots. Alternatively, the Adiponectin-CreERT line shows high efficiency and specificity for adipocytes, while the Pdgfr α -CreERUCL and Pdgfr α -CreERJHU lines do not efficiently target adipocyte precursor cells in the major adipose depots. Instead, we show that the Pdgfr α -Cre line is preferable for studies targeting adipocyte precursor cells *in vivo*.

Introduction

Adipose tissue is recognized as a vital player in the maintenance of energy balance, nutrient status, and metabolic homeostasis.¹ White adipose tissue (WAT) retains the ability to grow and shrink dramatically to meet the energetic needs of an organism; however, severe metabolic consequences can result from excessive WAT gain, the defining characteristic of obesity, or extreme loss of WAT mass, known as lipodystrophy.^{2,3} These include defects in glucose homeostasis, inflammation, and cardiovascular function.^{3–6} In order to fully understand these complex multi-organ pathologies, studies must be performed *in vivo*, and our ability to address the role of adipose tissue in these systems relies on the tools that have been developed to manipulate gene expression within adipose tissue *in vivo*.

Mature adipocytes compose the majority of the volume of adipose tissue, and the remaining cell populations in the tissue include blood cells, endothelial cells, various immune cell populations, and adipocyte precursors. Recently, the identification of specific murine white adipocyte precursor populations, including Lin⁻:CD29⁺:CD34⁺:Sca-1⁻:CD24⁺ adipocyte progenitor cells and Lin⁻:CD29⁺:CD34⁺:Sca-1⁻:CD24⁻ preadipocytes,⁷ has enabled further study of the adipocyte lineage *in vivo*. Furthermore, several groups have reported that platelet-derived growth factor receptor α (Pdgfr α) is expressed on adipocyte precursor cells in

WAT and traces all adipocytes in normal murine WAT depots,^{8,9} which indicates that this promoter is a useful tool for targeting the adipocyte cellular lineage.

The Cre/lox system of gene targeting has revolutionized the study of tissue-specific function *in vivo*. Cre recombinase can be integrated downstream of an endogenous promoter, often termed a “knock-in”, or it can be placed under control of a promoter fragment which is then integrated into the genome at a random site. The length of this promoter sequence and the location of the integration site can affect the expression pattern of the Cre transgene, and may affect the fidelity of Cre expression compared with the expression of the endogenous gene.¹⁰ Additionally, temporal control over gene expression can be achieved with inducible targeting models such as doxycycline-regulated Cre expression or tamoxifen-sensitive Cre. When applying these techniques, however, it is important to keep in mind the limitations and pitfalls of these approaches including the erroneous expression of Cre transgenes, the varying sensitivity of different genomic sites to Cre-mediated loxP recombination, and the potential for changes in Cre expression or efficacy over several generations of mouse colony maintenance.^{11,12}

To facilitate the study of adipocyte function in physiologically relevant contexts, several adipocyte-specific promoters have been generated to drive the expression of Cre recombinase in adipose tissues. Of these, the most commonly used Cre transgenes

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were created from the promoter of the fatty acid binding protein 4 (*Fabp4*) gene which encodes adipocyte protein 2 (aP2).¹³ Two separate lines (aP2-Cre^{BI} and aP2-Cre^{Salk}) were generated^{14,15} and widely used in studies to target adipocytes in vivo.¹⁶ However, these lines have been shown to have Cre activity in other tissues and cell types, including brain, endothelial cells, macrophages, adipocyte precursors and embryonic tissues.¹⁶⁻¹⁹ Alternatively, the promoter of the adipocyte-specific protein Adiponectin (encoded by *Adipoq*) has been used to generate Cre lines for the study of adipocyte function.^{20,21} These Adiponectin-Cre mouse lines have been shown by multiple groups to be highly specific to adipose tissue.^{16,18,20} To identify the most useful tools for targeting the adipocyte lineage in vivo, we sought to investigate the recombination efficiency and specificity of commonly used Cre lines, using a membrane-targeted dual fluorescent reporter model to quantitatively assess Cre recombinase activity in adipose tissues.

Here we characterize the recombination of specific cell populations within adipose tissue in the aP2-Cre^{BI} and aP2-Cre^{Salk} lines, as well as the tamoxifen-inducible Adiponectin-CreERT and Pdgfr α -CreERT lines. We find incomplete targeting of adipocytes and a lack of specificity with the aP2-Cre^{BI} and aP2-Cre^{Salk} lines, and remarkably low recombination efficiency in adipocyte precursors in Pdgfr α -CreER lines. Finally, we show that the Adiponectin-CreERT is a useful inducible model for targeting mature adipocytes, while the Pdgfr α -Cre is useful for studying the adipocyte lineage in vivo.

Results

To characterize the pattern of Cre expression in the aP2-Cre lines, we crossed each of these lines to the dual fluorescent reporter model (mT/mG) that we have previously used to perform lineage tracing of white adipose tissue.^{9,22} This reporter expresses membrane-targeted tdTomato (mT), and the expression of Cre recombinase results in the excision of the tdTomato cassette, which then permits the expression of membrane-targeted eGFP (mG). Since these fluorescent proteins are membrane-targeted, this model provides clear fluorescently labeled cellular boundaries, facilitating the identification of adipocytes in which Cre-mediated recombination has occurred. When we analyzed the pattern of Cre expression in several adipose depots of aP2-Cre^{BI}; mT/mG mice, we found that few adipocytes were labeled in WAT depots, while approximately half of the adipocytes in the intrascapular brown adipose tissue (iBAT) depot were labeled (Fig. 1A and B). Additionally, flow cytometry analysis indicated that a large percentage of endothelial cells were labeled in both WAT and BAT depots in the aP2-Cre^{BI} line, while the adipocyte precursor populations were not labeled in WAT depots (Fig. 1B). Further confocal analysis confirmed that the majority of eGFP-positive cells in SWAT co-stained with the endothelial stain GSIB₄ (Fig. 1C). Finally, we observed negligible labeling of both liver and skeletal muscle cells of these mice (data not shown). These data indicate that while this line was originally shown to efficiently label adipocytes,¹⁴ it now targets

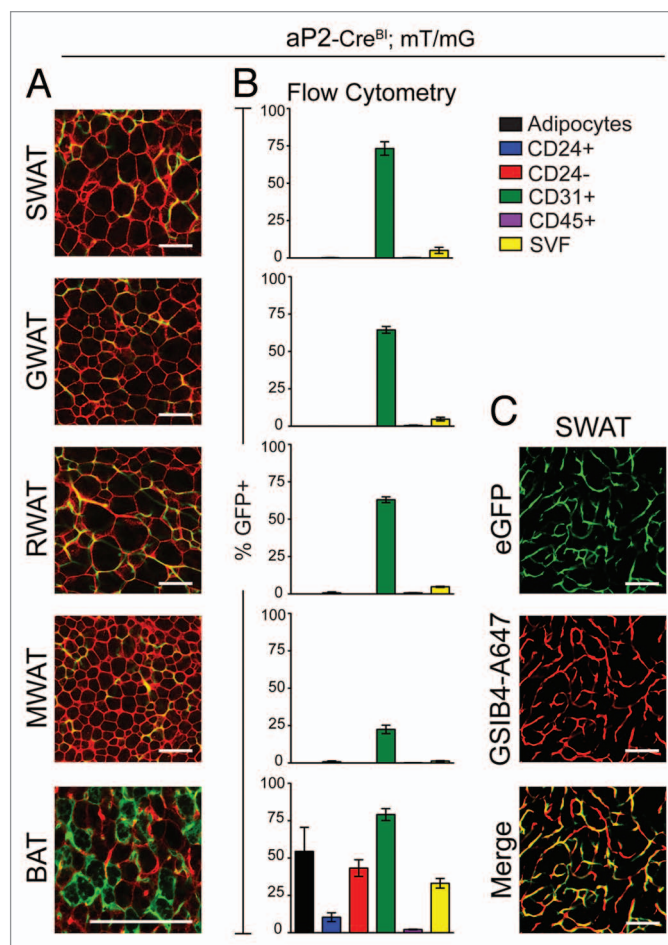


Figure 1. aP2-Cre^{BI} primarily labels endothelial cells and brown adipocytes. (A) Confocal microscopy of the indicated adipose tissue depots from aP2-Cre^{BI}; mT/mG mice. (B) Quantification of cells displaying Cre-mediated expression of eGFP in cell populations of the indicated adipose tissue depots ($n = 3$). (C) Confocal microscopy of SWAT from aP2-Cre^{BI}; mT/mG mice stained with isolectin GSIB₄ to label endothelial cells. Scale bars in (A and C) are 100 μ m. SWAT, subcutaneous WAT; GWAT, gonadal WAT; RWAT, retroperitoneal WAT; MWAT, mesenteric WAT; BAT, brown adipose tissue; SVF, stromal vascular fraction.

primarily brown adipocytes and endothelial cells within adipose tissue.

We next characterized Cre expression in the aP2-Cre^{Salk} line using the mT/mG fluorescent reporter. We found that the percentage of adipocytes labeled by this line in WAT depots was much higher than the aP2-Cre^{BI} line, and varied between 50 and 80 percent (Fig. 2A and B). Brown adipocytes were labeled to a similar degree (Fig. 2A and B). While there was some labeling in the adipocyte precursor populations in all depots, we also observed some labeling in blood lineage cells and significant labeling of CD31⁺ endothelial cells in all adipose depots analyzed (Fig. 2B), again indicating a lack of specificity for the adipocyte lineage in the aP2-Cre^{Salk} line. However, we observed negligible Cre-mediated recombination in cells of the liver and skeletal muscle of these mice (data not shown).

The promoter for adiponectin, a hormone secreted by mature adipocytes, has been used by multiple groups to

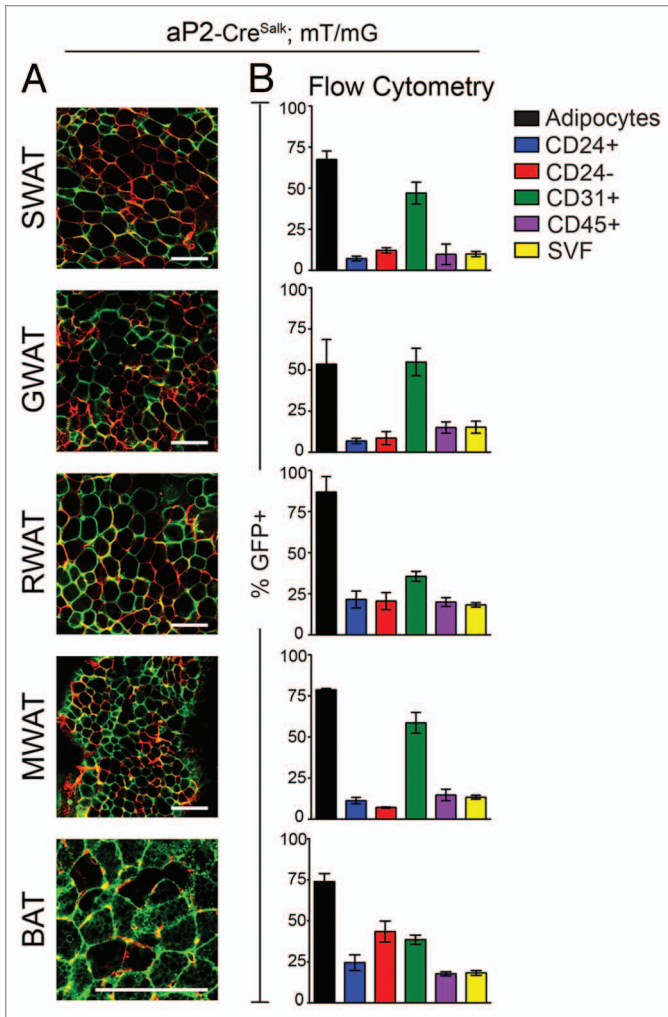


Figure 2. aP2-Cre^{Salk} labels multiple cell populations within adipose tissue. (A) Confocal microscopy of the indicated adipose tissue depots from aP2-Cre^{Salk}; mT/mG mice. (B) Quantification of cells displaying Cre-mediated expression of eGFP in cell populations of the indicated adipose tissue depots (n = 3). Scale bars in (A) are 100 μm. SWAT, subcutaneous WAT; GWAT, gonadal WAT; RWAT, retroperitoneal WAT; MWAT, mesenteric WAT; BAT, brown adipose tissue; SVF, stromal vascular fraction.

generate adipocyte-specific Cre lines.^{20,21} The most widely used Adiponectin-Cre^R line¹² has been shown several times to be specific to adipocytes,^{9,16,18} and useful for targeting mature adipocytes in vivo. To characterize inducible Cre expression in a new Adiponectin-CreER line generated with the same BAC used to create the Adiponectin-Cre^R mice, we crossed this line to the mT/mG reporter line and treated mice with 50 mg/kg tamoxifen for 6 d. Before tamoxifen treatment, no adipocyte labeling was observed (Fig. S1A), and after tamoxifen treatment, adipocyte recombination was nearly 100% in all WAT depots analyzed, with negligible recombination in any other cell population, and greater than 85% recombination was observed in brown adipocytes (Fig. 3A and B). Importantly, no cells within the adipose SVF displayed Cre-mediated recombination in this model, including the adipocyte precursor populations,

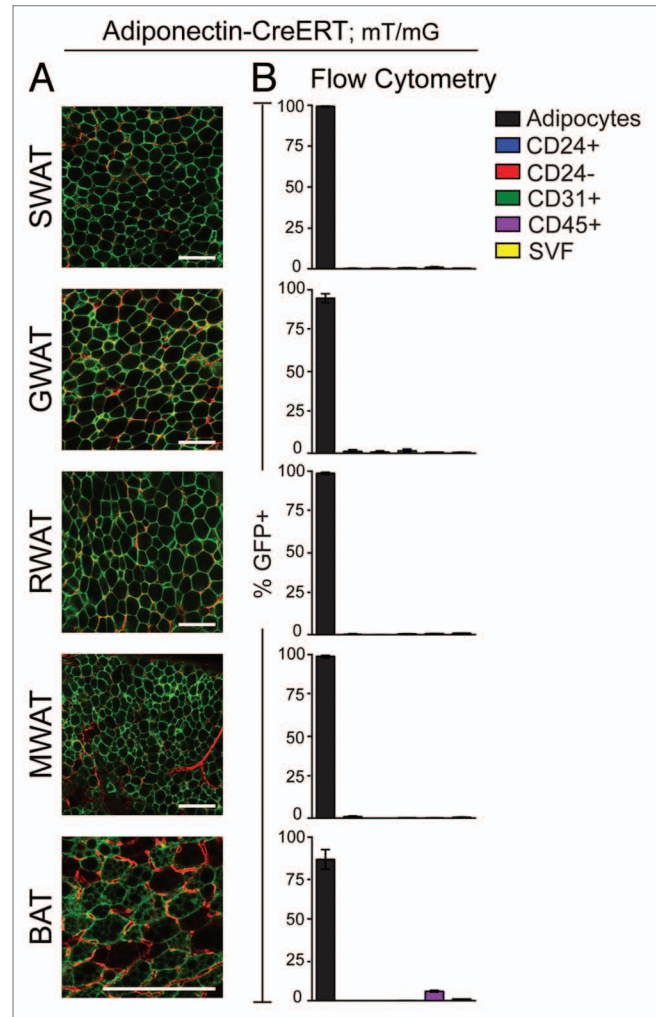


Figure 3. Adiponectin-CreERT labels mature adipocytes. (A) Confocal microscopy of the indicated adipose tissue depots from Adiponectin-CreERT; mT/mG mice after tamoxifen treatment. (B) Quantification of cells displaying Cre-mediated expression of eGFP in cell populations of the indicated adipose tissue depots (n = 3). Scale bars in (A) are 100 μm. SWAT, subcutaneous WAT; GWAT, gonadal WAT; RWAT, retroperitoneal WAT; MWAT, mesenteric WAT; BAT, brown adipose tissue; SVF, stromal vascular fraction.

(Fig. 3B), confirming the specificity of this promoter for mature adipocytes.

We and other groups have shown that Pdgfrα is expressed on adipocyte precursor populations within WAT,^{8,9} and that almost all of the adipocyte precursors are labeled in Pdgfrα-Cre; mT/mG mice. In addition, the vast majority of the cells displaying Cre-recombination within WAT SVF of Pdgfrα-Cre; mT/mG mice are adipocyte precursors.⁹ To determine whether this Cre line may be useful for metabolic studies targeting the adipocyte lineage, but not other major metabolic tissues, we assessed Cre-mediated recombination in the cells of the muscle and liver of Pdgfrα-Cre; mT/mG mice. We found low levels of recombination in both of these tissues (Fig. 4A), indicating that the muscle and liver are minimally targeted by the Pdgfrα promoter. These

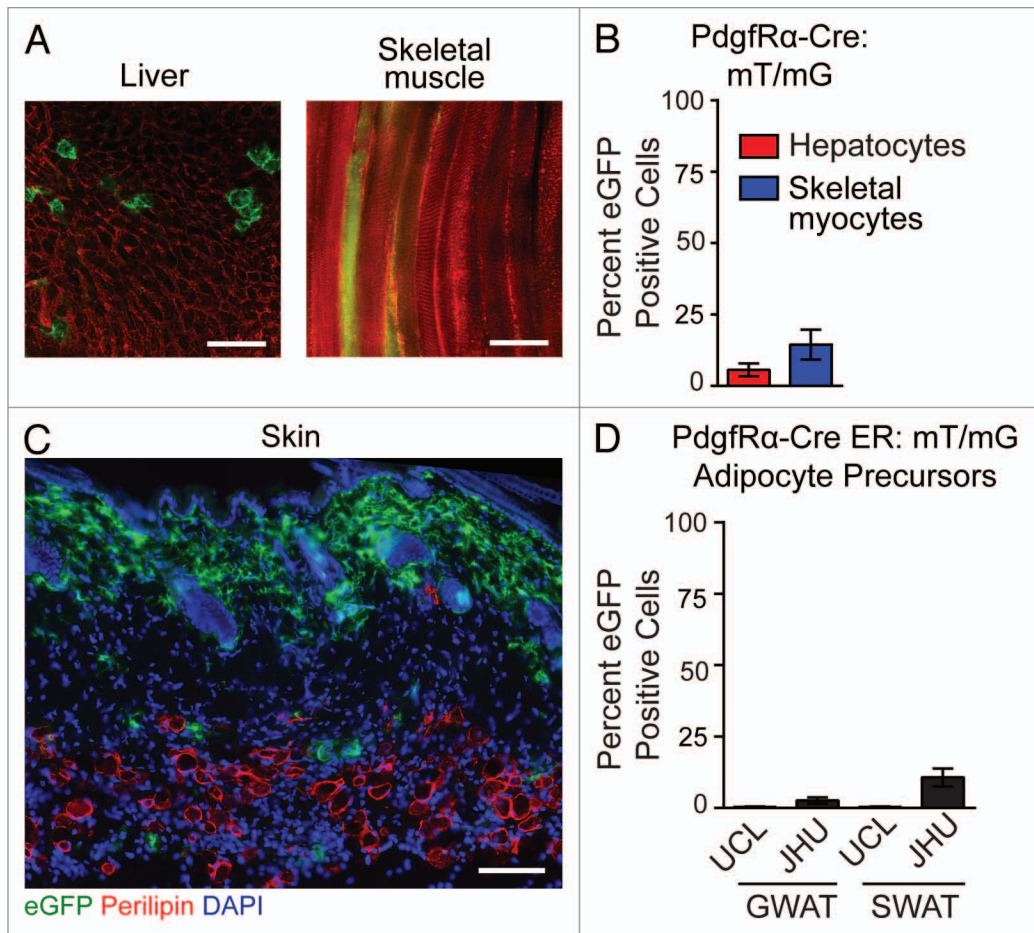


Figure 4. Pdgfr α -CreER does not efficiently label adipocyte precursor cells. (A) Confocal microscopy of the liver and skeletal muscle (gastrocnemius) from Pdgfr α -Cre; mT/mG mice. (B) Quantification of cells displaying Cre-mediated expression of eGFP in liver and skeletal muscle of Pdgfr α -Cre; mT/mG mice ($n = 3$). (C) Immunofluorescence image of the back skin of Pdgfr α -CreER^{UCL}; mT/mG mouse after tamoxifen injection showing recombination in fibroblastic cells of the upper dermis. Perilipin staining of adipocytes indicates the location of the adipocyte layer within the dermal compartment. Representative image from 2 animals. (D) Quantification of cells displaying Cre-mediated expression of eGFP in the adipocyte precursor population of the indicated depots of Pdgfr α -CreER^{UCL}; mT/mG and Pdgfr α -CreER^{JHU}; mT/mG mice after daily treatment of 50mg/kg tamoxifen for 6 d ($n = 3-4$). Scale bars in (A and C) are 100 μ m. SWAT, subcutaneous WAT; GWAT, gonadal WAT; SVF, stromal vascular fraction.

data suggest that the Pdgfr α -Cre line may be appropriate for functional studies of the adipocyte cellular lineage in vivo.

Inducible versions of the Pdgfr α -Cre mouse line have been generated by both the Richardson group²³ (Pdgfr α -CreER^{UCL}) and the Bergles group²⁴ (Pdgfr α -CreER^{JHU}). Both of these lines have previously been shown to label oligodendrocyte precursors in the brain.^{23,24} The Pdgfr α -CreER^{UCL} line has been used to label adipocyte lineage cells in WAT⁸ and fibroblast-like cells in the skin,²⁵ although with variable efficiency. To assess the potential utility of both of these lines for the study of adipocyte precursors in WAT depots in vivo, we treated Pdgfr α -CreER^{UCL}; mT/mG and Pdgfr α -CreER^{JHU}; mT/mG mice with 50 mg/kg tamoxifen daily for 6 d and subsequently analyzed the percentage of adipocyte precursor cells displaying Cre-mediated recombination in the skin and primary WAT depots of these mice. In tamoxifen-treated Pdgfr α -CreER^{JHU}; mT/mG mice, we observed a high level of GFP-positive cells in the dermal layer of the skin (Fig. 4B), a region known to contain Pdgfr α -Cre-expressing cells including

an adipogenic population;^{25,26} however, less than 1% of the intra-dermal adipocyte precursor cells were labeled (data not shown). When we analyzed the stromal vascular fraction of WAT in Pdgfr α -CreER^{UCL}; mT/mG mice, we observed very low rates of recombination (less than 3%) in adipocyte precursor populations of both GWAT and SWAT, with only marginally better recombination percentages in the SWAT of Pdgfr α -CreER^{JHU}; mT/mG mice (Fig. 4C). Treatment of Pdgfr α -CreER^{JHU}; mT/mG mice with a higher tamoxifen dose (300 mg/kg for 5 d), did not improve the recombination efficiency (Fig. S1C), indicating that Cre activity in adipocyte precursors in these lines is insufficient for assessing gene function in adipocyte precursors in vivo.

Discussion

Tools for the genetic targeting of adipose tissue in vivo are essential for the study of adipose function and metabolic disease.

Consistent with previous reports,^{16,17} our data show that the aP2-Cre^{BI} line and the aP2-Cre^{Salk} line are not specific to adipocytes. However, contrary to previous studies using cytoplasmic LacZ cre reporters,¹⁸ quantitative analysis of adipocyte labeling with the mT/mG reporter indicates that the aP2-Cre^{BI} line does not label the majority of adipocytes in WAT depots. Since this could be due to a shift in the Cre expression pattern in these lines over generations,¹² these data emphasize the importance of monitoring recombination within a line over time. Additionally, these data highlight the importance of determining the specificity of Cre-recombination to the cell types of interest in a particular tissue in studies of cellular lineage dynamics and cell function.

We and others have previously found that the Adiponectin-Cre^R effectively targets mature adipocytes,^{9,16,18,20} and we show here that the Adiponectin-CreER line can be used to efficiently target adipocytes in an inducible manner. This model can be applied in studies of adult animals in which developmental defects in adipose tissue could present confounding effects, and also provides the temporal control necessary to perform quantitative pulse-labeling studies when coupled with the mT/mG reporter.

The Pdgfr α -CreER model would also be a valuable tool for targeting adipocyte precursor cells. While one group has used very high doses of tamoxifen to achieve partial recombination in WAT-resident APs,⁸ we show here that in our hands neither of the existing Pdgfr α -CreER lines efficiently label this population, despite labeling of other Pdgfr α -expressing populations in the dermis (Fig. 4). Given that both of the promoter sequences used to generate the Pdgfr α -CreER lack a region of at least 60 kb upstream of the Pdgfr α gene compared with the Pdgfr α -Cre construct, this low labeling efficiency may be due to the lack of *cis*-regulatory elements that are normally necessary to drive expression from the Pdgfr α promoter in certain cell populations.^{23,24,27} Another potential factor in the difference in labeling between the Pdgfr α -CreER and Pdgfr α -Cre lines is differences in integration sites in the genome. Regardless of the reason, the low labeling efficiency in these Pdgfr α -CreER lines indicates that they are not useful for quantitative adipocyte lineage studies or for study of gene function in the adipocyte lineage.

Even when the promoter region used to drive transgene expression is large, differences in enhancer activity or positional effects of the insertion site can cause the expression of the transgene to differ from the endogenous gene. This appears to be the case for the Pdgfr α -Cre line, which efficiently labels adipocyte precursors.⁹ During murine embryonic development, staining for endogenous Pdgfr α and experiments utilizing a knock-in fluorescent reporter have shown that Pdgfr α is expressed in regions of the endoderm, mesoderm and ectoderm, with the most prominent expression occurring in mesodermal tissues.²⁸⁻³¹ Starting at embryonic day 8, Pdgfr α is expressed throughout the somites,²⁸ which give rise several mesodermal tissues including the skeletal muscle, dermis, and cartilage. However, in adult Pdgfr α -Cre mice, the broad recombination that would be expected given this embryonic expression pattern is not observed. For example, adult skeletal muscle displays a low level of Cre-mediated recombination in the Pdgfr α -Cre line. These data suggest that the

expression of Cre from the Pdgfr α promoter in the Pdgfr α -Cre model does not mimic these early embryonic expression patterns (Fig. 4). This restricted expression may be due to a lack of promoter and/or enhancer elements in the Pdgfr α -Cre genetic construct that are required to induce embryonic expression of the gene. The Pdgfr α -Cre model has been shown to label other cell populations such as the Muller glial cells of the retina,²⁷ but our data show that it is not significantly expressed in the parenchymal cells of other major metabolic organs such as the liver and skeletal muscle. These findings indicate that Pdgfr α -Cre should not have significant “off target” effects in other metabolic tissues. However, the known Cre expression from this promoter in other cell types, such as oligodendrocytes,²³ should be considered when interpreting results from this model.

Another caveat that must be considered when using either the Pdgfr α or the Adiponectin promoters for the study of adipose function is that these models express Cre recombinase in both white and brown adipocytes. There are not currently any models known to specifically target WAT without targeting BAT, and the promoters known to target BAT also target either muscle and subsets of white adipocytes³²⁻³⁴ or target both beige and brown adipocytes.³⁵ Therefore, we conclude that Pdgfr α -Cre is currently the best model available for the targeting of adipocyte lineage cells in adipose depots in vivo, while the Adiponectin-Cre^R and Adiponectin-CreER are effective for targeting mature adipocytes.

Materials and Methods

All animal studies followed guidelines issued by Yale University’s Institutional Animal Care and Use Committee (IACUC). aP2-Cre^{BI} mice were a generous gift from Dr Barbara Kahn, obtained in October 2013. The Pdgfr α -CreER^{UCL} mouse line²³ was independently obtained from Dr Anne Perl and Dr Dana McTigue. The aP2-Cre^{Salk} mice (005069), Pdgfr α -Cre mice (013148), Pdgfr α -CreER^{JHU} mice (018280), and mT/mG mice (007676) were purchased from Jackson Laboratories. AdiponectinCre-ERT mice (024671) are now available at Jackson Laboratories. Except for the Adiponectin-CreERT and Pdgfr α -CreER lines, all mice analyzed were males between 4 and 6 wk of age.

For Adiponectin-CreERT experiments, 8-wk-old male mice were given daily intraperitoneal injections of 50 mg/kg tamoxifen in vegetable oil for 6 d. Mice were then allowed to recover for one week, and then sacrificed. For Pdgfr α -CreER experiments, where indicated, mice between 5 and 10 wk of age were given either intraperitoneal injections of 50 mg/kg tamoxifen in vegetable oil daily for 6–7 d or oral gavage of 300 mg/kg tamoxifen in vegetable oil daily for 5 d.

Confocal microscopy and flow cytometry were performed as described.^{9,36,37} For immunofluorescence, 14 μ m skin sections were fixed with 4% paraformaldehyde and incubated with primary antibodies against GFP (chicken, Abcam, 1:1000) and perilipin A (goat, Abcam, 1:1000) overnight at 4 °C followed by incubation with alexa fluor-conjugated secondary antibodies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/adipocyte/article/29674/

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