

Methods of Making and Using iPSC-derived Mural Progenitor Cells via Activation of NKX3.1

CLAIM OF PRIORITY

This application claims the benefit of U.S. Provisional Application Serial No. 63/544,104, filed on October 13, 2023, U.S. Provisional Application Serial No. 63/653,051, filed on May 29, 2024, and U.S. Provisional Application Serial No. 63/695,667, filed on September 17, 2024. The entire contents of the foregoing are incorporated herein by reference.

GOVERNMENT SUPPORT STATEMENT

This invention was made with government support under Grant Number HL128452, awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND

Mural cells, including pericytes and smooth muscle cells (SMCs), are critical for vascular development, function, and stability. Dysregulation of mural cells can lead to vascular abnormalities, emphasizing the need for generating functional mural cells to explore novel therapeutic approaches in vascular disorders, tissue repair, and regenerative medicine. Human induced pluripotent stem cells (iPSCs) offer a promising means for obtaining patient-specific mural cells; however, conventional chemical-based differentiation methods are limited in scope and precision. Inducible transcription factors (TFs) have gained traction as a differentiation strategy, offering precise temporal control and the potential for simultaneous differentiation of multiple cell types. However, identifying TFs for mural cell differentiation remains challenging. Further, there is a pressing need to address vascular disorders in blood vessels that can cause a range of health problems, which can be severe and even prove fatal.

SUMMARY

This application is based, *inter alia*, on the discovery that NK3 Homeobox 1 (NKX3.1) determines mural cell lineage fate, and NKX3.1 activation in iPSC-derived mesodermal progenitors (MePCs) effectively generated iPSC-derived mural progenitor cells (iMPCs), offering a novel method for creating limitless functional mural cells for regenerative medicine.

Our group previously demonstrated the successful generation of vascular endothelial cells (iECs) from iPSCs using ETV2, a pioneer TF (Wang et al., *Sci Adv* 2020). Recently, we discovered that another TF, NK3 Homeobox 1 (NKX3.1), can be used to generate mural progenitor cells (iMPCs) from iPSCs. The data presented herein reveal efficient iMPC production upon transient activation of NKX3.1 in iPSC-derived mesodermal progenitors (MePCs). These iMPCs display critical mural cell characteristics (e.g., calcium influx, contractile properties, and extracellular matrix synthesis), aligning them with control mural cells like primary SMCs. In addition, we found that iMPCs mature into fully differentiated mural cells upon interaction with ECs. This interaction enhances iMPCs' capacity to modulate EC function, including the formation of vascular networks in vivo. Moreover, our single-cell RNA sequencing analysis substantiates the maturation of iMPCs and the resulting mural cell heterogeneity.

Our group has also developed a novel vascular organoid (VO) model that allows concurrent co-differentiation of iPSCs into iECs and iMPCs. This VO model facilitates the maturation of iMPCs and is anticipated to be instrumental in studying the mechanisms involved in mural cell reprogramming and maturation.

Described herein, *inter alia*, are method of making iPSC-derived mural progenitor cells (iMPCs) comprising:

contacting a population of induced pluripotent stem cells (iPSCs) with a nucleic acid encoding NK3 Homeobox 1 (NKX3.1) or a functional variant thereof;

converting the iPSCs to mesodermal progenitors (MePCs); and

inducing the MePCs to express NKX3.1 for a time period sufficient to generate iMPCs.

In some embodiments, the nucleic acid is a vector (e.g., a PiggyBac transposon vector or viral vector). In some embodiments, the vector is a viral vector (e.g., retroviral, lentiviral). In some embodiments, the nucleic acid comprises an inducible promoter that controls expression of

NKX3.1 (e.g., doxycycline-inducible promoter or other known in the art). In some embodiments, converting the iPSCs to MePCs comprises activating the Wnt pathway and/or activating the Nodal pathway for a time period sufficient (e.g., about 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, or 48 hours) to generate MePCs. In some embodiments, the time period sufficient to generate iMPCs is about 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, or 48 hours.

Also described herein are methods of generating a population of mural cells comprising pericytes, smooth muscle cells, and fibroblasts, the method comprising:

generating iMPCs using the method of any one of claims 1-6;

co-culturing the iMPCs with a population of endothelial cells (ECs or iECs) for a time period sufficient to generate a population of mural cells comprising pericytes, smooth muscle cells, and fibroblasts. In some embodiments, the time period sufficient to generate a population of mural cells comprising pericytes, smooth muscle cells, and fibroblasts is about 1, 2, 3, 4, 5, 6, or 7 days.

Also described herein are methods of increasing blood vessel formation, methods of vascular cell therapy, and/or methods of vascular generation or regeneration in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of the population of ECs and iMPCs. In some embodiments, the methods further comprising identifying a subject in need or increasing blood vessel formation, vascular cell therapy, vascular generation, and/or vascular regeneration.

Described herein, *inter alia*, are methods maturing the iMPCs into functional mural cell subtypes, including smooth muscle cells, pericytes, and fibroblasts, as well as methods of increasing vascular development, angiogenesis, and cell junction via administering the iMPCs to a subject. As shown herein, iMPCs mediate the formation of functional vessels when implanted with endothelial cells (ECs); thus, methods of administering iMPCs and ECs and methods of modeling vascular diseases (e.g., a 3D vascular organoid (VO)) and therapeutic vascularization comprising administering iMPCs and EC are also described herein.

In some embodiments, the methods described herein comprise the use of a nucleic acid encoding a NKX3.1 (e.g., UnitProtKB Q99801-1, UnitProtKB Q99801-2, UnitProtKB Q99801-3, UnitProtKB Q99801-4, UnitProtKB Q99801-5, PDB NP_006158.2, and PDB NP_001243268.1). In some embodiments, the nucleic acid is an exogenous DNA molecule or an exogenous RNA molecule. Nonlimiting examples of useful RNA are a modified mRNA (modRNA; e.g., US20230364267A1), a mRNA, or a functional variant thereof. Nonlimiting examples of useful DNA can be a vector, plasmid, transposon, or a functional variant thereof.

In some embodiments, the methods described herein comprise the use of a nucleic acid encoding a E26 transformation-specific variant 2 (*ETV2*). In some embodiments, the nucleic acid is an exogenous DNA molecule or an exogenous RNA molecule. Nonlimiting examples of

useful RNA are a modified mRNA (modRNA; e.g., US20230364267A1), a mRNA, or a functional variant thereof. Nonlimiting examples of useful DNA can be a vector, plasmid, transposon, or a functional variant thereof.

Also described herein are populations of cells comprising at least about 90, 91, 92, 93,
 5 94, 95, 96, 97, 98, or 99% iPSC-derived mural progenitor cells (iMPCs). In some embodiments, the iMPCs comprise a nucleic acid (optionally, an exogenous nucleic acid) encoding NKX3.1 or transiently expressed NKX3.1 (optionally, from a degradable nucleic acid (e.g., mRNA or modRNA)). In some embodiments, the iMPCs express PDGFR β (CD140b) and aminopeptidase N (CD13). In some embodiments, less than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1% of the iMPCs express
 10 a TRA1-81 antigen. In some embodiments, the iMPCs express *ACTA2*, *CNN1*, *TAGLN*, *MYOCD*, *MYH11*, *CSPG4*, *DES*, *PDE5A*, and *THY1*.

Also described herein are populations of cells comprising a ratio of about 1:3, 1:2, 2:3, 1:1, 3:2, 1:2, or 3:1 of endothelial cells (ECs): iPSC-derived mural progenitor cells (iMPCs). In some embodiments, the iMPCs comprise a nucleic acid (optionally, an exogenous nucleic acid)
 15 encoding NKX3.1 or transiently expressed NKX3.1 (optionally, from a degradable nucleic acid (e.g., mRNA or modRNA)). In some embodiments, the iMPCs express PDGFR β (CD140b) and aminopeptidase N (CD13). In some embodiments, less than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1% of the iMPCs express a TRA1-81 antigen. In some embodiments, the iMPCs express *ACTA2*, *CNN1*, *TAGLN*, *MYOCD*, *MYH11*, *CSPG4*, *DES*, *PDE5A*, and *THY1*. In some embodiments, the ECs
 20 comprise any one or more of iPSC-derived ECs (iECs), human umbilical vein endothelial cells (HUVECs), endothelial colony-forming cells (ECFCs), adipose tissue-derived ECs, or organ-specific endothelial cells. In some embodiments, the organ-specific endothelial cells are from an organ selected from: heart, muscle, kidney, testis, ovary, lymphoid, liver, pancreas, brain, lungs, bone marrow, spleen, large intestine, and small intestine. In some embodiments, the iECs
 25 comprise a nucleic acid (optionally, an exogenous nucleic acid) encoding ETV2 or transiently expressed ETV2 (e.g., from a degradable nucleic acid (e.g., mRNA or modRNA)). In some embodiments, the NKX3.1 expression is controlled by an inducible promoter and the ETV2 expression is controlled by an inducible promoter. In some embodiments, the inducible promoter for NKX3.1 and the inducible promoter for ETV2 are the same (optionally, doxycycline). In
 30 some embodiments, the inducible promoter for NKX3.1 and the inducible promoter for ETV2 are not the same.

Also described herein are populations of vascular organoid (VO) or three-dimensional (3D) cell cultures comprising the population of cell populations described herein.

Also described herein are methods of making a vascular organoid (VO) or a three-dimensional (3D) cell culture, the methods comprising:

(i) culturing a first population of iPSC-derived mesodermal progenitor cells (MePCs) comprising a nucleic acid (optionally, an exogenous nucleic acid) encoding ETV2, or a functional variant thereof, ("ETV2/MePCs"), with a second population of MePCs comprising a nucleic acid (optionally, an exogenous nucleic acid) encoding NKX3.1, or a functional variant thereof, ("NKX3.1/MePCs"),

wherein expression of NKX3.1 is controlled by an inducible promoter and expression of ETV2 is controlled by an inducible promoter;

(ii) inducing expression of NKX3.1 in the NKX3.1/MePCs, thereby creating iPSC-derived mural progenitor cells (iMPCs) and inducing expression of ETV2 in the ETV2/MePCs, thereby creating iPSC-derived endothelial cells (iECs); and

(iii) culturing the cells for a time period sufficient to generate a VO or 3D cell culture, wherein the VO or 3D cell culture comprises iECs that are h-CD31⁺ and iMPCs that are PDGFR β ⁺ and h-CD31⁻.

In some embodiments, the inducible promoter for NKX3.1 and the inducible promoter for ETV2 are the same (optionally, doxycycline). In some embodiments, the inducible promoter for NKX3.1 and the inducible promoter for ETV2 are not the same.

In some embodiments, the time period sufficient to generate a VO or 3D cell culture is about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 days.

In some embodiments, the step (i) culturing occurs for about 1 day or 2 days; wherein the step (i) culturing occurs for about 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, or 48 hours; and/or wherein the culturing occurs for a time period sufficient to generate aggregates comprising both the NKX3.1/MePCs and the ETV2/MePCs.

In some embodiments, the (i) culturing step comprises culturing the cells using non-adherent culture plates and an orbital shaker.

In some embodiments, the population of NKX3.1/MePCs and the population of ETV2/MePCs are mixed at a ratio of about 1:3, 1:2, 2:3, 1:1, 3:2, 1:2, or 3:1 of NKX3.1/MePCs:ETV2/MePCs.

In some embodiments, the VOs are uniform size and/or about 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, 350, 355, 360, 365, 370, 375, 380, 385, 390, 395, 400, 405, 410, 415, 420, 425, 430, 435, 440, 445, 450, 455, 460, 465, 470, 475, 480, 485, 490, 495, 500, 505, 510, 515, 520, 525, 530, 535, 540, 545, or 550 μm in diameter; and/or wherein the average diameter size of the VOs or 3D cell cultures is about 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, or 350 μm in diameter; and/or wherein the median size of the VOs or 3D cell cultures is about 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, or 350 μm in diameter; and/or the VOs or 3D cell cultures comprise about 1,000, 1,500, 2,000, 2,500, 2,550, 2,600, 2,650, 2,700, 2,750, 2,800, 2,850, 2,900, 2,950, 3,000, 3,050, 3,100, 3,150, 3,200, 3,250, 3,300, 3,350, 3,400, 3,450, 3,500, 4,000, 4,500, or 5,000 cells.

In some embodiments, the cells self-assembled into a network of CD31+ vascular structures containing mature α -SMA+ perivascular mural cells. In some embodiments, the VOs comprise a network of lumenized vessels with apical-basal polarization and/or wherein the VO comprises arterial, venous, and/or capillary ECs. In some embodiments, the VOs comprise ECs that are *CDH5*⁺ and *VWF*⁺ and mural cell that are *ACTA2*⁺, *MYH11*⁺, *TAGLN*⁺, and *CNN1*⁺. In some embodiments, the methods comprise the formation of an artery, a vein, a capillary, an arteriole, a venule, or any combination thereof.

Also described herein are vascular organoids (VOs) or 3D cell cultures made by any one of the methods described herein.

Also described herein are methods of making vascular organoids (VOs) or three-dimensional (3D) cell cultures, the methods comprising:

- (i) transfecting a population of iPSC-derived mesodermal progenitors (MePCs) with a nucleic acid (optionally, a DNA, an RNA, an mRNA, an modRNA) encoding NKX3.1 or a functional variant thereof, thereby creating a population of iPSC-derived mural progenitor cells (iMPCs);
- (ii) mixing the population of iMPCs with a population of ECs (optionally, at a ratio of about 1:3, 1:2, 2:3, 1:1, 3:2, 1:2, or 3:1 of iMPC:EC); and

(iii) culturing the cells for a time period sufficient to generate the VOs or 3D cell cultures, wherein the VOs or 3D cell cultures comprise iECs that are h-CD31⁺ and iMPCs that are PDGFR β ⁺ and h-CD31⁻.

In some embodiments, the ECs comprise any one or more of iPSC-derived ECs (iECs), human umbilical vein endothelial cells (HUVECs), endothelial colony-forming cells (ECFCs), adipose tissue-derived ECs, or organ-specific endothelial cells.

In some embodiments, the organ-specific endothelial cells are from an organ selected from: heart, muscle, kidney, testis, ovary, lymphoid, liver, pancreas, brain, lungs, bone marrow, spleen, large intestine, and small intestine.

In some embodiments, the methods further comprise a step of transfecting a population of iPSCs with a nucleic acid encoding ETV2 or a functional variant thereof to thereby create a population of ECs prior to mixing with the population of iMPCs.

In some embodiments, the VOs are uniform size and/or about 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, 350, 355, 360, 365, 370, 375, 380, 385, 390, 395, 400, 405, 410, 415, 420, 425, 430, 435, 440, 445, 450, 455, 460, 465, 470, 475, 480, 485, 490, 495, 500, 505, 510, 515, 520, 525, 530, 535, 540, 545, or 550 μ m in diameter; and/or wherein the average diameter size of the VOs or 3D cell cultures is about 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, or 350 μ m in diameter; and/or wherein the median size of the VOs or 3D cell cultures is about 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, or 350 μ m in diameter; and/or the VOs or 3D cell cultures comprise about 1,000, 1,500, 2,000, 2,500, 2,550, 2,600, 2,650, 2,700, 2,750, 2,800, 2,850, 2,900, 2,950, 3,000, 3,050, 3,100, 3,150, 3,200, 3,250, 3,300, 3,350, 3,400, 3,450, 3,500, 4,000, 4,500, or 5,000 cells.

In some embodiments, the cells self-assembled into a network of CD31⁺ vascular structures containing mature α -SMA⁺ perivascular mural cells. In some embodiments, the VOs comprise a network of lumenized vessels with apical-basal polarization and/or wherein the VO comprises arterial, venous, and/or capillary ECs. In some embodiments, the VOs comprise ECs that are CDH5⁺ and VWF⁺ and mural cell that are ACTA2⁺, MYH11⁺, TAGLN⁺, and CNN1⁺. In

some embodiments, the methods comprise the formation of an artery, a vein, a capillary, an arteriole, a venule, or any combination thereof.

Also described herein are vascular organoids (VOs) or 3D cell cultures made by any one of the methods described herein.

5 In some embodiments, the VOs or 3D cell cultures are uniform size; and/or wherein the VOs or 3D cell cultures are about 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, 350, 355, 360, 365, 370, 375, 380, 385, 390, 395, 400, 405, 410, 415, 420, 425, 430, 435, 440, 445, 450, 455, 460, 465, 470, 475, 480, 485, 490, 495, 500, 505, 510, 515, 520, 10 525, 530, 535, 540, 545, or 550 μm in diameter; and/or wherein the average diameter size of the VOs or 3D cell cultures is about 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, or 350 μm in diameter; and/or wherein the median size of the VOs or 3D cell cultures is about 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 15 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, or 350 μm in diameter; and/or the VOs or 3D cell cultures comprise about 1,000, 1,500, 2,000, 2,500, 2,550, 2,600, 2,650, 2,700, 2,750, 2,800, 2,850, 2,900, 2,950, 3,000, 3,050, 3,100, 3,150, 3,200, 3,250, 3,300, 3,350, 3,400, 3,450, 3,500, 4,000, 4,500, or 5,000 cells.

In some embodiments, the VOs or 3D cell cultures comprise CD31+ vascular structures containing mature α -SMA+ perivascular mural cells. In some embodiments, the VOs or 3D cell 20 cultures comprise a network of lumenized vessels with apical-basal polarization and/or arterial, venous, and/or capillary ECs.

In some embodiments, the VOs or 3D cell cultures comprise ECs that are *CDH5*⁺ and *VWF*⁺ and mural cell that are *ACTA2*⁺, *MYH11*⁺, *TAGLN*⁺, and *CNN1*⁺.

25 Also described herein, *inter alia*, are compositions comprising any of the population of cells described herein and/or any of the VOs or 3D cell cultures described herein. In some embodiments, any composition may further comprise one or more of an agent, an excipient, a matrix, or a gel. In some embodiments, any composition may further comprise a gel or matrix comprising a hydrogel. In some embodiments, any composition may further comprise a gel or 30 matrix comprising gelatin, collagen, fibrinogen, thrombin, fibrin, or any combinations thereof. In some embodiments, any composition may further comprise a gel or matrix comprising about 1.5

mg/mL collagen, about 30 µg/mL fibrinogen, and about 1 mg/mL human fibronectin. In some
embodiments, the matrix can contain collagen and/or fibrin. In some embodiments, fibrin is
formed with fibrinogen and thrombin, (optionally, about 50 µg/mL thrombin). In some
embodiments, any composition may further comprise a gel or matrix comprising any one or
5 more of gelatin, collagen, fibrinogen, laminin, entactin, or combinations thereof. In some
embodiments, any composition may further comprise a gel or matrix comprising laminin,
entactin, and collagen. In some embodiments, any composition may further comprise a gel or
matrix comprising about 5.25 mg/mL laminin, about 5.25 mg/mL entactin, and about 0.2 mg/mL
collagen IV. In some embodiments, any composition may further comprise a gel or matrix is
10 Matrigel™. Matrigel is known in the art (U.S. Pat. No. 4,829,000).

Also described herein, *inter alia*, are methods of transplanting any of the populations of
cells described herein, any of the VOs or 3D cell cultures described herein, and/or any of the
compositions described herein. In some embodiments, the method comprises administering to
the subject an effective amount of the population of cells, vascular organoids, or composition.

15 Also described herein, *inter alia*, are methods of increasing blood vessel formation
comprising administering to a subject in need thereof an effective amount of any of the
populations of cells described herein, any of the VOs or 3D cell cultures described herein, and/or
any of the compositions described herein. In some embodiments, the method comprises:

identifying a subject in need of increased blood vessel formation; and
20 administering to the subject an effective amount of any of the populations of cells
described herein, any of the VOs or 3D cell cultures described herein, and/or any of the
compositions described herein.

Also described herein, *inter alia*, are methods of increasing vascular generation or
vascular regeneration comprising administering to a subject in need thereof an effective amount
25 of any of the populations of cells described herein, any of the VOs or 3D cell cultures described
herein, and/or any of the compositions described herein. In some embodiments, the method
comprises:

identifying a subject at in need of vascular generation or vascular regeneration;
administering to the subject an effective amount of any of the populations of cells
30 described herein, any of the VOs or 3D cell cultures described herein, and/or any of the
compositions described herein.

Also described herein, *inter alia*, are methods of vascular cell therapy comprising administering to a subject in need thereof an effective amount of any of the populations of cells described herein, any of the VOs or 3D cell cultures described herein, and/or any of the compositions described herein. In some embodiments, the method comprises:

- 5 identifying a subject in need of vascular cell therapy;
administering to the subject an effective amount of any of the populations of cells described herein, any of the VOs or 3D cell cultures described herein, and/or any of the compositions described herein.

10 In some embodiments of any of the methods described herein, the subject or patient may be undergoing an organ transplant, selected to undergo an organ transplant, or need vascularization of an organ. In some embodiments, the organ is selected from the group consisting of skin, heart, kidney, testis, ovary, bone, lymph, liver, pancreas, brain, lungs, bone marrow, spleen, large intestine and small intestine.

15 In some embodiments of any of the methods described herein, vascularization comprises the formation of an artery, a vein, a capillary, an arteriole, a venule, or any combination thereof.

In some embodiments of any of the methods described herein, the subject has (or is at risk of having or developing) any one or more of the following: diabetes, diabetic retinopathy, an ischemic injury, a disease or disorder of the blood vessels, atherosclerosis, Age-related Macular Degeneration (AMD), Pulmonary Arterial Hypertension (PAH), Hereditary Hemorrhagic
20 Telangiectasia (HHT), peripheral artery disease (PAD), arteriovenous fistulas (e.g., dialysis patients), tumor angiogenesis, tumor metastasis, a stroke, and/or a wound (optionally, a chronic wound; e.g., a diabetic ulcer).

In some embodiments of any of the methods described herein, the methods normalize and/or correct aberrant vasculature; for example, where the vasculature lacks or has deficient
25 mural cells. In some embodiments of any of the methods described herein, the subject has a disorder characterized by vasculature that lacks or has deficient mural cells (optionally, diabetic retinopathy, tumor angiogenesis, tumor metastasis, stroke, ischemic injury, atherosclerosis, Age-related Macular Degeneration (AMD), Pulmonary Arterial Hypertension (PAH), Hereditary Hemorrhagic Telangiectasia (HHT), peripheral artery disease (PAD), arteriovenous fistulas (e.g.,
30 dialysis patients), and/or a wound (optionally, a chronic wound; e.g., a diabetic ulcer)

Without being bound by theory, tumors often exhibit abnormal vasculature characterized by a lack of proper mural cell coverage, leading to leaky and dysfunctional blood vessels. Introducing any of the iMPCs, cell populations, VOs, and compositions herein could help stabilize these vessels, improving the delivery of therapeutics, and/or reduce metastasis.

5 Without being bound by theory, in diabetic retinopathy, pericyte loss leads to weakened blood-retinal barriers, resulting in retinal ischemia and neovascularization. The iMPCs, cell populations, VOs, and/or compositions described herein can potentially restore the integrity of retinal vasculature and/or reduce the progression of the disease.

10 Without being bound by theory, after a stroke or ischemic injury, there is often a loss of vascular integrity and a need for vascular repair. The iMPCs, cell populations, VOs, and/or compositions described herein can aid in re-establishing stable blood vessels and/or promoting recovery of the affected tissue.

15 Without being bound by theory, in atherosclerosis, the stability of blood vessels is compromised due to inflammatory processes and endothelial dysfunction. The mural cells derived from iMPCs, iMPCs, cell populations, VOs, and/or compositions described herein can help reinforce vascular walls and/or mitigate the progression of atherosclerotic plaques.

20 Without being bound by theory, in wound healing, chronic wounds, such as diabetic ulcers, often suffer from poor vascularization and deficient mural cell coverage. The iMPCs, cell populations, VOs, and/or compositions described herein can enhance angiogenesis and vascular stability, promoting better wound healing outcomes.

25 Without being bound by theory, in Age-related Macular Degeneration (AMD), especially the wet form, choroidal neovascularization occurs with deficient pericyte support, leading to fragile and leaky vessels. The iMPCs, cell populations, VOs, and/or compositions described herein can help in providing the necessary support to these new vessels, reducing leakage, and/or reducing vision loss.

30 Without being bound by theory, Pulmonary Arterial Hypertension (PAH) is characterized by abnormal proliferation of pulmonary vascular cells and deficient pericyte coverage, leading to vascular remodeling and hypertension. The iMPCs, cell populations, VOs, and/or compositions described herein could stabilize these blood vessels and/or alleviate one or more symptoms (e.g., hypertension).

Without being bound by theory, Hereditary Hemorrhagic Telangiectasia (HHT) is a genetic disorder leading to abnormal blood vessel formation with deficient mural cell coverage, resulting in bleeding and arteriovenous malformations. The iMPCs, cell populations, VOs, and/or compositions described herein can potentially normalize these vessels and/or reduce bleeding episodes.

In some embodiments of any of the methods described herein, the population of cells, vascular organoid, or composition is administered to the subject, before, during, or after a cell transplant, tissue transplant, or organ transplant.

Also described herein are methods of tissue engineering (e.g., small-diameter vascular grafts). Tissue-engineered small-diameter vascular grafts are bioengineered constructs designed to replace damaged or diseased blood vessels. These grafts are typically created from scaffolds that are seeded with cells, such as smooth muscle cells (SMCs), which provide structural support and functionality. The cell populations described herein (e.g., NKX3.1-derived iMPCs and their mature mural cell derivatives) could be incorporated into these scaffolds as SMCs, facilitating the development of grafts that can be surgically implanted in patients as vessel replacements. Small-diameter vascular grafts are particularly relevant for clinical applications such as coronary artery bypass grafting (CABG), peripheral artery disease (PAD), and arteriovenous fistulas for dialysis patients.

In some embodiments, any of the populations of cells described herein, any of the VOs or 3D cell cultures described herein, and/or any of the compositions described herein (e.g., a composition comprising NKX3.1-derived iMPCs and/or their mature mural cell derivatives) are used as a cell source for tissue engineering applications, particularly in the development of small-diameter vascular grafts. For example, a population of iMPC-derived cells described herein can function as smooth muscle cells (SMCs) within the graft scaffold of a small-diameter vascular grafts and be surgically transplanted in a subject in need thereof (e.g., a subject in need to vessel replacement, a subject having or at risk of having coronary artery bypass grafting (CABG), a subject having or at risk of having peripheral artery disease (PAD), and a subject having or at risk of having arteriovenous fistulas (e.g., a dialysis patient). Without being bound by theory, the incorporation of iMPC cells into a structural scaffold enables the creation of functional, living vascular grafts that can be implanted to restore or replace damaged blood vessels, offering significant potential for these high-demand clinical scenarios.

In some embodiments of any of the methods described herein, the population of cells, vascular organoid, or composition is administered to the subject by direct injection into a blood vessel or subcutaneous, intradermal, intramuscular, intranodal, intravenous, intraprostatic, intratumor, intralymphatic, and intraperitoneal injection.

5 An “effective amount” is an amount sufficient to effect beneficial or desired results (e.g., an amount sufficient for the formation of an artery, a vein, a capillary, an arteriole, a venule, or any combination thereof in a patient or subject). For example, a therapeutic amount is one that achieves the desired therapeutic effect (e.g., an amount sufficient for the formation of an artery, a vein, a capillary, an arteriole, a venule, or any combination thereof; an amount sufficient to
10 increase blood flow in a patient or subject; an amount sufficient to ameliorate at least one symptom of a disease or condition in a patient or subject).

 As used herein, the term “about” means within a statistically meaningful range of a value, such as a stated concentration range, time frame, molecular weight, particle size, temperature or pH. Such a range can be within an order of magnitude, typically within 20%, more typically
15 within 10%, and even more typically within 5% of the indicated value or range. Sometimes, such a range can be within the experimental error typical of standard methods used for the measurement and/or determination of a given value or range. The allowable variation encompassed by the term “about” will depend upon the particular system under study, and can be readily appreciated by one of ordinary skill in the art. Whenever a range is recited within this
20 application, every whole number integer within the range is also contemplated as an embodiment of the present disclosure.

 An effective amount can be administered in one or more administrations, applications or dosages. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the
25 disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of any therapeutic population of cells, VO, 3D cell culture, composition described herein can include a single treatment or a series of treatments.

 A skilled artisan will be able to determine and identify a subject or patient suitable for
30 any of the methods described herein (e.g., a subject suffering from or at risk of having a stroke, a subject suffering from or at risk of having diabetes, a subject suffering from or at risk of having

diabetic retinopathy, a subject suffering from or at risk of having an ischemic injury, a subject suffering from or at risk of having a disease or disorder of the blood vessels, and/or a subject having or selected to have a transplant).

5 Mural cells are central to vascular integrity and function. Described herein is an innovative use of the transcription factor NKX3.1 to guide the differentiation of human induced pluripotent stem cells into mural progenitor cells (iMPCs). By transiently activating NKX3.1 in mesodermal intermediates, the methods described herein diverge from traditional growth factor-based differentiation techniques. This approach efficiently generates a robust iMPC population capable of maturing into diverse functional mural cell subtypes, including smooth muscle cells
10 and pericytes. These iMPCs exhibit key mural cell functionalities such as contractility, deposition of extracellular matrix, and the ability to support endothelial cell-mediated vascular network formation *in vivo*. These findings not only underscore the fate-determining significance of NKX3.1 in mural cell differentiation but also highlights the therapeutic potential of these iMPCs. We envision these insights could pave the way for a broader use of iMPCs in vascular
15 biology and regenerative medicine.

Additional methods of making, formulating, and administering VOs and 3D cell cultures are known in the art (*See*, e.g., US 2019/0376044 A1; US 2020/0199541 A1; US 2023/0287357 A1; US 2023/0174949 A1; US 2023/0364267 A1; US Pat. No. 11,214,768; WO 2022/226337; WO 2023/196683).

20 Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent
25 applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

DESCRIPTION OF DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

5 **FIGS. 1A-1O. Efficient differentiation of iPSCs into iMPCs using NKX3.1 activation.** (1A) A schematic of the two-step, feeder-free, chemically defined protocol used for mural cell differentiation. (1B) Flow cytometry analysis of CD13 and CD140b (mural cell markers) throughout differentiation stages showing conversion into CD140b+/CD13+ iMPCs with exceedingly high efficiency. (1C) Flow cytometry analysis of TRA1-81 (pluripotency marker) throughout differentiation stages. Negligible presence of undifferentiated iPSCs
10 expressing the TRA1-81 antigen. (1D) Immunofluorescence staining demonstrating the expression of mural-specific contractile proteins and cytoskeletal markers: α -SMA, SM22, Vimentin, and Calponin, with DAPI nuclear staining in iMPCs after one passage in culture (Scale bars: 50 μ m). (1E) mRNA expression (qPCR) of SMC markers and (1F) pericyte markers
15 in iMPCs, SMCs, and MSCs. The smooth muscle markers of SMC gene expression of 1E (n=4-7) and the pericyte markers showing pericyte-specific gene expression of 1F (n=4-7) are qRT-PCR quantification analysis depicted in bar graphs showing the mean $\pm$ SEM. (1G) NKX3.1 Expression: Time-course qRT-PCR analysis demonstrating NKX3.1 upregulation during differentiation (n=4-8; ***P<0.001; mean $\pm$ SEM). (1H) Morphological Progression: Phase-contrast microscopy revealing morphological evolution at various stages (Scale bars: 100 μ m;
20 insets 50 μ m). (1I) Differentiation schematic illustrating the stepwise differentiation from iPSCs to iMPCs, detailing mesodermal induction, mural cell specification, and expansion phases. (1J-1M) Doxycycline-induced expression of NKX3.1 in genetically engineered iPSCs and maintenance of pluripotency markers. (1J) Schematic illustrating the induction of NKX3.1
25 expression in human iPSCs upon doxycycline (Dox) treatment. Contrast image of an iPSC-Dox-NKX3.1 clone in culture. Scale bar: 200 μ m. (1K) Immunofluorescence imaging of iPSC-Dox-NKX3.1 clones without Dox (-Dox) and post 24 hours of Dox treatment (+Dox 24h). Cells are stained for NKX3.1 (green) with nuclei counterstained using DAPI (blue). Scale bars: 50 μ m. (1L) Representative immunofluorescence images demonstrating the maintenance of pluripotency
30 markers OCT4, SOX2, and NANOG in iPSC-Dox-NKX3.1 clones. Nuclei are counterstained with DAPI (blue). Scale bars: 50 μ m. (1M) qRT-PCR-based expression analyses of pluripotency

markers *OCT4*, *SOX2*, and *NANOG* in iPSC-Dox-NKX3.1 relative to parental iPSCs (n=4; unpaired two-tailed t-test; n.s. denotes nonsignificant differences; mean $\pm$ SEM). (1N-1O) Reproducibility of NKX3.1-induced differentiation in three independent iPSC lines. (1N) Flow cytometry analysis of CD13 and CD140b (mural cell markers) in iMPCs generated from three independent iPSC-Dox-NKX3.1 clones (BJ273, 3C18, and 11C23). (1O) qRT-PCR-based expression analyses of mural cell markers in iMPCs generated from iPSC-Dox-NKX3.1 clones 3C18 and 11C23 (n=3; mean $\pm$ SEM). For all FIG. 1A-1O experiments: *P<0.05, **P<0.01, ***P<0.001. All PCR data is normalized to GAPDH.

FIGS. 2A-2E. Comparative analysis of NKX3.1-induced iMPCs and chemically-induced iSMCs. (2A) Expression of NKX3.1 (top bar graphs) and TBXT (bottom bar graphs) (qPCR) in both the NKX3.1-induced (iMPCs; right column of graphs) and the chemically-induced (iSMCs; left column of graphs) differentiation protocols. Both protocols exhibit transient expression of NKX3.1 starting at 48 h. (n=4-6; ANOVA followed by Bonferroni's post-test analysis; mean $\pm$ SEM) (2B) mRNA expression (qPCR) of SMC markers and (2C) pericyte markers in iMPCs and iSMCs at 96 h. Analysis of qRT-PCR-based expression analyses of SMC markers in iMPCs and iSMCs in 2B and of pericyte markers in iMPCs and iSMCs in 2C are depicted in bar graphs showing the mean $\pm$ SEM (n=4-7; unpaired two-tailed t-test). (2D) Schematic of the chemically-induced protocol to generate iSMCs from iPSCs through MePC intermediates via exposure to PDGF-BB (10 ng/mL) and Activin A (2 ng/mL). (2E) Schematic depicting the dox-induced protocol to differentiate iPSCs into iMPCs via transient expression of NKX3.1. *P<0.05, **P<0.01, ***P<0.001; n.s. denotes non-significant differences.

FIGS. 3A-3G. Generation of iMPCs using a genomic footprint-free approach with modified mRNA. (3A) Schematic representation of the modRNA-induced protocol. (3B) Transfection of iPSC with modRNA and expression of NKX3.1 by qPCR after 48 h. (3C) FACS analysis indicating $\sim$ 95% conversion efficiency to iMPCs following modRNA-induced NKX3.1 activation in MePCs. (3D) mRNA expression (qPCR) of mural cell markers at 96 h. The qRT-PCR-based expression analyses of SMC and pericyte markers in iMPCs generated with Dox versus modRNA are depicted in bar graphs showing the mean $\pm$ SEM (n=5; unpaired two-tailed t-test; *P<0.05, n.s. denotes nonsignificant differences). (3E) Flow cytometric analyses of CD13 and CD140b (mural cell markers) expression in iMPCs at 96 hours generated with Dox versus modRNA. (3F) Schematic of timeline depicting the differentiation protocol from iPSCs through

mesodermal intermediates (MePCs) into induced Mural Progenitor Cells (iMPCs) using modRNA encoding for NKX3.1. **(3G)** Quantitative PCR analysis of *NKX3.1* expression over time after modRNA transfection (24 h, 48 h, and passages P1 and P2). Not transfected MePCs served as control (n=4; ANOVA followed by Bonferroni's post-test analysis; ***P<0.001, n.s. denotes non-significant differences). For all PCR analyses, expression values were normalized to the housekeeping gene *GAPDH*. **P<0.01.

FIGS. 4A-4J. Functional characterization and secretory profile of iMPCs. (4A)

Calcium imaging shows increased intracellular calcium in iMPCs in response to endothelin-1 and carbachol. **(4B)** Collagen contractility assay show comparable responses of iMPCs, MSCs, and iSMCs to U46619 (TXA2 analog; vasoconstrictive stimulus). Collagen Gel contraction image shown below the analysis of gel contractility (n=3; ***P<0.001; mean $\pm$ SEM) **(4C)** Increased fibronectin production in iMPCs upon TGF- β treatment, and its inhibition by TGF- β signaling inhibitor, SB31542. **(4D)** Angiogenesis protein array of conditioned media (CM) from iMPCs, SMCs, and MSCs. **(4E)** Secreted pro-angiogenic factors by Luminex protein assay: a multiplex assay of angiogenic factors in conditioned media, including FGF-2, HB-EGF, HGF, PLGF, VEGF-A, and VEGF-C (n=2; *P<0.05, **P<0.01, ***P<0.001; mean $\pm$ SEM). **(4F)** Calcium Imaging: Intracellular calcium flux in iMPCs visualized using a green fluorescent indicator following stimulation with endothelin-1, carbachol, or PBS. Pseudo-coloring indicates intensity, with blue and red representing lower and higher calcium levels, respectively (Scale bars: 40 μ m). Quantitative analysis of peak calcium uptake is shown on the right. **(4G)** Peak Calcium Response: Comparative uptake in MSCs, SMCs, and iMPCs upon endothelin-1 and carbachol stimulation, shown as delta fluorescence (n=5; ***P<0.001; mean $\pm$ SEM). **(4H-4I)** Fibronectin Deposition: Immunofluorescence staining of iMPCs treated with TGF β and TGF β inhibitor SB431542 (Scale bars: 100 μ m), with quantification of fibronectin intensity per cell shown in **4I** (n=6; **P<0.01, ***P<0.001; mean $\pm$ SEM). **(4J)** FN1 Expression: RT-qPCR analysis of FN1 normalized to GAPDH (n=4; ***P<0.001; mean $\pm$ SEM). *P<0.05, **P<0.01, ***P<0.001.

FIGS. 5A-5M. Modulation of EC function by iMPCs. (5A) EC proliferation under

indirect co-culture with iMPCs. Schematic diagram depicting ECs cultured with mural cells and proliferation assessment when co-cultured with SMCs, MSCs, and iMPCs (Right; n=7; *P<0.05; mean $\pm$ SEM). **(5B)** EC proliferation upon exposure to iMPC-conditioned media (CM-(iMPCs)).

Growth quantification in ECs exposed to 2-fold concentrated conditioned medium from SMCs, MSCs, and iMPCs ($***P < 0.001$; mean $\pm$ SEM). **(5C)** Enhancement of EC migration and re-endothelialization by CM-(iMPCs). Scratch assay comparing EC migration in conditioned medium from iMPCs and basal medium at 24 hours (Left; Scale bar: 200 μ m), with a quantified migration percentage of gap closure normalized to the basal medium control (Right; n=4; $***P < 0.001$; mean $\pm$ SEM). **(5D)** Capillary-like structure formation in three-dimensional cultures exposed to CM-(iMPCs). Tube formation assay on Matrigel using conditioned medium, with representative images (Scale bars: 200 μ m) and quantification of total tube length (Right; n=4; $**P < 0.01$; mean $\pm$ SEM). **(5E)** Blood perfusion in subcutaneous implants containing ECs + mural cells (SMCs, MSCs, or iMPCs) one week post-implantation into nude mice, with explanted grafts visually assessed at day 7 (Scale bar: 4 mm). **(5F)** H&E staining identifying perfused blood vessels in implants at day 7 (yellow arrows) (Scale bars: 50 μ m). H&E staining show the formation of perfused vessels containing murine erythrocytes in implants seeded with ECs + iMPCs, but not in implants with ECs alone. **(5G)** Microvessel density analysis per mm² area (n=7; $*P < 0.05$; mean $\pm$ SEM). Average microvessel densities at day 7 across implants with different mural cell populations. **(5H)** Staining of newly formed human vessels for human-specific CD31 and surrounding α -SMA-positive perivascular cells. **(5I)** Human Vessel Identification: IHC showing human-specific ECs (h-CD31+) and human perivascular cells (h-Vimentin+) (Scale bar: 50 μ m; inset 10 μ m). **(5J)** iMPC Tracing: GFP and α -SMA staining to track GFP-labeled iMPCs within the perivascular niche *in vivo* (Scale bars: 50 μ m; insets 10 μ m). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. **(5K)** Schematic depiction and fluorescent images showing the coculture of ECs and iMPCs (P1) within a microfluidic on-a-chip model. GFP-labeled iMPCs and DsRed-labeled ECs were embedded in a fibrin gel, and the formation of vascular structures was observed after 2 days. (Scale bars: 500 μ m). **(5L)** Immunofluorescent staining of the vascular network formed within the microfluidic chip. The ECs are marked by CD31 and VE-Cadherin (red), and the iMPCs are identified by α -SMA and SM22 (green). Nuclei are counterstained with DAPI (blue). The inset shows a magnified view of an endothelial lumen surrounded by mural cells (yellow arrowheads). (Scale bars: 100 μ m). **(5M)** Quantification of the percentage of human vessels with human mural cell coverage, comparing ECs implanted with SMCs, MSCs, and iMPCs (n=4; mean $\pm$ SEM). All experiments in this Figure used nascent iMPCs right after differentiation (96 h).

FIGS. 6A-6J. Maturation of iMPCs upon interaction with ECs. (6A) Schematic of iMPCs co-cultured with ECs for 7 days and then isolated as CD31⁺ cells for bulk RNA-seq analysis. The lower panel indicates up-regulated and down-regulated gene counts in co-cultured iMPCs (co-iMPCs) versus mono-cultured iMPCs. (6B) Principal Component Analysis of differentially expressed genes demonstrating that co-iMPCs exhibit closer transcriptional proximity to primary SMCs and MSCs than iMPCs. Transcriptional comparison of co-iMPCs with primary SMCs and MSCs relative to iMPCs and iPSCs (n=3). (6C) Gene Ontology (GO) analysis highlighting the significant enrichment of genes associated with mature mural cell functions in co-iMPCs. Up-regulated genes in co-iMPCs associated with mature mural cell functions. (6D) qPCR analysis confirming the significant upregulation of genes associated with mural cell markers in co-iMPCs. (6E) Transcriptomic Correlation: Pearson's correlation plot delineating transcriptional profiles among SMCs, co-iMPCs, MSCs, iMPCs, iSMCs, and iPSCs. (6F-6G) Marker Gene Expression: RT-qPCR analysis of SMC and pericyte markers, showing enhanced expression in co-iMPCs versus mono-cultured iMPCs (n=3-9; *P<0.05, **P<0.01, ***P<0.001; mean ± SEM). All PCR data is normalized to GAPDH. (6H) Immunofluorescence Characterization of co-iMPCs: co-iMPCs were sorted as CD31⁺ cells from the co-culture and stained for α-SMA (green) with 3G5 (pericyte marker, red) and nuclei counterstained with DAPI (blue), demonstrating the presence of both SMCs (α-SMA⁺/3G5⁻) and pericytes (α-SMA⁺/3G5⁺) (Scale bar: 100 μm). (6I) Gene Expression Heatmap: Differential gene expression patterns in co-iMPCs compared to iMPCs. *P<0.05, **P<0.01, ***P<0.001. (6J) Sorted cells stained for α-SMA (green) (green), MYH11 (red), and DAPI (blue). The co-localization of MYH11 and α-SMA is indicative of cells with a more mature SMC phenotype (yellow arrowheads). (Scale bar: 50 μm). All experiments in this Figure used nascent iMPCs right after differentiation (96 h).

FIGS. 7A-7C. Single-cell RNA sequencing analysis of mural cell heterogeneity. (7A) Time points of analysis during the differentiation protocol and after co-culture with ECs. (7B) Integrated clustering analysis using Seurat (UMAP plot). We identified 8 distinct clusters by manual annotation. (7C) UMAP plots with temporal emergence of clusters, showing the transition from iPSCs to MePCs and iMPCs, and the eventual maturation of iMPCs into distinct mural cell types (SMCs, pericytes, and fibroblasts) after co-culture with ECs.

FIGS. 8A-8G. Co-differentiation of iPSCs into iECs and iMPCs in 3D vascular organoids (VOs). (8A) Schematic representation of the VO method using dox-ETV2-iPSC and dox-NKX3.1-iPSC lines. (8B) Image illustrating uniform size (~200 μ m) of VOs generated in 5 days. (8C) Flow cytometry panel showing iECs (h-CD31+) and iMPCs (h-CD31-/PDGFR β +) in the VO post-differentiation. (8D) Image of self-assembled CD31+ vascular structures within the VO. (8E) Schematic representation of VOs implantation into the renal capsule of NSG mice. (8F) H&E-stained image indicating extensive network of perfused blood vessels within the grafts. (8G) Immunofluorescent staining confirming perfused microvessels lined by human-CD31+ iECs and surrounded by α -SMA+ perivascular mural cells.

FIGS. 9A-9C. Comparative maturation of iMPCs and iECs in our VO model. (9A) Schematic representation of VO enzymatic digestion process and sorting of CD31+ (VO-iECs) and CD31- (VO-iMPCs) cells. (9B) Increased expression of key endothelial markers (qPCR) in VO-iECs compared to 2D-iECs, signifying enhanced maturation. (9C) Significant upregulation of mural cell markers (qPCR) in VO- iMPCs, indicating improved maturation. ***P<0.001.

FIGS. 10A-10D. Transplantation of VOs into ischemic tissue improves blood flow and prevents necrosis. (10A) Schematic representation of the procedure for inducing hind limb ischemia in diabetic nude mice and the injection of VOs. Untreated ischemic mice served as control. (10B) Bioluminescent imaging showing successful engraftment of VOs in ischemic hind limbs. (10C) Laser Doppler imaging demonstrates a 50% blood flow recovery in the affected limbs at 2 weeks post-VO injection. (10D) Prevention of necrotic tissue development in mice receiving VOs. **P<0.01.

FIGS. 11A-11H. Delineation of mural cell heterogeneity and maturation in iMPCs via scRNA-seq. (11A) Differentiation and Co-Culture Timeline: Schematic cartoon depicting the progression from iPSCs through various stages to iMPCs and their subsequent co-culture with ECs, with an emphasis on the transition points sampled for scRNA-seq. c-SMCs and s-SMCs refer to contractile and synthetic SMCs, respectively. (11B) Cellular Clustering: UMAP projection displaying 17 identified clusters annotated into eight cell types, including iPSCs, MePCs, iMPCs, and various mural cells, based on gene expression markers. (11C) Differentiation Trajectory: UMAP visualization tracking the differentiation from iPSCs to iMPCs and the emergence of mural cell clusters. (11D) Marker Gene Expression: Dot plot summarizing the expression profiles of key markers across clusters, delineating cell identity. (11E) Gene

Expression Dynamics: Volcano plots displaying the up- and down-regulated genes in iMPCs after EC co-culture, with emphasis on genes related to contractility and ECM components. (11F)

Pseudotime Analysis: UMAP overlaid with pseudotime scores, indicating the developmental progression of cells. (11G)

Pseudotime Trajectory: Sequential UMAP plots showing the gradual transition from iPSCs to mature mural cells over time and the influence of EC co-culture. (11H)

Schematic Summary: Illustration summarizing the differentiation of iMPCs into specialized mural cells, highlighting the impact of EC interaction on iMPC maturation and the establishment of mural cell heterogeneity.

FIG. 12. Heatmap of differentially expressed genes across cell clusters. Heatmap delineating the top ten upregulated genes within each cell cluster—iPSCs, MePCs, iMPCs, and various mural cell subtypes—identified through single-cell RNA sequencing. The color spectrum (purple for lower, yellow for higher expression) highlights the gene expression magnitude. Rows indicate individual genes, and columns represent single cells, illustrating the unique expression profiles that define each cell population. This heatmap underscores the transcriptional diversity characteristic of cellular differentiation stages.

FIG. 13. UMAP visualization of select gene expression in cellular differentiation. This feature plot presents a UMAP visualization depicting the expression patterns of key genes across cell types derived from iPSCs. Each panel represents the expression of a specific gene in single-cell RNA sequencing data, with color intensity reflecting expression levels from low (gray) to high (blue). The genes displayed are markers indicative of pluripotent stem cells (*SOX2*, *POU5F1*), mesodermal progenitors (*MIXL1*, *TBXT*), induced mural progenitor cells (*NKX3.1*, *DES*), mural cells (*PDGFRB*, *NT5E*, *ACTA2*, *CNN1*, *COL1A1*), and endothelial cells (*PECAMI*), providing insights into the molecular signatures characteristic of each differentiation stage.

FIG. 14. Violin plot of marker gene expression in cellular subtypes. Violin plots representing the distribution of expression levels for selected marker genes within distinct cell clusters identified via single-cell RNA sequencing. The clusters include induced pluripotent stem cells (iPSCs), mesodermal progenitor cells (MePCs), induced mural progenitor cells (iMPCs), fibroblasts, pericytes, contractile smooth muscle cells (c-SMCs), synthetic smooth muscle cells (s-SMCs), endothelial cells (ECs), and endothelial cells post co-culture with iMPCs (co-ECs).

Each plot provides a visual representation of the density of cells at varying expression levels, offering insights into the transcriptional landscape characteristic of each cell subtype.

FIGS. 15A-15B. NKX3.1-induced differentiation of MePCs into iMPCs. (15A)

NKX3.1 Expression: Time-course qRT-PCR analysis demonstrating NKX3.1 upregulation during differentiation (n=9; ***P<0.001; mean $\pm$ SEM). **(15B)** Quantitative PCR analysis of mural cell marker expression with/without NKX3.1 induction. Gene expression of *ACTA2*, *CNN1*, *TAGLN*, *MYOCD*, *TPM1*, *MYH11*, *PDGFRB*, *CSPG4*, *DES*, *PDE5A*, and *THY1* in MePCs treated with doxycycline (+Dox) and without doxycycline (-Dox) for 48 hours to induce iMPCs. (n=4; unpaired two-tailed t-test; *P<0.05, **P<0.01, ***P<0.001; mean $\pm$ SEM). All PCR data is normalized to control (+dox).

FIGS. 16A-16C. Immunofluorescence analysis of mural cell markers in iMPCs.

(16A) Immunofluorescence staining of ECFCs and iMPCs for endothelial and mural cell markers. Control ECFCs show positive expression of EC markers (CD31, VE-Cadherin, vWF) and negative expression of mural cell markers (α -SMA, SM22, Calponin), which demonstrates antibody specificity. Nuclei are stained with DAPI (blue). Scale bars: 50 μ m. **(16B)** Immunofluorescence staining of iMPCs from three different iPSC lines (BJ273, 3C18, 11C23). iMPCs are positive for mural cell markers α -SMA, SM22, Calponin, and Vimentin (green). Nuclei are stained with DAPI (blue). **(16C)** Quantification of marker expression in iMPCs. The bar graph shows the percentage of cells positive for α -SMA, SM22, Calponin, and Vimentin in BJ273, 3C18, and 11C23 iMPCs (n=5; mean $\pm$ SEM). Scale bars: 50 μ m.

FIGS. 17A-17D. *In vivo* validation of iMPCs' capacity to facilitate HUVEC-mediated vascular network development. Subcutaneous implantation of HUVECs with iMPCs into nude mice. Implants with ECFCs + iMPCs served as controls. **(17A)** H&E staining identifying perfused blood vessels at day 7 (yellow arrowheads) (Scale bars: 50 μ m). **(17B)** Microvessel density in grafts at day 7 (unpaired two-tailed t-test; n.s. denotes non-significant differences; mean $\pm$ SEM). **(17C)** Human Vessel Identification: IHC shows human-specific ECs (h-CD31+) covered by human perivascular cells (h-Vimentin+) in grafts on day 7 (yellow arrowheads) (Scale bars: 50 μ m; inset 10 μ m). **(17D)** Quantification of the percentage of human vessels covered by human mural cells (n=4; unpaired two-tailed t-test; n.s. denotes non-significant differences; mean $\pm$ SEM).

FIG. 18. Quantitative PCR analysis of mural cell markers in iMPCs after co-culture with and without ECs. Gene expression of *ACTA2*, *CNN1*, *TAGLN*, *MYOCD*, *TPMI*, *MYH11*, and *CSPG4* in iMPCs co-cultured for 7 days with and without ECs. Data normalized to the condition without ECs (n=3, unpaired two-tailed t-test; *P<0.05, **P<0.01, ***P<0.001; mean +/- SEM).

FIGS. 19A-19D. UMAP visualization of select gene expression in MePCs and iMPCs. (19A) UMAP plots derived from single-cell RNA sequencing data capture the transition from mesodermal progenitor cells (MePCs) at day 2 to induced mural progenitor cells (iMPCs) at day 4 following NKX3.1 induction. (19B-19D) Feature plots illustrate the expression levels at days 2 and 4 of genes associated with (19B) paraxial mesoderm (*TBX6*, *MSGN1*), (19C) somites (*FOXC2*, *MEOX2*, *TCF15*), and (19D) sclerotome (*PAX9*, *SOX9*, *NKX3.2*). The + symbols indicate detectable gene expression, with the intensity denoted by the depth of color, from low (light gray) to high (dark purple).

FIG. 20. UMAP visualization of select gene expression in cellular differentiation.

This feature plot presents a UMAP visualization depicting the expression patterns of key genes across cell types derived from iPSCs. Each panel represents the expression of a specific gene in single-cell RNA sequencing data, with color intensity reflecting expression levels from low (gray) to high (blue). The genes displayed are markers indicative of pluripotent stem cells (*SOX2*, *POU5F1*), mesodermal progenitors (*MIXL1*, *TBXT*), induced mural progenitor cells (*NKX3.1*, *DES*), mural cells (*PDGFRB*, *NT5E*, *ACTA2*, *CNN1*, *COL1A1*), and endothelial cells (*PECAMI*), providing insights into the molecular signatures characteristic of each differentiation stage.

FIGS. 21A-21D. Comparative transcriptomic analysis of iMPC-derived mural cells with publicly available mural cell data. (21A, 21B) Heatmaps displaying Pearson correlation

coefficients for transcriptomic comparisons of iMPC-derived SMCs (clusters #6 and #7 in Fig.5) and pericytes (cluster #5 in Fig.5) with publicly available primary human SMCs (accession codes GSM7073879, GSM7073881, and GSM7073883) and brain pericytes datasets (accession codes GSM5293256, GSM5293257, and GSM5293258), respectively. Strong correlations (correlation coefficient ~0.6, p < 0.001) indicate a high degree of similarity in gene expression profiles. (21C, 21D) Bar graphs showing the number of iMPC-derived SMCs and pericytes that closely match with their respective cell types in The Tabula Sapiens Consortium scRNA-seq data. This analysis

confirms the resemblance of iMPC-derived mural cell types to both primary human cells and cells from a comprehensive single-cell atlas, underscoring the relevance of our differentiation model to in vivo counterparts.

FIGS. 22A-22E. Analysis of signaling pathways from ECs influencing mural cell maturation. (22A, 22B) CellChat analysis illustrating predicted ligand-receptor interactions between ECs and iMPCs based on scRNA-seq data. Pathways are color-coded by communication probability, with statistical significance denoted by symbols. **(22C)** Schematic of the co-culture setup for iMPCs with ECs, with treatments using inhibitors of TGF- β (SB431542) and NOTCH (DAPT) pathways, followed by cell sorting and qPCR analysis. **(22D)** Effect of TGF- β pathway inhibition on the expression of mural cell markers in co-cultured iMPCs (n=5; unpaired two-tailed t-test; **P<0.01, ***P<0.001; mean +/- SEM). **(22E)** Impact of NOTCH pathway inhibition on mural cell marker expression (n=5; unpaired two-tailed t-test; *P<0.05, **P<0.01, ***P<0.001; mean +/- SEM).

FIGS. 23A-23C. Analyses of DEGs between nascent and mature pericytes and contractile SMCs. (23A) Gene ontology (GO) analysis comparing nascent pericytes at day 4 and mature pericytes at day 11. The enriched pathways in day 11 pericytes include extracellular matrix organization, cellular response to TGF- β stimulus, and integrin-mediated signaling, indicating a more mature mural cell phenotype. **(23B)** Gene ontology (GO) analysis comparing cluster 5 (pericytes) and cluster 6 (contractile SMCs), showing enrichment in pathways related to extracellular matrix organization, cell-matrix adhesion, and TGF- β signaling in cluster 6. **(23C)** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis highlighting enriched pathways in cluster 6, including ECM-receptor interaction, vascular smooth muscle contraction, and TGF- β signaling. The size of the dots represents the number of genes, and the color indicates the adjusted p-value.

FIGS. 24A-24B. Analysis of gene regulatory networks (GRNs) in iPSC-derived mural cells. (24A) Dot plot highlighting the prevalence and average expression of various GRNs across different cell identities: SMCs, pericytes, MePCs, and iMPCs. **(24B)** Detailed network diagrams for each identified GRN (GRN-1 to GRN-13) illustrate the relationships between the regulatory genes within each network. Each network is color-coded to represent distinct functional clusters, with edges denoting regulatory interactions among the genes.

FIG. 25. Pseudotime Analysis: Differentiation trajectory from iMPCs to pericytes and SMCs, with annotations indicating distinct cell identities.

DETAILED DESCRIPTION

Effective *in vitro* models for studying vascular diseases require accurately representing multiple cell types. Mural cells are a crucial component of blood vessels and play a vital role in vascular development and function. Induced pluripotent stem cells (iPSCs) hold promise for generating mural cells, but current differentiating protocols rely on chemical or growth factor stimulation and are limited in scope and precision. Here, we present a method using transient activation of the transcription factor NKX3.1 to generate mural progenitor cells (iMPCs). These cells resemble primary mural progenitors in terms of transcriptomic and functional characteristics. In addition, secretome analysis reveals that iMPCs produce angiogenic factors similar to those produced by mesenchymal stem cells and vascular smooth muscle cells, thereby enhancing endothelial cell growth. Moreover, when co-cultured with endothelial cells, iMPCs are capable of maturing into functional mural cell subtypes, including smooth muscle cells, pericytes, and fibroblasts with upregulated genes related to vascular development, angiogenesis, and cell junction. Pericytes are primarily associated with microvessels, such as capillaries, while SMCs are more commonly found in larger vessels like arteries and veins. Mural cells contribute to vessel stabilization, blood flow regulation, endothelial cell quiescence, and the integrity of the blood-brain barrier. However, their dysregulation can lead to vascular abnormalities, including aberrant angiogenesis, vessel destabilization, and impaired vascular function. Consequently, the generation of functional mural cells is essential for understanding vascular function in health and diseases and for developing novel therapeutic approaches targeting perivascular cells.

Importantly, the *in vivo* experiments described herein demonstrate that iMPCs mediate the formation of functional vessels when implanted with endothelial cells, highlighting their potential for modeling vascular diseases and therapeutic vascularization. The findings here not only establish NKX3.1 as a mural cell fate-determining transcription factor but also highlight the potential of these progenitors in vascular biology research and the development of novel therapeutic strategies for vascular diseases.

Blood vessels are fundamental to mammalian development and homeostasis. However, creating a fully functional vascular system for solid organ regeneration remains a significant

challenge. Central to the formation of blood vessels are mural cells, including smooth muscle cells (SMCs) and pericytes, which regulate vascular development, stability, and function. Dysregulation of mural cells in various diseases can lead to vascular abnormalities. Furthermore, mural cells are instrumental in fostering blood vessel formation in therapeutic vascularization and vascular tissue engineering. Given their significance, there is a pressing need to develop methods for generating patient-specific mural cells. Doing so holds the promise of significantly advancing vascular regenerative medicine and addressing unmet needs in the treatment of vascular disorders.

Human induced pluripotent stem cells (iPSCs) represent a promising, noninvasive source of patient-specific mural cells. Existing differentiation strategies transition iPSCs through two distinct stages, regulated by Wnt, Nodal, TGF β , and PDGF signaling pathways. However, inducible transcription factors (TFs) are emerging as a more precise and versatile tool for differentiation. By using TFs, it is possible to gain precise temporal control and the potential for simultaneous differentiation of multiple cell types, an approach that has shown promise in tissue engineering and organoid systems.

The emergence of human induced pluripotent stem cells (h-iPSCs) provided a promising and noninvasive approach to obtaining patient-specific mural cells. Conventional methods for mural cell differentiation are largely based on vascular development and involve transitioning h-iPSCs through two distinct stages. Initially, h-iPSCs are differentiated into intermediate mesodermal progenitor cells (MePCs), regulated by Wnt and Nodal signaling pathways. Subsequently, the cells undergo SMC specification, primarily driven by TGF β and PDGF signaling. Despite advancements in chemically induced strategies, there has been growing interest in employing inducible transcription factors (TFs) for differentiation. Utilizing TF-based approaches offers several benefits, such as precise temporal control and the possibility of developing methods for the simultaneous differentiation of multiple cell types. However, the identification of a TF that can be effectively leveraged for mural cell differentiation remains elusive.

NK3 Homeobox 1 (NKX3.1) is a TF belonging to the NKX family of homeodomain-containing proteins and plays a pivotal role in the development and maintenance of various tissues, particularly in prostate and SMC differentiation. During SMC differentiation, NKX3.1 interacts with Serum Response Factor (SRF), a critical TF involved in regulating smooth muscle-

specific gene expression. Moreover, NKX3.1 cooperates with additional TFs and coactivators, such as GATA-6 and myocardin, further modulating the transcriptional activity of SRF and promoting the expression of smooth muscle-specific genes. Thus, molecular interactions mediated by NKX3.1 collectively contribute to establishing and maintaining the SMC phenotype. Nevertheless, the feasibility of utilizing NKX3.1 as a single fate-determining TF to guide iPSC differentiation into mural cells has not yet been investigated.

As described herein, transient activation of NKX3.1 in human MePCs robustly drives their differentiation into progenitor cells that closely resemble primary mural cells in their gene expression profiles and functional characteristics. These iPSC-derived mural progenitor cells (iMPCs) are capable of further maturation upon co-culture with endothelial cells (ECs), generating heterogeneous mural cell subpopulations that include pericytes and SMCs. Importantly, the iMPCs described herein exhibit robust angiogenic capacity and support EC engraftment in the form of functional vessels *in vivo*, highlighting their therapeutic potential for vascular repair and regenerative medicine applications. The study establishes NKX3.1 as a key regulator of mural cell differentiation from iPSCs and presents a novel strategy for generating mural progenitors, opening new avenues for understanding mural cell biology and developing innovative therapeutic approaches for vascular diseases.

The studies described herein revealed the capacity of the NKX3.1 to rapidly drive the differentiation of human MePCs into functionally competent iMPCs. These findings notably simplify the complexity associated with traditional chemically induced differentiation processes, which rely on a cascade of signaling molecules to achieve cell lineage specificity. The capability to streamline the iPSC-to-mural cell differentiation process into a four-day window through the transient induction of a single TF has broad implications for both basic research and translational applications.

NKX3.1 has been implicated in SMC differentiation during development through cooperative interactions with other TFs and coactivators like serum response factor and Myocardin. However, its potential to serve as a singular fate-determining TF for iPSC-to-mural cell differentiation remained an open question. We previously participated in a comprehensive, unbiased TF screen, which included over 1,500 human TFs across three human PSC lines. This screening uncovered 290 TFs capable of triggering differentiation into discernable lineages without requiring modifications in external soluble or biomechanical cues. Among the identified

TFs, NKX3.1 emerged as particularly notable for its ability to direct human iPSCs toward fibroblast-like cells. In this study, the direct activation of NKX3.1 in iPSCs, thereby bypassing the MePC intermediate stage, resulted predominantly in fibroblast-like cells but not in perivascular SMCs or pericytes. However, during embryogenesis, mural cell lineages are primarily derived from mesodermal progenitors. Indeed, mouse models previously demonstrated a highly orchestrated expression of *Nkx3.1*, initiating in the paraxial mesoderm at E7.5, and gradually concentrating adjacent to the dorsal aorta's endothelium by E9.5. By E11.5-15.5, *Nkx3.1* was co-expressed with smooth muscle myosin heavy chain (SM-MHC) in these regions. Zebrafish studies corroborated this mesodermal origin, showing *nkx3.1* expression in mesodermal precursors specific to the trunk pericyte lineage.

The present disclosure showed, *inter alia*, that activating NKX3.1 at the MePC stage, rather than directly in iPSCs, yields a more versatile mural progenitor cell population, which we termed iMPCs. By timing the activation of NKX3.1, we could transcend the production of merely fibroblast-like cells, generating a homogeneous population of mural progenitors capable of recapitulating the complexity of mural cell heterogeneity. Importantly, these iMPCs exhibit functional competence as perivascular cells upon interaction with vascular ECs. Thus, the differentiation strategy offers remarkable efficiency and closely mimics the native developmental pathways of mesoderm-derived mural cells. This NKX3.1 reprogramming paradigm is an ideal platform to probe the process of mural lineage specification. The simplicity and modularity of the approach afford customizability, and it can be further tailored to activate additional genes or pathways, thereby providing a highly adaptable means for generating an array of mural cell types and studying their diverse functional roles. This flexibility is a crucial advantage for research focused on elucidating the temporal aspects of gene function and their influence on differentiation. Our NKX3.1-driven system could become a versatile tool for *in vitro* disease modeling and drug discovery and, ultimately, enable efficient derivation of patient-specific mural cells for precision and regenerative medicine applications.

In recent years, there has been growing interest in employing inducible TFs for cell differentiation. Among the most significant advantages of TF-driven differentiation is the temporal control it provides. By modulating the expression of NKX3.1 in a time-specific manner, our system allows for precise dissection of the cellular and molecular events that occur at the stage of mural cell specification. This is invaluable for gaining insights into the detailed

mechanisms driving the generation of iMPCs from MePCs and offers a novel platform for interrogating the processes underlying cell fate decisions.

An additional advantage of TF-based approaches, like the one described herein, is the possibility of developing methods for the concurrent differentiation of multiple cell types.

5 Simultaneously differentiating human iPSCs into cells from different lineages in a controllable manner is not trivial because each cell type requires mutually incompatible differentiating conditions. However, an orthogonal differentiation approach that relies on specific TFs could override a broad range of media cues, enabling the simultaneous generation of different cell types. Indeed, recent studies have demonstrated the potential of orthogonal programming in
10 tissue engineering and organoid systems. For instance, Ng *et al.* used a model of cerebral organoids and showed the orthogonal differentiation of iPSCs into both neurons and oligodendrocytes via dox-induced transient activation of two TFs, ATOH1 and SOX9, respectively. Skylar-Scott *et al.* used an orthogonal differentiation approach to generate vascular ECs (via ETV2) and neurons (NGN1) from human iPSCs and produce vascularized and
15 patterned cortical organoids within days, demonstrating the applicability of orthogonal programming to the vasculature. However, while the list of TFs that support efficient cell differentiation into individual cell lineages continues to grow, the identification of TFs that can be effectively leveraged for mural cell differentiation within organoid systems remains elusive. Accordingly, we also investigated whether the inducible activation of NKX3.1 could enable the
20 incorporation of mural cells in such orthogonal programming efforts within diverse organoid models.

The data presented herein, *inter alia*, confirmed the validation of the functional competence of our iMPCs and their mural cell derivatives. Indeed, the functional aptitude of perivascular cells is critical for modulating EC behavior and, in turn, for their utility in vascular
25 therapies. While ECs inherently possess self-assembly capabilities into vascular structures, robust engraftment and functional vascularization *in vivo* necessarily require perivascular cell support. Traditionally, these accessory cells have been sourced from primary perivascular cells, including SMCs, pericytes, fibroblasts, and MSCs. However, recent advancements have pivoted towards utilizing pluripotent cells as a personalized and inexhaustible source for perivascular
30 cells.

While other protocols have successfully generated functionally competent perivascular cells from pluripotent stem cells, our study takes a unique TF-driven approach. Earlier chemically induced methods relied on embryoid body formation for spontaneous differentiation, although these were later deemed unspecific and inefficient. In contrast, more recent 2-D methods have proved considerably more efficient, initially inducing differentiation into an intermediate mesodermal stage via Wnt and Activin/Nodal pathways and subsequently transforming these cells into perivascular cells through the application of specific growth factors such as PDGF-BB and TGF- β . Building on these foundational methods, our NKX3.1-driven approach provides a streamlined procedure for generating functionally competent iMPCs, bypassing limitations commonly associated with media-induced methods, minimizing the risk of off-target effects and introducing a reproducible, robust framework that should facilitate standardizing the differentiation of iPSCs into mural cells.

Transcriptionally, the NKX3.1-induced iMPCs described herein align with a precursor population that closely resembles an immature pericyte phenotype. Although some pathways promoting mural cell differentiation are known, the genetic pathways that guide undifferentiated cells into mature mural cells remain incompletely elucidated. Our detailed examination of marker expression in iMPCs revealed a phenotype akin to nascent pericytes, marked by the expression of *CSPG4*, *PDGFRB*, and *DES*, but the absence of contractile proteins such as *ACTA2*, *CNN1*, and *TAGLN*. This phenotype is distinct from those observed in the mature cell populations obtained upon interaction with ECs. Instead, iMPCs serve as true progenitors that can differentiate entirely into pericytes and SMCs after a week of co-culturing with ECs. We substantiated this transformation from MePCs to iMPCs to terminal mural cell populations with our single-cell RNA sequencing and trajectory analyses.

Moreover, the comparative evaluations demonstrated that the gene expression profiles of our iMPC-derived SMCs and pericytes significantly align with established primary human mural cells and a detailed single-cell reference from The Tabula Sapiens Consortium, underscoring the relevance of our differentiation model to *in vivo* counterparts.

The transcriptional and functional analyses have also revealed a close alignment of iMPCs with primary MSCs, which are widely recognized as mural progenitors, although debates about their equivalence to pericytes persist. Indeed, our iMPCs exhibit traits consistent with chemically-induced mesenchymal progenitors identified in previous studies as PDGFR β +

CD271+ CD73- immature pericytes capable of differentiating into mature mural cells. Our work described herein confirms, *inter alia*, that transient activation of NKX3.1 in MePCs suffices to yield a population of iMPCs that function as mural progenitor cells exhibiting characteristics congruent with mesenchymal progenitors.

Thus, described herein, *inter alia*, are robust and efficient TF-driven methodologies for differentiating human iPSCs into functional iMPCs. By transiently activating NKX3.1 at an intermediary stage of differentiation, we have demonstrated remarkable differentiation efficiency as well as the functional competence of the resulting iMPCs. In addition, we have shown two distinct approaches for inducing NKX3.1: a Dox-inducible system and a genomic footprint-free modRNA method. The latter, being nonviral, nonintegrating, and inherently transient, has distinct translational advantages. From a clinical application standpoint, our method could provide a reliable pathway for generating patient-specific mural cells for regenerative medicine and disease modeling. Moreover, our iMPCs could offer therapeutic potential in conditions characterized by pericyte loss, such as diabetic retinopathy and stroke.

Beyond these immediate translational applications, the studies described herein serve as a foundational platform offering a standardized, reproducible approach for the derivation of mural cells from human iPSCs. Our studies revealed the singular capacity of the NKX3.1 to rapidly drive the differentiation of human MePCs into functionally competent iMPCs. This finding notably simplifies the complexity associated with traditional chemically induced differentiation processes, which rely on a cascade of signaling molecules to achieve cell lineage specificity. The capability to streamline the iPSC-to-mural cell differentiation process into a four-day window through the transient induction of a single TF has broad implications for both basic research and translational applications.

iPSC, MePCs, & MPCs and Methods of Making MPCs

Generally, the present disclosure is based, *inter alia*, on the discovery that NK3 Homeobox 1 (NKX3.1) determines mural cell lineage fate, and NKX3.1 activation in iPSC-derived mesodermal progenitors (MePCs) effectively generated iPSC-derived mural progenitor cells (iMPCs). This disclosure provides, *inter alia*, methods for making and using mural progenitor cells and compositions comprising mural progenitor cells.

The use of cells derived from human pluripotent stem cells, such as human induced pluripotent stem cells (iPSC) or embryonic stem cells (ESC), allow cells from any donor to be reprogrammed into a pluripotent, self-renewing state and thus allow the expansion of a homogeneous population of cells from any genetic background. The use of iPSCs overcome ethical and political considerations pertaining to hESC and can be generated from adult, terminally differentiated cells. Briefly, iPSCs have been generated by expression of several key genes shown to be required for full reprogramming, namely combinations of: Oct4, Sox2, Klf4, c-Myc, l-Myc, Lin28, and/or Nanog, for example one or more factors selected from Oct4, Sox2, Klf4, c-Myc, l-Myc, Lin28, and/or Nanog. Expression and differentiation analysis has shown iPSCs to be very close to ESCs at the molecular level with variations between clonal iPSC cultures of similar magnitude to those seen when comparing multiple ESC lines. iPSCs are a promising and noninvasive approach to obtaining patient-specific mural cells, and iPSCs may ultimately result in cell therapies generated from the patient's own cells in an autologous transplantation that may prevent graft rejection.

Additional information, including additional methods of isolating, making, and differentiating iPSCs and ESCs are known in the art (e.g., US 20240228951 A1; US 20240050483 A1; US 2022/0243174; US 20200385685 A1; US 20200182861 A1; US 2018/0371422; WO 2015/073625; US 2016/0002604; US 2014/0199274; US 2013/0052268; US 2012/0128655; and US 2009/0226401; as well as US 11,898,169; US 11,001,809; US 10,844,356; US 10,676,165; US 9,657,273; US 9,750,768; US 9,580,689; and US 9,376,664), each of the foregoing is incorporated herein by reference in its entirety.

Mural cells, which include pericytes and smooth muscle cells (SMCs), are essential components of blood vessels, playing critical roles in vascular development, stability, and function. Pericytes are primarily associated with microvessels, such as capillaries, while SMCs are more commonly found in larger vessels like arteries and veins. Mural cells contribute to vessel stabilization, blood flow regulation, endothelial cell quiescence, and the integrity of the blood-brain barrier.

Conventional methods for mural cell differentiation are largely based on vascular development and involve transitioning h-iPSCs through two distinct stages. Initially, h-iPSCs are differentiated into intermediate mesodermal progenitor cells (MePCs), regulated by Wnt and/or

Nodal signaling pathways. Subsequently, the cells undergo SMC specification, primarily driven by TGF β and PDGF signaling.

As demonstrated and discussed herein, utilizing TF-based approaches offers several benefits, such as precise temporal control and the possibility of developing methods for the simultaneous differentiation of multiple cell types. However, the identification of a TF that can be effectively leveraged for mural cell differentiation remains elusive.

NK3 Homeobox 1 (NKX3.1; e.g., UnitProtKB Q99801-1, UnitProtKB Q99801-2, UnitProtKB Q99801-3, UnitProtKB Q99801-4, UnitProtKB Q99801-5, PDB NP_006158.2, and PDB NP_001243268.1) is a TF belonging to the NKX family of homeodomain-containing proteins and plays a pivotal role in the development and maintenance of various tissues, particularly in prostate and SMC differentiation. During SMC differentiation, NKX3.1 interacts with Serum Response Factor (SRF), a critical TF involved in regulating smooth muscle-specific gene expression. Moreover, NKX3.1 cooperates with additional TFs and coactivators, such as GATA-6 and myocardin, further modulating the transcriptional activity of SRF and promoting the expression of smooth muscle-specific genes.

The data presented herein showed that transient activation of NKX3.1 in human MePCs robustly drives their differentiation into progenitor cells capable of contributing to mural cell lineage in their gene expression profiles and functional characteristics. Furthermore, the data showed these iPSC-derived mural progenitor cells (iMPCs) are capable of further maturation upon co-culture with endothelial cells (ECs), generating heterogeneous mural cell subpopulations that include pericytes and SMCs. Importantly, the iMPCs herein exhibit robust angiogenic capacity and support EC engraftment in the form of functional vessels *in vivo*, highlighting their therapeutic potential for vascular repair and regenerative medicine applications. The study described herein establishes NKX3.1 as a key regulator of mural cell differentiation from iPSCs and presents a novel strategy for generating mural progenitors, opening new avenues for understanding mural cell biology and developing innovative therapeutic approaches for vascular diseases.

In some embodiments, methods of making iPSC-derived mural progenitor cells (iMPCs) comprise or consist of: one or more of the following steps: contacting a population of induced pluripotent stem cells (iPSCs) with a nucleic acid encoding NK3 Homeobox 1 (NKX3.1) or a functional variant thereof; converting the iPSCs to mesodermal progenitors (MePCs); and

inducing the MePCs to express NKX3.1 for a time period sufficient to generate iMPCs. In some embodiments, the nucleic acid is a vector (e.g., a PiggyBac transposon vector or viral vector). In some embodiments, the vector is a viral vector (e.g., retroviral, lentiviral). In some embodiments, the nucleic acid comprises an inducible promoter that controls expression of NKX3.1 (e.g., doxycycline-inducible promoter or other known in the art). In some embodiments, converting the iPSCs to MePCs comprises activating the Wnt pathway and/or activating the Nodal pathway for a time period sufficient (e.g., about or at least 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, or 48 hours) to generate MePCs. In some embodiments, the time period sufficient to generate iMPCs is about or at least 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, or 48 hours.

Also described herein are methods of generating a population of mural cells comprising pericytes, smooth muscle cells, and fibroblasts. The methods comprise or consist of: one or more of the following steps: generating iMPCs using the methods as described herein; co-culturing the iMPCs with a population of endothelial cells (ECs or iECs) for a time period sufficient to generate a population of mural cells comprising pericytes, smooth muscle cells, and fibroblasts. In some embodiments, the time period sufficient to generate a population of mural cells comprising pericytes, smooth muscle cells, and fibroblasts is about or at least 1, 2, 3, 4, 5, 6, or 7 days.

In some embodiments, the methods described herein comprise the use of a nucleic acid encoding a NKX3.1 (e.g., UnitProtKB Q99801-1, UnitProtKB Q99801-2, UnitProtKB Q99801-3, UnitProtKB Q99801-4, UnitProtKB Q99801-5, PDB NP_006158.2, and PDB NP_001243268.1). In some embodiments, the NKX3.1 is a functional variant of NKX3.1. In some embodiments, the NKX3.1 is a wildtype NKX3.1. In some embodiments, the NKX3.1 is a human NKX3.1. In some embodiments, the nucleic acid is an exogenous DNA molecule or an exogenous RNA molecule. Nonlimiting examples of useful RNA are a modified mRNA (modRNA; e.g., US20230364267A1), a mRNA, or a functional variant thereof. Nonlimiting examples of useful DNA can be a vector, plasmid, transposon, or a functional variant thereof.

In some embodiments, the methods described herein comprise the use of a nucleic acid encoding a E26 transformation-specific variant 2 (*ETV2*). In some embodiments, the ETV2 is a functional variant of ETV2. In some embodiments, the ETV2 is a wildtype ETV2. In some embodiments, the ETV2 is a human ETV2. In some embodiments, the nucleic acid is an

exogenous DNA molecule or an exogenous RNA molecule. Nonlimiting examples of useful RNA are a modified mRNA (modRNA; e.g., US20230364267A1), a mRNA, or a functional variant thereof. Nonlimiting examples of useful DNA can be a vector, plasmid, transposon, or a functional variant thereof.

Also described herein are methods of making a vascular organoid (VO) or a three-dimensional (3D) cell culture. The methods comprise or consist of: one or more of the following steps:

(i) culturing a first population of iPSC-derived mesodermal progenitor cells (MePCs) comprising a nucleic acid (optionally, an exogenous nucleic acid) encoding ETV2, or a functional variant thereof, ("ETV2/MePCs"), with a second population of MePCs comprising a nucleic acid (optionally, an exogenous nucleic acid) encoding NKX3.1, or a functional variant thereof, ("NKX3.1/MePCs"),

wherein expression of NKX3.1 is controlled by an inducible promoter and expression of ETV2 is controlled by an inducible promoter;

(ii) inducing expression of NKX3.1 in the NKX3.1/MePCs, thereby creating iPSC-derived mural progenitor cells (iMPCs) and inducing expression of ETV2 in the ETV2/iPSCs, thereby creating iPSC-derived endothelial cells (iECs); and

(iii) culturing the cells for a time period sufficient to generate a VO or 3D cell culture, wherein the VO or 3D cell culture comprises iECs that are h-CD31+ and iMPCs that are PDGFR β + and h-CD31-.

In some embodiments, the inducible promoter for NKX3.1 and the inducible promoter for ETV2 are the same (optionally, a doxycycline inducible promoter). In some embodiments, the inducible promoter for NKX3.1 and the inducible promoter for ETV2 are not the same.

In some embodiments, the time period sufficient to generate a VO or 3D cell culture is about or at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 days.

In some embodiments, the step (i) culturing occurs for about 1 day or 2 days; wherein the step (i) culturing occurs for about or at least 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, or 48 hours; and/or wherein the culturing occurs for a time period sufficient to generate aggregates comprising both the NKX3.1/MePCs and the ETV2/MePCs.

In some embodiments, the (i) culturing step comprises culturing the cells using non-adherent culture plates and an orbital shaker.

In some embodiments, the population of NKX3.1/MePCs and the population of ETV2/MePCs are mixed at a ratio of about 1:3, 1:2, 2:3, 1:1, 3:2, 1:2, or 3:1 of NKX3.1/MePCs:ETV2/MePCs.

In some embodiments, described herein are methods of making vascular organoids (VOs) or three-dimensional (3D) cell cultures, the methods comprising:

(i) transfecting a population of iPSC-derived mesodermal progenitors (MePCs) with a nucleic acid (optionally, a DNA, an RNA, an mRNA, an modRNA) encoding NKX3.1 or a functional variant thereof, thereby creating a population of iPSC-derived mural progenitor cells (iMPCs);

(ii) mixing the population of iMPCs with a population of ECs (optionally, at a ratio of about 1:3, 1:2, 2:3, 1:1, 3:2, 1:2, or 3:1 of iMPC:EC); and

(iii) culturing the cells for a time period sufficient to generate the VOs or 3D cell cultures, wherein the VOs or 3D cell cultures comprise iECs that are h-CD31⁺ and iMPCs that are PDGFR β ⁺ and h-CD31⁻.

In some embodiments, the ECs comprise any one or more of iPSC-derived ECs (iECs), human umbilical vein endothelial cells (HUVECs), endothelial colony-forming cells (ECFCs), adipose tissue-derived ECs, or organ-specific endothelial cells.

In some embodiments, the organ-specific endothelial cells are from an organ selected from: heart, muscle, kidney, testis, ovary, lymphoid, liver, pancreas, brain, lungs, bone marrow, spleen, large intestine, and small intestine.

In some embodiments, the methods further comprise a step of transfecting a population of iPSCs with a nucleic acid encoding ETV2 or a functional variant thereof to thereby create a population of ECs prior to mixing with the population of iMPCs.

In some embodiments, the VOs are uniform size and/or about 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, 350, 355, 360, 365, 370, 375, 380, 385, 390, 395, 400, 405, 410, 415, 420, 425, 430, 435, 440, 445, 450, 455, 460, 465, 470, 475, 480, 485, 490, 495, 500, 505, 510, 515, 520, 525, 530, 535, 540, 545, or 550 μ m in diameter; and/or the average diameter size of the VOs or 3D cell cultures is about 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, or 350 μ m in diameter; and/or the median size of the

VOs or 3D cell cultures is about 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, or 350 μm in diameter; and/or the VOs or 3D cell cultures comprise about 1,000, 1,500, 2,000, 2,500, 2,550, 2,600, 2,650, 2,700, 2,750, 2,800, 2,850, 2,900, 2,950, 3,000, 3,050, 3,100, 3,150, 3,200, 3,250, 3,300, 3,350, 3,400, 3,450, 3,500, 4,000, 4,500, or 5,000 cells.

In some embodiments, the cells self-assembled into a network of CD31+ vascular structures containing mature $\alpha\text{-SMA}^+$ perivascular mural cells. In some embodiments, the VOs comprise a network of lumenized vessels with apical-basal polarization and/or wherein the VO comprises arterial, venous, and/or capillary ECs. In some embodiments, the VOs comprise ECs that are *CDH5*⁺ and *VWF*⁺ and mural cell that are *ACTA2*⁺, *MYH11*⁺, *TAGLN*⁺, and *CNN1*⁺. In some embodiments, the methods comprise the formation of an artery, a vein, a capillary, an arteriole, a venule, or any combination thereof.

Additional information about making cell populations, VOs, and 3D cell cultures is known in the art (*See*, e.g., US 2024/0287463 A1; US 2024/0287463 A1; US 2020/0182861 A1; US 2019/0376044 A1; US 2020/0199541 A1; US 2023/0287357 A1; US 2023/0174949 A1; US 2023/0364267 A1; US Pat. No. 11,214,768; WO 2024/133285; WO 2022/226337; and WO 2023/196683), each of the foregoing is incorporated herein by reference in its entirety.

Cell Compositions and Vascular Organoids (VOs)

In some embodiments, described herein, *inter alia*, are populations of cells comprising iPSC-derived mural progenitor cells (iMPCs) made using any of the methods described herein. In some embodiments, described herein, *inter alia*, are populations of cells (e.g., iPSCs, MePCs, and iMPCs) comprising a nucleic acid encoding a NKX3.1 (e.g., UnitProtKB Q99801-1, UnitProtKB Q99801-2, UnitProtKB Q99801-3, UnitProtKB Q99801-4, UnitProtKB Q99801-5, PDB NP_006158.2, and PDB NP_001243268.1). In some embodiments, the nucleic acid is an exogenous DNA molecule or an exogenous RNA molecule. Nonlimiting examples of useful RNA are a modified mRNA (modRNA; e.g., US20230364267A1), a mRNA, or a functional variant thereof. Nonlimiting examples of useful DNA can be a vector, plasmid, transposon, or a functional variant thereof.

Also described herein are populations of cells comprising at least about 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% iPSC-derived mural progenitor cells (iMPCs). In some embodiments,

the iMPCs comprise a nucleic acid (optionally, an exogenous nucleic acid) encoding NKX3.1 or transiently expressed NKX3.1 (optionally, from a degradable nucleic acid (e.g., mRNA or modRNA)). In some embodiments, the iMPCs express PDGFR β (CD140b) and aminopeptidase N (CD13). In some embodiments, less than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1% of the iMPCs express a TRA1-81 antigen. In some embodiments, the iMPCs express *ACTA2*, *CNN1*, *TAGLN*, *MYOCD*, *MYH11*, *CSPG4*, *DES*, *PDE5A*, and *THY1*.

Also described herein are populations of cells created by co-culturing the iMPCs with a population of endothelial cells (ECs or iECs) for a time period sufficient to generate a population of mural cells comprising pericytes, smooth muscle cells, and fibroblasts. In some embodiments, the population of cells comprises mural cells from iMPCs and a population of ECs (optionally, at a ratio of about 1:3, 1:2, 2:3, 1:1, 3:2, 1:2, or 3:1 of iMPC:EC).

In some embodiments, the ECs comprise any one or more of iPSC-derived ECs (iECs), human umbilical vein endothelial cells (HUVECs), endothelial colony-forming cells (ECFCs), adipose tissue-derived ECs, or organ-specific endothelial cells.

In some embodiments, the organ-specific endothelial cells are from an organ selected from: heart, muscle, kidney, testis, ovary, lymphoid, liver, pancreas, brain, lungs, bone marrow, spleen, large intestine, and small intestine.

In some embodiments, the methods further comprise a step of transfecting a population of iPSCs with a nucleic acid encoding ETV2 or a functional variant thereof to thereby create a population of ECs prior to mixing with the population of iMPCs. In some embodiments, described herein are populations of cells comprising a ratio of about 1:3, 1:2, 2:3, 1:1, 3:2, 1:2, or 3:1 of endothelial cells (ECs): iPSC-derived mural progenitor cells (iMPCs). In some embodiments, the iMPCs comprise a nucleic acid (optionally, an exogenous nucleic acid) encoding NKX3.1 or transiently expressed NKX3.1 (optionally, from a degradable nucleic acid (e.g., mRNA or modRNA)). In some embodiments, the iMPCs express PDGFR β (CD140b) and aminopeptidase N (CD13). In some embodiments, less than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1% of the iMPCs express a TRA1-81 antigen. In some embodiments, the iMPCs express *ACTA2*, *CNN1*, *TAGLN*, *MYOCD*, *MYH11*, *CSPG4*, *DES*, *PDE5A*, and *THY1*. In some embodiments, the ECs comprise any one or more of iPSC-derived ECs (iECs), human umbilical vein endothelial cells (HUVECs), endothelial colony-forming cells (ECFCs), adipose tissue-derived ECs, or organ-specific endothelial cells. In some embodiments, the organ-specific endothelial cells are from an

organ selected from: heart, muscle, kidney, testis, ovary, lymphoid, liver, pancreas, brain, lungs, bone marrow, spleen, large intestine, and small intestine. In some embodiments, the iECs comprise a nucleic acid (optionally, an exogenous nucleic acid) encoding ETV2 or transiently expressed ETV2 (e.g., from a degradable nucleic acid (e.g., mRNA or modRNA)). In some
5 embodiments, the NKX3.1 expression is controlled by an inducible promoter and the ETV2 expression is controlled by an inducible promoter. In some embodiments, the inducible promoter for NKX3.1 and the inducible promoter for ETV2 are the same (optionally, induced by doxycycline). In some embodiments, the inducible promoter for NKX3.1 and the inducible promoter for ETV2 are not the same.

10 In some embodiments, the methods described herein comprise the use of a nucleic acid encoding a E26 transformation-specific variant 2 (*ETV2*), or a functional variant thereof. In some embodiments, the nucleic acid is an exogenous DNA molecule or an exogenous RNA molecule. Nonlimiting examples of useful RNA are a modified mRNA (modRNA; e.g.,
US20230364267A1), a mRNA, or a functional variant thereof. Nonlimiting examples of useful
15 DNA can be a vector, plasmid, transposon, or a functional variant thereof.

Also described herein are 3D vascular organoids (VOs), populations of VOs, and three-dimensional (3D) cell cultures comprising any one or more of the cell populations described herein (e.g., a population of iMPCs; iMPCs co-cultured with a population of endothelial cells (ECs or iECs); a population of mural cells comprising a nucleic acid encoding NKX3.1). iMPCs
20 mediate the formation of functional vessels when implanted with endothelial cells (ECs).

Also described herein are VOs and 3D cell cultures made by any one of the methods described herein. In some embodiments, the VOs or 3D cell cultures comprise iECs that are h-CD31+ and iMPCs that are PDGFR β + and h-CD31-.

In some embodiments, the VOs are uniform size and/or about 180, 185, 190, 195, 200,
25 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, 350, 355, 360, 365, 370, 375, 380, 385, 390, 395, 400, 405, 410, 415, 420, 425, 430, 435, 440, 445, 450, 455, 460, 465, 470, 475, 480, 485, 490, 495, 500, 505, 510, 515, 520, 525, 530, 535, 540, 545, or 550 μ m in diameter; and/or wherein the average diameter size of the VOs or 3D cell cultures is about 180, 185, 190, 195,
30 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, or 350 μ m in diameter; and/or wherein the

median size of the VOs or 3D cell cultures is about 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, or 350 μm in diameter; and/or wherein the VOs or 3D cell cultures comprise about 2,500, 2,550, 2,600, 2,650, 2,700, 2,750, 2,800, 2,850, 2,900, 2,950, 3,000, 3,050, 3,100, 3,150, 3,200, 3,250, 3,300, 3,350, 3,400, 3,450, or 3,500 cells.

In some embodiments, the cells self-assembled into a network of CD31+ vascular structures containing mature $\alpha\text{-SMA}^+$ perivascular mural cells. In some embodiments, the VOs and 3D cell cultures comprise a network of lumenized vessels with apical-basal polarization and/or wherein the VO comprises arterial, venous, and/or capillary ECs. In some embodiments, the VOs and 3D cell cultures comprise ECs that are *CDH5*⁺ and *VWF*⁺ and mural cell that are *ACTA2*⁺, *MYH11*⁺, *TAGLN*⁺, and *CNN1*⁺.

In some embodiments, the VOs and 3D cell cultures comprise CD31+ vascular structures containing mature $\alpha\text{-SMA}^+$ perivascular mural cells. In some embodiments, the VOs or 3D cell cultures comprise a network of lumenized vessels with apical-basal polarization and/or arterial, venous, and/or capillary ECs.

Also described herein, *inter alia*, are compositions comprising the populations of cells described herein and/or any of the VOs described herein. In some embodiments, the composition may further comprise one or more of an agent, an excipient, a matrix, or a gel. In some embodiments, any composition may further comprise a gel or matrix comprising a hydrogel. In some embodiments, any composition may further comprise a gel or matrix comprising gelatin, collagen, fibrinogen, thrombin, fibrin, or any combinations thereof. In some embodiments, any composition may further comprise a gel or matrix comprising about 1.5 mg/mL collagen, about 30 $\mu\text{g/mL}$ fibrinogen, and about 1 mg/mL human fibronectin. In some embodiments, the matrix can contain collagen and/or fibrin. In some embodiments, fibrin is formed with fibrinogen and thrombin, (optionally, about 50 $\mu\text{g/mL}$ thrombin). In some embodiments, any composition may further comprise a gel or matrix comprising any one or more of gelatin, collagen, fibrinogen, laminin, entactin, or combinations thereof. In some embodiments, any composition may further comprise a gel or matrix comprising laminin, entactin, and collagen. In some embodiments, any composition may further comprise a gel or matrix comprising about 5.25 mg/mL laminin, about 5.25 mg/mL entactin, and about 0.2 mg/mL

collagen IV. In some embodiments, any composition may further comprise a gel or matrix is Matrigel™. Matrigel is known in the art (U.S. Pat. No. 4,829,000).

In some embodiments, any of the populations of cells, vascular organoids, or compositions can be formulated to be administered to the subject (e.g., subcutaneous, intradermal, intramuscular, intranodal, intravenous, intraprostatic, intratumor, intralymphatic, and intraperitoneal injection). In some embodiments, any of the populations of cells, vascular organoids, or compositions can be used to vascularize a tissue or organ prior to transplantation to the patient, and accordingly, composition described herein can also comprise cells and tissue from any one or more of the following skin, heart, kidney, testis, ovary, bone, lymph, liver, pancreas, brain, lungs, bone marrow, spleen, large intestine and small intestine.

Additional information and methods of formulating populations of cells, vascular organoids, or compositions are known in the art (*See*, e.g., US 2024/0287463 A1; US 2024/0287463 A1; US 2020/0182861 A1; US 2019/0376044 A1; US 2020/0199541 A1; US 2023/0287357 A1; US 2023/0174949 A1; US 2023/0364267 A1; US Pat. No. 11,214,768; WO 2024/133285; WO 2022/226337; and WO 2023/196683), each of the foregoing is incorporated herein by reference in its entirety.

Methods of Using the Cells and VOs

Described herein, *inter alia*, are methods of administering any of the populations of cells (e.g., iMPCs, iMPCs, including the mature mural cell derivatives, and ECs) and/or VOs described herein to a subject. Useful populations of cells and VOs are described throughout, for example, in the above section.

In some embodiments, the subject has, or is at risk of having or developing, a disease or disorder associated with a blood vessel disorder (e.g., leaky blood vessels, narrow blood vessels, coronary artery disease, peripheral arterial disease, cerebrovascular disease, renal artery stenosis, aortic aneurysm, cardiomyopathy, hypertensive heart disease, heart failure, pulmonary heart disease, cardiac dysrhythmias and valvular heart disease cerebral cavernous malformations, hemorrhagic strokes, hereditary hemorrhagic telangiectasias, or arteriovenous malformations). In some embodiments of any of the methods described herein, the subject has (or is at risk of having or developing) any one or more of the following: a metabolic disorder, diabetes, diabetic retinopathy, an ischemic injury, a disease or disorder of the blood vessels, atherosclerosis, Age-

related Macular Degeneration (AMD), Pulmonary Arterial Hypertension (PAH), Hereditary Hemorrhagic Telangiectasia (HHT), peripheral artery disease (PAD), arteriovenous fistulas (e.g., dialysis patients), tumor angiogenesis, tumor metastasis, a cancer, a metabolic disease, an immunological disease, a mitochondrial dysfunction disorder, a stroke, and/or a wound (optionally, a chronic wound; e.g., a diabetic ulcer).

In some embodiments, described herein are methods to treat, or reduce the risk of developing, any one or more of the following: a disease or disorder of the blood vessels, aberrant vasculature, leaky blood vessels, narrow blood vessels, coronary artery disease, peripheral arterial disease, cerebrovascular disease, renal artery stenosis, aortic aneurysm, cardiotoxicity (e.g., caused by chemotherapy), cardiomyopathy, hypertensive heart disease, heart failure, pulmonary heart disease, cardiac dysrhythmias, valvular heart disease cerebral cavernous malformations, hemorrhagic strokes, hereditary hemorrhagic telangiectasias, arteriovenous malformations, a metabolic disorder, diabetes, diabetic retinopathy, an ischemic injury, an IRI injury, atherosclerosis, Age-related Macular Degeneration (AMD), Pulmonary Arterial Hypertension (PAH), Hereditary Hemorrhagic Telangiectasia (HHT), peripheral artery disease (PAD), arteriovenous fistulas (e.g., dialysis patients), tumor angiogenesis, tumor metastasis, a cancer, a metabolic disease, a stroke, and/or a wound (optionally, a chronic wound; e.g., a diabetic ulcer).

Also described herein are methods of increasing blood vessel formation, methods of vascular cell therapy, and/or methods of vascular generation or regeneration in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of the population of ECs and iMPCs. In some embodiments, the methods further comprising identifying a subject in need or increasing blood vessel formation, vascular cell therapy, vascular generation, and/or vascular regeneration.

Described herein, *inter alia*, are methods of increasing vascular development, angiogenesis, and cell junction via administering the iMPCs to a subject. In some embodiments, described herein are methods of administering iMPCs and ECs (e.g., therapeutic vascularization) and methods of modeling vascular diseases (e.g., a 3D vascular organoid (VO)) are also described herein. Also described herein are methods of tissue engineering (e.g., small-diameter vascular grafts). Tissue-engineered small-diameter vascular grafts are bioengineered constructs designed to replace damaged or diseased blood vessels. These grafts are typically created from

scaffolds that are seeded with cells, such as smooth muscle cells (SMCs), which provide structural support and functionality. The cell populations described herein (e.g., NKX3.1-derived iMPCs and their mature mural cell derivatives) could be incorporated into these scaffolds as SMCs, facilitating the development of grafts that can be surgically implanted in patients as vessel replacements. Small-diameter vascular grafts are particularly relevant for clinical applications such as coronary artery bypass grafting (CABG), peripheral artery disease (PAD), and arteriovenous fistulas for dialysis patients.

In some embodiments, any of the populations of cells described herein, any of the VOs or 3D cell cultures described herein, and/or any of the compositions described herein (e.g., a composition comprising NKX3.1-derived iMPCs and/or their mature mural cell derivatives) are used as a cell source for tissue engineering applications, particularly in the development of small-diameter vascular grafts. For example, a population of iMPC-derived cells described herein can function as smooth muscle cells (SMCs) within the graft scaffold of a small-diameter vascular grafts and be surgically transplanted in a subject in need thereof (e.g., a subject in need to vessel replacement, a subject having or at risk of having coronary artery bypass grafting (CABG), a subject having or at risk of having peripheral artery disease (PAD), and a subject having or at risk of having arteriovenous fistulas (e.g., a dialysis patient). Without being bound by theory, the incorporation of iMPC cells into a structural scaffold enables the creation of functional, living vascular grafts that can be implanted to restore or replace damaged blood vessels, offering significant potential for these high-demand clinical scenarios.

In some embodiments, described herein are methods of transplanting any of the populations of cells described herein, any of the VOs or 3D cell cultures described herein, and/or any of the compositions described herein. In some embodiments, the method comprises administering to the subject an effective amount of the population of cells, vascular organoids, or composition.

In some embodiments, described herein are methods of increasing blood vessel formation comprising administering to a subject in need thereof an effective amount of any of the populations of cells described herein, any of the VOs or 3D cell cultures described herein, and/or any of the compositions described herein. In some embodiments, the method comprises: identifying a subject in need of increased blood vessel formation; and administering to the

subject an effective amount of any of the populations of cells described herein, any of the VOs or 3D cell cultures described herein, and/or any of the compositions described herein.

Also described herein, *inter alia*, are methods of increasing vascular generation or vascular regeneration comprising administering to a subject in need thereof an effective amount of any of the populations of cells described herein, any of the VOs or 3D cell cultures described herein, and/or any of the compositions described herein. In some embodiments, the method comprises: identifying a subject at in need of vascular generation or vascular regeneration; administering to the subject an effective amount of any of the populations of cells described herein, any of the VOs or 3D cell cultures described herein, and/or any of the compositions described herein.

Also described herein, *inter alia*, are methods of vascular cell therapy comprising administering to a subject in need thereof an effective amount of any of the populations of cells described herein, any of the VOs or 3D cell cultures described herein, and/or any of the compositions described herein. In some embodiments, the method comprises: identifying a subject in need of vascular cell therapy; administering to the subject an effective amount of any of the populations of cells described herein, any of the VOs or 3D cell cultures described herein, and/or any of the compositions described herein.

In some embodiments of any of the methods described herein, the subject or patient may be undergoing an organ transplant, selected to undergo an organ transplant, or need vascularization of an organ. In some embodiments, the organ is selected from the group consisting of skin, heart, kidney, testis, ovary, bone, lymph, liver, pancreas, brain, lungs, bone marrow, spleen, large intestine and small intestine. In some embodiments of any of the methods described herein, the population of cells, vascular organoid, or composition is administered to the subject, before, during, or after a cell transplant, tissue transplant, or organ transplant.

In some embodiments of any of the methods described herein, vascularization comprises the formation of an artery, a vein, a capillary, an arteriole, a venule, or any combination thereof.

In some embodiments of any of the methods described herein, the methods normalize and/or correct aberrant vasculature; for example, where the vasculature lacks or has deficient mural cells; where the vasculature lacks stable structure and/or function; and/or where there is leaking or narrowing of a blood vessel, etc. In some embodiments of any of the methods described herein, the subject has a disorder characterized by aberrant vasculature and/or

vasculature that lacks or has deficient mural cells (e.g., diabetic retinopathy, tumor angiogenesis, tumor metastasis, stroke, ischemic injury, atherosclerosis, Age-related Macular Degeneration (AMD), Pulmonary Arterial Hypertension (PAH), Hereditary Hemorrhagic Telangiectasia (HHT), peripheral artery disease (PAD), arteriovenous fistulas (e.g., dialysis patients), and/or a wound (e.g., a chronic wound; e.g., a diabetic ulcer).

Without being bound by theory, narrowing of blood vessels result from plaque build-up on the walls of the vessels and/or chronic inflammation, which can include conditions such as ischemic disease, peripheral artery disease, angina, heart attack, stroke, Reynaud's disease, Brueger's disease, hypertension, chemotherapeutic compromise, and erectile dysfunction.

Furthermore, each condition and blood vessel disease frequently results in distal vessel injury and/ or dysfunction that, in turn, complicates and, in many cases, exacerbates revascularization strategies and recovery from ischemic injury. The iMPCs, cell populations, VOs, and/or compositions described herein can potentially restore the integrity of the vasculature, help stabilize these vessels, reduce the severity of any injury caused by these conditions, and/or prevent further complications from arising as a result of the condition(s).

Without being bound by theory, tumors often exhibit abnormal vasculature characterized by a lack of proper mural cell coverage, leading to leaky and dysfunctional blood vessels. Introducing any of the iMPCs, cell populations, VOs, and compositions herein could help stabilize these vessels, improving the delivery of therapeutics, and/or reduce metastasis.

Without being bound by theory, in diabetic retinopathy, pericyte loss leads to weakened blood-retinal barriers, resulting in retinal ischemia and neovascularization. The iMPCs, cell populations, VOs, and/or compositions described herein can potentially restore the integrity of retinal vasculature and/or reduce the progression of the disease.

Without being bound by theory, after a stroke or ischemic injury, there is often a loss of vascular integrity and a need for vascular repair. The iMPCs, cell populations, VOs, and/or compositions described herein can aid in re-establishing stable blood vessels and/or promoting recovery of the affected tissue.

Without being bound by theory, in atherosclerosis, the stability of blood vessels is compromised due to inflammatory processes and endothelial dysfunction. The mural cells derived from iMPCs, iMPCs, cell populations, VOs, and/or compositions described herein can help reinforce vascular walls and/or mitigate the progression of atherosclerotic plaques.

Without being bound by theory, in wound healing, chronic wounds, such as diabetic ulcers, often suffer from poor vascularization and deficient mural cell coverage. The iMPCs, cell populations, VOs, and/or compositions described herein can enhance angiogenesis and vascular stability, promoting better wound healing outcomes.

5 Without being bound by theory, in Age-related Macular Degeneration (AMD), especially the wet form, choroidal neovascularization occurs with deficient pericyte support, leading to fragile and leaky vessels. The iMPCs, cell populations, VOs, and/or compositions described herein can help in providing the necessary support to these new vessels, reducing leakage, and/or reducing vision loss.

10 Without being bound by theory, Pulmonary Arterial Hypertension (PAH) is characterized by abnormal proliferation of pulmonary vascular cells and deficient pericyte coverage, leading to vascular remodeling and hypertension. The iMPCs, cell populations, VOs, and/or compositions described herein could stabilize these blood vessels and/or alleviate one or more symptoms (e.g., hypertension).

15 Without being bound by theory, Hereditary Hemorrhagic Telangiectasia (HHT) is a genetic disorder leading to abnormal blood vessel formation with deficient mural cell coverage, resulting in bleeding and arteriovenous malformations. The iMPCs, cell populations, VOs, and/or compositions described herein can potentially normalize these vessels and/or reduce bleeding episodes.

20 In some embodiments of any of the methods described herein, the population of cells, vascular organoid, or composition is administered to the subject by direct injection into a blood vessel or subcutaneous, intradermal, intramuscular, intranodal, intravenous, intraprostatic, intratumor, intralymphatic, and intraperitoneal injection.

25 An effective amount can be administered in one or more administrations, applications or dosages. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of any therapeutic population of cells, VO, 3D cell culture, composition described herein can include a
30 single treatment or a series of treatments.

A skilled artisan will be able to determine and identify a subject or patient suitable for any of the methods described herein (e.g., a subject suffering from or at risk of having a stroke, a subject suffering from or at risk of having diabetes, a subject suffering from or at risk of having diabetic retinopathy, a subject suffering from or at risk of having an ischemic injury, a subject
5 suffering from or at risk of having a disease or disorder of the blood vessels, and/or a subject having or selected to have a transplant).

Additional information about methods of formulating and administering cell populations, VOs and 3D cell cultures are known in the art (*See*, e.g., US 2024/0287463 A1; US 2024/0287463 A1; US 2020/0182861 A1; US 2019/0376044 A1; US 2020/0199541 A1; US
10 2023/0287357 A1; US 2023/0174949 A1; US 2023/0364267 A1; US Pat. No. 11,214,768; WO 2024/133285; WO 2022/226337; and WO 2023/196683; each of which is incorporated herein by reference in its entirety).

EXAMPLES

15 The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

METHODS

Generation of Doxycycline-inducible NKX3.1 iPSC lines

20 The doxycycline-inducible NKX3.1 (dox-NKX3.1) cell line was generated using the piggybac (PB) transposon and transposase system. The PB transposon vector harboring the NKX3.1 ORF was constructed using the Gateway cloning system. The PB super transposase (SBI, PB210PA-1) was purchased from SBI system biosciences. The PB dox-NKX3.1 transposon and transposase vectors were transfected via electroporation at 5:1 ratio into three
25 independent human induced pluripotent stem cells (hiPSCs) lines that were generated as previously reported. One microgram of super transposase and 5 µg of PB transposon were used to transfect the 2 million cells with use of the Neon electroporation system, following the vendor's guidelines (Invitrogen, MPK10096). Electroporation parameters were set at 1150 V for pulse voltage, and 30 ms for pulse width; two pulses were introduced. For 2 million cells, we used 3
30 mL of electrolytic buffer and 100-µL of resuspension buffer R in 100-µL reaction tips. The

electroporated cells were seeded on a Matrigel-coated dish in mTESR plus medium (STEMCELL Technologies, 100-0276) with 5 μ M Y27632 (Selleckchem, S1049). Positive cells were then selected by adding puromycin (InvivoGen, ant-pr-1) at 0.5 μ g/mL. The iPSC clones were collected by manual picking. The pluripotency and reactivity to doxycycline of clone 9 were validated by qPCR, and immunostaining, and used in further experiments of this application.

Differentiation of h-iPSCs into mural progenitor cells (S1-NKX3.1)

To generate mural progenitor cells using Dox-NKX3.1-iPSCs or modified NKX3.1 RNA, we followed a 2-step differentiation over 4-day period. On the first day, we seeded Dox-NKX3.1 or BJ-273 iPSCs on a Matrigel-coated 6 well-plate (Corning, cat# 354277) in 5 μ M Y27632 in mTESR plus medium at a seeding density of 200,000 cells/well. On the following day, the medium was switched to the 6 μ M of CHIR99021-containing S1 medium (Sigma-Aldrich, SML1046-25MG), which was formulated with 1X glutamax (ThermoFisher, cat# 35050061) and 60 μ g/mL ascorbic acid in Advanced DMEM/F-12 (Thermo Fisher Scientific, cat# 12634028). The cells were then continued to culture in fresh S1 medium with fresh CHIR99021 added each day. On day 3, the cells were treated with 5 μ g/mL of doxycycline (Sigma-Aldrich, D9891-10G) in S1 medium for additional two days with fresh doxycycline by changing the medium every day. For modified NKX3.1, on day 3 of differentiation, cells were dissociated by TrypLE (Thermo Fisher Scientific, cat# 12563029), and 5 μ g of RNA were transfected to 2 million cells with Neon electroporation as the same parameter set described above. Finally, the electroporated cells were plated on a Matrigel-coated 6-well plate in S1 medium. After four days of differentiation, iMPCs were maintained in SmGM-2 medium (Lonza, CC-3182) on 1% gelatin coating plate. The iMPCs are passaged twice a week until passage 2 (P2), after which the frequency is reduced to once a week. The split ratio ranges from 1:2 to 1:4, depending on the confluency.

modRNA synthesis and formulation

Chemically modRNA encoding NKX3.1 [modRNA(NKX3.1)] was generated by TriLink BioTechnologies LLC. In brief, modRNA(NKX3.1) was synthesized in vitro by T7 RNA polymerase-mediated transcription from a linearized DNA template, which incorporates the 5'

and 3' untranslated regions (UTRs) and a poly-A tail. Specifically, NKX3.1 (ORF:

ATGCTCAGGGTTCCGGAGCCGCGGCCCGGGGAGGCGAAAGCGGAGGGGGCCGCGC
CGCCGACCCCGTCCAAGCCGCTCACGTCCTTCCTCATCCAGGACATCCTGCGGGACG
GCGCGCAGCGGCAAGGCGGCCGCACGAGCAGCCAGAGACAGCGCGACCCGGAGCC
5 GGAGCCAGAGCCAGAGCCAGAGGGAGGACGCAGCCGCGCCGGGGCGCAGAACGAC
CAGCTGAGCACCGGGCCCCGCGCCGCGCCGGAGGAGGCCGAGACGCTGGCAGAGA
CCGAGCCAGAAAGGCACTTGGGGTCTTATCTGTTGGACTCTGAAAACACTTCAGGCG
CCCTTCCAAGGCTTCCCCAAACCCCTAAGCAGCCGCAGAAGCGCTCCCGAGCTGCCT
TCTCCCACTCAGGTGATCGAGTTGGAGAGGAAGTTCAGCCATCAGAAGTACCTGT
10 CGGCCCCCTGAACGGGGCCACCTGGCCAAGAACCTCAAGCTCACGGAGACCCAAGTG
AAGATATGGTTCCAGAACAGACGCTATAAGACTAAGCGAAAGCAGCTCTCCTCGGA
GCTGGGAGACTTGGAGAAGCACTCCTCTTTGCCGGCCCTGAAAGAGGAGGCCTTCTC
CCGGGCCTCCCTGGTCTCCGTGTATAACAGCTATCCTTACTACCCATACCTGTACTGC
GTGGGCAGCTGGAGCCCAGCTTTTTGGTAA; SEQ ID NO: 39; 705 bp) was cloned into the

15 mRNA expression vector pmRNA, which contains a T7 RNA polymerase promoter, an
unstructured synthetic 5'UTR, a multiple cloning site, and a 3'UTR that was derived from the
mouse β -globin 3' gene. In vitro transcriptional reaction (1-ml scale) was performed to generate
unmodified mRNA transcripts with wild-type bases and a poly-A tail. Cotranscriptional capping
with CleanCap Cap1 AGtrimer yields a naturally occurring Cap1 structure.

20 Deoxyribonucleasetreatment was used to remove DNA template. 5'-Triphosphate was removed
by phosphatase treatment to reduce innate immune response. After elution through a silica
membrane, the purified RNA was dissolved in ribonuclease-free sodium citrate buffer (1 mM,
pH 6.4).

25 **Isolation and maintenance of human MSCs, ECs, and VSMCs.**

Human mesenchymal stem cells (MSCs) were isolated from bone marrow as previously
described in Lin, R.-Z., Moreno-Luna, R., Li, D., Jaminet, S.-C., Greene, A.K., and Melero-
Martin, J.M. (2014). Human endothelial colony-forming cells serve as trophic mediators for
mesenchymal stem cell engraftment via paracrine signaling. *Proc. Natl. Acad. Sci.* 111, 10137–
30 10142, and maintained in MSCGM (Lonza, PT-3001) on 1% gelatin-coated plate. Human
primary vascular smooth muscle cells (SMCs) from pulmonary artery tissues were obtained from

Lonza (Lonza, CC-2581). The SMCs were cultured in SmGM-2 medium (Lonza, CC-3182) on 1% gelatin-coated plate (Sigma-Aldrich, G2500-500G). Endothelial colony-forming cells (ECFCs; herein referred to as ECs) were isolated from human umbilical cord blood and cultured in ECGM2 (Lonza, CC-3162) supplemented with 20% FBS (Genesee, 25-514) without hydrocortisone. All primary cells were used up to passage 10.

Flow cytometry analysis

Cells were dissociated into single-cell suspensions using TrypLE (Thermo Fisher Scientific, cat#12563029) and subsequently washed with FACS buffer formulated in PBS supplemented with 1% bovine serum albumin and 0.2 mM EDTA. For specific experiments, flow cytometry analysis was conducted after fixing the cells with 4% paraformaldehyde (PFA, Electron Microscope Sciences, cat# 15714-S). The staining procedure involved incubating the cells with the respective antibodies for 15 minutes on ice. Following incubation, the cells were washed three times with PBS buffer to remove any unbound antibodies. Flow cytometry analysis was performed using a BD Accuri C6 Plus flow cytometer (BD Biosciences), and the acquired data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR). Detailed information regarding the antibodies used in the staining procedure can be found in Table 2.

Immunofluorescence staining

Cells were seeded in tissue culture-treated polymer coverslip eight-well chamber slides (ibidi USA, Fisher Scientific, cat# 50-305-795) or 8-well chamber slides (ibidi USA, Fisher Scientific, NC1535706) at a seeding density of 2×10^4 cells/cm². On the following day, cells were fixed with 4% paraformaldehyde (PFA) and permeabilized with 100% cold methanol at -20°C or 0.2% Triton in PBS for 15 mins. After blocking with 10% of BSA for 30 mins at RT, primary antibodies were added and incubated for 1 hour at room temperature or overnight at 4°C. After washing three times with PBS, the cells were incubated with secondary antibodies and DAPI at room temperature for 30 minutes. The slides containing the stained cells were mounted using DAKO fluorescence mounting medium (Agilent, S302380-2) or directly imaged without mounting. Images were obtained using an Axio Observer Z1 inverted microscope (Carl Zeiss) and AxioVision Rel. 4.8 software. For phase contrast images, we used an AxioCam MRc5 camera with either a 5X or 10X objective lens. Validation of pluripotency was carried out using

OCT4, NANOG, and SOX2. Detailed information regarding the antibodies used in the staining procedure can be found in Table 2.

Intracellular Ca²⁺ flux detection assay

5 Intracellular calcium flux was measured and visualized by Fluo-4 Calcium imaging kit (Life Technologies, F10489) and we followed the manual that the vendor provided. Either pVSMC or Day4 iMPC were seeded in an 8-well chamber slide with SMGM-2 medium at 2000 cells per well density. On the following day, the cells were washed with live cell imaging solution (LCIS) buffer (ThermoFisher, A14291DJ). Fluo-4 AM loading solution was prepared by
10 adding 20mM glucose in live cell imaging solution (LCIS) buffer, which contained probenecid, power load and fluo-4. The 200 μ L loading solution was then added to cells and incubated for 30 mins at 37°C followed by 15 mins incubation at room temperature. After washing the cells, we replaced the loading solution with 20mM glucose-LCIS buffer with 10 mM carbachol (Millipore Sigma, PHR1511), 0.1 μ M endothelin1 (Millipore Sigma, E7764-10UG) or PBS and imaged
15 immediately. The fluorescence Images were obtained using an Axio Observer Z1 inverted microscope (Zeiss) for 5 mins with 5-second intervals and the relative fluorescence level of individual cells was analyzed by ImageJ with mean intensity and normalized by $(F-F_0)/F_0$.

Collagen gel contraction assay

20 Prior to the experiment, cells were serum starved in basal smooth muscle cell medium (SMCM, ScienceCell, 1101) which contained 0.1% FBS in the incubator overnight. The following day, 3 mL of ice-cold collagen solution was prepared by mixing 1.8 mL of 1x DMEM with 0.3 ml FBS and 0.75 ml of Bovine Collagen-1 in a 50 ml falcon tube. The solution was kept on ice to prevent solidification when adjusting the pH to 7.4 using 0.1N NaOH. A cell
25 suspension was prepared at 10⁶ cells/ml collagen solution and 10 μ L of cell-collagen suspension was plated in triplicate in angiogenesis μ -slide (ibidi, 81506). After plating the collagen-cell suspension, the plate was incubated at 37°C for 30 min. Once solidified, 40 μ L of SMGM2 media was added on top of the gels with a 10 μ M U46619 vasoconstrictor. Images were captured after 72 hours of incubation, with Axio Observer Z1 inverted microscope (Zeiss) at 4X objective and
30 ZEN 3.6 (blue edition) software. The surface area of collagen cells was quantified using ImageJ. The percentage of contraction was then calculated by comparing the final area to the initial area

on day 0, using the following formula: Percentage of original gel area = (Final Area / Initial Area) $\times$ 100.

Fibronectin deposit assay

5 A total of 1×10^4 induced mural progenitor cells (iMPCs) were seeded onto eight-well chamber slides (ibidi USA, Fisher Scientific, cat# 50-305-795) or 24-well plates in Smooth Muscle Growth Medium-2 (SMGM-2). The next day, the SMGM-2 medium was replaced with fresh SMGM-2 containing either 0, 2.5, 10 or 100 ng/mL of transforming growth factor-beta (TGF β) (Prospec, CYT-716), along with or without TGF β inhibitor, SB431542 (Santa Cruz
10 Biotech, sc-204265). The cells were then treated for 72 hours. To assess fibronectin expression, the cells were fixed with 4% PFA and stained with a fibronectin antibody (Abcam, Ab2413) in a blocking solution (1.5% BSA solution) without permeabilization followed by blocking for 30 mins. Subsequently, after three times of washing, a goat anti-rabbit-488 secondary antibody was applied to facilitate detection with DAPI. Images were obtained using an Axio Observer Z1
15 inverted microscope (Zeiss) and ZEN 3.6 (blue edition) software and quantified the fibronectin deposit by measuring the green fluorescence intensity and dividing the number of cells which is measured by DAPI. The quantification was performed by Image J.

Mural-endothelia cell co-culture assay

20 To perform the co-culture assay of endothelial cells, at day 4 of differentiation, induced mural progenitor cells (iMPCs), human mesenchymal stem cells (MSCs), and endothelial colony-forming cells (ECFCs) were harvested using 300 μ L of TrypLE. A total of 5×10^4 iMPCs or MSCs and the same number of ECFCs were pre-mixed and seeded onto a 6-well plate coated with 1% gelatin. The co-culture was carried out for a duration of 7 days in Endothelial Growth
25 Medium-2 (EGM-2, Lonza, CC-3162). Following the 7-day co-culture period, CD31-positive cells were selectively removed using magnetic bead-based sorting (Invitrogen, 11155D). The CD31-positive endothelial cells (ECs) were separated and discarded using a magnetic separator, while the negative fractions were isolated for further analysis.

Transwell co-culture assay

30

The transwell co-culture experiment was conducted using a 48-well permeable transwell plate with a 3 μm pore size (Corning, CLS3415). One day before co-culture, 1×10^3 endothelial colony-forming cells (ECFCs) were seeded in the bottom well of the transwell using K-medium. On the following day, 1×10^4 iMPCs at day 4 of differentiation were seeded on the top insert in basal Endothelial Basal Medium-2 (Lonza, EBM-2) supplemented with 5% Fetal Bovine Serum (FBS). After three days of co-culture, the ECFCs in the bottom well were fixed and stained with DAPI. Subsequently, 4 images per well were captured using an Axio Observer Z1 inverted microscope (Carl Zeiss) and AxioVision Rel. 4.8 software and the images were analyzed using ImageJ software. The analysis included the functions: Threshold at 50, Fill Holes, Watershed, and Analyze Particles size bigger than 10 pixels to quantify the stained cells.

Endothelial cell growth assay in conditioned media

At day 4 of differentiation, induced mural progenitor cells (iMPCs) were subjected to treatment with 2mL of basal medium of Endothelial Basal Medium-2 (Lonza, 190860) supplemented with 5% FBS (Genesee, 25-514) for 24 hours on a 6 well-tissue culture plate. Subsequently, 12 mL of the cultured medium was collected and filtered through a 0.22 μm filter (VWR, 76479-016). The filtered medium was then concentrated using a 3kDa cut-off centrifugal concentrator (Millipore, UFC900324) at 10,000 rpm for 45 minutes. To re-constitute the 2-fold concentrated samples, up to 6 mL of fresh EBM-2 medium was added. For the endothelial cell (EC) growth test, 96-well plates were utilized, with 1000 cells seeded per well in K-medium, formulated using Endothelial Cell Growth Medium-2 (ECGM2, Lonza, CC-3162) supplemented with 20% FBS (Genesee, 25-514) without hydrocortisone. The following day, the medium was switched to either Basal medium or the 2X conditioned medium obtained as described above for further experimentation.

Tube formation assay

Human ECFCs on Matrigel were used to perform tube formation assay. Matrigel (Corning, 354277) was thawed overnight at 4°C and 200 μL was distributed on each well of the 24-well plate. After incubating 30 mins in 37°C, ECFCs were dissociated with TrypLE (ThermoFisher, 12563029) and resuspended at 2×10^5 per mL and then distributed 50 μL of cell suspension on top of solidified matrigel. Either basal medium with 0.5% FBS (control) or

conditioned medium was used to culture. After 24 hours of incubation, the images were obtained at phase contrast 5X and analyzed using Angiogenesis Analyzer plugin in ImageJ software.

Wound healing assay

5 5×10^4 ECFCs were seeded each well in a 24-well plate, and cultured until 100 percent confluence. Using 1000P tips, we scratched one line in the center of the well and changed the medium to either basal medium of ECM-2 with 5% FBS or conditioned medium collected as described in EC growth assay. After 24 hours of incubation, the scratched area was imaged by phase contrast 5X and analyzed the area using ImageJ.

Angiogenic array

10 The Proteome Profiler kit (R&D systems, ARY007) was used to analyze the expression profiles of 55 angiogenesis-related proteins. Two-fold concentrated conditioned medium was prepared as described above and followed the instructions in the manufacturer's manual. In brief, 15 2 mL of blocking buffer (array buffer 7) was added to the membrane and incubated for one hour on a rocking platform shaker. During blocking, 1 mL of each sample was mixed with 0.5 mL of dilution buffer (array buffer 4), and 15 uL detection antibody cocktail and then incubated for one hour. After the blocking buffer was removed, the sample-antibody mixture was distributed and incubated overnight at 4°C on a rocking shaker. The membranes were washed three times with 20 20mL for 10 mins. Prior to streptavidin-HRP addition, a 4-well multi-dish was cleaned with distilled water and dried thoroughly. 2mL of diluted streptavidin-HRP in array buffer 5 was added to the membrane in a 4well dish and incubated for 30 mins on a rocking shaker. Followed by 3 times wash, 1 mL of Chemi-reagent evenly distributed onto the membrane. Lastly, we covered the membrane with the top sheet of the plastic protector and smoothed out the air 25 bubbles, and incubated for 1 min before imaging. For image analysis, array spots were segmented using Cellpose. Expression levels were determined by calculating the product of spot size and intensity. Normalization of values for each experiment was performed by dividing them with the negative control value.

Angiogenic factors multiplex analysis

The protein concentration of 17 angiogenic factors was analyzed by the standard curve of the Human Angiogenesis 17-Plex Discovery Assay. The human angiogenesis 17-plex discovery assay was performed by Eve Technologies, Canada. Luminex xMAP technology was used for multiplexed quantification. The multiplexing analysis was performed using the Luminex™ 200 system (Luminex, Austin, TX, USA) by Eve Technologies Corp. (Calgary, Alberta). Seventeen markers were simultaneously measured in the samples using Eve Technologies' Human Angiogenesis & Growth Factor 17-Plex Discovery Assay® (MilliporeSigma, Burlington, Massachusetts, USA) according to the manufacturer's protocol. The 17-plex consisted of Angiopoietin-2, BMP-9, EGF, Endoglin, Endothelin-1, FGF-1, FGF-2, Follistatin, G-CSF, HB-EGF, HGF, IL-8, Leptin, PLGF, VEGF-A, VEGF-C, VEGF-D. Assay sensitivities of these markers range from 0.2 – 42.8 pg/mL for the 17-plex. Individual analyte sensitivity values are available in the MilliporeSigma MILLIPLEX® MAP protocol.

Quantitative reverse transcription PCR

RNA was isolated with either an RNeasy kit (Qiagen, cat# 74106) or SYBR™ Green Cells-to-CT™ Kit (ThermoFisher Scientific, cat# 4402954). The cDNA was prepared using either reverse transcriptase III (Thermo Fisher Scientific, cat# 4368814) or SYBR™ Green Cells-to-CT™ Kit according to the manufacturer's instructions. Quantitative PCR was performed using SYBR Green Master Mix (Thermo Fisher Scientific, A25776), and detection was achieved using the QuantStudio™ 3 Real-Time PCR System, 96-well (Thermo Fisher Scientific, cat# A28567). The expression of target genes was normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Real-time PCR primer sequences are listed in Table 1.

Statistical analyses: Unless otherwise stated, data were expressed as means ± SEM of the mean. For comparisons between two groups, means were compared using unpaired two-tailed Student's t-tests. Comparisons between multiple groups were performed by analysis of variance (ANOVA) followed by Bonferroni's post-test analysis. All statistical analyses were performed using GraphPad Prism v.9 software (GraphPad Software Inc.).

In vitro vascular network-forming 'on-a-chip' assay

We utilized the idenTx 3 Chip and Holder from AIM Biotech Pte. Ltd., following a slightly modified version of the manufacturer's guidelines. ECFCs and iMPCs were combined at

a 3:1 ratio, resulting in 1.2×10^5 cells in 10 μL , and embedded in a hydrogel solution. This hydrogel consisted of 6 mg/mL fibrinogen (Sigma, F8630) in 1x PBS at 37°C and 50 U/mL thrombin (Sigma, T4648) in 1x PBS. The mixture was seeded into the chip's cell/gel channel. The culture medium was EGM-2, supplemented with 5% FBS and 50 ng/mL VEGF. For cell preparation, cells were suspended in a medium-diluted thrombin solution (4 U/mL), mixed with the fibrinogen solution to achieve final concentrations of 2 U/mL thrombin and 3 mg/mL fibrinogen, and applied to the chip's gel channel, allowing it to polymerize at 37°C for 30 minutes. After polymerization, 15 μL of culture medium was added to both media channels. To create a flow gradient, the medium volume was adjusted to 70 μL on one side and 50 μL on the other, with daily medium changes to maintain cell viability. The chip was kept at 37°C in a 5% CO₂ environment.

Animal experiments

All animal experiments were performed at Boston Children's Hospital in accordance with the institutional guideline approved by Institutional Animal Care and Use Committee (IACUC) protocol 20-12-4327R. For the *in-vivo* vascular network forming assay, we purchased the six-week-old Athymic nude mice (Foxnl/nu mice) from Envigo and reared them according to the following immune-deficient mice guidelines.

In vivo vascular network-forming assay

The *in vivo* vascular network forming assay was carried out by co-transplanting human endothelial colony-forming cells (ECFCs) or Human umbilical vein endothelial cells (HUVEC, ATCC, CRL-1730), and mural cells in collagen mixed with fibrinogen gel, as previously described in Nowak-Sliwinska, P., et al. (2018). Consensus guidelines for the use and interpretation of angiogenesis assays. *Angiogenesis* 21, 425–532. 10.1007/s10456-018-9613-x. In brief, the collagen/fibrinogen gel solution was prepared by combining 1.5 mg/mL collagen (Trevigen, cat# 3442-050-01), 30 $\mu\text{g/mL}$ fibrinogen (Sigma-Aldrich, F8630-1G), 1mg/mL human fibronectin (Millipore-Sigma, F0895-2MG) plus 25 mM HEPES and 10% FBS on ice. The two cell types, ECFCs or HUVECs (0.8×10^6) and one of the mural cells iMPCs, pSMCs, bm-MSC (1.2×10^6) were pre-mixed in 200 μL of pH-neutral gel solution, and loaded into a 30G syringe by using a 1 mL pipette. Mice were anesthetized with isoflurane, subcutaneously injected

with 50 μ L of 50 μ g/mL thrombin (Sigma-Aldrich, T4648), then with 200 μ L of cell-loaded gel into the same site. Cell-gel implants were harvested after 1 week to analyze vascular formation.

Histology and immunofluorescence staining

5 Explanted grafts were fixed overnight in 10% buffered formalin and were washed in 70% ethanol. Fixed ex-grafts were embedded in paraffin and sectioned at 7 μ m. H&E-stained sections were used to assess micro-vessel density. The number of vessels per area (vessels/ mm^2) of graft was counted as the average number of erythrocyte-filled vessels (vessels/ mm^2) in H&E-stained sections. For immunostaining, sections were deparaffinized via xylene for 10 mins and
10 sequential immersion in ethanol and underwent antigen retrieval in citric buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 30 min at 95 °C. Sections were then blocked for 30 min in 5% BSA, and incubated with primary and secondary antibodies each for 1 hour at RT. Human-specific anti-CD31 antibodies were used to stain human blood vessels and perivascular mural cells were immunostained with anti- α SMA antibodies. Anti-GFP or human-specific Vimentin
15 antibodies were used to trace iPSC. The antibodies are detailed in Table 2.

Bulk RNA sequencing

 RNA extraction, library preparation, and sequencing were conducted at Azenta Life Sciences (South Plainfield, NJ, USA) as follows: Total RNA was extracted from fresh frozen
20 cell pellet samples using Qiasymphony RNA kit following manufacturer's instructions (Qiagen, Hilden, Germany).

Library preparation with PolyA selection and Illumina sequencing

 RNA samples were quantified using Qubit 2.0 Fluorometer (Life Technologies, Carlsbad,
25 CA, USA) and RNA integrity was checked using Agilent TapeStation 4200 (Agilent Technologies, Palo Alto, CA, USA). RNA sequencing libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit for Illumina using the manufacturer's instructions (NEB, Ipswich, MA, USA). Briefly, mRNAs were initially enriched with Oligod(T) beads. Enriched mRNAs were fragmented for 15 minutes at 94 °C. First-strand and second-strand
30 cDNA were subsequently synthesized. cDNA fragments were end-repaired and adenylated at 3'ends, and universal adapters were ligated to cDNA fragments, followed by index addition and

library enrichment by PCR with limited cycles. The sequencing library was validated on the Agilent TapeStation (Agilent Technologies, Palo Alto, CA, USA), and quantified by using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) as well as by quantitative PCR (KAPA Biosystems, Wilmington, MA, USA). The sequencing libraries were clustered on two flow cells. After
5 clustering, the flowcell was loaded on the Illumina instrument 4000 according to the manufacturer's instructions. The samples were sequenced using a 2x150bp Paired-End (PE) configuration. Image analysis and base calling were conducted by the Control software. Raw sequence data (.bcl files) generated by the sequencer were converted into fastq files and demultiplexed using Illumina's bcl2fastq 2.17 software. One mismatch was allowed for index
10 sequence identification.

Analysis of RNA sequencing data

After investigating the quality of the raw data, sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality using Trimmomatic v.0.36. The
15 trimmed reads were mapped to the *Homo sapiens* reference genome available on ENSEMBL using the STAR aligner v.2.5.2b. The STAR aligner is a splice-aligner that detects splice junctions and incorporates them to help align the entire read sequences. BAM files were generated as a result of this step. Unique gene hit counts were calculated by using feature Counts from the Subread package v.1.5.2. Only unique reads that fall within exon regions were counted.

20 After extraction of gene hit counts, the gene hit counts table was used for downstream differential expression analysis. Using DESeq2, a comparison of gene expression between the groups of samples was performed. The Wald test was used to generate p-values and Log2 fold changes. Genes with adjusted p-values < 0.05 and absolute log2 fold changes > 1 were called as differentially expressed genes for each comparison. A gene ontology analysis was performed on
25 the statistically significant set of genes by implementing the software GeneSCF. The goa_human or mouse GO list was used to cluster the set of genes based on their biological process and determine their statistical significance. A PCA analysis was performed using the "plotPCA" function within the DESeq2 R package. The plot shows the samples in a 2D plane spanned by their first two principal components. The top 500 genes, selected by highest row variance, were
30 used to generate the plot.

Single-cell RNA sequencing

Cell and library preparation

Cells were treated with 300µL of TrypLE for 3 minutes at 37°C, followed by pipetting several times using a p1000 pipette. Subsequently, the dissociated cells were filtered through a FACS filter cell strainer. Single-cell RNA samples were prepared using the Chromium Next 10X genomics technique (10xgenomics, PN-1000128, PN-1000127) according to the manufacturer's protocol. Quantification of DNA samples was conducted using a Qubit 2.0 Fluorometer, and quality control assessment was performed at the Harvard core facility using Agilent TapeStation D5000. RNA sequencing libraries were subsequently prepared using the dual index kit (10xgenomics, PN-1000213) according to the manufacturer's instructions. Validation of sequencing libraries was carried out using Agilent TapeStation D1000 at the Harvard core facility.

Illumina sequencing

Illumina Sequencing was performed by Medgenome (CA, USA). Libraries were sequenced via Illumina Novaseq 6000 sequencer (Illumina, San Diego, CA). 150 PE reads were generated for a total of ~503 GB of data. Illumina raw BCL sequencing files were processed through the CellRanger software (10x Genomics) for generating FASTQ files and count matrixes (support.10xgenomics.com/single-cell-gene-expression/software/overview/welcome). Feature-barcode matrices were obtained from "cellranger count" for all the samples.

Dataset quality control

The single-cell sequencing datasets were processed using 10x Genomics Cell Ranger (version 7.1.0) tool sets. For each sample, the "cellranger count" pipeline was employed to quantify gene expression from the FASTQ files, including reads alignment, filtering, barcode scanning, and UMI counting. During this step, the GRCh38 human genome (version refdata-gex-GRCh38-2020-A) served as a reference. Subsequently, the filtered feature barcode matrix files were imported into the Seurat package (version 4.1.0) for quality control, analysis, and exploration. To eliminate doublets, the DoubletFinder package (version 2.0.3) was applied. Only cells expressing more than 200 and fewer than 9,000 unique genes, with mitochondrial percentages below 20%, were retained for further analysis.

Cell clustering

Datasets normalization, scaling, dimension reduction, cell clustering, and differentially expressed genes (DEGs) identification were performed using the Seurat package (version 4.1.0). In brief, the global-scaling normalization method “LogNormalize” was applied to normalize the feature expression measurements for each cell, and then “ScaleData” function was used for linear transformation. The top 2,000 most variable genes were identified using the “FindVariableFeatures” function to perform principal component analysis (PCA). The “FindNeighbors” function was used for construct a K-nearest neighbor (KNN) graph based on the first 15 principal components (PCs), and the “FindClusters” function was performed to cluster cells into different populations by the graph. DEGs for each population were identified using the “FindAllMarkers” function with default parameters.

Trajectory inference

To delineate the cell differential trajectory, Monocle3 (version 1.3.4) was employed for single-cell trajectory analysis. Throughout the analysis, the PCA dimension reduction algorithm and Log normalization method were used for preprocessing steps. The “reduce_dimension” function was then applied for dimensionality reduction using the UMAP algorithm. To cluster cells, the “cluster_cells” function was employed using the Leiden clustering method. Following preprocessing, dimension reduction, and cell clustering, the trajectory was built by the “learn_graph” function. The resulting trajectory structure was visualized by the “plot_cells” function.

Comparative transcriptomic analysis of iMPC-derived mural cells with publicly available mural cell data

We employed the "Scanorama" algorithm to compare iMPC-derived pericytes/SMCs with publicly available mural cell data (Hie, B., et. al., (2019) Nat. Biotechnol. 37, 685–691). The reference dataset used was "TS_Vasculature" from The Tabula Sapiens Consortium (Consortium*, T.S., Jones, R.C., Karkanias, J., Krasnow, M.A., Pisco, A.O., Quake, S.R., Salzman, J., Yosef, N., Bulthaupt, B., Brown, P., et al. (2022). The Tabula Sapiens: A multiple-organ, single-cell transcriptomic atlas of humans. Science 376, eabl4896). For each

pericyte/SMC in our scRNA-seq dataset, we identified the most similar cell type in the reference dataset and counted these occurrences. Using edgeR (version 4.0.16) for pseudo-bulk differential expression analysis, we calculated the Spearman coefficient to assess similarity between iMPC-derived and primary pericytes/SMCs. The results were visualized with Pheatmap (version 1.0.12), using ECFCs as a negative control (Robinson, M.D., et. al., (2009) *Bioinformatics* 26, 139–140).

Cell-cell interaction analysis

We used the R packages CellChat (version 1.6.1) and Monocle (version 2.22.0) to profile cell-cell communication and cell trajectory, respectively (Jin, S., et. al., (2021) *Nat Commun* 12, 1088).

Gene Regulator Network analysis

The R package hdWGCNA was utilized to construct specific co-expression networks across cellular hierarchies (Morabito, S., et. al., (2023) *Cell Rep. Methods* 3, 100498).

Statistical analysis

Except where specifically mentioned, data were presented as means $\pm$ standard error of the mean (SEM). When comparing two groups, mean values were compared using unpaired two-tailed Student's t-tests. Multiple group comparisons were conducted through analysis of variance (one-way ANOVA) followed by Bonferroni correction. No exclusion criteria were applied to any of the analyses. All statistical calculations were performed using GraphPad Prism v.9 software (GraphPad Software Inc.). Statistical significance was set at $P < 0.05$.

Table 1. Sequences of primers used for RT-qPCR

Gene	Forward primer (5'-3')	Reverse primer (3'-5')
<i>GAPDH</i>	CATGTTTCGTCATGGGTGTGAACCA (SEQ ID NO:1)	ATGGCATGGACTGTGGTCATGAGT (SEQ ID NO:2)
<i>OCT4</i>	GGGCTCTCCCATGCATTCAAAC (SEQ ID NO:3)	CACCTTCCCTCCAACCAAGTTGC (SEQ ID NO:4)
<i>SOX2</i>	CACATGAAGGAGCACCCGGATTAT (SEQ ID NO:5)	GTTTCATGTGCGCGTAACTGTCCAT (SEQ ID NO:6)
<i>NANOG</i>	AGTCCCAAAGGCAAACAACCCACT	TGCTGGAGGCTGAGTATTTCTGTC

	TC (SEQ ID NO:7)	TC (SEQ ID NO:8)
<i>TBXT</i>	GTGCTGTCCCAGGTGGCTTACAGA TG (SEQ ID NO:9)	CCTTAACAGCTCAACTCTAACTACT TG (SEQ ID NO:10)
<i>ACTA2</i> (Aortic smooth muscle actin, alpha2)	GCCAAGCACTGTCAGGAATC (SEQ ID NO:11)	GGGTACTTCAGGGTCAGGAT (SEQ ID NO:12)
<i>TAGLN</i> (Transgelin, SM22 alpha)	GTCTGGGGAAAGCTCCT (SEQ ID NO:13)	ATGTCTGGGGAAAGCTCCT (SEQ ID NO:14)
<i>MYOCD</i> (Myocardin)	ACGGATGCTTTTGCCTTTGAA (SEQ ID NO:15)	AACCTGTCTGAAGGGGTATCTG (SEQ ID NO:16)
<i>CNN1</i> (Calponin)	GAACGTGGGAGTGAAGTACGC (SEQ ID NO:17)	CAGCCCAATGATGTTCCGC (SEQ ID NO:18)
<i>MYH11</i> (smooth muscle myosin heavy chain)	GGTCACGGTTGGGAAAGATGA (SEQ ID NO:19)	GGGCAGGTGTTTATAGGGTT (SEQ ID NO:20)
<i>PDGFRB</i>	TCTTTGTGCCAGATCCCACC (SEQ ID NO:21)	AGTGCAACGTCCCCTTTCTT (SEQ ID NO:22)
<i>CSPG4</i>	GCCACGTTGTCAGTCGATG (SEQ ID NO:23)	CCCATAGGGGACCTCTAGGG (SEQ ID NO:24)
<i>PDE5A</i>	GCAGAGTCCTCGTGCAGATAA (SEQ ID NO:25)	GTCTAAGAGGCCGGTCAAATTC (SEQ ID NO:26)
<i>NDUFA4L2</i>	ATGATCGGCTTAATCTGCCTG (SEQ ID NO:27)	TCCGGGTTGTTCTTTCTGTCC (SEQ ID NO:28)
<i>DES</i> (DESMIN)	GACGTGGATGCAGCTACTCTA (SEQ ID NO:29)	GGAACGCGATCTCCTCGTTG (SEQ ID NO:30)
<i>TPM1</i>	TTGAGAGTCGAGCCCCAAAAG (SEQ ID NO:31)	CATATTTGCGGTCTGGCATCTT (SEQ ID NO:32)
<i>THY1</i>	GCCTAACGGCCTGCCTAGT (SEQ ID NO:33)	GGGTGAACTGCTGGTATTCTCAT (SEQ ID NO:34)
<i>NKX3.1</i>	TCACGTCCTTCCTCATCCAG (SEQ ID NO:35)	AAGACCCCAAGTGCCTTTCT (SEQ ID NO:36)
<i>FN1</i>	AAACCAATTCTTGAGCAGG (SEQ ID NO:37)	CCATAAAGGGCAACCAAGAG (SEQ ID NO:38)

Table 2. List of Antibodies

Antibody	SOURCE	IDENTIFIER
PE-anti-CD13	Biolegend	RRID: AB_314179 Cat # 301703
APC-anti-CD140b	Biolegend	RRID:AB_2162787 Cat# 323608

APC-anti-Hu-TRA-1-81 (Podocalyxin)	Invitrogen eBioscience	RRID:AB_1059790 5 Cat# 17-8883-42
Monoclonal Mouse anti-Actin, a-Smooth Muscle	Sigma	RRID:AB_476701 Cat# A2547
Rabbit anti-alpha Smooth Muscle Actin	Abcam	RRID:AB_2223021 Cat# AB5694
Polyclonal Rabbit anti-TAGLN/Transgelin (SM22)	Abcam	RRID:AB_443021 Cat# Ab14106
Monoclonal Mouse anti-Hu-Vimentin	Abcam	RRID:AB_306239 Cat# Ab8069
Mouse anti-Calponin	Santa Cruz Biotechnology	RRID:AB_781770 Cat# Sc-58707
Anti-NKX3.1	Abcam	RRID:AB_2861392 Cat# Ab196020
Rabbit anti-OCT4	Stemgent	RRID:AB_2167689 Cat# 09-0023
Rabbit anti-SOX2	Stemgent	RRID:AB_2195775 Cat# 09-0024
Rabbit anti-NANOG	Stemgent	RRID:AB_2298294 Cat# 09-0020
Rabbit anti-Fibronectin	Abcam	RRID:AB_2262874 Cat# Ab2413
Rabbit anti-GFP	Abcam	RRID:AB_305564 Cat# AB6556
Mouse anti-hu- Anti-CD31/PECAM-1 Antibody (JC70)	Santa Cruz Biotechnology	RRID:AB_629040 Cat# sc-53411
Donkey anti-mouse IgG, AlexaFluor-488	Invitrogen	RRID:AB_141607 Cat# A21202
Donkey anti-mouse IgG, AlexaFluor-674	Invitrogen	RRID:AB_162542 Cat# A-31571
Donkey anti-rabbit IgG, AlexaFluor-594	Invitrogen	RRID:AB_2535792 Cat# A21206
Goat anti-rabbit IgG, AlexaFluor-488	Invitrogen	RRID:AB_2576217 Cat# A-11034

Example 1: Transient NKX3.1 activation efficiently differentiates MePCs into iMPCs

We initially identified NKX3.1 in a screening conducted in collaboration with Dr. George Church at Harvard (Ng, A. H. M. et al. A comprehensive library of human transcription factors for cell fate engineering. Nat Biotechnol 39, 510–519 (2020)). To evaluate the differentiation potential of NKX3.1, we first genetically engineered human iPSCs to express NKX3.1 in response to doxycycline (Dox) using a piggyBac transposon system. Puromycin-selected clones were screened for homogeneous expression of NKX3.1 upon administration of Dox (**FIGS. 1J-**

10). The engineered clones (termed iPSC-Dox-NKX3.1) remained pluripotent and maintained the expression of pluripotency markers OCT4, SOX2, and NANOG at comparable levels to the parental iPSC counterpart (**FIGS. 1J-1O**). Next, we engineered a human iPSC line with a dox-inducible NKX3.1 transgene and developed a new feeder-free protocol (**Figs. 1A and 1I**). The first 48-hour step converts iPSCs into mesodermal progenitors (MePCs) via Wnt and Nodal pathways. The second step transiently activates NKX3.1 for 48 h, yielding iMPCs. Flow cytometry confirmed mural cell presence by analyzing PDGFR β (CD140b) and aminopeptidase N (CD13) expression. Puromycin-selected clones were screened for homogeneous expression of NKX3.1 upon administration of Dox (**Figs. 1J-1M**). The engineered clones (termed iPSC-Dox-NKX3.1) remained pluripotent and maintained the expression of pluripotency markers OCT4, SOX2, and NANOG at comparable levels to the parental iPSC counterpart (**Fig. 1M**). As discussed above, the two-dimensional, feeder-free, and chemically defined protocol that relies on a timely transition of iPSCs through two distinct stages, each lasting 48 hours (**Figs. 1A and 1I**). The first step, which entails the conversion of iPSCs into intermediate MePCs, is mediated by the activation of Wnt signaling pathways using the glycogen synthase kinase 3 inhibitor CHIR99021 and is characterized by the transient activation of the TF TBXT (**FIG. 2A, bottom panels**). The second step, again, involves the activation of NKX3.1 for 48 hours via the provision of Dox in the absence of any growth factors (**Figs. 1G and 15A**). Thereafter, the resulting cells, herein termed iMPCs, were grown in a serum-containing smooth muscle growth medium (SMGM) for additional passages.

During the 4 days of differentiation, we observed significant morphological changes in the cells that progressively resembled those of mesenchymal cell types (**Fig. 1H**). Moreover, iMPCs morphologically resembled mural cells in culture, displaying a characteristic stellate shape (**Fig. 1H**). We traced the presence of mural populations during differentiation using flow cytometry by analyzing the expression of PDGFR β (CD140b), a general mural cell marker, and aminopeptidase N (CD13), known to be expressed in mural cells *in vivo*.

The protocol effectively converted iPSCs into CD140b⁺/CD13⁺ iMPCs (~99% efficiency; **Fig. 1B**), with less than 1% of cells expressing the TRA1-81 antigen, indicating minimal undifferentiated iPSCs (**Fig. 1C**). After one passage in culture, iMPCs expressed mural-specific contractile proteins, including alpha-smooth muscle actin (α -SMA), calponin, transgelin (SM22), and vimentin (**Fig. 1D**). Comparison of iMPCs mRNA expression with control human

primary pulmonary vascular SMCs and bone marrow-derived mesenchymal stem cells (MSCs) confirmed mural cell specification (**Figs. 1E-1F**). Expression of SMC markers in iMPCs was comparable to or higher than in SMCs and MSCs (**Fig. 1E**). Specifically, the expression of selected smooth muscle markers was either equal to (e.g., *ACTA2*, *TPMI*) or significantly higher (*CNN1*, *TAGLN*, *MYOCD*, *MYH11*) in iMPCs compared to SMCs and MSCs (**Fig. 1E**). Pericyte markers expression was similar or upregulated in iMPCs (*CSPG4*, *DES*, *NDUFA4L2*, *PDE5A*, and *THY1*), except *PDGFRB*, which was higher in MSCs (**Fig. 1F**).

This effective conversion of MePCs into PDGFR β ⁺ iMPCs via activation of NKX3.1 was reproducible across iPSC lines from three distinct cellular origins (**Figs. 1N-1O**).

After differentiation, indirect immunofluorescence confirmed the expression of mural-specific contractile cytoskeletal proteins in iMPCs, including alpha-smooth muscle actin (α -SMA), calponin, transgelin (SM22), and vimentin (**Fig. 1D**). The expression of these mural cell markers was highly uniform (>90%) and reproducible across iMPCs derived from three distinct iPSC lines (**Figs. 1N-1O and 16A-16C**). Importantly, in the absence of Doxycycline, expression levels of mural cell markers were significantly lower, supporting NKX3.1's role in mural cell specification (**Figs. 1N-1O and 15B**).

It is important to note that during the differentiation of MePCs to iMPCs, the expression of NKX3.1 was only transient (**Figs. 1G, 2A, and 15A**). This transitory expression enabled the possibility of using chemically modified mRNA (modRNA), thus developing a genomic footprint-free protocol. Indeed, transfection of unmodified iPSCs with modRNA encoding NKX3.1 enabled robust transient expression of NKX3.1 (**Figs. 3A-3G**). Moreover, activation of NKX3.1 with modRNA in MePCs efficiently produced iMPCs that were indistinguishable from those generated by the Dox-inducible protocol, including a robust expression of mural cell markers (**Figs. 3A-3G**).

Example 2. Comparing our TF-induced method with a standard chemically-induced differentiation protocol

We compared iMPCs generated via our NKX3.1-induced protocol to a chemical induction protocol originally reported by Patsch et al. in 2015. This protocol generates SMCs (herein termed iSMCs) from the same MePCs but uses PDGF-BB (10 ng/mL) and Activin A (2 ng/mL) for 48 h (**Fig. 2A**). Both iMPCs and iSMCs exhibited a similar pattern of transient

NKX3.1 expression (**Fig. 2A**). Cells during both the NKX3.1-induced and chemically-induced protocols exhibited a similar sequential pattern of transient expression of *TBXT* and *NKX3.1*, coinciding with their transition through mesodermal and mural cell differentiation stages, respectively (**Fig. 2A**).

SMC marker expression was consistent in both iMPCs and iSMCs, except for *ACTA2* and *MYOCD*, which were significantly higher in iSMCs (**Fig. 2B**). Conversely, pericyte markers were more prevalent in iMPCs (**Fig. 2C**). In short, both protocols successfully generate mural cells; however, the chemically-induced approach predominantly yields cells with an SMC phenotype, whereas NKX3.1 induction generates a more diverse iMPC population that likely includes SMCs and pericytes.

It is important to note that our protocol for generating iSMCs was inspired by, rather than directly following, the protocol described by Patsch et al. We adopted only the mesodermal to mural cell differentiation aspects of their protocol (i.e., the use of PDGF-BB and Activin A) to enable a comparable mesodermal stage and effectively compare the outcome of our NKX3.1 induction protocol with a chemically induced protocol.

Together, these data suggest that while both protocols are effective at generating mural cells, the chemically-induced method preferentially produces cells consistent with an SMC phenotype, whereas the NKX3.1-induced protocol generates iMPCs with characteristics of both SMCs and pericytes.

In summary, the transient activation of NKX3.1 expression in MePCs (via a Dox-inducible system or modRNA) effectively and efficiently converted human iPSCs into cells exhibiting a distinct mural cell phenotype.

Example 3. A genomic footprint-free approach for deriving iMPCs with modified mRNA

NKX3.1 expression is only required transiently (**Fig. 2A**), creating an opportunity to use chemically modified mRNA (modRNA) and a genomic footprint-free protocol (modRNA is always transient) (**Figs. 3A and 3F**). To this end, we designed modRNA encoding NKX3.1 (TriLink) and verified that the transfection of unmodified iPSCs with this modRNA resulted in a pronounced transient expression of NKX3.1 (**Fig. 3B**). The use of modRNA to generate iMPCs was effective (**Figs. 3A-3G**).

Indeed, activating NKX3.1 in MePCs generated iMPCs with high efficiency ($\sim 95\%$ conversion measured by flow cytometry; **Fig. 3C**), indistinguishable from those produced by the Dox-inducible protocol and exhibiting robust expression of mural cell markers (**Fig. 3D**). Overall, activating NKX3.1 (either via Dox or modRNA) efficiently converts human MePCs into
5 iMPCs that display a robust mural cell phenotype. Moreover, activation of NKX3.1 with modRNA in MePCs efficiently produced iMPCs that were indistinguishable from those generated by the Dox-inducible protocol, including a robust expression of mural cell markers (**Figs. 3A-3G**).

In summary, the transient activation of NKX3.1 expression in MePCs (via a Dox-inducible system or modRNA) effectively and efficiently converted human iPSCs into cells
10 exhibiting a distinct mural cell phenotype (**Figs. 1A-3G**).

Example 4. Contractile and secretory competence of iMPCs

We evaluated several functional attributes of our NKX3.1-induced iMPCs, focusing on
15 mural cell-associated characteristics like calcium influx, contractility, extracellular matrix synthesis, and EC interaction. iMPC contractility was tested using vasoconstrictive agents. Calcium imaging showed endothelin-1 and carbachol increased intracellular calcium in iMPCs, similar to control primary MSCs and SMCs (**Figs. 4A, 4F, and 4G**). The three-dimensional collagen contractility assay further confirmed their response to a vasoconstrictive stimulus
20 (U46619; a thromboxane A2 (TXA2) analog that acts as a potent vasoconstrictor), as iMPCs contracted similarly to MSCs and iSMCs (**Fig. 4B**). These findings indicate that iMPCs can respond to vasoconstrictive stimuli, thus confirming iMPCs exhibit a crucial functional characteristic of *in vivo* mural cells.

Extracellular fibronectin deposition in iMPCs was assessed after TGF- β treatment. TGF- β
25 led to significant fibronectin production, which was inhibited by TGF- β signaling inhibitor SB31542 (**Figs. 4C, 4H, 4I, and 4J**). This increase in fibronectin production, evident at both the protein (**Figs. 4C, 4H, and 4I**) and mRNA (**Fig. 4J**) levels. Additionally, the introduction of small molecules that inhibit TGF- β signaling (SB431542) effectively prevented fibronectin production, thereby confirming that fibronectin deposition in iMPCs is mediated by TGF- β . The
30 capacity to deposit extracellular fibronectin represents a key functional property of mural cells.

Lastly, we also explored iMPCs' ability to interact with ECs by producing angiogenic factors. Central to mural cell function is their capacity to interact with ECs by producing angiogenic factors. We investigated the ability of iMPCs to modulate EC behavior through the secretion of paracrine pro-angiogenic factors and compared it to that of SMCs and MSCs by examining their respective conditioned media using an angiogenesis protein array and quantitative Luminex assay (**Figs. 4D-4E**). Angiogenesis protein array (**Fig. 4D**) and Luminex protein assay (**Fig. 4E**) of conditioned media from iMPCs, SMCs, and MSCs revealed that iMPCs secreted several pro-angiogenic factors. Notably, iMPCs secreted various pro-angiogenic factors, including VEGF-A, PLGF, HB-EGF, HGF, several members of the IGFBP family, as well as members of the serine protease inhibitor (serpin) superfamily of proteins (Serpine E1 and Serpin F1) and urokinase-type plasminogen activator (uPA), among others. While some factors were more abundant in iMPCs (e.g., PLGF), others were less prominent in iMPCs compared to primary SMCs (e.g., VEGF-A and FGF2). Nevertheless, the overall pro-angiogenic secretome of iMPCs was consistent with what is expected for mural cells.

Together, these results suggest that our NKX3.1-induced iMPCs mimic some of the functional attributes typically associated with mural cells, including contractile responses, the ability to deposit fibronectin, and the secretion of angiogenic factors.

Example 5. Modulation of EC function by iMPCs: in vitro and in vivo assays

We studied the influence of iMPC-secreted proteins on EC activity using human umbilical cord blood-derived endothelial colony-forming cells (ECFCs, referred to herein as ECs) in three *in vitro* assays (**Figs. 5A-5D**). Both indirect co-culture with iMPCs (**Fig. 5A**) and exposure to iMPC-conditioned media (CM-(iMPCs) for 72 hours (**Fig. 5B**) promoted EC proliferation, comparable to results obtained with CM-(SMCs) and CM-(MSCs). In both assays, the number of ECs exposed to CM-(iMPCs) for 72 hours was significantly higher than the number observed when cells were exposed to a basal control medium. Moreover, CM-(iMPCs) enhanced EC migration and EC ability re-endothelialize scratched monolayers (scratch assay; **Fig. 5C**), as well as assemble into capillary-like structure formation in three-dimensional cultures (**Fig. 5D**). Overall, our data indicate that iMPCs effectively modulate EC function *in vitro* through the secretion of paracrine factors, and their capacity to influence EC activity is comparable to that of control mural SMCs and MSCs.

To evaluate a more physiologically relevant assay, we used an established *in vitro* model that cocultures iMPCs directly with ECs in a three-dimensional (3D) hydrogel. This microphysiological system—a microfluidic ‘on-a-chip’ model—facilitates the dynamic interaction of cells and the formation of a microvascular network through vasculogenesis. First, we combined GFP-labeled iMPCs and DsRed-labeled ECs within a fibrin gel and examined the ability of the iMPCs to enable vascular morphogenesis (**Fig. 5J**). This setup led to the formation of vascular structures lined by the DsRed⁺ ECs within 2 days (**Fig. 5J**). Furthermore, immunofluorescent staining confirmed the formation of a vascular network within the chip with a continuous endothelial lining marked by CD31 and VE-Cadherin and the presence of α -SMA⁺ and SM22⁺ iMPCs serving as perivascular cells adjacent to some of the EC-lined lumens (**Fig. 5K**). This ‘on-a-chip’ model confirmed the potential of iMPCs, when cocultured with ECs, to assemble complex vascular networks, hence supporting their functionality as mural cells.

To evaluate *in vivo* functionality iMPCs (e.g., function as perivascular cells and support *in vivo* blood vessel formation), we subcutaneously implanted ECs with iMPCs into immunodeficient mice (**Figs. 5E-5L**) using our established model. One week post-implantation, we removed the implants and analyzed them for the formation of human-specific vascular networks. Grafts containing mural cells (SMCs, MSCs, or iMPCs) exhibited evidence of blood perfusion (**Fig. 5E**), and histological examinations revealed the presence of perfused vessels containing murine erythrocytes (**Fig. 5F**), with no signs of hemorrhage or thrombosis (i.e., platelet aggregation and uniform fibrin deposition), indicating proper functionality. Indeed, H&E staining confirmed that all implants seeded with mural cells had formed numerous perfused blood vessels containing murine erythrocytes (**Fig. 5F**), while grafts with ECs alone failed to form perfused vessels. There were no significant differences in the average microvessel densities across grafts with different mural cell populations (**Fig. 5G**). This quantification of the average microvessel densities at day 7 confirmed there were no statistically significant differences between implants prepared with each of the different mural cell populations (**Fig. 5G**). The ability of iMPCs to support vascular networks *in vivo* was corroborated using another source of primary ECs. Grafts containing human umbilical vein endothelial cells (HUVECs) and iMPCs yielded mature and perfused vessels, as evidenced by human-specific CD31 staining and the presence of human perivascular cells (**Figs. 17A-17D**).

Most of the vessels within the grafts stained positive for human-specific CD31, confirming they were lined by the implanted human ECs (**Fig. 5H**). The human vessels were perfused, thus indicating they had connected with the murine host blood vessels (**Fig. 5H**). Moreover, α -SMA-positive perivascular cells surrounded all human blood vessels within the implants (**Fig. 5H**), confirming the contribution of iMPCs to the perivascular compartment of blood vessels.

The generation of EC-lined vascular structures depended on the presence of mural cells. Perfused vessels stained positively for human-specific CD31, indicating that the newly formed human vasculature had established functional anastomoses with murine host blood vessels (**Fig. 5I**). Perivascular involvement of α -SMA-expressing iMPCs was confirmed by human-specific vimentin staining observed in cells surrounding the human EC-lined microvessels (**Fig. 5I-5J**). In designated experiments, we employed GFP-labeled iMPCs to track their *in vivo* location. Double staining of GFP and α -SMA revealed that, after 7 days *in vivo*, GFP-expressing iMPCs were primarily detected in proximity and immediately adjacent to luminal structures (**Fig. 5J**), indicating their structural participation in the perivascular compartment of newly formed blood vessels. Quantification of mural cell investment revealed that a substantial majority (>90%) of the human vessels exhibited perivascular coverage, with a significant proportion of these vessels being invested by the transplanted iMPCs (**Fig. 5L**).

In conclusion, our results demonstrate that iMPCs can modulate EC function, both *in vitro* and *in vivo*, on par with control mural SMCs and MSCs.

Example 6. Maturation of iMPCs upon interaction with ECs

The maturation of mural progenitor cells during vascular development is largely contingent on their interaction with ECs. Interactions between mural progenitor cells and ECs play a pivotal role in vascular blood vessel development, maturation, and stabilization. Concurrently, these interactions drive the mural progenitors to mature into terminally differentiated mural cell types. We set out to determine if our iMPCs could similarly mature upon co-culture with ECs. Thus, we co-cultured iMPCs with ECs for 7 days, resulting in co-iMPCs. Following this, we isolated the co-cultured ECs and iMPCs (herein referred to as co-iMPCs) via MACS as CD31-negative cells and subjected them to gene expression analysis via bulk RNA-seq (**Fig. 6A**). On a global scale, there were hundreds of genes differentially

expressed between iMPCs and co-iMPCs. Upon close inspection, the comparison revealed thousands of differentially expressed genes between iMPCs and co-iMPCs (**Fig. 6A**). Principal components analysis of these differentially expressed genes showed that co-iMPCs exhibited transcriptional proximity to primary mural cells (specifically, SMCs and MSCs) more closely than did iMPCs prior to co-culture with ECs (**Fig. 6B**). This finding suggested the occurrence of a mural cell maturation process.

Moreover, hierarchical clustering analysis showed that co-iMPCs aligned transcriptionally more closely with primary SMCs and MSCs than iMPCs (**Fig. 6I**). Pairwise correlation (**Fig. 6E**) and principal component analyses (**Fig. 6B**) further confirmed this hierarchical association.

To further understand the transcriptional differences between iMPCs and co-iMPCs, we performed gene ontology (GO) enrichment analysis. Notably, we observed significant enrichment in co-iMPCs for genes associated with mature mural cell functions (**Fig. 6C**). These included genes associated with extracellular matrix organization, regulation of vasculature development, regulation of angiogenesis, smooth muscle contraction, connective tissue development, and response to TGF β (**Fig. 6C**). Additionally, qPCR analysis confirmed significant upregulation of several genes associated with mural cells, including both SMC and pericyte markers, in co-iMPCs (**Figs. 6D, 6F, and 6G**). These included SMC-associated genes, such as *ACTA2*, *CNN1*, *TAGLN*, *MYOCD*, and *TPM1* (**Figs. 6D and 6F**), as well as pericyte-related genes like *CSPG4* and *PDE5A* (**Figs. 6D and 6G**). Of note, control iMPCs cultured in the same media for seven days without ECs did not exhibit the upregulation of mature mural markers observed when co-cultured with ECs (**Fig. 18**). This general upregulation pattern in co-iMPCs mirrored that observed in primary MSCs after a seven-day co-culture with ECs, underscoring the widely recognized progenitor role of MSCs.

Moreover, immunofluorescence staining of co-iMPCs confirmed the separate presence of both 3G5+ pericytes (the 3G5 ganglioside antigen is expressed on the cell surface of pericytes) and α -SMA+/3G5- SMCs (**Fig. 6H**). This 3G5 ganglioside antibody was previously validated to accurately label pericytes in both culture and clinical samples. Moreover, studies have corroborated that 3G5 is not found in vascular SMCs and have utilized the 3G5 antibody for pericyte identification and isolation across various tissues, including human skin and mouse hearts. Thus, the 3G5 ganglioside is accepted as a reliable marker for identifying pericytes.

Lastly, it is important to note that iMPCs exhibited only minimal MYH11 expression (a mature SMC marker) before co-culture with ECs at both the mRNA and protein levels (**Figs. 6F and 6J**). This is consistent with the well-documented observation that MYH11 expression is generally subdued in SMCs when cultured in isolation. Instead, robust expression of MYH11 is typically reported in vivo, in freshly isolated cells, or in coculture systems that facilitate interactions with ECs. Indeed, upon 7-day coculture of iMPCs with ECs, we observed some cells displayed high levels of both MYH11 and α -SMA, while others exhibited high MYH11 but low α -SMA (**Fig. 6J**), suggesting a heterogeneous mixture of mural cell phenotypes. This protein-level evidence supports the presence of MYH11+ SMCs among the generated mural cells and reinforces the contextual dependency of MYH11 expression in SMCs.

Collectively, these findings were consistent with the concept of iMPC maturation into mural cells upon interaction with ECs. These results indicate that NKX3.1-induced iMPCs are not passive EC function regulators. Instead, they display an active, dynamic response to EC interaction that leads to a significant maturation into mural cells. This ability to differentiate, characterized by a distinct upregulation of mature mural cell-associated genes, underscores the progenitor nature of iMPCs.

Example 7. iMPC maturation and mural cell heterogeneity

Next, we conducted single-cell RNA sequencing (scRNA-seq) to investigate whether EC co-culture facilitates iMPC maturation and unveils mural cell heterogeneity. These experiments investigate the extent to which iMPCs can recapitulate mural cell heterogeneity and employed scRNA-seq to examine the differentiation of iPSCs into iMPCs as well as the interaction between iMPCs and ECs (**Figs. 7A-7C and 11A-11H**). Cells were examined at various points in the differentiation protocol (days 0, 2, and 4) and after seven days of co-culture with ECs (day 11) (**Figs. 7A and 11A**). Specifically, we sampled four critical stages of our differentiation protocol corresponding to day 0 (iPSCs), day 2 (MePCs after mesodermal differentiation), day 4 (iMPCs after NKX3.1 activation), and day 11, following a seven-day co-culture of iMPCs with ECs (**Figs. 7A-7C, 11A-14, and 19A-20**). The goal was to determine whether co-culturing with ECs enhances iMPC maturation and diversifies the mural cell population into recognizable perivascular cell types.

Post co-culture, ECs and iMPCs were denoted as co- ECs and co-iMPCs, respectively. Through our 10X Genomics platform, we generated data on 10,000 cells per time point (at each differentiation stage). Seurat v325 facilitated normalization between time points and subsequent cell clustering into an integrated analysis (**Figs. 7B and 11B**). This analysis yielded 19 distinct clusters, 15 of which were manually annotated into eight to nine discrete groups, including iPSCs (annotated by expression of *OCT4*, *NANOG*, *SOX2*), MePCs (*TBXT*, *MIXL1*), iMPCs (*CSPG4*, *PDGFRB*), ECs and co-ECs (*PECAMI*, *CDH5*), SMCs (*ACTA2*, *CNN1*, *TAGLN*), pericytes (*NT5E*, *TAGLN*, *CSPG4*, *PDGFRB*), and fibroblasts (*COL1A1*, *COL1A2*, *TAGLN*) (see UMAP plot, **Figs. 7B and 11B**). The discrete cell populations were identified based on the expression of characteristic cellular markers (**Figs. 7B, 11B, 11D, 12-14, and 19A-20**). For example, iMPCs (Cluster #3 in **Figs. 11B and 11C**) exhibited high levels of *NKX3.1*, expressed *CSPG4*, *PDGFRB*, and *DES*, but had reduced expressions of genes encoding for CD73 (*NT5E*) and contractile proteins (*ACTA2*, *CNN1*, and *TAGLN*), and the fibroblast-like population (Cluster #4 in **Figs. 11B and 11C**) was characterized by high *NKX3.1*, *PDGFRB*, *ACTA2*, and *PDGFRA* expression (**Figs. 7B-7C, 11B-11G, 12-14, and 19A-20**). The annotated populations also included iPSCs (Cluster #1 in **Figs. 11B and 11C**, marked by *OCT4*, *NANOG*, and *SOX2*) and MePCs (Cluster #2 in **Figs. 11B and 11C**, expressing *TBX6*, *MSGN1*, *MIXL1* and *TBXT*) (**Figs. 7B-7C, 11B-11G, 12-14, and 19A-20**). Of note, the expression of *PDGFRA* was prominent in cluster #4 compared to the other annotated mural cell populations, which aligns with common criteria used in the field for identifying fibroblasts (Lendahl, U., et. al., (2022) Nat. Commun. 13, 3409).

Next, we assessed each cluster emergence over time (**Figs. 7C and 11C**). The temporal evolution of these clusters was consistent with the progression from iPSCs (day 0) to MePCs (day 2) and then to iMPCs (day 4) (**Figs. 7C and 11C**).

Additionally, we analyzed our scRNA-seq data for markers associated with paraxial mesoderm (*TBX6*, *MSGN1*), somites (*FOXC2*, *MEOX2*, *TCF15*), and sclerotome (*PAX9*, *SOX9*, *NKX3.2*) (**Figs. 19A-19D**). At the mesodermal stage (MePCs at day 2, before *NKX3.1* activation), *TBX6* and *MSGN1* were detectable, aligning with their expected expression in early mesodermal differentiation. However, post *NKX3.1* activation at day 4 (iMPCs), these markers were not prominently expressed, suggesting a transition away from a general mesodermal identity towards a more defined lineage. Markers associated with somite and sclerotome

differentiation showed negligible expression on day 2, and only *FOXC2* and *SOX9* showed some expression on day 4 in iMPCs (**Figs. 19A-19D**). This pattern indicates a minimal influence of *NKX3.1* activation on inducing somite or sclerotome identities directly from MePCs.

We also analyzed interactions between iMPCs and ECs. After 7 days of co-culture with ECs, iMPCs matured into three distinct mural cell subpopulations (clusters #5, #6, and #7 at day 11; **Figs. 7B, 7C, 11B, 11C**). These mural cell clusters no longer expressed *NKX3.1*, confirming its transient activation, but uniformly expressed general perivascular markers *PDGFRB* and *NT5E* (CD73). Of note, while iMPCs resembled nascent pericytes, mural cell clusters after co-culture with ECs resembled mature perivascular cells, including pericytes (cluster #5), contractile SMCs (c-SMCs; cluster #6), and synthetic SMCs (s-SMCs; cluster #7). These clusters, while sharing the expression of *PDGFRB*, exhibited important differences, particularly with respect to genes associated with contractile proteins and ECM production (**Fig. 11E**). Indeed, a direct comparison of differentially expressed genes revealed a significant upregulation in genes encoding for cell contractility (e.g., *ACTA2*, *CNN1*, *TAGLN*) and ECM proteins (e.g., *FNI*, *COL1A1*, *COL1A2*) in SMCs compared to pericytes (Cluster #5) (**Fig. 11E**), which is consistent with their perivascular roles *in vivo*. Meanwhile, a direct comparison between the two clusters of SMCs revealed a clear distinction between the contractile (e.g., upregulation of *ACTA2*, *MYL9*, *TAGLN*) and the synthetic (e.g., *FNI*, *COL5A1*, *COL4A1*) phenotypes of c-SMCs and s-SMCs, respectively (**Fig. 11E**), consistent with the previous description of these two types of SMC manifestations.

Additionally, we expanded our comparative analysis to evaluate the similarity between our iMPC-derived mural cells (i.e., after co-culture with ECs) and primary mural cells. First, we conducted comparative analyses with publicly available bulk RNA datasets to provide a more precise context. We specifically compared our scRNA-seq data from cells characterized as SMCs (**Figs. 7A-7C**; clusters #6 and #7 in **Figs. 11A-11H**) to human aortic SMCs in public datasets. Similarly, our cells identified as pericytes (**Figs. 7A-7C**; cluster #5 in **Figs. 11A-11H**) were compared to public datasets of primary human brain pericytes. Pearson correlation analysis of these comparisons demonstrated robust correlations (correlation coefficient ~0.6, $p < 0.001$) for both sets of comparisons, indicating a substantial transcriptional alignment of our derived mural cells with public datasets (**Figs. 21A-21B**).

Furthermore, to establish an additional unbiased benchmark, we used the comprehensive Tabula Sapiens Consortium's vasculature dataset. This dataset encompasses a diverse array of vascular endothelial and mural cell types. By overlaying our scRNA-seq data at day 11 (i.e., mural cells generated from iMPCs after 7 days of co-culture with ECs), we observed that our cells identified as SMC-like clusters (**Figs. 7A-7C**; clusters #6 and #7 in **Figs. 11A-11H**) exhibited substantial overlap with the reference SMCs (4,832 of our SMCs matched the reference Tabula Sapiens SMCs; **Fig. 21C**). Similarly, most of our cells categorized as pericytes (**Figs. 7A-7C**; cluster #5 in **Figs. 11A-11H**) closely matched with reference pericytes (808 of our pericytes matched the reference Tabula Sapiens pericytes; **Fig. 21D**). This analysis indicates that most of our SMCs align strongly with established reference SMCs. It also shows that cells from our pericyte cluster show more similarity to the reference pericytes than to SMCs or fibroblasts.

These comparative evaluations demonstrate that the gene expression profiles of our iMPC-derived SMCs and pericytes exhibit significant congruence with established primary human mural cells and a detailed single-cell reference from The Tabula Sapiens Consortium, suggesting the relevance of our differentiation model to *in vivo* counterparts.

In order to gain further insights into the signals involved in mural cell differentiation and maturation, we examined the cell non-autonomous signals derived from ECs that promote mural cell maturation. Using CellChat to analyze our scRNA-seq data, we identified several key signaling pathways, notably NOTCH and TGF- β , which are known to be implicated in vascular and mural cell development (**Figs. 22A-22B**). To confirm the functional importance of these pathways, we conducted in vitro assays where iMPCs were co-cultured with ECs in the presence of specific pathway inhibitors (**Fig. 22C**). The inhibition of NOTCH signaling with DAPT significantly impaired the maturation of iMPCs in both pericyte and SMC phenotypes, confirming the role of NOTCH in mural cell maturation (**Fig. 22D**). Similarly, inhibition of TGF- β signaling with SB431542 selectively disrupted SMC maturation, indicating its pivotal role in this process while preserving a pericyte-like phenotype (**Fig. 22E**).

Our analysis also included examining the differential gene expression profiles between nascent and more mature pericytes (clusters #5 at days 4 and 11, respectively). We identified distinct gene signatures differentiating early-stage pericytes from their mature counterparts. GO pathway analyses of these DEGs revealed that day 11 pericytes exhibited significant enrichment in genes associated with extracellular matrix organization, cellular adhesion, and TGF- β

signaling pathways, indicative of a mature mural cell phenotype (**Figs. 23A-23C**). In contrast, days 4 pericytes showed enriched expression in genes linked to cell proliferation, regulation of cell differentiation, and Wnt signaling pathways, reflecting their developmental stage closer to mesodermal progenitors (see DEGs). Moreover, GO and KEGG analyses of differential gene expression between pericytes (cluster #5) and c-SMCs (cluster #6) confirmed significant enrichment in functions associated with extracellular matrix organization, cell-matrix adhesion, cellular contractility, and various signaling pathways related to TGF- β signaling in c-SMCs (**Figs. 23A-23C**), indicative of the contractile and structural roles typically associated with SMCs.

Next, we performed an in-depth analysis of our scRNA-seq data to elucidate the gene regulatory networks (GRNs) driving the differentiation of our mural cell populations from MePCs into iMPCs, pericytes, and SMCs (**Figs. 24A-24B**). Using a combination of transcription factor motif enrichment analysis and gene expression correlation mapping, we identified distinct GRNs that govern the progression from MePCs to mature mural cells, including pericytes and SMCs (**Fig. 24A**). This approach leveraged regulatory elements predicted to be active in each cell state, providing a dynamic view of the transcriptional controls that shape cell fate decisions. For instance, in early-stage MePCs, these networks included regulators such as MIXL1 and MSX1, which are pivotal during mesodermal specification (GRN9, **Fig. 24B**). Later, several GRNs were highly active in iMPCs compared to mature SMCs and pericytes (GRN4 and GRN12, **Fig. 24B**), suggesting that genes included in these GRNs, such as TIMP1, TGFB1, JAK, and PIEZO1, could be influenced by NKX3.1. As the cells progressed toward a more defined mural cell fate, we observed a transition in the active GRN10, with an increased representation of motifs related to TGF- β signaling (TGFB2), ECM production, and contractile function—key aspects of mature mural cell phenotypes (**Fig. 24B**).

Lastly, our trajectory analyses (**Fig. 11F**) with pseudotime plots provided further insights into the temporal evolution from iPSCs to MePCs and subsequently to iMPCs (**Fig. 11G**). This analysis also confirmed that iMPC interaction with ECs promoted the development of the various mature mural cell subpopulations, starting with pericytes and progressing to c-SMCs and s-SMCs (**Fig. 11G**). Additional pseudotime analysis provided a more precise visualization of the developmental trajectory and maturation stages of different cell subsets derived from iMPCs (**Fig. 25**). This pseudo-time trajectory analysis indicated that the pericyte cluster appears

temporally closer to the iMPCs than the SMC clusters, suggesting an earlier stage in pericyte development. This is consistent with the notion that iMPCs resemble nascent pericytes. On day 11, the pericyte cluster (#5) manifests earlier than the synthetic SMC cluster (#7) (**Fig. 25**), highlighting a developmental hierarchy. Furthermore, our analyses revealed insights into the origins of s-SMCs during co-culture maturation stages. Pseudotime trajectory analysis suggests that synthetic SMCs (s-SMCs) represent a later stage of mural cell differentiation, emerging from contractile SMCs (c-SMCs) under the influence of continuous endothelial interaction, highlighting the dynamic interplay of cell-autonomous and non-autonomous signals in mural cell diversification (**Fig. 25**).

As anticipated, cell populations on days 0, 2, and 4 were relatively homogeneous, reflecting a synchronized transition from iPSCs to MePCs and iMPCs (**Figs. 7C and 11C**). Of note, analysis of co-iMPCs on day 11 indicated that following co-culture with ECs, iMPCs matured into three distinguishable mural cell types, namely SMCs, pericytes, and fibroblasts (**Figs. 7C and 11C**). These data were consistent with the notion of iMPC maturation upon interaction with ECs, resulting in mural cell heterogeneity.

Thus, iMPCs act as true mural cell progenitors; after a week of co-culturing with ECs, the iMPCs diversified into distinct perivascular mural cell subpopulations, including pericytes and SMCs. This finding suggests that EC interaction is pivotal in the maturation of iMPCs, allowing for a robust recapitulation of mural cell heterogeneity (cartoon depictions in **Fig. 11H**).

Example 8. Effective co-differentiation of iPSCs into iECs and iMPCs in a 3D vascular organoid (VO) model

Leveraging our previously established method for iEC generation via ETV2, we explored the concurrent differentiation of iPSCs into iECs and iMPCs using ETV2 and NKX3.1, respectively. This led to the development of a new 3D VO model (**Fig. 8A**). Briefly, we used our genetically engineered dox-ETV2-iPSC and dox-NKX3.1-iPSC lines. After conversion into MePCs over three days, cells from these two iPSC lines (1:1 mixture) were aggregated in 3D using non-adherent culture plates and an orbital shaker. Then, the cells readily differentiated into iECs (h-CD31+) and iMPCs (h-CD31-/PDGFR β +) upon exposure to Dox for 3 days (**Fig. 8C**). Moreover, the cells self-assembled into a robust network of CD31+ vascular structures containing mature α -SMA+ perivascular mural cells (**Fig. 8D**). This method can rapidly generate

numerous VOs of uniform size (~200 μm) (**Fig. 8B**), exhibiting attributes consistent with proper vascular development, including a robust network of lumenized vessels with proper apical-basal polarization and diverse endothelial heterogeneity with arterial, venous, and capillary ECs (not shown).

5

Example 9. VO engraftment and formation of perfused vascular networks

We evaluated the capacity of our VOs to form functional blood vessels *in vivo*. To this end, we used a renal capsule model of implantation into immunodeficient NSG mice (1,000 VO/mouse; **Figs. 8E-8G**). On day 14, examination of the explants suggested robust
 10 vascularization of the grafts. Indeed, histological (H&E) analysis revealed that the grafts had an extensive network of perfused blood vessels (**Fig. 8F**). The microvessels were primarily lined by the h-iECs, as confirmed by the expression of human-specific CD31 (**Fig. 8G**), and contained mouse erythrocytes, indicating the formation of functional anastomoses with the host circulatory system. Also, the human vessels were surrounded by $\alpha\text{-SMA}^+$ perivascular mural cells (**Fig.**
 15 **8G**), a sign of vessel maturity and stability. In summary, we have developed a novel method for efficient co-differentiation of endothelial and mural cells in a VO model. The VOs, when implanted *in vivo*, form robust, functional vascular networks. This VO model will be instrumental for our studies to determine the mechanisms underlying the maturation of iMPCs.

20 ***Example 10. Maturation of iMPCs and iECs in our VO model***

We explored the maturation of VO-derived iMPCs and iECs (referred to as VO-iMPCs and VO-iECs) compared to 2D monoculture- differentiated iMPCs and iECs. Following enzymatic digestion of the VOs (day 5) and MACS sorting, we obtained CD31+ VO-iECs and CD31- VO-iMPCs (**Fig. 9A**). qPCR comparison of key endothelial and mural cell marker
 25 expression revealed significant upregulation of various EC markers (*CDH5*; *VWF*) in VO-iECs (**Fig. 9B**) and several mural cell markers (*ACTA2*, *MYH11*, *TAGLN*, *CNN1*) in VO-iMPCs (**Fig. 9C**).

This supports the notion that the orthogonal co-differentiation of endothelial and mural cells in our VO model, involving the activation of ETV2 and NKX3.1, promotes the maturation
 30 of the resultant iMPCs and iECs.

Example 11. Transplantation of VOs into ischemic tissue

To assess the efficacy of VO engraftment in ischemic conditions, we utilized our murine model of hind limb ischemia. Immune-deficient athymic nude mice, aged 10 weeks, were rendered diabetic through a single intraperitoneal injection of streptozotocin (STZ; 220 mg/kg).

5 A week post-STZ injection, the diabetic mice were anesthetized using isoflurane. After achieving anesthesia, 7-0 silk sutures were employed to tie off the proximal and deep femoral artery and vein, with the intervening vessels excised to obstruct blood flow completely. Subsequently, 1,000 VOs, each containing a luciferase reporter in the iECs, were resuspended in 50 μ L of Matrigel and injected into the muscle at the site of the femoral artery and vein ligation
10 (**Fig. 10A**). The engraftment of the VOs in the ischemic hind limbs was successfully visualized through bioluminescence on days 1 and 7 post-injection (**Fig. 10B**). Significantly, the VO engraftment led to improved blood flow in the ischemic limbs, with Laser Doppler imaging showing a 50% recovery of blood flow at 2 weeks post-injection (**Fig. 10C**). Furthermore, the engraftment prevented the development of necrotic tissue in the mice receiving VOs (**Fig. 10D**).
15 In stark contrast, untreated control mice exhibited severe necrosis and compromised blood flow in their ischemic limbs. In summary, our data suggest the potential of VOs to engraft and serve as therapeutic agents *in vivo*, preventing necrosis and restoring blood flow in a hind limb ischemia model in diabetic mice.

REFERENCES CITED

1. Eelen, G., Treps, L., Li, X. & Carmeliet, P. Basic and Therapeutic Aspects of Angiogenesis Updated. *Circ Res* 127, 310–329 (2020).
2. Potente, M., Gerhardt, H. & Carmeliet, P. Basic and Therapeutic Aspects of Angiogenesis. *Cell*
25 146, 873–887 (2011).
3. Rafii, S., Butler, J. M. & Ding, B.-S. Angiocrine functions of organ-specific endothelial cells. *Nature* 529, 316–325 (2016).
4. Cleaver, O. & Dor, Y. Vascular instruction of pancreas development. *Development* 139, 2833–2843 (2012).
- 30 5. Augustin, H. G. & Koh, G. Y. Organotypic vasculature: From descriptive heterogeneity to functional pathophysiology. *Science* 357, eaal2379 (2017).

6. Majesky, M. W. Vascular Development. *Arteriosclerosis Thrombosis Vasc Biology* 38, e17–e24 (2018).
7. Margolis, E. A., Friend, N. E., Rolle, M. W., Alsberg, E. & Putnam, A. J. Manufacturing the multiscale vascular hierarchy: progress toward solving the grand challenge of tissue engineering. *Trends Biotechnol* (2023) doi: 10.1016/j.tibtech.2023.04.003.
8. Rouwkema, J. & Khademhosseini, A. Vascularization and Angiogenesis in Tissue Engineering: Beyond Creating Static Networks. *Trends Biotechnol* 34, 733–745 (2016).
9. Armulik, A., Genové, G. & Betsholtz, C. Pericytes: Developmental, Physiological, and Pathological Perspectives, Problems, and Promises. *Dev Cell* 21, 193–215 (2011).
10. Bergers, G. & Song, S. The role of pericytes in blood-vessel formation and maintenance. *Neuro-oncology* 7, 452–464 (2005).
11. Jain, R. K. Molecular regulation of vessel maturation. *Nat Med* 9, nm0603–685 (2003).
12. Armulik, A., Abramsson, A. & Betsholtz, C. Endothelial/Pericyte Interactions. *Circ Res* 97, 512–523 (2005).
13. Gaengel, K., Genové, G., Armulik, A. & Betsholtz, C. Endothelial-Mural Cell Signaling in Vascular Development and Angiogenesis. *Arteriosclerosis Thrombosis Vasc Biology* 29, 630–638 (2009).
14. Basatemur, G. L., Jorgensen, H. F., Clarke, M. C. H., Bennett, M. R. & Mallat, Z. Vascular smooth muscle cells in atherosclerosis. *Nat Rev Cardiol* 16, 727–744 (2019).
15. Hellström, M. et al. Lack of Pericytes Leads to Endothelial Hyperplasia and Abnormal Vascular Morphogenesis. *J Cell Biology* 153, 543–554 (2001).
16. Majesky, M. W. Developmental Basis of Vascular Smooth Muscle Diversity. *Arteriosclerosis Thrombosis Vasc Biology* 27, 1248–1258 (2007).
17. Melero-Martin, J. M. et al. Engineering Robust and Functional Vascular Networks In Vivo With Human Adult and Cord Blood–Derived Progenitor Cells. *Circ Res* 103, 194–202 (2008).
18. Lin, R.-Z. et al. Human endothelial colony-forming cells serve as trophic mediators for mesenchymal stem cell engraftment via paracrine signaling. *Proc National Acad Sci* 111, 10137–10142 (2014).
19. Au, P., Tam, J., Fukumura, D. & Jain, R. K. Bone marrow–derived mesenchymal stem cells facilitate engineering of long-lasting functional vasculature. *Blood* 111, 4551–4558 (2008).
20. Chen, X. et al. Rapid Anastomosis of Endothelial Progenitor Cell–Derived Vessels with Host Vasculature Is Promoted by a High Density of Cotransplanted Fibroblasts. *Tissue Eng Pt A* 16, 585–94 (2010).

21. Takahashi, K. et al. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* 131, 861–872 (2007).
22. Park, I.-H. et al. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 451, 141 (2008).
- 5 23. Patsch, C. et al. Generation of vascular endothelial and smooth muscle cells from human pluripotent stem cells. *Nat Cell Biol* 17, 994–1003 (2015).
24. Shen, M., Quertermous, T., Fischbein, M. P. & Wu, J. C. Generation of Vascular Smooth Muscle Cells From Induced Pluripotent Stem Cells: Methods, Applications, and Considerations. *Circ Res* 128, 670–686 (2021).
- 10 25. Ng, A. H. M. et al. A comprehensive library of human transcription factors for cell fate engineering. *Nat Biotechnol* 39, 510–519 (2020).
26. Wang, K. et al. Robust differentiation of human pluripotent stem cells into endothelial cells via temporal modulation of ETV2 with modified mRNA. *Sci Adv* 6, eaba7606 (2020).
27. Skylar-Scott, M. A. et al. Orthogonally induced differentiation of stem cells for the programmatic patterning of vascularized organoids and bioprinted tissues. *Nat Biomed Eng* 1–14 (2022)
- 15 doi:10.1038/s41551-022-00856- 8.
28. Clevers, H. Modeling Development and Disease with Organoids. *Cell* 165, 1586–1597 (2016).
29. Lancaster, M. A. & Knoblich, J. A. Organogenesis in a dish: Modeling development and disease using organoid technologies. *Science* 345, 1247125 (2014).
- 20 30. Velasco, S. et al. Individual brain organoids reproducibly form cell diversity of the human cerebral cortex. *Nature* 570, 523–527 (2019).
31. Neumeyer, J. et al. Bioengineering hemophilia A–specific microvascular grafts for delivery of full-length factor VIII into the bloodstream. *Blood Adv* 3, 4166–4176 (2019).
32. Mack, C. P. Signaling Mechanisms That Regulate Smooth Muscle Cell Differentiation. *Arteriosclerosis Thrombosis Vasc Biology* 31, 1495–1505 (2011).
- 25 33. Tanaka, M., Lyons, G. E. & Izumo, S. Expression of the Nkx3.1 homobox gene during pre and postnatal development. *Mech Develop* 85, 179–182 (1999).
34. Tanaka, M. et al. Vertebrate homologs of tinman and bagpipe: Roles of the homeobox genes in cardiovascular development. *Dev Genet* 22, 239–249 (1998).
- 30 35. Kilens, S. et al. Parallel derivation of isogenic human primed and naive induced pluripotent stem cells. *Nat Commun* 9, 360 (2018).
36. Wu, J. & Izpisua Belmonte, J. C. Dynamic Pluripotent Stem Cell States and Their Applications. *Cell Stem Cell* 17, 509–525 (2015).

37. Palpant, N. J. et al. Generating high-purity cardiac and endothelial derivatives from patterned mesoderm using human pluripotent stem cells. *Nat Protoc* 12, 15–31 (2016).
38. Yang, X., Pabon, L. & Murry, C. E. Engineering Adolescence. *Circ Res* 114, 511–523 (2014).
39. Warren, E. & Gerecht, S. Beyond the endothelium: the role of mural cells in vascular biology: in vitro systems to study endothelial/pericyte cell interactions. *Vasc Biology* 5, e220021 (2023).
40. Wimmer, R. A., Leopoldi, A., Aichinger, M., Kerjaschki, D. & Penninger, J. M. Generation of blood vessel organoids from human pluripotent stem cells. *Nat Protoc* 14, 3082–3100 (2019).
41. Wimmer, R. A. et al. Human blood vessel organoids as a model of diabetic vasculopathy. *Nature* 565, 505–510 (2019).
42. Melero-Martin, J. M. Human Endothelial Colony-Forming Cells. *Csh Perspect Med* a041154 (2022) doi:10.1101/cshperspect.a041154.
43. Chen, Y. et al. Functional Human Vascular Network Generated in Photocrosslinkable Gelatin Methacrylate Hydrogels. *Adv Funct Mater* 22, 2027–2039 (2012).
44. Lin, R.-Z. et al. Host non-inflammatory neutrophils mediate the engraftment of bioengineered vascular networks. *Nat Biomed Eng* 1, 0081 (2017).
45. Kang, K.-T., Lin, R.-Z., Kuppermann, D., Melero-Martin, J. M. & Bischoff, J. Endothelial colony forming cells and mesenchymal progenitor cells form blood vessels and increase blood flow in ischemic muscle. *Sci Rep-uk* 7, 770 (2017).
46. Landt, S. G. et al. ChIP-seq guidelines and practices of the ENCODE and modENCODE consortia. *Genome Res* 22, 1813–1831 (2012).
47. Guo, L. et al. Resolving Cell Fate Decisions during Somatic Cell Reprogramming by Single-Cell RNA-Seq. *Mol Cell* 73, 815–829.e7 (2019).
48. Paik, D. T. et al. Large-Scale Single-Cell RNA-Seq Reveals Molecular Signatures of Heterogeneous Populations of Human Induced Pluripotent Stem Cell-Derived Endothelial Cells. *Circ Res* 123, 443–450 (2018).
49. Liu, Z. et al. Single-cell transcriptomics reconstructs fate conversion from fibroblast to cardiomyocyte. *Nature* 551, 100 (2017).
50. Butler, A., Hoffman, P., Smibert, P., Papalexi, E. & Satija, R. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat Biotechnol* 36, 411–420 (2018).
51. Street, K. et al. Slingshot: cell lineage and pseudotime inference for single-cell transcriptomics. *Bmc Genomics* 19, 477 (2018).
52. Ji, Z. & Ji, H. TSCAN: Pseudo-time reconstruction and evaluation in single-cell RNA-seq analysis. *Nucleic Acids Res* 44, e117–e117 (2016).

53. Qiu, X. et al. Reversed graph embedding resolves complex single-cell trajectories. *Nat Methods* 14, 979–982 (2017).
54. Aibar, S. et al. SCENIC: single-cell regulatory network inference and clustering. *Nat Methods* 14, 1083–1086 (2017).
- 5 55. Chen, J. et al. VEGF amplifies transcription through ETS1 acetylation to enable angiogenesis. *Nat Commun.* 8, 383 (2017).
56. Zhou, P. et al. Mapping cell type-specific transcriptional enhancers using high affinity, lineage-specific Ep300 bioChIP-seq. *Elife* 6, e22039 (2017).
57. Bailey, T. et al. Practical Guidelines for the Comprehensive Analysis of ChIP-seq Data. *Plos*
- 10 *Comput Biol* 9, e1003326 (2013).
58. Sherwood, R. I. et al. Discovery of directional and nondirectional pioneer transcription factors by modeling DNase profile magnitude and shape. *Nat Biotechnol* 32, 171–178 (2014).
59. Gilbert, L. A. et al. Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. *Cell* 159, 647–661 (2014).
- 15 60. Mandegar, M. A. et al. CRISPR Interference Efficiently Induces Specific and Reversible Gene Silencing in Human iPSCs. *Cell Stem Cell* 18, 541–553 (2016).
61. Habib, N. et al. Massively parallel single-nucleus RNA-seq with DroNc-seq. *Nat Methods* 14, 955–958 (2017).
62. Gustafsson, C., Paepe, A. D., Schmidl, C. & Månsson, R. High-throughput ChIPmentation: freely
- 20 scalable, single day ChIPseq data generation from very low cell-numbers. *Bmc Genomics* 20, 59 (2019).
63. Skene, P. J., Henikoff, J. G. & Henikoff, S. Targeted in situ genome-wide profiling with high efficiency for low cell numbers. *Nat Protoc* 13, 1006–1019 (2018).
64. Natsume, T., Kiyomitsu, T., Saga, Y. & Kanemaki, M. T. Rapid Protein Depletion in Human Cells by Auxin- Inducible Degron Tagging with Short Homology Donors. *Cell Reports* 15, 210–218
- 25 (2016).
65. Rylova, S. N., Randhawa, P. K. & Bautch, V. L. Chapter 6 In Vitro Differentiation of Mouse Embryonic Stem Cells Into Primitive Blood Vessels. *Methods Enzymol* 443, 103–117 (2008).
66. Risau, W. et al. Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. *Development* 102, 471–478 (1988).
- 30 67. Jakobsson, L., Kreuger, J. & Claesson-Welsh, L. Building blood vessels—stem cell models in vascular biology. *J Cell Biology* 177, 751–755 (2007).
68. Bassil, R. et al. Improved modeling of human AD with an automated culturing platform for iPSC neurons, astrocytes and microglia. *Nat Commun* 12, 5220 (2021).

69. Lach, R. S. et al. Nucleation of the destruction complex on the centrosome accelerates degradation of β -catenin and regulates Wnt signal transmission. *Proc National Acad Sci* 119, e2204688119 (2022).
70. Stratman, A. N., Malotte, K. M., Mahan, R. D., Davis, M. J. & Davis, G. E. Pericyte recruitment
5 during vasculogenic tube assembly stimulates endothelial basement membrane matrix formation. *Blood* 114, 5091–5101 (2009).
71. Jin, S. et al. Inference and analysis of cell-cell communication using CellChat. *Nat Commun* 12, 1088 (2021).
72. Shamsi, F., Zheng, R., Ho, L.-L., Chen, K. & Tseng, Y.-H. Comprehensive analysis of
10 intercellular communication in thermogenic adipose niche. *Biorxiv* 2022.09.28.509990 (2022) doi:10.1101/2022.09.28.509990.
73. Cui, K. et al. Epsin Nanotherapy Regulates Cholesterol Transport to Fortify Atheroma Regression. *Circ Res* 132, e22–e42 (2023).
74. Zheng, R. et al. MEBOCOST: Metabolite-mediated Cell Communication Modeling by Single
15 Cell Transcriptome. *Biorxiv* 2022.05.30.494067 (2022) doi:10.1101/2022.05.30.494067.
75. No, D., Yao, T. P. & Evans, R. M. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc National Acad Sci* 93, 3346–3351 (1996).
76. Loffredo, F. & Lee, R. T. Therapeutic Vasculogenesis. *Circ Res* 103, 128–130 (2008).
77. Wang, K., Lin, R.-Z. & Melero-Martin, J. M. Bioengineering human vascular networks: trends
20 and directions in endothelial and perivascular cell sources. *Cell Mol Life Sci* 76, 421–439 (2019).
78. Melero-Martin, J. M. et al. In vivo vasculogenic potential of human blood-derived endothelial progenitor cells. *Blood* 109, 4761–4768 (2007).
79. Shepherd, B. R., Jay, S. M., Saltzman, M. W., Tellides, G. & Pober, J. S. Human Aortic Smooth
25 Muscle Cells Promote Arteriole Formation by Coengrafted Endothelial Cells. *Tissue Engineering Part A* 15, 165–173 (2009).
80. Maier, C. L., Shepherd, B. R., Yi, T. & Pober, J. S. Explant Outgrowth, Propagation and Characterization of Human Pericytes. *Microcirculation* 17, 367–380 (2010).
81. Hendrickx, B. et al. Integration of Blood Outgrowth Endothelial Cells in Dermal Fibroblast Sheets Promotes Full Thickness Wound Healing. *Stem Cells* 28, 1165–1177 (2010).
- 30 82. Lin, R.-Z., Moreno-Luna, R., Zhou, B., Pu, W. T. & Melero-Martin, J. M. Equal modulation of endothelial cell function by four distinct tissue-specific mesenchymal stem cells. *Angiogenesis* 15, 443–455 (2012).
83. Chen, K. H., Boettiger, A. N., Moffitt, J. R., Wang, S. & Zhuang, X. Spatially resolved, highly multiplexed RNA profiling in single cells. *Science* 348, aaa6090 (2015).

84. Moffitt, J. R. et al. High-performance multiplexed fluorescence in situ hybridization in culture and tissue with matrix imprinting and clearing. *Proc National Acad Sci* 113, 14456–14461 (2016).
85. Yano, C. F., Bertollo, L. A. C. & Cioffi, M. de B. Fluorescence In Situ Hybridization (FISH). *Springer Protoc Handbooks* 429–443 (2016) doi:10.1007/978-3-662-52959-1_44.
- 5 86. Ramos, J. M. Fluorescent In Situ Hybridization (FISH). *Methods Mol Biology Clifton N J* 2422, 179–189 (2021).
87. Daneman, R., Zhou, L., Kebede, A.A., and Barres, B.A. (2010). Pericytes are required for blood–brain barrier integrity during embryogenesis. *Nature* 468, 562–566. 10.1038/nature09513.
88. Schlaeger, T.M., Daheron, L., Brickler, T.R., Entwisle, S., Chan, K., Cianci, A., DeVine, A.,
10 Ettenger, A., Fitzgerald, K., Godfrey, M., et al. (2015). A comparison of non-integrating reprogramming methods. *Nat. Biotechnol.* 33, 58–63. 10.1038/nbt.3070.
89. Lin, R.-Z., Moreno-Luna, R., Li, D., Jaminet, S.-C., Greene, A.K., and Melero-Martin, J.M. (2014). Human endothelial colony-forming cells serve as trophic mediators for mesenchymal stem cell engraftment via paracrine signaling. *Proc. Natl. Acad. Sci.* 111, 10137–10142. 10.1073/pnas.1405388111.
- 15 90. Zheng, G.X.Y., Terry, J.M., Belgrader, P., Ryvkin, P., Bent, Z.W., Wilson, R., Ziraldo, S.B., Wheeler, T.D., McDermott, G.P., Zhu, J., et al. (2017). Massively parallel digital transcriptional profiling of single cells. *Nat. Commun.* 8, 14049. 10.1038/ncomms14049.
91. Consortium, I.H.G.S., Research., W.I. for B.R., Center for Genome, Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., et al. (2001). Initial sequencing and analysis of the human genome. *Nature* 409, 860–921. 10.1038/35057062.
- 20 92. Hao, Y., Hao, S., Andersen-Nissen, E., Mauck, W.M., Zheng, S., Butler, A., Lee, M.J., Wilk, A.J., Darby, C., Zager, M., et al. (2021). Integrated analysis of multimodal single-cell data. *Cell* 184, 3573–3587.e29. 10.1016/j.cell.2021.04.048.
93. McGinnis, C.S., Murrow, L.M., and Gartner, Z.J. (2019). DoubletFinder: Doublet Detection in Single-Cell RNA Sequencing Data Using Artificial Nearest Neighbors. *Cell Syst.* 8, 329–337.e4. 10.1016/j.cels.2019.03.003.
- 25 94. Cao, J., Spielmann, M., Qiu, X., Huang, X., Ibrahim, D.M., Hill, A.J., Zhang, F., Mundlos, S., Christiansen, L., Steemers, F.J., et al. (2019). The single-cell transcriptional landscape of mammalian organogenesis. *Nature* 566, 496–502. 10.1038/s41586-019-0969-x.
- 30 95. Song, S., Ewald, A.J., Stallcup, W., Werb, Z., and Bergers, G. (2005). PDGFR β + perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival. *Nat. Cell Biol.* 7, 870–879. 10.1038/ncb1288.
96. Dorn, G.W., and Becker, M.W. (1993). Thromboxane A2 stimulated signal transduction in vascular smooth muscle. *J. Pharmacol. Exp. Ther.* 265, 447–456.

97. Low, E.L., Baker, A.H., and Bradshaw, A.C. (2019). TGF β , smooth muscle cells and coronary artery disease: a review. *Cell. Signal.* 53, 90–101. 10.1016/j.cellsig.2018.09.004.
98. Siekmann, A.F. (2023). Biology of vascular mural cells. *Development* 150. 10.1242/dev.200271.
99. Folkman, J., and D'Amore, P.A. (1996). Blood Vessel Formation: What Is Its Molecular Basis? *Cell* 87, 1153–1155. 10.1016/s0092-8674(00)81810-3.
100. Orlova, V.V., Drabsch, Y., Freund, C., Petrus-Reurer, S., Hil, F.E. van den, Muenthaisong, S., Dijke, P. ten, and Mummery, C.L. (2018). Functionality of Endothelial Cells and Pericytes From Human Pluripotent Stem Cells Demonstrated in Cultured Vascular Plexus and Zebrafish Xenografts. *Arter., Thromb., Vasc. Biol.* 34, 177–186. 0.1161/atvbaha.113.302598.
101. Bianco, P., Robey, P.G., and Simmons, P.J. (2008). Mesenchymal Stem Cells: Revisiting History, Concepts, and Assays. *Cell Stem Cell* 2, 313–319. 10.1016/j.stem.2008.03.002.
102. Owens, G.K., Kumar, M.S., and Wamhoff, B.R. (2004). Molecular Regulation of Vascular Smooth Muscle Cell Differentiation in Development and Disease. *Physiol. Rev.* 84, 767–801. 10.1152/physrev.00041.2003.
103. Beamish, J.A., He, P., Kottke-Marchant, K., and Marchant, R.E. (2010). Molecular Regulation of Contractile Smooth Muscle Cell Phenotype: Implications for Vascular Tissue Engineering. *Tissue Eng. Part B: Rev.* 16, 467–491. 10.1089/ten.teb.2009.0630.
104. Carmeliet, P., and Jain, R.K. (2011). Molecular mechanisms and clinical applications of angiogenesis. *Nature* 473, 298. 10.1038/nature10144.
105. Cheung, C., Bernardo, A.S., Trotter, M.W.B., Pedersen, R.A., and Sinha, S. (2012). Generation of human vascular smooth muscle subtypes provides insight into embryological origin-dependent disease susceptibility. *Nat Biotechnol* 30, 165–173. 10.1038/nbt.2107.
106. Loh, K.M., Ang, L.T., Zhang, J., Kumar, V., Ang, J., Auyeong, J.Q., Lee, K.L., Choo, S.H., Lim, C.Y.Y., Nichane, M., et al. (2014). Efficient Endoderm Induction from Human Pluripotent Stem Cells by Logically Directing Signals Controlling Lineage Bifurcations. *Cell Stem Cell* 14, 237–252. 10.1016/j.stem.2013.12.007.
107. Carson, J.A., Fillmore, R.A., Schwartz, R.J., and Zimmer, W.E. (2000). The Smooth Muscle γ -Actin Gene Promoter Is a Molecular Target for the Mouse bagpipe Homologue, mNkx3-1, and Serum Response Factor*. *J. Biol. Chem.* 275, 39061–39072. 10.1074/jbc.m006532200.
108. Sun, Q., Taurin, S., Sethakorn, N., Long, X., Imamura, M., Wang, D.-Z., Zimmer, W.E., Dulin, N.O., and Miano, J.M. (2009). Myocardin-dependent Activation of the CArG Box-rich Smooth Muscle γ -Actin Gene. *J Biol Chem* 284, 32582–32590. 10.1074/jbc.m109.033910.
109. Nishida, W., Nakamura, M., Mori, S., Takahashi, M., Ohkawa, Y., Tadokoro, S., Yoshida, K., Hiwada, K., Hayashi, K., and Sobue, K. (2002). A Triad of Serum Response Factor and the GATA and

- NK Families Governs the Transcription of Smooth and Cardiac Muscle Genes*. *J. Biol. Chem.* 277, 7308–7317. 10.1074/jbc.m111824200.
110. Yoshida, T., Sinha, S., Dandré, F., Wamhoff, B.R., Hoofnagle, M.H., Kremer, B.E., Wang, D.-Z., Olson, E.N., and Owens, G.K. (2003). Myocardin Is a Key Regulator of CArG-Dependent Transcription of Multiple Smooth Muscle Marker Genes. *Circ. Res.* 92, 856–864.
- 10.1161/01.res.0000068405.49081.09.
111. Hoggatt, A.M., Kim, J.-R., Ustiyan, V., Ren, X., Kalin, T.V., Kalinichenko, V.V., and Herring, B.P. (2013). The Transcription Factor Foxf1 Binds to Serum Response Factor and Myocardin to Regulate Gene Transcription in Visceral Smooth Muscle Cells*. *J. Biol. Chem.* 288, 28477–28487.
- 10.1074/jbc.m113.478974.
112. Wang, G., Jacquet, L., Karamariti, E., and Xu, Q. (2015). Origin and differentiation of vascular smooth muscle cells. *J. Physiol.* 593, 3013–3030. 10.1113/jp270033.
113. Rajan, A.M., Ma, R.C., Kocha, K.M., Zhang, D.J., and Huang, P. (2020). Dual function of perivascular fibroblasts in vascular stabilization in zebrafish. *PLoS Genet.* 16, e1008800.
- 10.1371/journal.pgen.1008800.
114. Almalki, S.G., and Agrawal, D.K. (2016). Key transcription factors in the differentiation of mesenchymal stem cells. *Differentiation* 92, 41–51. 10.1016/j.diff.2016.02.005.
115. Fernandopulle, M.S., Prestil, R., Grunseich, C., Wang, C., Gan, L., and Ward, M.E. (2018). Transcription Factor–Mediated Differentiation of Human iPSCs into Neurons. *Curr. Protoc. Cell Biol.* 79, e51. 10.1002/cpcb.51.
116. Xie, C.-Q., Zhang, J., Villacorta, L., Cui, T., Huang, H., and Chen, Y.E. (2007). A Highly Efficient Method to Differentiate Smooth Muscle Cells From Human Embryonic Stem Cells. *Arter., Thromb., Vasc. Biol.* 27, e311–e312. 10.1161/atvbaha.107.154260.
117. Ferreira, L.S., Gerecht, S., Shieh, H.F., Watson, N., Rupnick, M.A., Dallabrida, S.M., Vunjak-Novakovic, G., and Langer, R. (2007). Vascular Progenitor Cells Isolated From Human Embryonic Stem Cells Give Rise to Endothelial and Smooth Muscle–Like Cells and Form Vascular Networks In Vivo. *Circ Res* 101, 286–294. 10.1161/circresaha.107.150201.
118. Xie, C., Ritchie, R.P., Huang, H., Zhang, J., and Chen, Y.E. (2011). Smooth Muscle Cell Differentiation In Vitro. *Arteriosclerosis Thrombosis Vasc Biology* 31, 1485–1494.
- 10.1161/atvbaha.110.221101.
119. Sumi, T., Tsuneyoshi, N., Nakatsuji, N., and Suemori, H. (2008). Defining early lineage specification of human embryonic stem cells by the orchestrated balance of canonical Wnt/ β -catenin, Activin/Nodal and BMP signaling. *Development* 135, 2969–2979. 10.1242/dev.021121.

120. Kumar, A., D'Souza, S.S., Moskvina, O.V., Toh, H., Wang, B., Zhang, J., Swanson, S., Guo, L.-W., Thomson, J.A., and Slukvin, I.I. (2017). Specification and Diversification of Pericytes and Smooth Muscle Cells from Mesenchymoangioblasts. *Cell Reports* 19, 1902–1916. 10.1016/j.celrep.2017.05.019.
121. Caplan, A.I. (2008). All MSCs Are Pericytes? *Cell Stem Cell* 3, 229–230.
5 10.1016/j.stem.2008.08.008.
122. Crisan, M., Yap, S., Casteilla, L., Chen, C.-W., Corselli, M., Park, T.S., Andriolo, G., Sun, B., Zheng, B., Zhang, L., et al. (2008). A Perivascular Origin for Mesenchymal Stem Cells in Multiple Human Organs. *Cell Stem Cell* 3, 301–313. 10.1016/j.stem.2008.07.003.
123. Caporarello, N., D'Angeli, F., Cambria, M.T., Candido, S., Giallongo, C., Salmeri, M.,
10 Lombardo, C., Longo, A., Giurdanella, G., Anfuso, C.D., et al. (2019). Pericytes in Microvessels: From “Mural” Function to Brain and Retina Regeneration. *Int. J. Mol. Sci.* 20, 6351. 10.3390/ijms20246351.

OTHER EMBODIMENTS

- 15 It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of making iPSC-derived mural progenitor cells (iMPCs) comprising:
contacting a population of induced pluripotent stem cells (iPSCs) with a nucleic acid
encoding NK3 Homeobox 1 (NKX3.1) or a functional variant thereof;
5 converting the iPSCs to mesodermal progenitors (MePCs); and
inducing the MePCs to express NKX3.1 for a time period sufficient to generate iMPCs.
2. The method of claim 1, wherein the nucleic acid is a DNA molecule (optionally, vector (e.g.,
a PiggyBac transposon vector or viral vector)) or an RNA (e.g., mRNA or a modified
mRNA).
- 10 3. The method of claim 2, wherein the vector is a viral vector (e.g., retroviral, lentiviral).
4. The method of any one of claims 1-3, wherein the nucleic acid comprises an inducible
promoter that controls expression of NKX3.1 (e.g., doxycycline-inducible promoter).
5. The method of any one of claims 1-4, wherein the converting the iPSCs to MePCs comprises
activating the Wnt pathway and/or activating the Nodal pathway for a time period sufficient
15 (e.g., about 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, or 48
hours) to generate MePCs.
6. The method of any one of claims 1-5, wherein the time period sufficient to generate iMPCs is
about 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, or 48 hours.
7. A method of making iPSC-derived mural progenitor cells (iMPCs) comprising inducing the
20 MePCs to express NK3 Homeobox 1 (NKX3.1) or a functional variant thereof for a time
period sufficient to generate iMPCs.
8. The method of claim 7, wherein the MePCs comprise a nucleic acid encoding NKX3.1 or a
functional variant thereof.
9. The method of claim 7, wherein the inducing comprises transfecting the MePCs with a
25 nucleic acid encoding NKX3.1 or a functional variant thereof.
10. The method of any one of claims 8-9, wherein the nucleic acid is a DNA (optionally, a
plasmid, a vector, a viral vector) or an RNA molecule (optionally, an mRNA or a modified
RNA (modRNA)).
11. The method of any one of claims 8-10, wherein the nucleic acid comprises an inducible
30 promoter that controls expression of NKX3.1 (e.g., doxycycline-inducible promoter).

12. The method of claim 11, wherein the inducing comprises contacting the cells with the agent to activate the inducible promoter (e.g., doxycycline).
13. The method of any one of claims 7-12, wherein the time period sufficient to generate iMPCs is about 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, or 48 hours.
14. A method of generating a population of mural cells comprising pericytes, smooth muscle cells, and fibroblasts, the method comprising:
 - generating iMPCs using the method of any one of claims 1-13;
 - co-culturing the iMPCs with a population of endothelial cells (ECs) for a time period sufficient to generate a population of mural cells comprising pericytes, smooth muscle cells, and fibroblasts.
15. The method of claim 14, wherein the time period sufficient to generate a population of mural cells comprising pericytes, smooth muscle cells, and fibroblasts is about 1, 2, 3, 4, 5, 6, or 7 days.
16. A method of increasing blood vessel formation in a subject comprising administering to the subject a therapeutically effective amount of the population of ECs and iMPCs.
17. A population of cells generated by the method of any one of claims 1-13.
18. A population of cells comprising at least 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% iPSC-derived mural progenitor cells (iMPCs).
19. The population of cells of claim 18, wherein the iMPCs comprise a nucleic acid (optionally, an exogenous nucleic acid) encoding NKX3.1 or transiently express NKX3.1 (optionally, from a degradable nucleic acid (e.g., mRNA or modRNA)).
20. The population of cells of claim 18 or claim 19, wherein the iMPCs express PDGFR β (CD140b) and aminopeptidase N (CD13).
21. The population of cells of any one of claims 18-20, wherein less than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1% of the iMPCs express a TRA1-81 antigen.
22. The population of cells of any one of claims 18-21, wherein the iMPCs express *ACTA2*, *CNN1*, *IAGLN*, *MYOCD*, *MYH11*, *CSPG4*, *DES*, *PDE5A*, and *THY1*.
23. A population of cells comprising a ratio of about 1:3, 1:2, 2:3, 1:1, 3:2, 1:2, or 3:1 of endothelial cells (ECs): iPSC-derived mural progenitor cells (iMPCs).

24. The population of cells of claim 23, wherein the iMPCs comprise a nucleic acid (optionally, an exogenous nucleic acid) encoding NKX3.1 or transiently expressed NKX3.1 (optionally, from a degradable nucleic acid (e.g., mRNA or modRNA)).
25. The population of cells of claim 23 or claim 24, wherein the iMPCs express PDGFR β (CD140b) and aminopeptidase N (CD13).
26. The population of cells of any one of claims 23-25, wherein less than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1% of the iMPCs express a TRA1-81 antigen.
27. The population of cells of any one of claims 23-26, wherein the iMPCs express *ACTA2*, *CNN1*, *TAGLN*, *MYOCD*, *MYH11*, *CSPG4*, *DES*, *PDE5A*, and *THY1*.
28. The population of cells of any one of claims 23-27, wherein the ECs comprise any one or more of iPSC-derived ECs (iECs), human umbilical vein endothelial cells (HUVECs), endothelial colony-forming cells (ECFCs), adipose tissue-derived ECs, or organ-specific endothelial cells.
29. The method of claim 28, wherein said organ-specific endothelial cells are from an organ selected from: heart, muscle, kidney, testis, ovary, lymphoid, liver, pancreas, brain, lungs, bone marrow, spleen, large intestine, and small intestine.
30. The population of cells of claim 28 or 29, wherein the iECs comprise a nucleic acid (optionally, an exogenous nucleic acid) encoding ETV2 or transiently expressed ETV2 (optionally, from a degradable nucleic acid (e.g., mRNA or modRNA)).
31. The population of cells of any one of claims 28-30, wherein the NKX3.1 expression is controlled by an inducible promoter and the ETV2 expression is controlled by an inducible promoter.
32. The population of cells of claim 31, wherein the inducible promoter for NKX3.1 and the inducible promoter for ETV2 are the same (optionally, doxycycline).
33. The population of cells of claim 31, wherein the inducible promoter for NKX3.1 and the inducible promoter for ETV2 are not the same.
34. A vascular organoid (VO) or three-dimensional (3D) cell culture comprising the population of cells of any one of claims 18-33.
35. A method of making a vascular organoid (VO) or a three-dimensional (3D) cell culture, the method comprising:

(i) culturing a first population of iPSC-derived mesodermal progenitor cells (MePCs) comprising a nucleic acid (optionally, an exogenous nucleic acid) encoding ETV2, or a functional variant thereof, ("ETV2/MePCs") with a second population of MePCs comprising a nucleic acid (optionally, an exogenous nucleic acid) encoding NKX3.1, or a functional variant thereof, ("NKX3.1/MePCs")

wherein expression of NKX3.1 is controlled by an inducible promoter and expression of ETV2 is controlled by an inducible promoter;

(ii) inducing expression of NKX3.1 in the NKX3.1/MePCs, thereby creating iPSC-derived mural progenitor cells (iMPCs) and inducing expression of ETV2 in the ETV2/MePCs, thereby creating iPSC-derived endothelial cells (iECs); and

(iii) culturing the cells for a time period sufficient to generate a VO or 3D cell culture, wherein the VO or 3D cell culture comprises iECs that are h-CD31+ and iMPCs that are PDGFR β + and h-CD31-.

36. The method of claim 35, wherein the inducible promoter for NKX3.1 and the inducible promoter for ETV2 are the same (optionally, doxycycline).

37. The method of claim 35, wherein the inducible promoter for NKX3.1 and the inducible promoter for ETV2 are not the same.

38. The method of any one of claims 35-37, wherein the time period sufficient to generate a VO or 3D cell culture is about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 days.

39. The method of any one of claims 35-38, wherein the step (i) culturing occurs for about 1 day or 2 days; wherein the step (i) culturing occurs for about 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, or 48 hours; and/or wherein the culturing occurs for a time period sufficient to generate aggregates comprising both the NKX3.1/MePCs and the ETV2/MePCs.

40. The method of any one of claims 35-39, wherein the (i) culturing step comprises culturing the cells using non-adherent culture plates and an orbital shaker.

41. The method of any one of claims 35-40, wherein the population of NKX3.1/MePCs and the population of ETV2/MePCs are mixed at a ratio of about 1:3, 1:2, 2:3, 1:1, 3:2, 1:2, or 3:1 of NKX3.1/MePCs:ETV2/MePCs.

42. A method of making a vascular organoid (VO) or a three-dimensional (3D) cell culture, the method comprising:

(i) transfecting a population of iPSC-derived mesodermal progenitors (MePCs) with a nucleic acid (optionally, an RNA, an mRNA, an modRNA) encoding NKX3.1 or a functional variant thereof, thereby creating a population of iPSC-derived mural progenitor cells (iMPCs);

5 (ii) mixing the population of iMPCs with a population of ECs (optionally, at a ratio of about 1:3, 1:2, 2:3, 1:1, 3:2, 1:2, or 3:1 of iMPC:EC); and

(iii) culturing the cells for a time period sufficient to generate a VO or 3D cell culture, wherein the VO or 3D cell culture comprises iECs that are h-CD31⁺ and iMPCs that are PDGFR β ⁺ and h-CD31⁻.

10 43. The method of claim 42, wherein the ECs comprise any one or more of iPSC-derived ECs (iECs), human umbilical vein endothelial cells (HUVECs), endothelial colony-forming cells (ECFCs), adipose tissue-derived ECs, or organ-specific endothelial cells.

44. The method of claim 42, wherein said organ-specific endothelial cells are from an organ selected from: heart, muscle, kidney, testis, ovary, lymphoid, liver, pancreas, brain, lungs,
15 bone marrow, spleen, large intestine, and small intestine.

45. The method of claim 42 further comprising a step of transfecting a population of iPSCs with a nucleic acid encoding ETV2 or a functional variant thereof to thereby create a population of ECs prior to mixing with the population of iMPCs.

46. The method of any one of claims 35-45, wherein the VOs are uniform size and/or about
20 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, 350, 355, 360, 365, 370, 375, 380, 385, 390, 395, 400, 405, 410, 415, 420, 425, 430, 435, 440, 445, 450, 455, 460, 465, 470, 475, 480, 485, 490, 495, 500, 505, 510, 515, 520, 525, 530, 535, 540, 545, or 550 μ m in diameter; and/or wherein the average diameter size of the VOs or 3D
25 cell cultures is about 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, or 350 μ m in diameter; and/or wherein the median size of the VOs or 3D cell cultures is about 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340,
30 345, or 350 μ m in diameter; and/or the VOs or 3D cell cultures comprise about 1,000, 1,500, 2,000, 2,250, 2,500, 2,550, 2,600, 2,650, 2,700, 2,750, 2,800, 2,850, 2,900, 2,950, 3,000,

3,050, 3,100, 3,150, 3,200, 3,250, 3,300, 3,350, 3,400, 3,450, 3,500, 3,750, 4,000, 4,250, 4,500, 4,750, or 5,000 cells.

47. The method of any one of claims 35-46 wherein the cells self-assembled into a network of CD31+ vascular structures containing mature α -SMA+ perivascular mural cells.
- 5 48. The method of any one of claims 35-47, wherein the VOs comprise a network of lumenized vessels with apical-basal polarization and/or wherein the VO comprises arterial, venous, and/or capillary ECs.
49. The method of any one of claims 35-48, wherein the VO comprises ECs that are *CDH5*+ and *VWF*+ and mural cell that are *ACTA2*+, *MYH11*+, *TAGLN*+, and *CNN1*+
- 10 50. The VO or 3D cell culture made by any one of the methods of claims 35-49.
51. The VO or 3D cell culture of claim 50, wherein the VOs or 3D cell cultures are uniform size; and/or wherein the VOs or 3D cell cultures are about 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, 350, 355, 360, 365, 370, 375, 380, 385, 390, 395, 400, 405, 410, 415, 420, 425, 430, 435, 440, 445, 450, 455, 460, 465, 470, 475, 480, 15 485, 490, 495, 500, 505, 510, 515, 520, 525, 530, 535, 540, 545, or 550 μ m in diameter; and/or wherein the average diameter size of the VOs or 3D cell cultures is about 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, or 350 μ m in diameter; 20 and/or wherein the median size of the VOs or 3D cell cultures is about 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, or 350 μ m in diameter; and/or the VOs or 3D cell cultures comprise about 1,000, 1,500, 2,000, 2,250, 2,500, 2,550, 2,600, 2,650, 2,700, 2,750, 2,800, 2,850, 2,900, 2,950, 3,000, 3,050, 3,100, 3,150, 3,200, 3,250, 25 3,300, 3,350, 3,400, 3,450, 3,500, 3,750, 4,000, 4,250, 4,500, or 5,000 cells.
52. The VO or 3D cell culture of claim 50 or 51, wherein the VO or 3D cell culture comprises CD31+ vascular structures containing mature α -SMA+ perivascular mural cells.
53. The VO or 3D cell culture of any one of claims 50-52, wherein the VO or 3D cell culture comprises a network of lumenized vessels with apical-basal polarization and arterial, venous, 30 and/or capillary ECs.

54. The VO or 3D cell culture of any one of claims 50-53, wherein the VO or 3D cell culture comprises EC markers (*CDH5*; *VWF*) in VO-iECs (**Fig. 9B**) and several mural cell markers (*ACTA2*, *MYH11*, *TAGLN*, *CNN1*) in VO-iMPCs
55. A composition comprising the population of cells of any one of claims 17-33 or the VO or 3D cell culture of any one of claims 34 and 50-54.
56. The composition of claim 55 further comprising any one or more of an agent, an excipient, a matrix, or a gel.
57. The composition of claim 56, wherein the gel or matrix comprises a hydrogel.
58. The composition of claim 56, wherein the gel or matrix comprises gelatin, collagen, fibrinogen, thrombin, fibrin, or combinations thereof.
59. The composition of claim 56, wherein the gel or matrix comprises about 1.5 mg/mL collagen, about 30 µg/mL fibrinogen, and about 1 mg/mL human fibronectin.
60. The composition of claim 56, wherein the gel or matrix comprises any one or more of gelatin, laminin, entactin, collagen, fibrinogen, and combinations thereof.
61. The composition of claim 56, wherein the gel or matrix comprises laminin, entactin, and collagen.
62. The composition of claim 56, wherein the gel or matrix comprises about 5.25 mg/mL laminin, about 5.25 mg/mL entactin, and about 0.2 mg/mL collagen IV.
63. The composition of claim 56, wherein the gel or matrix is Matrigel™.
64. A method of transplanting the population of cells of any one of claims 17-33, the vascular organoid of any one of claims 34 and 50-54, or the composition of any one of claims 55-63 in a subject comprising:
administering to the subject an effective amount of the population of cells, vascular organoid, or composition.
65. A method of increasing blood vessel formation in a subject, comprising:
identifying a subject in need of increased blood vessel formation; and
administering to the subject an effective amount of the population of cells of any one of claims 17-33, the vascular organoid of any one of claims 34 and 50-54, or the composition of any one of claims 55-63.
66. A method of increasing vascular generation or vascular regeneration in a subject comprising:
identifying a subject at in need of vascular generation or vascular regeneration;

administering to the subject an effective amount of the population of cells of any one of claims 17-33, the vascular organoid of any one of claims 34 and 50-54, or the composition of any one of claims 55-63.

67. A method of vascular cell therapy comprising:

5 identifying a subject in need of vascular cell therapy;
administering to the subject an effective amount of the population of cells of any one of claims 17-33, the vascular organoid of any one of claims 34 and 50-54, or the composition of any one of claims 55-63.

68. The method of any one of claims 16 and 64-67, wherein the subject has (or is at risk of
10 having or developing) any one or more of the following: diabetes, diabetic retinopathy, an ischemic injury, a disease or disorder of the blood vessels, atherosclerosis, Age-related Macular Degeneration (AMD), Pulmonary Arterial Hypertension (PAH), Hereditary Hemorrhagic Telangiectasia (HHT), peripheral artery disease (PAD), arteriovenous fistulas (e.g., dialysis patients), tumor angiogenesis, tumor metastasis, a stroke, and/or a wound
15 (optionally, a chronic wound; e.g., a diabetic ulcer).

69. The method of any one of claims 16 and 64-68, wherein the population of cells, vascular organoid, or composition is administered to the subject, before, during, or after a cell transplant, tissue transplant, or organ transplant.

70. The method of any one of claims 16 and 64-69, wherein the population of cells, the
20 vascular organoid, or the composition is administered to the subject by direct injection into a blood vessel or subcutaneous, intradermal, intramuscular, intranodal, intravenous, intraprostatic, intratumor, intralymphatic, and intraperitoneal injection.