

## REVIEW

# Sorting out adipocyte precursors and their role in physiology and disease

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**The ability to maintain and expand the pool of adipocytes in adults is integral to the regulation of energy balance, tissue/stem cell homeostasis, and disease pathogenesis. For decades, our knowledge of adipocyte precursors has relied on cellular models. The identity of native adipocyte precursors has remained unclear. Recent studies have identified distinct adipocyte precursor populations that are physiologically regulated and contribute to the development, maintenance, and expansion of adipocyte pools in mice. With new tools available, the properties of adipocyte precursors can now be defined, and the regulation and function of adipose plasticity in development and physiology can be explored.**

Adipocytes (“fat cells”) are critical regulators of several aspects of normal physiology, including energy balance, nutrient homeostasis, and tissue homeostasis (Rosen and Spiegelman 2014). White adipocytes represent the principle site for energy storage in mammals. These cells accumulate excess energy in the form of intracellular triglycerides packaged into large single lipid droplets. White adipocytes are also endocrine cells; adipocytes secrete numerous hormones and cytokines that impact several aspects of physiology, including nutrient homeostasis, inflammation, and reproductive biology (Ouchi et al. 2011). Classical brown (referred to here as “brown”) and beige/BRITE (referred to here as “beige”) adipocytes comprise a second major class of adipocytes that function to convert chemical energy into heat. They are characterized by their abundance of mitochondria, multilocular fat droplet appearance, and specific expression of uncoupling protein 1 (UCP1) (Rosen and Spiegelman 2014). Brown adipose tissue (BAT) likely evolved to help defend animals against the cold. Historically, BAT has been referred to as the “hibernating organ” due to its function in maintaining body temperature in hibernating animals and newborns. Beyond its role in regulating thermogenesis, brown and beige adipocytes have a major role in regulating

glucose and lipid homeostasis (Seale et al. 2011; Kajimura et al. 2015; Shao et al. 2016).

Interest in adipocyte biology has increased dramatically over the last 20 years, coincident with the global obesity epidemic and the realization that adipocytes play a broader role in physiology than envisioned previously (Rosen and Spiegelman 2014). The field has witnessed great progress in revealing the many functions of adipocytes in physiology; however, the basic developmental biology of adipose tissue has lagged behind. Below, we highlight recent efforts to identify native adipose precursor populations that contribute to adipose development and the physiological recruitment of white, brown, and beige adipocytes in postnatal animals. We focus primarily on recent insight gained from mouse studies and discuss new tools that will help the field move forward. Furthermore, we summarize new data that reveal the complexities of adipose tissue development and expansion *in vivo*.

## Development of white adipocytes and their precursors

### *Anatomical distribution of white adipose depots and fetal origins*

Some of the earliest studies of adipocyte development dated back to the late 19th century. Notable anatomists such as Toldt and Flemming expressed seemingly opposing views of the origin of adipocytes (discussed by Wasserman 1965, republished in Wasserman 2011). Flemming argued that adipocytes originated from loose connective tissue and that nearly all fibroblast-like cells had the capacity to become lipid-laden cells. As such, Flemming implied that adipocytes were not truly distinct cells worth considerable observation. Toldt and, later, Wasserman believed that adipose tissue, like other tissues and organs, followed a developmental pattern in which fat cells emerge from specific precursor cells at select time points. In particular, seminal work from Wasserman (1926) and others illustrated that adipocytes first emerge in conjunction with dense

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networks of blood vessels, forming what was called the “primitive organ.” In recent years, a number of studies have used modern molecular/genetic approaches to demonstrate the dependence of adipocytes on angiogenesis for tissue development (Han et al. 2011; Hong et al. 2015). In fact, some studies have suggested a possible endothelial origin of adipocytes; however, reported lineage tracing studies are conflicting (Tran et al. 2012; Berry and Rodeheffer 2013).

White adipocytes are found throughout the body; however, they are generally organized into distinct tissues or “depots.” Today, it is clear that adipose tissues emerge and develop in a specific spatiotemporal manner. Adipose depots are broadly classified as “subcutaneous” or “visceral,” reflecting their general anatomical localization; however, each individual subcutaneous or visceral depot develops at different fetal stages. In humans, nascent adipose tissue first appears in the second trimester of gestation as “primitive fat lobules” that accumulate first in the head and neck, then in the trunk, and later in the limbs (Poissonnet et al. 1984). Likewise, murine white adipose depots are specified and develop at various stages of life (Han et al. 2011; Jiang et al. 2014). Subcutaneous inguinal adipocytes emerge perinatally; most intra-abdominal depots take form after birth (Wang et al. 2013).

The precise developmental origin of white adipose tissue (WAT) during fetal development is still unclear; however, lineage tracing in mice indicates that subcutaneous and intra-abdominal depots emanate from distinct mesenchymal lineages (Chau et al. 2014). Adipocytes in visceral, but not subcutaneous or brown, adipose depots descend from cells expressing the mesothelial cell marker Wilms tumor 1 (WT1). These data suggest the lateral plate mesoderm as the fetal origin of visceral adipose tissues. However, the precise origin of adipose tissue may vary from depot to depot. For instance, in mice, craniofacial, but not peripheral, subcutaneous WAT depots originate from neuroectoderm rather than mesodermal structures (Billon and Dani 2012). Adding to the complexity, individual depots may contain adipocytes that arise through distinct lineages (Sanchez-Gurmaches and Guertin 2014). Molecular and cellular studies indicate that anatomically distinct depots are also functionally distinct and express differing molecular programs (Macotela et al. 2012; Wu et al. 2012; Lee et al. 2013; Morgan-Bathke et al. 2015). As a result, the simple designation of white adipocytes as “subcutaneous” or “visceral” is likely too simplistic. Each anatomically distinct depot may actually represent a developmentally and functionally distinct form of adipose tissue.

#### *Maintenance and expansion of WAT in adults*

During the first year of life, adipose tissue continues to expand considerably through cellular hypertrophy and the recruitment of new adipocytes (cellular hyperplasia) (Knittle et al. 1979). In adulthood, adipocyte turnover occurs at a rate of ~10% per year (Spalding et al. 2008). In rodents, the degree of turnover may be higher, varying from depot to depot (Wang et al. 2013; Rivera-Gonzalez et al. 2016). A remarkable and unique property of adipose tissue

is the tremendous capacity to expand or contract in different physiological settings. The capacity to expand is most evident in the setting of obesity, where adipose tissue mass can reach upward of 60% of body weight in the morbidly obese (Ortega et al. 2010). The ability of adipose tissue to shrink and then re-expand is perhaps best highlighted by the response of mammary adipose tissue to pregnancy and lactation. During pregnancy and the lactation period, adipocytes are replaced by milk-producing alveolar structures and then reappear as the alveolar structures involute at weaning (Cinti 2009).

The manner by which adipose tissue expands is of great clinical significance (for review, see Hepler and Gupta 2016). Preferential expansion of subcutaneous depots is associated with protection against cardiometabolic disease in obesity (Lee et al. 2013; Karpe and Pinnick 2015). Preferential expansion of visceral depots correlates well with insulin resistance and diabetes. Moreover, expansion of adipose tissue mass through adipocyte hyperplasia is more metabolically favorable than through an increase in cell size (hypertrophy) (Lee et al. 2010). Hyperplasia occurs through the recruitment of adipocyte precursors that undergo adipogenesis and is associated with adipose health and a delayed onset of insulin resistance. As such, *de novo* adipocyte differentiation in adults appears critical for the maintenance and expansion of adipose depots (Gustafson et al. 2009).

Lineage tracing studies have revealed that the mode of adipose depot expansion in mice fed a high-fat diet occurs in a depot- and sex-dependent manner (Wang et al. 2013; Kim et al. 2014; Jeffery et al. 2015). Epididymal adipose expansion in adult male mice fed a high-fat diet occurs through both adipocyte hypertrophy and hyperplasia, while the inguinal (subcutaneous) depot in these same animals expands almost exclusively through cellular hypertrophy (Wang et al. 2013). In female mice, adipocyte hyperplasia occurs in both the perigonadal WAT and, to some variable extent, the inguinal WAT (Jeffery et al. 2016). A better understanding of how adipose precursors are activated in obesity may lead to therapeutic strategies to alter body fat distribution and/or adipose tissue health.

In recent years, it has become increasingly clear that adipocyte accumulation in areas beyond the major depots plays an important role in physiology and pathology (Shook et al. 2016). Adipocytes accumulate in the skin below the dermis (dermal WAT, referred to here as “dWAT”) and play a critical role in dermal homeostasis (Alexander et al. 2015; Kruglikov and Scherer 2016). This relatively unexplored depot expands in response to cold exposure, wounding, and bacterial infection. Adipose lineage cells in the skin also respond to hair follicle cycling and regulate follicle homeostasis (Festa et al. 2011). Marrow adipose tissue (MAT), located in various skeletal regions, appears to contribute to local skeletal remodeling, hematopoietic regulation, and systemic metabolism (Scheller et al. 2016). Marrow adipocytes are a developmentally distinct white adipocyte population. MAT, but not other WAT depots, is derived from cells once expressing *Osx1* (Berry et al. 2015). MAT expansion occurs in response to various pharmacological or physiological triggers, such

as glucocorticoids, anti-diabetic thiazolidinediones, estrogen deficiency, obesity, and aging. Likewise, adipocytes also accumulate in the skeletal muscle at sites of injury (Joe et al. 2010; Uezumi et al. 2010). The accumulation of adipocytes in skeletal muscle can impact the regenerative capacity and function of muscle. Similarly, a defining feature of arrhythmogenic cardiomyopathy is the progressive replacement of the myocardium with fibrous tissue and adipocytes (Samanta et al. 2016). The developmental origin and exact properties and function of these lesser-studied adipocytes are still being unraveled; however, it is clear that the ability of new adipocytes to emerge in these tissues is critical for tissue homeostasis and perhaps systemic metabolism.

#### *White adipocyte precursors: in vitro models*

Much of our knowledge of the cellular aspects of adipocyte differentiation emanate from the use of cell culture models. Mouse embryonic fibroblasts (MEFs) from mid-stage to late stage embryos can be readily induced to undergo adipogenesis in response to a hormonal/pharmacological cocktail consisting of dexamethasone, iso-butyl-methyl-xanthine, and insulin. This observation lends credence to the views of Flemming that adipocytes can and readily do emerge from ubiquitous fibroblast-like cells. Similarly, adherent fibroblast-like cells within cultures of the adipose stromal vascular fraction (SVF) have been propagated and differentiated into mature adipocytes in vitro (Poznanski et al. 1973; Van et al. 1976; Van Robin and Roncari 1977). Other mesenchymal lineages, such as osteoblasts, myoblasts, and chondrocytes, can also differentiate from cultures of the adherent adipose SVF (Cawthorn et al. 2012). As such, the adherent SVF from adipose tissue is often described as “adipose stem cells” or “mesenchymal stem cells” (Cawthorn et al. 2012). Importantly, cultured MEFs or SVF cells are heterogeneous; whether these cultures contain cells that truly reflect native adipocyte precursors is not certain.

In the 1970s, Green and Kehinde (1974) derived 3T3-L1 cells as a clonal subline of 3T3 immortalized MEFs from Swiss mice. 3T3-L1 cells are viewed as being a “determined” preadipocyte cell line; the cells are morphologically indistinguishable from less adipogenic or nonadipogenic fibroblasts but are highly committed to the adipocyte lineage. The isolation of such committed cells implied that preadipocytes were specialized fibroblasts with a stably unique molecular program. Preadipocyte cell lines have been powerful models for the study of adipocyte determination and differentiation. *Ppar $\gamma$* , the “master regulator” of adipocyte differentiation, was originally discovered through the use of 3T3 cells (Chawla and Lazar 1994; Tontonoz et al. 1994). Today, there are dozens of transcriptional regulators implicated in the process of 3T3-L1 adipocyte differentiation. Excellent reviews have been written on this matter (Farmer 2006; Cristancho and Lazar 2011); however, it is now clear that anatomically distinct adipocytes represent, at least to some degree, distinct lineages. As such, whether cultured preadipocyte cell lines represent any particular

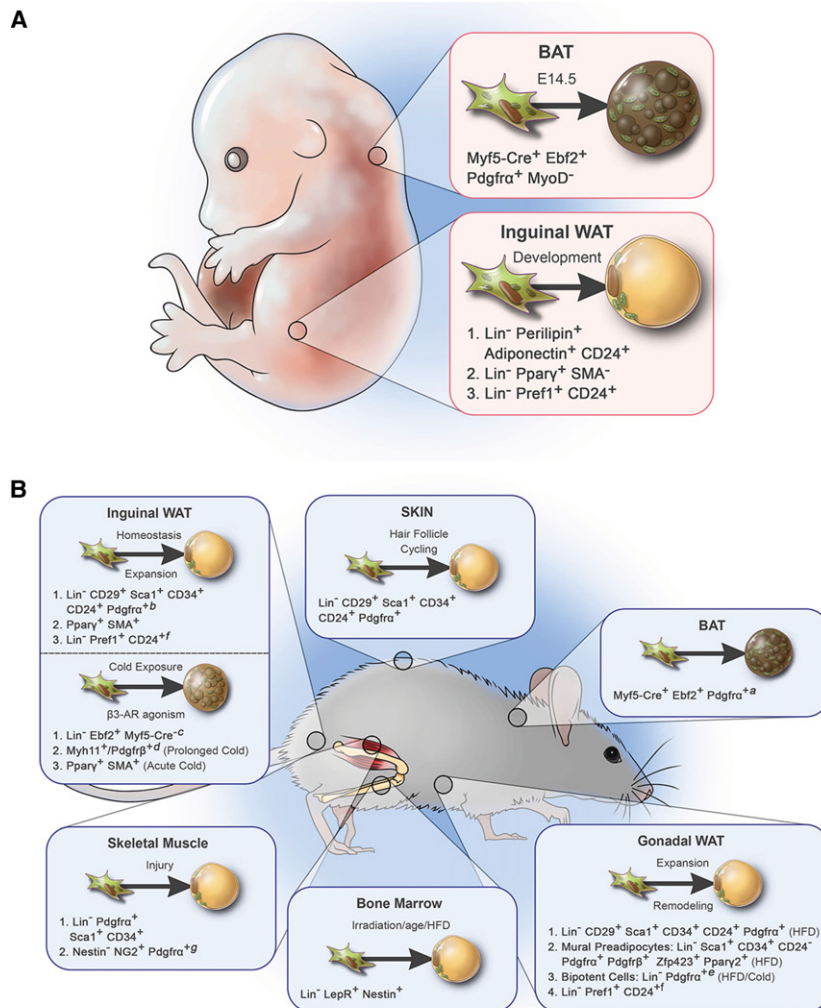
type of native adipocyte precursor remains unclear. Here, we summarize recent efforts to identify and characterize native adipose precursors, highlighting their regulation and potential contribution to the adipose lineage and physiology (summarized in Fig. 1).

#### *Native adipocyte precursors: identity, regulation, and function*

***CD24<sup>+</sup> and CD24<sup>-</sup> adipocyte progenitor cells*** Several years ago, Friedman and colleagues (Rodeheffer et al. 2008) developed a strategy to prospectively purify adipocyte progenitor cells (APCs) from freshly isolated murine adipose SVFs. These investigators combined antibodies raised against combinations of accepted stem cell surface proteins with fluorescent-activated cell sorting (FACS) to isolate and characterize two functionally distinct APC subpopulations. Both populations are devoid of endothelial and hematopoietic markers (CD31 and CD45, respectively) and positive for common mesenchymal stem cell markers *Sca1*, *CD34*, and *CD29* but distinguishable by the expression of *CD24*. The *CD24<sup>+</sup>* APCs represent a highly proliferative and stem cell-like primitive adipose progenitor population, whereas *CD24<sup>-</sup>* APCs appear to represent a more committed “preadipocyte” population that expresses lineage-selective genes such as *Ppar $\gamma$ 2* and *C/ebpa*. *CD24<sup>+</sup>* APCs give rise to *CD24<sup>-</sup>* APCs in vivo during development, en route to adipocyte differentiation (Berry and Rodeheffer 2013). These data reveal the existence of hierarchical populations of adipose precursors exhibiting different levels of commitment.

In adult animals, *CD24<sup>+</sup>* APCs are relatively rare in comparison with the more committed *CD24<sup>-</sup>* APC population; however, the *CD24<sup>+</sup>* population appears highly regulated by a number of physiological settings. The frequency of these cells is relatively higher in lipodystrophic animals (Rodeheffer et al. 2008). In addition, *CD24<sup>+</sup>* APCs are regulated by high-fat diet feeding in a sex- and depot-dependent manner (Jeffery et al. 2015, 2016). *CD24<sup>+</sup>* APCs undergo rapid and transient proliferation within days of high-fat diet feeding, selectively in depots exhibiting a hyperplastic response. Importantly, these data suggest that adipocyte precursor proliferation is triggered by diet rather than obesity per se. Transplantation studies indicate that the abilities of precursors to proliferate and undergo differentiation upon high-fat diet feeding are largely regulated by the local microenvironment (Jeffery et al. 2016). The notion that depot-specific signals control local adipogenesis is highlighted by recent studies of dWAT, where *CD24<sup>+</sup>* APCs are present and undergo proliferation and differentiation in association with hair follicle cycling (Rivera-Gonzalez et al. 2016). Maintenance of the *CD24<sup>+</sup>* APC pool in dWAT, but not in gonadal or inguinal WAT, is dependent on local *Pdgfa* (platelet-derived growth factor  $\alpha$ ) signaling. Moreover, hair follicle transit-amplifying cells regulate proliferation of *CD24<sup>+</sup>* APCs and dermal adipogenesis through secretion of sonic hedgehog (Zhang et al. 2016).

The FACS-based method to isolate *CD24<sup>+</sup>* and *CD24<sup>-</sup>* APCs provides a convenient approach to purify and study

**Footnotes:**

- Contribution to maintenance and expansion of BAT in adults is unclear.
- Cells contribute to adipocyte hyperplasia in females but not in males undergoing high fat diet feeding.
- Highly committed to beige lineage upon cold exposure; however, lineage tracing not yet reported. Localization in WAT is unknown.
- Lineage tracing indicates beige cells arise from cells expressing these markers after 2 weeks of cold exposure; exact beige precursor subpopulation has not been purified.
- Gonadal Pdgfra<sup>+</sup> cells are bipotent *in vitro*, giving rise to both white and UCP1<sup>+</sup> adipocytes.
- Adipose expansion upon HFD depends on these cells; however, lineage tracing not reported.
- Two types of pericytes have been characterized in the injured skeletal muscle using transgenic reporter mice: *Nestin-GFP/NG2-DsRed*<sup>+</sup> (type-1) and *Nestin-GFP/NG2-DsRed*<sup>+</sup> (type-2). Type 1 pericytes express Pdgfra. Type-2 pericytes participate in muscle regeneration, while type-1 contribute to fat accumulation.

**Figure 1.** Adipocyte precursor heterogeneity in mice. (A) Characterized fetal adipose precursor populations: Three adipose progenitor populations have been characterized in the developing inguinal WAT depot. The degree of overlap in these populations is unclear. Seale and colleagues (Wang et al. 2014) have identified a brown adipocyte progenitor population in BAT that is marked by myogenic factor 5 (*Myf5*)-Cre and expresses platelet-derived growth factor  $\alpha$  (Pdgfra) and Ebf2. These cells emerge at embryonic day 10.5 (E10.5) and are devoid of MyoD at this stage, suggesting that brown adipocyte lineage commitment has already occurred. (B) Tissue-resident adipose precursors in adult mice: Adipose precursor populations have been identified in multiple tissues and are listed as they were identified and characterized. There is likely a degree of overlap in the populations. Additional populations may exist based on published lineage tracing results.

adipose precursors from various rodent models with easily obtainable commercial reagents. One limitation to the approach lies in the inability to visualize these cells *in vivo*

in their natural environment; multiple cell surface markers are needed to detect these specific cells. As such, it is still unclear where CD24<sup>+</sup> APCs reside and are regulated

within the adipose depots. Recently reported BrdU-based “pulse-chase” lineage tracing experiments indicate a contribution of CD24<sup>+</sup> APCs to adipocyte hyperplasia in obesity (Jeffery et al. 2015, 2016); however, it remains unclear to what extent adipocyte differentiation originates from the CD24<sup>+</sup> versus CD24<sup>-</sup> APC population. As pointed out by Jeffery et al. (2016), most newly formed adipocytes accumulating following the onset high-fat diet feeding appear to originate from nonproliferating APCs. More precise lineage tracing and manipulation of CD24<sup>+</sup> cells is needed to better understand their requirement in adult physiology.

*Pdgfra<sup>+</sup> cells* There has long been interest in identifying muscle-resident precursors for adipocytes and fibrocytes accumulating in injured skeletal muscle. In 2010, independent studies from the laboratories of Uezumi et al. (2010) and Joe et al. (2010) reported the presence of a population of stromal cells distinct from muscle progenitors that give rise to fibroblasts and adipocytes upon chemical-induced muscle injury in mice. These cells, termed “fibroadipogenic progenitors” (FAPs), can be identified by the expression of *Pdgfra* along with *Sca1* and *CD34* and are activated to proliferate in response to injury. Interestingly, transplantation assays revealed that FAPs differentiate into adipocytes upon transplantation into injured, but not healthy, skeletal muscle. These data further highlight the importance of the microenvironment in dictating a proadipogenic response.

Granneman and colleagues (Lee et al. 2012) focused on identifying thermogenic adipocyte precursors in adipose depots that respond to  $\beta$ 3 adrenergic receptor ( $\beta$ 3AR) signaling. Proliferating precursors stimulated by  $\beta$ 3AR activation can be defined also by the expression of *Pdgfra<sup>+</sup>*; *CD34<sup>+</sup>*; *Sca1<sup>+</sup>*. These cells are bipotent in vitro—capable of differentiating into white and beige adipocytes. *Pdgfra<sup>+</sup>* progenitors reside in close proximity to capillaries but have dendritic processes that extend away from the vasculature and do not share a basement membrane with endothelial cells. Importantly, lineage tracing indicated that newly formed adipocytes emerging in response to high-fat diet feeding descend from cells expressing *Pdgfra*. In fact, nearly all adipocytes in the body reside within the *Pdgfra* lineage (i.e., targeted by *Pdgfra-Cre*), and CD24<sup>+</sup> and CD24<sup>-</sup> APCs actively express this marker (Berry and Rodeheffer 2013). As such, the isolation of *Pdgfra<sup>+</sup>*; *CD31<sup>-</sup>*; *CD45<sup>-</sup>* cells represents a convenient strategy to enrich for adipose precursors within adipose depots and other tissues.

*Adipose tissue mural cells* Another approach commonly used to locate stem/progenitor populations is to follow the expression of lineage-determining factors. Graff and colleagues (Tang et al. 2008) reasoned that the expression of *Ppar $\gamma$* , the most critical adipogenic factor, would facilitate the identification of adipose precursors. Tang et al. (2008) generated reporter mice in which the *Ppar $\gamma$*  locus drives GFP or LacZ expression. The investigators demonstrated that *Ppar $\gamma$ <sup>+</sup>* cells reside within the blood vessel walls of adult WAT and resemble mural cells (pericytes

and vascular smooth muscle cells). These cells express several mural cell markers, such as *Pdgfr $\beta$* , NG2 (neural/glia antigen 2), and SMA ( $\alpha$  smooth muscle actin). Furthermore, they uniformly express *CD34* and *Sca1*. Importantly, these cells contribute to WAT homeostasis in lean mice (chow-fed), with an estimate of 10%–20% of new adipocytes forming per month depending on the WAT depots examined (Tang et al. 2008; Jiang et al. 2014). This observation was significant, since it provided the first molecular evidence of the hypotheses put forth by Wasserman (1960) and others (Napolitano 1963; Cinti et al. 1984) decades ago that adipose progenitors reside in or near the vasculature of adipose tissue and resemble pericytes. These data provide evidence that the adipocyte precursor niche is created by the microenvironment of the vasculature.

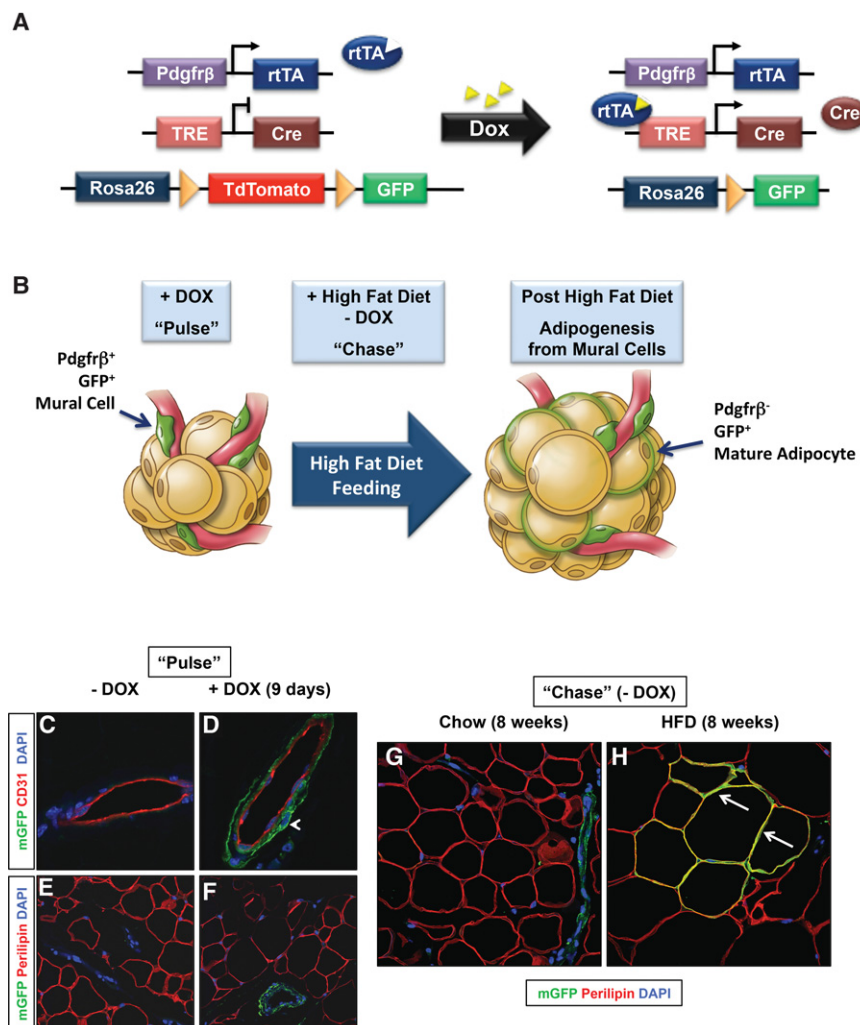
The hypothesis of a perivascular origin of adipogenesis is also supported by mural cell expression of *Zfp423* (Gupta et al. 2012; Vishvanath et al. 2016). *Zfp423* is a multi-zinc finger transcription factor that regulates preadipocyte *Ppar $\gamma$*  expression and adipocyte differentiation in vitro and in vivo (Gupta et al. 2010). Using *Zfp423* reporter mice (transgenic *Zfp423<sup>GFP</sup>* reporter mice), we localized the expression of GFP to a distinct subset of endothelial and mural cells in adipose depots (Gupta et al. 2012). Nonendothelial *Zfp423<sup>+</sup>* stromal cells express the mural cell marker *Pdgfr $\beta$*  as well as the aforementioned APC markers *Pdgfra*, *CD34*, and *Sca1*. The expression of *Ppar $\gamma$*  and other adipose lineage-selective genes is enriched in *Zfp423<sup>+</sup>* mural cells (Vishvanath et al. 2016). Furthermore, most *Zfp423<sup>+</sup>* mural cells were devoid of *CD24* expression, suggesting that they are among the committed preadipocyte population. The enrichment of *Ppar $\gamma$*  suggests that this population at least overlaps with the mural cell population examined by Graff and colleagues (Tang et al. 2008). *Zfp423<sup>+</sup>* mural cells undergo adipocyte differentiation in vitro more readily than *Zfp423<sup>-</sup>* mural cells. Importantly, the abundance of *Zfp423<sup>+</sup>* mural cells is physiologically regulated in a sex- and depot-dependent manner. In male mice, the frequency of *Zfp423<sup>+</sup>* mural cells is higher and more robustly activated by high-fat diet feeding in the gonadal depot than in the inguinal depot; this correlates well with the hyperplastic potential of the depots in obesity (Vishvanath et al. 2016). A number of questions remain unanswered about this precursor population. It is unclear whether these cells proliferate prior to undergoing differentiation or whether the number of *Zfp423<sup>+</sup>* mural cells increases by virtue of activating *Zfp423* expression in additional *Pdgfr $\beta$ <sup>+</sup>* cells. Furthermore, it remains unclear what signals activate these cells and/or *Zfp423* expression in response to high-fat diet feeding.

The identification of perivascular adipose precursors is consistent with historical observations that primitive adipocytes are associated with the vasculature. However, direct lineage tracing evidence in support of this hypothesis has been lacking. In the modern era of developmental biology, bona fide lineage tracing is best achieved by using an inducible Cre/lox recombination system for “pulse-chase” lineage tracing. In this approach, a defined cell

population is irreversibly marked by the expression of a Cre-dependent reporter (usually LacZ or fluorescent protein) at a selected time ("pulse labeling"). This is achieved by transient activation of Cre recombinase. Marked cells are then detected at later time points to determine how the originally labeled precursors contribute to specific cell types ("chase"). Recently, we and others used lineage tracing models to evaluate the contribution of mural preadipose populations to the maintenance and expansion of the adipocyte pool in adults (Jiang et al. 2014; Berry et al. 2016; Vishvanath et al. 2016). Jiang et al. (2014) used SMA-CreERT2 mice to evaluate the contribution of SMA<sup>+</sup> mural cells to the maintenance of adipose tissue mass in lean animals. Lineage tracing indicated that the adipocyte pool in adult mice is maintained at least in part through differentiation of SMA<sup>+</sup> mural APCs. In fact, these cells appear critical for maintaining adipose tissue mass; inducible deletion of *Pparγ* in these cells leads to progressive lipotrophy and a lipodystrophic-like metabolic phenotype (Jiang et al. 2014).

Our group has recently derived a novel doxycycline-inducible mural cell lineage tracking system in which the

*Pdgfrβ* promoter ultimately drives Cre expression (Fig. 2; Vishvanath et al. 2016). Using this system (termed the "MuralChaser" mouse), we asked whether *Pdgfrβ*<sup>+</sup> mural cells contribute to adipocyte hyperplasia associated with diet-induced obesity. Indeed, we identified several mural cell-derived adipocytes in visceral WAT depots formed during the high-fat diet feeding period (Vishvanath et al. 2016). In agreement with prior lineage tracing studies described above, adipocyte hyperplasia was not observed in the inguinal WAT depot of male mice (Wang et al. 2013). These data provide direct genetic evidence that perivascular preadipocytes contribute to adipocyte hyperplasia in obesity. This is in line with fate-mapping studies of perivascular *nestin*<sup>+</sup> cells in the adipose tissue (Nestin-Cre; *Rosa26R*<sup>tdTomato</sup>), revealing the presence of labeled gonadal adipocytes following high-fat diet (Iwayama et al. 2015). It is currently unclear whether the adipogenic capacity of perivascular preadipocytes cells is critical for healthy WAT expansion in the setting of obesity. Functional manipulation of adipogenic signaling pathways (e.g., *Pparγ* deletion) in *Pdgfrβ*<sup>+</sup> cells will help clarify the importance of this population.



**Figure 2.** Tracking de novo adipocyte differentiation using the MuralChaser model. (A) Genetic alleles comprising the MuralChaser lineage tracing system. In the presence of doxycycline (DOX), *rtTA* activates *Cre* expression. *Cre* excises the loxP-flanked membrane *tdTomato* cassette and allows constitutive activation of membrane GFP (*mGFP*) reporter expression. (B) "Pulse-chase" lineage tracing approach. Doxycycline-containing food was administered to MuralChaser mice for 9 d to label *Pdgfrβ*<sup>+</sup> cells ("pulse"). Animals were then switched to a high-fat diet (HFD) or chow diet for 8 wk in the absence of doxycycline (chase). *mGFP*<sup>+</sup> adipocytes represent de novo differentiated fat cells formed during the 8-wk period. (C,D) Administration of doxycycline to MuralChaser mice for 9 d resulted in doxycycline-dependent *mGFP* labeling (arrowhead) of *Pdgfrβ*<sup>+</sup> cells lining the endothelium (CD31<sup>+</sup>) of adipose tissue ("pulse"). (E,F) *mGFP* labeling was not found in mature adipocytes (perilipin<sup>+</sup>). (G,H) Following 8 wk of high-fat diet feeding, numerous *mGFP*<sup>+</sup> perilipin<sup>+</sup> adipocytes were observed (arrows). These cells represent de novo differentiated adipocytes formed during the 8-wk period. Portions of this figure were reproduced with data from Vishvanath et al. (2016), with permission from Elsevier.

*Bone marrow LepR<sup>+</sup> perivascular cells*

Adipogenesis occurs within skeletal bone marrow in response to a number of physiological and pharmacological conditions. Adipocyte accumulation is strongly associated with decreased osteogenesis; however, the signals that influence the balance between the two lineages have remained unclear. Morrison and colleagues (Zhou et al. 2014) identified a subpopulation of multipotent perivascular stem cells defined by the expression of the leptin receptor (LepR). LepR<sup>+</sup> cells emerge postnatally and represent a majority of the colony-forming cells in the bone marrow (Zhou et al. 2014). They actively express *Pdgfra*, *Pdgfrβ*, and *Nestin* and are targeted by *Prx1-Cre* mice. These cells are quiescent but proliferate robustly after injury. Lineage analysis indicates that LepR<sup>+</sup> perivascular cells are multipotent, giving rise to most adipocytes and osteoblasts accumulating after irradiation or fracture (Zhou et al. 2014). LepR<sup>+</sup> cells also give rise to marrow adipocytes accumulating with age. Importantly, LepR<sup>+</sup> cells serve as a hematopoietic stem cell niche, secreting cytokines that promote stem cell maintenance (Ding et al. 2012).

Recently, Yue et al. (2016) determined that LepR signaling itself plays an important role in the cell fate decision of LepR<sup>+</sup> cells. Leptin signaling in *Prx1* lineage cells promotes marrow adipogenesis and inhibits osteogenesis in response to high-fat diet feeding. Interestingly, loss of LepR signaling had no impact on adipogenesis induced by irradiation. This indicates that multiple mechanisms control the fate of LepR<sup>+</sup> cells in skeletal bone marrow.

*Potential circulating bone marrow-derived progenitors* It is generally assumed that adipocyte precursors reside in close proximity to the sites of adipocyte accumulation. A number of studies have now challenged this assumption, suggesting that circulating bone marrow-derived cells may differentiate into adipocytes under certain conditions (Ryden et al. 2015; Gavin et al. 2016). This hypothesis emerged largely from the work of Klemm and colleagues (Majka et al. 2010) that demonstrated the production of adipocytes from bone marrow-derived progenitor cells in the major fat depots of mice. Bone marrow progenitor (BMP)-derived adipocytes preferentially accumulated in visceral fat depots of mice in response to high-fat diet feeding or treatment with the *Pparγ* agonist rosiglitazone. This hypothesis has been elegantly explored in humans as well. Gavin et al. (2016) and Ryden et al. (2015) demonstrated the presence of donor BMP-derived adipocytes in human subjects undergoing bone marrow transplantation, contributing to up to 10% of the adipocyte population in adipose tissues. Some lineage tracing studies in mice have not supported the notion of a myeloid origin of adipocytes, at least during normal development (Berry and Rodeheffer 2013). However, it is certainly plausible that, under specific insults such as bone marrow irradiation and transplantation, these BMPs give rise to a subset of adipocytes and contribute to adipose expansion or remodeling. The isolation and characterization of these putative BMPs will be critical for the advancement of this hypothesis.

*Adipose Dlk1/Pref1<sup>+</sup> cells* Perhaps the first described “preadipocyte marker” is the preadipocyte-secreted EGF repeat-containing protein Dlk1/Pref1 (Smas and Sul 1993; Smas et al. 1997; Hudak and Sul 2013). Studies originally performed in cell lines indicated that *Dlk1* is expressed in preadipocytes, where it acts as a molecular brake on adipogenesis. Dlk1 is expressed in only the preadipocyte and not the mature adipocyte; thus, in principle, the expression of this gene can help localize APCs in vivo. Indeed, lineage tracing using *Dlk1-CreERT2* mice reveals that dermal adipocytes descend from embryonic Dlk1<sup>+</sup> cells. Hudak et al. (2014) used tetracycline-inducible systems to isolate, localize, and trace Dlk1<sup>+</sup> cells. They revealed that Dlk1<sup>+</sup> cells isolated from WAT are highly adipogenic. These cells exhibited the cell surface profile of CD24<sup>+</sup> APCs and did not express mural cell markers or *Zfp423* and *Pparγ*, suggesting that this population is a very early mesenchymal precursor that likely overlaps with the CD24<sup>+</sup> APCs identified by Friedman and colleagues (Rodeheffer et al. 2008).

Dlk1<sup>+</sup> precursors first appear in the dorsal mesenteric region around embryonic day 10.5 (E10.5) of mouse development and give rise to inguinal and gonadal WAT (Hudak et al. 2014). Ablation of fetal Dlk1<sup>+</sup> cells through *Dlk1*-driven diphtheria toxin expression leads to postnatal lipodystrophy. This study did not report Dlk1<sup>+</sup> lineage tracing results under obesogenic conditions in adult mice; however, ablation of Dlk1<sup>+</sup> cells in adult animals prevented the normal expansion of WAT depots following high-fat diet feeding. Interestingly, inguinal WAT also failed to fully expand in mice lacking Dlk1<sup>+</sup> cells. This depot expands predominantly by adipocyte hypertrophy rather than adipocyte hyperplasia. As such, whether the failure of WAT depots to expand in these animals is due to the loss of adipocyte hyperplasia or the loss of the Dlk1<sup>+</sup> cells per se is unclear. Other functions of Dlk1<sup>+</sup> precursors in WAT may exist.

*Fetal vs. adult adipose precursors*

An emerging concept in the field is the notion that the precursor populations used in the initial development of adipose tissue (“organogenesis”) are molecularly and developmentally distinct from precursor populations used in adult animals for the maintenance and expansion of adipose depots. As described above, LepR<sup>+</sup> stem cells in the bone marrow represent adult progenitors that emerge postnatally. In WAT depots of adults, *Pparγ*<sup>+</sup> precursors reside in a periendothelial position and express mural cell markers; however, fetal *Pparγ*<sup>+</sup> APCs are devoid of SMA expression and, like fetal Dlk1<sup>+</sup> cells, do not lie in a classic mural position (Tang et al. 2008; Jiang et al. 2014). Furthermore, fetal adipose precursors appear to be molecularly distinct from adult precursors. Hong et al. (2015) revealed that proliferating CD24<sup>+</sup> APCs within the fetal developing inguinal WAT depot surprisingly expressed perilipin and adiponectin. In adults, perilipin and adiponectin expression is almost exclusively confined to the mature adipocyte. Perilipin<sup>+</sup> CD24<sup>+</sup> APCs were found in proximity to the vasculature but not in direct contact with the endothelium. Collectively, these data point to a model in

which adipose precursors adopt a mural cell phenotype postnatally as they reside in their vascular niche. Interestingly, Jiang et al. (2014) provided data to suggest that adult mural precursors may be entirely distinct in origin, specified to an adipose fate even before the adipose precursors contributing to the fetal development of WAT.

Recent genetic analyses of adipogenic regulators indicate that the regulatory mechanisms required for adipose tissue formation during fetal/early postnatal development are distinct from those required in adult animals for the recruitment of new adipocytes. For example, *Akt2* is dispensable for adipose tissue development but required for  $CD24^+$  APC proliferation in postnatal animals (Jeffery et al. 2015). Moreover, *C/ebpa* is surprisingly dispensable for the maturation and maintenance of adipose tissue formed during fetal and early postnatal development (Wang et al. 2015). However, when *C/ebpa* inactivation is triggered in adipocytes of adult mice, white, but not beige, adipogenesis is impaired. These results illustrate the complexity of adipose development *in vivo* and stress the need to study adipose tissue development in various physiological contexts.

## Development of thermogenic adipocytes and their precursors

### *Development of brown adipose tissue*

In rodents, BAT depots form during fetal development prior to most other adipose depots (for review, see Wang and Seale 2016). Clusters of interscapular brown adipocytes are detectable by E14.5. The thermogenic capacity of BAT depots is fully functional by birth, providing newborns with the ability to acclimatize to the cold. In adult mice, the major BAT depots are located in the dorsal anterior region and consist of the interscapular, cervical, and axillary BAT. These depots can expand further through adipocyte hyperplasia in animals challenged to prolonged cold exposure (Lee et al. 2015b). Infant humans also have interscapular and perirenal BAT that has a molecular profile similar to that of rodent interscapular BAT (Lidell et al. 2013).

Fate-mapping studies in mice indicate that most brown adipocytes in the dorsal BAT depots are likely of paraxial mesoderm origin. Inducible lineage analyses showed that cells marked by homeobox gene *engrailed 1* (*En1*) expression at E8.5–E9.5 give rise to brown adipocytes in anterior depots (Atit et al. 2006). Similar studies indicate that brown adipocytes also descend from cells once expressing myogenic factor 5 (*Myf5*) and paired box 7 (*Pax7*), which encode two transcription factors that define myogenic precursors and regulate skeletal myogenesis (Seale et al. 2008; Lepper and Fan 2010). These data were surprising, as BAT and WAT were long believed to derive from a common “adipoblast.” The notion that BAT and skeletal muscle are closely related in development is supported by molecular and functional analyses (Timmons et al. 2007). Brown adipocyte precursor cells express many skeletal muscle-specific genes. In addition, the mitochondrial proteome of brown adipocytes is more similar to that of

muscle cells than to white adipocytes (Forner et al. 2009). Importantly, manipulation of key transcription factors and microRNAs reveals that muscle cells and brown fat cells can readily undergo a cell fate switch (for review, see Wang and Seale 2016).

### *Recruitment of beige adipocytes in adults*

It has long been recognized that WAT depots of cold-exposed rodents can undergo extensive remodeling and adopt a thermogenic phenotype elicited by the emergence of  $UCP1^+$  multilocular adipocytes (Loncar 1991). However, the aforementioned lineage tracing reveals that most  $UCP1^+$  cells within WAT depots are not derived from a  $Myf5^+$  lineage, suggesting a developmental origin largely distinct from the fetal-derived brown adipocytes (Seale et al. 2008; Sanchez-Gurmaches and Guertin 2014). Cold-induced multilocular  $UCP1^+$  adipocytes appear to be molecularly and likely functionally distinct from brown adipocytes present in brown adipose depots. Beige cells appear to exhibit properties of both white and brown adipocytes; therefore, these cells are now widely designated as “beige adipocytes.” Cells closely resembling beige adipocytes are present in adult humans, particularly in supraclavicular and paravertebral regions (Sharp et al. 2012; Shinoda et al. 2015).

A great number of pharmacological and physiological stimuli can trigger beige adipocyte accumulation in rodents and humans beyond cold exposure, including cancer cachexia, gastric bypass, exercise, and burn trauma (Kir et al. 2014; Neinast et al. 2015; Sidossis et al. 2015; Stanford et al. 2015). Great progress has been made in elucidating the transcriptional machinery and signaling cascades driving brown and beige adipocyte accumulation; however, in most cases, the actual cellular origin of the  $UCP1^+$  adipocytes accumulating under physiological conditions or in genetic models has remained unclear. Multiple lineage tracing studies now indicate that  $UCP1^+$  cells naturally appear rapidly and arise predominantly, but not exclusively, by *de novo* beige adipogenesis in response to  $\beta 3AR$  agonism or cold exposure (Wang et al. 2013; Berry et al. 2016; Shao et al. 2016; Vishvanath et al. 2016). It has also been observed that beige adipocytes have the capacity to revert to a white adipocyte phenotype; the cells lose *UCP1* expression and become unilocular when animals are returned to thermoneutral conditions following cold exposure (Rosenwald et al. 2013). These same adipocytes can then revert back to  $UCP1^+$  cells upon re-exposure to cold. This interconversion of phenotypes is often referred to as “transdifferentiation” (Barbatelli et al. 2010). Classically, transdifferentiation most often refers to a direct conversion of one differentiated cell type into another in the absence of dedifferentiation; this type of event is difficult to prove experimentally. Some have postulated that the white-to-beige phenotype switching may simply reflect beige adipocytes in active and inactive (“dormant”) thermogenic states (Kajimura et al. 2015). It should be noted that current adipocyte lineage tracing approaches do not distinguish between these two possibilities. The *adiponectin* promoter used for adipocyte targeting is expressed



in nearly all types of adipocytes. Clear molecular markers discriminating between unilocular white and unilocular dormant beige adipocytes are still needed. As such, the current data support at least two general mechanisms that lead to the natural formation of beige adipocytes during cold exposure: (1) de novo differentiation from precursors and (2) conversion of unilocular adipocytes in multilocular UCP1<sup>+</sup> adipocytes (activation of “dormant” unilocular beige adipocytes and/or transdifferentiation).

There is now tremendous interest in elucidating the cellular origin of beige adipocytes. Long et al. (2014) first revealed that beige adipocytes express a smooth muscle-like gene program akin to how classical brown adipocytes exhibit a skeletal muscle-like gene signature. Functional studies also suggest a lineage relationship between beige adipocytes and smooth muscle cells. Stromal vascular cells deficient in *Myocardin-related transcription factor A (MRTFA)* exhibit less of a smooth muscle-like phenotype and more readily undergo beige adipogenesis (McDonald et al. 2015). Multiple lines of lineage tracing evidence further suggest a smooth muscle origin of beige adipocytes (Long et al. 2014; Berry et al. 2016). Inguinal beige adipocytes rapidly emerging (within 7 d) upon cold exposure descend from cells expressing SMA. Furthermore, at least a subpopulation of beige adipocytes formed in cold-exposed mice originates from Myh11<sup>+</sup> cells (Myh11-CreERT2 lineage tracing) and mural cells expressing Pdgfr $\beta$  (MuralChaser lineage tracing). However, beige adipocytes originating from Myh11<sup>+</sup> and Pdgfr $\beta$ <sup>+</sup> cells appeared only after 2 wk of cold exposure (Berry et al. 2016; Vishvanath et al. 2016). These data suggest that multiple waves of beige adipogenesis may occur, each drawing on somewhat distinct perivascular precursor populations.

#### *Brown and beige adipocyte precursors*

Prospective approaches for isolating brown and beige adipose precursors are also starting to emerge (Wang et al. 2014). Wang et al. (2014) revealed that fetal brown adipogenic precursor activity is confined to Pdgfr $\alpha$ <sup>+</sup> cells among the *Myf5*-Cre lineage-marked cells. The committed brown preadipose fraction of this population can be further selected by the expression of *Ebf2*, a key regulator of brown adipocyte determination (Rajakumari et al. 2013). *Ebf2*-expressing cells purified from *Ebf2*-GFP reporter embryos or adult brown fat tissue from *Ebf2*-GFP reporter mice uniformly differentiate into brown adipocytes but not muscle cells. Analysis of *Ebf2* expression during fetal development indicates that *Ebf2* expression was first detected in *Myf5* lineage cells in anterior somites. At this stage, *Ebf2* and *MyoD* were expressed in distinct *Myf5* lineage cells, suggesting that brown adipocyte and myogenic precursors have undergone lineage commitment by the time that *Ebf2* protein expression is detectable.

Analysis of clonal cell lines derived from cultured inguinal WAT SVF first indicated the presence of cells that can differentiate into either white or dormant beige adipocytes with thermogenic potential. This suggests the pres-

ence of committed beige precursors within the adult adipose stromal compartment (Wu et al. 2012). However, selective markers for the prospective isolation of native beige cell precursors from adipose tissues have been elusive. Wang et al. (2014) revealed that *Ebf2* expression could also identify beige adipose precursors in the inguinal WAT depot of mice. Upon cold exposure or  $\beta$ 3AR agonism, the frequency of *Ebf2*<sup>+</sup>;Pdgfr $\alpha$ <sup>+</sup> cells in the inguinal WAT, but not gonadal WAT, depot increases. *Ebf2*<sup>+</sup>;Pdgfr $\alpha$ <sup>+</sup>, but not *Ebf2*<sup>-</sup>;Pdgfr $\alpha$ <sup>+</sup>, cells isolated from cold-exposed mice differentiate in Ucp1<sup>+</sup> adipocytes, further suggesting that these cells represent native beige adipose precursors (Wang et al. 2014).

A number of critical questions remain regarding the exact identity of beige adipocyte precursors and their relationship to white adipocyte precursors. In particular, it remains unclear whether *Ebf2*<sup>+</sup> and *Ebf2*<sup>-</sup> Pdgfr $\alpha$ <sup>+</sup> cells represent distinct mesenchymal cell types/lineages that diverged much earlier in development. Alternatively, beige and white adipocytes may originate from bipotent/multipotent Pdgfr $\alpha$ <sup>+</sup> progenitors to give rise to *Ebf2*<sup>+</sup> and *Ebf2*<sup>-</sup> preadipocytes. Importantly, it is unclear how *Ebf2*<sup>+</sup> precursors fit into the lineage tracing results described above; the exact localization of these cells and the cell type that they represent in vivo are unclear.

It also remains unclear how beige precursors are activated to differentiate upon activation of the sympathetic nervous system.  $\beta$ 3AR expression appears confined largely to the mature adipocytes; therefore, non-cell-autonomous mechanisms are likely in place. A number of studies in recent years now suggest that catecholamine-triggered beige adipogenesis is coordinated and facilitated by the innate immune system. Lee et al. (2016) demonstrate that bipotent gonadal WAT Pdgfr $\alpha$ <sup>+</sup> cells localize to sites of M2 macrophages upon cold exposure and suggest that lipids from a subpopulation of adjacent macrophages may trigger adipogenesis through activation of *Ppar $\gamma$* . Lee et al. (2015a) and Brestoff et al. (2015) unveiled an adipose type-2 innate immune response driven by the sympathetic nervous system in response to cold exposure. In this proposed model, ILC2s, eosinophils, and macrophages produce cytokines such as IL-13, IL-4, and Met-enkephalin, all of which act directly on adipose precursors to drive proliferation and/or differentiation (Qiu et al. 2014; Brestoff et al. 2015; Lee et al. 2015a). The exercise-induced myokine Meteorin-like stimulates beige adipocyte accumulation in inguinal WAT through an eosinophil-/macrophage-dependent mechanism (Rao et al. 2014). Thus, this immune cell relay mechanism also appears operative in exercise-induced beiging. Additional insight into the exact identity of beige precursors and the development of more specific tools to target these cells will be essential to further our understanding of beige adipocyte formation in adult animals.

#### **Perspectives, limitations, and future directions**

Adipose tissue is unique in its remarkable capacity to expand or remodel in various physiological settings.

Adipocytes are known mostly for their role in energy metabolism; however, it is now clear that the emergence of adipocytes in tissues beyond adipose impacts local tissue regeneration, homeostasis, stem cell activity, and pathologies. There is now considerable interest in harnessing the tremendous plasticity of the adipose lineage for therapeutic benefit. Potential avenues include lineage reprogramming of energy-storing white adipocytes to energy-burning brown/beige adipocytes and/or altering the development of white adipocytes to impact adipose distribution and tissue regeneration in adults. A deeper understanding of the developmental origins of all adipocytes and the identities of their precursors will be critical to these efforts.

For decades, adipose biologists have relied exclusively on immortalized or primary cultures of preadipose cells induced to differentiate *in vitro* by robust proadipogenic stimuli. As a result, many have perhaps grown accustomed to thinking of the adipose life cycle in terms of “preadipocytes” and “mature adipocytes.” Here we describe recent efforts to identify native adipose precursor populations leading to the development, maintenance, and expansion of adipocytes in different physiological settings. Some of these different studies may appear conflicting, particularly as they relate to exact cell types that APCs represent as well as their localization *in vivo* (i.e., periendothelial or not periendothelial). In our view, most of these data are not mutually exclusive, since we cannot rule out the possibility that multiple populations may exist. Here we present each population as it was identified and characterized; however, it is important to note that there is likely significant overlap in the reported precursor populations. Furthermore, stable APC populations with intermediate phenotypes may exist, reflecting an adipose precursor hierarchy (e.g., CD24<sup>+</sup> and CD24<sup>-</sup> APCs). Alternatively, each precursor population may exhibit a specialized role in adipose biology. The exact precursor population drawn on to undergo adipogenesis may depend on the sex, location, age, or proadipogenic stimulus. At the very least, the studies described here indicate that adipose precursors, much like the adipocytes themselves, are likely heterogeneous. The vast complexity of the adipose lineage is increasingly more apparent as lineage tracing results continue to be reported. As such, additional distinct precursor populations are likely to emerge.

Studies pertaining to dermal and bone marrow stem cells indicate that one should not assume that the sole function of APCs is to give rise to adipocytes. Cells similar or related to the APC populations described here have also been implicated in the regulation of inflammation, angiogenesis, and stem cell maintenance through cytokine production. A major challenge going forward will be to determine the exact requirement of the various precursor populations described here. This will be best achieved by the development of genetic tools for temporal control of gene expression in these populations. Current inducible Cre/loxP models, such as the MuralChaser mice, may prove useful in exploring the adipose precursor niche, determining how the precursors are regulated by the microenvironment, and examining the functional conse-

quences of manipulating these cells on adipose development and their neighboring cells.

The development of genetic tools and FACS-based approaches to isolate adipose precursors gives plenty of reason for optimism in this growing field; however, there are still significant limitations to the current approaches that must be addressed going forward. In fact, some of these limitations may explain the inherent discrepancies in published lineage tracing results. Notably, many lineage tracing studies have used transgenic lines in which Cre activity is constitutive. The use of constitutively active Cre lines can be limiting in a number of ways. When bred to a Cre-dependent *Rosa26R* reporter strain, reporter expression is subsequently activated in all descending cells; this occurs regardless of whether descending cells continue to express the promoter of interest. Therefore, it becomes difficult to directly assess whether adipogenesis originates from cells actively expressing the promoter of interest during development. Moreover, the lack of temporal control over Cre activity prevents the use of these strains to determine the contribution of putative precursors to adipocyte hyperplasia under specific physiological conditions postnatally. Another important limitation is in the nature of the promoters ultimately driving Cre expression. The expression of *Pdgfr $\beta$* , *Pdgfra*, *Ppar $\gamma$*  (all of which have been used for lineage tracing), and other markers is not entirely restricted to one cell type in mice. This can become an issue in attempts to manipulate gene expression in different precursor populations, as confounding nonadipose phenotypes may emerge that indirectly affect adipogenesis. Unfortunately, a single marker that unambiguously identifies native APCs has yet to be identified.

As described above, genetic “pulse-chase” lineage tracing is currently the most precise way to establish clear lineage relationships. These approaches most often use doxycycline-inducible/suppressible systems for regulated Cre expression or animals expressing tamoxifen-inducible CreERT2. Both systems have been powerful in this field; however, they, too, have concerning limitations. Tamoxifen is a well-known inhibitor of estrogen receptor activity. The estrogen receptor itself plays an important role in adipose tissue remodeling (Davis et al. 2013; Palmer and Clegg 2015). At certain doses, tamoxifen can elicit detrimental effects on adipose tissue and remain localized in the adipocyte nucleus well beyond removal of the ligand (Ye et al. 2015). These effects include rapid cell death followed by adipose regeneration via *de novo* adipocyte differentiation. Liu et al. (2015) made similar observations and suggested that tamoxifen triggers adipocyte death through the inappropriate accumulation of reactive oxygen species. Moreover, Kloting and colleagues (Hesselbarth et al. 2015) observed that tamoxifen treatment itself impacts body composition and glucose and lipid homeostasis and alters adipose tissue cellularity. Notably, the investigators observed that tamoxifen treatment triggers substantial browning of the subcutaneous depot. This artificial turnover/adipogenesis is significant, as it can directly confound the interpretation of lineage tracing experiments.

There are also adverse effects of chronic doxycycline treatment on metabolism. Auwerx and colleagues (Chatzisprou et al. 2015; Moullan et al. 2015) demonstrated that doxycycline negatively impacts mitochondrial function. Doxycycline can also impact the gut microbiota in humans; the direct impact of this on adipose biology is unclear but potentially significant (Angelakis et al. 2014). Furthermore, doxycycline can regulate the function of matrix metalloproteinases (Stechmiller et al. 2010). Nevertheless, we are presently unaware of data that would suggest that short-term doxycycline exposure directly impacts adipose tissue turnover in a manner that would confound lineage tracing studies. Moreover, doxycycline leaves the system shortly after removal of doxycycline-containing food or water; expression of the widely used TRE-Cre transgene turns off within 24 h of removing doxycycline (Wang et al. 2013). This precise and verifiable temporal control over Cre expression is essential for lineage tracing analysis. As such, it is our position that the Tet-on systems are preferable over the tamoxifen-based Cre induction systems when performing adipose lineage tracing studies. At the very least, great caution must be taken in interpreting lineage tracing results when using tamoxifen; its action on the estrogen receptor in adipose tissue and its potential to trigger cell turnover must be considered.

Our discussion here focused on recent advancements in murine adipose precursor biology. It is important to note that some of the representative subcutaneous and visceral depots explored in mouse models are not direct correlates of human depots commonly studied. Human adipose precursors certainly share at least some characteristics with the murine populations studied here. Of note, an increasing number of studies point to the mural cell compartment of the vasculature as a home for mesenchymal stem cells and/or adipose precursors in various human tissues (Crisan et al. 2008; Zimmerlin et al. 2010; Yuan et al. 2014). Better FACS-based approaches allowing for more selective purification of human adipocyte precursor subpopulations will be needed. New strategies to isolate human adipose precursors may also help in exploring adipogenesis in disorders beyond obesity, including lipodystrophy.

It is increasingly apparent that the developmental biology of adipose tissues is quite complex. Considerably more effort, facilitated by more specific genetic tools and antibodies, will be needed to fully sort out the adipose lineage and the role of adipose precursors in various aspects of physiology and disease. In the end, further insight into the biology of adipose precursors may lead to novel therapeutic strategies that target the adipose lineage and combat various diseases.

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