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Characterization of the adipocyte cellular lineage in vivo

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Abstract

Mature adipocytes are generated through the proliferation and differentiation of precursor cells. Our prior studies identified adipocyte progenitors in white adipose tissue (WAT) as Lin −:CD29+:CD34+:Sca-1+:CD24+ (CD24+) cells that are capable of generating functional WAT[1]. Here, we employ several Cre recombinase mouse models to identify the adipocyte cellular lineage in vivo. While it has been proposed that white adipocytes are derived from endothelial[2] and hematopoietic[3, 4] lineages, we find that neither of these lineages label white adipocytes. However, platelet-derived growth factor receptor α (*PdgfR*α*)-Cre* trace labels all white adipocytes. Analysis of WAT from *PdgfR*α*-Cre* reporter mice identifies CD24+ and Lin −:CD29+:CD34+:Sca-1+:CD24− (CD24−) cells as adipocyte precursors. We show that CD24+ cells generate the CD24− population in vivo and the CD24− cells express late markers of adipogenesis. From these data we propose a model where the CD24+ adipocyte progenitors become further committed to the adipocyte lineage as CD24 expression is lost, generating CD24− preadipocytes. This characterization of the adipocyte cellular lineage will facilitate study of the mechanisms that regulate WAT formation in vivo and WAT mass expansion in obesity.

The number of mature adipocytes in white adipose tissue (WAT) of adults is tightly regulated, despite their continual turnover[5]. As mature adipocytes are post-mitotic[6, 7], change in adipocyte number occurs via disruption of the balance between rates of adipogenesis and adipocyte death. Therefore, characterization of the adipocyte cellular lineage is required for mechanistic understanding of WAT homeostasis and growth.

Various methods have been used to study adipocyte precursors ex vivo and in vivo. One common method is to culture the whole stromal-vascular fraction (SVF) from adipose tissues and select cell populations by their adherence to plastic[8, 9]. The cells derived from this method are referred to as preadipocytes or adipocyte-derived stem cells. However, these

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cells have not been shown to have de novo adipogenic capacity in vivo and their relationship to adipocyte lineage cells in vivo is not known.

Alternatively, several groups used fluorescence-activated cell sorting (FACS) in a prospective approach to identify adipogenic cell populations from various tissues[1, 10-12]. Two cell populations derived from WAT, defined by the marker profiles Lin −:CD34+:CD29+:Sca-1+:CD24+ (CD24+) and Lin−:CD34+:CD29+:Sca-1+:CD24− (CD24−), are adipogenic in vitro but only the CD24+ population is capable of generating a functional WAT depot upon transplantation into a residual WAT depot of lipodystrophic mice[1], indicating that the CD24+ population contains adipocyte progenitors. Cells with similar marker profiles have been shown to be adipogenic within the skin[10] and skeletal muscle[11].

Genetic approaches have also been used to investigate the adipocyte cellular lineage. A previous study showed, through crossing *Cadherin-5 (Cdh5)-Cre* mice into reporter lines that express cytoplasmic β-galactosidase and GFP, that *Cdh5-Cre* labels mature adipocytes[2, 13], suggesting an endothelial origin for white adipocytes as *Cdh5* labels endothelial lineages[14]. However, for studies of WAT the cellular specificity of reporters that stain the cytoplasm is difficult to delineate given the paucity of cytoplasm in mature adipocytes and the high vascularity of WAT. To overcome this limitation, we employed a mouse strain harboring a fluorescent –membrane dTomato/membrane eGFP (*mT/mG*) Cre reporter construct that marks Cre excision by a heritable switch from membrane targeted tdTomato expression to membrane targeted eGFP expression[15]. We crossed this reporter to several mouse lines expressing Cre recombinase from various promoters to more specifically determine the identity of the adipocyte precursors. Whole mount confocal microscopy of WAT from *Adiponectin-Cre:mT/mG* mice demonstrates GFP expression in mature adipocytes of all WAT depots assayed, with no GFP fluorescence in the absence of Cre expression, indicating that the *mT/mG* reporter model is appropriate for lineage tracing of mature adipocytes (Fig. 1a and Supplemental Fig. 1a). Flow cytometry analysis of the SVF from *mT/mG* models (Supplemental Fig. 2a) demonstrates that this model is also suitable for the study of potential precursor populations. However, flow cytometry analysis of *Adiponectin-Cre:mT/mG* WAT shows there are no GFP+ cells in the SVF (Fig. 1c, Supplemental Fig. 1b), indicating that the *Adiponectin* promoter is not active in immature adipocyte lineage cells and thus, *Adiponectin-Cre* mice are not useful for identification of adipocyte precursors in adult WAT.

We next generated *Cdh5-Cre:mT/mG* mice, using the same mouse line used in the previous study[2], to determine if adipocyte precursors within the SVF are derived from *Cdh5* expressing cells. While CD31+ endothelial cells were almost completely labeled by *Cdh5-Cre*, mature adipocytes from WAT depots and brown adipose tissue did not express GFP in this model (Figs. 1a-c, Supplemental Fig. 1). Analysis of another endothelial lineage marker, endothelial-specific receptor tyrosine kinase (*Tie2*)*-Cre*, produced similar results (Fig. 1a,c, Supplemental Fig. 1), indicating that adipocytes are not derived from endothelial cells under normal conditions. Due to the high vascularity of WAT and the tight association of capillaries with mature adipocytes, some adipocytes are completely surrounded by GFP+ vasculature, giving the appearance that the adipocyte may be GFP+. However, labeling

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endothelial cells with isolectin GSIB4 clearly shows that the GFP signal is derived from endothelial cells in *Cdh5-Cre* and *Tie2-Cre* and the mature adipocytes are dTomato+ (Fig. 1b and Supplemental Fig. 1c).

It has also been proposed that adipocytes are derived from hematopoietic lineages[16-22], and recent studies of transplant and injury models have shown that at least some adipocytes are derived from circulating cells of hematopoietic origin[3, 4]. However, *Tie2-Cre* labels nearly all CD45+ cells in WAT, yet does not label mature adipocytes. Similarly, Vav 1 oncogene (*Vav1*)-*Cre*, another marker of hematopoietic lineages[14, 23], fails to label adipocytes despite near complete label of CD45+ cells in WAT (Fig.1a,c, Supplemental Fig. 1a,b). These data indicate that adipocytes are not normally derived from hematopoietic lineages.

To determine if endothelial or hematopoietic lineages contribute mature adipocytes in the context of obesity we placed *Cdh5-Cre* and *Vav1-Cre mT/mG* reporter mice on a high fat diet (HFD). Following 10 weeks of HFD, no GFP positive adipocytes were observed in the *Cdh5-Cre* WAT, while adipocyte-like GFP positive structures are present in *Vav1-Cre* WAT (Supplemental Fig. 3). However, all of these GFP positive structures are multi-nucleated and stain positive for macrophage markers F4/80, CD11b, and CD45 (Supplemental Fig. 3a-d) indicating that the GFP fluorescence is not derived from adipocyte membranes but from macrophages forming crown like structures[24]. These data indicate that hematopoietic and endothelial lineage cells do not contribute to mature adipocyte formation in HFD-induced obesity.

Previous studies demonstrated that PdgfRα is expressed in adipogenic cells from skeletal muscle[11, 12]. In addition, PdgfRα is expressed in WAT-resident cells that produce brown-like adipocytes in response to beta-adrenergic stimulation and white adipocytes upon HFD feeding[25]. Therefore, we determined if *PdgfRα* labels mature adipocytes during normal formation of WAT. *PdgfRα-Cre* labels all mature adipocytes in all major WAT depots, as indicated by GFP+ adipocyte membranes (Fig. 1a, Supplemental Fig. 1a). As *PdgfRα* is not expressed in mature adipocytes[25] (Fig. 1d), the GFP expression observed in adipocytes of *PdgfRα-Cre:mT/mG* mice is due to lineage tracing.

We next examined the labeling of SVF cells in *PdgfRα-Cre* mice to identify potential adipocyte precursors. The SVF of *PdgfRα-Cre:mT/mG* mice contains a low percentage of GFP+ cells in CD31+ and CD45+ populations. In contrast, both the CD24+ and CD24− cells are nearly completely traced by *PdgfRα-Cre* (Fig. 1c, Supplemental Fig. 2b) and PdgfRα is expressed in these cell populations (Fig. 1d-f, Supplemental Fig. 2b). This data suggests that both the CD24+ and the CD24− populations are part of the *in vivo* adipocyte cellular lineage.

Therefore, we next examined the relationship between the CD24+ and CD24− cells in vivo. During development of subcutaneous WAT (SWAT), lipid filling of differentiating adipocytes is not observed until birth[26]. Flow cytometry analysis of embryonic SWAT (e15.5-e18.5) shows there is a large population of cells with the cell surface marker profile of CD24+ adipogenic precursors (Fig 2a,b). These embryonic CD24+ cells are 98.62 ± 1.08% PdgfRα+ and are adipogenic in cell culture (Fig 2c), suggesting that they are

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adipocyte progenitors. Prior to birth there is no discernable CD24− population (Fig 2a). Upon birth (P0) there is a shift in the population profiles, with the appearance of a large population CD24− cells and a simultaneous reduction in the amount of CD24+ cells to levels similar to those observed in adult WAT (Fig. 2a). These population dynamics during development of WAT suggests that the CD24+ cells may generate the CD24− cell population.

To test this hypothesis we employed an A-Zip transplantation model similar to the assay previously used to demonstrate the capacity of CD24+ cells to reconstitute functional WAT[1]. In these assays, dTomato-labeled CD24+ cells from *mT/mG* mice were transplanted into the undeveloped WAT of A-Zip mice and injected tissues were analyzed before the appearance of mature adipocytes, 1-4 days post-injection. When dTomato+:CD24− cells are transplanted, dTomato+ cells recovered one day post-injection are nearly all CD24− with no dTomato+ cells in the CD24+ gate, demonstrating the assay accurately identifies the CD24− population (Fig. 2f, g). In contrast, when dTomato+:CD24+ cells are transplanted, 51.7% of dTomato+ cells are CD24− after one day, while four days post-injection 91.4% of dTomato + cells are CD24− (Fig. 2d, e). The increasing appearance of CD24− donor cells over time after transplantation of CD24+ adipocyte progenitors indicates that the CD24+ adipocyte progenitors generate the CD24− adipogenic cell population.

The finding that the CD24− cell population is derived from the CD24+ adipocyte progenitors, coupled with the reduced ability of the CD24− population to generate functional adipose tissue in vivo[1], suggests that the CD24− cell population contains cells that are further differentiated towards mature adipocytes. Thus, we performed gene expression analysis on sorted cells to investigate the relative state of differentiation in these adipocyte precursor populations. Real-time PCR shows that CD24 mRNA is enriched in the CD24+ population, validating the sorting of these populations (Fig. 3a,b). *CD24* is also highly expressed in unfractionated SVF as *CD24* is expressed in several other cell types in SVF, such as B lymphocytes[27], and only 0.61% ± 0.12 of SVF cells that express CD24 display the complete cell surface marker profile of adipocyte progenitors. The late adipogenic markers *Pparγ2* and *C/ebpα* are enriched in the CD24− population while markers of mature adipocytes, *Adiponectin* and *Perilipin*, are only expressed in isolated mature adipocytes (Fig 3c).

The enrichment of late adipogenic markers in CD24− cells suggests that this population may contain cells that are committed to an adipogenic fate. Thus, we tested the adipogenic capacity of the CD24+ and CD24− cells in a previously developed in vivo adipocyte commitment assay[28, 29]. PdgfRα+ populations (Fig. 4a, Supplemental Fig. 2b) were sorted from *leptin-luciferase* mice[30] and injected subcutaneously over the sternum of wild type mice, a region where adipose tissue does not normally form. While the PdgfRα+:CD24+ adipocyte progenitors and PdgfRα+:Lin+ cells did not produce luciferase signal, the transplanted PdgfRα+CD24− cells produced significant luciferase signal after 6 weeks, indicative of the formation of mature adipocytes (Fig. 4b, c).

Despite the inability of the CD24+ adipocyte progenitors to form mature adipocytes outside of the WAT microenvironment (Fig. 4b, c), previous work has shown that this cell

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population is capable of reconstituting a functional WAT depot that rescues hyperglycemia when transplanted into the residual perigonadal WAT (GWAT) of A-Zip mice[1]. The same study also showed that the CD24− cell population has limited adipogenic capacity in this WAT microenvironment. To more directly assess the capacity of CD24− cells to form adipocytes, we performed sternum injections of PdgfRα+:CD24− cells from *Adiponectin-Cre:mT/mG* mice. These injections resulted in the formation of lipid filled, *Adiponectin* expressing mature adipocytes (Fig. 4d,e). However, the adipose tissue formed by differentiation of these PdgfRα+:CD24− cells is several fold smaller than the tissue observed in GWAT reconstitution by CD24+ adipocyte progenitors[1] in A-Zip mice (Fig. 4d, e). In addition, the adipose tissue resulting from the injection of PdgfRα+:CD24− cells above the sternum of A-Zip mice does not rescue their hyperglycemia (Fig. 4f). The ability of the PdgfRα+:CD24− population to form small amounts of adipose tissue both within and outside of the WAT microenvironment indicates that this population harbors cells that are further to committed to adipogenesis yet have limited adipogenic capacity as compared to CD24+ adipocyte progenitors.

Taken together our data characterize a previously unappreciated cellular lineage of adipocytes within WAT. Our recent prospective studies identified the CD24+ population as a rare cell type in WAT that is capable of forming a functional WAT depot in vivo[1]. We show here that these CD24+ adipocyte progenitors are indeed a component of the adipocyte lineage in vivo, becoming further committed to the adipocyte lineage as they lose CD24 expression, forming a CD24− preadipocyte population in the course of differentiating into mature adipocytes in vivo (Fig. 4g).

Our data also indicate that adipocytes are derived from *PdgfR*α expressing cells during the normal establishment of WAT. Previous data has demonstrated that adipocytes formed in response to β-Adrenergic stimulation and HFD are also derived from PdgfRα+ cells that share other markers with the preadipocytes described here, including Sca-1 and CD34[25], although this previous study did not investigate the role of the CD24+ adipocyte progenitors. Using Pdgfrα as a single marker of adipocyte precursors, this previous study found that Pdgfrα+ cells are associated with the vasculature[25]. However, we have shown that the Pdgfrα+ population also contains non- adipogenic cell populations (Fig. 4b, c). Therefore, Pdgfrα expression alone cannot definitively identify adipocyte progenitors within WAT. Similarly, CD24 cannot be used as a sole marker of adipocyte progenitors as only 0.61% of CD24+ cells within WAT are adipocyte progenitors (Supplemental Fig. 4a, b). Additionally, only 17.42% of Pdgfrα+;CD24+ cells within WAT are adipocyte progenitors (Supplemental Fig. 4a, b). These Pdgfrα+;CD24+ cells are dispersed throughout WAT and are not preferentially localized to vasculature (Supplemental Fig. 4c), indicating that precise localization of adipocyte progenitors requires more exclusive markers.

While many factors are known to affect adipocyte formation in vitro, the precise role of these factors in adipocyte dynamics in vivo remains uncharacterized. For example, the expression of Pdgfrα on adipocyte precursors suggests that PDGF signaling plays a role in WAT mass regulation *in vivo*. However, previous studies of the role of PDGF in adipogenesis in vitro are conflicting, with PDGF signaling either inducing[31] or inhibiting[32] adipogenesis. The identification of the adipocyte cellular lineage in vivo and the

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characterization of *PdgfR*α*-Cre* mice as a model for genetic manipulation of adipocyte precursors will now permit detailed studies of the cellular and molecular mechanisms that regulate adipogenesis and WAT mass in vivo under various conditions, including the establishment of the adipose tissue 'set point'[33] during development and the expansion of WAT mass in obesity. Determining the mechanisms involved in these different states of WAT mass regulation may lead to the development of novel therapeutic strategies for controlling WAT mass.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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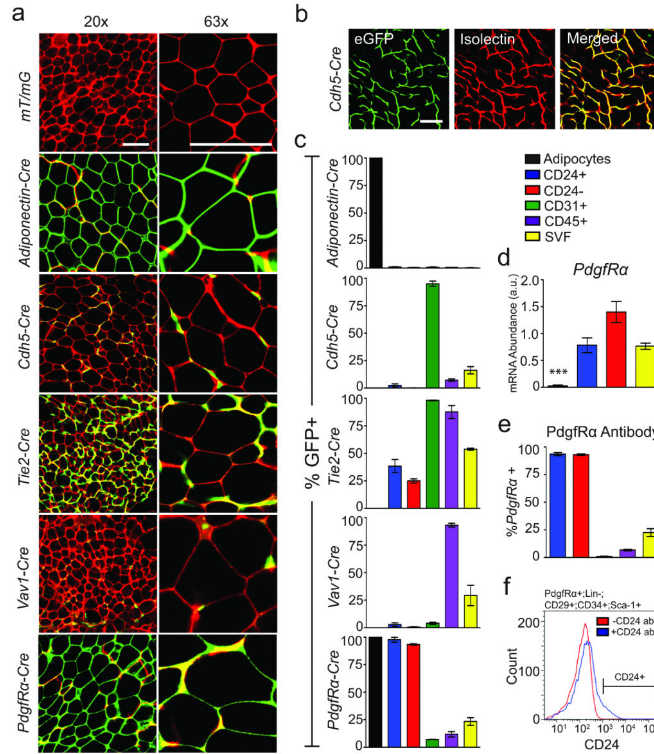

Figure 1.

Adipocytes are derived from PdgfRα+ precursor cells in subcutaneous WAT. (**a**) Confocal images of whole-mounted SWAT from indicated 4-week old Cre:*mT/mG* male mice (red: membrane-targeted dTomato; green: membrane-targeted eGFP, indicating Cre excision of dTomato). (**b**) Confocal images of membrane targeted eGFP and Isolectin GS-IB4 Alexa Fluor 647 staining endothelial cells of *Cdh5-Cre:mT/mG* SWAT. (**c**) Quantification of flow cytometry analysis of SVF populations from indicated 4-week old Cre:*mT/mG* mice (n=3). (**d**) Quantification of qPCR analysis of *PdgfRα* in mature adipocytes and FACS sorted SVF, Lin−:CD29+:CD34+:Sca-1+:CD24+ (CD24+) and Lin−:CD29+:CD34+:Sca-1+:CD24− (CD24−) cell populations (n=5 RNA extractions from independently isolated cell samples, *** $p<0.001$). (**e**) Quantification of flow cytometry analysis of anti-PdgfRα-PE antibody staining in indicated cell populations from 6-week old male C57BL/6 SWAT (n=3 SWAT SVF preparations). (**f**) A histogram of the distribution of CD24 staining in PdgfRα+:Lin −:CD29+:CD34+:Sca-1+ cells from (e). All error bars represent S.E.M. All scale bars represent 100 μm.

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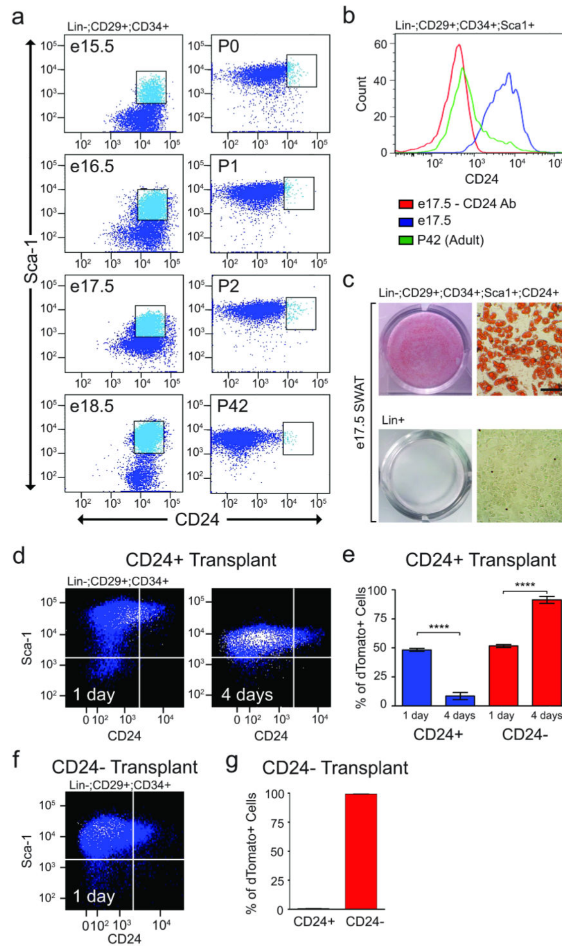

Figure 2.

CD24+ adipocyte progenitors give rise to CD24− cells in vivo. (**a**) Flow cytometry plots of SVF from a time course SWAT development in C57BL/6 mice at the indicated embryonic (e) and post-natal (P) days. Dot plots show Lin−:CD29+:CD34+ cells. (**b**) CD24 staining in Lin−:CD29+:CD34+:Sca1+ cells from e17.5 and P42 SWAT. (**c**) Oil Red O staining of differentiated FACS sorted cell populations from e17.5 SWAT. Scale bar represents 100 μm. (**d**) Representative images of flow cytometry plots of SWAT Lin−:CD29+:CD34+ cells from 6-week old *Rag2-/-;IL2Rγ-/-;A-Zip* mice at indicated days post transplantation of 100,000 FACS-isolated dTomato+, Lin−:CD29+:CD34+:Sca-1+:CD24+ (CD24+) cells from 6-week old *mT/mG* mice. Plots show overlay of dTomato+ transplanted cells (white) on *Rag2-/-;IL2Rγ-/-;A-Zip* recipient SVF cells (blue). (**e**) Quantification of flow cytometry analysis of dTomato+ CD24+ transplantations into *Rag2-/-;IL2Rγ-/-;A-Zip* mice (n=4 transplantations, **** $p<0.0001$, error bar represent S.E.M.). (**f**). Representative flow cytometry plot of SWAT Lin−:CD29+:CD34+ cells from 6-week old *Rag2-/-;IL2Rγ-/-;A-Zip* mice at 1 day post transplantation of 100,000 FACS isolated dTomato+, CD24− cells from 6-week old *mT/mG* mice. The dot plot shows overlay of dTomato+ transplanted cells (white) on *Rag2-/-;IL2Rγ-/-;A-Zip* recipient SVF cells (blue). (**g**) Quantification of flow cytometry analysis of dTomato+ CD24− transplantations into *Rag2-/- ;IL2Rγ-/-;A-Zip* mice (n=3 transplants, error bars represent S.E.M.).

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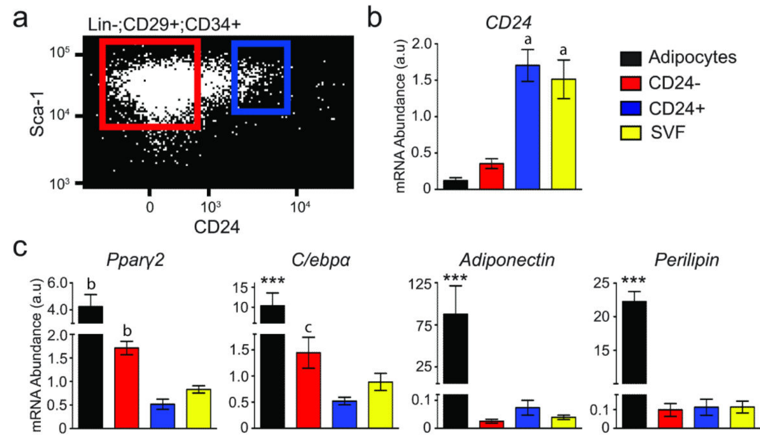

Figure 3.

CD24− cells express late adipogenic genes. (**a**) A representative dot plot is shown with boxes representing the sort parameters for the CD24+ (blue) and CD24− (red) populations. (**b,c**) qPCR of CD24 expression, late adipogenic and mature adipocyte specific genes in purified mature adipocytes and FACS isolated cell populations (n=5 RNA extractions from independently isolated cell samples; *** $p<0.001$ vs. all other populations; [a] $p<0.001$ vs. CD24− and adipocytes (Adi); [b] $p<0.001$ vs. CD24+ and SVF; [c] $p<0.001$ vs. Adi and $p<0.05$ vs. CD24+; error bars represent S.E.M.).

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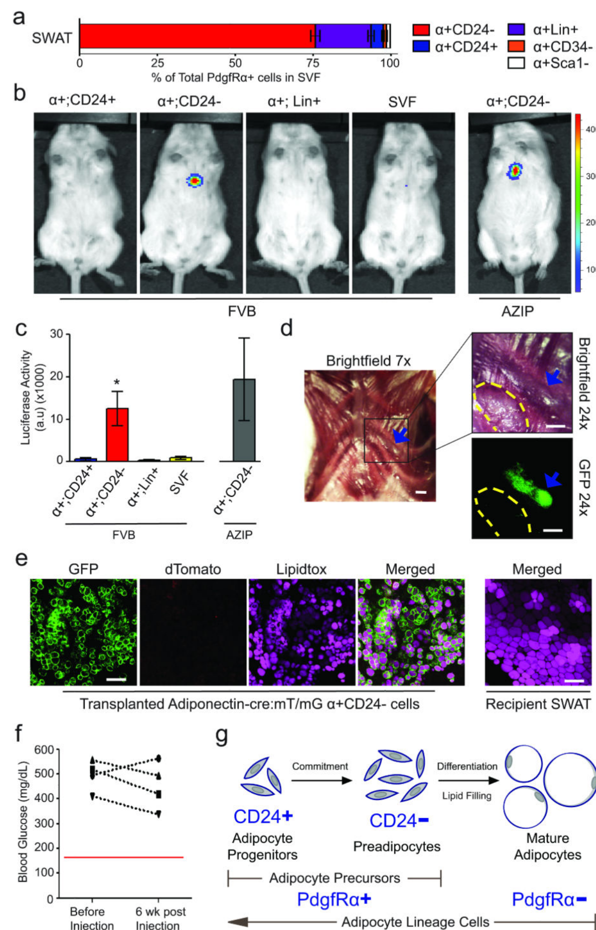

Figure 4.

CD24– cells are further committed to an adipogenic fate. (**a**) Breakdown of PdgfRα+ (α+) SVF into subpopulations. Flow cytometry plots and percentages are shown in Supplemental Figure 2b. α+:CD24–, α+:CD24+, and α+:Sca1– are Lin–:CD29+:CD34+. α+:CD34– are Lin–:CD29+. (**b**) Luminescence in 12-week old FVBN/J or A-Zip mice at 6 weeks post subcutaneous sternum transplantation of indicated FACS isolated populations from 6-week old *leptin-luciferase* BAC transgenic mice. 50,000 cells were transplanted per experiment. (**c**) Quantification of luminescence from (b) (n=5 for α+:CD24+ and α+:Lin+ transplants into FVB; n=10 for α+:CD24– and SVF transplants into FVB; n=4 for α+:CD24– transplants into AZIP. *p<.05, error bars represent S.E.M.). (**d**) Brightfield and fluorescent images of tissue formed 6 weeks post subcutaneous sternum transplantation of 2×10^5 *Adiponectin-Cre:mT/mG* α+:CD24– cells. Blue arrow indicates tissue formed from transplanted cells. Yellow dashes outline a small piece of recipient SWAT placed on top of sternum as a negative imaging control. Scale bars represent 1 mm. (**e**) Representative confocal images of Lipidtox stained tissues from (**d**). Scale bars represent 100 μm. (**f**) Blood glucose measurement of A-Zip mice before and 6 weeks post subcutaneous sternum transplantation of 50,000 α+CD24– as shown in (**b**) and (**c**). (**g**) A model of in vivo adipogenesis.