Defining the lineage of thermogenic perivascular adipose tissue

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Brown adipose tissue (BAT) is specialized for heat production and has garnered much attention because of its capacity to counteract metabolic disease1–12. Brown adipocytes have large numbers of mitochondria that contain uncoupling protein 1 (UCP1). On activation, UCP1 uncouples the mitochondrial proton gradient from ATP synthesis, creating an electrochemical driving force to burn large amounts of fatty acids, glucose and other substrates for heat production. Additional thermogenic mechanisms, including creatine- and calcium-driven futile cycles, also operate in adipocytes13,14.

Multiple discrete thermogenic fat deposits in humans can be activated by cold exposure or β-adrenergic activators9–12. Infants have a BAT depot in the interscapular region, analogous to the location of the largest BAT depot in many rodent species13,14,15,16. However, the interscapular BAT (iBAT) pad regresses and becomes undetectable in adults17. Adult humans retain BAT deposits in the supraclavicular region and in several depots surrounding large blood vessels, known as PVAT14,18,19. For example, UCP1+ adipocytes are present within the carotid sheath and mediastinal cavity of humans14,18,19. PVAT in the thoracic peri-aortic region of humans increases glucose uptake in response to systemic administration of a β3-adrenergic receptor agonist, a key functional attribute of BAT1. Therefore, understanding the ontogeny of thermogenic PVAT may reveal therapeutic targets to expand brown fat mass for combating obesity and metabolic disease.

Here, we performed a detailed analysis of peri-aortic adipose tissue development and maintenance. We found that the aortic PVAT of mice forms perinatally and possesses characteristics of classical BAT. Single-cell transcriptomic analysis of the aorta and associated adventitia in the perinatal period identified several distinct cell types, including preadipocytes and mesenchymal progenitor cells. Cell differentiation assays and genetic lineage tracing studies show that fibroblastic progenitor cells mediate aortic PVAT organogenesis. Interestingly, the aortic adventitia of adult mice lacks fibroblastic preadipocytes (Pdgfra+, Ly6a+ and Pparg−), but contains a population of adipogenic smooth muscle cells (Myh11+, Pdgfra− and Pparg+), contributing to periadventitial adipocyte formation. Similarly, human PVAT contains presumptive fibroblastic and smooth muscle–like adipocyte progenitor cells, as revealed by single-nucleus RNA sequencing. Together, these studies define distinct populations of progenitor cells for thermogenic PVAT, providing a foundation for developing strategies to augment brown fat activity.

Results

Aortic PVAT expresses an EBF2-dependent classical brown fat programme. The aorta of adult mice is surrounded by multiple lobes of PVAT, all of which are enclosed by a layer of fascia that defines an anatomical compartment (Fig. 1a). Histologically, perivascular adipocytes have densely eosin-stained cytoplasm and contain multiple lipid droplets, resembling brown adipocytes in iBAT (Fig. 1a). Single-cell transcriptomic analysis of the aorta and associated adventitia (Fig. 1a) showed high similarity between the aortic PVAT and the brown fat marker gene Ucp1 (Fig. 1b). The thermogenic character of aortic PVAT was further recruited by cold exposure. Aortic PVAT from cold-exposed (4 °C for 1 week) mice had reduced lipid stores and expressed higher levels of thermogenesis-related genes (Cycs, Ppargc1a and Ucp1), as compared to PVAT from mice housed at thermoneutrality (30°C; Fig. 1c,d).

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The transcription factor early B cell factor 2 (EBF2) is a critical regulator of thermogenic adipocyte development\textsuperscript{21–24}. To determine if EBF2 controls PVAT fate, we analysed thoracic aortas from mice lacking Ebf2 expression in adipocytes (Ebf2\textsuperscript{-/-}) and littermate control mice (Ebf2\textsuperscript{+/+}). Aortic PVAT from Ebf2\textsuperscript{-/-} mice displayed a whitened morphology, characterized by increased lipid deposition (Fig. 1e). Ebf2 mutant tissue also showed markedly reduced expression of brown-fat-specific genes, including an ~80% reduction of Ucp1 and lower levels of Cysc (Fig. 1f). In contrast to white adipose tissue (WAT) depots, iBAT is relatively resistant to inflammation triggered by a high-fat or Western diet\textsuperscript{29}. To determine if PVAT is protected from diet-induced inflammation, we fed C57BL/6 mice a Western diet for 16 weeks (Fig. 1g). Aortic PVAT from Western diet-fed mice displayed enlarged lipid droplets (Fig. 1h)\textsuperscript{29}. Identification of fibroblast heterogeneity in developing aortic structures from embryonic day (E) 18 embryos (just before birth) revealed many of the phenotypic properties of classical BAT.

Identification of fibroblast heterogeneity in developing aortic PVAT. We next sought to identify the progenitor cells responsible for the genesis of PVAT. Histological examination of thoracic aorta revealed fibroblast heterogeneity in developing aortic structures. Consistent with this observation, there was a dramatic rise in macrophage and pro-inflammatory genes (‘M1-like macrophage’) and decreased levels of anti-inflammatory (‘M2-like macrophage’) gene expression in the expression levels of various inflammatory genes in thoracic PVAT. The small rise of F4/80 levels is consistent with our prior study showing a decrease in macrophages after high-fat-diet feeding\textsuperscript{29}. Collectively, these findings demonstrate that aortic PVAT possesses many of the phenotypic properties of classical BAT.

Identification of fibroblast heterogeneity in developing aortic PVAT. Consistent with this observation, there was a dramatic rise in macrophage and pro-inflammatory genes (‘M1-like macrophage’) and decreased levels of anti-inflammatory (‘M2-like macrophage’) genes (Fig. 1i). By contrast, Western diet feeding did not affect the expression levels of various inflammatory genes in thoracic PVAT. The small rise of F4/80 levels is consistent with our prior study showing an increase in macrophages after high-fat-diet feeding\textsuperscript{29}. Collectively, these findings demonstrate that aortic PVAT possesses many of the phenotypic properties of classical BAT.

Fig. 1 | Aortic PVAT expresses an EBF2-regulated classical brown fat programme. a, H&E staining of thoracic aorta and iBAT from C57BL/6 mice (scale bar, 100 μm). Representative images of n = 5 experiments. b, mRNA levels of indicated genes in thoracic PVAT, iBAT and iWAT from C57BL/6 mice (n = 5 mice per group; mean ± s.e.m.). One-way analysis of variance (ANOVA) with Holm–Sidak multiple corrections (Adipoq: P = 0.00228; Ucp1: P = 0.00005). c, d, H&E staining (c) and mRNA levels of indicated genes (d) in thoracic aorta from C57BL/6 mice housed at thermoneutrality (TN) or 4 °C for 1 week (n = 6 mice per group; mean ± s.e.m.; scale bar, 100 μm). Two-sample, two-sided t-test (Cysc: P = 0.0000188; Pparc1a: P = 0.00017; Ucp1: P = 0.000000747). e, f, H&E staining of thoracic aorta (e) and mRNA levels of indicated genes in aortic PVAT (f) from Ebf2\textsuperscript{+/+} and Ebf2\textsuperscript{-/-} mice (n = 5 mice per group; mean ± s.e.m.; scale bar, 362.3 μm). Two-sample, two-sided t-test (Eb2: P = 0.000194; Cysc: P = 0.000144; Ucp1: P = 0.002233). g, Body weights of C57BL/6 mice fed normal Chow or Western diet for 16 weeks (n = 8 mice per group; mean ± s.e.m.). Two-sample repeated-measures ANOVA with Holm–Sidak multiple correction (P < 0.0001). h, i, H&E staining of thoracic aorta (h) and mRNA levels of indicated genes in eWAT and thoracic PVAT (i) from above mice. (n = 6 mice per group; mean ± s.e.m.). Two-sample, two-sided t-test (F4/80: P = 0.0347); eWAT (F4/80: P = 0.00271; Tnf: P = 0.00420; Fcgr1: P = 0.000052; Nos2: P = 0.0334; Ptg21: P = 0.000027; Retnla: P = 0.000052). NS, not significant (P > 0.05); **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. Lu, vessel lumen; Mac, macrophage.
regulation' and 'striated muscle contraction'. Intermediate cells were enriched for the 'Hedgehog signalling' pathway. Progenitor cells were enriched for genes involved in 'complement activation' and 'endochondral ossification'. Preadipocytes were notable for their enriched expression of genes related to 'electron transport chain', 'oxidative phosphorylation' and 'tricarboxylic acid cycle', corresponding to gene programmes that are critical for the activity of thermogenic adipocytes.

The adipogenic potential of sorted cell populations was investigated ex vivo. FACS-purified cell populations were plated and treated with a standard adipogenic differentiation cocktail. SMCs and intermediate cells displayed almost no capacity to...
undergo adipocyte differentiation, as assayed by BODIPY staining for lipid accumulation (Fig. 3d,e). By contrast, progenitor cells and preadipocytes efficiently formed adipocytes, with the preadipocytes displaying the highest levels of adipogenesis (Fig. 3d,e). Overall, these studies reveal a gradient of adipogenic competency from non-adipogenic SMCs to highly adipogenic preadipocytes.

**Adipogenic fibroblasts represent the major source of aortic PVAT in neonates.** The contribution of smooth muscle and fibroblastic cells to perivascular adipocyte development was examined in vivo using genetic lineage tracing models (Fig. 4a). We first used *Myh11-Cre* mice to test if SMCs give rise to peri-aortic adipocytes. *Myh11* is a specific marker of SMCs and its expression was restricted to SMCs in our scRNA-seq dataset (Extended Data Fig. 3a). Analysis of *Myh11-Cre; tdTomato* reporter mice revealed uniform labelling of aortic SMCs (Fig. 4b). We did not detect any tdTomato+ fibroblasts or adipocytes around the aorta at P3, implying that SMCs do not develop into adipocytes during the genesis of this depot (Fig. 4b).

*Pdgfra* is expressed in all peri-aortic adventitial fibroblasts, including intermediate cells, progenitors and preadipocytes (Extended Data Fig. 3a). To evaluate the contribution of these cell populations to adipocytes, we utilized tamoxifen-inducible *Pdgfra-CreER; GFP* reporter mice (*Pdgfra-CreER; mTmG*). We administered one dose of tamoxifen to pregnant females on E18 to induce GFP expression in *Pdgfra* (fibroblast) cells. Analysis of embryos 24 h later (pulse) revealed GFP expression in many fibroblastic cells surrounding the aorta (Fig. 4c). At later time points (chase), many mature adipocytes were marked by GFP expression, demonstrating that perivascular adipocytes initially develop from embryonic *Pdgfra*+ fibroblastic cells (Fig. 4c).

RNA-seq analysis identified *Vipr2* as a selective marker gene of intermediate cells relative to the other fibroblast subpopulations (Extended Data Fig. 3b). We used *Vipr2-Cre; GFP* (*Vipr2-Cre; mTmG*) reporter mice to determine if intermediate cells develop into adipocytes. At P3, GFP expression was confined to fibroblastic cells associated with the outer SMC layer of the aorta, consistent with an intermediate cell identity. In adult mice, we observed a similar pattern of GFP expression in fibroblasts cells around the aorta, but an absence of GFP expression in adipocytes (Fig. 4d).

*Penk* expression was enriched in progenitors and preadipocytes, relative to other fibroblasts (Extended Data Fig. 3a). In P3 *Penk-Cre; GFP* (*Penk-Cre; mTmG*) reporter mice, GFP was expressed in peri-aortic fibroblast cells, with GFP labelling of adipocytes becoming widespread in adulthood (Fig. 4e). Collectively, these experiments demonstrate that peri-aortic adipose tissue develops from fibroblastic progenitors and preadipocytes.

**Comparative analysis of thermogenic and white adipogenic progenitors.** We next sought to determine if perivascular progenitor cells were stably committed to the thermogenic lineage. The adipogenic cell populations from thoracic aorta (progenitor and preadipocyte) and preadipocytes from interscapular WAT (iWAT) were plated and induced to undergo adipocyte differentiation. The PVAT and iWAT-derived cultures expressed similar levels of general adipocyte marker genes (*Adipoq* and *Pparg2*), indicating comparable adipogenic competency (Fig. 5a and Extended Data Fig. 4a). Notably, PVAT progenitors and preadipocytes but not iWAT preadipocytes adopted a thermogenic gene programme (*Dio2* and *Ucp1*) following adipocyte differentiation (Fig. 5a and Extended Data Fig. 4a).

Our previous work identified a lineage hierarchy in WAT, comprising multipotent progenitors, and preadipocytes committed to the adipocyte lineage. We found that the developing PVAT contains transcriptionally similar adipogenic populations: progenitor cells expressing genes such as *Pit16, Cd55* and *Dpp4*, and preadipocytes expressing commitment markers *Pparg* and *Lpl*. To search for conserved gene programmes controlling these two cellular states, we performed bulk RNA-seq on freshly sorted progenitors and preadipocytes from aortic PVAT and iWAT. Principal-component analysis showed that tissue origin accounted for much of the variance in the expression of the 500 most variable genes (Fig. 5b). We defined a consensus gene signature of progenitors, consisting of genes selectively expressed in progenitors versus preadipocytes from both PVAT and iWAT (Fig. 5c and Extended Data Fig. 4b). Pathway analysis of the progenitor consensus gene signature identified enrichment of prostaglandin synthesis, transforming growth factor-β (TGFβ) and WNT signalling (Fig. 5c). The consensus preadipocyte genes were enriched for ‘PPAR signalling’, ‘adipogenesis’ (*Pparg, Cd36* and *Rxrg*), as well as an unexpected signature of cell cycle activity (*Cmm2, Cdc45* and *Rpa1*) (Fig. 5d and Extended Data Fig. 4e). PVAT preadipocytes specifically expressed genes related to the electron transport chain, oxidative phosphorylation and the tricarboxylic acid cycle, including many genes important for the function (*Cox5a, Cox7a2* and *Ucp1*) and transcriptional control (*Pparg1a and Ersla*) of brown adipocytes (Fig. 5d,e and Extended Data Fig. 4d). These data support a model in which uncommitted progenitor cells (enriched for expression of anti-adipogenic WNT and TGFβ pathways) develop into preadipocytes, with specific induction of a thermogenic gene programme in PVAT preadipocytes (Fig. 5f).

**Identification of adipogenic smooth muscle cells in adult PVAT.** To determine if the cell types observed in developing PVAT were also present in adult mice, we performed scRNA-seq analysis on the stromal vascular fraction of thoracic aorta from 13-week-old mice. Many of the cell groups identified in adult aorta were analogous to perinatal cell types (Fig. 6a and Extended Data Fig. 5a). Clustering analysis revealed three populations of adult fibroblastic cells (all *Pdgfra*7), two of which were present in perinatal tissue: *Pit16*/Ly6a+ progenitors and *Bace2*/Clec11a+ intermediate cells (Fig. 6b).

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**Figure 2** | **Identification of multiple fibroblast populations in developing aortic PVAT.** a, H&E staining of retroperitoneum en bloc from E18 and P3 CD1 mice (scale bar, 517.6 μm). Representative of n = 3 experiments. b, mRNA levels of indicated genes in thoracic aorta from E18 and P3 CD1 mice (n = 3 biological replicates of pooled aortas; mean ± s.e.m.). Two-sample, two-sided t-test (*Adipoq: P = 0.000011; Lep: P = 0.000618; Pparg2: P = 0.000016; Ucp1: p = 0.00002). c, Uniform manifold approximation and projection (UMAP) of gene expression in 17,957 cells from thoracic aorta of P3 CD1 mice. d, UMAP showing expression of smooth muscle marker genes (left) and immunostaining of PDGFRA (red) and MYH11 (green) in sections of E18 thoracic aorta (right; scale bar, 271.8 μm). Representative of n = 2 experiments. e, UMAP showing expression of adipocyte marker genes (left) and immunostaining of PLIN1 (red) in P3 thoracic aorta (scale bar, 543.5 μm). Representative of n = 3 experiments. f, UMAP showing expression of preadipocyte marker genes and immunostaining of PPARy (red) and PDGFRA (green) in P3 thoracic aorta. Arrowhead shows a preadipocyte (scale bar, 135.9 μm). Representative of n = 2 experiments. g, UMAP showing expression of intermediate cell marker genes and mRNA in situ hybridization of Bace2 (red) in P3 thoracic aorta. Arrowheads indicate intermediate cells (scale bar, 145 μm). Representative of n = 2 experiments. h, UMAP showing expression of the progenitor marker gene *Pit16* (left) and mRNA in situ hybridization of (1) *Pit16* (green; middle) and (2) Clec11a (red) and *Pit16* (green; right) in P3 thoracic aorta. Arrowheads in (L) inset indicate progenitors. White arrowhead in (R) inset points to an intermediate cell. Yellow arrowhead shows progenitor cell (scale bar, 291 μm (L) and 145 μm (R)). Representative of n = 2 experiments. i, Model for thoracic aorta tissue organization at P3. DAPI (blue) was used to stain nuclei. NS, P > 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.
The third fibroblast population (‘unknown fibroblasts’) was defined by selective expression of S100a4. In situ hybridization analysis of Ly6a, Pi16 and Bace2 showed that the spatial localization of intermediate and progenitor cells was conserved in adults, with intermediate cells located immediately adjacent to the aortic smooth muscle and progenitors located more distant radially (Extended Data Fig. 5b,c). Notably, we did not detect a fibroblastic preadipocyte (Pparg+/Pdgfra+) population in adult aorta. However, we identified a new cluster of SMCs (SMC 2) that expressed canonical smooth muscle markers Myh11, Acta2 and Tagln2, along with adipocyte markers like Pparg, Lpl and Fabp4 (Fig. 6c). Cd81, a recently described marker of smooth muscle-related beige fat progenitor cells.
cells in WAT<sup>−</sup>, was widely expressed in multiple cell types, whereas the brown adipogenic regulator PRDM16 was selectively expressed in SMCs (Extended Data Fig. 5d).

We developed a FACS strategy to purify the following cell populations: progenitors (LY6A<sup>+</sup>), preadipocytes (PreAd; LY6A<sup>−</sup>; CD142<sup>−</sup>), SMCs (LY6A<sup>−</sup>CD142<sup>−</sup>CD200<sup>+</sup>), intermediate cells (Int; LY6A<sup>−</sup>CD142<sup>+</sup>CD200<sup>+</sup>) and mesothelium (meso; LY6A<sup>−</sup>CD142<sup>+</sup>CD200<sup>−</sup>CD317<sup>−</sup>). Representative plots from ten separate experiments. See the full strategy in Extended Data Fig. 2a, b.

Expression maps of Seurat-generated cluster-defining genes mapped onto sorted-cell RNA-seq results (n = 3 biological replicates per group). c, Pathway analysis of cluster-defining genes for each cell population. Cluster-defining genes are significantly differentially expressed genes with log2 fold change (LFC) > 1.5 in every pairwise comparison from sorted-cell bulk RNA-seq (n = 3 biological replicates). The plots show −log<sub>10</sub> unadjusted P values. P values were calculated using Enrichr with WikiPathways 2019 annotations and the hypergeometric test; P<sub>adj</sub> values for multiple comparisons were calculated with Benjamini–Hochberg. P<sub>adj</sub> values in the order they appear on graphs: SMCs (8.70 × 10<sup>−4</sup>, 1.73 × 10<sup>−4</sup>, 0.012645474, 0.078202676, 0.093946235 and 0.093946235); intermediate (0.03706, 0.019056, 0.025219 and 0.060584); PreAd (1.97 × 10<sup>−4</sup>−49, 6.31 × 10<sup>−27</sup>, 2.74 × 10<sup>−20</sup>, 9.95 × 10<sup>−18</sup>, 5.46 × 10<sup>−16</sup> and 1.37 × 10<sup>−13</sup>), SIDS, sudden infant death syndrome; GPCRs, G-protein-coupled receptors; LCFA, long-chain fatty acid; BODIPY, lipid (green) and Hoechst (DNA; blue) staining of cell cultures following differentiation with adipogenic cocktail (representative image of five separate experiments). Scale bars, 500 μm (whole well) and 100 μm (zoom).
Fig. 4 | Adipogenic fibroblasts are the major source of aortic adipocytes in neonates. a, Schematic of cell types in perinatal aorta and corresponding marker genes. Created with BioRender.com. b, Staining of PLIN1 (green) and RFP (red) in sections of P3 thoracic aorta from Myh11-Cre−; tdTom (control) and Myh11-Cre+; tdTom reporter mice. Arrowheads in inset indicate labelled SMCs (white) and unlabelled adipocytes (yellow). (n = 2 Cre−; n = 3 Cre+; scale bar, 271.8 μm). c, Staining of GFP (green) and PLIN1 (red) in sections of thoracic aorta from Pdgfra-CreER−; mTmG (control) and Pdgfra-CreER+; mTmG mice collected 24 h (pulse E18) and 8 weeks (adult chase) after tamoxifen treatment. Arrowheads in inset 1 show labelled fibroblasts; arrowheads in inset 2 show GFP+ adipocytes (pulse: n = 1 Cre−; n = 3 Cre+; chase: n = 1 Cre−; n = 2 Cre+; scale bar, 271.8 μm). d, Staining of GFP (green) in sections of P3 and adult thoracic aorta from Vipr2-Cre−; mTmG and Vipr2-Cre+; mTmG mice. Arrowheads indicate GFP-expressing intermediate cells. (P3: n = 3 mTmG−; n = 4 mTmG+; adult: n = 1 mTmG−; n = 4 mTmG+; scale bar, 290 μm (P3), 724.7 μm (adult)). e, Staining of GFP (green) in sections of P3 and adult thoracic aorta from Penk-Cre−; mTmG and Penk-Cre+; mTmG+ mice. Yellow arrowheads show GFP+ fibroblasts; white arrowheads show GFP+ adipocytes (P3: n = 1 Cre−; n = 3 Cre+; adult: n = 1 Cre−; n = 3 Cre+; scale bar, 145 μm (P3), 724.7 μm (adult)). DAPI (blue) was used to stain nuclei.
progenitors and unknown fibroblasts (PDGFRα−CD200−), SMC-1 (MCAM+CD200+) and SMC-2 (MCAM+CD200−; Fig. 6d and Extended Data Fig. 5e,f). The expression of cluster-defining genes from the single-cell transcriptomes was mapped onto the sorted cell transcriptomes, demonstrating a high level of concordance (Fig. 6e from the single-cell transcriptomes was mapped onto the sorted cell Extended Data Fig. 5e,f). The expression of cluster-defining genes (MCAM≤conserved ontogeny of adipocyte development. Created with Biorender.com. NS, 0.01, ***P 0.0001.

to promote adipocyte turnover38. Rosiglitazone treatment greatly peri-aortic fat. To further assess the adipogenic activity of SMCs, displayings no adipogenic potential (Fig. 7a). The adult fibroblast populations displayed similar adipogenic activity to their neonatal counterparts, with progenitor cells undergoing robust adipocyte differentiation, and intermediate cells displaying no adipogenic potential (Fig. 7a).

To determine if SMCs can contribute to perivascular adipocyte development in adult animals, we analysed the aortas of adult Myh11-Cre; tdTomato reporter animals. Under baseline conditions, tdTomato+ adipocytes were detected, albeit at low levels, in peri-aortic fat. To further assess the adipogenic activity of SMCs, we treated Myh11-Cre; tdTomato reporter mice with rosiglitazone to promote adipocyte turnover44. Rosiglitazone treatment greatly increased the number of tdTomato+PLIN1+ adipocytes in thoracic PVAT (Fig. 7b). Importantly, rosiglitazone did not induce MYH11 expression in any adipocytes, strongly suggesting that SMCs underwent adipocyte differentiation (Extended Data Fig. 6a).

RNA-seq analyses of freshly sorted cells identified Trpv1 as a gene expressed specifically in SMC-2 cells compared to SMC-1 cells and fibroblastic (PDGFRα+) cells (Extended Data Fig. 6b). We used Trpv1-Cre; GFP reporter mice to examine the adipogenic activity of SMC-2 cells. In adult mice housed at thermoneutrality (30°C) or following cold exposure at 4°C for 1 week, GFP was detected in a subset of aortic PVAT adipocytes (Extended Data Fig. 6c).

Adipogenic activity of smooth muscle cells in adult PVAT. We investigated the adipogenic differentiation competency of FACS-purified SMC and fibroblastic populations isolated from peri-aortic adipose tissue of adult mice. CD200+ SMCs (SMC-1) did not undergo adipogenesis, whereas CD200− SMCs (SMC-2) efficiently differentiated into lipid-droplet-containing adipocytes (Fig. 7a). The adult fibroblast populations displayed similar adipogenic activity to their neonatal counterparts, with progenitor cells undergoing robust adipocyte differentiation, and intermediate cells displaying no adipogenic potential (Fig. 7a).

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Altogether, these findings demonstrate that SMC-2 cells contribute to perivascular adipocyte formation in adult animals.

We investigated the anatomical localization of the two SMC subtypes in thoracic aortic tissues. Immunostaining for the pan-SMC marker TAGLN identified SMC cells within the adipose tissue proper, as well as in the aortic media around the lumen (Extended Data Fig. 6d). Histological analysis of the aorta in longitudinal section identified blood vessels coursing through the adipose tissue (Extended Data Fig. 6e). To determine if the adipogenic Pparγ+CD200− SMCs (SMC-2) were selectively enriched in the aortic adventitia, we separated adventitia/adipose tissue and aortic media, and isolated cells for flow cytometry analysis (Fig. 7c). The adventitia was highly enriched for CD200− progenitor cells, and the cleaned aorta was enriched for CD200− intermediate cells, verifying the separation strategy. Notably, there was also striking enrichment of CD200− (adipogenic) SMCs (SMC-2) in the adventitia samples, whereas the cleaned aorta was enriched for CD200+ SMCs (SMC-1).

To test if fibroblastic cells also generate new adipocytes in adult PVAT, we tracked the fate of Pdgfra+ cells using Pdgfra-CreER; GFP reporter mice (Extended Data Fig. 6f). These mice were treated with tamoxifen to induce GFP expression in fibroblasts, followed by treatment with rosiglitazone. Under these conditions, we observed many GFP-marked adipocytes in aortic PVAT, showing that fibroblastic cells can undergo de novo adipocyte formation (Extended Data Fig. 6f). Interestingly, cold exposure did not noticeably increase the development of new aortic PVAT adipocytes from either SMCs (Trpv1−; Extended Data Fig. 6c) or fibroblastic cells (Pdgfra−; Extended Data Fig. 6g), whereas Pdgfra+ cells generated many new adipocytes in iBAT under these conditions (Extended Data Fig. 6g).

We identified the Hedgehog-related gene Gli1 as a selective marker gene of intermediate fibroblast cells in adult aorta (Extended Data Fig. 6b). To determine if intermediate cells can generate adipocytes, we performed lineage analysis with Gli1-CreER; GFP mice (Extended Data Fig. 6h). Upon tamoxifen administration, GFP expression was induced in intermediate fibroblasts lining the aortic smooth muscle and was not expressed in any adipocytes (Extended Data Fig. 6h). Following rosiglitazone treatment, we did not detect GFP+ adipocytes, suggesting that intermediate cells are not a major source of new adipocytes in adult animals.

Single-nucleus transcriptomic analyses of human PVAT. To determine if human PVAT contains comparable cell types to those discovered in mice, we carried out single-nucleus transcriptomic analysis on aortic PVAT samples. Integrated analysis of single-nucleus transcriptomes from three individuals identified numerous cell clusters, many of which corresponded to readily identifiable cell types on the basis of marker gene expression.

Fig. 5 | Comparative gene expression profiling of white and brown adipogenic cells. a. mRNA levels of indicated genes in differentiated adipocytes derived from the following cell types of P3 CD1 mice: iWAT preadipocytes, thoracic perivascular progenitors and thoracic perivascular preadipocytes. (n = 4 independent wells from pooled FACS populations per group; mean ± s.e.m.). One-way ANOVA followed by two-sided pairwise comparisons with Holm–Sidak correction. P values in order: iWAT PreAd versus Prog, iWAT PreAd versus PreAd, Prog versus PreAd. Adipon (0.4198, 0.3825 and 0.7582); Pparγ (0.0019, 0.3125 and 0.0059); Dio2 (0.0002, 0.0021 and 0.0705); Ucp1 (0.0583, 0.0003 and 0.0034). b. Principal-component analysis (PCA) of the top 500 differentially expressed genes in sorted-cell RNA-seq (n = 3 biological replicates per cell type). One PVAT PreAd data point includes two overlapping replicates. c. Venn diagram of genes enriched in progenitor populations relative to preadipocyte populations from iWAT and PVAT. Pathway analysis of genes commonly enriched in both depots (significantly differentially expressed and LFC > 0). Plots show −log_{10} unadjusted P values. P values were calculated using clusterProfiler with WikiPathways 2019 annotations using the hypergeometric distribution. P values for multiple comparisons were calculated with false discovery rate (FDR). P values in order: 0.002835, 0.01171, 0.01171, 0.028483, 0.070511 and 0.077592. d. Venn diagram of genes enriched in preadipocyte populations relative to progenitor populations. Pathway analysis of genes commonly enriched in both depots (significantly differentially expressed and LFC > 0; left) and genes unique to PVAT (right). Graphs and P values calculated as in c. e. z-score split heat map of representative genes from Gene Ontology analysis. Gene expression levels are calculated between cell types (that is, PVAT progenitors versus PVAT PreAd) within a depot of origin (n = 3 biological replicates per cell type). f. Model for conserved ontogeny of adipocyte development. Created with Biorender.com. NS, P > 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.
(Extended Data Fig. 7a,b). For the subsequent analysis, we separated out the immune and endothelial cells, focusing our attention on adipocytes and any potential adipocyte progenitor groups. Mature adipocytes were marked by ADIPOQ expression, with a subset of these cells expressing the brown fat markers UCP1, EBF2 and PRDM16 (Fig. 7d,e and Extended Data Fig. 7d). We also identified three groups of PDGFRα-expressing fibroblasts (Fig. 7d,f). Among these, CD55+ fibroblasts clustered the farthest away from adipocytes and expressed enriched levels of several genes typical of mouse iWAT and PVAT progenitors, including CD55 and Pi16 (Fig. 7d,f and Extended Data Fig. 7f). The remaining two groups of fibroblasts expressed enriched levels of adipogenic genes including PPARg and clustered near adipocytes, likely corresponding to preadipocytes (that is, PreAd-1 and PreAd-2). The two subgroups of preadipocytes expressed elevated levels of certain genes, exemplified by the selective expression of COL15A1 in PreAd-1 and COL4A4 in PreAd-2 (Extended Data Fig. 7d). ‘Transitional cells’, expressing lower levels of PDGFRα and increasing levels of PPARg, connected putative iWAT PreAd

![Ex vivo differentiated adipocytes](image)

- **a** PCA of top 500 variable genes
- **b** Commonly enriched in progenitors
- **c** Commonly enriched in PreAds
- **d** Common progenitor
- **e** Common PreAd
- **f** Developmental model
pseudopocytes with adipocytes. These results suggest that human PVAT and mouse fat depots have remarkably similar fibroblastic adipocyte precursor cell lineage hierarchies.

We also identified two closely connected clusters of SMC-related cells that we called ‘SMCs’ and ‘PPARγ-expressing SMC-like cells’. Both cell groups expressed many SMC-selective genes including NOTCH3, PDGFRb and TRPC6 (refs. 32,39; Fig. 7d,g and Extended Data Fig. 7c,d). Pathway analysis identified enrichment of smooth muscle-related terms in both cell groups, relative to all other cell groups (Extended Data Fig. 7e). The PPARγ+ SMC-like cells were enriched for many adipogenesis pathways relative to SMCs (Fig. 7h). Interestingly, the SMC population specifically expressed the canonical smooth muscle gene MYH11, whereas the PPARγ+ SMC-like cells expressed enriched levels of PPARγ and other adipogenic genes (Fig. 7d,f,g and Extended Data Fig. 7c). Finally, we analysed a publicly available single-nucleus transcriptome dataset generated from deep neck BAT23, corresponding to PVAT near the common carotid artery. Like the aortic PVAT, deep neck BAT contained PPARγ+ pseudopocytes and presumptive progenitor cells marked by many common marker genes, including CD55 and PI16 (Extended Data Fig. 7g–j). We also detected a PPARγ+ SMC-like population that expressed many SMC and adipogenic genes but lacked MYH11 expression (Extended Data Fig. 7g–j), similar to the aortic PVAT population. Together, these data suggest that PPARγ+ SMC-like cells represent adipocyte progenitor cells that may be functionally analogous to the adipogenic SMC-2 cells in mouse aorta.

Discussion

Aortic PVAT represents a unique type of thermogenic fat depot that is conserved in rodents and adult humans. This fat depot exhibits many of the defining properties of IBAT in mice, including high levels of UCP1 expression, responsiveness to cold exposure, dependence on the brown fat transcription factor EBF2 and resistance to obesity-induced inflammation.

Aortic brown fat cells become organized into recognizable depots in the immediate postnatal period between E18 and P3. We postulated that this period of time would involve large shifts in progenitor cell phenotypes, correlating with the burst of adipogenesis. Rather, we observed a striking similarity in the stromal cell composition of the depot immediately before birth (when no adipocytes were present) and after birth (when many adipocytes were present). This finding suggests that perivascular adipocyte progenitor cells are seeded earlier in development and remain poised to undergo adipogenic differentiation until receiving adipogenic cues. The period of time between E18 to P3 therefore presents an opportunity to identify the native signals that trigger adipogenesis.

Cell lineage tracking studies using the SMC-restricted Myh11-Cre driver indicated that differentiated SMCs do not contribute to aortic PVAT formation during the fetal and early postnatal period (Fig. 7i). This result contrasts with prior studies suggesting that perivascular adipocytes originate from SMCs based on lineage tracing studies using Tagln (Sm22)-Cre41,42. Tagln-Cre+ cells give rise to nearly all perivascular structures, including aortic smooth muscle, adventitial fibroblasts and adipocytes. We assume that Tagln-Cre is activated during early embryonic development in a cell population that does not express Myh11 and is fated for a perivascular-restricted but not an SMC-restricted fate. Consistent with this notion, cells expressing Tagln at E8.5 develop into thermogenic adipocytes42.

Aortic adventitial tissue in perinatal mice contains two distinct groups of adipogenic fibroblasts: progenitors and preadipocytes. Progenitors were marked by expression of Cd55, Pi16 and Dpp4, whereas preadipocytes expressed adipocyte-related transcripts, including the adipogenic transcription factor Pparg. Analogous mesenchymal populations, expressing many of the same marker genes, are also present in subcutaneous WAT51. Pathway analysis identified enrichment of the anti-adipogenic WNT and TGFβ signalling pathways in both PVAT and WAT progenitors44–46. These pathways likely play crucial roles in maintaining progenitor cell identity, at least in part, via blocking adipocyte lineage progression.

We also identified a common gene signature of PVAT (brown) and WAT preadipocytes, including many genes associated with adipogenesis, such as Lpl and Cid36. The common preadipocyte signature was also significantly enriched for genes linked to cell cycle progression. In this regard, adipocyte differentiation of preadipocyte cell lines requires a round of post-confluent mitosis47–49. However, it remains unclear if the cell cycle gene profile identified in preadipocytes relates to cell cycle entry, exit or progression. Additionally, many genes associated with fatty acid oxidation and mitochondrial biogenesis were selectively expressed in PVAT (brown) compared to WAT preadipocytes, indicating that metabolic specialization is already established at the preadipocyte stage.

We identified another interesting fibroblastic cell type in aortas, which we termed intermediate cells due to their anatomical location in between the vascular SMCs and adventitial progenitors. Intermediate cells expressed enriched levels of Hedgehog signalling genes. Previous studies identified a discrete population of sonic Hedgehog-responsive cells located underneath the aorta, likely corresponding to intermediate cells44–49. These cells did not undergo adipocyte differentiation in vitro or in vivo. Understanding the physiological function(s) of intermediate cells remains an area of great interest. The intimate association of these cells with the outer layer of aortic smooth muscle suggests a potential role in modulating vascular biology. In this regard, a recent report suggests that fibroblastic cells surrounding the vasculature have the capacity to differentiate into SMCs after severe vessel injury50. We did not detect these cells in human aortic PVAT, likely because of their close association with the vessel, which was not included in these samples.

Adult PVAT in mouse also possessed a distinctive subset of adipogenic SMCs that expressed Pparg and other adipocyte-related genes. This Pparg+ SMC population, marked by the expression of Trpv1 and Myh11, displays robust potential to differentiate into adipocytes. Related to this finding, elegant work by the Jiang et al. identified distinct lineages of embryonic and adult adipocyte progenitors in WAT51. Intriguingly, they showed that adult adipocytes

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**Fig. 6 | Identification of adipogenic smooth muscle cells in adult PVAT.** a, UMAP of gene expression in 6,753 cells from adult thoracic aortas of pooled 13-week-old male CD1 mice. b, UMAPs showing expression of fibroblast marker genes. c, UMAPs showing expression of SMC marker genes. d, FACS isolation of fibroblast and SMC populations. Live Lin+ cells were gated to isolate the following cell populations: intermediate cells (PDGFRα+MCAM+CD200+), progenitors (PDGFRα+MCAM+CD200−) and SMC-1 (PDGFRα+MCAM−CD200−) and SMC-2 (PDGFRα+MCAM−CD200−). Representative image of six separate experiments. See the full strategy in Extended Data Fig. 5f. e, Heat map of Seurat-generated cluster-defining genes mapped onto sorted-cell RNA-seq results (n = 3 biological replicates per group). f, Pathway analysis of cluster-defining genes for each cell population. ACE, angiotensin-converting enzyme; EDA, ectodysplasin; GDNF, glial cell line-derived neurotrophic factor. Cluster-defining genes were significantly differentially expressed genes with LFC > 1.5 in every pairwise comparison (n = 3 biological replicates). Graphs show −log10 unadjusted P values. P values were calculated using clusterProfiler with WikiPathways 2019 annotations and the hypergeometric distribution. P quánet values for multiple comparisons were calculated with FDR. P quánet values in order: intermediate cells (0.027783, 0.029414, 0.034167, 0.093761, 0.105357 and 0.105357), progenitors (0.026565, 0.026565, 0.029454, 0.078199, 0.078199 and 0.181118), SMC-1 (6.64×10−4, 6.64×10−3, 0.040965, 0.040965 and 0.269701) and SMC-2 (0.000933, 0.001561, 0.004055, 0.008551, 0.047207 and 0.047207).
in WAT arise via Acta2-expressing mural cells, which they identified as SMCs. It remains unclear if the Acta2+ cells in WAT or SMCs identified here in PVAT progress through a fibroblastic intermediate (that is, preadipocyte) or differentiate directly into adipocytes. It is important to note that adult mouse PVAT also contains progenitor cells, a fibroblast population with the potential to produce adipocytes. Notably, cold exposure did not induce high levels of new adipocyte differentiation from either SMCs or progenitor cells in aortic PVAT, as compared to other fat depots52–54. This finding suggests that PVAT expandability is somehow constrained. One possibility is that cold may act mainly on preadipocyte cells, which are depleted in adult mouse PVAT (Fig. 6a). Alternatively, these findings may suggest that the browning of the PVAT depot following cold exposure is mainly driven by the activation of thermogenic programmes in pre-existing mature fat cells. Together, our study suggests that both SMCs and progenitor cells contribute to the maintenance of

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**Figure a**

- Schwann cells
- Unknown fibroblasts
- Progenitor cells
- Immune cells
- Mesotheium
- Intermediate cells
- SMC2
- Endothelial cell 1
- Endothelial cell 2

**Figure b**

- Fibroblastic cells
- Intermediate cells
- Known fibroblasts
- Intermediate cells
- Unknown fibroblasts

**Figure c**

- Myh11
- Acta2
- Tagln
- Pparg
- Lpl
- Fabp4

**Figure d**

- Live Lin−
- MCAM+
- PDGFRα+

- CD200−; MCAM+
- MCAM−; PDGFRα+

- CD200−; PDGFRα+
- CD200+; PDGFRα+

**Figure e**

- Single-cell transcriptomes
- Sorted-cell bulk transcriptomes

- SMC-1
- SMC-2
- Int
- Prog

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**Figure f**

- Intermediate cells
- Hedgehog signaling pathway
- Endothelial calcium signaling
- Primary focal segmental glomerulosclerosis
- EDA signaling in hair follicle development
- GDNF–RET signaling axis

- Progenitor cells
- Chemokine signaling pathway
- Lung fibrosis
- Nucleotide GPCRs
- WNT signaling pathway
- Retinoic acid metabolism
- ACE inhibitor pathway

- SMC-1
- Focal adhesion
- Integrin-mediated cell adhesion
- Myometrial relaxation and contraction pathways
- Osteoclast
- Calcium regulation in the cardiac cell

- SMC-2
- Adipogenesis genes
- Calcium regulation in the cardiac cell
- Non-odorant GPCRs
- Striated muscle contraction
- Monoamine GPCRs
- PPAR signaling pathway

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adult PVAT in mice (Fig. 7i). Further studies are needed to determine if these populations are recruited by different stimuli.

Aortic PVAT in adult humans, unlike that in mice, possesses two presumptive populations of fibroblastic ‘preadipocyte’ cells, closely connected to mature adipocytes. A third type of fibroblast represents putative progenitor cells, clustering closely with preadipocytes and expressing many of the same marker genes as their counterparts in mice, including CD55, DPP4 and P116. We also identified...
a presumptive population of adipogenic SMC-related cells in human PVAT. Interestingly, however, these 'PPARγ' SMC-like cells in human PVAT, while clustering closely with SMCs and displaying enriched levels of many smooth muscle pathways, did not express the canonical smooth muscle marker MYH11. We speculate that SMCs may give rise to adipogenic 'SMC-like' cells in human PVAT, coupled with the loss of some mature smooth muscle characteristics.

In summary, this work defines the progenitor cells responsible for the development and maintenance of PVAT, a distinct thermogenic depot conserved in rodents and humans. Based on preclinical studies in rodent models, BAT-targeted therapies for the treatment of human obesity and metabolic disease will depend on the ability to increase brown fat mass, either by transplantation of brown fat cells or by inducing brown fat cell formation in situ. The identification of the native progenitors and developmental ontogenies of thermogenic adipocytes, which are retained in adult humans, provides a critical foundation for therapeutic strategies aimed at augmenting brown fat.

Methods

Mice. All animal experiments were performed according to procedures approved by the University of Pennsylvania's Institutional Animal Care and Use Committee (approval no. 805649). Mice were housed under the care of University of Pennsylvania University Laboratory Animal Resources. Animals were raised at room temperature on standard Chow diet with a 12-h light–dark cycle. All mouse housing and husbandry occurred at room temperature (22 °C) unless otherwise specified. For thermoneutral acclimatization, 4- to 5-week old mice were housed at room temperature on standard chow with a 12-h light–dark cycle. All mouse handling and experiment procedures were performed under aseptic conditions. For the development and maintenance of PVAT, a distinct thermogenic depot conserved in rodents and humans. Based on preclinical studies in rodent models, BAT-targeted therapies for the treatment of human obesity and metabolic disease will depend on the ability to increase brown fat mass, either by transplantation of brown fat cells or by inducing brown fat cell formation in situ. The identification of the native progenitors and developmental ontogenies of thermogenic adipocytes, which are retained in adult humans, provides a critical foundation for therapeutic strategies aimed at augmenting brown fat.
Heat maps represent either LFC versus the row mean or row score as indicated. Underlying data for heat maps came from the DESeq2 regularized log transformation of the normalized count values using the DESeq2 function ‘log2’. Figures showing side-by-side scRNA-seq and bulk RNA-seq heat maps contain the exact same genes in the same order on both heat maps. For Figs. 2b and 6c, ‘cluster-defining genes’ were generated using the Seurat v3 function FindAllMarkers; these gene lists were thresholded at the indicated average LFC cut-off and then plotted using the function DimHeatmap. Analogous heat maps were then generated to show DESeq2 analysis of bulk RNA-seq data of sorted cells. For Extended Data Fig. 5g, a similar process was performed using pairwise comparisons between the indicated groups, using the Seurat function FindMarkers for scRNA-seq data and a DESeq2 pairwise analysis for bulk RNA-seq data. Extended Data Figs. 2d and 3i correspond to the same data in Figs. 2b and 6c, respectively, showing the total number of genes plotted for each cluster in the heat maps, as well as how many Seurat v3 clusters and doublets were filtered out in the bulk RNA-seq data (enriched/concordant), and how many of the Seurat v3 cluster-defining genes were downregulated in the bulk RNA-seq data (de-enriched/discordant).

Single-cell RNA sequencing. Single cells were isolated from dissected aortas and flow sorted to isolate live (DAPI−) cells and remove debris. Cells were collected as either CD45+ (immune) or CD45− (non-immune) cells. For adult scRNA-seq, CD45+ cells were mixed with CD45− cells to achieve a proportion of ~20%. Single-cell transcriptomes were collected using the 10x Genomics platform with v2 (peripheral) and v5 (adult) chemistry (10x Genomics). The libraries were prepared following the manufacturer’s Illumina library procedure, processed using the Cell Ranger pipeline and R Studio using Seurat (v3)30. Cells were filtered based on their expression of a minimum number of genes (E18/P3: 500 < nFeatureRNA < 3,000; adult: 250 < nFeatureRNA < 4,000) and mitochondrial genome (<10%) abundance for each dataset individually. Regression was performed using the ScaleData function for the following variables: percentage of mitochondrial reads, cell cycle phase (G2M.Score/S.Score), % percent.mito, and differential gene expression between clusters was performed using the FindMarkers function. For all datasets, immune cells were collapsed into one cluster for simplicity. For the perinatal dataset, two clusters of mesothelial cells were collapsed to generate one mesothelial cluster. For the adult dataset, initial clustering revealed that SMC population 1 separated into two clusters, but the effect size of gene expression between the two groups was small, so they were collapsed to form SMC population 1. Feature plots, dot plots and heat maps were generated using Seurat.

E18/P3 combined analysis. Dataset integration was performed using the Seurat functions FindIntegrationAnchors followed by IntegrateData. Scaling and regression were performed using the Seurat ScaleData function for the following variables: percentage of mitochondrial reads and cell cycle phase (G2M.Score/S.Score). Dimensionality reduction was performed in Seurat using UMAP and differential gene expression between clusters was performed using the FindMarkers function. For all datasets, immune cells were collapsed into one cluster for simplicity. For the perinatal dataset, two clusters of mesothelial cells were collapsed to generate one mesothelial cluster. For the adult dataset, initial clustering revealed that SMC population 1 separated into two clusters, but the effect size of gene expression between the two groups was small, so they were collapsed to form SMC population 1. Feature plots, dot plots and heat maps were generated using Seurat.

Human aortic PVAT single-nucleus RNA sequencing: individual sample analysis. The reads were processed using the Cell Ranger pipeline and R Studio using Seurat (v3)30. To exclude low-quality nuclei and doublets, ‘cluster-defining genes’ were generated using the Seurat v3 function FindAllMarkers; these gene lists were thresholded at the indicated average LFC cut-off and then plotted using the function DimHeatmap. Analogous heat maps were then generated to show DESeq2 analysis of bulk RNA-seq data of sorted cells. For Extended Data Fig. 5g, a similar process was performed using pairwise comparisons between the indicated groups, using the Seurat function FindMarkers for scRNA-seq data and a DESeq2 pairwise analysis for bulk RNA-seq data. Extended Data Figs. 2d and 3i correspond to the same data in Figs. 2b and 6c, respectively, showing the total number of genes plotted for each cluster in the heat maps, as well as how many Seurat v3 clusters and doublets were filtered out in the bulk RNA-seq data (enriched/concordant), and how many of the Seurat v3 cluster-defining genes were downregulated in the bulk RNA-seq data (de-enriched/discordant).

Deep peak BAT analysis. Previously published deep peak BAT single-nucleus RNA-seq data were reanalyzed using the same protocol described herein. Endothelial cells and immune cells that were identified by Sun et al. and by expression of PECAM1, VWF, CDH5 (endothelial), PTPRC and MRCI1 (immune) were filtered out.

Differential gene expression between clusters was performed using FindAllMarkers on the subsetted, reclustered Seurat object. This Seurat object was used for all single-gene UMAP feature plots showing expression of selected genes on the integrated dataset were generated for the RNA assay using Seurat DotPlot, FeaturePlot and VlnPlot functions, respectively.

Generation of single-cell suspensions from mouse aorta. Aortas were isolated from mice, minced and placed into digestion medium (DMEM, collagenase D: 6.1 mg ml−1 (Roche), dispase II; 2.4 mg ml−1 (Roche)) and layered on ice constant agitation at 200 r.p.m. To enrich for cell populations with differential sensitivity to digestion, we used the following procedures: For embryonic/newborn mice, 20% of the digestion was quenched at 15 min, 20% at 20 min and the remaining 60% at 25 min (embryos/perinatal). For adult aortas, 50% of the digestion was quenched at 20 min, 20% at 30 min and the remaining 30% of digestion was performed for 60 min. The reaction was quenched at 60 min after digestion of stripped aortas, PVAT was removed from the aorta and digested separately from the cleaned aorta. Tissue digestions were quenched with an equal volume of complete medium (DMEM/10% FBS). Dissociated cells were suspended using a P1000 pipette and filtered through a 100-μm filter. Cells were then pelleted at 400g for 4 min, and red blood cells were lysed in 155 mM NH4Cl, 12 mM NaHCO3, and 0.1% low data were performed in FlowJo (10.6.2). The following antibodies were used to generate dot plots, single-gene UMAP feature plots and single-gene violin plots using Seurat’s DotPlot, FeaturePlot and VlnPlot functions, respectively.

Isolation of human aortic PVAT nuclei. Human peri-aortic fat tissue was obtained from patients undergoing coronary artery bypass grafting surgery (males aged 65, 65 and 61 years (average, 64 years); body mass index (BMI): 23.5, 23.9 and 35.3 (average, 28.2). This work was performed at Maine Medical Center under the approval of Maine Medical Center IRB no. 1396116–4, and informed consent was obtained from all study participants. The isolation and purification of nuclei was adapted from work by Hu et al. 34. Briefly, 40–80 mg of frozen human PVAT was homogenized using a glass homogenizer (Sigma–Aldrich; 7–9 ml) on ice in 2.5 ml of homogenization buffer (0.32 M sucrose (Sigma), RNase-free and DNase-free ultra-pure grade), 5 mM CaCl2 (Sigma), 3 mM MgAc2 (Sigma–Aldrich), 10 mM Tris-HCl (pH 8.0; Fisher Chemical), 0.1% Triton X-100 (Amresco), 0.1 mM EDTA (Thermo Fisher) and protease inhibitor cocktail (Roche) and layered into ultracentrifuge tubes (Beckman Coulter) over 12 ml sucrose cushion (1.8 M sucrose, 10 mM Tris-HCl (pH 8.0; Fisher Chemical), 3 mM MgAc2 (Sigma–Aldrich) and protease inhibitor cocktail (Roche) and topped with 14.5 ml of homogenization buffer. Samples were ultra-centrifuged (Beckman Coulter, L-70K) using a swinging-bucket rotor (Beckman Coulter, SW28) at 25,000 r.p.m. for 2 h at 4 °C. The supernatant was aspirated to remove debris, and the nuclear pellet was resuspended in 1 ml of DPBS (containing) with RNase inhibitor (Lonza) and incubated for 20 min of incubation on ice. nuclei were filtered through a 100-μm cell strainer and pelleted at 5,000 r.p.m. for 10 min at 4 °C. Nuclei were resuspended in 2% BSA (GoldBio) in DPBS, spun again and filtered 2× through a 40-um mini strainer (plusSelect). Nuclei were stained with Trypan blue, counted on a haemocytometer and visually inspected for intact morphology before 10x loading at the Center for Analytical Genomics Sequencing Core (Children’s Hospital of Philadelphia, PA) using the following reagents: 10x Genomics: v3.1 Next GEM kit; sequence: Illumina NovoSeq 6000; and flow cell: SP 100 cycles v1.5.

Flow cytometry and sorting. Isolated cells were suspended in FACS buffer (HBSS, 3% FCS, 0.04% sodium azide (Thermo)). Cells were stained in FACS buffer with antibodies (see below) for 1 h at 4 °C in the dark. Cells were washed 2× with cold FACS buffer and sorted on a FACS Aria cell sorter (BD Biosciences) with a 100-μm nozzle as previously described. 35 All compensation was performed at the time of acquisition in BD FACS DIVA software (8.0.1) using single stained cells or compensation beads (BioLegend, A10497). Downstream analysis and visualization of flow cytometry and sorting data was performed using FlowJo (Version 10.6.2). The following antibodies and dilutions were used for FACS protocols: P3 aorta: Lr6α-BV711 (1:100; BioLegend, 108131; RRID: AB_2562241), CD17-PerCp-Cy5.5 (1:100; BioLegend, 127022; RRID: AB_2566667), CD200-4F4 (1:100; Bio-Rad, MCA1958A488), CD45-APC/Cy7 (1:1,000; BioLegend, 303115; RRID: AB_312980), CD31-APC/Fire750 (1:1,000; BioLegend, 102528; RRID: AB_2721491), Ten19-APC/Cy7 (1:1,000; BioLegend, 116223; RRID: AB_2137788), CD124-FlC46 (1:1,000; Sino Biological), FVS510 (1:100; BD Biosciences, 654406); P3 iWAT: CD45-APC/Cy7 (1:1,000; BioLegend, 103115; RRID: AB_312980), CD31-APC/Fire750 (1:1,000; Sino Biological), 50413-R001), CD26(DPP4)-FITC (1:100; BioLegend, 302704),
ICAM1–PE/Cy7 (1:100; BioLegend, 353116); adult aorta: CD200–AF488 (1:100; Bio-Rad, MCA1958A488), CD45–APC/Cy7 (1:1,000; BioLegend, 103115; RRID: AB_213980), CD231–APC/Fire750 (1:1,000; BioLegend, 105258; RRID: AB_2721491), TenC–APC/Cy7 (1:1,000; BioLegend, 116223; RRID AB_2137788), MCAM–PerCP/Cy5.5 (1:100; BioLegend, 134709; RRID: AB_11204083), PDGFRα–PE/Cy7 (1:200; BioLegend, 135912; RRID: AB_2715974) and DAPI (1:10,000; Roche, 1023627601).

Cell culture. Sorted-purified cells were plated on 384-well CellBIND plates (Sigma-Aldrich, CLS3770) and cultured in DMEM/F12 supplemented with 10% FBS and Primocin (50 µg ml⁻¹). Cells were plated at a density of 10,000–30,000 cells per well and induced to differentiate into adipocytes within 3 d of initial plating. Cell density and time until differentiation was kept consistent within each experiment. The adipogenic differentiation cocktail consisted of 1 µM dexamethasone (Sigma, D5727), 0.5 µM isobutylylmalonyl-CoA (Sigma, S7287), 125 nM indomethacin, 20 nM insulin and 1 nM T3 for 48 h. Following 2 d of induction, the medium was changed to 20 nM insulin and 1 nM T3. Medium was changed every 2 d for six more days.

Adipogenesis assays and quantification. Adipogenesis was assessed by staining with BODIPY 493/503 (Invitrogen, D39292) for lipid accumulation and Hoechst 33342 (Thermo Fisher, 62249) for nuclei. Briefly, cells were differentiated in 384-well tissue culture plates (Sigma-Aldrich, CLS3770), fixed with 4% paraformaldehyde, stained and imaged on a Keyence inverted microscope (BZ-X710) using Keyence BZ-X viewer software (1.3.1.1) with the indicated filters for DAPI (excitation, 360–400 nm; emission, 460–500 nm; Keyence, OP-87762) and GFP (excitation, 470–40 nm; emission, 525–50 nm; Keyence, OP-87763). Images were acquired at >200 in a 7×7 tiled grid and stitched to capture the entirety of each well. Tiling and stitching were performed with Keyence BZ-X analyzer software (1.3.0.3). Image quantification was conducted automatically in ImageJ (version 1.52E) using a macro that performed the following steps: (1) split images into component channels; (2) for the nuclei channel, applied a 3-sigma Gaussian blur, performed thresholding to identify signal above background, performed watershed to segmentation and counted the number of nuclei; (3) for the lipid channel, applied a 2-sigma Gaussian blur, performed thresholding to identify signal above background and counted the area (number of pixels) with signal above threshold. The amount of adipogenesis was calculated as the lipid area divided by the number of nuclei.

Statistical analysis. No power calculations were performed before initiation of the study. No mice were omitted from the study. All individual data points were plotted to assay normality. Experiments from a common pool of digested tissue or independent pools processed separately are indicated in the text. Statistical testing was performed for comparisons between more than two groups. Two-way t-tests were performed for comparisons between two groups. Statistical testing plotted to assay normality. Experiments from a common pool of digested tissue or independent pools processed separately are indicated in the text. Statistical testing was performed for comparisons between more than two groups. Two-way t-tests were performed for comparisons between two groups.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Further information and requests for resources and reagents, including unique/stable reagents generated in this study, are available from the corresponding author without restriction. Sequencing data and code are available on the Gene Expression Omnibus under accession GSE164528.

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Author contributions
A.R.A., A.P.S. and P.S. were responsible for conceptualization, data analysis and writing. A.R.A. and A.P.S. contributed equally and conducted the majority of the experiments and carried out the bioinformatics analyses. C.D.H. and M.N.A. prepared the human aortic PV AT samples for perinatal single-cell datasets. K.S. provided sequencing reagents and key preparation for perinatal single-cell datasets. K.S. provided sequencing reagents and key experimental insight. K.B. assisted with data analysis. L.L. obtained and provided human peri-aortic PVAT samples.

Competing interests
The authors declare no competing interests.

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Extended Data Fig. 1 | Single cell transcriptional profiling of aortas from E18 and P3 mice. (a) UMAPs of gene expression in cells from E18 and P3 thoracic aorta of pooled CD1 mice. (b–f) UMAPs showing expression of indicated genes for: smooth muscle cells (SMC) (b); Intermediate cells (c); Progenitors (d); Preadipocytes (e); and Adipocytes (f). (g) Expression dotplot of indicated genes for cell clusters from P3 aorta.
Extended Data Fig. 2 | Purification of smooth muscle and fibroblast cells from aorta (Related to Fig. 3). a, FACS isolation of fibroblastic and smooth muscle cell (SMC) populations. Dissociated cells were gated on: (1) SSC-A and FSC-A to exclude debris; (2) FSC-H vs. FSC-W then SSC-H vs. SSC-W to isolate single cells; and (3) live (FVS510- Lin- (CD45-, CD31-, Ter119-) cells. Depicted sort gates were used to isolate the following populations: Progenitors [LY6A high], Preadipocytes (PreAd) [LY6A(-), CD142 mid; CD200(-)]; SMCs [LY6A(-); CD142(-); CD200(+)], Intermediate Cells (Int) [LY6A(-); CD142(+); CD200(+), CD317(-)], Meso (Mesothelium) [LY6A(-); CD142(+); CD200(+), CD317(+)] (Representative images from n=10 expts). Related to experiments shown in Figs. 3a and 5. b, Violin plots showing expression of genes used in the sorting strategy. c, Staining of CD200 (red), MYH11 (green), and DAPI (blue) in P3 thoracic aorta. White arrowhead shows an Intermediate cell. Yellow arrowhead shows an SMC (scale bar, 65 μm; Lu: lumen). Representative of n=1 experiment. d, Expression concordance of cluster-defining genes (scRNAseq) in corresponding sorted cell populations (from bulk RNAseq).

| Comparison        | SMC | Int. Cell | Prog. | PreAd |
|-------------------|-----|-----------|-------|-------|
| Total Genes       | 331 | 163       | 193   | 215   |
| Enriched (Concordant) | 255 | 161       | 192   | 151   |
| De-enriched (Discordant) | 76  | 2         | 1     | 64    |
| No change (LFC=0) | 0   | 0         | 0     | 0     |
Extended Data Fig. 3 | Expression profiles for Cre-driver mouse lines (Related to Fig. 4). a, UMAPs showing expression of genes used for Cre and CreER driver mouse strains. b, Vipr2 expression levels in indicated cell types from sorted cell bulk RNAseq analysis. (One way ANOVA followed by two sided pairwise comparisons with Holm Sidak correction. n=3 biologically independent samples per group; mean ±/− SEM). Statistical Testing: ****p≤0.0001.
Extended Data Fig. 4 | Gene profiling analyses of iWAT vs. PVAT progenitors and preadipocytes (Related to Fig. 5). a, mRNA levels of indicated genes in differentiated primary adipocytes from: iWAT preadipocytes, aortic PVAT progenitors, aortic PVAT preadipocyte cells (all from P3 CD1 mice). Second experimental replicate performed on different day (n = 2 PVAT Pread; n = 3 PVAT Progenitor; n = 4 iWAT Pread independent wells per group from pooled FACS samples; mean±/− SEM). b, Z-Score split heatmap of genes from overlaps depicted in Fig. 5c,d. Gene expression levels are calculated between cell types (that is PVAT Progenitors vs PVAT Pread) within a tissue of origin (n = 3 biological replicates). c, Expression heatmap of all cell cycle genes (used by Seurat) in sorted progenitor and preadipocyte cells from iWAT and thoracic aortic PVAT. Data comes from DESeq2 normalized count data (bulk RNAseq). d, Pathway analysis of genes enriched in preadipocytes from PVAT vs. iWAT (significantly differentially expressed and LFC>0). Graph plots -log10 unadjusted p values. P values were calculated using clusterProlifer with WikiPathways2019 annotations using the hypergeometric distribution. Padjusted for multiple comparisons was calculated with FDR. Padjusted in order (2.48E-34, 1.07E-20, 1.31E-15, 2.97E-13, 1.37E-12, 2.01E-10, 1.71E-07, 2.66E-07, 1.84E-05).
Extended Data Fig. 5 | Purification and analysis of aorta-associated cells from adult animals (Related to Fig. 6). a, Expression dot plot of indicated genes in cell clusters from adult aorta. b, mRNA in situ hybridization of Ly6a (green) in adult aorta. Arrowhead in inset shows progenitor cell. DAPI (blue) stains nuclei. (scale bar, 362.3 μm). Lu: lumen. Representative of n=2 experiments. c, mRNA in situ hybridization of Pi16 (green) and Bace2 (red). Yellow arrowhead shows intermediate cell (scale bar, 145 μm). Representative of n=2 experiments. d, UMAPs showing Cd81 and Prdm16 expression. e, Violin plots showing expression of indicated genes used for sorting. f, FACS isolation of fibroblasts and smooth muscle cells (SMCs). To exclude debris and isolate single live cells, we gated on: (1) SSC-A and FSC-A; (2) FSC-H vs FSC-W; and (3) SSC-H vs SSC-W. We then gated on Live (DAPI-); Lin- (CD45-,CD31-,TER119-) cells. Further selection was used to isolate: Intermediate Cells [PDGFRα+ MCAM(-), CD200+]; Progenitors [PDGFRα+, MCAM+, CD200+]; SMC1 [PDGFRα-, MCAM+, CD200+]; SMC2 [PDGFRα-, MCAM+, CD200-]. (Representative image of 6 separate experiments). Related to experiments shown in Figs. 6d and 7a,c. g, Expression of Seurat cluster-defining genes in the sorted cell bulk RNAseq datasets. Gene levels in sorted cell populations were compared to the row mean and annotated as “enriched” or “de-enriched” (Log2 FC>0.25). h,i, Expression heatmap of Seurat cluster-defining genes mapped on to sorted-cell RNASeq results for fibroblast (H) and SMC populations (I) (n=3).
Extended Data Fig. 6 | Analysis of aortic PVAT in adult mice (Related to Fig. 7). a, Staining for MYH11 (red) and PLIN (green) in aortas of Rosiglitazone (Rosi)-treated mice, scale bar 200 μm. Representative of n=1 experiment. Scale bar, 200 μm. b, mRNA levels of Trpv1 and Gli1 in sorted cell populations. c, Staining of GFP (green) and either PLIN1 or UCP1 (red) in aortas of male Trpv1-Cre; mTmG mice housed at thermoneutrality or following 1 week of 4C cold exposure. Scale bar, 100 μm. d, Staining of TAGLN (red) in adult aorta. White arrowheads show adventitial SMCs. Yellow arrowhead indicates parenchymal SMCs of the aorta. Representative of n=2 experiments. Scale bar, 271.8 μm. e, H&E staining of adult aorta. Arrowheads show adventitial blood vessel. f, Staining of GFP (green) in aortas of Pdgfra-CreER-; mTmG+ mice following a 5-day pulse of Tamoxifen and a 2.5 week treatment with DMSO or Rosi. Arrowheads in GFP+ adipocytes (n=1 Cre−; n=4 DMSO; n=5 Rosi; scale bar, 543.5 μm). g, Staining of GFP (green) in aortas of Pdgfra-CreER+; mTmG+ mice following a 5-day pulse of Tamoxifen and 2-week chase at room temperature or 4C (cold). Representative of n=1 experiment. Scale bar, 100 μm. h, Staining of GFP (green) in aortas of CreER- CTL and Gli1-CreER+; mTmG+ mice following a 5-day pulse of Tamoxifen and 2.5-week treatment with DMSO or Rosi. (n=1 Cre−; n=3 Cre+; DMSO; n=3 Cre+; Rosi scale bar, 543.5 μm). DAPI (blue) was used to stain nuclei.
Extended Data Fig. 7 | Single nucleus transcriptomic analyses of human PVAT (Related to Fig. 7). a, UMAP of gene expression in 18,758 nuclei from adult human peri-aortic PVAT (Full dataset, n=3 humans, integrated analysis). b, Expression dotplot of indicated genes in dataset from (A). c,d, Violin plots (c) and UMAPs (d) showing expression of select genes, corresponding to the subclustered dataset in Fig. 7d. e, Pathway analysis of cluster defining genes (average logFC>0.5) in SMC and PPARg+ SMC-like cells compared to all other clusters in the dataset from Fig. 7d. Graph plots -log10 unadjusted p values. P values were calculated using clusterProfiler with WikiPathways2019 annotations using the hypergeometric distribution. Padjusted for multiple comparisons was calculated with FDR. Padjusted in order SMC (1.59E-07, 2.50E-06, 2.75E-06, 0.000106107, 0.000968522, 0.000968522, 0.001899498); PPARg + SMC Like Cells (5.75E-05, 0.000135397, 0.003396457, 0.00971142, 0.00971142, 0.010635022, 0.010635022). f, Violin plots showing expression of select marker genes in the fibroblastic populations from Fig. 7d. g, UMAP of gene expression in 7285 nuclei from adult human deep neck BAT (subclustered to remove immune and endothelial cells, n=16 humans, integrated analysis, re-analyzed data from: Sun et al 40). h-j, UMAPs (h), expression dotplot (i) and violin plots (j) showing expression of select genes in cells from the dataset above (g).
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

- scRNA-Seq: Single cell transcriptomes were collected using the 10X Genomics platform with v2 (perinatal) and v3 (adult) chemistry (10X Genomics, Pleasanton, CA). The libraries were sequenced using an Illumina HiSeq 4000.
- qPCR: Real-time PCR was performed on an ABI7500HT PCR machine using SYBR green fluorescent dye (Applied Biosystems).
- Bulk RNA-Seq Data collection: Bulk RNA sequencing from sorted progenitors was performed at GeneWiz with the following procedure. RNA was extracted using Trizol LS (Invitrogen) and quantified using Qubit Fluorometer (2.0, Life Technologies, Carlsbad, CA) and TapeStation. RNA sequencing libraries were made using the SMART-Seq v4 Ultra Low Input Kit. Samples were sequenced on an Illumina HiSeq 4000 with a 2x150 Paired End (PE) configuration.
- Imaging: Images were acquired on a Keyence inverted microscope (BZ-X710) using Keyence BZ-X Viewer Software (1.3.1.1). Image tiling and stitching were performed with Keyence BZ-X Analyzer software (1.3.0.3).
- Flow Cytometry: FACS and sorting experiments used a BD-FACS Aria (BD Biosciences) running BD FACs DIVA software (8.0.1.0) to set gates and compensation.

Data analysis

- qPCR: Real-time PCR was performed on an ABI7500HT PCR machine using SYBR green fluorescent dye (Applied Biosystems). Fold changes were calculated in Microsoft Excel (2016) using the delta-delta-CT method, with TaTa Binding Protein (Tbp) mRNA serving as a normalization control; statistical tests and data visualizations were done in GraphPad Prism (Versions 7-9).
- Bulk RNA-Seq: FASTQ files were aligned to mm10 using STAR (2.5.2a) with quantMode = GeneCounts. The unstranded genecounts for each sample were analyzed for differential gene expression using the R (3.6.3) package DESeq2 (1.26.0). In general, for each experiment involving an RNA seq dataset, the DESeq model was built with the design “[CellType] where “CellType” refers to the different sorted populations, with all CellTypes included in the model. Pairwise differential expression was calculated using the DESeq2 function “results” with thresholds of alpha=0.1, Log2 Fold change thresholds were used as indicated. ENSEMBL ID’s were converted to gene symbols using the R package org.Mm.eg.db (3.10.0). Pathway analysis was performed via WikiPathways with annotations for Mus Musculus using the package clusterProfiler (3.13.3) and / WikiPathways (1.6.1) [Figures 5, 6 and 7, 54, 57]. Pathway analysis was performed using Enrichr (https://maayanlab.cloud/Enrichr/) WikiPathways 2019 Mouse for Figure 3.
scRNA-Seq: The reads were processed with CellRanger (v3.1) pipeline then analyzed in R (3.6.3) using Seurat (v3).
Imaging: Image quantification was performed in ImageJ (version 1.52E). Calculations on this data were performed in R (3.6.3). Statistical tests and data visualizations were done in GraphPad Prism (7-9).
Flow Cytometry: Downstream analysis and visualization of flow data was performed in Flowjo (10.6.2).

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All sequencing data, including raw Fastq files and analysis outputs are deposited at GEO under GSE164528

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
No power calculations were performed prior to initiation of the study to determine sample sizes. Sample sizes were chosen based on previous experience estimating gene expression variability between mice (Angueira, Cell Reports 2020). These sample sizes have previously been able to detect biologically meaningful effect sizes in mice in vivo.

Data exclusions
No data were excluded from analysis

Replication
Replicates were performed as indicated in the text for in vivo and in vitro experiments. Replication of findings in micrographs are indicated in figure legends. All attempts at replication were successful.

Randomization
Cells/animals were randomly assigned to treatments by investigators.

Blinding
Investigators were not blinded to experimental groups for IHC analysis to allow proper thresholding for GFP and RFP staining in Cre- controls. For other experiments, investigators were blinded to group allocation during data collection and analysis.

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Materials & experimental systems

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- [ ] Clinical data
- [ ] Dual use research of concern

Methods

- [x] Involved in the study

- [x] ChiP-seq
- [x] Flow cytometry
- [ ] MRI-based neuroimaging

Antibodies

P3 Aorta: LY6A-BV711 (1:100 Biologend-108131 RRID:AB_2562241), CD317-PerCp-Cy5.5 (1:100 Biologend-127022 RRID:AB_2566647), CD200-AF488 (1:100 Biorad-MCA1958AA488), CD45-APC/Cy7 (1:1000, Biologend-103115 RRID:AB_312980),
Validation

Sorting antibodies used in the strategies for P3FAT, P3 IAT, and Adult PVT, were validated by sequencing sorted populations and ensuring populations expressed similar gene signature to populations identified on single cell analysis. All sorting strategies included fluor-minus-one controls for each step of the gating procedure to set antibody thresholds. Inclusion of reporter or cre- mice was used to validate GFP and RFP antibodies. For staining, antibodies were validated based on appropriate expression pattern. i.e. Perilipin-1 in cells with adipocyte morphology; GFP expression limited to mice that express Cre and reporter allele.

Animals and other organisms

Policy information about studies involving animals ARRIVE guidelines recommended for reporting animal research

Laboratory animals

CS7/B6 mice and CD1 mice were used in this study. CS7/B6N (RRID:IMSR_TAC:B6) mice were obtained from Taconic. CD1 (RRID:IMSR_CRL:024) mice were obtained from Charles River. The following strains were obtained from the Jackson Laboratory: mTmG (strain name: B6.129(Cg-Gt(ROSA)26Sortm4(ACTB-tdTomato,EGFP)Luo/J), RRID:IMSR_JAX:007676), tdTom (strain name: B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J, RRID:IMSR_JAX:007914), Myh11-Cre [strain name: B6.Cg-Tg(Myh11-cre,Efgfp)2Mly/J, RRID:IMSR_JAX:007742], Pdgfra-CreER (strain name: B6.129S1-Pdgfratm1.1(cincreERT2)Bly/J, RRID:IMSR_JAX:032770), Gli1 Cre/R (strain name: Gli1tm3(creERT2)Aj/J, RRID:IMSR_JAX:007913), Pten-Cre (strain name: B6.129S1-Ptentm2(creERT2)Ily/J, RRID:IMSR_JAX:001921), Vop2-Cre [strain name: B6.Cg-Tg(Vop2om1.1(cre)Hze/J, RRID:IMSR_JAX:031592) and Tp53-Cre (strain name: B6.129-Tp53tm1(cre)Bly/J, RRID:IMSR_JAX:017769). The following stock was generated by Patrick Seale: AdipoqCre; Ebf2loxP/loxP [Angueira et al., 2020]. For perinatal experiments, male and female mice were used at indicated timepoint. For adult sorting and single cell experiments, male 13 week old CD1 mice were used. For aged lineage tracing (into adulthood), male and female mice were used for Myh11-Cre experiment, while female mice were used for PdgfraCreER experiment. Adult (male or female mice) Tp53-Cre mice were used for lineage tracing. Adult mice were ages 8-17 weeks old.

Mice were housed under the care of University of Pennsylvania University Laboratory Animal Resources (ULAR). Animals were raised at room temperature on standard chow with a 12-hour light/dark cycle. All mouse housing and husbandry occurred at RT (22°C) unless specified otherwise. For thermoneutral acclimation, 4- to 5-week old mice were housed at 30°C for one month. For chronic cold exposure, 8- to 10-week-old mice were pair-housed in cages at 4oC for one week.

Wild animals

No wild animals were used in the study.

Field-collected samples

No field-collected samples were used in the study.

Ethics oversight

Mice were housed under the care of University of Pennsylvania University Laboratory Animal Resources (ULAR), which provides both basic husbandry and veterinary care. All animal experiments were performed according to procedures approved by the University of Pennsylvania’s Institutional Animal Care and Use Committee (Approval #805649).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics

3 males aged 61-65 with BMIs ranging from 23.5-35.3 undergoing cardiac surgery.

Recruitment

Patients undergoing cardiac surgery at the Maine Medical Center. This strategy selected for patients undergoing cardiac surgery, which is not representative of the general population, but enriched for patients with older patients with coronary atherosclerotic disease which could limit interpretation.

Ethics oversight

Maine Medical Center: IRB #1396116-4

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Flow Cytometry

Plots

- Confirm that:
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  - The axis scales are clearly visible, include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Aorta were isolated from mice, minced and placed into digestion medium (DMEM, Collagenase D: 6.1mg/ml Roche), Dispase II: 2.4 mg/ml (Roche) and placed at 37°C with constant agitation at 200 rpm. To enrich for cell populations with differential sensitivity to digestion, we utilized the following procedures, for embryonic/newborn mice, 20% of the digestion was quenched at 15 minutes, 20% at 20 minutes and the remaining 60% at 25 minutes (embryonic/perinatal). For adult aorta 50% of the digestion was quenched at 30 minutes, 50% at 60 minutes. For stripped aorta flow cytometry analysis, perivascular adipose tissue was removed from the aorta and digested separately from the cleaned aorta. Tissue digestions were quenched with an equal volume of complete medium (DMEM/10% FBS). Dissociated cells were suspended using P1000 pipette and filtered through a 100μm filter. Cells were then pelleted at 400 g for 4 min and RBCs were lysed in 155mM NH4Cl, 32mM NaHCO3, and 0.1mM EDTA for 4 min. An equal volume of complete medium was added and the cells were filtered through a 40μm filter for downstream analyses.

Isolated cells were suspended in FACS buffer (HBSS, 3% 0.45μm filtered FBS, Fischer). Cells were stained in FACS buffer with antibodies (see below) for 1 h at 4°C in the dark. Cells were washed 2× with cold FACS buffer. The following sets of antibodies were used for FACS protocols.

- P3 Aorta: LEY A-BV711 (1:100 Biologend-108313, RRID:AB_2562341), CD31-PerCP-Cy5.5 (1:100 Biologend-127022 RRID:AB_2566647), CD200-APC (1:100 Biologend-MCA1558A488), CD45-APC-Cy7 (1:1000, Biologend-103115 RRID:AB_312986), CD31-APC/Fire750 (1:1000 Biologend-102528 RRID:AB_2721491), Ter119-APC/Cy7 (1:1000 Biologend 115229 RRID:AB_2137788), DI42-CF647 (1:1000 Sino Biological), FVS510 (1:1000 BD Biosciences 65406).
- P3 IWAT: CD45-APC/Cy7 (1:1000, Biologend-103115 RRID:AB_312986), CD31-APC/Fire750 (1:1000 Biologend-102528 RRID:AB_2721491), CD142-CF647 (1:1000 Sino Biological, cat#PS013-K010), CD16 (BD)FITC (Biologend, San Diego, CA, cat# 302704 1:100), ICAM1-PE-Cy7 (Biologend cat# 353116 1:100).

Adult Aorta: CD200-APC (1:100 Biologend-MCA1558A488), CD45-APC-Cy7 (1:1000, Biologend-103115 RRID:AB_312986), CD31-APC/Fire750 (1:1000 Biologend-102528 RRID:AB_2721491), Ter119-APC/Cy7 (1:1000 Biologend-116223 RRID:AB_2137788), MCAM-PerCP/Cy5.5 (1:1000 Biologend-134709 RRID:AB_11704083), PDGFRA-PE-Cy7 (1:1000 Biologend-135512 RRID:AB_2715974), and DAPI (1:10000 Roche 10235279001).

Instrument

BD FACS Aria II cell sorter (BD Biosciences) with a 1.0 micron nozzle.

Software

All compensation was performed at the time of acquisition in BD FACS DIVA 8.0.1 software using single stained cells or compensation beads (Biologend catalog no. A10697). FACS plots appearing in figures were generated in FlowJo v10.6.2.

Cell population abundance

Cell population abundance is indicated on each FACS plot. Purity was assessed for each FACS strategy by sequencing sorted populations and comparing their transcriptomes to scRNA seq populations, assessing the validity of the FACS strategy.

Gating strategy

FACS strategies were designed on the basis of scRNA seq expression data. Every experiment included fluoro-minus-one (FM0) controls for each gate. Negative gates were set on the basis of FMO staining within each experiment.

- P3 Aorta: Initial Gate: SSC-A vs FSC-A, Singlet Gates: FSC-H vs FSC-W, SSC-H vs SSC-W. Live Non Immune, Non Endothelial, Non Erythroid Gate: FVS510 vs CD45, CD31, Ter119. Negative cells from the prior gate cells were gated to isolated the following cell populations: Aorta Progenitors (Ly6a high) which were sorted. Ly6a negative cells were then gated on CD142 and CD200 to isolate Smooth Muscle Cells [CD142+, CD200+] and Aorta Progenitors [CD142 Intermediate, CD200-]. CD142+ CD200+ double positive cells were further gated on the basis of CD31 to separate mesothelial cells (CD31+) from Intermediate Cells (CD31-).

- Adult Aorta: Initial Gate: SSC-A vs FSC-A, Singlet Gates: FSC-H vs FSC-W, SSC-H vs SSC-W. Live Non Immune, Non Endothelial, Non Erythroid Gate: DAPI vs CD45, CD31, Ter119. Negative cells from the prior gate cells were gated to isolated the following cell populations: Intermediate Cells [PDGFRα+, MCAM-, CD200-], Smooth Muscle Cells-1 (SMC-1) [PDGFRα+, MCAM-, CD200-], SMC-2 [PDGFRα+, MCAM+, CD200-].

- P3 IWAT: Initial Gate: SSC-A vs FSC-A, Singlet Gates: FSC-H vs FSC-W, SSC-H vs SSC-W. Live Non Immune, Non Endothelial, Non Erythroid Gate: DAPI vs CD45, CD31, Ter119. Negative cells from the prior gate cells were gated to isolated the following cell populations: CD142+ cells [CD142+] which were sorted. CD142 Low cells were then gated on DPP4 and ICAM1 to isolate: DPP4+ Progenitors [CD142-, DPP4+, ICAM1-] and ICAM1+ Progenitors [CD142-, DPP4-, ICAM1+].

*Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary information.*