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Are Perivascular Adipocyte Progenitors Mural Cells or Adventitial Fibroblasts?

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We would like to thank Gupta and colleagues (Vishvanath et al., 2017) for their interest in our recent publication concerning the role of pericytes as mesenchymal stem cells (MSCs), in which we used *Tbx18-CreERT2* to label pericytes in multiple organs, including fat depots, to examine potential fate switching of pericytes in response to aging or injury, or, in the case of fat depots, in response to a high-fat diet (HFD) regimen (Guimarães-Camboa et al., 2017). We agree with Vishvanath et al. that our conclusions concerning fate plasticity of pericytes relate only to the particular experimental regimens that we employed. However, we also argue that in combination, our findings and previous related work provide strong evidence that the cells acting as adipogenic progenitors in vivo are adventitial fibroblasts and not mural cells because previous lineage labeling strategies involving *SMA-CreERT2, Pdgfrb-rTA*, and *Nestin-Cre* label both adventitial cells and mural cells, whereas our *Tbx18-CreERT2* specifically labels mural cells and, unlike in the previous studies, does not show contribution to adipogenesis.

At the start of our study, we found that fluorescence-activated cell sorting (FACS)-purified *Tbx18*-expressing pericytes behave as MSCs in vitro, including the capacity to adopt an adipocyte cell fate, and we were then interested to explore whether these cells also behave as MSCs in vivo. We looked at the potential contribution of *Tbx18*-expressing pericytes to adipogenesis in vivo using a *Tbx18-CreERT2* that selectively marks mural cells (pericytes and vascular smooth muscle cells). Adipogenesis, the generation of new fat cells from adipose progenitors, occurs throughout the lifetime of the animal for adipocyte turnover (reviewed by Hepler and Gupta, 2017). We investigated the potential contribution of *Tbx18-CreERT2* pericytes to this ongoing process by labeling pericytes in male adult animals fed a normal chow diet at 8 weeks of age, and looked at perigonadal (epididymal) and inguinal white adipose tissue (WAT) as well as scapular brown adipose tissue (BAT) 8 weeks and 2 years post-pericyte labeling. We did not see any appreciable labeling of adipocytes by *Tbx18-CreERT2* lineages during aging. We also investigated the same fat depots in male

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mice following 6 weeks on a HFD designed to model human obesity and did not see contribution of *Tbx18-CreERT2*-labeled pericytes to adipocytes. We therefore concluded that in our in vivo experiments, the *Tbx18-CreERT2* lineage mural cells do not contribute to adipogenesis. We did not have space in the original manuscript to provide an in-depth discussion of our results in the context of recent literature, and we welcome the opportunity for further discussion in the current response.

White Adipose Progenitors: Mural Cells or Adventitial Fibroblasts?

As discussed by Vishvanath et al., several decades of studies on the growth of adipose depots consistently suggest that de novo adipogenesis takes place in close proximity to the vasculature, and has led to the inference that the adipogenic progenitor is associated with the vascular wall. The exact nature of this progenitor has, however, remained controversial. The wall of blood vessels is composed of three concentric cellular layers: tunica intima (endothelial cells that delimitate the vascular lumen), tunica media (composed of mural cells: pericytes or vascular smooth muscle), and tunica adventitia (external layer of fibroblastic cells). In larger vessels, distinction between cells of the tunica media and tunica adventitia is relatively easy. However, in small vessels distinguishing between distinct perivascular cell types, pericytes and adventitial fibroblasts, can be challenging, as adventitial fibroblasts can be closely juxtaposed to endothelium and pericytes. Additionally, pericytes and fibroblasts have a similar mesenchymal phenotype with abundant cytoplasmic projections. PDGFRβ is commonly used as a marker of mural cells, and PDGFRα as a marker of fibroblasts. However, the use of these markers alone is not sufficient to unequivocally discriminate between these cell types, as certain populations of fibroblasts express PDGFRβ, and certain populations of mural cells express PDGFRα. Importantly, we have found that in epididymal WAT, PDGFRβ is expressed by most, if not all, adventitial fibroblasts (Figure S1).

Histological studies in human fat (Lin et al., 2008) provided a relatively simple strategy to distinguish all cell types of the vascular wall based on expression of three cell-surface antigens: CD31, CD34, and CD146. Endothelial cells are triple positive (CD31+CD34+CD146+), mural cells are single positive for CD146 (CD31− CD34−CD146+), and adventitial cells are single positive for CD34 (CD31−CD34+CD146−). Friedman and colleagues utilized a multistep FACS protocol to purify cells from the stromal vascular fraction of mouse adipose tissue and showed that CD31−CD45−CD34+ cells have in vitro adipogenic potential (Rodeheffer et al., 2008). Based on data from these studies and others, the current consensus antigen signature for progenitors of white adipocytes is CD31−CD45−CD34+Sca1+ PDGFRα+ (Hepler and Gupta, 2017). This signature is typical of an adventitial fibroblast and distinct from the signature of a mural cell (Figure S1). We performed extensive histological characterization of *Tbx18*-expressing cells and defined their surface antigen profile as CD31− CD45− CD34− Sca1− PDGFRα− PDGFRβ+ CD146+, a classic signature of mural cells. As such, our findings that mural cells do not significantly contribute as progenitors of white adipocytes is consistent with much of the literature on the identity of the adipogenic progenitor as assayed in vitro.

Lineage Studies with Cre Lines Labeling Perivascular Cells

Cre mice that have recently been used to label perivascular cells to examine their contribution to adult adipogenesis include a constitutive *Pdgfrβ-Cre* (Tang et al., 2008), an inducible *Smooth Muscle Actin (SMA)-CreERT2* (Jiang et al., 2014), an inducible *Pdgfra-CreERT2* (Lee et al., 2012), a constitutive *Nestin-Cre* (Iwayama et al., 2015), and an inducible *Pdgfi*β-rtTA (Vishvanath et al., 2016). Based on analysis of the phenotypes of labeled cells, it seems to us that all of these studies included lineage labeling of adventitial fibroblasts.

The Graff lab initially conducted lineage tracing of mural cells using the constitutive *Pdgfrβ-Cre*, showing extensive labeling of white adipocytes (Tang et al., 2008). However, we showed that labeling by this *Pdgfrβ-Cre* is not confined to mural cells and instead occurs in multiple cell types (Guimarães-Camboa et al., 2017). Later work from the Graff lab utilized an *SMA-CreERT2*, which, when induced at postnatal day 30 (P30) and "chased" at P60, resulted in labeling of adipocytes. Histological analyses performed 2 days following induction at P30 demonstrated that, at this initial time, adipocytes were not labeled, and that some lineage-labeled cells co-localized with perivascular smooth muscle actin-expressing cells. Importantly, however, FACS analyses showed that at the initial time of labeling about half of the lineage-labeled cells had an adventitial fibroblast signature of PDGFRα+CD34+ (Jiang et al., 2014).

Cells that were lineage labeled by *Pdfgra-CreERT2* were shown to give rise to both "beige" adipocytes in the context of browning of WAT (45% of multilocular adipocytes labeled in epididymal WAT), and white adipocytes in epididymal WAT following 8 weeks of either chow (1.8% of epididymal white adipocytes labeled) or high-fat feeding (25% of epididymal white adipocytes labeled) (Lee et al., 2012). The initially labeled cells did not include adipocytes, were often found near blood vessels, and were positive for PDGFRα and CD34, and therefore appear to be adventitial fibroblasts. The extent of contribution of PDGFRα cells to fat in these studies may be underestimates, as approximately 50% of PDGFRα cells were labeled at baseline.

The constitutive *Nestin-Cre* selectively labels perivascular and adventitial cells, but not adipocytes, in 3-week-old mice. Approximately 60%–80% of *Nestin-Cre* lineage-traced cells in inguinal or epididymal WAT had an adipocyte precursor/adventitial fibroblast cell-surface signature (CD34+CD29+PDGFRα+). *Nestin-Cre* lineage-traced mice fed on normal chow diet for 12 subsequent weeks (15-week-old mice) showed a slight increase in adipocyte labeling in epididymal WAT, while those fed a HFD during the same period showed labeling of 50% of adipocytes in epididymal WAT (Iwayama et al., 2015).

Induction of *Pdgfi*β-rtTA in adult animals does not lineage label adipocytes but does label approximately 50%–70% of PDGFRβ cells in perigonadal WAT and inguinal WAT, respectively, as determined by FACS analyses. Histological analyses showed labeling of both periendothelial cells and adventitial fibroblasts by *Pdgfi*β-rtTA, consistent with known expression of PDGFRβ in both mural cells and adventitial fibroblasts (Figure S1). Adult males were kept on chow or HFD for 8 weeks post-labeling and results demonstrated

labeling of adipocytes only in epididymal WAT, with a slight increase observed in adipocyte labeling for animals on normal chow, and 10% of adipocytes being labeled for animals on a HFD (Vishvanath et al., 2016).

Because the *SMA-CreERT2, Pdgfrβ-rtTA*, and *Nestin-Cre* alleles used in the above studies label both mural cells and adventitial fibroblasts, they cannot be used to discriminate between these two perivascular populations in terms of adipogenic potential. In contrast, *Tbx18-CreERT2* selectively labels mural cells, and not adventitial cells, thus allowing for specific examination of mural cell contribution to adipogenesis. Notably, in our aging studies of mice on a chow diet, we did not see appreciable contribution of *Tbx18-CreERT2*-labeled mural cells to adipocytes within epididymal WAT either at 8 weeks or 2 years post-induction of *Tbx18-CreERT2* labeling (Guimarães-Camboa et al., 2017). These results are in contrast to those with *Pdgfra-CreERT2, Pdgfr*β-rtTA, and *Nestin-Cre*, where some increased labeling of epididymal WAT adipocytes was observed from 8 to 12 weeks post-labeling in animals on normal chow.

When taken together with results obtained with *Pdgfra-CreERT2*, which selectively labels adventitial fibroblasts, we argue that the combined findings discussed above are consistent with adventitial fibroblasts, and not mural cells, being in vivo adipogenic progenitors. However, we also would like to highlight that, as mentioned in our discussion, our results "apply exclusively to bona fide pericytes and vascular smooth muscle and do not exclude that other cell lineages located within or in the vicinity of the vascular niche might act as tissue-resident progenitors."

Involvement of Mural Cells in Other Adipogenic Processes

Although we studied the main BAT depot in our aging studies, as Vishvanath et al. pointed out, we did not address the potential role of *Tbx18CreERT2*-labeled pericytes in "browning" of white fat, as we did not employ treatments that induce browning of white fat. Therefore, we fully agree that it remains a possibility that mural cells contribute to beige fat cells induced in WAT in response to cold treatment or β-adrenergic agonists. Additionally, Vishvanath et al. raise an important point that the HFD we employed, despite inducing substantial increase in fat depots, may not have been extreme enough to provoke substantial de novo adipogenesis. To try to mimic events occurring in human obesity, we used a 12.5% milk fat, 1.25% cholesterol "Western" diet (42% of kilocalories drawn from fat) that has been previously used to promote nutritionally induced obesity in mice. Studies in the last few years using a lard diet (60% of kilocalories drawn from fat) have shown that in males, HFD-induced adipogenesis occurs selectively in epididymal WAT, with amplification of fat progenitors occurring within the first couple of days of HFD, and appearance of new adipocytes occurring between 4 and 7 weeks of HFD feeding (reviewed by Hepler and Gupta, 2017). In our study, we examined epididymal WAT at 6 weeks post-onset of our HFD.

We look forward to continued discussion with the groups of Gupta and Spiegelman in a coordinated effort to further investigate whether mural cells, in addition to adventitial fibroblasts, are adipose progenitors in contexts other than those we initially examined. As

Tbx18-CreERT2 offers a unique opportunity to specifically and efficiently label mural cells within adipose depots, and following the feedback from Vishvanath et al., we have initiated studies to determine the contribution of mural cells to adipogenesis in the context of a lard HFD, and in situations where WAT undergoes browning.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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