A single-cell transcriptomic inventory of murine smooth muscle cells

Graphical abstract

Highlights
- A comparative single-cell resource of SMCs from four murine organs
- Characterization of inter- and intra-organ molecular heterogeneity of SMC subtypes
- Identification and molecular characterization of venous SMC and intermediate cells
- Publicly available database for gene-by-gene and organ-by-organ exploration

Authors
Lars Muhl, Giuseppe Mocci, Riikka Pietilä, ..., Michael Vanlandewijck, Urban Lendahl, Christer Betsholtz

Correspondence
lars.muhl@ki.se (L.M.), christer.betsholtz@igp.uu.se (C.B.)

In brief
This study presents a comprehensive resource of vascular and non-vascular smooth muscle cells (SMCs) from four adult murine organs at single-cell resolution. Here, Muhl et al. identify inter- and intra-organ heterogeneities of arterial SMCs, specific transcriptional profiles of venous SMC and intermediate cells, and functional and regulatory hallmarks of SMC subtypes.
A single-cell transcriptomic inventory of murine smooth muscle cells

Lars Muhl,1,5,* Giuseppe Mocci,1 Riikka Pietilä,2 Jianping Liu,1 Liqun He,2 Guillem Genové,1 Stefanos Leptidis,1 Sonja Gustafsson,1 Byambajav Buyandelger,1 Elisabeth Raschperger,1 Emil M. Hansson,1 Johan L.M. Björkegren,1,3 Michael Vanlandewijck,1,2 Urban Lendahl,1,4 and Christer Betsholtz1,2,*

1Department of Medicine (Huddinge), Karolinska Institutet, 141 57 Huddinge, Sweden
2Department of Immunology, Genetics and Pathology, Rudbeck Laboratory, Uppsala University, 751 85 Uppsala, Sweden
3Department of Genetics and Genetic Science, Icahn Institute for Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
4Department of Cell and Molecular Biology, Karolinska Institutet, 171 77 Stockholm, Sweden
5Lead contact
*Correspondence: lars.muhl@ki.se (L.M.), christer.betsholtz@igp.uu.se (C.B.)
https://doi.org/10.1016/j.devcel.2022.09.015

SUMMARY

Smooth muscle cells (SMCs) execute important physiological functions in numerous vital organ systems, including the vascular, gastrointestinal, respiratory, and urogenital tracts. SMC differ morphologically and functionally at these different anatomical locations, but the molecular underpinnings of the differences remain poorly understood. Here, using deep single-cell RNA sequencing combined with in situ gene and protein expression analysis in four murine organs—heart, aorta, lung, and colon—we identify a molecular basis for high-level differences among vascular, visceral, and airway SMC, as well as more subtle differences between, for example, SMC in elastic and muscular arteries and zonation of elastic artery SMC along the direction of blood flow. Arterial SMC exhibit extensive organotypic heterogeneity, whereas venous SMC are similar across organs. We further identify a specific SMC subtype within the pulmonary vasculature. This comparative SMC cross-organ resource offers insight into SMC subtypes and their specific functions.

INTRODUCTION

Smooth muscle cells (SMCs) are essential for a vast array of organ functions. In contrast to skeletal muscle cells, SMC lack striation, do not fuse into multinucleated myotubes and respond to exogenous stimuli leading to Ca2+-dependent contraction propagated via gap junctions (Pogoda et al., 2019). The physiological roles of SMC span developmental organogenesis (Jaslove and Nelson, 2018), uptake, and distribution of oxygen and nutrients by the airways, gastrointestinal (GI) system and vasculature (Fisher, 2010; Liu and Gomez, 2019; Spencer et al., 2021), urine storage/discharge by the bladder (Andersson and Ameer, 2004), and contraction of the uterus during delivery (Shynlova et al., 2009).

SMC organize into anatomical structures with different architectures reflecting the diverse physiological tasks. Vascular SMC wrap the endothelial cells to stabilize the vessel structure and regulate the tube diameter (Reho et al., 2014). Vascular SMC coverage ranges from the multilayered media of large arteries to a sparse coating of veins by morphologically distinct SMC (Vanhouette and Janssens, 1978). In capillaries, a SMC-related cell type—the pericyte—provides partial coverage of the endothelial tubing (Armulik et al., 2011). Visceral SMC of the GI tract display a distinct organization with both circularly and longitudinally oriented SMC bundles, allowing swallowing, stretch resistance, peristaltic movements, and mucus secretion from goblet cells (Greenwood and Davison, 1987). Tracheal SMC stabilize the airways to ensure proper airflow (Zhang and Gunst, 2008). The anatomical differences are paralleled by functional differences. Visceral SMC display coordinated contraction (single unit), whereas vascular SMC contract individually (multunits). Also, the contraction profile of visceral SMC is fast (phasic), whereas it is slow (tonic) for vascular SMC (Himpens et al., 1988).

SMC malfunction contributes to several diseases. Vascular SMC respond to injury during atherosclerosis resulting in a shift from a contractile to a synthetic phenotype (Liu and Gomez, 2019; Wirka et al., 2019). Dysregulated visceral SMC contraction is a hallmark of gastroparesis and constipation (Camilleri et al., 2017). Structural remodeling and hyper-responsiveness of airway SMC contribute to asthma and other pulmonary diseases (Jones et al., 2016; Lauzon and Martin, 2016).

Despite the differences in cell morphology and tissue organization, we lack insight into the common and heterogeneous molecular traits of different SMC subtypes. SMC are notoriously missing or under-represented in thus far published single-cell RNA sequencing (scRNA-seq) data (He et al., 2020; Tabula Muris et al., 2018), probably reflecting inefficient SMC isolation using
Figure 1. Single-cell RNA sequencing of SMC
(A) IF staining for CD31 (all endothelial cells) and EMCN (capillary and venous endothelial cells; Paz and D’Amore, 2009) together with reporters Acta2GFP and Cspg4DsRED in heart (upper panel), lung (middle panel), and colon (lower panel). Arrows indicate venous SMC. Arrowheads indicate arterial SMC. Open arrows indicate airway SMC (lung) or visceral (muscularis) SMC (colon). Open arrowheads indicate interstitial (mucosal) SMC. Boxed areas are magnified in the right panels. Nuclei are stained by Hoechst 33342. Scale bars, 100 μm.

(B) Schematic representation of reporter gene expression in different populations of SMC from heart, lung, and colon.

(legend continued on next page)
standard protocols. The lack of known SMC-specific cell surface markers limits prospects for antibody-based enrichment of SMC. In the present study, we therefore utilized transgenic SMC reporter mice to capture vascular, visceral, and airway SMC from heart, lung, and colon for scRNA-seq analysis, and we mapped the anatomical location of the various SMC subtypes using immunofluorescence (IF) and in situ RNA hybridization (ISH). Our data demonstrate common and distinct transcriptomic signatures of different SMC subtypes, along the proximo-distal axis of large arteries, between arteries and veins, and between arteries from different organs. Collectively, the data provide a resource for improved understanding of SMC diversity, function, and regulation.

RESULTS

Transcriptional demarcation and core gene signature of SMC

We found that two transgenic reporter alleles in combination, Acta2GFP (Acta2 encodes smooth muscle actin x2) and Cspg4dsRED (Cspg4 encodes chondroitin sulfate proteoglycan 4/NG2), labeled SMC broadly in tissue sections and allowed discrimination between SMC populations as follows: Arterial SMC were positive for both Acta2GFP and Cspg4dsRED, venous and non-vascular SMC were positive for Acta2GFP but negative for Cspg4dsRED, and pericytes were positive for Cspg4dsRED but largely negative for Acta2GFP (Figures 1A and S1A). Using compound Acta2GFP::Cspg4dsRED reporter mice, we sorted cells from the heart, lung, and colon (Figures 1B and S1B). These organs were selected to provide representation of vascular, visceral, and airway SMC (Figures 1A–1C). Aortic SMC were isolated separately and without reporters (Figure S1C). We applied Smart-Seq2 (Picelli et al., 2014) to achieve the deepest possible RNA-seq information from individual cells. Besides SMC, we collected other stromal cell types, such as endothelial cells, pericytes, and fibroblasts, and we included certain previous single-cell transcriptomes generated with Smart-Seq2 (Muhl et al., 2020, 2022; Vanlandewijck et al., 2018) in the analysis (Figure 1C). In total, 9,813 single-cell transcriptomes passed quality thresholds: 3,062 from heart, 675 from aorta, 2,817 from lung, and 3,259 from colon (Figures 1D–1F and S1D–S1G). We used the pagoda2 algorithm (Fan et al., 2016) to define cell clusters and uniform manifold approximation and projection (UMAP) (Becht et al., 2018), sorting points into neighborhoods (SPINs) (Tsafir et al., 2005), and bar plots for data visualization (Figures S1D–S1G and S2). The bar plot visualizations of gene expression can be explored organ-by-organ and gene-by-gene at http://betsholtzlab.org/Publications/SMC/database.html.

We first demarcated SMC transcriptomes from those of other stromal cell types for each organ system separately. Broad cell type annotations were guided by known marker gene expression (Armulk et al., 2011; Fisher, 2010; Vanlandewijck et al., 2018) and previously assigned multi-gene signatures for discrimination between mural cells (pericytes and SMC) and fibroblasts (Muhl et al., 2020; Figures S1D and S1E). SMC molecular hallmarks included well-established markers such as Acta2, Tagln, Cnn1, and Myh11 (Fisher, 2010) combined with the lack of or very low expression of fibroblast markers such as Pdgfra and of endothelial cell markers such as Pecam1 (Figures S3A–S3C). To identify common SMC-enriched genes, we assigned transcripts using stringent threshold criteria (see STAR Methods). In this way, a signature of 12 genes common to all SMC was identified: Ppp1r12b, Lmod1, Myf9, Tpm2, Actg2, Dmpk, Cnn1, Tagln, Myh11, Mylk, Acta2, and Fna (Figures 1G–1I and S4A–S4D; Table S1). Several of these genes encode known constituents of the contractile apparatus, as corroborated by gene ontology (GO) analysis (Figure S4E). The protein phosphatase 1 regulatory subunit 12B (MYPT2; encoded by Ppp1r12b), has been suggested to be specifically expressed in heart, skeletal muscle, and brain, in contrast to the widely expressed MYPT1 isoform encoded by Ppp1r12a (Ito et al., 2022). However, our data highlight Ppp1r12b as part of the pan-SMC signature, implicating a universal role of MYPT2 in SMC contraction through the regulation of myosin light-chain phosphorylation.

SMC organotypicity

Our data also implicated the existence of SMC subpopulation-specific gene expression profiles (Figures 1G–1I; Table S1). To explore those in further detail, we collected all cells defined as SMC from all organ datasets and combined them for further in-depth analysis. This dataset, consisting of 471 cells from heart, 572 from aorta, 831 from lung, and 906 from colon (Figures 2A, 2B, and S3), was re-clustered into 10 clusters representing putative SMC subtypes. Eight of these clusters contained cells mainly from a single organ. Two of the clusters contained cells from two or three organs, respectively, indicating that certain SMC subtypes span organ boundaries (Figures 2A and 2B; Table S2).

We next assessed the differences between the clusters and assigned each cluster to a specific SMC subtype or set of subtypes (Figures 2A and 2B). These annotations, for which experimental validation is provided in subsequent paragraphs, identified that vascular and non-vascular SMC occupied distinct regions of the UMAP landscape (Figure 2C), reflecting qualitative as well as quantitative gene expression differences. For example, Notch3, a known marker for vascular SMC (Domenga et al., 2004; Prakash et al., 2002), marked all vascular SMC clusters (#1,2,3,5,6,7,8), whereas the hedgehog signaling modulator Hhip marked all non-vascular SMC clusters (#4,9,10) (Figure 2C). A display of the top 50 enriched transcripts for each cluster marked unique, shared, and cluster subset-specific gene expression patterns, the latter demonstrating heterogeneity also within the individual SMC clusters (Figure 2D). Examples...
Figure 2. SMC subtype-specific gene expression
(A) UMAP visualization of the inter-organ SMC dataset color coded for organ of origin and pagoda2 cluster annotation (compare with Figure S3D).
(B) Bar plot visualization of pagoda2 clusters, color coded for organ of origin, showing Acta2 levels in individual cells (bars). The cell order in each cluster is determined by SPIN. The cellular composition of each cluster is given.
(C) The expression levels for Notch3 and Hhip are presented as bar plots and superimposed on the UMAP landscape (blue: high, gray: low).
(D) Heatmap showing the 50 most enriched genes for each of the ten pagoda2 clusters of the inter-organ SMC dataset. Single and double asterisks indicate the location of Notch3 and Hhip, respectively.
(E) The expression levels of representative genes from (D) are shown as bar plots or superimposed on the UMAP landscape (blue: high, gray: low).
of transcripts enriched in individual, or combinations of clusters included Itgb1 (aortic SMC), Offr78 (heart and colon arterial SMC), Chrm2 (airway SMC), Actc1 (lung arterial SMC), Odrd1 (venous and visceral SMC), Bdkrb2 (interstitial SMC), and Shisa3 (visceral SMC) (Figure 2E).

The cluster dispersal in UMAP display further suggested extensive organotypicity among the vascular SMC. Interestingly, although arterial SMC clustered largely according to organ origin, venous SMC formed a single cluster (#7) suggesting a relative homogeneity (Figures 2A and 2B).

Vascular SMC

Our analysis identified distinct clusters of airway, interstitial, visceral, and vascular SMC. Below, we first characterize the various subtypes of vascular SMC in the order of elastic arteries to muscular arteries to veins.

SMC in the elastic arteries

A key function of large elastic arteries is to distend at systole and recoil at diastole, thereby keeping blood pressure and perfusion constant over the cardiac cycle. Consistent with a molecular specialization of elastic artery SMC, we found aortic SMC localized to clusters #1 and #2, which together form a separate island in the UMAP landscape (Figure 2A). 83.2% (564 of 678 cells) of the cells in these clusters originated from dissected aortas (aortic arch and descending aorta until the 3rd rib), whereas the remaining cells originated from isolated hearts containing the aortic root. Aortic SMC exhibited a distinct expression profile compared with other SMC populations, exemplified by Sost (encoding sclerostin [SOST]) and Npnt (encoding nephronectin [NPNT]) expression (Figures 3A and SSA). SOST is a glycoprotein involved in bone growth (Li et al., 2019). SOST IF analysis confirmed its expression in the aortic media and absence in smaller arterial branches (Figure 3B). IF analysis of NPNT, an extracellular matrix (ECM)-localized integrin ligand, and the integrin α-8 subunit (ITGA8) further corroborated the identity of clusters #1 and #2 cells as aortic SMC (Figure SSA).

Different origins and hemodynamics have been suggested to correlate with SMC heterogeneity along the proximo-distal aortic axis (Pfaltzgraff and Bader, 2015). Putative aortic SMC from heart preparations distributed mainly to cluster #1, whereas aorta-derived SMC distributed to both cluster #1 and #2 (Figures 2A, 2B, and SSA), suggesting that cluster #1 represents proximal SMC and cluster #2 distal SMC within the aortic segment analyzed. Differential gene expression analysis between clusters #1 and #2 identified molecular differences exemplified by Pkp4, Mylk4, and Foxc2 expression in the distal SMC (Figure 3C). GO analysis showed an enrichment of coagulation-related terms in the proximal SMC (cluster #1) and terms related to developmental processes in the distal SMC (cluster #2, Figure 3D), possibly reflecting the differences in blood flow at the two locations (turbulent at the outflow tract and laminar further distally). The proximal aortic SMC (cluster #1) showed signs of zonation in both SPIN and UMAP displays (Figures 3E and 3F), i.e., a gradual change in gene expression from the aortic root toward the aortic arch and further into the thoracic aorta.

SMC located at the aortic root were enriched with Dcn, Des, Fbn1, and Lum, suggesting a higher resemblance to fibroblasts and pericytes than more distally located aortic SMC. Conversely, the distal SMC were enriched with Aqp1, Npr1, Sfrp2, and Sost (Figures 3E, 3F, and SSA). Combined IF analysis of SOST and desmin (DES) confirmed the predicted zonation of aortic SMC from the outflow tract toward the thoracic aorta (Figures 3G, 3H, and SSD; Dobnikar et al., 2018; Kan et al., 2021).

The pulmonary circulation is a high-volume and a low-pressure system (Towsley, 2012). The right cardiac ventricle pumps de-oxygenized blood into the lungs via the pulmonary arteries and oxygenated blood returns to the left atrium through the lung veins. Pulmonary arteries are elastic similar to the aorta. Cells within cluster #5 were annotated as pulmonary SMC. They were the closest neighbors of the aortic SMC in the UMAP landscape (Figure 2A) and showed gene expression similarity to aortic SMC (Figures 2D and 2E). Like the aortic SMC, the pulmonary arterial SMC exhibited molecular zonation along their proximo-distal axis, as identified by both SPIN and UMAP displays (Figures 4A and 4B). Along this axis, several transcripts, for example, Thbs1, Tbx5, and S1pr3, distributed distally, whereas Efhd1, Lgr6, Sfrp2, Sost, and neural cell adhesion molecule 1 (Ncam1) distributed proximally (Figures 4A–4C). The molecular zonation was confirmed at the protein level by IF for SOST and NCAM1 (Figures 4D, 4E, S5E, and 5F). We found both gene expression similarities (Sost and Ncam1) and differences between aortic and pulmonary arterial SMC (Figure 4F), the latter possibly reflect adaptations to the differences in blood pressure and flow (Saouti et al., 2010). For example, aortic SMC exhibited
Figure 4. Characterization of arterial SMC

(A) Heatmap showing differentially expressed genes along the SPIN range of cluster #5 (pulmonary arterial SMC) of the inter-organ SMC dataset. The arbitrary split of the range into three equal parts was done to facilitate comparison between the most different cells located at the SPIN range ends.

(legend continued on next page)
enriched expression of Uchl1 (encoding a ubiquitin hydrolase), Sorbs2 (encoding sorbin and SH3 domain-containing 2, a protein associated with cardiomyopathy) (Ding et al., 2020) and Emb (encoding embigin, a transmembrane cell adhesion molecule suggested to modulate neuromuscular junction formation) (Lain et al., 2009). Pulmonary arterial SMC instead expressed Rgs2 (encoding a G-protein signaling regulator), Kcnk3 (encoding the potassium channel subfamily K member 3 [TASK-1]), and Ednrb (encoding endothelin receptor type B) (Figures 4F and 4G), the latter two suggested to be involved in pulmonary diseases (Murtaza et al., 2017; Tabeling et al., 2022). GO analysis of the differentially expressed genes showed terms related to development/differentiation and sensory perception enriched in aortic SMC and terms related to cell proliferation enriched in pulmonary arterial SMC (Figure S5G).

Common and organotypic features of muscular arterial SMC
From the elastic arteries, blood distributes further into and within the organs via muscular arteries. Muscular arterial SMC are the most abundant vascular SMC subtype and therefore usually referred to as “arterial SMC.” These cells are morphologically well characterized and readily identifiable in tissues through their morphology and expression of Acta2 and Csgp4 (Figures 1A and S1A). SMC clusters #3 and #8 contained arterial SMC from heart and colon, respectively. These clusters were neighbors but separated in the UMAP landscape, indicating organotypicity (Figure 2A). A comparison between elastic arterial SMC (from aorta and lung) and muscular arterial SMC (from heart and colon) showed extensive gene expression differences (Figure 4H). Elastic arterial SMC expressed for example high levels of Elnt (encoding elastin, the principal component of elastic laminae). Conversely, muscular arterial SMC expressed Olfr78 (encoding a G-protein coupled receptor [GPCR] and putative hypoxia sensor) (Chang et al., 2013) and Pln (encoding phospholamban, a Ca²⁺ regulator in cardiomyocytes) (Simmelman and Jones, 1998; Figures 4H, 4I, and S5H). GO analysis of differentially expressed genes between elastic and muscular arterial SMC showed an enrichment of terms related to ECM and development in elastic arterial SMC and GPCR signaling and ion transport in muscular arterial SMC (Figures 4J and 4K). ISH localized the expression of Olfr78 to coronary arteries (Figure S5I). In colon, the expression of Olfr78 and S1pr3 (encoding a GPCR for sphingosine-1-phosphate) co-localized with arterial structures (Figure S5J).

Collectively, these data confirm the identity of clusters #3 and #8 as muscular arterial SMC and identify common features of muscular arterial SMC that distinguish them from elastic arterial SMC of pulmonary arteries and the aorta. Nevertheless, SMC of muscular arteries showed organotypicity (Figure 4L), which may in part reflect distinct developmental origins as indicated by the Hoxa10 and Gata4 expression patterns and in part different metabolic regulation of ion fluxes. In line with the latter notion, Abcc8, expressed by arterial SMC in colon, but not heart (Figures 4L and 4M), encodes the sulphohydrase receptor 1 (SUR1), a component of the ATP-sensitive potassium channel that regulates glucose-stimulated insulin secretion in pancreatic beta cells (Shiota et al., 2002).

Common and organotypic features of venous SMC
Following gas and nutrient exchange in capillaries, blood passes via venules to veins and back to the heart and lung for re-oxygenation. Compared with arteries, veins have larger luminal diameter and thinner SMC coat. In marked contrast to arterial SMC, the venous SMC collected into a single cluster (#7) with limited UMAP dispersion, regardless of organ origin (Figure 5A; see also Figures 2A and 2B and Table S2). In addition to a common Csgp4-negative phenotype (Figures 1A, 1B, and S1A), we found enriched expression of Chrdl1 (encoding chordin like 1, a bone morphogenetic protein [BMP] antagonist) (Nakayama et al., 2001) and Sdc1 (encoding syndecan 1, a heparin sulfate proteoglycan cell surface receptor [CD138] involved in immune functions) (Teng et al., 2012) in venous SMC (Figures S5B and S5C). ISH confirmed the presence of Chrdl1 in venous SMC in all analyzed organs (Figures 5D–5H), and SDC1 IF was observed on the SMC surface in the azygos vein and in heart and colon veins (Figures S6A and S6B), together confirming cluster #7 as venous SMC. GO analysis of venous SMC-enriched genes identified terms related to wound healing, ECM organization, and negative regulation of cell migration (Figure 5I). Despite the cross-organ similarity between venous SMC, certain organotypicity was still present. For example, cardiac venous SMC expressed the expression of Sost on a section of the lung. Arrowheads indicate SOST positive arterial SMC. Boxed areas 1–3 are shown magnified in the right panel.
Figure 5. Characterization of venous SMC
(A) UMAP visualization color coded for organ of origin (the same as Figure 2A). The boxed area highlights the location of venous SMC (cluster #7) and is shown magnified in the right panel, depicting the cells for their origin (heart and aorta, lung, colon).
expressed Rgs5, Colec11, and Fapb4, which are also markers of cardiac pericytes (Muhl et al., 2020). Pulmonary venous SMC were instead enriched for Rgs2, Vdflr, Fads1, and Fads2 (the latter two encoding fatty-acid desaturases) (Figure S6C), genes also expressed by lung arterial SMC (compare with Figures 4F–4I). It is conceivable that the different gene expression patterns in venous SMC from heart and lung may reflect metabolic specializations or adaptations of the vascular beds. Venous SMC from the colon on the other hand showed enriched expression of ECM-encoding transcripts such as Tnc (encoding tenacin C) and Hmcdn2 (encoding hemicentin 2) (Figure S6C).

**Intermediate SMC**

Cluster #6 was located in-between the lung arterial (cluster #5) and venous (cluster #7) SMC clusters in the UMAP landscape (but far from the airway SMC [cluster #4] as separately discussed below) (Figure 5j; see also Figures 2A and 2B). We therefore named cluster #6 “intermediate cells,” a term used previously (Townsley, 2012). When analyzed together with all lung cells and without the presence of cells from other organs, the intermediate cells formed part of the lung venous SMC cluster (Table S2). However, compared with lung venous SMC, intermediate cells expressed higher levels of Gja4, Vtn, and Hgd1b, which are known to be enriched in pericytes (He et al., 2016, 2018; Vanlandewijck et al., 2018) (Figure S6D). Compared with lung pericytes, intermediate cells expressed typical vascular SMC genes, such as Eln, Tagln, Myh11, and Acta2, but their expression of Cspg4 was low similar to venous SMC (Figure S6E). Of note, most intermediate cells (37 of 44 cells) were collected through the Acta2-positive/Cspg4-negative sorting protocol (Tables S3 and S4). Together, these observations led us to hypothesize that intermediate cells are SMC located at terminal arterioles and/or post-capillary venules, sites previously suggested to harbor a specialized SMC subtype (Townsley, 2012). Intermediate cells expressed high levels of Adora1 (encoding adenosine A1 receptor) and Colba1 (encoding type-VIII collagen α1 [COL8A1]). IF analysis of COL8A1 showed expression in SMC of small caliber non-capillary lung vessels (Figures 5K and S6F), supporting a location at terminal arterioles and/or post-capillary venules. Intermediate cells expressed high levels of Notch3 and Pdgfrb (Figure S6F), a profile previously suggested for SMC involved in the progression of pulmonary hypertension (Li et al., 2009; Sheikh et al., 2015; Steffes et al., 2020). The high and specific expression of Adora1, a vascular pro-inflammatory modulator in mouse allergic asthma (Ponnoth et al., 2010) and culprit in pulmonary arterial hypertension (Ålenkar et al., 2017), further points to intermediate cell involvement in pulmonary disease. Future systematic investigation to identify the exact location(s) of intermediate cells within the pulmonary vascular tree and their mechanistic role(s) in pulmonary vascular remodeling is warranted in the search for new principles for the treatment of lung disease.

**The unique features of non-vascular SMC**

We identified putative non-vascular, Hhip-positive, SMC in the lung (airway SMC, cluster #4) and colon (interstitial and visceral SMC in clusters #9 and #10, respectively), which showed distinct and organotypic distribution within the UMAP landscape of the inter-organ SMC dataset (Figure 6A).

**Airway SMC**

In addition to the expression of Hhip (Danopoulos et al., 2020), airway SMC (cluster #4) showed enriched expression of Chrm2 (encoding the muscarinic cholinergic receptor 2), Foxf1 (encoding the forkhead box transcription factor F1), Gja1 (encoding the gap-junction protein 1/connexin 43), and Cldn1 (encoding the tight junction protein claudin 1), the latter a suggested marker of airway SMC (Fujita et al., 2011; Figures 6B and 6C; see also Figures 2D and 2E). Kcnj8, which is expressed by pericytes (Armulik et al., 2011) and plays a role in SMC differentiation in the brain (Ando et al., 2022), was also highly expressed by airway SMC (Figure 6B). ISH for Chrm2 and Hhip (Figures 6D, S6G, S6H, and S6I) and IF for connexin 43 (GJA1) and H Hipp confirmed the identity of cluster #4 cells as airway SMC (Figures 6E and S6H). The contractile potential of these cells is associated with, for example, asthma (Doeing and Solway, 2013; James et al., 2018), emphasizing the importance of identifying targets amenable to specific pharmacological targeting. GO analysis of airway SMC-enriched genes identified terms related to epithelial and organ morphogenesis (Figure S6I), underscoring the potential importance of airway SMC for proper lung structure (Jasiose and Nelson, 2018).

**Common and specific features of visceral and interstitial SMC**

The intestine contains visceral SMC in several distinct anatomical structures. A circularly oriented SMC layer, the muscularis mucosae and muscularis externa. In...
addition, a network of interstitial SMC is present in the colonic mucosa (Muhl et al., 2020). Visceral and interstitial SMC were clearly distinct from vascular SMC in colon (Figure 6F), as shown by their enriched expression of for example Agt (encoding angiotensinogen), Tgfb1 (encoding the matricellular protein transforming growth factor β-induced), and Adamdec1 (encoding the intestine-specific metallo-endopeptidase decisin 1) (Figures 6F and 6G). Furthermore, transcriptional differences between visceral and visceral SMC were found (Figure 6H). Interstitial SMC showed enriched expression of for example Rgs2, Acta1, Cpxm2, Tntrsf19, and F2r (the latter encoding the thrombin receptor [PAR1]). Visceral SMC were instead defined by their enriched expression of Grem2 (encoding gremlin 2, a BMP signaling antagonist), Shisa3 (encoding a transmembrane modulator of wingless/integration [WNT] and fibroblast growth factor [FGF] signaling), Foxp2 (encoding the forkhead box transcription factor P2), and (the venous SMC marker) Chrdl1 (Figures 6H and 6I compare with Figure 5). Interestingly, the differential expression of Lamb1 and Lamb2 (encoding the basement membrane components laminin β1 and β2) in interstitial and visceral SMC, respectively, suggests specific ECM requirements in the colonic mucosa and muscularis compartments (Figure 6H). ISH for Grem2 and Chrdl1 and IF for HHIP, SHISA3, and NPNT confirmed the identity of clusters #9 and #10 as interstitial and visceral SMC, respectively (Figures 6I–6L and compare with Figure 5). GO analysis of differentially expressed genes between interstitial and visceral SMC identified terms related to blood circulation, blood vessel diameter maintenance, or wound healing enriched in interstitial SMC (Figure 6C), whereas terms related to Wnt/beta-catenin signaling or immune-cell proliferation enriched in visceral SMC (Figure 6F). In summary, these data illustrate common and specific attributes among non-vascular SMC in the colon that demarcate them from vascular SMC and each other.

**Transcriptional heterogeneity underlying SMC subtype function and regulation**

SMC contraction mechanisms differ from those of striated muscle cells and are controlled by a plethora of signaling molecules, receptors, and exogenous stimuli (Reho et al., 2014). The molecular diversity among SMC identified by the present study may be expected to reflect specific differences in the physiological functions and their regulation in different vascular and non-vascular SMC populations. Below, we describe and discuss the enrichment of biological process GO terms, and more specifically, the differential distribution of transcription factors, GPCRs, and certain other signaling modalities between different subtypes of SMC.

**Biological processes**

**GO**

To compare GO terms globally across the different SMC subtypes, we used the list of cluster-specific differentially expressed genes, yielding enriched GO terms in eight of the ten SMC clusters (Figures 7A; Table S5). For example, the aortic SMC (cluster #2) showed terms related to angiogenesis and vasculature as well as ECM (Figure 7A compare with Figures 3D, 4J, and 5G). In contrast, the muscular arterial SMC (cluster #3 and cluster #8) displayed terms associated with blood circulation, ion homeostasis, and GPCRs (Figure 7A compare with Figure 4K). Venous SMC (cluster #7) showed terms related to inflammation or immune response, among others (Figure 7A compare with Figure 5I). Intermediate cells (cluster #6) exhibited few significantly enriched terms related to chemotaxis, Rho-signaling, and cell-cell adhesion (Figure 7A). Airway SMC (cluster #4) exhibited enriched terms related to muscle morphogenesis and development as well as regulation of blood circulation and response to FGF (Figure 7A compare with Figure 5I), whereas interstitial or visceral SMC (cluster #9 and cluster #10, respectively) showed enrichment of terms related to development and morphogenesis as well as ECM organization or Wnt signaling pathway, respectively (Figure 7A compared with Figures 5C and 5D).

**Transcription factors**

Transcription factors drive sub-specialization of many cell types. To investigate the impact of transcription factors on molecular differences between SMC subtypes, we applied the single-cell regulatory network inference and clustering (SCENIC) algorithm (Aibar et al., 2017) to identify active gene regulatory networks (GRNs) within different SMC subpopulations. Clustering of the
Figure 7. Specification of SMC subtypes

(A) Dot plots showing the GO result of differentially expressed genes of each specific cluster compared with all other clusters.

(B) Heatmap showing differentially expressed GPCR-encoding genes across the inter-organ SMC dataset.

Figure continued on next page.

Open Access

Resource

Developmental Cell

57, 2426–2443, October 24, 2022

Developmental Cell
SMC populations based on GRN activity provided a similar SMC cluster distribution and relationships as previously established by hierarchical clustering (Figures S7E and S7F compare with Figure S3D). Gene-by-gene comparison of selected transcription factor families further identified distinct expression patterns (see Figures S7G–S7I for a detailed account). The expression profiles of members of the T-box transcription factor (TBX) family (Papaloannou, 2014) reflected organotypicity (Figure S7G). For example, SMC of the large pulmonary elastic arteries (right part of cluster #5) were negative for the expressions of Tbx4 and Tbx5 (compare with Figures 4A and 4B), suggesting a different developmental history of these SMC compared with other pulmonary SMC populations (Lee et al., 2016). The expression of Gata5 recapitulated the pattern seen from Tbx4 and Tbx5, whereas Gata3 was expressed (albeit weakly) in aortic SMC and was reciprocal to Tbx4 and Tbx5 in pulmonary arterial SMC, suggesting a lineage relationship between aortic and pulmonary elastic arterial SMC (Figure S7H). NK homeobox (Nkx) transcription factors are known to play a pivotal role during development and tissue patterning (McMahon, 2000; Stanfel et al., 2005). Expression of Nkx2-3 was colon specific, whereas Nkx3-2 was restricted to visceral SMC of the colon musculature (Figure S7I). Of note, we found expression of Nkx2-5 specifically in SMC of the aortic root, whereas expression of Nkx6-1 was enriched in more distally located aortic SMC, in line with the observed SMC zonation from the cardiac outflow tract toward the thoracic aorta (Figure S7I compare with Figures 3C–3H) (Dobnikar et al., 2018; Gittenberger-de Groot et al., 1999; Kan et al., 2021). SMC located in different layers of the aortic wall have previously been shown to originate from the cardiac neural crest (inner layers) or second heart field (outer layers) (Sawada et al., 2017), adding additional complexity to ascending aortic SMC diversity.

**GPCRs**

Innervation of SMC by the autonomous nervous system provides crucial physiological regulation (Sheng and Zhu, 2018; Webb, 2003). GPCR are key transducers of signals from the nervous system to control SMC function (Jacoby et al., 2006). Based on a curated list of targetable GPCR (https://www.guidetopharmacology.org; in total 356 GPCR genes), we found 162 SMC-expressed GPCR (Table S6), 43 of which exhibited differential expression between SMC subtypes (Figure 7B). Expression of the adrenergic receptors Adra1a and Adra2a was particularly pronounced in arterial SMC in colon, whereas Adra1d showed the strongest expression in aortic and pulmonary arterial (i.e., elastic) SMC (Figures 7B and 7C). Conversely, the expression of Adra2b was strong in heart and colon arterial SMC (Figures 7B and 7C), suggesting differential adrenergic regulation of elastic and muscular SMC (Sheng and Zhu, 2018). Sctr (encoding the secretin receptor) showed specific expression in colon arterial SMC. Additionally, Chrm2 was specifically expressed by airway SMC, indicating cholinergic regulation of bronchial tone and lumen (Figures 7B and 7C; see also Figures 2D, 2E, 6B, and 6C). Tacr2 (encoding tachykinin receptor 2) and Bdkrb2 (encoding bradykinin receptor beta-2) showed differential expression in interstitial and visceral SMC of the colon, respectively (see also Figures 2D and 2E), in line with the fine-tuned regulation of intestinal contraction (Steinhoff et al., 2014). Of note, deletion of Tacr2 results in disturbed gastric emptying (Mao et al., 2017), emphasizing its importance for proper GI function. SMC of the muscularis propria displayed high levels of the adenosine receptor Adora1 (Figures 7B and 7C), similar to the intermediate cells of the pulmonary vasculature (Figures S6D and S6F).

**Growth factors, morphogens, and Ca²⁺-handling**

The BMP and WNT signaling pathways are major contributors to tissue patterning and homeostasis. Of the 553 genes compiled in the GO terms BMP signaling pathway and WNT signaling pathway, we found 434 to be expressed in the SMC dataset, of which 79 were differentially expressed between SMC subtypes (Figure 7D). These differences are exemplified by enriched expression of Sulf1 in aortic SMC, Rspos3 in visceral SMC, and Sal1 in interstitial SMC (Figures 7D and 7E). Scx (encoding the transcription factor scleraxis, implicated in the regulation of BMP and WNT signaling) was expressed in aortic SMC and airway SMC (Figures 7D and 7E), suggesting specific ECM requirements at these anatomic sites (De Micheli et al., 2020). Interestingly, the Notch pathway-regulated gene Nrarp (Krebs et al., 2001) was specifically expressed in peripheral arterial SMC and intermediate cells of the lung (Figures 7D and 7E), suggesting enhanced Notch signaling in SMC of muscular, but not elastic, arteries.

SMC contraction is controlled by the cytosolic Ca²⁺ concentration (Webb, 2003), regulated among others by the calsequestrin (CASQ) calcium-buffering proteins. We found Casq1 and Casq2 (encoding CASQ1 and 2, respectively) to be differentially expressed between SMC populations (Figure S7J). The expression pattern of Casq1 was similar to that of Adra1d (encoding the adrenergic receptor α1d), possibly indicating a functional link between these genes in the regulation of contractility in distinct SMC populations (Figures 7C and S7J).

**DISCUSSION**

We provide molecular insights into SMC diversity across different organs, ranging from high-level molecular signatures demarcating SMC from non-SMC and vascular from nonvascular SMC to more subtle signatures reflecting organotypic specialization and subtype diversification. We also provide evidence for molecular zonation of SMC along the heart-to-pulmonary axis of elastic arteries and identify that arterial SMC display profound organotypicity (Gittenberger-de Groot et al., 1999), whereas venous SMC are considerably more similar across organs. Finally, we molecularly identify intermediate cells, a SMC subtype of the pulmonary circulation.

Our transcriptomic inventory of SMC, which is provided as an appended database allowing online access to gene-by-gene expression patterns at http://betsholtzlab.org/Publications/SMC/database.html, is important for several aspects of SMC...
research. First, the resource provides several markers to identify the various SMC subtypes in different organs. Second, new research tools, such as novel Cre-driver and reporter mouse lines, can be generated based on the information, allowing for improved lineage-tracing and cell sorting approaches. Third, the resource is relevant for decoding the role of SMC subtypes in various physiological contexts. Distinct SMC subtypes can now be identified, traced, and explored in organ development, aging, or disease. For example, the spatial organization (zonation) of SMC at the cardiac outflow tract and pulmonary arteries/arterioles suggests that distinct SMC subpopulations may contribute differentially to physiological functions as well as pathological mechanisms, and an in-depth analysis could provide clues relevant to understanding specific roles for diseases, such as atherosclerosis (Alencar et al., 2020; Misra et al., 2018; Pan et al., 2020), aortic dissection, and aneurysm (MacFarlane et al., 2019; Yassine et al., 2017) or pulmonary vascular remodeling (Sheikh et al., 2015; Steffes et al., 2020). Furthermore, the organotypicity of arterial SMC suggests specific functions of SMC in the respective tissue environment, for example, with regard to ion fluxes or ECM composition and homeostasis. In contrast, venous SMC exhibited only minor heterogeneity, suggesting more similar physiological requirements of the venous system across organs. Tissue-specific supporting structures, such as the pulmonary myocardium (Millino et al., 2000; Mommersteeg et al., 2007), may accommodate venous structure and stability, thus mitigating the need for specialized venous SMC subtypes.

Finally, the provided resource will prove useful in exploring novel targets for pharmacological therapy development, and we exemplify this by providing evidence for subtype-specific expression of GPCRs, WNTs, BMPs, and Ca²⁺-regulators, classes of proteins for which drugs have been developed for other medical purposes and which potentially can be repurposed for diseases related to SMC dysfunction. The specific targeting of only disease-relevant SMC subpopulations would be particularly appealing, as it may reduce adverse side-effects (Hauser et al., 2007; Livshits and Kalinkovich, 2021). In conclusion, this transcriptomic inventory of SMC subtypes will prove useful to further explore the multifaceted roles of different SMC populations and hopefully inspire new research exploring the functions of SMC subtypes in health and disease.

**Limitations of the study**

Although we present transcriptomic and histological analyses of SMC from four different murine tissues and annotate up to ten different SMC populations, we cannot formally exclude the possibility that other SMC populations may have been lost during the cell isolation process and therefore are not present in this resource. We also cannot exclude that the transgenic reporter used to sort SMC may have influenced the transcriptome of the cells, for example, through toxicity of the expressed fluorescent proteins. Our data should therefore be viewed as a first-generation SMC transcriptome resource that should be challenged and further developed through alternative methods and SMC analyses of other organs and species.

Other limitations that are generic to studies of this type include the process of cell type annotations and the criteria for inclusion into or exclusion from a pre-existing cell class. What should count as an SMC and as a certain SMC type or subtype? It is tempting to use the cluster assignment as an unbiased classification method, but it should be remembered that the clustering results depend on the parameters/variables set for the clustering algorithm, and the heterogeneity of the input data. For example, when all lung cells were compared, the pagoda2 algorithm split the lung arterial SMC into two clusters (Figure 1E), whereas the same cells formed a single cluster when all SMC were compared (Figure 2A; Table S2). Nevertheless, our subsequent analysis confirmed the heterogeneity in this cluster and linked it to proximo-distal zonation of the SMC (Figure 4B). In spite of this heterogeneity, we count, arbitrarily, lung arterial SMC as one (of 10) SMC subtypes identified.

We have used IF, ISH, and transgenic reporters to link the unique transcriptomes identified by scRNA-seq to cells with distinct anatomical locations. Although an essential part of the cell type annotation process, this procedure is biased and dependent on, for example, the number of available reagents and their quality. Other data used for the annotation of SMC included anatomical landmarks based on pre-existing views, as well as data from other cell types. For example, our discrimination between arterial and venous SMC depends on the venous (but not arterial) endothelial expression of endomucin (EMCN) (Figures 1A and S1A) and the expression of Slc6a2 (Figure 5G) specifically in venous endothelial cells of the lung. Our choice of antibodies or ISH probes against SMC subtypes in this study was done to the best of our judgment; however, new or improved reagents or animal models may allow for more refined analyses in the future.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Animals
- **METHOD DETAILS**
  - Preparation of single cell solution from mouse tissues
  - Fluorescent activated cell sorting (FACS)
  - Library preparation and sequencing
  - Previously published datasets
  - Sequence data processing
  - Gene ontology analysis
  - Immunofluorescence staining
  - In situ hybridization (RNAscope®)
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
  - Statistics and reproducibility
- **ADDITIONAL RESOURCES**

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.devcel.2022.09.015.
ACKNOWLEDGMENTS

We thank Professor Moustapha Hassan and the Pre-Clinical Laboratories (PKL)—Karolinska University Hospital Huddinge, as well as the Karolinska Institutet MedH flux activated cell sorting (FACS) facility, Cecilia Olsson, Pia Peterson, Jana Chmielniakova, and Helene Leksell for technical help. This study was supported by grants from Magn. Bergvalls Foundation (L.M.: 2020-03735, 2021-04275), the Swedish Cancer Society (C.B.: 2018/449, 2018/1154, 21 1714 Pj), the Swedish Research Council (C.B.: 2015-00550, U.L.: 2019-0286), Knut and Alice Wallenberg Foundation (C.B.: 2015.0030, 2020.0057), the Louise Jeantet Medical Prize (C.B.), The Anders Jahre Medical Prize (C.B.), the Innovative Medicines Initiative (C.B.: IM2PACT-807015), and the Wenner-Gren foundations Fellow program (E.M.H.).

AUTHOR CONTRIBUTIONS

L.M., U.L., and C.B. conceived the study and designed the project and experiments. L.M., G.M., and R.P. performed the experiments with assistance from J.L. and G.G. L.M., G.M., and S.L. designed and performed the FACS experiments. J.L., S.G., B.B., and E.R. performed the sample preparations for scRNA-seq and J.L. performed the sequencing. L.M., G.M., and L.P.H. performed bioinformatic analysis. L.H. constructed the online database. L.M., G.M., and L.H. analyzed and interpreted the bioinformatic data. E.M.H. and J.L.M.B. supervised the work of S.L. and G.M., respectively. L.M., U.L., and C.B. wrote the paper with significant input from G.M. and R.P. L.M. created the figures with help from G.M. and R.P. All authors reviewed and edited the text.

DECLARATION OF INTERESTS

C.B. holds a research grant from AstraZeneca BioPharmaceuticals R&D. U.L. holds a research grant from Merck KGaA but receives no personal remuneration from them.

REFERENCES

Albar, S., Gonzalez-Blas, C.B., Moerman, T., Huynh-Thu, V.A., Imrichova, H., Huiselmanns, G., Rambow, F., Marine, J.C., Geurts, P., Aerts, J., et al. (2017). SCENIC: single-cell regulatory network inference and clustering. Nat. Methods 14, 1083–1086. https://doi.org/10.1038/nmeth.4463.
Alencar, A.K.N., Montes, G.C., Barreiro, E.J., Sudo, R.T., and Zapata-Sudo, G. (2017). Adenosine receptors as drug targets for treatment of pulmonary arterial hypertension. Front. Pharmacol. 8, 858. https://doi.org/10.3389/fphar.2017.00858.
Alencar, G.F., Owsiany, K.M., Karnewar, S., Sukhavasi, K., Mocci, G., Nguyen, A.T., Williams, C.M., Shamsuzzaman, S., Mokry, M., Henderson, C.A., et al. (2020). Stem cell pluripotency genes Kif4 and Oct4 regulate complex SMC phenotypic changes critical in late-stage atherosclerotic lesion pathogenesis. Circulation 142, 2045–2059. https://doi.org/10.1161/CIRCULATIONAHA.120.048672.
Andersson, K.E., and Arner, A. (2004). Urinary bladder contraction and relaxation: physiology and pathophysiology. Physiol. Rev. 84, 935–986. https://doi.org/10.1152/physrev.00038.2003.
Ando, K., Tong, L., Peng, D., Vázquez-Liebana, E., Chiyoda, H., He, L., Liu, J., Kawakami, K., Mochizuki, N., Fukuhara, S., et al. (2022). KCNJ8/ABCC9-containing K-ATP channel modulates brain vascular smooth muscle development and neurovascular coupling. Dev. Cell 57, 1383–1399.e7. https://doi.org/10.1016/j.devcel.2022.04.019.
Armulik, A., Genové, G., and Betsholtz, C. (2011). Pericytes: developmental, physiological and pathologic perspectives, problems, and promises. Dev. Cell 21, 193–215. https://doi.org/10.1016/j.devcel.2011.07.001.
indications. Nat. Rev. Drug Discov. 16, 829–842. https://doi.org/10.1038/nrd.2017.178.
He, L., Vanityandwijk, M., Måe, M.A., Andrae, J., Ando, K., Del Gaudio, F., Nahar, K., Lebouvier, T., Lavita, B., Gouveia, L., et al. (2018). Single-cell RNA sequencing of mouse brain and lung vascular and vessel-associated cell types. Sci. Data 5, 180160. https://doi.org/10.sdata.2018.160.
He, L., Vanityandwijk, M., Rascherperger, E., Andalousi Måe, M., Jung, B., Lebouvier, T., Ando, K., Hofmann, J., Keller, A., and Betscholt, C. (2016). Analysis of the brain mural cell transcriptome. Sci. Rep. 6, 35108. https://doi.org/10.1038/srep35108.
He, S., Wang, L.H., Liu, Y., Li, Y.O., Chen, H.T., Xu, J.H., Peng, W., Lin, G.W., Wei, P.P., Li, B., et al. (2020). Single-cell transcriptome profiling of an adult human cell atlas of 15 major organs. Genome Biol. 21, 294. https://doi.org/10.1186/s13059-020-02210-0.
Himpens, B., Matthys, G., Somlyo, A.V., Butler, T.M., and Somlyo, A.P. (1988). Cytoplasmic free calcium, myosin light chain phosphorylation, and force in phasic and tonic smooth muscle. J. Gen. Physiol. 92, 713–729. https://doi.org/10.1085/jgp.92.6.713.
Ito, M., Okamoto, R., Ito, H., Zhe, Y., and Dohi, K. (2022). Regulation of myosin light-chain phosphorylation and its roles in cardiovascular physiology and pathophysiology. Hypertens. Res. 45, 40–52. https://doi.org/10.1007/s10992-021-01773-y.
Jacoby, E., Bouhelal, R., Gerspacher, M., and Seuwen, K. (2006). The 7 TM domain protein and CUB domain containing 2 (TCUB2) is an essential component of the VE-cadherin junctional complex. J. Clin. Investig. 118, 1083–1094. https://doi.org/10.1172/JCI25347.
Krebs, L.T., Deftos, M.L., Bevan, M.J., and Gridley, T. (2001). The nrarp gene encodes an ankyrin-repeat protein that is transcriptionally regulated by the parathyroid hormone receptor 1. J. Biol. Chem. 276, 35108–35115. https://doi.org/10.1074/jbc.M108861200.
Lee, S., Wang, L.H., Liu, M., and Gomez, D. (2019). Smooth muscle cell phenotypic diversity. Arterioscler. Thromb. Vasc. Biol. 39, 1715–1723. https://doi.org/10.1161/ATVBAHA.119.312131.
Livahtis, G., and Kalinovich, A. (2021). Receptors for pro-resolving mediators as a therapeutic tool for smooth muscle remodeling-associated disorders. Pharmacol. Res. 164, 105340. https://doi.org/10.1016/j.phrs.2020.105340.
Lun, A.T., McCarthy, D.J., and Marioni, J.C. (2016). A step-by-step workflow for low-level analysis of single-cell RNA-seq data with Biocductor. F1000Res 5, 2122. https://doi.org/10.12688/f1000research.9501.2.
MacFarlane, E.G., Parker, S.J., Shin, Y.J., Kang, B.E., Ziegler, S.G., Creamer, T.J., Bagircizadeh, R., Bedja, D., Chen, Y., Calderon, J.F., et al. (2019). Lineage-specific events underlie aortic root aneurysm pathogenesis in Loeys-Dietz syndrome. J. Clin. Investig. 129, 659–675. https://doi.org/10.1172/JCI123547.
Mao, Y.L., Shen, C.L., Zhou, T., Ma, B.T., Tang, L.Y., Wu, W.T., Zhang, H.X., Liu, H.L., Xu, W.X., and Wang, Z.G. (2017). Ablation of TαrC1 in mice leads to gastric emptying disturbance. Neurogastroenterol. Motil. 29. https://doi.org/10.1111/nmo.13131.
Mcmahan, A.P. (2000). Neural patterning: the role of Nkx genes in the ventral spinal cord. Genes Dev. 14, 2261–2264. https://doi.org/10.1101.gad.840800.
Millino, C., Sarinella, F., Tiveron, C., Villa, A., Sartore, S., and Ausoni, S. (2000). Cardiac and smooth muscle cell contribution to the formation of the murine pulmonary veins. Dev. Dyn. 218, 414–425. https://doi.org/10.1002/1097-0177(200007)218:3<414::AID-DVDY1002>3.0.CO;2-H.
Misra, A., Feng, Z., Chandran, R.R., Kabir, I., Rotlián, N., Aryal, B., Sheikh, A.Q., Ding, L., Qin, L., Fernández-Hernando, C., et al. (2018). Integrin β3 regulates clonality and fate of smooth muscle-derived atherosclerotic plaque cells. Nat. Commun. 9, 2073. https://doi.org/10.1038/s41467-018-04447-7.
Molmersteeg, M.T., Brown, N.A., Prall, O.W., de Gier-de Vries, C., Harvey, R.P., Moorman, A.F., and Christoffels, V.M. (2007). Ptx2 and Nkx2-5 are required for the formation and identity of the pulmonary myocardium. Circ. Res. 101, 902–909. https://doi.org/10.1161/CIRCRESAHA.107.161182.
Muhl, L., Genové, G., Leptidis, S., Liu, J., He, L., Mocci, G., Sun, Y., Gustafsson, S., Buyandelger, B., Chivukula, I.V., et al. (2020). Single-cell analysis uncovers fibroblast heterogeneity and criteria for fibroblast and mural cell identification and discrimination. Nat. Commun. 11, 3953. https://doi.org/10.1038/s41467-020-17740-1.
Nakayama, N., Han, C.E., Scully, S., Nishinakamura, R., He, C., Zeni, L., Yamane, H., Chang, D., Yu, Y., Yokota, T., and Wen, D. (2001). A novel chordin-like protein inhibitor for bone morphogenetic proteins expressed preferentially in mesenchymal cell lineages. J. Biol. Chem. 276, 38925–38930. https://doi.org/10.1074/jbc.M108491200.
Pan, H., Xue, C., Auerbach, B.J., Fan, J., Bashore, N.C., Cui, J., Jones, D., Trignano, S.B., Liu, W., Shi, J., et al. (2020). Single-cell genomics reveals a progenitor cell state during smooth muscle cell phenotypic switching and potential therapeutic targets for atherosclerosis in mouse and human. Circulation 142, 2060–2075. https://doi.org/10.1161/CIRCULATIONAHA.120.048378.
Papaioannou, V.E. (2014). The T-box gene family: emerging roles in development. Cell. Mol. Life Sci. 71, 401–416. https://doi.org/10.1007/s00018-013-1244-2.
Picelli, S., Faridani, O.R., Björklund, A.K., Winberg, G., Sagasser, S., and Sandberg, R. (2014). Full-length RNA-seq from single cells using Smart-seq2. Nat. Protoc. 9, 171–181. https://doi.org/10.1038/nprot.2014.006.

Pogoda, K., Kameritsch, P., Mannell, H., and Pohi, U. (2019). Connexins in the control of vasomotor function. Acta Physiol. Oxf. 225, e13108. https://doi.org/10.1111/apha.13108.

Ponnath, D.S., Nadeem, A., Tilley, S., and Mustafa, S.J. (2010). Involvement of A1 adenosine receptors in altered vascular responses and inflammation in an allergic mouse model of asthma. Am. J. Physiol. Heart Circ. Physiol. 299, H81–H87. https://doi.org/10.1152/ajpheart.01090.2009.

Prakash, N., Hansson, E., Betsholtz, C., Mitsiadis, T., and Lendahl, U. (2002). Mouse notch 3 expression in the pre- and postnatal brain: relationship to the stroke and dementia syndrome CADASIL. Exp. Cell Res. 278, 31–44. https://doi.org/10.1006/excr.2002.5544.

Reho, J.J., Zheng, X., and Fisher, S.A. (2014). Smooth muscle contractility diversity in the control of regional circulations. Am. J. Physiol. Heart Circ. Physiol. 306, H160–H172. https://doi.org/10.1152/ajpheart.00493.2013.

Saouli, N., Westerhof, N., Postmus, P.E., and Vonk-Noordegraaf, A. (2019). The arterial load in pulmonary hypertension. Eur. Respir. Rev. 19, 197–203. https://doi.org/10.1183/16000617.0002210.

Sawada, H., Rateri, D.L., Moorleghen, J.J., Majesky, M.W., and Daugherty, A. (2012). A vascular niche of cell types and zonation in the brain vasculature. Nature 497, 475–480. https://doi.org/10.1038/nature12259.

Sheng, Y., and Zhu, L. (2018). The crosstalk between autonomic nervous system and blood vessels. Int. J. Physiol. Pathophysiol. Pharmacol. 10, 17–28.

Shiota, W., Larsson, O., Shulkin, K.D., Shiota, M., Efanov, A.M., Hoy, M., Lindner, J., Koopiwut, S., Junnti-Berggren, L., Gromada, J., et al. (2002). Sulfonylurea receptor type 1 knock-out mice have intact feeding-stimulated insulin secretion despite marked impairment in their response to glucose. J. Biol. Chem. 277, 37176–37183. https://doi.org/10.1074/jbc.M206757200.

Shynlova, O., Tsui, P., Jaffer, S., and Lye, S.J. (2009). Integration of endocrine and mechanical signals in the regulation of myometrial functions during pregnancy and labour. Eur. J. Obstet. Gynecol. Reprod. Biol. 144, S2–S10. https://doi.org/10.1016/j.ejogrb.2009.02.044.

Simmerman, H.K., and Jones, L.R. (1998). Phospholamban: protein structure, mechanism of action, and role in cardiac function. Physiol. Rev. 78, 921–947. https://doi.org/10.1152/physrev.1998.78.4.921.

Spencer, N.J., Costa, M., Hibberd, T.J., and Wood, J.D. (2021). Advances in colonic motor complexes in mice. Am. J. Physiol. Gastrointest. Liver Physiol. 320, G12–G29. https://doi.org/10.1152/ajpgi.00317.2020.

Stanfel, M.N., Moses, K.A., Schwartz, R.J., and Zimmer, W.E. (2005). Regulation of organ development by the NKX-homeodomain factors: an NKX code. Cell. Mol. Life Sci. Noisy-Le-Grand Suppl 51, OL785–799.

Sterfels, L.C., Frisstad, A.A., Andruska, A., Boehm, M., McGlenn, M., Zhang, F., Zhang, W., Hou, D., Tian, X., Miquerol, L., et al. (2020). A notch3-marked sub-population of vascular smooth muscle cells is the cell of origin for occlusive pulmonary vascular lesions. Circulation 142, 1545–1561. https://doi.org/10.1161/CIRCULATIONAHA.120.054750.

Steinhoff, M.S., von Mentzer, B., Geppetti, P., Pothisoulakis, C., and Bunnett, N.W. (2014). Tachykinins and their receptors: contributions to physiological control and the mechanisms of disease. Physiol. Rev. 94, 265–301. https://doi.org/10.1152/physrev.00031.2013.

Tabeling, C., González Calera, C.R., Lienau, J., Höppner, J., Tschernig, T., Kershaw, O., Gubitier, B., Naujoks, J., Herbert, J., Opitz, B., et al. (2022). Endothelin B receptor immunodynamics in pulmonary arterial hypertension. Front. Immunol. 13, 895501. https://doi.org/10.3389/fimmu.2022.895501.

Tabula Muris Consortium; Overall coordination; Logistical coordination; Organ collection and processing; Library preparation and sequencing; Computational data analysis; Cell type annotation; Writing group; Supplemental text writing group; Principal investigators (2018). Single-cell transcriptomics of 20 mouse organs creates a Tabula Muris. Nature 562, 367–372. https://doi.org/10.1038/s41586-018-0590-4.

Teng, Y.H., Aquino, R.S., and Park, P.W. (2012). Molecular functions of syndecan-1 in disease. Matrix Biol. 31, 3–16. https://doi.org/10.1016/j.matbio.2011.10.001.

Townesley, M.I. (2012). Structure and composition of pulmonary arteries, capillaries, and veins. Compr. Physiol. 2, 673–709. https://doi.org/10.1002/cphy.c100081.

Tsafriri, D., Tsafriri, I., Ein-Dor, L., Zuck, O., Noterman, D.A., and Domany, E. (2005). Sorting points into neighborhoods (SPIN): data analysis and visualization by ordering distance matrices. Bioinformatics 21, 2301–2308. https://doi.org/10.1093/bioinformatics/bti329.

Vanhoutte, P.M., and Janssens, W.J. (1978). Local control of venous function. Microvasc. Res. 16, 196–214. https://doi.org/10.1016/0026-2862(78)90055-9.

Vranlandewijck, M., He, L., Mæ, M.A., Andrae, J., Ando, K., Del Gaudio, F., Nahar, K., Lebouvier, T., Laví, B., Gouveia, L., et al. (2018). A molecular atlas of cell types and zonation in the brain vasculature. Nature 554, 475–480. https://doi.org/10.1038/nature25739.

Webb, R.C. (2003). Smooth muscle contraction and relaxation. Adv. Physiol. Educ. 27, 201–206. https://doi.org/10.1152/advan.2003.00025.2003.

Wirka, R.C., Wagh, D., Paik, D.T., Pianci, M., Nguyen, T., Miller, C.L., Kundu, R., Nagoa, M., Coller, J., Koyano, T.K., et al. (2019). Atheroprotective roles of smooth muscle cell phenotypic modulation and the TCF21 disease gene as revealed by single-cell analysis. Nat. Med. 25, 1280–1289. https://doi.org/10.1038/s41591-019-0512-5.

Wu, T., Hu, E., Xu, S., Chen, M., Guo, P., Dai, Z., Feng, T., Zhou, L., Tang, W., Zhan, L., et al. (2021). clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. Innovation (Camb) 2, 100141. https://doi.org/10.1016/j.innov.2021.100141.

Yassine, N.M., Shahram, J.T., and Body, S.C. (2017). Pathogenic mechanisms of bicuspid aortic valve aortopathy. Front. Physiol. 8, 687. https://doi.org/10.3389/fphys.2017.00687.

Yokota, T., Kawakami, Y., Nagai, Y., Ma, J.X., Tsai, J.Y., Kincade, P.W., and Steinberg, R. (2014). Notch1 regulates smooth muscle cell fate decisions. Proc. Am. Thorac. Soc. 11, 13–22. https://doi.org/10.1513/pats.2004-0346.

Zhang, W., and Gunst, S.J. (2008). Interactions of airway smooth muscle cells with their tissue matrix: implications for contraction. Proc. Am. Thorac. Soc. 5, 32–39. https://doi.org/10.1513/pats.200704-048VS.

Developmental Cell Resource
## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Alpha-SMA           | Sigma  | C6198; RRID: AB_476856 |
| Alpha-SMA           | Sigma  | F3777; RRID: AB_476977 |
| CD31                | R&D Systems | AF3628; RRID: AB_2161028 |
| CD31                | Abcam  | ab28364; RRID: AB_726362 |
| CD31                | BD Bioscience | 550274; RRID: AB_383571 |
| CD31                | BD Bioscience | 561814; RRID: AB_10893351 |
| CD31                | BD Bioscience | 561813; RRID: AB_10896651 |
| CNN1                | Abcam  | ab216651 |
| Connexin 43 (GJA1)  | Cell Signalling Tech. | 3512; RRID: AB_2294590 |
| Desmin (DES)        | Abcam  | ab32362; RRID: AB_731901 |
| Endomucin (EMCN)    | eBioscience | 14-5851-82; RRID: AB_891527 |
| HHIP                | R&D Systems | AF1568; RRID: AB_2116693 |
| Integrin alpha-8 (ITGA8) | R&D Systems | AF4076; RRID: AB_2296280 |
| Ly-6A/E (SCA1)      | eBioscience | 17-5981-81 |
| Nephronectin (NPNT) | R&D Systems | AF4298; RRID: AB_10646643 |
| Neural cell adhesion molecule 1 (NCAM1) | R&D Systems | AF2408; RRID: AB_442152 |
| NG2 (CSPG4)         | Millipore | AB5320; RRID: AB_91789 |
| PDGFRbeta           | eBioscience | 17-1401-81; RRID: AB_529482 |
| Sclerostin (SOST)   | R&D Systems | AF1589; RRID: AB_2195345 |
| Shisa family member 3 (SHISA3) | Novus Biologicals | NBP2-22340 |
| Syndecan-1 (SDC1)   | R&D Systems | AF3190 |
| Type-VIII collagen alpha-1 (COL8A1) | Novus Biologicals | NBP2-13856 |
| **Critical commercial assays** | | |
| RNAscope Protease III | ACD | 322337 |
| RNAscope Protease IV | ACD | 322336 |
| RNAscope Fluorescent Multiplex Assay | ACD | 320851 |
| RNAscope Fluorescent Multiplex Assay V2 | ACD | 323110 |
| RNAscope 4-Plex Ancillary kit | ACD | 323120 |
| **Deposited data**  |        |            |
| Raw and analyzed data | This paper | GSE210106 |
| Raw and analyzed data | Vanlandewijck et al., 2018 | GSE99235 |
| Raw and analyzed data | Muhl et al., 2020 | GSE150294 |
| Raw and analyzed data | Muhl et al., 2022 | GSE198592 |
| **Experimental models: Organisms/strains** | | |
| Mouse: Tg(Pdgfrb-eGFP) JN169Gsat/’Mmucd’ | Gensat.org; This paper | RRID: MMRRRC_031796-UCD |
| Mouse: Tg(Cspg4-DsRed.T1)1Akik/J | The Jackson Laboratory | RRID: IMSR_JAX:008241 |
| Mouse: Tg(Acta2-GFP)1P1K | Yokota et al., 2006; MMRRRC; This paper | RRID: MMRRRC_041573-MU |
| Mouse: B6.129S4-Pdgfratm11(EGFP)Sor/J | The Jackson Laboratory | RRID: IMSR_JAX:007669 |
| **Oligonucleotides** | | |
| RNAscope probe: Mm-Acta2 | ACD | 319531-C2 |
| RNAscope probe: Mm-Acta2 | ACD | 319531-C3 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further data and information about resources and reagents are available from the corresponding author, Lars Muhl (lars.muhl@ki.se), upon reasonable request.

Materials availability
This study did not generate new unique reagents or models.

Data and code availability
- All data to support the findings of this study are included in the paper and the supplemental information and freely available as a searchable database at http://betsholtzlab.org/Publications/SMC/database.html. Heatmaps included in the study for which the included genes are not depicted in the figures can be accessed as supplemental files thought the online database. The scRNA-seq raw-data from previously published datasets is available at NCBI’s Gene Expression Omnibus database under the accession numbers GSE99235, GSE150294 and GSE198592. The scRNA-seq raw-data from the previously unpublished cells of this study are available at NCBI’s Gene Expression Omnibus database under the accession number GSE210106.
- No code was developed for this study. Details about the used code are available from the lead contact upon reasonable request.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon reasonable request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
All animal experiments have been carried out in accordance with the Swedish legislation as well as local regulations and guidelines for animal welfare. All mouse experiments were approved by the local authority: Linköpings Animal Research committee (Linköpings
METHOD DETAILS

Preparation of single cell solution from mouse tissues

All tissues (except aorta as detailed below) were treated with the same protocol as described before (Muhl et al., 2020): animals were euthanized by cervical dislocation and organ(s) of interest (heart, lung, colon) were dissected out and placed into ambient-tempered PBS solution (DBPS, ThermoFisher Scientific). For cell isolation from the aorta, tissues were perfused with DBPS through the left ventricle. The aorta was cleaned from the adventitia and dissected out from the aortic arch to the 3rd rib and placed into ambient DBPS. For each aorta-sample the aortae from two mice were pooled. Tissues were then cut into smaller pieces with scalpel or scissors and incubated in dissociation buffer (Skeletal muscle dissociation kit from Miltenyi Biotec, supplemented with 1 mg/ml Collagenase type I, type II and type IV-S, and 2 mg/ml of Elastase all from Sigma-Aldrich) at 37 °C shaking at 500 – 800 rpm. To support dissociation, mechanical disintegration by pipetting was applied in three to four cycles with 10 min intervals. Remaining debris was removed by passing the cell suspension through a 70 μm cell strainer (Corning) which was subsequently washed with 5 ml DMEM (ThermoFisher Scientific) to recover cells adherent to the surface. Thereafter, the cells were pelleted by centrifugation at 300 x g for 5 min. Supernatant was removed and the cell pellet was resuspended in 1 ml FACS buffer (DPBS supplemented with 0.5% bovine serum albumin, 2 mM EDTA, 25 mM HEPES). Labeling of endothelial cells or non-smooth muscle stromal cells was done using fluorophore-conjugated antibodies directed against either CD31 (PECAM1), or SCA1 (LY6A/E) (see Table S7). For aortic SMC sorting from reporter gene negative mice, the cells were labeled with calcine green AM (ThermoFisher Scientific) as viability indicator, and for PDGFRβ by an antibody as described above. Cell labeling was done at room temperature (RT) for 20 min in small volume (100 – 200 μl). Thereafter, ≥ 1 ml of FACS buffer was added to dilute out any non-bound antibodies and cell suspensions were placed on ice until further processing.

Fluorescent activated cell sorting (FACS)

Antibody labeled cell suspensions were analyzed on a BD FACSMelody or BD FACSaria III (Becton Dickinson Biosciences) cell sorter, equipped with a 100 μm nozzle. Single-cells meeting the criteria as described below were collected by deposition into 384-well plates containing 2.3 μl lysis buffer (0.2% Triton X-100, 2 U/ml RNase inhibitor, 2 mM dNTP, 1 μM Smart-dT30VN, ERCC 1:4 x 10⁶ dilution) per well. Importantly, the analysis on the cell sorter was not the basis for cell type identification, but for the enrichment of target cell populations dependent on antibody labeling (CD31, SCA1 or PDGFRβ) or reporter gene expression (Acta2GFP / Cspg4dsRED). For sorting of single cells into 384-well plates, first a gate for forward scatter-area/ side scatter area (FCS-A/SSC-A, linear scale) was set generally to include most events, except events showing low values and likely represent debris or red blood cells. As second step, doublet discrimination was implemented using SSC-A/SSC-height and FCS-A/FCS-height with lavish thresholds to ensure the presence of smooth muscle cell populations in the sorting sample and prevent the bias to cells with a uniform shape after tissue dissociation. Thereafter, cells were analyzed for their fluorescent signals, separating them dependent on their antibody labeling and reporter gene expression patterns. Fluorescent signals were controlled by “fluorescence minus one” samples, without antibody labeling or from reporter gene negative animals.

Endothelial cells were collected from mice with different genotypes, such as PdgfraH2BGFP, PdgfrbGFP, Acta2GFP or Cspg4dsRED, based on their positive signal for anti CD31 labeling, while being negative for any of the used reporter gene constructs. SMC populations were collected based on their positive signal for Acta2GFP and a simultaneous negative signal for CD31 or SCA1 (Table S4). The SCA1 antibody was chosen due to simultaneous labeling of endothelial cells and other stromal cells, such as fibroblasts, whereas SMC populations are negative for the expression of Ly6a (encoding SCA1), according to our previous studies (Muhl et al., 2020). Further enrichment or discrimination of specific SMC populations was done combining the reporter gene signal for Acta2GFP with the reporter gene signal for Cspg4dsRED, expecting non-vascular SMC and/or venous SMC to become enriched when collecting Acta2GFP-positive and Cspg4dsRED-negative subpopulations. Aortic SMC were collected on the basis of viability (calcine green AM), a positive antibody-based labeling for PDGFRβ and their distribution in the FCS-A/SSC-A scatter plot. Exemplary scatter plots (prepared using FlowJo software, version 10.8.1) describing the two FACS strategies are shown in Figures S1B and S1C. Of note, using different cell sorting and selection strategies may cause skewing of the cell subpopulation capture. Nevertheless, the data shows that aortic SMC collected from the aorta displayed comparable transcriptomes to aortic SMC collected from heart preparations using the reporter gene-based cell selection and sorting protocol. Before the sorting, the plates were briefly centrifuged
to ensure the lysis buffer in the bottom of the well. Of note, the correct deposition of the selected droplet (single cell) was controlled by test-spotting (aiming) of beads, or cell populations of control samples onto the seal of the respective 384-well plate. If necessary, the plate holder position was adjusted for centered deposition of the droplets for each plate. The sample-stand and plate-holder were maintained at 4°C during analysis and sorting. Sorted plates were sealed and immediately placed on dry-ice and stored at −80°C, until further processing.

**Library preparation and sequencing**

Single-cell cDNA libraries were prepared according to the previously established protocol for Smart-Seq2 (Picelli et al., 2014). In brief, poly-adenylated mRNA was transcribed to cDNA using oligo(dT) primer and SuperScript II reverse transcriptase (ThermoFisher Scientific). Synthesis of second strand cDNA was achieved using a template switching oligo, and the double stranded cDNA was then amplified using polymerase chain reaction (PCR) for 23–26 cycles. After bead purification, overall cDNA quality was controlled (QC) by analyzing randomly selected single cell samples (wells) on a 2100 Bioanalyzer with a DNA High sensitivity chip (Agilent Biotechnologies). When the sample (plate) passed the QC, the cDNA was fragmented and tagged (tagmented) using Tn5 transposase, and each single cell sample (well) was uniquely indexed using Illumina Nextera XT index kits (set A-D). Thereafter, the indexed single-cell samples from one plate were pooled to be sequenced together on one lane of a HiSeq3000 sequencer (Illumina), using dual indexing and 50 base read-length.

**Previously published datasets**

Single cell transcriptomes from published mouse datasets were used in the study. We used 1441 cells isolated from the lung and previously published in Vanlandewijck et al. (2018), 1,277 cells isolated from the heart and 1,644 cells from the colon previously published in Muhl et al. (2020), as well as 478 cells isolated from the heart and previously published in Muhl et al. (2022). Transcriptomes of previously published cells (in total 4,840) were generated with the Smart-Seq2 protocol and raw-sequence processing was adjusted before (Muhl et al., 2020). In brief, low-quality cells (≤50,000 counts library size, ≤1500 expressed genes, >10% mitochondrial genes or ERCC counts) were removed from the dataset. Cells with >10,000 expressed genes were also removed from the dataset as potential doublets. Low expressed genes were removed from the dataset with the following criteria: expression in at least three cells with a count value >20 and a cumulative counts value of >300. Cells that exhibited a transcriptome with clear signs of cross cell type contamination were also removed from the dataset. These filtering resulted in a dataset of in total of 3737 cells from the heart & aorta, 2817 cells from the lung, and 3259 cells from the colon, collected from n = 16 mice, plus 2 samples containing a pool of two individual mice each (excluding lung cells integrated from Vanlandewijck et al., 2018).

The dataset (equally performed for each organ dataset separately) was organized in R-software using the SingleCellExperiment package (version 1.8.0) (Lun et al., 2016), and calculation of dataset attributes, such as PCA (nPcs=50, n.dgenes=3000) and nearest neighbor clustering, was performed using the pagoda2 (pathway and gene set overdispersion analysis, https://github.com/hms-dbmi/pagoda2) R-software package. Dimensional reduction visualization was done using the UMAP function (UMAP: uniform manifold approximation and projection). For the construction of bar plot graphs, hierarchical clustering of the determined clusters, done on the basis of average gene expression values per cluster, was used to order clusters (Figures 1G–1I), each SMC cluster was compared to the remaining cells of the dataset and stringent criteria were used for gene qualification: 100 counts per cell as threshold for gene detection, expression in ≥30% of cells per cluster (%) expressing the gene with ≥100 counts (dot-size). The 90-gene signature scores (Figure S1D) for each cluster were calculated using the normalized expression of the genes included in the 90-gene signature (Muhl et al., 2020) for each cell and the individual scores were used to calculate the average expression score per cluster for fibroblast and mural genes, respectively.

The monocle R-software package (Trapnell et al., 2014) was used for differential gene expression analysis. The DifferentialGeneTest function was applied, comparing two groups. Dependent on the purpose as well as the expected level of heterogeneity between the two analyzed groups the thresholds for gene qualification were adjusted to obtain reasonable results. For SMC subpopulation enriched gene identification (Figures 1G–1I), each SMC cluster was compared to the remaining cells of the dataset and stringent criteria were used for gene qualification: 100 counts per cell as threshold for gene detection, expression in >30% of cells per group and a greater or equal to twofold (log2) difference in expression was required for gene selection. Venn diagrams were
used for cross-reference of obtained gene-lists and the VennDiagram R-software package (https://CRAN.R-project.org/package=VennDiagram) was used. For the SMC subtype-specific over-represented genes (Figure 2D) the respective SMC subpopulation was compared to all other SMC populations analyzed in the inter-organ SMC dataset. Due to high variance between the cell numbers of SMC subpopulations, we randomly selected a given number of cells (smallest cell number of compared clusters) from those clusters with higher cell number, otherwise the same thresholds for gene qualification were used as described above for Figures 1G–1I. The top 50 enriched expressed genes (ordered by fold-change) per SMC subtype were selected for presentation in the heatmap. For differential gene expression analysis between the aortic SMC clusters of the inter-organ SMC dataset (cluster #1, #2), a fold change of >1 log2 and average expression >25 counts was used as criteria (Figure 3C). For the differential gene expression analysis within the proximal aortic SMC cluster #1 as well as the pulmonary arterial SMC cluster #5 of the inter-organ SMC dataset along their respective SPIN range, cells of the first third were compared to cells of the last third of the cluster. Criteria for gene qualification were fold change >1 log2 and expression in >30% of cells with >100 counts, to identify marker genes that can be used for in situ visualization (Figures 3E and 4A). For differential gene expression analysis between elastic arterial SMC from the aorta (cluster #1, #2) and lung (cluster #5), as well as the differential gene expression analysis between the combined elastic arterial SMC from aorta and lung (cluster #1, #2, #5) compared to the combined muscular arterial SMC from the heart and colon (cluster #3, #8) the same criteria as described above for Figures 1G–1I were used were applied (Figures 4F and 4H). For differential expression analysis between heart (cluster #3) and colon (cluster #8) arterial SMC, the top 50 genes, ordered by fold change, with an average expression >25 counts in the respective cluster are shown in the heatmap (Figure 4L). For identification of venous SMC enriched genes, cells from cluster #7 were compared to all other vascular SMC (clusters #1, #2, #3, #5, #6, #8) and the same criteria as described above for Figures 1G–1I were applied (Figure 5B). For determination of organotypic expressed genes between venous SMC (cluster #7), cells originating from either heart, lung or colon were compared to the remaining cells, and genes with fold change >1 log2 and average expression >25 counts are displayed in the heatmap (Figure S6C). For the identification of differentially expressed genes between intermediate cells (cluster #6) and venous SMC from the lung (Figure S6D) as well as between intermediate cells (cluster #6) and pericytes from the lung (cluster #5 in lung dataset) (Figure S6E) the same criteria as described above for Figures 1G–1I were used. For identification of airway SMC enriched genes, cells from cluster #4 (airway SMC) were compared to all other SMC of the lung and the top 50 genes, sorted by fold change (lowest fold change = 2.1 log2), expressed in >30% with >100 counts are shown in the heatmap (Figure 6B). For differential gene expression analysis between vascular and non-vascular SMC of the colon (Figure 6F), and between interstitial SMC (cluster #9) and visceral SMC (cluster #10) from the colon (Figure 4H) the same criteria as described for Figures 1G–1I were applied. To determine overall differential gene expression within the inter-organ dataset, each cluster was compared to the remaining dataset. In order to retain also lower expressed genes, the threshold for gene qualification was set to >25 counts average expression per cluster. Genes with fold change >2 log2 were selected. The combined list of (unique) enriched genes per cluster was used as basis for identification of differentially expressed GPCR (Figure 7B) as well as differentially expressed members of the BMP and WNT signaling pathways (Figure 7D). A list of GPCR (G-protein coupled receptors) was obtained from https://www.guidetopharmacology.org, containing in total 356 GPCR, categorized with biopharmaceutical targets (Table S7). The lists for members of the BMP and WNT signaling pathways were collected from the gene ontology terms BMP signaling pathway (GO: 0030509) and WNT signaling pathway (GO:0016055), respectively. For calculation of transcription factor regulatory network activity, the SCENIC R-software package (Aibar et al., 2017) was used according to the authors recommendations. The cluster definition was retained from pagoda2 analysis of the inter-organ SMC dataset.

Gene ontology analysis
For gene ontology (GO) analysis the clusterProfiler R-software package (Wu et al., 2021) was used. The enrichGO function was applied to identify enriched GO terms belonging to the Biological Process subontology. The list of all genes retained in the inter-organ SMC dataset after the removal of low expressed genes (see above) was used as reference ("gene universe"). Terms with an adjusted p-value < 0.05 (pvalueCutoff=0.05, pAdjustMethod="BH" (BH=Benjamini-Hochberg)) were selected and the top 10 terms (min-GSSize=10, maxGSSize=500), after using the simplify function to omit redundant terms (cutoff=0.7), are displayed in the respective dot plots. As input, gene lists obtained from differential gene expression analysis as described above were used. Details, such as gene qualification thresholds are described above and/or in the respective figure legends.

Immunofluorescence staining
Standard procedures for immunofluorescence (IF) staining were applied. In brief, tissues were harvested from euthanized mice as described above and if not otherwise stated immersion fixed with 4% formaldehyde solution (Histolab) for 4 – 12h at 4°C, followed by immersion in 20-30% sucrose/PBS for at least 24h, at 4°C. For cryo-sectioning, tissues were embedded in cryo-medium (NEG50) and sectioned on a CryoStat NX70 (ThermoFischer Scientific) into 14 μm or 30 μm thick sections, collected on SuperFrost Plus glass slides (Metzler Gläser). Until further processing, sections were stored at -80°C. For staining, the sections were placed on RT and allowed to dry for 15 min. Thereafter, the sections were briefly washed in PBS and then incubated for at least 60 min at RT with blocking buffer (Serum-free protein blocking solution, DAKO), supplemented with 0.2% Triton X-100 (Sigma-Aldrich). After the blocking, the sections were sequentially incubated with primary antibodies and the corresponding fluorescently conjugated secondary antibodies, according to the manufacturers’ recommendations (Table S7). For nuclear (DNA) stain, Hoechst 33342 (trihydrochloride, trihydrate, ThermoFisher Scientific) was applied at 10 μg/ml together with the secondary antibodies. Thereafter, the sections were mounted with ProLong® Gold mounting medium (ThermoFisher Scientific). Micrographs were acquired using a Leica TCS SP8 confocal
microscope with LAS X software (version: 3.5.7.23225, Leica Microsystems). The acquired images were graphically processed and adjusted individually for brightness and contrast using the ImageJ/FIJI software (Schindelin et al., 2012) for optimal visualization. Images are, if not otherwise stated, presented in the figures as maximum intensity projections of acquired z-stacks covering the thickness of the section.

**In situ hybridization (RNAscope®)**

For fluorescent multiplex in situ hybridization (ISH) the RNAscope® system (Advanced Cell Technologies) was applied. Either the Fluorescent Multiplex Assay kit (Figure S7A) or the Fluorescent Multiplex Assay V2 kit with TSA-amplification (Figures 5D–5H, 6D, 6J, SS1, SSJ, and S6H) was used according to the manufacturer’s recommendations. For one set of lung samples, the tissues were briefly (1-2 min) perfused (via the right ventricle) with 1% para-FA/PBS, followed by inflation with cryo-medium (NEG50)/PBS solution (1:1). After dehydration, the sections were prepared using Pretreat 3 solution (fresh frozen lung), or Pretreat 4 solution (all other tissues) for 15 - 30 min at RT. RNAscope® probes (Table S7) were applied according to the manufacturer’s recommendations for 2h at 40°C. After completion of the protocol, the sections were mounted using ProLong® Gold mounting medium and micrographs were acquired and processed for visualization as described above (see immunofluorescent staining).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Statistics and reproducibility**

For gene ontology (GO) analysis a significance threshold of p=0.05 was used after adjusting the p-value using the Benjamini-Hochberg (BH) procedure implemented in the clusterProfiler R-software package (Wu et al., 2021). All antibody immunofluorescence experiments have been performed at least two times using identical or varying antibody combinations. Antibody immunofluorescent experiment were carried out on tissue samples of at least two individual mice. All RNAscope® ISH experiments have been performed at least two times using identical or varying combinations of probes, in total analyzing tissue samples from at least two individual mice.

**ADDITIONAL RESOURCES**

The scRNA-seq data of this paper is publicly accessible as a searchable database at: http://betsholtzlab.org/Publications/SMC/database.html.

Heatmaps included in the paper, for which not all included genes are depicted within the figures, can be accessed as supplemental files through the same internet page as the searchable database.