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- (71) Applicant: **CHILDREN'S HOSPITAL MEDICAL CENTER** [US/US]; 3333 Burnet Ave., Cincinnati, Ohio 45229 (US).
- (72) Inventors: **GUO, Ziyuan**; 1023 Aristides Drive, Union, Kentucky 41091 (US). **DAO, Lan**; 7022 Lookout Ct., Liberty Twp., Ohio 45011 (US).
- (74) Agent: **GAURAV, Apurv** et al.; Norton Rose Fulbright US LLP, 98 San Jacinto Boulevard, Suite 1100, Austin, Texas 78701 (US).
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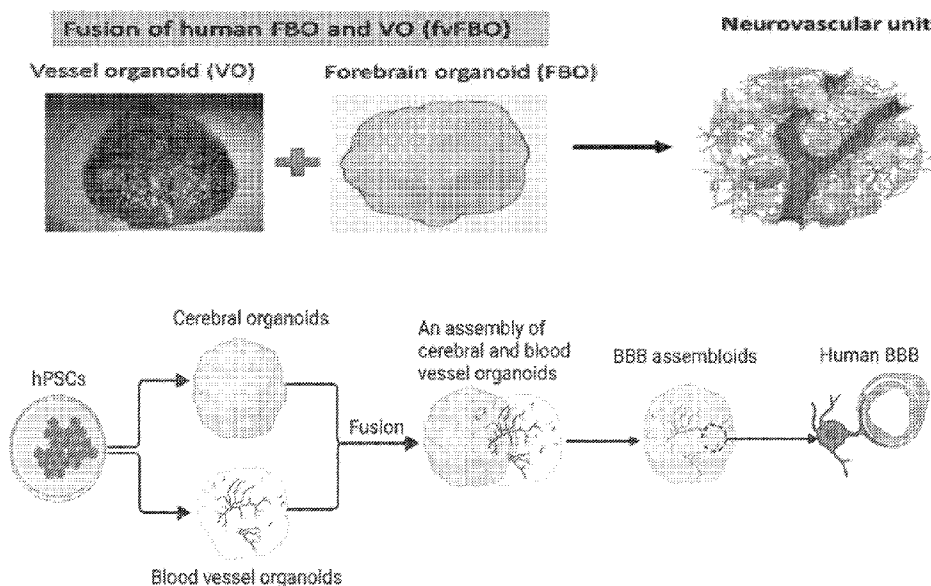


FIG. 1A

(57) Abstract: Disclosed herein are organoid models of the blood-brain barrier, optionally having a disease associated feature, such as a cerebral cavernous malformation (CCM)-like feature, compositions comprising the same, methods for making the same from pluripotent stem cells, and methods of using the same. These organoids exhibit the complex organization of cells including neurons, endothelial cells, glial cells, and pericytes resembling the natural blood-brain barrier structure. The organoids may also exhibit disease like characteristics, such as having a cerebral cavernous malformation (CCM)-like features. These organoids may be used for studying the functions of the blood-brain barrier and cerebrovascular disorders.

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VASCULARIZED BRAIN ORGANOIDS AND METHODS OF MAKING AND USING THE SAME

STATEMENT REGARDING FEDERALLY SPONSORED R&D

[0001] This invention was made with government support under NS134628, NS122169, and MH132038 awarded by the National Institutes of Health (NIH), National Heart, Lung & Blood Institute (NHLBI). The government has certain rights to the invention.

CROSS REFERENCE TO RELATED APPLICATIONS

[0002] This application claims the benefit of priority of U.S. Provisional Patent Application Serial No. 63/510,463 filed on June 27, 2023, and U.S. Provisional Patent Application Serial No. 63/645,794 filed on May 10, 2024, the contents of each of which are hereby incorporated by reference in their entirety.

FIELD OF THE INVENTION

[0003] Aspects of the present disclosure relate generally to organoid compositions exhibiting a blood-brain barrier structure, and methods of making and use thereof. Embodiments disclosed herein include human models of cerebral cavernous malformation (CCM) using patient-induced pluripotent stem cell-derived blood-brain barrier assembloids and CCM primary tissue organoids, and methods of making and use thereof.

BACKGROUND

[0004] The blood-brain barrier (BBB) is a biological structure of great importance, as it selectively allows or prevents the crossing of molecules and other substances from the blood into the central nervous system. This offers protection against pathogens and immune-related components such as immune cells, antibodies, and cytokines to shield the central nervous system from the effects of peripheral immune function. However, this also results in difficulty in pharmaceutical molecules from accessing the central nervous system, including the brain, limiting their therapeutic efficacy unless specifically designed to cross the BBB. Accordingly, the BBB is an essential component to be considered during drug development, and there is a great need for robust *in vitro* and/or *in vivo* models to study the BBB.

[0005] CCMs are a common inherited cerebrovascular disease that affects nearly 1 in 200 people with prevalence in youth; they represent a major cause of stroke and neurological defect in children, adolescents, and young adults. Genetic studies in humans have shown that CCMs are traced to the loss of one of the CCM risk genes (KRIT1, CCM2 and PDCD10). Animal models with conditional deletion of CCM genes in brain ECs recapitulate lesion phenotypes and reveal loss-of-function (LOF) of CCM genes correlates with lesion burdens. However, there is still no drug treatment for CCMs. This is largely because of lack of faithful and highly expandable models for validating and screening candidate therapeutics in humans.

[0006] Although rodent models having the conditional deletion of CCM genes in brain endothelial cells (ECs) have been explored, such rodent models fall significantly short of modeling the unique physiology of the human BBB. Specifically, the molecular signature and functional properties of the human BBB is highly distinct from those of rodent models. Such significant disparities in drug permeability between the human BBB and non-human models contribute to a high failure rate in clinical trials for preclinical drugs, and further underscores the urgent need for human BBB models in advancing translational research.

[0007] Although human induced pluripotent stem cells (iPSCs)-based models of brain endothelial-like cells have been explored for the purpose of investigating molecular permeability and studying barrier dysfunction caused by genetic disturbances such as in CCM, such models are less suited for modeling the fully formed BBB given their dimensional limitations and disparities in developmentally relevant differentiation trajectories, lack of a definitive brain endothelial identity and unwanted acquisition of epithelial fate. There are also an absence of, and therefore a need for, *in vitro* systems that delineate how primitive ECs are specified to become BBB-specific ECs or that model the interactions occurring between the primitive capillary plexus and neuroepithelium tissues to coordinate human neurovascular development.

[0008] There is thus an urgent demand for robust *in vitro* and/or *in vivo* human models capable of accurately replicating cavernoma phenotypes and facilitating the study of CCMs.

SUMMARY

[0009] Disclosed herein are compositions comprising and methods for producing vascularized brain organoids (e.g., blood-brain barrier (BBB) assembloids), optionally having a cerebral cavernous malformation (CCM)-like feature. In some embodiments, the methods comprise:

culturing a blood vessel organoid and a brain organoid for a period of time until the blood vessel organoid and the brain organoid fuse together and blood vessels of the blood vessel organoid infiltrate the brain organoid. In some embodiments, neurons of the brain organoid innervate the blood vessels of the blood vessel organoid that have infiltrated the organoid, thereby forming the vascularized brain organoid. In some embodiments, the vascularized brain organoid comprises a blood-brain barrier that is formed between the brain organoid and all or a portion of the blood vessels of the blood vessel organoid that have infiltrated the brain organoid. In some embodiments, the blood-brain barrier comprises endothelial cells linked with tight junctions, astrocytes, pericytes, smooth muscle, and/or fibroblast. In some embodiments, the vascularized brain organoid comprises a basement membrane covering blood-brain barrier.

[0010] Also disclosed herein are the vascularized brain organoids, optionally having a CCM-like features. Also provided herein are vascularized brain organoids produced according to the methods disclosed herein. Also provided herein are compositions for use in methods of making vascularized brain organoids. Also provided herein are methods of screening drug candidates utilizing compositions of the disclosure.

[0011] An aspect of the disclosure is a method of making a CCM primary tissue organoid. In some embodiments, the method comprises culturing primary cavernomas tissue in culture medium until CCM primary tissue organoids form.

[0012] Exemplary embodiments of the present disclosure are provided in the following numbered embodiments:

[0013] Embodiment 1: A method for producing a vascularized brain organoid, comprising: culturing a blood vessel organoid and a brain organoid for a period of time until the blood vessel organoid and the brain organoid fuse together and blood vessels of the blood vessel organoid infiltrate the brain organoid; wherein neurons of the brain organoid innervate the blood vessels of the blood vessel organoid that have infiltrated the organoid, thereby forming the vascularized brain organoid; and wherein the vascularized brain organoid comprises a blood-brain barrier that is formed between the brain organoid and all or a portion of the blood vessels of the blood vessel organoid that have infiltrated the brain organoid.

[0014] Embodiment 2: The method of embodiment 1, wherein the blood-brain barrier comprises endothelial cells linked with tight junctions, astrocytes, pericytes, and/or smooth muscle cells.

[0015] Embodiment 3: The method of embodiment 2, wherein the endothelial cells express CD31, GLUT-1 and PDGFR- β ; the tight junctions comprise claudin-5, ZO-1 and cadherin 5; the astrocytes express S100B, GFAP, and AQP4; the pericytes express PDGFR- β , α SMA and NG2, and/or the smooth muscle cells express SMA.

[0016] Embodiment 4: The method of embodiment 2 or 3, wherein the endothelial cells form a continuous basement membrane and express collagen IV.

[0017] Embodiment 5: The method of any one of the preceding embodiments, wherein the vascularized brain organoid comprises cells selected from the group consisting of neural progenitors, proliferative astrocytes, GABAergic neurons, glutamatergic neurons, proliferative cells, brain vascular endothelial cells, vascular leptomeningeal cells, perivascular adipocytes, and tendon cells.

[0018] Embodiment 6: The method of any one of the preceding embodiments, wherein the cells of the vascular brain organoid are identified by cell-type specific gene expression markers.

[0019] Embodiment 7: The method of any one of the preceding embodiments, wherein the blood vessels comprise capillaries.

[0020] Embodiment 8: The method of embodiment 7, wherein the capillaries are ensheathed by pericytes and end-feet of astrocytes.

[0021] Embodiment 9: The method of any one of the preceding embodiments, wherein the brain organoid is a forebrain organoid, a midbrain organoid, a hypothalamus organoid, a hippocampus organoid, a spinal cord organoid, or a striatal brain organoid.

[0022] Embodiment 10: The method of any one of the preceding embodiments, wherein the blood vessel organoid and the brain organoid are contacted and/or cultured in a basement membrane matrix or component thereof, optionally Matrigel.

[0023] Embodiment 11: The method of any one of the preceding embodiments, wherein the blood vessel organoid and the brain organoid are cultured for a period of time that is between about 1- 70 days.

[0024] Embodiment 12: The method of any one of the preceding embodiments, wherein the blood vessel organoid and the brain organoid are cultured for a period of time that is about 30-70 days.

[0025] Embodiment 13: The method of any one of the preceding embodiments, wherein the blood vessel organoid and the brain organoid are cultured for a period of time that is about 30-60.

[0026] Embodiment 14: The method of any one of the preceding embodiments, wherein the blood vessel organoid and the brain organoid are cultured with agitation, optionally shaking, for at least a portion of the period of time.

[0027] Embodiment 15: The method of any one of the preceding embodiments, wherein the blood vessel organoid and the brain organoid are cultured: without agitation for about 1-14 days; and subsequently with agitation for about 1-70 days.

[0028] Embodiment 16: The method of any one of the preceding embodiments, wherein the blood vessel organoid and the brain organoid are cultured: without agitation for about 4-10 days; and subsequently with agitation for about 30-70 days.

[0029] Embodiment 17: The method of any one of the preceding embodiments, wherein the blood vessel organoid and the brain organoid are cultured: without agitation for about 4-10 days; and subsequently with agitation for about 1-40 days.

[0030] Embodiment 18: The method of any one of the preceding embodiments, wherein the blood vessel organoid and the brain organoid are cultured in a medium that promotes neuronal growth and/or vascular growth.

[0031] Embodiment 19: The method of any one of the preceding embodiments, wherein the blood vessel organoid and the brain organoid are cultured in a medium that comprises growth factors that promote neuronal growth and/or growth factors that promote vascular growth.

[0032] Embodiment 20: The method of embodiment 19, wherein the growth factors that promote neuronal growth comprise a cAMP pathway activator, ascorbic acid, brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), or any combination thereof.

[0033] Embodiment 21: The method of embodiment 19 or 20, wherein the growth factors that promote vascular growth comprise growth serum, a vascular endothelial growth factor (VEGF) pathway activator, a fibroblast growth factor (FGF) pathway activator, or any combination thereof.

[0034] Embodiment 22: The method of any one of the preceding embodiments, wherein culturing the blood vessel organoid and the brain organoid comprises: culturing the blood vessel organoid and the brain organoid without agitation for about 4-10 days; and subsequently culturing the organoids of with agitation for about 1-40 days; wherein the organoids of are cultured with and without agitation in a medium comprising growth factors that promote neuronal growth and/or

growth factors that promote vascular growth; optionally wherein the agitation comprises shaking; optionally wherein the growth factors that promote neuronal growth comprise a cAMP pathway activator, ascorbic acid, BDNF, GDNF, or any combination thereof; optionally wherein the growth factors that promote vascular growth comprise growth serum, a VEGF pathway activator, an FGF pathway activator, or any combination thereof; optionally wherein the blood vessel organoid and the brain organoid are cultured in a basement membrane matrix or component thereof, optionally Matrigel.

[0035] Embodiment 23: The method of embodiment 22, wherein the cAMP pathway activator is cAMP.

[0036] Embodiment 24: The method of embodiment 22 or 23, wherein the cAMP pathway activator is provided at a concentration that is between about 10-150 μ M.

[0037] Embodiment 25: The method of any one of embodiments 22-24, wherein the cAMP pathway activator is provided at a concentration that is between about 20-100 μ M.

[0038] Embodiment 26: The method of any one of embodiments 22-25, wherein the ascorbic acid is provided at a concentration that is between about 50 - 300 μ M.

[0039] Embodiment 27: The method of any one of embodiments 22-26, wherein the ascorbic acid is provided at a concentration that is between about 50 - 300 μ M.

[0040] Embodiment 28: The method of any one of embodiments 22-27, wherein the ascorbic acid is provided at a concentration that is between about 150 - 250 μ M.

[0041] Embodiment 29: The method of any one of embodiments 22-28, wherein the BDNF is provided at a concentration that is between about 1 -30 ng/mL.

[0042] Embodiment 30: The method of any one of embodiments 22-29, wherein the BDNF is provided at a concentration that is between about 15 - 25 ng/mL.

[0043] Embodiment 31: The method of any one of embodiments 22-30, wherein the GDNF is provided at a concentration that is between about 1-30 ng/mL.

[0044] Embodiment 32: The method of any one of embodiments 22-31, wherein the GDNF is provided at a concentration that is between about 15 - 25 ng/mL.

[0045] Embodiment 33: The method of any one of embodiments 22-32, wherein the growth serum is fetal bovine serum (FBS).

[0046] Embodiment 34: The method of any one of embodiments 22-33, wherein the growth serum is provided at a concentration that is between about 0.5%-20%.

[0047] Embodiment 35: The method of any one of embodiments 22-34, wherein the growth serum is provided at a concentration that is between about 12%-18%.

[0048] Embodiment 36: The method of any one of embodiments 22-35, wherein the VEGF pathway activator is VEGF.

[0049] Embodiment 37: The method of any one of embodiments 22-36, wherein the VEGF pathway activator is provided at a concentration that is between about 10-150 ng/mL.

[0050] Embodiment 38: The method of any one of embodiments 22-37, wherein the VEGF pathway activator is provided at a concentration that is between about 80-120 ng/mL.

[0051] Embodiment 39: The method of any one of embodiments 22-38, wherein the FGF pathway activator is FGF2.

[0052] Embodiment 40: The method of any one of embodiments 22-39, wherein the FGF pathway activator is provided at a concentration between about 10-150 ng/mL.

[0053] Embodiment 41: The method of any one of embodiments 22-40, wherein the FGF pathway activator is provided at a concentration between about 80-120 ng/mL.

[0054] Embodiment 42: The method of any one of the preceding embodiments, wherein the blood vessel organoid and/or the brain organoid are derived from pluripotent stem cells, optionally embryonic stem cells or induced pluripotent stem cells.

[0055] Embodiment 43: The method of any one of the preceding embodiments, wherein the blood vessel organoid has been produced according to a method comprising: causing, for a first period of time, an angiogenic sprout to activate an FGF pathway, a VEGF pathway, and, optionally, a Wnt pathway of the angiogenic sprout, while the angiogenic sprout is in growth serum.

[0056] Embodiment 44: The method of embodiment 43, wherein the first period of time is between about 1-30 days.

[0057] Embodiment 45: The method of embodiment 43 or 44, wherein the first period of time is between about 10-30 days.

[0058] Embodiment 46: The method of any one of embodiments 43-45, wherein the first period of time is between about 5-25 days.

[0059] Embodiment 47: The method of any one of embodiments 43-46, wherein the angiogenic sprout has been produced according to a method comprising: a) causing, for a second period of time, the pluripotent stem cells to activate a Wnt pathway and a BMP pathway of the

pluripotent stem cells to form vascular lineage cells; and b) causing, for a third period of time, the vascular lineage cells to activate a VEGF pathway and a second cAMP pathway of the vascular lineage cells to form the angiogenic sprout.

[0060] Embodiment 48: The method of embodiment 47, wherein the second period of time is between about 1-5 days.

[0061] Embodiment 49: The method of embodiment 47 or 48, wherein the second period of time is about 3 days.

[0062] Embodiment 50: The method of any one of embodiments 47-49, wherein the third period of time is between about 1-4 days.

[0063] Embodiment 51: The method of any one of embodiments 47-50, wherein the third period of time is about 2 days.

[0064] Embodiment 52: The method of any one of embodiments 47-51, wherein the BMP pathway is activated via a BMP pathway activator, wherein the BMP pathway activator is BMP4.

[0065] Embodiment 53: The method of any one of embodiments 47-52, wherein the BMP pathway activator is provided at a concentration that is between about 10-100 ng/mL.

[0066] Embodiment 54: The method of any one of embodiments 47-53, wherein the BMP pathway activator is provided at a concentration that is between about 20-70 ng/mL.

[0067] Embodiment 55: The method of any one of embodiments 47-54, wherein the Wnt pathway is activated via a Wnt pathway activator, wherein the Wnt pathway activator is CHIR99021.

[0068] Embodiment 56: The method of any one of embodiments 47-55, wherein the Wnt pathway is activated via a Wnt pathway activator provided at a concentration that is between about 1-20 μ M.

[0069] Embodiment 57: The method of any one of embodiments 47-56, wherein the Wnt pathway is activated via a Wnt pathway activator provided at a concentration that is between about 1-12 μ M.

[0070] Embodiment 58: The method of any one of embodiments 47-57, wherein the Wnt pathway is activated via a Wnt pathway activator provided at a concentration that is between about 4-20 μ M.

[0071] Embodiment 59: The method of any one of embodiments 47-58, wherein the second cAMP pathway is activated via a cAMP pathway activator comprising forskolin.

[0072] Embodiment 60: The method of any one of embodiments 47-59, wherein the second cAMP pathway is activated via a second cAMP pathway activator provided at a concentration that is between about 0.5 - 4 μ M.

[0073] Embodiment 61: The method of any one of embodiments 47-60, wherein the second cAMP pathway is activated via a second cAMP pathway activator provided at a concentration that is between about 1-3 μ M.

[0074] Embodiment 62: The method of any one of embodiments 43-61, wherein the blood vessel organoid differs from a blood vessel organoid that has been produced without causing the angiogenic sprout to activate the Wnt pathway by having increased expression of blood-brain barrier-specific endothelial markers, optionally GLUT-1 and ZO-1.

[0075] Embodiment 63: The method of any one of the preceding embodiments, wherein the brain organoid has been contacted with LIF and growth serum to induce astrocyte formation in the brain organoid.

[0076] Embodiment 64: The method of any one of the preceding embodiments, wherein the brain organoid has been produced according to a method comprising: causing, for a first period of time, pluripotent stem cells to inhibit a BMP pathway, a TGF-beta pathway, and a Wnt pathway of the pluripotent stem cells to form neuroectoderm cells; causing, for a second period of time, the neuroectoderm cells to inhibit a second TGF-beta pathway and activate a Wnt pathway of the neuroectoderm cells to form neuroepithelium cells; contacting the neuroepithelium cells with insulin for a third period of time to form cerebral tissue organoids; and for a fourth period of time: contacting the cerebral tissue organoid with GDNF, BDNF, and ascorbic acid, and activating a cAMP pathway activator of the cerebral tissue organoid to form the brain organoid.

[0077] Embodiment 65: The method of embodiment 64, wherein the brain organoid has been produced according to a method further comprising: contacting the cerebral tissue organoid with LIF and growth serum for a portion of the fourth period of time to induce astrocyte proliferation in the brain organoid.

[0078] Embodiment 66: The method of embodiment 64 or 65, wherein the first period of time is between about 1-14 days.

[0079] Embodiment 67: The method of any one of embodiments 64-66, wherein the first period of time is between about 5-10 days.

[0080] Embodiment 68: The method of any one of embodiments 64-67, wherein the second period of time is between about 1-14 days.

[0081] Embodiment 69: The method of any one of embodiments 64-68, wherein the second period of time is between about 5-10 days.

[0082] Embodiment 70: The method of any one of embodiments 64-69, wherein the third period of time is between about 20-70 days.

[0083] Embodiment 71: The method of any one of embodiments 64-70, wherein the third period of time is between about 50-70 days.

[0084] Embodiment 72: The method of any one of embodiments 64-71, wherein the fourth period of time is between about 7-70 days.

[0085] Embodiment 73: The method of any one of embodiments 64-72, wherein the fourth period of time is between about 20-60 days.

[0086] Embodiment 74: The method of any one of embodiments 65-73, wherein the portion of the fourth period of time is between about 7-21 days.

[0087] Embodiment 75: The method of any one of embodiments 64-74, wherein the BMP pathway is inhibited via a BMP pathway inhibitor comprising LDN-193189.

[0088] Embodiment 76: The method of any one of embodiments 64-75, wherein the BMP pathway is inhibited via a BMP pathway inhibitor provided at a concentration that is between about 0.1-2 μM .

[0089] Embodiment 77: The method of any one of embodiments 64-76, wherein the BMP pathway is inhibited via a BMP pathway inhibitor provided at a concentration that is between about 0.5-1.5 μM .

[0090] Embodiment 78: The method of any one of embodiments 64-77, wherein the TGF-beta pathway is inhibited via a TGF-beta pathway inhibitor and the second TGF-beta pathway is inhibited via a second TGF-beta pathway inhibitor.

[0091] Embodiment 79: The method of any one of embodiments 64-78, wherein the TGF-beta pathway is inhibited via a TGF-beta inhibitor comprising A83-01.

[0092] Embodiment 80: The method of any one of embodiments 64-79, wherein the TGF-beta pathway is inhibited via a TGF-beta pathway inhibitor provided at a concentration that is between about 0.5-4 μM .

[0093] Embodiment 81: The method of any one of embodiments 64-80, wherein the TGF-beta pathway is inhibited via a TGF-beta pathway inhibitor provided at a concentration that is between about 1-3 μM .

[0094] Embodiment 82: The method of any one of embodiments 64-81, wherein the second TGF-beta pathway is inhibited via a TGF-beta pathway inhibitor comprising SB-431542.

[0095] Embodiment 83: The method of any one of embodiments 64-82, wherein the second TGF-beta pathway is inhibited via a second TGF-beta pathway inhibitor provided at a concentration that is between about 0.1-2 μM .

[0096] Embodiment 84: The method of any one of embodiments 64-83, wherein the second TGF-beta pathway is inhibited via a second TGF-beta pathway inhibitor provided at a concentration that is between about 0.5-1.5 μM .

[0097] Embodiment 85: The method of any one of embodiments 64-84, wherein the Wnt pathway is inhibited via a Wnt pathway inhibitor comprising IWR-1.

[0098] Embodiment 86: The method of any one of embodiments 64-85, wherein the Wnt pathway is inhibited via a Wnt pathway inhibitor provided at a concentration that is between about 0.5-5 μM .

[0099] Embodiment 87: The method of any one of embodiments 64-86, wherein the Wnt pathway is inhibited via a Wnt pathway inhibitor provided at a concentration that is between about 2-4 μM .

[0100] Embodiment 88: The method of any one of embodiments 64-87, wherein the Wnt pathway is activated via a Wnt pathway activator comprising CHIR99021.

[0101] Embodiment 89: The method of any one of embodiments 64-88, wherein the Wnt pathway is activated via a Wnt pathway activator provided at a concentration that is between about 0.1-2 μM .

[0102] Embodiment 90: The method of any one of embodiments 64-89, wherein the Wnt pathway is activated via a Wnt pathway activator provided at a concentration that is between about 0.5-1.5 μM .

[0103] Embodiment 91: The method of any one of embodiments 64-90, wherein the insulin is provided at a concentration that is between about 0.5-5 $\mu\text{g/mL}$.

[0104] Embodiment 92: The method of any one of embodiments 64-91, wherein the insulin is provided at a concentration that is between about 1-3 $\mu\text{g/mL}$.

[0105] Embodiment 93: The method of any one of embodiments 65-92, wherein the LIF is provided at a concentration that is between about 1-20 mg/mL.

[0106] Embodiment 94: The method of any one of embodiments 65-93, wherein the LIF is provided at a concentration that is between about 5-15 mg/mL.

[0107] Embodiment 95: The method of any one of embodiments 64-94, wherein the brain organoid comprises astrocytes that express S100B, GFAP, and AQP4.

[0108] Embodiment 96: The method of any one of the preceding embodiments, wherein the blood vessel organoid and/or the brain organoid are human.

[0109] Embodiment 97: The method of any one of the preceding embodiments, wherein the blood vessel organoid and/or the brain organoid have been derived from a subject, optionally a human subject.

[0110] Embodiment 98: The method of embodiment 97, wherein the subject is afflicted by a cerebrovascular disease or a disease associated with blood-brain barrier dysfunction, optionally wherein the cerebrovascular disease or disease associated with blood-brain barrier dysfunction comprises cerebral cavernous malformation, Alzheimer's disease, or amyotrophic lateral sclerosis.

[0111] Embodiment 99: The method of any one of the preceding embodiments, wherein the blood vessel organoid and/or the brain organoid comprises cells having a genetic mutation associated with cerebral cavernous malformation (CCM) and/or are from a subject suffering from CCM; optionally wherein the blood vessel organoid but not the brain organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM; optionally wherein the brain organoid but not the blood vessel organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM; optionally wherein both the blood vessel organoid and the brain organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM.

[0112] Embodiment 100: The method of any one of the preceding embodiments, wherein the blood vessel organoid and/or the brain organoid are derived from pluripotent stem cells having a genetic mutation associated with CCM and/or from a subject suffering from CCM.

[0113] Embodiment 101: The method of embodiment 99 or 100, wherein the genetic mutation associated with CCM is a deletion or loss of function mutation of one or more of KRIT1, CCM2 and/or PDCD10.

[0114] Embodiment 102: The method of embodiment 101, wherein the genetic mutation associated with CCM is induced into the cell by a genetic modification technique, optionally using CRISPR.

[0115] Embodiment 103: A vascularized brain organoid produced by the method of any one of the preceding embodiments.

[0116] Embodiment 104: The vascularized brain organoid of embodiment 103, comprising a CCM-like feature, wherein the CCM-like feature is one or more of the following, as compared to a normal vascularized brain organoid: a cluster of enlarged endothelial channels, optionally arranged back-to-back; an upregulation of one or more lesion-marker genes, optionally in endothelial cells, optionally not in neuronal cells; an upregulation of tip cell and/or tumor tip cell markers, optionally in endothelial cells, optionally not in neuronal cells; a longer and/or a wider tip cell when evaluated in an angiogenesis assay; an expression of VEGFA and/or IGF2 in GABAergic neurons; a decrease in or an elimination of vascular smooth muscle cells (vSMCs); an increase in the number of astrocytes; a reduction in the expression of smooth muscle actin (SMA) protein, optionally measured by immunostaining; a reduction in expression of a tight junction protein; and/or a disassembled basement membrane.

[0117] Embodiment 105: The vascularized brain organoid of embodiment 104, wherein the CCM-like feature is a disruption, as compared to a normal vascularized brain organoid, of the blood-brain barrier that is formed between the brain organoid and all or a portion of the blood vessels of the blood vessel organoid that have infiltrated the brain organoid.

[0118] Embodiment 106: A vascularized brain organoid comprising a CCM-like feature and endothelial cells linked with tight junctions, astrocytes, and pericytes, optionally wherein the vascularized brain organoid is produced by the method of any one of embodiments 1-103, and optionally wherein the vascularized brain organoid having the CCM-like feature is any one of embodiments 104 or 105.

[0119] Embodiment 107: A vascularized brain organoid comprising a brain organoid and a blood vessel organoid, wherein at least a portion of the blood vessels of the blood vessel organoid have infiltrated the brain organoid, and neurons of the brain organoid innervate at least a portion of the infiltrating blood vessels.

[0120] Embodiment 108: The vascularized brain organoid of any one of embodiments 103-107, wherein the vascularized brain organoid has disease features, optionally wherein the disease features are CCM-like features.

[0121] Embodiment 109: The vascularized brain organoid of any one of embodiments 103-108, comprising a blood-brain barrier that is formed between the brain organoid and all or a portion of the blood vessels that have infiltrated the brain organoid.

[0122] Embodiment 110: The vascularized brain organoid of any one of embodiments 103-109, comprising gene expression markers indicative of excitatory neurons, inhibitory neurons, neural progenitors (NPs), astrocytes, endothelial cells (ECs), pericytes, mesenchymal stem cells (MSCs), smooth muscle cells (SMCs), and fibroblasts.

[0123] Embodiment 111: The vascularized brain organoid of any one of embodiments 103-110, comprising endothelial cells linked with tight junctions, astrocytes, pericytes, and smooth muscle cells, wherein the endothelial cells express CD31, GLUT-1 and PDGFR- β ; the tight junctions express claudin-5, ZO-1 and cadherin 5; the astrocytes express S100B, GFAP, and AQP4; the pericytes express PDGFR- β , α SMA and NG2; and/or the smooth muscle cells express SMA.

[0124] Embodiment 112: The vascularized brain organoid of any one of embodiments 103-111, wherein the endothelial cells form a continuous basement membrane and express collagen IV.

[0125] Embodiment 113: The vascularized brain organoid of any of embodiments 103-112 comprising cells selected from the group consisting of neural progenitors, proliferative astrocytes, GABAergic neurons, glutamatergic neurons, proliferative cells, brain vascular endothelial cells, vascular leptomeningeal cells, perivascular adipocytes, and tendon cells.

[0126] Embodiment 114: The vascularized brain organoid of embodiment 113, wherein the cells are identified by cell-type specific gene expression markers.

[0127] Embodiment 115: The vascularized brain organoid of any of embodiments 103-114, wherein the blood vessels comprise capillaries.

[0128] Embodiment 116: The vascularized brain organoid of any of embodiments 103-115, wherein the capillaries are ensheathed by pericytes and end-feet of the astrocytes.

[0129] Embodiment 117: The vascularized brain organoid of any of embodiments 103-116, wherein the brain organoid is a forebrain organoid, a midbrain organoid, a hypothalamus organoid, a hippocampus organoid, a spinal cord organoid, and/or a striatal brain organoid.

[0130] Embodiment 118: The vascularized brain organoid of any of embodiments 103-117, wherein the blood vessel organoid and the brain organoid were contacted and/or cultured in a basement membrane matrix or component thereof, optionally Matrigel.

[0131] Embodiment 119: The vascularized brain organoid of any of embodiments 103-118, wherein the vascularized brain organoid comprises cells having one or more genetic mutations and/or one or more phenotypes associated with neurodegenerative disorders, prion disease, Huntington disease, Alzheimer's disease, Fragile X syndrome, Parkinson's disease, amyotrophic lateral sclerosis (ALS), dementia, multiple sclerosis, encephalitis, epilepsy, infections, anxiety, bipolar disorder, depression, post-traumatic stress disorder, schizophrenia, attention deficit disorder, autism spectrum disorder, dyslexia, stroke, seizure, viral infection, bacterial infection, parasitic infection, traumatic brain injury, and/or cancer.

[0132] Embodiment 120: The vascularized brain organoid of any of embodiments 103-119, wherein the vascularized brain organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM.

[0133] Embodiment 121: The vascularized brain organoid of any of embodiments 103-120, wherein the vascularized brain organoid is derived from pluripotent stem cells having a genetic mutation associated with CCM and/or from a subject suffering from CCM.

[0134] Embodiment 122: The vascularized brain organoid of embodiment 121, wherein the genetic mutation associated with CCM is a deletion or loss of function mutation of one or more of KRIT1, CCM2 and/or PDCD10.

[0135] Embodiment 123: The vascularized brain organoid of embodiment 121 or 122, wherein the genetic mutation associated with CCM is induced into the cell by a genetic modification technique, optionally using CRISPR.

[0136] Embodiment 124: The vascularized brain organoid of any one of embodiments 105-123 comprising one or more CCM-like features, wherein the CCM-like feature is one or more of the following, as compared to a normal vascularized brain organoids and/or a normal brain tissue: a cluster of enlarged endothelial channels, optionally arranged back-to-back; an upregulation of one or more lesion-marker genes, optionally in endothelial cells, optionally not in neuronal cells;

an upregulation of tip cell and/or tumor tip cell markers, optionally in endothelial cells, optionally not in neuronal cells; a longer and/or a wider tip cell when evaluated in an angiogenesis assay; an expression of VEGFA and/or IGF2 in GABAergic neurons; a decrease in or an elimination of vascular smooth muscle cells (vSMCs); an increase in the number of astrocytes; a reduction in the expression of smooth muscle actin (SMA) protein, optionally measured by immunostaining; a reduction in expression of a tight junction protein; and/or a disassembled basement membrane.

[0137] Embodiment 125: The vascularized brain organoid of any one of the preceding embodiments, comprising one or more CCM-like feature, wherein the CCM-like feature is a disruption of the blood-brain barrier that is formed between the brain organoid and all or a portion of the blood vessels of the blood vessel organoid that have infiltrated the brain organoid, as compared to normal vascularized brain organoids and/or normal brain tissue.

[0138] Embodiment 126: The vascularized brain organoid of any one of the preceding embodiments, wherein the vascularized brain organoid exhibits one or more of the following features, as compared to the blood vessel organoids prior to culturing the blood vessel organoid and the brain organoid: reduced endothelial fenestration; increased expression of drug pumps; and/or reduced expression of an immune cell adhesion molecule marker.

[0139] Embodiment 127: The vascularized brain organoid of embodiment 126, wherein the reduced endothelial fenestration comprises reduced expression or non-expression of PLVAP.

[0140] Embodiment 128: The vascularized brain organoid of any one of embodiments 126 or 127, wherein the increased expression of drug pumps comprises increased expression of glucose transporter 1 (GLUT1), P-glycoprotein (P-gp), and/or one or more tight junction proteins.

[0141] Embodiment 129: The vascularized brain organoid of embodiment 128, wherein the one or more tight junction proteins comprises one or more of Claudin-5 or ZO-1.

[0142] Embodiment 130: The vascularized brain organoid of any one of embodiments 126-129, wherein the reduced expression of immune cell adhesion molecule marker comprises the reduced expression or non-expression of immune cell adhesion molecule 1 (ICAM-1).

[0143] Embodiment 131: The vascularized brain organoid of any one of the preceding embodiments, wherein the vascularized brain organoid exhibits the following features, as compared to the blood vessel organoids prior to culturing the blood vessel organoid and the brain organoid: reduced permeability; and increased trans-endothelial resistance.

[0144] Embodiment 132: The vascularized brain organoid of embodiment 131, wherein the increased trans-endothelial resistance comprises an increased transepithelial/trans-endothelial electrical resistance (TEER) value.

[0145] Embodiment 133: The vascularized brain organoid of embodiment 132, wherein an increase in TEER value of the vascularized brain organoid, as compared to a TEER value of the blood vessel organoids priori to culturing the blood vessel organoid and the brain organoid, is between about 850-1200 $\Omega \cdot \text{cm}^2$.

[0146] Embodiment 134: The vascularized brain organoid of embodiment 133, wherein an increase in TEER value of the vascularized brain organoid, as compared to a TEER value of the blood vessel organoids priori to culturing the blood vessel organoid and the brain organoid, is between about 950-1100 $\Omega \cdot \text{cm}^2$.

[0147] Embodiment 135: The vascularized brain organoid of any one of the preceding embodiments, wherein the vascularized brain organoid exhibits the following features, as compared to the brain organoid priori to culturing the blood vessel organoid and the brain organoid: increased numbers of mature astrocytes; more mature astrocytes; increased numbers of mature endothelial cells; and/or more mature endothelial cells.

[0148] Embodiment 136: A method of making a CCM primary tissue organoid, the method comprising: culturing primary cavernomas tissue in a culture medium and on ultra-low attachment plates with agitation, until CCM primary tissue organoids form.

[0149] Embodiment 137: The method of embodiment 136, wherein said culturing primary cavernomas tissue in culture medium until organoids form is for a period of time of about 1-2 weeks.

[0150] Embodiment 138: The method of any one of embodiments 136-137, wherein the method further comprises expanding the CCM primary tissue organoids by dissecting the CCM primary tissue organoids into pieces about 0.5 mm in diameter, and culturing the about 0.5 mm in diameter pieces until CCM primary tissue organoids form.

[0151] Embodiment 139: The method of any one of embodiments 136-138, wherein the method optionally comprises: a) incubating primary cavernomas tissue, as pieces having a diameter of about 0.5 mm, in red blood cell lysis buffer, optionally neutralizing the red blood cell lysis buffer at the end of the incubation; b) culturing, in ultra-low attachment plates with agitation, the primary cavernomas tissue pieces following the incubation in red blood cell lysis buffer,

optionally following the neutralization of the red blood cell lysis buffer; c) culturing at about 37°C, 5% CO₂ and/or 95% air; and/or d) changing the culture medium about every 3 days.

[0152] Embodiment 140: The method of any one of embodiments 136-139, wherein the culture media comprises about 10 ng/mL BDNF, about 10 ng/mL of GDNF, about 7.5% FBS, about 50 ng/mL VEGF-A, and about 50 ng/mL FGF-2, optionally wherein the culture media is half M4 complete media and half StemPro-34 SFM complete media, optionally further comprising about 0.025 mM cAMP, and about 0.1 mM of Ascorbic acid.

[0153] Embodiment 141: The method of any one of embodiments 136-140, wherein the cavernomas tissue is human.

[0154] Embodiment 142: The method of any one of embodiments 136-141, wherein the CCM-like feature is one or more of the following, as compared to a normal brain tissue: a cluster of enlarged endothelial channels, optionally arranged back-to-back; an upregulation of one or more lesion-marker genes, optionally in endothelial cells, optionally not in neuronal cells; an upregulation of tip cell and/or tumor tip cell markers, optionally in endothelial cells, optionally not in neuronal cells; a longer and/or a wider tip cell when evaluated in an angiogenesis assay; an expression of VEGFA and/or IGF2 in GABAergic neurons; a decrease in or an elimination of vascular smooth muscle cells (vSMCs); an increase in the number of astrocytes; a reduction in the expression of smooth muscle actin (SMA) protein, optionally measured by immunostaining; a reduction in expression of a tight junction protein; and/or a disassembled basement membrane.

[0155] Embodiment 143: A CCM primary tissue organoid made by the method of any one of embodiments 136-142.

[0156] Embodiment 144: A CCM primary tissue organoid comprising a CCM-like feature, optionally made by the method of any one of embodiments 136-143.

[0157] Embodiment 145: The CCM primary tissue organoid of any one of the preceding embodiments, wherein the CCM-like feature is one or more of the following, as compared to a normal brain tissue: a cluster of enlarged endothelial channels, optionally arranged back-to-back; an upregulation of one or more lesion-marker genes, optionally in endothelial cells, optionally not in neuronal cells; an upregulation of tip cell and/or tumor tip cell markers, optionally in endothelial cells, optionally not in neuronal cells; a longer and/or a wider tip cell when evaluated in an angiogenesis assay; an expression of VEGFA and/or IGF2 in GABAergic neurons; a decrease in or an elimination of vascular smooth muscle cells (vSMCs); an increase in the number of

astrocytes; a reduction in the expression of smooth muscle actin (SMA) protein, optionally measured by immunostaining; a reduction in expression of a tight junction protein; and/or a disassembled basement membrane.

[0158] Embodiment 146: The CCM primary tissue organoid of any one of the preceding embodiments, wherein the CCM-like feature is a disruption of the blood-brain barrier as compared to normal brain tissue.

[0159] Embodiment 147: A method of treating a cerebrovascular disease or a disease associated with blood-brain barrier dysfunction in a subject in need thereof, comprising administering to the subject the vascularized brain organoid of any one of embodiments 105-135, or a portion or fragment thereof, or the CCM primary tissue organoid of any one of embodiments 144-146, or a portion or fragment thereof.

[0160] Embodiment 148: A method of screening, comprising contacting the vascularized brain organoid of any of embodiments 105-135, or the CCM primary tissue organoid of any one of embodiments 144-146, or portions or fragments thereof, with a candidate compound or composition, and assessing the effects of the candidate compound or composition on the vascularized brain organoid, the CCM primary tissue organoid, or portions thereof.

[0161] Embodiment 149: The method of embodiment 148, wherein the effects comprises transport of the candidate compound or composition across the blood-brain barrier of the vascularized brain organoid, the CCM primary tissue organoid, or portions thereof.

[0162] Embodiment 150: The method of embodiment 148 or 149, wherein the vascularized brain organoid and/or CCM primary tissue organoid is a model for a cerebrovascular disease or a disease associated with blood-brain barrier dysfunction, and assessing the effects of the candidate compound or composition on the vascularized organoid and/or CCM primary tissue organoid comprises assessing the effects of the candidate compound or composition on the cerebrovascular disease or the disease associated with blood-brain barrier dysfunction.

[0163] Embodiment 151: The method of any one of embodiments 148-150, wherein the vascularized brain organoid and/or CCM primary tissue organoid has been produced from cells derived from a subject, optionally wherein the cells derived from the subject are induced pluripotent stem cells.

[0164] Embodiment 152: The method of embodiment 151, wherein the subject has or is disposed to develop the cerebrovascular disease or the disease associated with blood-brain barrier dysfunction.

[0165] Embodiment 153: The method of any one of embodiments 148-152, wherein the cerebrovascular disease or the disease associated with blood-brain barrier dysfunction is CCM.

[0166] Embodiment 154: The method of any one of embodiments 148-153, the cerebrovascular disease or the disease associated with blood-brain barrier dysfunction is a cerebrovascular disease or a disease associated with blood-brain barrier dysfunction in addition to CCM.

[0167] Embodiment 155: A cell culture media comprising, a first media component that promotes neuronal growth, and a second media component that promotes vascular growth.

[0168] Embodiment 156: The cell culture media of embodiment 155, wherein the first and/or second media component comprise added growth factors that promote neuronal growth and/or promote vascular growth.

[0169] Embodiment 157: The cell culture media of embodiment 155 or 156, wherein the added growth factors that promote neuronal growth comprise, consist essentially of, or consist of a cAMP pathway activator, ascorbic acid, BDNF, and/or GDNF.

[0170] Embodiment 158: The cell culture media of any one of embodiments 155-157, wherein the added growth factors that promote vascular growth comprise, consist essentially of, or consist of growth serum, a VEGF pathway activator, and/or an FGF pathway activator.

[0171] Embodiment 159: The cell culture media of any one of embodiments 155-158, further comprising a basement membrane matrix or component thereof.

[0172] Embodiment 160: The cell culture media of any one of embodiments 155-159, wherein the added cAMP pathway activator is cAMP.

[0173] Embodiment 161: The cell culture media of any one of embodiments 155-160, wherein the added cAMP pathway activator is at a concentration that is between about 10-150 μM .

[0174] Embodiment 162: The cell culture media of any one of embodiments 155-161, wherein the added cAMP pathway activator is at a concentration that is between about 20-100 μM .

[0175] Embodiment 163: The cell culture media of any one of embodiments 155-162, wherein the added ascorbic acid is at a concentration that is between about 50-300 μM .

- [0176]** Embodiment 164: The cell culture media of any one of embodiments 155-163, wherein the ascorbic acid is provided at a concentration that is between about 150 - 250 μ M.
- [0177]** Embodiment 165: The cell culture media of any one of embodiments 155-164, wherein the added BDNF is at a concentration that is between about 1-30 ng/mL.
- [0178]** Embodiment 166: The cell culture media of any one of embodiments 155-165, wherein the BDNF is provided at a concentration that is between about 15 - 25 ng/mL.
- [0179]** Embodiment 167: The cell culture media of any one of embodiments 155-166, wherein the added GDNF is at a concentration that is between about 1-30 ng/mL.
- [0180]** Embodiment 168: The cell culture media of any one of embodiments 155-167, wherein the GDNF is provided at a concentration that is between about 15 - 25 ng/mL.
- [0181]** Embodiment 169: The cell culture media of any one of embodiments 155-168, wherein the added growth factors are not xenogeneic to human cells, and/or are of good manufacturing practices (GMP) grade.
- [0182]** Embodiment 170: The cell culture media of any one of embodiments 155-169, wherein the added growth serum is fetal bovine serum (FBS).
- [0183]** Embodiment 171: The cell culture media of any one of embodiments 155-170, wherein the added growth serum is at a concentration that is between about 0.5%-20%.
- [0184]** Embodiment 172: The cell culture media of any one of embodiments 155-171, wherein the growth serum is provided at a concentration that is between about 12%-18%.
- [0185]** Embodiment 173: The cell culture media of any one of embodiments 155-172, wherein the VEGF pathway activator is VEGF.
- [0186]** Embodiment 174: The cell culture media of any one of embodiments 155-173, wherein the added VEGF pathway activator is at a concentration that is between about 10-150 ng/mL.
- [0187]** Embodiment 175: The cell culture media of any one of embodiments 155-174, wherein the VEGF pathway activator is provided at a concentration that is between about 80-120 ng/mL.
- [0188]** Embodiment 176: The cell culture media of any one of embodiments 155-175, wherein the FGF pathway activator is FGF2.
- [0189]** Embodiment 177: The cell culture media of any one of embodiments 155-176, wherein the added FGF pathway activator is at a concentration that is between about 10-150 ng/mL.
- [0190]** Embodiment 178: The cell culture media of any one of embodiments 155-177, wherein the FGF pathway activator is provided at a concentration between about 80-120 ng/mL.

[0191] Embodiment 179: The cell culture media of any one of embodiments 155-178, comprising about 10 ng/mL BDNF, about 10 ng/mL of GDNF, about 7.5% FBS, about 50 ng/mL VEGF-A, and about 50 ng/mL FGF-2, optionally wherein the culture media is, or is about, half M4 complete media and is, or is about, half StemPro-34 SFM complete media, optionally further comprising about 0.025 mM cAMP, and about 0.1 mM of Ascorbic acid.

[0192] Embodiment 180: The cell culture media of any one of embodiments 155-179, comprising a combination of half M4 complete medium and half StemPro-34 SFM complete medium, wherein the M4 complete medium comprises 0.05 mM of cAMP, 0.2 mM of Ascorbic acid, 20 ng/mL of BDNF, and 20 ng/mL of GDNF and M4 base medium, and wherein the StemPro-34 SFM complete medium comprises the StemPro-34 SFM base medium, supplemented with 15% FBS, 100 ng/mL VEGF-A, and 100 ng/mL FGF-2.

[0193] Embodiment 181: The cell culture media of any one of embodiments 155-180, further comprising blood vessel organoids and/or brain organoids, optionally wherein the blood vessel organoids and/or brain vessel organoids are derived from pluripotent stem cells, optionally embryonic stem cells or induced pluripotent stem cells.

[0194] Embodiment 182: A cell culture media for generating a vascularized brain organoid, the cell culture media comprising: a base endothelial cell (EC) media; a vascular endothelial growth factor A (VEGF-A); and an endothelial cell growth supplement (ECGS).

[0195] Embodiment 183: The cell culture media of embodiment 182, wherein the base EC media comprises FBS.

[0196] Embodiment 184: The cell culture media of embodiment 182 or 183, wherein the base EC media comprises FBS at a concentration of about 0.5% to about 20% of the cell culture media.

[0197] Embodiment 185: The cell culture media of any one of embodiments 181-184, wherein the ECGS comprises acidic FGF.

[0198] Embodiment 186: The cell culture media of any one of embodiments 181-185, wherein the ECGS comprises acidic FGF at a concentration of about 10 to 500 ng/ml.

[0199] Embodiment 187: The cell culture media of any one of embodiments 181-186, wherein the VEGF-A is at a concentration of about 10 to 500 ng/ml of cell culture media.

[0200] Embodiment 188: The cell culture media of any one of embodiments 181-187, wherein the VEGF-A is at a concentration of about 50 to 200 ng/ml of cell culture media.

[0201] Embodiment 189: The cell culture media of any one of embodiments any one of embodiments 181-188, further comprising: Heparin.

[0202] Embodiment 190: The cell culture media of embodiment 189, wherein the Heparin has a concentration of about 0 to 20 µg/ml of the cell culture media.

[0203] Embodiment 191: The cell culture media of embodiment 189 or 190, wherein the Heparin has a concentration of about 5 to 15 µg/ml of the cell culture media.

[0204] Embodiment 192: The cell culture media of any one of embodiments 181-191, wherein the base EC media comprises DMEM/F12.

[0205] Embodiment 193: A composition comprising the cell culture media of any one of embodiments 181-192, and further comprising: a vascularized brain organoid.

[0206] Embodiment 194: The composition of embodiments 193, wherein the vascularized brain organoid is produced by any one of embodiments 1-102.

[0207] Embodiment 195: The composition of embodiment 193 or 194, further comprising blood vessel organoids and/or brain organoids, optionally wherein the blood vessel organoids and/or brain vessel organoids are derived from pluripotent stem cells, optionally embryonic stem cells or induced pluripotent stem cells.

[0208] Embodiment 196: The composition of any one of embodiments 193-195, wherein the vascularized brain organoid comprises cells having one or more genetic mutations and/or one or more phenotypes associated with Fragile X syndrome.

[0209] Embodiment 197: The composition of embodiment 196, wherein the cells having the one or more phenotypes associated with Fragile X syndrome results in enlarged capillary perimeters and/or diameters in the vascularized organoid, compared to cells not having one or more genetic mutations and/or one or more phenotypes associated with Fragile X syndrome.

[0210] Embodiment 198: The composition of embodiment 196 or 197, wherein the cells having the one or more genetic mutations associated with Fragile X syndrome comprises cells having an upregulation of an angiogenic growth factor, compared to cells not having the one or more genetic mutations and/or one or more phenotypes associated with Fragile X syndrome.

[0211] Embodiment 199: The composition of any one of embodiments 196-198, wherein the cells having the one or more genetic mutations associated with Fragile X syndrome comprises cells having reduction of an Claudin-5, glucose transporter 1 (GLUT-1), and P-glycoprotein (P-gp),

compared to cells not having the one or more genetic mutations and/or one or more phenotypes associated with Fragile X syndrome.

[0212] Embodiment 200: The composition of any one of embodiments 196-199, wherein the cells having the one or more genetic mutations associated with Fragile X syndrome comprises cells with a hyperactivation of mTOR signaling pathway, compared to cells not having the one or more genetic mutations and/or one or more phenotypes associated with Fragile X syndrome.

[0213] Embodiment 201: The composition of any one of embodiments 196-200, wherein the cells having the one or more genetic mutations associated with Fragile X syndrome comprises cells with a downregulation of Wnt signaling pathway, compared to cells not having the one or more genetic mutations and/or one or more phenotypes associated with Fragile X syndrome.

[0214] Embodiment 202: The composition of any one of embodiments 193-201, wherein the vascularized brain organoid comprises cells having one or more genetic mutations and/or one or more phenotypes associated with Alzheimer's Disease.

[0215] Embodiment 203: The composition of any one of embodiments 193-202, wherein the cells having the one or more genetic mutations associated with Alzheimer's Disease comprises cells having reduction of an Claudin-5, glucose transporter 1 (GLUT-1), and P-glycoprotein (P-gp), compared to cells not having the one or more genetic mutations and/or one or more phenotypes associated with Alzheimer's Disease.

[0216] Embodiment 204: The composition of any one of embodiments 193-203, further comprising a viral vector, wherein the vascularized brain organoid exhibits one or more phenotypes associated with infection by the viral vector.

[0217] Embodiment 205: The composition of embodiment 204, wherein the one or more phenotypes associated with infection by the viral vector comprises: infected neurons; infected neural progenitors; and/or infected astrocytes.

[0218] Embodiment 206: A kit comprising means for performing the method according to any one of embodiments 1-102 and 148-154.

[0219] Embodiment 207: A kit comprising the vascularized brain organoid or CCM primary tissue organoid, or means for generating the vascularized brain organoid or CM primary tissue organoid of any one of embodiments 103-135.

[0220] Embodiment 208: A kit comprising the cell culture media, or means for generating the cell culture media, of any one of embodiments 155-192.

[0221] Embodiment 209: Use of the method, the vascularized brain organoid, the CCM primary tissue organoid, or cell culture media of any one of the preceding embodiments as a medicament, means for treatment and/or prevention of a disease, means for diagnosis, and/or medical research tool..

[0222] In addition to the features described herein, additional features and variations will be readily apparent from the following descriptions of the drawings and exemplary embodiments. It is to be understood that these drawings depict embodiments and are not intended to be limiting in scope.

BRIEF DESCRIPTION OF THE DRAWINGS

[0223] In addition to the features described herein, additional features and variations will be readily apparent from the following descriptions of the drawings and exemplary embodiments. It is to be understood that these drawings depict embodiments and are not intended to be limiting in scope.

[0224] **FIGS. 1A-1K** are schematics and images showing the assembly of human pluripotent stem cells (hPSCs)- derived cerebral cortical and blood vessel organoids to form vascularized brain organoids, according to example embodiments of the present disclosure.

[0225] **FIG. 1A** is a set of schematics showing an example method for generating vascularized brain organoids from human pluripotent stem cells (hPSCs). As shown in **FIG. 1A**, cerebral organoids and blood vessel organoids are generated separately and then assembled to mimic neurovascular co-development. For example, based on methods described herein, cerebral organoids and blood vessel organoids may undergo fusion to form an assembly of cerebral and blood vessel organoids, which is then cultured to form BBB assembloids.

[0226] **FIG. 1B** is a set of confocal images (e.g., brightfield and fluorescent images) showing an embodiment where an example of cerebral organoids, forebrain organoids, are differentiated from pluripotent stem cells. The fluorescent images show that, at an early stage of differentiation (Day 26), the forebrain organoids express the neuronal cell marker class III beta-tubulin (Tuj1) and neural stem cell marker SRY-Box transcription factor 2 (Sox2). The images also show that, at a later stage of differentiation (Day 61), the forebrain organoids express the neuronal cell markers B-cell lymphoma/leukemia 11B (BCL11B; Ct1p1) and T-Box brain transcription factor 1 (Tbr1).

[0227] **FIG. 1C.** is a set of confocal images showing an embodiment where the forebrain organoids differentiated from the pluripotent cells (as referenced in FIG. 1B) are cultured to induce astrocyte formation. The fluorescent images show that the forebrain organoids contain cells that are positive for astrocyte markers S100 calcium binding protein B (S100B) and glial fibrillary acidic protein (GFAP). Furthermore, the fluorescent images show that induced astrocytes of human cerebral cortical organoids displayed extensive cell soma and processes (arrowhead) and end-feet, as captured by the fluorescent labeling of aquaporin (AQP4). AQP4 is a selectively permeable water channel that is known to be characteristic feature in the brain.

[0228] **FIGS. 1D and 1E** are each a set of confocal images showing a whole-mount staining of blood vessel organoids for endothelial cells and pericytes on Day 15 derived from the H9-GFP line ($n = 4$ cultures). The endothelial cells are shown based on the marker CD31 and pericytes are shown based on the marker PDGFR- β . As previously discussed, vascular brain organoids are formed by culturing blood vessel organoids with cerebral organoids. Thus, the development of blood vessel organoids is an important aspect for the formation of vascular brain organoids. **FIG. 1E** is a closeup of **FIG. 1D**.

[0229] **FIG. 1F** is a set of representative images of cerebral-blood vessel assembloids at 7 days after assembling Day 15 H9-RFP vessel organoid (VO) and Day 75 H9-GFP cerebral organoid (CO) with excessive angiogenic front (arrows, red) at the periphery of fusion ($n = 5$ cultures). As shown, aspects of the cerebral-blood vessel assembloids stem from the cerebral organoid whereas other aspects of the cerebral-blood vessel assembloids stem from the vessel organoid.

[0230] **FIG. 1G** shows an extended vascular network in the cerebral organoid (as shown by the dashed line) from 14 and 28 days ($n = 4$ cultures). The expansion of the vascular network from Day 14 to Day 28 is indicative of actively growing brain vessels.

[0231] **FIG. 1H** is a box and whisker plot showing the time course analysis of vessel length in cerebral organoids after assembling with blood vessel organoids. As shown in FIG. 1H, the total length of the vessel length increases with time.

[0232] **FIG. 1I** is a set of confocal fluorescent images showing the development of CD31-positive brain capillaries within the forebrain organoid (see Brain Capillaries In fvFBO), and close association of GFAP-positive neural cells with the brain capillaries (See Neural Cells Innervate Brain Capillary). As shown in **FIG. 1I**, the development of CD31-positive brain capillaries within the forebrain organoid can be seen based on the appearance of CDH5 and GFAP markers.

Furthermore, the appearance of GFAP markers indicate the GFAP-positive neural cells innervating the brain capillaries.

[0233] **FIG. 1J** is a schematic illustrating a method of forming vessel organoids according to an example embodiment of the present disclosure. As shown in **FIG. 1J**, in at least one embodiment, vessel organoids may be derived from human pluripotent stem cells (hPSCs), whereby the HPSCs may be induced to form mesoderm using CHIR99021 and/or BMP-4. At about day 3, the mesoderm may be induced to form vascular lineage cells using VEGF-A and/or forsklin. At about day 5, the vascular lineage cells may be differentiated to vessel sprouts (e.g., angiogenic sprouts) using VEGF-A and/or FGF2, while being immersed in about 15% growth serum (e.g., about 15% FBS). At about Dat 10, the vessel sprouts may be matured to form vessel organoids using VEGF-A and/or FGF2 while being immersed in about 15% growth serum (e.g., about 15% FBS). In some embodiments, CHIR99021 may be used in the vessel sprouting and maturation processes.

[0234] **FIG. 1K** is a set of images showing an embodiment of brightfield and fluorescent images of blood vessel organoids differentiated from pluripotent stem cells according to the exemplary schematic of **FIG. 1J**. The fluorescent images show cells expressing GFP, which the initial pluripotent stem cells were engineered to express.

[0235] **FIGS. 2A-2L**. are a set of images, tables, and illustrations showing how the BBB assembloids described herein are able to recapitulate molecular, cellular, functional, and transcriptomic signatures of the *in vivo* human BBB, according to example embodiments of the present disclosure.

[0236] Specifically, **FIGS. 2A-2C** is a set of images showing that the majority of endothelial cells (ECs) of the BBB assembloids are able to express BBB markers GLUT1, ZO-1, and Claudin-5 by day 30 post-assembling with brain organoids (n = 3 cultures). The arrows indicate junctional-like structures in the BB assembloids.

[0237] **FIG. 2D** is a set of tables showing a quantification of these BBB marker expressions (for GLUT1, ZO-1, and Claudin-5) in blood vessel organoids (Vo), BBB assembloids, and human brain tissues (HB). As shown from these tables, the BBB assembloids were found to express these markers at similar levels to *in vivo* human BBB, thus showing that the BBB assembloids described herein can effectively replicate *in vivo* human BBB in various structural and/or functional aspects.

[0238] **FIG. 2E.** is a set of images of a brain endothelium of a BBB assembloid (CD31) covered by continuous Collagen IV basement membrane ($n = 3$ cultures), which is consistent with brain endothelium found in *in vivo* human BBB. **FIGS. 2F and 2G** are a set of images of orthogonal sections of the BBB assembloids showing GFAP-labeled astrocytic processes and AQP-4-labeled end-feet ensheathing capillary wall (arrow, green, $n = 3$ cultures). The presence of astrocytic processes and the end-feed ensheathing capillary walls show that the BBB assembloids are able to replicate the phenotypic features found in *in vivo* human BBB. **FIG. 2H** is a set of images showing a vascular network stained for CDH5 extending capillaries (arrow) into the cerebral organoid, which shows a tight innervation of the capillaries (derived from the blood vessel organoids) with the PDGFR- β -labeled pericyte processes (derived from the brain organoids) on Day 20 ($n = 3$ cultures). The composite image indicates a merging of the capillaries and the PDGFR- β -labeled pericyte processes. The innervation in this BBB assembloid thus replicates the innervation found in *in vivo* human BBB.

[0239] **FIG. 2I.** is a schematic showing cellular components and structures of BBB assembloids. As shown in **FIG. 2I**, the cellular components and structures may include but are not limited to endothelial cells, pericytes, tight junctions, neurons, astrocytes of neurons, astrocytic end feet, as well as blood vessel networks (e.g., capillaries) ensheathed by the astrocytic end feet

[0240] **FIG. 2J.** is a schematic showing a process for transepithelial/trans-endothelial electrical resistance (TEER) measurement in blood vessel organoids and hBBB assembloids ($n = 3$, three independent batches). As will be discussed herein, various embodiments of BBB assembloids of the present disclosure have increased trans-endothelial resistance relative to traditional brain organoids. In some embodiments, the increased trans-endothelial resistance may comprise or may be characterized by the TEER measurement.

[0241] **FIG. 2K** is a graph of a uniform manifold approximation and projection (UMAP) of single-cell transcriptome of human BBB assembloids ($n = 3$ batches, 28,062 cells) arranged by clusters.

[0242] **FIG. 2L** is a plot displaying a transcriptome-wide comparison of ECs in BBB assembloids with organ-specific ECs generated by the Tabula Muris Consortium. The similarity score is calculated by the Wilcoxon test. Scale bars: 200 nm in (C), 50 μm in (A), (B), and (D)-(G).

[0243] FIGS. 3A-3G are a set of images, graphs, and schematics showing the acquisition of BBB-specific markers via Wnt signaling activation, according to example embodiments of the present disclosure. **FIG. 3A.** is a schematic illustrating a process of isolating brain and vascular cells from BBB assembloids for transcriptomic analysis. **FIG. 3B** is a heatmap showing the statistical distance between RNA-seq datasets for three replicates from each group: cerebral organoid (CO), blood vessel organoid (VO), brain cells from Day 30 BBB assembloids without GFP (BBB-GFP⁻), and vessel cells from Day 30 BBB assembloids with GFP (BBB-GFP⁺). **FIG. 3C** is a volcano plot illustrating an example differential gene expression analysis between BBB-GFP⁺ cells and VO, with fold change > 2 and adjusted P-value < 0.01. **FIG. 3D** is a bubble map showing an example KEGG pathway enrichment analysis of differentially expressed genes (DEGs) between BBB-GFP⁺ cells and VO, displaying the top 30 enriched pathways. The color of the dot corresponds to the adjusted P-value, while the dot size represents the number of DEGs in the pathway. **FIG. 3E** is a heatmap displaying the differential expression of Wnt signaling genes between VO and BBB-GFP⁺ cells. The different shadings indicate higher and lower relative expression levels. **FIGS. 3F-3G** are a set of confocal images showcasing the remarkable elevation of GLUT1 and ZO-1 expression in vessel organoids (Vos) in the absence of neural tissue following CHIR 99021 treatment (n = 3 culture). Scale bars: 100 μ m in (**FIG. 3F**) and (**FIG. 3G**). ***p < 0.001. Data are shown as mean $\pm$ SEM.

[0244] FIGS. 4A-4E are sets of graphs and images showing the spatial transcriptomics analysis and development of BBB assembloids, according to example embodiments of the present disclosure. Specifically, **FIG. 4A-4B** are graphs showing the spatial clustering of spots and cluster-cell type associations for the BBB assembloids. Spots in each region of interest (ROI) are labeled by cell type. The spots then were used as the centroids of the nearest neighbor to assign spatial clusters to the other spots. **FIG. 4C** is a table thus showing the spatial domains in the ROI, each labeled with a cell type, as well as the statistical association between spatial clusters (columns) and cell types (rows). The radius of each dot shows the odds ratio. The shade of the dots shows the statistical significance of the association ($-\log_{10}$ p-value, chi-square test), ranging from not significant to highly significant. The yellow color shows the p-value threshold used to call an association significant (p-value = 0.015). For visualization purposes, a cutoff of 0.001 was used to represent the p-values in the dotplot. **FIGS. 4D-4E** are confocal images demonstrate the co-localization of endothelium (CD 31) with GABAergic neurons (GAD 67) and smooth muscle cells

(SMA) with glutamatergic neurons (CaMKII), respectively. Scale bars: 50 μ m in (D), (E). Data: mean $\pm$ SEM. The images show the development of phenotypic features of BBB assembloids (e.g., endothelium, GABAergic neurons) based their respective marker expressions.

[0245] **FIGS. 5A-5H** are a set of schematics, images, and graphs modeling cerebral cavernous malformations (CCMs) using patient-derived BBB assembloids according to example embodiments of the present disclosure. **FIG. 5A** is a representation illustrating the process of generating and comparing BBB assembloids derived from unaffected healthy controls and CCM patients. **FIG. 5B** is a set of sample confocal images showing the brain endothelium (stained by CD31) in BBB assembloids derived from controls and CCM patients. Notably, clusters of enlarged endothelial channels arranged back-to-back are observed in CCM BBB assembloids, signifying cavernous formation. Quantification of the average vessel width and lesion area in control and CCM BBB assembloids are also shown ($n = 3$ cultures). **FIG. 5C** is a set of images comparing ZO-1 expression in control and CCM BBB assembloids, with reduced ZO-1 observed in CCMs. The arrows highlight brain endothelium (shown via CD31 staining). **FIG. 5D** is a set of tables showing the quantification of ZO-1, Claudin-5, and Coll IV intensity in control and CCM BBB assembloids ($n = 3$ cultures). As shown in **FIG. 5D**, CCM samples expressed lower levels of ZO-1, Claudin-5, and Coll IV. **FIG. 5E** is a schematic showing a procedure for resecting primary cavernomas tissue, with confocal image showing enlarged endothelial channels in primary cavernomas ($n = 3$ cultures). **FIG. 5F** is a set of images showing a cascular network (based on CD31 expression) and Claudin-5 in postmortem brain tissue and primary cavernomas, with reduced Claudin-5 in cavernomas indicating BBB breakdown ($n = 3$ brain sections and 3 primary cavernomas sections). **FIG. 5G** is a set of confocal images of brain endothelium (CD31) and basement membrane (Coll IV) in brain tissue and primary cavernomas, with disrupted Coll IV expression in cavernomas indicating basement membrane disassembly ($n = 3$ brain sections and 3 primary cavernomas sections). **FIG. 5H** is a graph showing the quantification of vessel parameters and Claudin-5/Coll IV intensity ($n = 3$ brain sections and 3 primary cavernomas sections). Scale bars: 50 μ m in (B), (C), and (E)-(G). *** $p < 0.001$. Data: mean $\pm$ SEM.

[0246] **FIGS. 6A-6G.** is a set of graphs showing single cell transcriptomic analyses that reveal abnormal transcriptional profiles in vascular cell populations of CCM tissues, and reveal altered neuro-vascular interaction in CCM BBB assembloids as compared to control BBB assembloids. For example, **FIG. 6A-6B** is a UMAP visualization displaying cell states from control BBB

assembloids (n = 3 batches, 23,848 cells) and CCM BBB assembloids (n = 3 batches, 22,445 cells) colored by cell clusters (A) and by groups (B). **FIG. 6C** is a set of violin plots generated from the integrated dataset showing characteristic marker genes of each identified cell population in panel A. **FIG. 6D** is a heatmap illustrating selected known CCM lesion markers, presenting the average log fold change ($p_{adj} < 0.001$) of CCM versus control BBB assembloids. **FIG. 6E** is a heatmap demonstrating the average log fold change ($p_{adj} < 0.001$) of CCM versus control BBB assembloids for selected tip cell and tumor tip cell markers. **FIG. 6F** is a representative plot exhibiting comprehensive ligand-receptor pairs between neural and vascular cells for both control and CCM BBB assembloids. Sender cells in the center and receptor cells on the edges are colored by cell clusters, and the connections between sender and receptor cells are colored by ligand-receptor pairs. The size of rectangles represents interaction scores, and the width of connection lines represents probability scores. Notice the significant shift of ligand-receptor expression and top responsive vascular cells in CCM BBB assembloids compared with controls. **FIG. 6G** is a graph showing the top 10 ligand-receptor pairs between neural and vascular cells for both control and CCM BBB assembloids. The color and size of dots represent cell-cell communication (CCC) scores.

[0247] FIGS. 7A-7J are a set of graphs, schematics, and images showing the developmental loss of vascular smooth muscle cells in CCMs according to an example embodiment of the present disclosure. **FIG. 7A** is a set of UMAP plots of neuro-vascular cell clusters in BBB assembloids, with vascular smooth muscle cells (vSMCs) indicated by arrows. As shown in **FIG. 7A**, there is considerably less vSMCs in the CCM samples. **FIG. 7B** is a table showing the differential cell composition between control and CCM BBB assembloids, which also highlights the loss of vSMCs in CCMs (as indicated by the arrow). **FIG. 7C** is a STREAM visualization of developmental trajectories of mural cells, arranged by cell types and groups. **FIG. 7D** is a schematic depiction of vSMC loss from mesenchymal progenitors in CCMs as compared to normal mesenchymal progenitors. **FIGS. 7E-7G** are a set of confocal images and quantification revealing reduced smooth muscle actin expression in primary cavernomas tissue compared to healthy brain tissue (n = 3 samples). **FIGS. 7H-7J** are a set of images and quantification showing comparable nerve/glial-antigen 2 (NG2) expression between primary cavernomas tissue and healthy brain tissue (n = 3 samples). Scale bars: 50 μ m in (E), (F), (H), and (I). *** $p < 0.001$. NS denotes no significance. Data: mean $\pm$ SEM.

[0248] **FIGS. 8A-8L** are a set of schematics and images showing an example generation and characterization of cerebral organoids with astrocytic induction and blood vessel organoids according to example embodiments of the present disclosure. **FIG. 8A** is a schematic showing an example protocol and example timeline for the formation of cerebral organoids with astrocytic induction. **FIG. 8B** is a set of sample images showing the formation of cerebral organoids at different time points ($n = 3$ cultures). **FIG. 8C** is a set of representative images of cerebral organoids with or without astrocytic induction. Furthermore, **FIG. 8C** shows that human cerebral organoids after astrocytic induction remarkably increase the number of astrocytes labeled by S100B and GFAP compared to human cerebral organoids without astrocytic induction ($n = 3$ cultures). **FIG. 8D** is a 3D image of an example enriched GFAP+ human astrocytes in a tissue-cleared cerebral organoid after astrocytic induction ($n = 3$ cultures). **FIG. 8E** is a schematic showing an example protocol and example timeline for blood vessel organoid differentiation. **FIG. 8F** is a set of sample phase and fluorescence images showing the developmental timeline for blood vessel organoids, with mesoderm induction within 5 days, vascular lineage induction from Day 5 to 10, and blood vessel organoid formation after Day 15 ($n = 4$ cultures). **FIG. 8G** is a set of confocal images from vascular networks stained for CD31 to label endothelial cells, and stained for PDGFR- β to identify pericytes ($n = 3$ cultures). **FIG. 8G** shows the formation of a lumen from the z angle (as shown by the arrowhead). **FIGS. 8H-8L** are a set of images of example blood vessel organoids stained for CD31, GLUT1, ZO-1, Claudin-5, PLVAP, and immune cell adhesion molecule marker 1 (ICAM-1) ($n = 4$ cultures). As discussed, CD31 expression is used to identify endothelial cells. GLUT1 expression is an indicator of glucose transporter, and ZO-1 and Claudin-5 expression is an indicator of tight junction formation. Furthermore, PLVAP expression is an indicator of endothelial fenestration. ICAM-1 expression is an indicator of immune cell adhesion molecule. Scale bars: 50 μm in (H)-(L), 100 μm in (C) and (G), 200 μm in (D), 500 μm in (B) and (F).

[0249] **FIGS. 9A-9O** are a set of images and graphs characterizing and comparing example BBB assembloids with in vivo human BBB, according to example embodiments of the present disclosure. **FIG. 9A** shows low-magnification images of Day 30 BBB assembloids stained for ZO-1 ($n = 3$ cultures), with arrows indicating an endothelium. **FIGS. 9B-9D** are sets of images showing histological sections of human brain tissue ($n = 4$ brain sections) stained for BBB-specific markers: Claudin-5, ZO-1, GLUT1, and Coll IV. Arrows highlight junctional-like structures. **FIG. 9E** is a

Western blot analysis and quantification of Occludin, GLUT1, P-gp, and GAPDH in vessel organoids (VO), BBB assembloids, and human brain tissue (HB). As shown in FIG. 9E, the expression levels of the aforementioned markers in the BBB assembloids appear to closely align with those of the human brain tissue. **FIGS. 9F-9G** are images of Day 30 BBB assembloids stained for PLVAP and ICAM-1 (n = 3 cultures). **FIG. 9H** includes transmission electron microscopy images showing an ultrastructure of BBB assembloids (n = 3 cultures). In particular, FIG. 9H shows tight junctions (TJ), adherence junctions (AJ), and lumens (star) in the ultrastructure of the BBB assembloids. **FIG. 9I** is a set of confocal images demonstrating vascularization (arrows) of neuroepithelial rosettes (star) in BBB assembloids (n = 3 cultures). **FIG. 9J** is a set of confocal images of Day 30 BBB assembloids displaying neurons with extensive neurites (arrows) adjacent to brain capillaries (n = 3 cultures). **FIG. 9K** is an embodiment of a heat map showing the relative expression of cell markers in the cell types identified in the single cell RNA sequencing of the fused vascularized forebrain organoids. NPs1: neural progenitors; Ast: astrocytes; PAs: proliferative astrocytes, GABAs: GABAergic neurons; GluNs: glutaminergic neurons; VLMCs: vascular leptomeningeal cells; Brain VEs: brain vascular endothelial cells; VEs2: vascular endothelial cells 2; PAs: perivascular adipocytes; TCs: tendon cells; PCs: proliferative cells. **FIG. 9L** is a set of confocal images illustrating Day 21 and 45 BBB assembloids stained for Sox2 and LUM, with quantitative analysis revealing developmental decline of mesenchymal stem cells. **FIG. 9M** is a graph of a UMAP representation of the single-cell transcriptome of human BBB assembloids (n = 3 batches, 28,062 cells) color-coded by batch repeats. **FIGS. 9N-9O** are sets of confocal images of BBB assembloids stained for venous marker EphB4 and arterial marker EphrinB2. ***p < 0.001. Data presented as mean $\pm$ SEM. Scale bars: 200 nm in (H), 50 μ m in (B)-(D), (F), abd (J)-(M), 100 μ m in (I), 500 μ m in (A).

[0250] **FIGS. 10A-10E** are sets of graphs and images showing how vascular cells (e.g., from vessel organoids (VO) promote neurodevelopment, according to example embodiments of the present disclosure. **FIG. 10A** is a graph showing a Principal Component Analysis (PCA) of the normalized RNAseq data (transcripts per million, TPM) derived from the following groups: cerebral organoid (CO), blood vessel organoid (VO), brain cells isolated from Day 30 BBB assembloids without GFP (BBB-GFP-), and vascular cells isolated from Day 30 BBB assembloids with GFP (BBBGFP+). As shown in FIG. 10A, the PCA reveals two clusters – a vascular and a neural cluster, respectively, showing transcriptomic similarity within each cluster. Furthermore,

each cluster had two sub-clusters as shown, distinguished (in PC2) by the culture condition. **FIGS. 10B-10C** are representative confocal images depicting DMSO- and CHIR-treated blood vessel organoids (VO) and BBB assembloids stained for CD31 and LEF-1. Quantitative analysis indicates canonical Wnt activation, as evidenced by the increase in LEF-1 expression when assembled with neural tissues (as shown in **FIG. 10B**) as well as following CHIR administration (as shown in **FIG. 10C**). **FIG. 10D** is a volcano plot visualizing the differential gene expression analysis between BBB-GFP⁺ cells and CO, with fold change > 2 and adjusted P-value < 0.01. **FIG. 10E** is a graph showing a Gene Ontology (GO) term analysis of differentially expressed genes (DEGs) between BBB-GFP⁺ cells and CO, encompassing molecular function (MF), cellular component (CC), and biological process (BP), revealing the top 30 enrichment terms. The shade of the shape represents the adjusted P-value, while the size of the shape represents the gene ratio, indicating the percentage of total DEGs in the given GO term. ***p < 0.001. Data are shown as mean ± SEM. Scale bars: 50 μm.

[0251] **FIGS. 11A-11D** are sets of images showing an example characterization of cerebral and blood vessel organoids from unaffected health controls and CCM patients, according to example embodiments of the present disclosure. **FIG. 11A** is a set of sample images of cerebral organoids (COs) derived from both unaffected health controls and CCM patients at different time points (n = 3 cultures). **FIG. 11B** is a set of sample phase and fluorescence images illustrating an example developmental timeline for blood vessel organoids (VOs) derived from unaffected health controls and CCM patients. As shown in **FIG. 11B**, an example process involves mesoderm induction within about 5 days, vascular lineage induction from about Day 5 to about Day 10, and blood vessel organoid formation after about Day 15 (n = 4 cultures). **FIG. 11C** is a set of confocal images comparing a control and CCM vessel organoids (Vos) stained for CD31, revealing similar brain capillary morphology between the two groups (n = 3 cultures). **FIG. 11D** is a set of sample images showcasing control and CCM BBB assembloids, with the blood vessel cells visualized by GFP (n = 10 cultures). The “Cos” refer to cerebral organoids, “Vos” refer to blood vessel organoids, and “CCM” denotes patients with cerebral cavernous malformation. Scale bars: 100 μm in (C), 250 μm in (D), 500 μm in (A) and (B).

[0252] **FIGS. 12A-12D** are sets of graphs, images, and tables showing and comparing BBB markers in control and CCM BBB assembloids, according to example embodiments of the present disclosure. In particular, **FIG. 12A** is a UMAP visualization of integrated analysis of control and

CCM BBB assembloids arranged by clusters, while **FIG. 12B** is a heatmap generated from the integrated dataset showing characteristic marker genes of each identified cell population. Furthermore, **FIGS. 12C-12D** compare sprout and tip cell phenotypes in control and CCM BBB assembloids **FIG. 12C** is a set of sample images demonstrating enhanced sprout formation in CCM compared with control in an in vitro angiogenesis assay using a collagen-cultrex matrix. Tip cells are indicated by arrows ($n = 5$ cultures). **FIG. 12D** is a graph showing a quantification of the average width and total branch length of sprouts. Scale bar: 10 μm in (E). $*p < 0.05$, $***p < 0.001$. Data are shown as mean $\pm$ SEM.

[0253] FIGS. 13A-13E are a set of graphs showing a single-cell transcriptomics analysis revealing altered neuro-vascular interaction in control and CCM BBB assembloids, according to example embodiments of the present disclosure. Specifically, **FIGS. 13A-13B** are representative plots illustrating ligand-receptor pairs involved in communication among neurons to neurons, vascular to neurons, and vascular to vascular in both control and CCM BBB assembloids, as predicted by CellPhoneDB (**FIG. 13A**). Additionally, ligand-receptor pairs involved in communication among neurons to neurons, neurons to vascular, vascular to neurons, and vascular to vascular in both control and CCM BBB assembloids are depicted, as predicted by CellChat (**FIG. 13B**). Sender cells are displayed in the center, receptor cells on the edges, colored by cell clusters, and the connections between sender and receptor cells are colored based on the specific ligand-receptor pairs. The size of rectangles represents interaction scores, and the width of connection lines represents probability scores. Note the consistent patterns of predicted cell-cell interactions across both methodologies. Notice the significant shift in ligand-receptor expression, top sending vascular cell, and top responsive vascular cells in CCM BBB assembloids compared to controls. **FIGS. 13C-13D** Top 10 ligand-receptor pairs identified in the communication among neurons to neurons, vascular to neurons, and vascular to vascular for both control and CCM BBB assembloids, as predicted by CellPhoneDB (**FIG. 13C**). Additionally, the Top 10 ligandreceptor pairs identified in the communication among neurons to neurons, neurons to vascular, vascular to neurons, and vascular to vascular for both control and CCM BBB assembloids are depicted, as predicted by CellChat (**FIG. 13D**). The color and size of dots represent cell-cell communication (CCC) scores. **FIG. 13E** is a set of sample confocal images and quantification revealing a significant increase in VEGFR2 expression on CCM endothelium compared to controls ($n = 4$ cultures). Scale bar: 50 μm in (E). $***p < 0.001$. Data are shown as mean $\pm$ SEM.

[0254] FIGS. 14A-14D are sets of images and graphs showing distinct cell composition between control and CCM BBB assembloids, according to example embodiments of the present disclosure. FIG. 14A is a set of UMAP plots of neuro-vascular cell subclusters of BBB assembloids for each group, i.e., the control BBB assembloids and the CCM BBB assembloids. FIG. 14B is a STREAM visualization of developmental trajectories of mural cells by stream plots for both control and CCM BBB assembloids. While the top plot is coded by cell types, the bottom plot is coded based on the two groups. At a given pseudotime, the width of each branch is proportional to the total number of cells. FIG. 14C is a set of sample confocal images and quantification of LUM and CD31 staining for human postmortem brain tissue derived from healthy subjects and primary cavernomas tissue (n = 3 brain sections and 3 primary cavernomas tissue sections). FIG. 14D is a set of confocal images showing the expression of nerve/glial-antigen 2 (NG2) and CD31 staining for in human postmortem brain tissue derived from healthy subjects and CCM organoids. In particular, the images show that mural cells marked by the expression of NG2 and LUM remain unchanged between CCM and normal BBB organoids. Scale bar: 50 μ m in (C). ***p < 0.001. Data are shown as mean $\pm$ SEM.

[0255] FIGS. 15A-15G are a set of graphs illustrating a transcriptomic analysis of vascularized brain organoids, according to example embodiments of the present disclosure.

[0256] FIG. 15A depicts an embodiment of single cell RNA sequencing of fused vascularized forebrain organoids. The data shows the presence of multiple cell types that make up the cerebrovascular network, including neurons, astrocytes, and endothelial cells.

[0257] FIG. 15B depicts an embodiment of the single cell RNA sequencing data showing the presence of multiple sub-clusters of endothelial cells, suggesting the presence of a diverse population of cells in the fused vascularized forebrain organoids.

[0258] FIG. 15C depicts an embodiment of a heat map showing the relative expression of cell markers in the cell types identified in the single cell RNA sequencing of the fused vascularized forebrain organoids. NPs1: neural progenitors; Ast: astrocytes; PAs: proliferative astrocytes, GABAs: GABAergic neurons; GluNs: glutaminergic neurons; VLMCs: vascular leptomeningeal cells; Brain VEs: brain vascular endothelial cells; VEs2: vascular endothelial cells 2; PAs: perivascular adipocytes; TCs: tendon cells; PCs: proliferative cells.

[0259] **FIG. 15D** depicts an embodiment of a violin plot showing the expression of various neuronal and vascular markers in the cell types identified in the single cell RNA sequencing of the fused vascularized forebrain organoids.

[0260] **FIG. 15E** depicts an embodiment of a map showing relative prevalence of potential interactions between protein receptors and ligands expressed by two different cell types identified in the single cell RNA sequencing of the fused vascularized forebrain organoids. The data points shown here relate to crosstalk from vascular cell types to vascular cell types. NPs1: neural progenitors 1; NPs2: neural progenitors 2; PPs: proliferative progenitors; GABAs: GABAergic neurons; GluNs: glutaminergic neurons; VLMCs: vascular leptomeningeal cells; VEs1: vascular endothelial cells 1; VEs2: vascular endothelial cells 2; Pas: perivascular adipocytes; TCs: tendon cells; PCs: proliferative cells.

[0261] **FIG. 15F** depicts an embodiment of a map showing relative prevalence of potential interactions between protein receptors and ligands expressed by two different cell types identified in the single cell RNA sequencing of the fused vascularized forebrain organoids. The data points shown here relate to crosstalk from neural cell types to vascular cell types.

[0262] **FIG. 15G** depicts an embodiment of a map showing relative prevalence of potential interactions between protein receptors and ligands expressed by two different cell types identified in the single cell RNA sequencing of the fused vascularized forebrain organoids. The data points shown here relate to crosstalk from vascular cell types to neural cell types.

[0263] **FIGS. 16A-16G** are graphs showing the spatial transcriptomics analysis of BBB assembloids, according to example embodiments of the present disclosure. In particular, **FIGS 16A-16D** are graphs illustrating spots in the region of interest (ROI) labeled by cell type. **FIG. 16A**. The 2,380 spots associated with each cell type in the legend. Red colors are used for vascular cell types, blue colors are used for brain cell types. **FIG. 16B** is a graph showing the subset of 692 spots associated with vascular (EC-related) cell types. In **FIG. 16C**, the graph shows the 143,480 spots in the ROI, each labeled with a cell type. The 2,380 spots in **FIG. 16A** were used as the centroids of the nearest neighbor to assign cell types to the other spots. **FIG. 16D** is a graph showing the subset of 42,549 spots associated with vascular (EC-related) cell types. **FIG. 16E-G** are graphs showing the spatial clustering of spots and cluster-cell type associations. **FIG. 16E** is a graph showing the statistical association between spatial clusters (columns) and cell types (rows). The radius of each dot shows the odds ratio. The color of the dots shows the statistical significance

of the association ($-\log_{10}$ p-value, chi-square test), ranging from blue (not significant) to orange (highly significant). The yellow color shows the p-value threshold used to call an association significant (p-value = 0.015). For visualization purposes, a cutoff of 0.001 was used to represent the p-values in the dotplot. **FIG. 16F** is a graph where the 2,380 spots are labeled by spatial cluster and associated cell types in the legend. Red colors are used for vascular cell types, blue colors are used for brain cell types. **FIG. 16G** is a graph showing all the 143,480 spots in the ROI, each labeled by cluster. The 2,380 spots in **FIG. 16F** were used as the centroids of the nearest neighbor to assign spatial clusters to the other spots.

[0264] **FIGS. 17A-17D** are sets of images and graphs showing an example hypervascularization of BBB assembloids as a result of Fragile X Syndrome (FXS), according to example embodiments of the present disclosure. **FIG. 17A** is a confocal image of a Day 30 BBB assembloid derived from an FXS subject. The GFP-labeled vascular cells in the BBB assembloid express CD31 and GLUT-1 as shown in the image, which is an indicator of an endothelium and a glucose transport protein. **FIG. 17B** is a graph showing a Western blot analysis of Fragile X mental retardation protein (FMRP) expression in BBB assembloids derived from five healthy controls and four FXS subjects. As will be discussed herein, FXS results from expanded trinucleotide (CGG) repeats in the fragile X mental retardation syndrome 1 gene (FMR1) gene, leading to transcriptional silencing and loss of FMRP. The absence of FMRP in the four FXS subjects in **FIG. 17B** is consistent with the transcriptional silencing and loss of FMRP. **FIGS. 17C-17D** are a set of sample confocal images and statistical analyses showing hypervascularization in FXS BBB assembloids. In **FIG. 17C**, the CD31 marker is used as an indicator of an endothelium. In **FIG. 17D**, the CHD5 marker is used as an indicator of endothelium. (n = 3 biological repeats. *** indicates P-value < 0.001.).

[0265] **FIG. 18A-18H** are sets of images and graphs showing a breakdown in BBB, which is characteristic of FXS, according to example embodiments of the present disclosure. In particular, **FIGS. 18A-18B** are sample images and statistical analyses showing a reduction in the expression of Claudin-5, which is a marker of junction-like structures in the endothelium. Such structures are shown in arrows. Specifically, **FIG. 18A** compares BBB assembloids of a FXS sample to a control sample whereas **FIG. 18B** compares a mouse model to a wild type. As shown in **FIG. 18A**, the reduced expression of the marker Claudin-5 is also evident in the disassembly of the junction-like structures as shown by the arrows. **FIGS. 18C-18E** are sample images showing GLUT-1

expression on endothelium (arrows) in BBB assembloids of FXS and control samples (**FIG. 18C**), in Fmr1 Knockout mice (e.g., knockout mice with the activated fragile x mental retardation gene) and wild types (**FIG. 18D**), and in FXS postmortem brain tissues (**FIG. 18E**). As shown by these graphs, GLUT1 expression is reduced in FXS samples. This is validated by statistical analysis present in **FIGS. 18F**. **FIG. 18G** is a graph of a Western blot analysis showing expression reduction of BBB tight junction and transporter proteins (Occludin, GAPDH, P-gp, and GLUT-1) in FXS samples compared to the control samples. **FIG. 18H** is a graph showing the expression reduction of GLUT-1, Occludin, and P-gp in the FXS samples, compared to the control samples, when normalized to GAPDH expressions. n = 3 biological repeats for BBB assembloids, mouse models, and postmortem brain tissues. *** indicate P-value < 0.001.

[0266] **FIGS. 19A-19C** are a set of graphs showing a transcriptomic analysis of BBB markers found in vascularized brain organoids derived from FXS patients as compared to control samples healthy vascularized brain organoids, according to example embodiments of the present disclosure. Specifically, **FIG. 19A** is a uniform manifold approximation and projection (UMAP) of single-cell transcriptome arranged by clusters; **FIG. 19B** is a set of violin plots showing characteristic marker genes of each identified cell clusters; and **FIG. 19C** is a heatmap displaying known BBB tight junction and transporter, angiogenic growth factors and receptors, mTOR signaling, and Wnt signaling genes, showing average log fold change (padj < 0.001) in FXS versus healthy control BBB assembloids.

[0267] **FIGS. 20A-20F** are a set of graphs and images showing mTOR hyperactivation and Wnt inhibition, characteristic of FXS, according to example embodiments of the present disclosure. **FIGS. 20A-20B** are graphs of a Western blot analysis showing elevated phosphorylation of Akt (to form pAkt) and S6 (to form pS6) in patient-derived lymphoblastoid cells LCLs from FXS patients compared to a control group (**FIG. 20A**) and increased phosphorylation of 110β (to form p110β) in Fmr1 KO mice (**FIG. 20B**). The phosphorylated compounds p110β, phospho-S6 (pS6), and phospho-Akt (pAkt) are downstream effectors of the mTOR pathway, thus indicating mTOR hyperactivation in FXS. **FIGS. 20C-20D** are a set of sample images displaying increased vascular endothelial growth factor receptor 2 (VEGFR2) expression in FXS BBB assembloids, which is also an indicator of hyperactivation of the mTOR pathway. **FIG. 20E** is a graph of a Western blot analysis showing reduced lymphoid enhancer factor (LEF) expression in FXS BBB assembloids as compared to a control group. **FIG. 20F** is a

table that also shows the reduction in LEF expression, normalized to GAPDH. As LEF is a downstream transcriptional factor of the Wnt signaling pathways, the reduction indicates an inhibition of the Wnt signaling pathway in FXS samples. n = 4 or 5 biological replicates. *** indicate P-value < 0.001. * indicate P-value < 0.05.

[0268] **FIGS. 21A-21E** are a set of graphs showing how FMRP target mRNAs encoding mTOR and Wnt components, which is characteristic of FXS, according to example embodiments of the present disclosure. **FIG. 21A** is a graph of a Western blot of flag immunoprecipitation of flag-tagged mCherry-FMRP and flag-tagged mCherry, along with their inputs. The blot was detected with FMRP (green) and β -Actin (red). **FIG. 21B** is a table indicating mRNA enrichment of p110 β , β -Actin, and β -catenin by RT-PCR in FMRP samples compared to mCherry samples (control). **FIGS. 21C-21D** are tables of RT-PCR quantification showing that p110 β significantly increased in puromycin-sensitive polysome fractions from Fmr1 Knockout mice compared to wild type (WT). PSD95 and NR1 mRNAs served as positive and negative controls, respectively. **FIG. 21E** is a graph of an mRNA stability assay in control siRNA (siCTR) and FMR1 siRNA (siFMR1) HEK293T cells. RNA was isolated at the indicated time points after actinomycin D treatment, and the stability of CTNNB1 mRNAs was analyzed by RT-qPCR. *** indicate P-value < 0.001. ** indicate P-value < 0.01. * indicate P-value < 0.05.

[0269] **FIG. 22** is a schematic diagram showing the biochemical pathways leading to the phenotypic effects of FXS on BBB, as well as a method of using BBB assembloids and vascular organoids to model FXS, according to example embodiments of the present disclosure. As shown in **FIG. 22**, BBB assembloids produced using methods described herein may be used to model various phenotypes associated with FXS, such as hypervascularization and BBB breakdown. As further shown in **FIG. 22**, hypervascularization may be based on an expression of vascular endothelial growth factor triggering, via VEGFR, a pathway causing an upregulation in P13K, AKT, mTORC1, eIF4E, and VEGF, which may trigger angiogenesis. Furthermore, an inhibition of mTORC1 may reverse and/or attenuate this process. Rapamycin is presented as a solution for inhibiting mTORC1, and thus reversing mTOR hyperactivation. As will be discussed herein, Rapamycin treatment (10 nM for 2 weeks) administered on Day 30 BBB assembloids derived from CCM patients can ameliorate disease-related hypervascularization and reduce VEGFR expression in these assembloids. **FIG. 22** also shows that BBB breakdown is a result of an inhibition of the Wnt signaling pathway. Given the crucial role of the Wnt/ β -catenin signaling pathway in BBB

formation and integrity, pharmacological activation of Wnt signaling using Wnt activators like CHIR99021 and clinically available Wnt activators such as lithium can rescue BBB integrity in FXS.

[0270] **FIG. 23** is a set of confocal images and a table showing the ability of rapamycin to treat diseases affected by hyperactivation of the mTOR signaling pathway, such as FXS and cerebral cavernomas malformation (CCM), according to example embodiments of the present disclosure. The sample images and statistical analysis demonstrate that rapamycin significantly reduces vessel width and VEGFR expression in CCM BBB assembloids. $n = 4$ cultures. *** indicate P-value < 0.001 .

[0271] **FIG. 24** is a set of confocal images showing the expression of BBB markers in BBB samples from Alzheimer's Disease (AD) patients compared to normal patients (control), according to example embodiments of the present disclosure. Specifically, the top confocal images show a reduction of expression of the tight junction marker (Claudin-5) in AD BBB samples as compared to the control. The bottom confocal images show a reduction of expression of the glucose transporter protein (GLUT-1) in AD BBB samples as compared to the control.

[0272] **FIGS. 25A-25B** are a set of graphs of a transcriptomic analysis of BBB markers found in vascularized brain organoids derived from Alzheimer's Disease (AD) patients as compared to control samples healthy vascularized brain organoids, according to example embodiments of the present disclosure. Specifically, **FIG. 25A** is a UMAP visualization displaying cell clusters from a control sample of vascularized brain organoid derived from healthy BBB assembloids ($n = 2$, 17,540 cells) and a sample of vascularized brain organoid derived from AD BBB assembloids ($n = 2$, 18,573 cells); while **FIG. 25B** is a set of violin plots showing characteristic marker genes of each identified cell clusters. The Single-cell transcriptomics analysis shows markers for each neurovascular and blood-brain barrier (BBB) unit, including astrocytes (Ast), neural progenitors (NPs), GABAergic neurons (GABAs), mesenchymal stem cells (MSCs), endothelial cells (ECs), pericytes (Peri), vascular smooth muscle cells (vSMCs), fibroblasts (Fib), and proliferative cells (PCs).

[0273] **FIGS. 26A-26C** is a set of tables and graphs comparing cell to cell communications between AD BBB assembloids and healthy BBB assembloids (control), according to example embodiments of the present disclosure. **FIG. 26A** is a bar plot showing the number and strength of global cell-cell communications between the AD and control samples. **FIGS. 26B-26C** are

graphs showing the differential ligand-receptor interactions in terms of number (**FIG. 26B**) and strength (**FIG. 26C**) between AD and the controls. The thick and thin lines indicate reduced and increased interactions, respectively. Using the CellChat R package, altered cell-cell communications were predicted for the AD samples. As shown in **FIGS. 26A-26C**, there is a global decrease in the number and strength of cell communications in AD BBB assembloids, indicating disconnections among neurovascular cells underlying AD pathology.

[0274] **FIGS. 27A-27C** are sets of sample images showing the ability of different viral vectors to infect various aspects of BBB assembloids, according to example embodiments of the present disclosure. The viral vectors being compared are rAAV.eB and rAAV9, both labeled with GFP. These vectors were micro-injected into the vessels of the two groups of BBB assembloids, and the viral infections were assessed two weeks later. In particular, the use of the marker DCX in **FIG. 27A** captures the ability of viral vectors to infect neuron. The use of the marker SOX2 in **FIG. 27B** captures the ability of viral vectors to infect neural progenitors. The use of the marker S100B in **FIG. 27C** captures the ability of viral vectors to infect astrocytes. **FIGS. 27A-27C** show that there was a significant increase in the infection rate of human neurons, astrocytes, and neural progenitors by rAAV.eB compared to rAAV9, indicating higher translational potential for rAAV.eB.

DETAILED DESCRIPTION

[0275] The blood-brain barrier (BBB) offers a significant boundary to limit the exposure of the central nervous system from the rest of the body by regulating transport of essential molecules such as oxygen, carbon dioxide, and nutrients, but preventing the crossing of other molecules and larger biological entities such as cells and pathogens. The BBB is mediated by the formation of tight junctions between endothelial cells that make up blood vessels and capillaries in the brain. Additional cells in proximity such as astrocytes and pericytes also support the endothelial cells to maintain the BBB. For a model of the BBB to be representative of the natural structure, this highly organized structure forming a boundary between vascular and neuronal portions of the model is necessary.

[0276] Described herein are human blood-brain barrier models produced by vascularizing human brain organoids. These blood-brain barrier models may be used to model and study brain vascular disorders. These vascularized brain organoids may be transplanted *in vivo*, such as in the cortex of a mouse, to reconstitute active brain perfusion and integrate the organoid with living animals for advanced functional and *in vivo* study of the blood-brain barrier. These organoids also serve as a powerful drug screening platform to evaluate drug delivery across the blood-brain barrier.

[0277] In some embodiments, provided herein are data demonstrating the acquisition of important BBB signatures in vascularized brain organoids (aka BBB assembloids) described herein, including the reduction of endothelial fenestration, expression of drug pumps, and/or reduction of immune cell adhesion molecule marker expression. In some embodiments, BBB assembloids of the disclosure have a reduction in fenestration relative to traditional brain organoids (e.g., contemporary brain organoids, control brain organoids, contemporary 3D “hBBB” models). In some embodiments, BBB assembloids of the disclosure have increased expression of drug pumps relative to traditional brain organoids. In some embodiments, BBB assembloids of the disclosure have a reduction of immune cell adhesion molecule marker expression relative to traditional brain organoids. For example, the reduced endothelial fenestration may comprise reduced expression or non-expression of PLVAP. The increased expression of drug pumps may comprise increased expression of glucose transporter 1 (GLUT1) and one or more tight junction proteins. In some aspects, the one or more tight junction proteins comprises one or more of Claudin-5 or ZO-1. Furthermore, in some aspects, the reduced expression of immune cell adhesion molecule marker may comprise the reduced expression or non-expression of immune cell adhesion molecule 1 (ICAM-1).

[0278] In some embodiments, provided herein are data demonstrating the functional maturation of BBB assembloids of the disclosure, including mature phenotypes such as reduced permeability and/or increased trans-endothelial resistance. In some embodiments, BBB assembloids of the disclosure have reduced permeability relative to traditional brain organoids. In some embodiments, BBB assembloids of the disclosure have increased trans-endothelial resistance relative to traditional brain organoids. In some embodiments, the increased trans-endothelial resistance may comprise or may be characterized by an increased transepithelial/trans-endothelial electrical resistance (TEER) value. For example, the increase in TEER value of the vascularized

brain organoid, as compared to a TEER value of the blood vessel organoids prior to culturing the blood vessel organoid and the brain organoid, may be about 850, 860, 870, 880, 890, 900, 910, 920, 930, 940, 950, 960, 970, 980, 990, 1000, 1010, 1020, 1030, 1040, 1050, 1060, 1070, 1080, 1090, 1100, 1110, 1120, 1130, 1140, 1150, 1160, 1170, 1180, 1190, or 1200 $\Omega \cdot \text{cm}^2$, or any value within a range defined by any two of the aforementioned values, optionally about 1022.1 $\Omega \cdot \text{cm}^2$.

[0279] In some embodiments, provided herein are data demonstrating that BBB assembloids of the disclosure acquire transcriptomic profiles indistinguishable, or near indistinguishable, relative to those of human brain tissues. In some embodiments, BBB assembloids of the disclosure have increased numbers of mature astrocytes, more mature astrocytes, increased numbers of mature endothelial cells, and/or more mature endothelial cells, relative to traditional brain organoids. In some embodiments, BBB assembloids of the disclosure have acquired astrocytic and endothelial cell gene expression patterns indistinguishable, or near indistinguishable, relative to those of human brain tissues.

[0280] In some embodiments, provided herein are new and improved blood vessel media and/or culture protocols for creation of blood vessel organoids, wherein the new media and/or culture protocols improve the expression of select proteins relative to blood vessel organoids produced through traditional means. In some embodiments, blood vessel organoids provided herein have increased levels of complex and highly branched CD31-positive endothelial networks. In some embodiments, blood vessel organoids provided herein have increased tight interactions with pericytes as characterized by the molecular marker PDGFR β and/or tube-like structures featuring discernible lumens. In some embodiments, provided herein are compositions comprising a mixture of cerebral organoid maturation media, and blood vessel organoid maturation media (aka BBB maturation media), which can facilitate and/or maintain both neuroepithelial and vascular endothelial cellular identities.

[0281] In some embodiments, provided herein are methods of using BBB assembloids to study disease and/or disorder states such as but not limited to, neurodegenerative disorders, prion disease, Huntington disease, Alzheimer's disease, Fragile X syndrome, Parkinson's disease, amyotrophic lateral sclerosis (ALS), dementia, multiple sclerosis, encephalitis, epilepsy, infections, anxiety, bipolar disorder, depression, post-traumatic stress disorder, schizophrenia, attention deficit disorder, autism spectrum disorder, dyslexia, stroke, seizure, viral infection, bacterial infection, parasitic infection, traumatic brain injury, and/or cancer.

[0282] Also provided herein are methods of utilizing BBB assembloids to determine the capacity of one or more substances to cross the blood brain barrier, including but not limited to, for example, bacterial agents, viral agents, parasitic agents, toxins, retrovirus, lentivirus, adenovirus, adeno-associated virus (AAV), alphavirus, herpesvirus, liposomes, synthetic small molecules, naturally occurring small molecules, proteins, polypeptides, carbohydrates, and/or lipids.

[0283] In some embodiments, BBB assembloids provided herein comprise numerous advantages over traditional brain organoids and/or alternative human neuro-vascular co-culture and BBB models. In some embodiments, BBB assembloids provided herein comprise a complete neurovascular unit that includes various types of neurons, neural progenitors, astrocytes, endothelial cells, pericytes, mesenchymal stem cells, vascular smooth muscle cells, and fibroblasts. In some embodiments, BBB assembloids provided herein recapitulate key molecular, cellular, anatomical, functional, and transcriptomic characteristics of the BBB, including 1) the formation of capillary walls by endothelial cells through the assembly of tight-junction complexes, 2) endothelial acquisition of BBB-specific transporter expression and absence of fenestration marker expression, 3) pericyte processes and astrocytic end-feet ensheathing the endothelium, 4) a basement membrane covering the endothelium, 5) the establishment of BBB functionality with reduced permeability, and 6) endothelial cells that express a BBB-specific transcriptome. In some embodiments, BBB assembloids provided herein recapitulate more elaborate brain vascularization processes in neuro-vascular co-development. In some embodiments, BBB assembloids provided herein have robust Wnt signaling that drives endothelial acquisition of BBB features. In some embodiments, technologies provided herein, such as BBB assembloids, methods of making the same, and methods of using the same, model with robustness the *in vivo* human BBB, and provide highly valuable tools for myriad studies, including but not limited to the study of BBB development, BBB pathology, human disease, injury, and CNS drug development.

[0284] The following are non-limiting embodiments of methods and compositions of vascularized brain organoids (aka BBB assembloids) and CCM primary tissue organoids.

An assembly of brain and blood vessel organoids

[0285] The development of the central nervous system (CNS) involves the growth of blood vessels that contribute to the formation of the blood-brain barrier (BBB). However, it is challenging to pattern human pluripotent stem cells (hPSCs) to neuroectoderm and vascular

mesoderm simultaneously in one culture. Accordingly, to overcome the limitation and better understand the interactions between neuroepithelium and primitive capillary plexus tissues in human neurovascular development and BBB formation, various embodiments of the present disclosure describe a multistep protocol designed to generate cerebral brain and blood vessel organoids separately and assemble them to mimic neurovascular co-development (e.g., as shown in FIG. 1A). Cerebral organoids are characterized by self-organization of complex tissue architectures similar to the developing mammalian brain. An improved cerebral organoid protocol (e.g., as shown in FIG. 8A) is used to induce human embryonic stem cells (ESCs) H9 to cerebral organoids (e.g., as shown in FIG. 8B). Different developmental stages of cerebral organoids were stained with neural progenitor marker Sox2, mature neuron marker Tuj1, and cortical layer markers Ctip2 and Tbr1 (e.g., as shown in FIG. 1B), which indicate successful induction of neuroepithelial cell fate. Astrocytes are an important component of BBB and can regulate the interactions between blood flow and neuronal activities by extending their end-feet to wrap endothelium. However, contemporary cerebral organoids can only produce a limited number of immature astrocytes (e.g., as shown in FIG. 8C), and it could take up to 20 months to resemble primary human astrocytes. Therefore, the cerebral organoid protocol described herein was further optimized by adding leukemia inhibitory factor (LIF) and 15% fetal bovine serum (FBS) to promote astrocytic differentiation and maturation for BBB modeling (e.g., as shown in FIG. 8A). The astrocytic induction protocol produced a substantial number of astrocytes compared to non-induced organoids, and these induced human astrocytes highly expressed glial fibrillary acidic protein (GFAP), a type III intermediate filament protein that is almost absent in non-induced brain organoids at Day 90 (e.g., as shown in FIG. 8C). These results suggest that induced astrocytes possess more extensive cell processes. Moreover, induced human astrocytes also expressed aquaporin-4 (AQP4) on their end-feet (e.g., as shown in FIG. 1C) and exhibited a comprehensive astrocytic network in a tissue-cleared three dimensional (3D)-imaged cerebral organoid after astrocytic induction (e.g., as shown in FIG. 8D). Overall, the presently disclosed optimized cerebral organoid protocol with astrocytic induction can produce a comprehensive cortical structure with abundant mature astrocytes.

[0286] To incorporate vascular cells into the presently disclosed neurovascular co-development system, an improved methodology was used to produce blood vessel organoids (e.g., as shown in FIG. 1F). Briefly, H9 ESCs were induced to mesoderm by CHIR99021 and BMP4

and furthered to vascular lineage by VEGF-A and forskolin (e.g., as shown in FIG. 1F). Next, the organoids were embedded into a collagen-cultrex matrix supplemented with VEGF-A, FGF2, and 15% FBS to promote angiogenesis and vessel sprouting (e.g., as shown in FIGS. 8E and 8F). Finally, the cell aggregates were extracted from the gel matrix to allow for self-assembly of blood vessel organoids (e.g., as shown in FIG. 8G). By day 20, whole-mount confocal images revealed the formation of complex and highly branched endothelial networks labeled with CD31 (e.g., as shown in FIG. 1D). These self-assembled 3D endothelial networks showed tight interactions with pericytes, as defined by the molecular marker PDGFR β (e.g., as shown in FIGS. 1D and 8G). Additionally, these networks formed tube-like structures with a discernible lumen (e.g., as shown in FIG. 8G), demonstrating the successful generation of 3D self-organized blood vessel organoids.

[0287] To recreate neuro-vascular co-development, blood vessel organoids and cerebral organoids that had undergone astrocytic induction were assembled on parafilm and embedded in a Matrigel droplet (see Materials and Methods). The assembled organoids were then cultured in a mixture of half cerebral organoid maturation media and half blood vessel organoid maturation media to maintain both neuroepithelial and vascular endothelial identities. Within 4 to 6 days after the assembly, vessel cells actively invaded the neuroepithelium (e.g., as shown in FIG. 1F), indicating active angiogenesis in cerebral organoids. Blood vessel organoids further vascularized cerebral organoids by extending endothelial tubes labeled by CD31 in a developmental manner (as shown in FIGS. 1F-1I) and eventually merged into a single organoid around 21 days after assembly (as shown in FIG. 1G), suggesting the formation of capillary networks in cerebral organoids.

[0288] The present disclosure further describes one or more embodiments for assembling hPSCs-derived brain and blood vessel organoids into human BBB (hBBB) assembloids. In an example experiment, the hBBB assembloids, of which example embodiments are presently described, were found to mimic features of the human BBB as evidenced by presence of brain-specific ECs with specialized tight junctions and transporter expression. Additionally, the hBBB assembloids exhibited reduced endothelial fenestrations, pericyte processes and astrocytic end-feet ensheathing the endothelium, characteristic transcriptional programs, and increased transendothelial electrical resistance indicative of low permeability. The example experiment included generating iPSCs from patients carrying LOF mutations in the CCM1 gene. When BBB assembloids were derived from these CCM iPSCs, clusters of enlarged endothelial channels were observed, arranged back-to-back, resembling *in vivo* cavernoma phenotypes. Single-cell

transcriptomic analysis revealed CCM-related angiogenesis, disrupted neurovascular interaction, and an altered developmental trajectory of mural cells, suggesting an intrinsic molecular and cellular pathology underlying CCMs. Finally, patient-derived BBB assembloids were benchmarked against primary cavernomas tissues removed from neurosurgery. The presently disclosed models allow improved studies of the human BBB and facilitate the development of new therapeutics for CCMs and other cerebrovascular diseases.

Assembloids exhibiting key biology of BBB

[0289] Brain microvascular endothelial cells (BMECs) possess BBB-specific features different from the endothelium outside of brains, which enable many essential functions for BBB. To investigate whether neuroepithelial tissues could induce endothelial cells to acquire BBB-specific features during neuro-vascular co-development, BBB-specific markers were analyzed on the endothelium. By day 21, majority of the endothelial cells expressed BBB-specific markers such as glucose transporter 1 (Glut-1) and tight junction proteins such as Claudin-5 and ZO-1 (e.g., as shown in FIGS. 2A-2D and 9A), indicating that the endothelial cells were differentiating towards a BBB-specific fate. Transmission electron microscopy revealed the typical ultrastructure of the BBB, including tight junction (TJ) and adherence junction (AJ) complexes (e.g., as shown in 9H), suggesting that human BMECs formed capillaries through tight junctions. Furthermore, the brain endothelium was covered by a continuous basement membrane, a crucial structure for regulating angiogenesis and maintaining the BBB which was defined by the molecular marker Collagen IV (e.g., as shown in Figure 2E). The BBB is a highly organized structure forming a boundary between the vascular and neuronal portions of the brain; it consists of not only endothelial cells but also pericytes processes and astrocytic end-feet coverage. Interestingly, it was observed that astrocytic processes labeled by GFAP were well-aligned with endothelial tubes (e.g., as shown in FIG. 2F), and human astrocytes extended their end-feet labeled by AQP-4 to wrap up the abluminal capillary surface (e.g., as shown in FIG. 2G). The newly form capillaries were not only ensheathed by astrocytic processes but also by human pericytes (stained for PDGFR- β , e.g., as shown in FIG. 2H). Furthermore, neuronal and neural progenitor innervation was observed (e.g., as shown in FIGS. 9I-9J). The presence of these important components in cerebral-vessel assembly was summarized in Figure 9L, highlighting the striking resemblance of the *in vivo* human BBB-like structure. This included endothelial cells forming the capillary wall, connected via tight junctions, as well as the presence of a basement membrane, astrocytic end-feet, pericyte

ensheathment, and neuronal innervation (e.g., as shown in FIG. 2I all closely mirroring the characteristics of the BBB in the human brain (e.g., as shown in FIG. 9B-9D). Herein, this microphysiological system is also referred to as BBB assembloids.

[0290] To comprehensively decipher the cell populations present in BBB assembloids at the transcriptomic level, single-cell RNA sequencing (scRNA-seq) was performed on Day 30 BBB assembloids derived from H9 with three replicate cultures. The sequencing data was aligned and quantified using Cell Ranger (10x Genomics) to obtain raw count data. The R package Seurat (version 4) was used to normalize the raw count data and DoubletFinder was applied to remove doublet cells in the scRNA-seq data (see Materials and Methods). After doublet cell removal, a total of 28,062 cells were extracted for further analysis (n = three cultures). FindClusters was applied to identify differentially expressed gene markers for each cell cluster (see Materials and Methods). Clusters of excitatory neurons, inhibitory neurons, neural progenitors (NPs), astrocytes, endothelial cells (ECs), pericytes, mesenchymal stem cells (MSCs), smooth muscle cells (SMCs), fibroblast, and other features of BBB assembloids were identified (e.g., as shown in FIGS. 2K-2L) suggesting that BBB assembloids can model a complete neurovascular unit. The single-cell transcriptomics analysis revealed a similar cell population between biological replicas (e.g., as shown in FIG. 9L), indicative of the reliability of the protocol. Importantly, gene expression between ECs in BBB assembloids were compared with organ-specific ECs generated by the Tabula Muris Consortium. It was found that ECs in BBB assembloids presented an identical gene expression pattern with BMECs but not with other organ-specific ECs (e.g., as shown in FIG. 2L), confirming the endothelial acquisition of brain-specific transcriptomic signatures in BBB assembloids.

Gain of Wnt signaling pathways is important for the endothelial acquisition of BBB properties

[0291] To investigate the signaling pathways that propel the specification of brain endothelial cells, Fluorescence-Activated Cell Sorting (FACS) was performed to isolate vascular cells from Day 30 BBB assembloids, which were GFP-positive, and their transcriptomes were compared to those of unassembled blood vessel organoids (VOs) using bulk RNA sequencing (RNA-seq). A similar approach was applied to neural tissues by comparing GFP-negative neural cells to unassembled cerebral organoids (COs). An illustrative diagram is shown in Figure 3A. A heatmap of the Pearson correlation coefficient between samples and principal components (PCA) analysis

revealed vascular and neural clusters and two sub-clusters each respectively, distinguished by culture condition (assembloids versus unassembled organoids; FIGS. 3B and 10A), indicating that vascular gene expression changes were induced by the co-culture with neuroepithelial tissues. To delineate the gene regulatory program underlying the specification of brain endothelial cells, differentially expressed genes (DEGs) between GFP⁺ vascular cells derived from BBB assembloids and unassembled VCs were examined. It was found that more than three thousand genes were significantly changed (Figure 10E, fold change > 2 and P adjusted value < 0.01). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed DEGs were related to pathways of neurodegeneration and prion disease, including Huntington disease, Parkinson disease, Alzheimer's disease, Amyotrophic lateral sclerosis, etc. This suggests that the alteration of neuro-vascular cross-talks is implicated in neurodegenerative disorders. Moreover, several signaling pathways, including Wnt and Notch, were significantly altered in vascular cells of the assembloids (e.g., as shown in Figure 3D). The Wnt signaling pathway previously showed its important role in the specification and differentiation of brain endothelial cells in animal models. A heatmap of gene expression demonstrated that over 40 Wnt signaling pathways-related genes were significantly changed, including Wnt ligands (WNT5A, WNT2B, WNT16, WNT7B, and WNT9A) and frizzled (FZD) receptors (FZD6, FZD9, and FZD10), most of which were upregulated (e.g., as shown in FIG. 3E), suggesting that the gain of Wnt signaling pathways may lead to the brain specification of ECs. CHIR, an agonist of Wnt, was administered to blood vessel organoids through vessel sprouts and network formations to validate these results, and, as expected, CHIR remarkably enhanced Glut1 and ZO-1 expression in blood vessel organoids in absence of neural tissue compared with DMSO-treated ones (FIGS. 3F-3G). This indicates that gain of Wnt signaling pathways alone could drive the endothelial acquisition of BBB properties, such as expression of glucose transporter and tight-junction protein.

[0292] DEGs between GFP⁺ neural cells derived from BBB assembloids and unassembled COs were also examined to assess how the vascular tissue modulates neurodevelopment. It was found that more than two thousand genes were significantly changed (e.g., as shown in FIG. 10D, log fold change > 2 and P adjusted value < 0.01). Gene Ontology (GO) analysis showed DEGs were related to synapse organization, ion channel activity, neuron projection guidance, axonogenesis, etc. (e.g., as shown in Figure 10E), suggestive of the important regulatory roles of vascular tissue in neurodevelopment and brain function.

Spatially revealing the neuro-vascular interactions in BBB assembloids

[0293] After conducting single cell analyses, it was found that the BBB assembloid model closely resembled a complete neurovascular unit with various cell clusters, including excitatory neurons, inhibitory neurons, NPs, astrocytes, ECs, MSCs, pericytes, SMCs, fibroblasts, and others (as shown in Figure 9K). Additionally, the molecular mechanism underlying the co-development of the neuro-vascular system and the specification of brain ECs were delineated using bulk transcriptomics analysis (as illustrated via Figures 3A-3G and 10A-10E). However, the lack of spatial transcriptomics information hindered the understanding of the neuro-vascular interactions in the BBB assembloids. To address this limitation, a High Fidelity (HiFi)-slide sequencing technology, which was developed as part of the Human BioMolecular Atlas Program (HuBMAP) and has passed HuBMAP's rigorous quality controls was utilized. In short, HiFi-slide technology captures organoid's transcriptome on the repurposed Illumina flow cells, ligates the organoid's RNA with the previously synthesized DNA with submicron density on the flow cells, and co-sequences the organoid's RNA with the spatial-location-resolved DNA, allowing for the spatially resolved transcriptome of a piece of tissue at an incredibly high resolution.

[0294] To spatially resolve the transcriptome of BBB assembloids, a section of the assembloid was placed on the HiFi slide, which consists of 66 tiles placed on a rectangular matrix (11 rows and 6 columns), with each tile covering approximately 1 mm² of surface (~1.18 mm x 0.85 mm). A single tile overlapping a smooth part of the tissue was selected as Region of Interest (ROI) and was used for data analysis. The entire sequencing run consisted of around 217 million raw read pairs, with a total of over 178 million (around 82% of the total reads) spatially resolved pairs. Regarding the ROI, there were 143,480 spatially resolved locations with RNA (called from now on "spots") with an average 1-dimensional distance of 0.7 μ m (approximately 14 spatially resolved spots in 10 μ m²) and over 2.2 million spatially resolved RNAs (approximately 227 spatially resolved RNAs in 10 μ m²).

[0295] Spots in the spatial transcriptomics data were labeled by cell type using a set of 59 marker genes derived from scRNA-seq analysis. Cell types were assigned to spots expressing the corresponding marker genes. Then, the remaining spots were assigned to the cell type of their nearest neighbor spot (centroid of nearest neighbor algorithm). It was found that 54 out of the 59 marker genes were expressed across 2,380 spots (e.g., as shown in FIGS. 4A-4C, and FIG. 16A), with a total of 43,659 expressed genes in those spots. Astrocytes (Ast), endothelial cells (EC,

consisting of two groups: EC.1 expressing only EC markers, and EC.2 expressing both EC and Ast markers), GABAergic neurons (GABA), glutamatergic neurons (GluN), mural cells (MC, including cells expressing common markers of Peri, MSC and Fib), neural progenitors (NP), proliferative cells (PC), pericytes (Peri), smooth muscle cells (SMC), fibroblasts (Fib), and mesenchymal stem cell (MSC) were cell types present in the BBB assembloids. To discern the vascular structure of the BBB assembloids, cell types were grouped into two anatomical categories, namely vascular (represented in red) and neural cells (represented in blue). 692 of 2,380 spots were assigned to vascular cells (e.g., as shown in FIG. 16B). Furthermore, the rest of the spots were assigned to the cell type of the nearest spot, creating an approximately single-cell resolution spatial map of the BBB assembloids (e.g., as shown in FIG. 16C). Vascular cells-only spots were shown in FIG. 16D. From FIG. 16C-16D, the vascular structure of the BBB assembloids clearly emerges.

[0296] Next, spatial clustering of the 2,380 spots expressing cell type marker genes was performed. This analysis considered both transcriptome and spatial information of spots simultaneously to identify biologically significant patterns (see Materials and Methods). 12 spatial domains were extracted and statistically assigned them to groups of cell types (Figure 16E). With spatial clusters colored using the same notation as above, vascular cell types were in red and neural cell types were in blue (Figure 16F). Finally, the 143,480 spots in the ROI were labeled by cluster, revealing the distinct structure of spatial clusters (Figure 16G). Notably, cluster 2 was identified as where endothelial cells (EC.2) spatially colocalized with GABAergic neurons and cluster 3 was identified as where smooth muscle cells spatially colocalized with glutamatergic neurons, conveying the extensive neuro-vascular interactions with the spatial patterns in BBB assembloids.

Using BBB assembloids and cavernomas organoids to model CCMs

[0297] It was next investigated whether BBB assembloids could be utilized to model cerebrovascular defects, such as cerebral cavernous malformations (CCMs). CCMs are clusters of thin-walled, dilated blood vessels which can induce neurological symptoms if they rupture; these abnormalities in brain vasculature are caused by mutations in KRIT1 (otherwise known as CCM1), CCM2, or PDCD10 (otherwise known as CCM3) genes. Loss of function mutations in any of these genes can lead to the development of CCMs. To assess this, cerebral and blood vessel organoids were generated from iPSCs of two CCMs patients with carrying CCM1 mutations and three control subjects. Differentiation defects of CCM1 iPSCs into cerebral or blood vessel organoids were not observed (e.g., as shown in FIGS. 11A-11C). This is consistent with the clinical observation that

CCMs are primarily found in CNS vasculature. To investigate vascular defects under brain conditions, BBB assembloids were assembled by combining cerebral and blood vessel organoids derived from CCM1 iPSCs and compared with controls (e.g., as shown in FIG. 5A and 11D). Vessel morphology was assessed on Day 30. Confocal images of BBB assembloids sections revealed clusters of enlarged endothelial channels arranged back-to-back in CCMs but not in controls (Figure 15B), implying the formation of cavernous malformations *in vitro*.

[0298] For further validation of these results, first-in-class cavernomas organoids were developed. Primary cavernomas tissues were extracted via neurosurgery. A multi-step protocol was developed to culture primary cavernomas tissues in suspension culture (e.g., as explained via Figure 5A-5H; see Materials and Methods). Primary cavernomas tissues formed spheroids within seven days of the first microdissection and could be further expanded by mechanical dissection, resulting in cavernomas organoids (FIG. 5E). Encouragingly, both primary and first passage of cavernomas organoids exhibited distinct clusters of ECs resembling the cavernous malformation phenotype, which is identical to BBB assembloids derived from CCM1 patients (FIG. 5B). Furthermore, cavernomas organoids demonstrated a significant reduction in the expression of tight junction proteins and exhibited disassembled basement membranes (FIGS. 5F-5H), indicating a breakdown of BBB similar to animal models. Consequently, both patient iPSC-derived BBB assembloids and primary cavernomas tissue-derived organoids represent invaluable *in vitro* models that faithfully reproduce CCMs' morphological phenotypes, enabling comprehensive investigations into the underlying pathology of CCMs in the context of human genetics.

Single cell transcriptomics analysis revealing CCM-related molecular changes in a cell-type-specific manner

[0299] To investigate which cell type(s) could be responsible for the formation of cavernomas, scRNA-seq analysis was performed on Day 30 BBB assembloids derived from CCM1 patients and compared them with controls. scRNA-seq libraries were generated using the 10X Genomics platform and analyzed the data using the R package Seurat (version 4) after quality control (see Materials and Methods). In total, 23,848 cells from three control BBB assembloids and 22,445 cells from three CCM BBB assembloids were analyzed jointly. Through unsupervised clustering, 12 cell clusters and 22 cell sub-clusters were distinguished and visualized using 'uniform manifold approximation and projection' (UMAP, FIG. 6A). As expected, both control and CCM BBB assembloids (FIG. 6B) were capable of producing most of the neurovascular cells, including

excitatory neurons, inhibitory neurons, NPs, astrocytes, ECs, pericytes, MSCs, SMCs, fibroblasts, etc. (FIG. 6A). These results were comparable with the cell clusters identified from H9-derived BBB assembloids in FIGS. 2A-2L, furthering the reliability of the protocol across distinct ESCs and iPSCs lines. The expression profiles with cell-type markers were shown in FIG. 6C.

[0300] To decipher the gene regulatory programs underlying CCM pathology in a cell-type specific manner, the gene expression data for each cell cluster of BBB assembloids were merged, and differential analysis was performed using Wilcoxon rank-sum test with Bonferroni correction by comparing CCM to control BBB assembloids. First, the previously reported lesion-marker genes within each cell cluster were checked. Interestingly, almost all lesion-marker genes were upregulated in vascular cell clusters, particularly ECs but not in neural cell clusters (FIG. 6D), which is indicative of a dominant autonomous endothelial pathology underling CCMs. Moreover, a set of tip cell and tumor tip cell markers was dramatically increased in ECs clusters, particularly in ECs1 (FIG. 6E), suggesting an enhanced angiogenesis in CCM BBB assembloids. To validate these results further, the tip cells were examined and quantified in gel angiogenesis assay (FIG. 12C) where CCM brain ECs exhibited a significant longer and wider tip cells than control ones (FIG. 12D). To determine which cell(s) drive CCM-related angiogenesis, a ligand-receptor mediated cell-cell communication analysis was performed on single-cell transcriptomics data (see Materials and Methods). A comprehensive intercellular network of potential ligand-receptor interactions among neural and vascular clusters was built, and dramatically distinct ligand-receptor pairs were found in neural to vascular, vascular to vascular, and vascular to neural sender-to-receiver groups between CCMs and controls, but not in neural to neural sender-to-receiver group (FIGS. 6F and 13A). This highlighted an altered neuro-vascular interaction in CCMs. The top 10 ligand-receptor pairs were identified among all groups, and as a result, CCM but not control GABAs expressed VEGFA and IGF2 to receivers, ECs1, ECs2, MSCs1, MSCs2, etc. (FIG. 6G). VEGFA was highly implicated in CCM pathology, and IGF-1 was also shown to promote angiogenesis and remodel brain vasculature. No angiogenetic factors were found in top 10 ligand-receptors pairs of other groups (e.g., as shown in FIG. 13C). This suggests an important role of GABAergic neurons in driving CCM-related angiogenesis, possibly through stimulation of VEGF and IGF.

Developmental loss of vascular smooth muscle cells in CCMs

[0301] Mural cells, which include pericytes and vascular smooth muscle cells (vSMCs), are specialized cells that play an important role in the development and maintenance of blood vessels, including those in the brain. However, unlike the extensive studies of ECs in CCM animal models, there are very few studies on mural cells, which restricts the understanding of the autonomous roles of mural cells in CCM pathology. Surprisingly, a significant loss of vSMCs were found in CCM BBB assembloids through a side-by-side comparison of control and CCM UAMPs (e.g., as shown in FIG. 7A and 14B). The absolute cell percentage in control and CCM BBB assembloids is shown in FIG. 7B. During brain vascular development, mesenchymal stem cells (MSCs) can give rise to pericytes, vSMCs, and fibroblasts. Therefore, a developmental trajectory was reconstructed from MSCs to pericytes, vSMCs, and fibroblasts (e.g., as shown in FIGS. 7C and 14B) by plotting single cells based on their pseudotime (see Materials and Methods). Intriguingly, CCM MSCs exhibited a defect in differentiating into vSMCs in comparison with control MSCs (e.g., as shown in FIGS. 7C and 14B), suggesting that loss-of-function mutations in CCM1 could result in developmental loss of vSMCs in CCMs (e.g., as shown in FIG. 7D).

[0302] To validate the developmental loss of vSMCs in CCMs, immunostaining was conducted for smooth muscle actin (SMA). The presently disclosed analysis revealed a pronounced reduction in SMA expression within primary cavernomas assembloids compared to brain sections derived from healthy controls (FIGS. 7E-7G). However, nerve/glial-antigen 2 (NG2)-labeled mural cells remained unchanged between cavernomas tissue and healthy brain sections (e.g., as shown in FIG. 14D). This finding provides compelling evidence of the specifically impaired development of vSMCs associated with CCMs. Interestingly, it was observed that control vSMCs were the most interactive vascular cells with both neural and vascular clusters, regardless of whether they acted as senders or receivers (e.g., as shown in FIG. 6F and 13A). However, in CCMs, VEs2 replaced vSMCs and acted as the most interacting vascular cells, completely altering neuro-vascular ligand-receptor interaction patterns in CCMs compared to controls (FIGS. 6F and 13A). Without being bound by any theory, it is contemplated that this alteration in neuro-vascular interactions may be due to the developmental loss of vSMCs in CCMs. These data suggest an important mural cell developmental defect in CCMs, which may contribute to CCM pathology, such as consequential alteration of neuro-vascular interactions.

[0303] Thus, a three-dimensional microphysiological system, also referred to herein as BBB assembloids, was successfully generated, which incorporates vascular and perivascular cells into neural tissue and mimics a *bona fide* blood-brain barrier (BBB). Compared to contemporary human neuro-vascular co-culture and BBB models, various embodiments of the presently disclosed systems have several advantages. Firstly, in some embodiments it comprises a complete neurovascular unit, including various types of neurons, neural progenitors, astrocytes, endothelial cells, pericytes, mesenchymal stem cells, vascular smooth muscle cells, and fibroblasts, which enables a comprehensive study of neuro-vascular interplay in development and disease. Secondly, various embodiments of the presently disclosed system exhibit key molecular, cellular, anatomical, and transcriptomic characteristics of the BBB, including the formation of capillary walls by endothelial cells through the assembly of tight-junction complexes, pericyte processes and astrocytic end-feet ensheathing the endothelium, a basement membrane covering the endothelium, and endothelial acquisition of BBB-specific transcriptomes. A reliable resemblance to the *in vivo* human BBB can facilitate the study of BBB pathology in human disease and drug development targeting the BBB. Thirdly, in some embodiments, the modular system described herein captures more elaborate brain vascularization processes in neuro-vascular co-development. By analyzing the transcriptomics of isolated vascular cells from assembloids and unassembled blood vessel organoids, it was discovered that the gain of Wnt signaling pathways could drive endothelial acquisition of BBB features. Assembling organ-specific organoids with blood vessel organoids provides a general modularity and facilitates understanding of endothelial acquisition of organ-specific features in development.

[0304] Cerebral cavernous malformations (CCMs) are a common inherited cerebrovascular disease with no drug treatment available. Although previous genetic and transgenic animal studies revealed three disease-causing genes and several downstream effector pathways, *in vitro* human CCMs models are significantly understudied, hindering drug development studies using human models. To address this gap, induced pluripotent stem cells derived from CCM patients carrying CCM1 mutations were generated and patient-derived BBB assembloids were generated. Cavernomas organoids derived from primary cavernomas tissues removed from neurosurgery (aka CCM primary tissue organoids) were also generated, in order to serve as a benchmark for *in vitro* human CCMs models. Both CCM BBB assembloids and cavernomas organoids exhibited a typical cavernomas phenotype with clusters of enlarged endothelial channels arranged back-to-back and

BBB breakdown—consistent with the *in vivo* lesion phenotype in CCMs. With these faithful and potentially highly expandable CCM models in humans, high throughput screening can identify novel therapeutic candidates for CCMs.

[0305] BBB assembloids derived from CCM patients offer a unique opportunity to study how the loss of CCM genes alters cerebrovascular development in humans. Unexpectedly, it was found that in some embodiments CCM mesenchymal stem cells alter their developmental trajectories and fail to give rise to vascular smooth muscle cells (vSMCs), as benchmarked by cavernomas organoids. This developmental loss of vSMCs is implicated in the abnormal neuro-vascular crosstalk in CCMs, indicating a new cellular pathology underlying CCMs. In addition, inhibitory GABAergic neurons were identified as a major resource for releasing pro-angiogenic factors, including VEGF and IGF, to endothelial cells by a ligand-receptor interaction analysis, indicating a novel molecular mechanism driving angiogenesis in CCMs. Thus, these important molecular and cellular mechanisms underlying CCMs provide novel therapeutic targets in humans.

Terms

[0306] In the following detailed description, reference is made to the accompanying drawings, which form a part hereof. In the drawings, similar symbols typically identify similar components, unless context dictates otherwise. The illustrative embodiments described in the detailed description, drawings, and claims are not meant to be limiting. Other embodiments may be utilized, and other changes may be made, without departing from the spirit or scope of the subject matter presented herein. It will be readily understood that the aspects of the present disclosure, as generally described herein, and illustrated in the Figures, can be arranged, substituted, combined, separated, and designed in a wide variety of different configurations, all of which are explicitly contemplated herein.

[0307] Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood when read in light of the instant disclosure by one of ordinary skill in the art to which the present disclosure belongs. For purposes of the present disclosure, the following terms are explained below.

[0308] The disclosure herein uses affirmative language to describe the numerous embodiments. The disclosure also includes embodiments in which subject matter is excluded, in full or in part, such as substances or materials, method steps and conditions, protocols, or procedures.

[0309] The articles “a” and “an” are used herein to refer to one or to more than one (for example, at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

[0310] By “about” is meant a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that varies by as much as 10% to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length.

[0311] Throughout this specification, unless the context requires otherwise, the words “comprise,” “comprises,” and “comprising” will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. By “consisting of” is meant including, and limited to, whatever follows the phrase “consisting of.” Thus, the phrase “consisting of” indicates that the listed elements are required or mandatory, and that no other elements may be present. By “consisting essentially of” is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase “consisting essentially of” indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they materially affect the activity or action of the listed elements.

[0312] The terms “individual”, “subject”, or “patient” as used herein have their plain and ordinary meaning as understood in light of the specification, and mean a human or a non-human mammal, e.g., a dog, a cat, a mouse, a rat, a cow, a sheep, a pig, a goat, a non-human primate, or a bird, e.g., a chicken, as well as any other vertebrate or invertebrate. The term “mammal” is used in its usual biological sense. Thus, it specifically includes, but is not limited to, primates, including simians (chimpanzees, apes, monkeys) and humans, cattle, horses, sheep, goats, swine, rabbits, dogs, cats, rodents, rats, mice, guinea pigs, or the like.

[0313] The terms “effective amount” or “effective dose” as used herein have their plain and ordinary meaning as understood in light of the specification, and refer to that amount of a recited composition or compound that results in an observable effect. Actual dosage levels of active ingredients in an active composition of the presently disclosed subject matter can be varied so as to administer an amount of the active composition or compound that is effective to achieve the desired response for a particular subject and/or application. The selected dosage level will depend upon a variety of factors including, but not limited to, the activity of the composition, formulation,

route of administration, combination with other drugs or treatments, severity of the condition being treated, and the physical condition and prior medical history of the subject being treated. In some embodiments, a minimal dose is administered, and dose is escalated in the absence of dose-limiting toxicity to a minimally effective amount. Determination and adjustment of an effective dose, as well as evaluation of when and how to make such adjustments, are contemplated herein.

[0314] The terms “function” and “functional” as used herein have their plain and ordinary meaning as understood in light of the specification, and refer to a biological, enzymatic, or therapeutic function.

[0315] The term “inhibit” as used herein has its plain and ordinary meaning as understood in light of the specification, and may refer to the reduction or prevention of a biological activity. The reduction can be by a percentage that is, is about, is at least, is at least about, is not more than, or is not more than about, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, or an amount that is within a range defined by any two of the aforementioned values. As used herein, the term “delay” has its plain and ordinary meaning as understood in light of the specification, and refers to a slowing, postponement, or deferment of a biological event, to a time which is later than would otherwise be expected. The delay can be a delay of a percentage that is, is about, is at least, is at least about, is not more than, or is not more than about, 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or an amount within a range defined by any two of the aforementioned values. The terms inhibit and delay may not necessarily indicate a 100% inhibition or delay. A partial inhibition or delay may be realized.

[0316] As used herein, the term “isolated” has its plain and ordinary meaning as understood in light of the specification, and refers to a substance and/or entity that has been (1) separated from at least some of the components with which it was associated when initially produced (whether in nature and/or in an experimental setting), and/or (2) produced, prepared, and/or manufactured by the hand of man. Isolated substances and/or entities may be separated from equal to, about, at least, at least about, not more than, or not more than about, 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98%, about 99%, substantially 100%, or 100% of the other components with which they were initially associated (or ranges including and/or spanning the aforementioned values). In some embodiments, isolated agents are, are about, are at least, are at least about, are not more than, or are not more than about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about

96%, about 97%, about 98%, about 99%, substantially 100%, or 100% pure (or ranges including and/or spanning the aforementioned values). As used herein, a substance that is “isolated” may be “pure” (e.g., substantially free of other components). As used herein, the term “isolated cell” may refer to a cell not contained in a multi-cellular organism or tissue.

[0317] As used herein, “in vivo” is given its plain and ordinary meaning as understood in light of the specification and refers to the performance of a method inside living organisms, usually animals, mammals, including humans, and plants, as opposed to a tissue extract or dead organism.

[0318] As used herein, “ex vivo” is given its plain and ordinary meaning as understood in light of the specification and refers to the performance of a method outside a living organism with little alteration of natural conditions.

[0319] As used herein, “in vitro” is given its plain and ordinary meaning as understood in light of the specification and refers to the performance of a method outside of biological conditions, e.g., in a petri dish or test tube.

[0320] The terms “nucleic acid” or “nucleic acid molecule” as used herein have their plain and ordinary meaning as understood in light of the specification, and refer to polynucleotides, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), oligonucleotides, those that appear in a cell naturally, fragments generated by the polymerase chain reaction (PCR), and fragments generated by any of ligation, scission, endonuclease action, and exonuclease action. Nucleic acid molecules can be composed of monomers that are naturally-occurring nucleotides (such as DNA and RNA), or analogs of naturally-occurring nucleotides (e.g., enantiomeric forms of naturally-occurring nucleotides), or a combination of both. Modified nucleotides can have alterations in sugar moieties and/or in pyrimidine or purine base moieties. Sugar modifications include, for example, replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, and azido groups, or sugars can be functionalized as ethers or esters. Moreover, the entire sugar moiety can be replaced with sterically and electronically similar structures, such as aza-sugars and carbocyclic sugar analogs. Examples of modifications in a base moiety include alkylated purines and pyrimidines, acylated purines or pyrimidines, or other well-known heterocyclic substitutes. Nucleic acid monomers can be linked by phosphodiester bonds or analogs of such linkages. Analogs of phosphodiester linkages include phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, or phosphoramidate. The term “nucleic acid molecule” also includes so-called “peptide nucleic

acids,” which comprise naturally-occurring or modified nucleic acid bases attached to a polyamide backbone. Nucleic acids can be either single stranded or double stranded. “Oligonucleotide” can be used interchangeable with nucleic acid and can refer to either double stranded or single stranded DNA or RNA. A nucleic acid or nucleic acids can be contained in a nucleic acid vector or nucleic acid construct (e.g. plasmid, virus, retrovirus, lentivirus, bacteriophage, cosmid, fosmid, phagemid, bacterial artificial chromosome (BAC), yeast artificial chromosome (YAC), or human artificial chromosome (HAC)) that can be used for amplification and/or expression of the nucleic acid or nucleic acids in various biological systems. Typically, the vector or construct will also contain elements including but not limited to promoters, enhancers, terminators, inducers, ribosome binding sites, translation initiation sites, start codons, stop codons, polyadenylation signals, origins of replication, cloning sites, multiple cloning sites, restriction enzyme sites, epitopes, reporter genes, selection markers, antibiotic selection markers, targeting sequences, peptide purification tags, or accessory genes, or any combination thereof.

[0321] A nucleic acid or nucleic acid molecule can comprise one or more sequences encoding different peptides, polypeptides, or proteins. These one or more sequences can be joined in the same nucleic acid or nucleic acid molecule adjacently, or with extra nucleic acids in between, e.g. linkers, repeats or restriction enzyme sites, or any other sequence that is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, or 300 bases long, or any length in a range defined by any two of the aforementioned lengths. The term “downstream” on a nucleic acid as used herein has its plain and ordinary meaning as understood in light of the specification and refers to a sequence being after the 3’-end of a previous sequence, on the strand containing the encoding sequence (sense strand) if the nucleic acid is double stranded. The term “upstream” on a nucleic acid as used herein has its plain and ordinary meaning as understood in light of the specification and refers to a sequence being before the 5’-end of a subsequent sequence, on the strand containing the encoding sequence (sense strand) if the nucleic acid is double stranded. The term “grouped” on a nucleic acid as used herein has its plain and ordinary meaning as understood in light of the specification and refers to two or more sequences that occur in proximity either directly or with extra nucleic acids in between, e.g. linkers, repeats, or restriction enzyme sites, or any other sequence that is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,

17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, or 300 bases long, or any length in a range defined by any two of the aforementioned lengths, but generally not with a sequence in between that encodes for a functioning or catalytic polypeptide, protein, or protein domain.

[0322] The nucleic acids described herein comprise nucleobases. Primary, canonical, natural, or unmodified bases are adenine, cytosine, guanine, thymine, and uracil. Other nucleobases include but are not limited to purines, pyrimidines, modified nucleobases, 5-methylcytosine, pseudouridine, dihydrouridine, inosine, 7-methylguanosine, hypoxanthine, xanthine, 5,6-dihydrouracil, 5-hydroxymethylcytosine, 5-bromouracil, isoguanine, isocytosine, aminoallyl bases, dye-labeled bases, fluorescent bases, or biotin-labeled bases.

[0323] The terms “peptide”, “polypeptide”, and “protein” as used herein have their plain and ordinary meaning as understood in light of the specification and refer to macromolecules comprised of amino acids linked by peptide bonds. The numerous functions of peptides, polypeptides, and proteins are known in the art, and include but are not limited to enzymes, structure, transport, defense, hormones, or signaling. Peptides, polypeptides, and proteins are often, but not always, produced biologically by a ribosomal complex using a nucleic acid template, although chemical syntheses are also available. By manipulating the nucleic acid template, peptide, polypeptide, and protein mutations such as substitutions, deletions, truncations, additions, duplications, or fusions of more than one peptide, polypeptide, or protein can be performed. These fusions of more than one peptide, polypeptide, or protein can be joined in the same molecule adjacently, or with extra amino acids in between, e.g. linkers, repeats, epitopes, or tags, or any other sequence that is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, or 300 bases long, or any length in a range defined by any two of the aforementioned lengths. The term “downstream” on a polypeptide as used herein has its plain and ordinary meaning as understood in light of the specification and refers to a sequence being after the C-terminus of a previous sequence. The term “upstream” on a polypeptide as used herein has its plain and ordinary meaning as understood in light of the specification and refers to a sequence being before the N-terminus of a subsequent sequence.

[0324] The term “purity” of any given substance, compound, or material as used herein has its plain and ordinary meaning as understood in light of the specification and refers to the actual

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abundance of the substance, compound, or material relative to the expected abundance. For example, the substance, compound, or material may be at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% pure, including all decimals in between. Purity may be affected by unwanted impurities, including but not limited to nucleic acids, DNA, RNA, nucleotides, proteins, polypeptides, peptides, amino acids, lipids, cell membrane, cell debris, small molecules, degradation products, solvent, carrier, vehicle, or contaminants, or any combination thereof. In some embodiments, the substance, compound, or material is substantially free of host cell proteins, host cell nucleic acids, plasmid DNA, contaminating viruses, proteasomes, host cell culture components, process related components, mycoplasma, pyrogens, bacterial endotoxins, and adventitious agents. Purity can be measured using technologies including but not limited to electrophoresis, SDS-PAGE, capillary electrophoresis, PCR, rtPCR, qPCR, chromatography, liquid chromatography, gas chromatography, thin layer chromatography, enzyme-linked immunosorbent assay (ELISA), spectroscopy, UV-visible spectrometry, infrared spectrometry, mass spectrometry, nuclear magnetic resonance, gravimetry, or titration, or any combination thereof.

[0325] The term “yield” of any given substance, compound, or material as used herein has its plain and ordinary meaning as understood in light of the specification and refers to the actual overall amount of the substance, compound, or material relative to the expected overall amount. For example, the yield of the substance, compound, or material is, is about, is at least, is at least about, is not more than, or is not more than about, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% of the expected overall amount, including all decimals in between. Yield may be affected by the efficiency of a reaction or process, unwanted side reactions, degradation, quality of the input substances, compounds, or materials, or loss of the desired substance, compound, or material during any step of the production.

[0326] As used herein, “pharmaceutically acceptable” has its plain and ordinary meaning as understood in light of the specification and refers to carriers, excipients, and/or stabilizers that are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed or that have an acceptable level of toxicity. A “pharmaceutically acceptable” “diluent,” “excipient,” and/or “carrier” as used herein have their plain and ordinary meaning as understood in light of the specification and are intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like,

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compatible with administration to humans, cats, dogs, or other vertebrate hosts. Typically, a pharmaceutically acceptable diluent, excipient, and/or carrier is a diluent, excipient, and/or carrier approved by a regulatory agency of a Federal, a state government, or other regulatory agency, or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, including humans as well as non-human mammals, such as cats and dogs. The term diluent, excipient, and/or “carrier” can refer to a diluent, adjuvant, excipient, or vehicle with which the pharmaceutical composition is administered. Such pharmaceutical diluent, excipient, and/or carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin. Water, saline solutions and aqueous dextrose and glycerol solutions can be employed as liquid diluents, excipients, and/or carriers, particularly for injectable solutions. Suitable pharmaceutical diluents and/or excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. A non-limiting example of a physiologically acceptable carrier is an aqueous pH buffered solution. The physiologically acceptable carrier may also comprise one or more of the following: antioxidants, such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, such as serum albumin, gelatin, immunoglobulins, hydrophilic polymers such as polyvinylpyrrolidone, amino acids, carbohydrates such as glucose, mannose, or dextrans, chelating agents such as EDTA, sugar alcohols such as mannitol or sorbitol, salt-forming counterions such as sodium, and nonionic surfactants such as TWEEN®, polyethylene glycol (PEG), and PLURONICS®. The composition, if desired, can also contain minor amounts of wetting, bulking, emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, sustained release formulations and the like. The formulation should suit the mode of administration.

[0327] Cryoprotectants are cell composition additives to improve efficiency and yield of low temperature cryopreservation by preventing formation of large ice crystals. Cryoprotectants include but are not limited to DMSO, ethylene glycol, glycerol, propylene glycol, trehalose, formamide, methyl-formamide, dimethyl-formamide, glycerol 3-phosphate, proline, sorbitol, diethyl glycol, sucrose, triethylene glycol, polyvinyl alcohol, polyethylene glycol, or hydroxyethyl starch. Cryoprotectants can be used as part of a cryopreservation medium, which include other components such as nutrients (e.g. albumin, serum, bovine serum, fetal calf serum [FCS]) to

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enhance post-thawing survivability of the cells. In these cryopreservation media, at least one cryoprotectant may be found at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 0.01%, 0.05%, 0.1%, 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, or any percentage within a range defined by any two of the aforementioned numbers.

[0328] Additional excipients with desirable properties include but are not limited to preservatives, adjuvants, stabilizers, solvents, buffers, diluents, solubilizing agents, detergents, surfactants, chelating agents, antioxidants, alcohols, ketones, aldehydes, ethylenediaminetetraacetic acid (EDTA), citric acid, salts, sodium chloride, sodium bicarbonate, sodium phosphate, sodium borate, sodium citrate, potassium chloride, potassium phosphate, magnesium sulfate sugars, dextrose, fructose, mannose, lactose, galactose, sucrose, sorbitol, cellulose, serum, amino acids, polysorbate 20, polysorbate 80, sodium deoxycholate, sodium taurodeoxycholate, magnesium stearate, octylphenol ethoxylate, benzethonium chloride, thimerosal, gelatin, esters, ethers, 2-phenoxyethanol, urea, or vitamins, or any combination thereof. Some excipients may be in residual amounts or contaminants from the process of manufacturing, including but not limited to serum, albumin, ovalbumin, antibiotics, inactivating agents, formaldehyde, glutaraldehyde, β -propiolactone, gelatin, cell debris, nucleic acids, peptides, amino acids, or growth medium components or any combination thereof. The amount of the excipient may be found in composition at a percentage that is, is about, is at least, is at least about, is not more than, or is not more than about, 0%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 100% w/w or any percentage by weight in a range defined by any two of the aforementioned numbers.

[0329] The term “pharmaceutically acceptable salts” has its plain and ordinary meaning as understood in light of the specification and includes relatively non-toxic, inorganic and organic acid, or base addition salts of compositions or excipients, including without limitation, analgesic agents, therapeutic agents, other materials, and the like. Examples of pharmaceutically acceptable salts include those derived from mineral acids, such as hydrochloric acid and sulfuric acid, and those derived from organic acids, such as ethanesulfonic acid, benzenesulfonic acid, p-toluenesulfonic acid, and the like. Examples of suitable inorganic bases for the formation of salts

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include the hydroxides, carbonates, and bicarbonates of ammonia, sodium, lithium, potassium, calcium, magnesium, aluminum, zinc, and the like. Salts may also be formed with suitable organic bases, including those that are non-toxic and strong enough to form such salts. For example, the class of such organic bases may include but are not limited to mono-, di-, and trialkylamines, including methylamine, dimethylamine, and triethylamine; mono-, di-, or trihydroxyalkylamines including mono-, di-, and triethanolamine; amino acids, including glycine, arginine and lysine; guanidine; N-methylglucosamine; N-methylglucamine; L-glutamine; N-methylpiperazine; morpholine; ethylenediamine; N-benzylphenethylamine; trihydroxymethyl aminoethane.

[0330] Proper formulation is dependent upon the route of administration chosen. Techniques for formulation and administration of the compounds described herein are known to those skilled in the art. Multiple techniques of administering a compound exist in the art including, but not limited to, enteral, oral, rectal, topical, sublingual, buccal, intraaural, epidural, epicutaneous, aerosol, parenteral delivery, including intramuscular, subcutaneous, intra-arterial, intravenous, intraportal, intra-articular, intradermal, peritoneal, intramedullary injections, intrathecal, direct intraventricular, intraperitoneal, intranasal or intraocular injections. Pharmaceutical compositions will generally be tailored to the specific intended route of administration.

[0331] As used herein, a “carrier” has its plain and ordinary meaning as understood in light of the specification and refers to a compound, particle, solid, semi-solid, liquid, or diluent that facilitates the passage, delivery and/or incorporation of a compound to cells, tissues and/or bodily organs.

[0332] As used herein, a “diluent” has its plain and ordinary meaning as understood in light of the specification and refers to an ingredient in a pharmaceutical composition that lacks pharmacological activity but may be pharmaceutically necessary or desirable. For example, a diluent may be used to increase the bulk of a potent drug whose mass is too small for manufacture and/or administration. It may also be a liquid for the dissolution of a drug to be administered by injection, ingestion or inhalation. A common form of diluent in the art is a buffered aqueous solution such as, without limitation, phosphate buffered saline that mimics the composition of human blood.

[0333] The term “% w/w” or “% wt/wt” as used herein has its plain and ordinary meaning as understood in light of the specification and refers to a percentage expressed in terms of the weight of the ingredient or agent over the total weight of the composition multiplied by 100. The term “%

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v/v” or “% vol/vol” as used herein has its plain and ordinary meaning as understood in the light of the specification and refers to a percentage expressed in terms of the liquid volume of the compound, substance, ingredient, or agent over the total liquid volume of the composition multiplied by 100.

Stem Cells

[0334] The term “totipotent stem cells” (also known as omnipotent stem cells) as used herein has its plain and ordinary meaning as understood in light of the specification and are stem cells that can differentiate into embryonic and extra-embryonic cell types. Such cells can construct a complete, viable organism. These cells are produced from the fusion of an egg and sperm cell. Cells produced by the first few divisions of the fertilized egg are also totipotent.

[0335] The term “embryonic stem cells (ESCs),” also commonly abbreviated as ES cells, as used herein has its plain and ordinary meaning as understood in light of the specification and refers to cells that are pluripotent and derived from the inner cell mass of the blastocyst, an early-stage embryo. For purpose of the present disclosure, the term “ESCs” is used broadly sometimes to encompass the embryonic germ cells as well.

[0336] The term “pluripotent stem cells (PSCs)” as used herein has its plain and ordinary meaning as understood in light of the specification and encompasses any cells that can differentiate into nearly all cell types of the body, i.e., cells derived from any of the three germ layers (germinal epithelium), including endoderm (interior stomach lining, gastrointestinal tract, the lungs), mesoderm (muscle, bone, blood, urogenital), and ectoderm (epidermal tissues and nervous system). PSCs can be the descendants of inner cell mass cells of the preimplantation blastocyst or obtained through induction of a non-pluripotent cell, such as an adult somatic cell, by forcing the expression of certain genes. Pluripotent stem cells can be derived from any suitable source. Examples of sources of pluripotent stem cells include mammalian sources, including human, rodent, porcine, and bovine.

[0337] The term “induced pluripotent stem cells (iPSCs),” also commonly abbreviated as iPS cells, as used herein has its plain and ordinary meaning as understood in light of the specification and refers to a type of pluripotent stem cells artificially derived from a normally non-pluripotent cell, such as an adult somatic cell, by inducing a “forced” expression of certain genes. hiPSC refers to human iPSCs. In some methods known in the art, iPSCs may be derived by transfection of

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certain stem cell-associated genes into non-pluripotent cells, such as adult fibroblasts. Transfection may be achieved through viral transduction using viruses such as retroviruses or lentiviruses. Transfected genes may include the master transcriptional regulators Oct-3/4 (POU5F1) and Sox2, although other genes may enhance the efficiency of induction. After 3-4 weeks, small numbers of transfected cells begin to become morphologically and biochemically similar to pluripotent stem cells, and are typically isolated through morphological selection, doubling time, or through a reporter gene and antibiotic selection. As used herein, iPSCs include first generation iPSCs, second generation iPSCs in mice, and human induced pluripotent stem cells. In some methods, a retroviral system is used to transform human fibroblasts into pluripotent stem cells using four pivotal genes: Oct3/4, Sox2, Klf4, and c-Myc. In other methods, a lentiviral system is used to transform somatic cells with OCT4, SOX2, NANOG, and LIN28. Genes whose expression are induced in iPSCs include but are not limited to Oct-3/4 (POU5F1); certain members of the Sox gene family (e.g., Sox1, Sox2, Sox3, and Sox15); certain members of the Klf family (e.g., Klf1, Klf2, Klf4, and Klf5), certain members of the Myc family (e.g., C-myc, L-myc, and N-myc), Nanog, LIN28, Tert, Fbx15, ERas, ECAT15-1, ECAT15-2, Tcf1, β -Catenin, ECAT1, Esg1, Dnmt3L, ECAT8, Gdf3, Fth117, Sal14, Rex1, UTF1, Stella, Stat3, Grb2, Prdm14, Nr5a1, Nr5a2, or E-cadherin, or any combination thereof. Other methods of producing induced pluripotent stem cells as conventionally known in the art are also envisioned.

[0338] The term "precursor cell" as used herein has its plain and ordinary meaning as understood in light of the specification and encompasses any cells that can be used in methods described herein, through which one or more precursor cells acquire the ability to renew itself or differentiate into one or more specialized cell types. In some embodiments, a precursor cell is pluripotent or has the capacity to becoming pluripotent. In some embodiments, the precursor cells are subjected to the treatment of external factors (e.g., growth factors) to acquire pluripotency. In some embodiments, a precursor cell can be a totipotent (or omnipotent) stem cell; a pluripotent stem cell (induced or non-induced); a multipotent stem cell; an oligopotent stem cells and a unipotent stem cell. In some embodiments, a precursor cell can be from an embryo, an infant, a child, or an adult. In some embodiments, a precursor cell can be a somatic cell subject to treatment such that pluripotency is conferred via genetic manipulation or protein/peptide treatment. Precursor cells include embryonic stem cells (ESC), embryonic carcinoma cells (ECs), epiblast stem cells (EpiSC), and induced pluripotent stem cells.

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[0339] In developmental biology, cellular differentiation is the process by which a less specialized cell becomes a more specialized cell type. As used herein, the term “differentiation” or “directed differentiation” describes a process through which a less specialized cell becomes a particular specialized target cell type. The particularity of the specialized target cell type can be determined by any applicable methods that can be used to define or alter the destiny of the initial cell. Exemplary methods include but are not limited to genetic manipulation, chemical treatment, protein treatment, and nucleic acid treatment.

[0340] The term “feeder cell” as used herein has its plain and ordinary meaning as understood in light of the specification and refers to cells that support the growth of pluripotent stem cells, such as by secreting growth factors into the medium or displaying on the cell surface. Feeder cells are generally adherent cells and may be growth arrested. For example, feeder cells are growth-arrested by irradiation (e.g. gamma rays), mitomycin-C treatment, electric pulses, or mild chemical fixation (e.g. with formaldehyde or glutaraldehyde). However, feeder cells do not necessarily have to be growth arrested. Feeder cells may serve purposes such as secreting growth factors, displaying growth factors on the cell surface, detoxifying the culture medium, or synthesizing extracellular matrix proteins. In some embodiments, the feeder cells are allogeneic or xenogeneic to the supported target stem cell, which may have implications in downstream applications. In some embodiments, the feeder cells are mouse cells. In some embodiments, the feeder cells are human cells. In some embodiments, the feeder cells are mouse fibroblasts, mouse embryonic fibroblasts, mouse STO cells, mouse 3T3 cells, mouse SNL 76/7 cells, human fibroblasts, human foreskin fibroblasts, human dermal fibroblasts, human adipose mesenchymal cells, human bone marrow mesenchymal cells, human amniotic mesenchymal cells, human amniotic epithelial cells, human umbilical cord mesenchymal cells, human fetal muscle cells, human fetal fibroblasts, or human adult fallopian tube epithelial cells. In some embodiments, conditioned medium prepared from feeder cells is used in lieu of feeder cell co-culture or in combination with feeder cell co-culture. In some embodiments, feeder cells are not used during the proliferation of target stem cells.

Cell Differentiation

[0341] In some embodiments, known methods for producing downstream cell types from pluripotent cells (e.g., iPSCs or ESCs) are applicable to the methods described herein. In some embodiments, pluripotent cells are derived from a morula. In some embodiments, pluripotent stem

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cells are stem cells. Stem cells used in these methods can include, but are not limited to, embryonic stem cells or induced pluripotent stem cells. Embryonic stem cells can be derived from the embryonic inner cell mass or from the embryonic gonadal ridges. Embryonic stem cells or germ cells can originate from a variety of animal species including, but not limited to, various mammalian species including humans.

[0342] In some embodiments, the pluripotent stem cells are treated with one or more small molecule compounds, activators, inhibitors, or growth factors for a time that is, is about, is at least, is at least about, is not more than, or is not more than about, 6 hours, 12 hours, 18 hours, 24 hours, 36 hours, 48 hours, 60 hours, 72 hours, 84 hours, 96 hours, 120 hours, 150 hours, 180 hours, 240 hours, 300 hours or any time within a range defined by any two of the aforementioned times, for example 6 hours to 300 hours, 24 hours to 120 hours, 48 hours to 96 hours, 6 hours to 72 hours, or 24 hours to 300 hours. In some embodiments, more than one small molecule compounds, activators, inhibitors, or growth factors are added. In these cases, the more than one small molecule compounds, activators, inhibitors, or growth factors can be added simultaneously or separately.

[0343] In some embodiments, the pluripotent stem cells are cultured in growth media that supports the growth of stem cells. In some embodiments, the pluripotent stem cells are cultured in stem cell growth media. In some embodiments, the stem cell growth media is RPMI 1640, DMEM, DMEM/F12, or Advanced DMEM/F12. In some embodiments, the stem cell growth media comprises fetal bovine serum (FBS). In some embodiments, the stem cell growth media comprises FBS at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 0%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20%, or any percentage within a range defined by any two of the aforementioned concentrations, for example 0% to 20%, 0.2% to 10%, 2% to 5%, 0% to 5%, or 2% to 20%. In some embodiments, the stem cell growth media does not contain xenogeneic components. In some embodiments, the growth media comprises one or more small molecule compounds, activators, inhibitors, or growth factors.

[0344] In some embodiments, pluripotent stem cells are prepared from somatic cells. In some embodiments, pluripotent stem cells are prepared from biological tissue obtained from a biopsy. In some embodiments, the pluripotent stem cells are cryopreserved. In some embodiments, the somatic cells are cryopreserved. In some embodiments, pluripotent stem cells are prepared from PBMCs. In some embodiments, human PSCs are prepared from human PBMCs. In some

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embodiments, pluripotent stem cells are prepared from cryopreserved PBMCs. In some embodiments, PBMCs are grown on a feeder cell substrate. In some embodiments, PBMCs are grown on a mouse embryonic fibroblast (MEF) feeder cell substrate. In some embodiments, PBMCs are grown on an irradiated MEF feeder cell substrate.

[0345] In some embodiments, iPSCs are expanded in cell culture. In some embodiments, iPSCs are expanded in Matrigel. In some embodiments, the iPSCs are expanded in cell culture comprising a ROCK inhibitor (e.g. Y-27632).

[0346] In some embodiments, pluripotent stem cells, mesoderm, vascular lineage cells, ectoderm, neural lineage cells, or any combination thereof, are contacted with a Wnt pathway activator or Wnt pathway inhibitor. For example, the pluripotent stem cells, mesoderm, vascular lineage cells, ectoderm, neural lineage cells, or any combination thereof, activate the Wnt pathway or inhibit the Wnt pathway. In some embodiments, the Wnt pathway activator comprises a Wnt protein. In some embodiments, the Wnt protein comprises a recombinant Wnt protein. In some embodiments, the Wnt pathway activator comprises Wnt1, Wnt2, Wnt2b, Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, Wnt8a, Wnt8b, Wnt9a, Wnt9b, Wnt10a, Wnt10b, Wnt11, Wnt16, BML 284, IQ-1, WAY 262611, or any combination thereof. In some embodiments, the Wnt pathway activator comprises a GSK3 pathway inhibitor. In some embodiments, the Wnt pathway activator comprises CHIR99021, CHIR 98014, AZD2858, BIO, AR-A014418, SB 216763, SB 415286, aloisine, indirubin, alsterpaullone, kenpaullone, lithium chloride, TDZD 8, or TWS119, or any combination thereof. In some embodiments, the Wnt pathway inhibitor comprises IWR-1, C59, PNU 74654, KY-02111, PRI-724, FH-535, DIF-1, or XAV939, or any combination thereof. In some embodiments, the cells are not treated with a Wnt pathway activator or Wnt pathway inhibitor. The Wnt pathway activator or Wnt pathway inhibitor provided herein may be used in combination with any of the other growth factors, pathway activators, or pathway inhibitors provided herein.

[0347] In some embodiments, pluripotent stem cells, mesoderm, vascular lineage cells, ectoderm, neural lineage cells, or any combination thereof, are contacted with an FGF pathway activator. For example, the pluripotent stem cells, mesoderm, vascular lineage cells, ectoderm, neural lineage cells, or any combination thereof activate the FGF pathway. In some embodiments, the FGF pathway activator comprises an FGF protein. In some embodiments, the FGF protein comprises a recombinant FGF protein. In some embodiments, the FGF pathway activator

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comprises one or more of FGF1, FGF2, FGF3, FGF4, FGF4, FGF5, FGF6, FGF7, FGF8, FGF8, FGF9, FGF10, FGF11, FGF12, FGF13, FGF14, FGF15 (FGF19, FGF15/FGF19), FGF16, FGF17, FGF18, FGF20, FGF21, FGF22, or FGF23. In some embodiments, the cells are not treated with an FGF pathway activator. The FGF pathway activator provided herein may be used in combination with any of the other growth factors, pathway activators, or pathway inhibitors provided herein.

[0348] In some embodiments, pluripotent stem cells, mesoderm, vascular lineage cells, ectoderm, neural lineage cells, or any combination thereof, are contacted with a BMP pathway activator or BMP pathway inhibitor. For example, the pluripotent stem cells, mesoderm, vascular lineage cells, ectoderm, neural lineage cells, or any combination thereof activate the BMP pathway or inhibit the BMP pathway. In some embodiments, the BMP pathway activator comprises a BMP protein. In some embodiments, the BMP protein is a recombinant BMP protein. In some embodiments, the BMP pathway activator comprises BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8a, BMP8b, BMP10, BMP11, BMP15, IDE1, or IDE2, or any combination thereof. In some embodiments, the BMP pathway inhibitor comprises Noggin, Dorsomorphin, RepSox, LY364947, LDN-193189, SB-431542, or any combination thereof. In some embodiments, the cells are not treated with a BMP pathway activator or BMP pathway inhibitor. The BMP pathway activator or BMP pathway inhibitor provided herein may be used in combination with any of the other growth factors, pathway activators, or pathway inhibitors provided herein.

[0349] In some embodiments, pluripotent stem cells, mesoderm, vascular lineage cells, ectoderm, neural lineage cells, or any combination thereof, are contacted with a VEGF pathway activator. For example, the pluripotent stem cells, mesoderm, vascular lineage cells, ectoderm, neural lineage cells, or any combination thereof activate the VEGF pathway. In some embodiments, the VEGF pathway activator comprises one or more of VEGF or GS4012. In some embodiments, the cells are not treated with a VEGF pathway activator. The VEGF pathway activator provided herein may be used in combination with any of the other growth factors, pathway activators, or pathway inhibitors provided herein.

[0350] In some embodiments, pluripotent stem cells, mesoderm, vascular lineage cells, ectoderm, neural lineage cells, or any combination thereof, are contacted with a TGF-beta (TGF-b) pathway activator or TGF-b pathway inhibitor. For example, the pluripotent stem cells,

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mesoderm, vascular lineage cells, ectoderm, neural lineage cells, or any combination thereof activate the TGF- β pathway or inhibit the TGF- β pathway. In some embodiments, the TGF- β family comprises bone morphogenetic protein (BMP), growth and differentiation factor (GDF), anti-Müllerian hormone, Activin, and Nodal pathways. In some embodiments, the TGF- β pathway activator comprises TGF- β 1, TGF- β 2, TGF- β 3, Activin A, Activin B, Nodal, a BMP, IDE1, IDE2, or any combination thereof. In some embodiments, the TGF- β pathway inhibitor comprises A8301, RepSox, LY365947, SB-431542, or any combination thereof. In some embodiments, the cells are not treated with a TGF- β pathway activator or TGF- β pathway inhibitor. The TGF- β pathway activator or TGF- β pathway inhibitor provided herein may be used in combination with any of the other growth factors, pathway activators, or pathway inhibitors provided herein.

[0351] In some embodiments, pluripotent stem cells, mesoderm, vascular lineage cells, ectoderm, neural lineage cells, or any combination thereof, are contacted with a cAMP pathway activator. For example, the pluripotent stem cells, mesoderm, vascular lineage cells, ectoderm, neural lineage cells, or any combination thereof inhibit the cAMP pathway. In some embodiments, the cAMP pathway activator comprises forskolin or cAMP. In some embodiments, the cells are not treated with a cAMP pathway activator. The cAMP pathway activator provided herein may be used in combination with any of the other growth factors, pathway activators, or pathway inhibitors provided herein.

[0352] In some embodiments, pluripotent stem cells, mesoderm, vascular lineage cells, ectoderm, neural lineage cells, or any combination thereof, are contacted with ascorbic acid. In some embodiments, the cells are not treated with ascorbic acid. Ascorbic acid as provided herein may be used in combination with any of the other growth factors, pathway activators, or pathway inhibitors provided herein.

[0353] In some embodiments, pluripotent stem cells, mesoderm, vascular lineage cells, ectoderm, neural lineage cells, or any combination thereof, are contacted with leukemia inhibitory factor (LIF). In some embodiments, the cells are not treated with LIF. LIF as provided herein may be used in combination with any of the other growth factors, pathway activators, or pathway inhibitors provided herein.

[0354] In some embodiments, pluripotent stem cells, mesoderm, vascular lineage cells, ectoderm, neural lineage cells, or any combination thereof, are contacted with glial cell line-derived neurotrophic factor (GDNF). In some embodiments, the cells are not treated with GDNF.

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GDNF as provided herein may be used in combination with any of the other growth factors, pathway activators, or pathway inhibitors provided herein.

[0355] In some embodiments, pluripotent stem cells, mesoderm, vascular lineage cells, ectoderm, neural lineage cells, or any combination thereof, are contacted with brain-derived neurotrophic factor (BDNF). In some embodiments, the cells are not treated with BDNF. BDNF as provided herein may be used in combination with any of the other growth factors, pathway activators, or pathway inhibitors provided herein.

[0356] In some embodiments, for any of the small molecule compounds, pathway activators, pathway inhibitors, or growth factors, the cells are contacted for a time that is, is about, is at least, is at least about, is not more than, or is not more than about, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, 18 hours, 24 hours, 36 hours, 48 hours, 60 hours, 72 hours, 84 hours, 96 hours, 120 hours, 150 hours, 180 hours, 240 hours, 300 hours or any time within a range defined by any two of the aforementioned times, for example 1 hour to 300 hours, 24 hours to 120 hours, 48 hours to 96 hours, 6 hours to 72 hours, or 24 hours to 300 hours. In some embodiments, more than one small molecule compounds, activators, inhibitors, or growth factors are added. In these cases, the more than one small molecule compounds, activators, inhibitors, or growth factors can be added simultaneously or separately.

[0357] In some embodiments, the PSCs are differentiated into mesoderm cells. In some embodiments, the PSCs are differentiated to vascular lineage cells. In some embodiments, the PSCs are differentiated to blood vessel organoids. In some embodiments, the PSCs are differentiated into ectoderm cells. In some embodiments, the PSCs are differentiated to neural lineage cells. In some embodiments, the PSCs are differentiated to cortical organoids.

[0358] In some embodiments, any of the cells disclosed herein may be cryopreserved for later use. In some embodiments, the cells are cryopreserved according to methods generally known in the art.

Methods of making blood vessel organoid

[0359] . A schematic for an improved method for producing blood vessel organoids from pluripotent stem cells is depicted in **FIG. 1J according to example embodiments of the present disclosure**. The methods may involve the use of a Wnt pathway activator such as CHIR99021

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during differentiation to produce endothelial cells that resemble those that are found in brain blood vessels.

[0360] Disclosed herein are methods of producing blood vessel organoids. In some embodiments, the methods comprise contacting an angiogenic sprout with a Wnt pathway activator (e.g., activating the Wnt pathway), an FGF pathway activator (e.g., activating the FGF pathway), a VEGF pathway activator (e.g., activating the VEGF pathway), and optionally a growth serum, for a first period of time; thereby forming the blood vessel organoid. In some embodiments, the methods comprise contacting an angiogenic sprout with an FGF pathway activator, a VEGF pathway activator, optionally a Wnt pathway activator, and optionally a growth serum, for a first period of time; thereby forming the blood vessel organoid.

[0361] In some embodiments, the angiogenic sprout is derived from pluripotent stem cells, for example, induced pluripotent stem cells. In some embodiments, the angiogenic sprout is cultured in a basement membrane matrix. In some embodiments, the angiogenic sprout is cultured in collagen I and/or Matrigel. In some embodiments, the first period of time is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 days, or any number of days within a range defined by any two of the aforementioned number of days, for example, 1-30 days, 1-10 days, 5-20 days, 10-30 days, or 5-25 days. In some embodiments, the first period of time is 5 days or at least 5 days. In some embodiments, the angiogenic sprout has been produced according to a method comprising: a) causing, for a second period of time, the angiogenic sprout to activate the Wnt pathway and the BMP pathway (e.g., contacting pluripotent stem cells with a Wnt pathway activator and a BMP pathway activator) to form vascular lineage cells; and b) causing, for a third period of time, the angiogenic sprout to activate the VEGF pathway and a cAMP pathway (e.g., a second cAMP pathway) (e.g., contacting the vascular lineage cells with a VEGF pathway activator and a second cAMP pathway activator); thereby forming the angiogenic sprout. In some embodiments, the vascular lineage cells are cultured in a basement membrane matrix. In some embodiments, the vascular lineage cells are cultured in collagen I and/or Matrigel. In some embodiments, the second period of time is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, or 5 days, or any number of days within a range defined by any two of the aforementioned number of days, for example, 1-5 days, 1-3 days, or 3-5 days. In some embodiments, the second period of time is 3 days. In some embodiments, the third period of

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time is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, or 4 days, or any number of days within a range defined by any two of the aforementioned number of days, for example, 1-4 days, 1-2 days, or 2-4 days. In some embodiments, the third period of time is 2 days.

[0362] In some embodiments, the BMP pathway activator is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 ng/mL, or any concentration within a range defined by any two of the aforementioned concentrations, for example, 10-100 ng/mL, 10-30 ng/mL, 30-100 ng/mL, or 20-70 ng/mL. In some embodiments, the BMP pathway activator is provided at a concentration of 30 ng/mL or about 30 ng/mL. In some embodiments, the BMP pathway activator is BMP4.

[0363] In some embodiments, the Wnt pathway activator is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 μ M, or any concentration within a range defined by any two of the aforementioned concentrations, for example, 1-20 μ M, 1-12 μ M, 4-12 μ M, 4-20 μ M, 2-6 μ M, or 10-15 μ M. In some embodiments, the Wnt pathway activator is provided at a concentration of 4 μ M or about 4 μ M. In some embodiments, the Wnt pathway activator is provided at a concentration of 12 μ M or about 12 μ M. In some embodiments, the Wnt pathway activator is CHIR99201.

[0364] In some embodiments, the second cAMP pathway activator is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, or 4 μ M, or any concentration within a range defined by any two of the aforementioned concentrations, for example, 0.5-4 μ M, 0.5-2 μ M, 2-4 μ M or 1-3 μ M. In some embodiments, the second cAMP pathway activator is provided at a concentration of 2 μ M or about 2 μ M. In some embodiments, the second cAMP pathway activator is forskolin.

[0365] In some embodiments, the growth serum is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20%, or any concentration within a range defined by any two of the aforementioned concentrations, for example, 0.5%-20%, 0.5%-5%, 1%-15%, 10-15%, 15-20%, or 12-18%. In some embodiments, the

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growth serum is provided at 15% or about 15%. In some embodiments, the growth serum is provided at 1% or about 1%. In some embodiments, the growth serum is fetal bovine serum (FBS).

[0366] In some embodiments, the VEGF pathway activator is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, or 150 ng/mL, or any concentration within a range defined by any two of the aforementioned concentrations, for example 10-150 ng/mL, 10-100 ng/mL, 100-150 ng/mL, or 80-120 ng/mL. In some embodiments, the VEGF is provided at 100 ng/mL or about 100 ng/mL. In some embodiments, the VEGF pathway activator is VEGF.

[0367] In some embodiments, the FGF pathway activator is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, or 150 ng/mL, or any concentration within a range defined by any two of the aforementioned concentrations, for example 10-150 ng/mL, 10-100 ng/mL, 100-150 ng/mL, or 80-120 ng/mL. In some embodiments, the FGF pathway activator is provided at 100 ng/mL or about 100 ng/mL. In some embodiments, the FGF pathway activator is FGF2.

[0368] In some embodiments, the blood vessel organoid differs from a blood vessel organoid that has been produced without contacting the cells of step b) with the Wnt pathway activator in step c) by having increased expression of blood-brain barrier-specific endothelial markers. For example, the cells of step b) may activate the Wnt pathway in step c) by having increased expression of blood-brain barrier-specific endothelial markers. In some embodiments, the blood-brain barrier-specific endothelial markers comprise glucose transporter 1 (GLUT-1) and zonula occludens-1 (tight junction protein-1; ZO-1). In some embodiments, the blood vessel organoid comprises endothelial cells that express CD31 and pericyte cells, or progenitors thereof, that express PDGFR- β .

[0369] In some embodiments, including any of the embodiments disclosed above and elsewhere herein, the blood vessel organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM. In some embodiments, the blood vessel organoid but not the brain organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM. In some embodiments, both the blood vessel organoid and the brain organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM. In some embodiments, the blood vessel organoid

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is derived from pluripotent stem cells having a genetic mutation associated with CCM and/or from a subject suffering from CCM. In some embodiments, the genetic mutation associated with CCM is a deletion or loss of function mutation of one or more of KRIT1, CCM2 and/or PDCD10. In some embodiments, the genetic mutation associated with CCM is induced into the cell by a genetic modification technique, optionally using CRISPR.

Methods of making brain organoid

[0370] A schematic for an exemplary method for producing dorsal forebrain organoids, which are a type of cortical (brain) organoid, is provided in **FIG. 8A** according to example embodiments of the present disclosure. These methods can be adapted to produce cortical organoids of alternative types, such as midbrain, striatal brain, hypothalamus, hippocampal, or spinal cord organoids. The methods may involve the use of LIF and/or fetal bovine serum during differentiation to induce formation of astrocytes in the brain organoids.

[0371] Disclosed herein are methods of producing brain organoids. In some embodiments, the methods comprise a) inhibiting a BMP pathway (e.g., contacting pluripotent stem cells with a BMP pathway inhibitor), inhibiting a TGF-beta pathway (e.g., contacting the pluripotent stem cells with a TGF-beta pathway inhibitor), and inhibiting the Wnt pathway (e.g., contacting the pluripotent stem cells with a Wnt pathway inhibitor) for a first period of time to form neuroectoderm cells; b) inhibiting a TGF-beta pathway (e.g., a second TGF-beta pathway) and activating a Wnt pathway (e.g., contacting the neuroectoderm cells of step a) with a second TGF-beta pathway inhibitor and a Wnt pathway activator) for a second period of time to form neuroepithelium cells; c) contacting the neuroepithelium cells of step b) with insulin for a third period of time to form cerebral tissue organoids; and d) contacting the cerebral tissue organoids of step c) with GDNF, BDNF, ascorbic acid, and a cAMP pathway activator (e.g., to activate the cAMP pathway) for a fourth period of time to form the brain organoid. In some embodiments, the cerebral tissue organoid is further contacted with LIF and growth serum for a portion of the fourth period of time to induce astrocyte proliferation in the brain organoid. In some embodiments, the first period of time is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days, or any number of days within a range defined by any two of the aforementioned number of days, for example, 1-14 days, 1-7 days, 7-14 days, or 5-10 days. In some embodiments, the first period of time is 7 days. In some embodiments, the second period of time is, is about, is

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at least, is at least about, is not more than, or is not more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days, or any number of days within a range defined by any two of the aforementioned number of days, for example, 1-14 days, 1-7 days, 7-14 days, or 5-10 days. In some embodiments, the second period of time is 7 days. In some embodiments, the third period of time is, is about, is at least, is at least about, is not more than, or is not more than about, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, or 70 days, or any number of days within a range defined by any two of the aforementioned number of days, for example, 20-70 days, 20-60 days, 40-70 days, or 40-60 days. In some embodiments, the third period of time is 56 days. In some embodiments, the fourth period of time is, is about, is at least, is at least about, is not more than, or is not more than about, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, or 70 days, or any number of days within a range defined by any two of the aforementioned number of days, for example, 7-70 days, 7-50 days, 20-60 days, or 20-70 days. In some embodiments, the portion of the fourth period of time is, is about, is at least, is at least about, is not more than, or is not more than about, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 17, 18, 19, 20, or 21 days, or any number of days within a range defined by any two of the aforementioned number of days, for example, 7-21 days, 7-14 days, 14-21 days, or 10-18 days. In some embodiments, the portion of the fourth period of time is 14 days. In some embodiments, the portion of the fourth period of time is at the beginning of the fourth period of time. In some embodiments, the brain organoid comprises cells that express Tuj1, Sox2, Ctip1, Tbr1, or any combination thereof. In some embodiments, the brain organoid comprises astrocytes that express S100B, GFAP, and AQP4.

[0372] In some embodiments, the BMP pathway inhibitor is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2 μ M, or any concentration within a range defined by any two of the aforementioned concentrations, for example, 0.1-2 μ M, 0.1-1 μ M, 1-2 μ M, or 0.5-1.5 μ M. In some embodiments, the BMP pathway inhibitor is provided at a concentration of 1 μ M or about 1 μ M. In some embodiments, the BMP pathway inhibitor is LDN-193189.

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[0373] In some embodiments, the TGF-beta pathway inhibitor and the second TGF-beta pathway inhibitor is the same or different. In some embodiments, the TGF-beta pathway inhibitor is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, or 4 μM , or any concentration within a range defined by any two of the aforementioned concentrations, for example, 0.5-4 μM , 0.5-2 μM , 2-4 μM , or 1-3 μM . In some embodiments, the TGF-beta pathway inhibitor is provided at a concentration of 2 μM or about 2 μM . In some embodiments, the second TGF-beta pathway inhibitor is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2 μM , or any concentration within a range defined by any two of the aforementioned concentrations, for example, 0.1-2 μM , 0.1-1 μM , 1-2 μM , or 0.5-1.5 μM . In some embodiments, the second TGF-beta pathway inhibitor is provided at a concentration of 1 μM or about 1 μM . In some embodiments, the TGF-beta pathway inhibitor and/or the second TGF-beta pathway inhibitor is A83-01. In some embodiments, the TGF-beta pathway inhibitor and/or the second TGF-beta pathway inhibitor is SB-431542.

[0374] In some embodiments, the Wnt pathway inhibitor is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, or 5 μM , or any concentration within a range defined by any two of the aforementioned concentrations, for example, 0.5-5 μM , 0.5-3 μM , 3-5 μM , or 2-4 μM . In some embodiments, the Wnt pathway inhibitor is provided at a concentration of 3 μM or about 3 μM . In some embodiments, the Wnt pathway inhibitor is IWR-1.

[0375] In some embodiments, the Wnt pathway activator is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2 μM , or any concentration within a range defined by any two of the aforementioned concentrations, for example, 0.1-2 μM , 0.1-1 μM , 1-2 μM , or 0.5-1.5 μM . In some embodiments, the Wnt pathway activator is provided at a concentration of 1 μM or about 1 μM . In some embodiments, the Wnt pathway activator is CHIR99021.

[0376] In some embodiments, the insulin is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, or 5 $\mu\text{g/mL}$, or any concentration within a range defined by any two of the aforementioned

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concentrations, for example, 0.5-5 µg/mL, 0.5-2.5 µg/mL, 2.5-5 µg/mL, or 1-3 µg/mL. In some embodiments, the insulin is provided at a concentration of 2.5 µg/mL or about 2 µg/mL.

[0377] In some embodiments, the LIF is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 mg/mL, for example, 1-20 mg/mL, 1-10 mg/mL, 10-20 mg/mL, or 5-15 mg/mL. In some embodiments, the LIF is provided at a concentration of 10 mg/mL or about 10 mg/mL.

[0378] In some embodiments, including any of the embodiments disclosed above and elsewhere herein, the brain organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM. In some embodiments, the brain organoid but not the blood vessel organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM. In some embodiments, both the brain organoid and the blood vessel organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM. In some embodiments, the brain organoid is derived from pluripotent stem cells having a genetic mutation associated with CCM and/or from a subject suffering from CCM. In some embodiments, the genetic mutation associated with CCM is a deletion or loss of function mutation of one or more of KRIT1, CCM2 and/or PDCD10. In some embodiments, the genetic mutation associated with CCM is induced into the cell by a genetic modification technique, optionally using CRISPR.

Methods of making vascularized brain organoids

[0379] Disclosed herein are methods for producing a vascularized brain organoid. In some embodiments, the methods comprise: culturing a blood vessel organoid and a brain organoid for a period of time (e.g., after contacting a blood vessel organoid and a brain organoid) until the blood vessel organoid and the brain organoid fuse together and blood vessels of the blood vessel organoid infiltrate the brain organoid, where neurons of the brain organoid innervate the blood vessels of the blood vessel organoid that have infiltrated the organoid, thereby forming the vascularized brain organoid. In some embodiments, the vascularized brain organoid comprises a blood-brain barrier that is formed between the brain organoid and all or a portion of the blood vessels of the blood vessel organoid that have infiltrated the brain organoid. In some embodiments, the blood-brain barrier comprises endothelial cells linked with tight junctions, astrocytes, and pericytes. In some

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embodiments, the blood-brain barrier comprises endothelial cells linked with tight junctions, astrocytes, pericytes, and/or smooth muscle cells. In some embodiments, the endothelial cells express CD31, GLUT-1 and PDGFR- β . In some embodiments, the tight junctions comprise claudin-5. In some embodiments, the endothelial cells express CD31, GLUT-1 and PDGFR- β . In some embodiments, the tight junctions comprise claudin-5, ZO-1 and cadherin 5. In some embodiments, the astrocytes express S100B, GFAP, and AQP4. In some embodiments, the pericytes express PDGFR- β , α -smooth muscle actin (α SMA), and neural/glial antigen 2 (NG2). In some embodiments the smooth muscle cells express SMA. In some embodiments, the endothelial cells form a continuous basement membrane and express collagen IV. In some embodiments, the vascularized brain organoid comprises cells selected from the group consisting of neural progenitors, proliferative astrocytes, GABAergic neurons, glutamatergic neurons, proliferative cells, brain vascular endothelial cells, vascular leptomeningeal cells, perivascular adipocytes, and tendon cells. In some embodiments, the vascularized brain organoid comprises cells selected from the group consisting of neural progenitor cells, GABAergic neurons, glutamatergic neurons, proliferative astrocytes, proliferative GABAergic neurons, mesenchymal stem cells, endothelial cells, pericytes, vascular smooth muscle cells, fibroblast, and proliferative cells. In some embodiments, the cells of the vascular brain organoid are identified by cell-type specific gene expression markers. In some embodiments, the cells of the vascular brain organoid are identified by cell-type specific gene expression markers. In some embodiments, the blood vessels comprise capillaries. In some embodiments, the capillaries are ensheathed by pericytes and end-feet of the astrocytes. In some embodiments, the brain organoid is a forebrain organoid, a midbrain organoid, a hypothalamus organoid, a hippocampus organoid, a spinal cord organoid, or a striatal brain organoid. In some embodiments, the blood vessel organoid and the brain organoid are contacted and/or cultured in a basement membrane matrix or component thereof, optionally Matrigel. In some embodiments, the blood vessel organoid and the brain organoid are cultured for a period of time that is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, or 70 days, or any number of days within a range defined by any two of the aforementioned days, for example, 1-70 days, 1-50 days, 30-70 days, or 30-60 days. In some embodiments, the blood vessel organoid and the brain organoid are cultured

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with agitation for at least a portion of the period of time. In some embodiments, the agitation comprises shaking. In some embodiments, the blood vessel organoid and the brain organoid are cultured: 1) without agitation for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days, or any number of days within a range defined by any two of the aforementioned days, for example, 1-14 days, 1-7 days, 7-14 days, or 5-10 days; and subsequently 2) with agitation for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, or 70 days, or any number of days within a range defined by any two of the aforementioned days, for example, 1-70 days, 1-50 days, 30-70 days, or 30-60 days. In some embodiments, the blood vessel organoid and the brain organoid are cultured in a medium that promotes neuronal growth and/or vascular growth. In some embodiments, the blood vessel organoid and the brain organoid are cultured in a medium that comprises growth factors that promote neuronal growth and/or growth factors that promote vascular growth. In some embodiments, the growth factors that promote neuronal growth comprise a cAMP pathway activator, ascorbic acid, BDNF, GDNF, or any combination thereof. In some embodiments, the growth factors that promote vascular growth comprise growth serum, a VEGF pathway activator, an FGF pathway activator, or any combination thereof. In some embodiments, the blood vessel organoid has been produced according to methods provided herein or adaptations of methods generally known in the art. In some embodiments, the brain organoid has been produced according to methods provided herein or adaptations of methods generally known in the art.

[0380] In some embodiments, culturing the blood vessel organoid and the brain organoid comprises: a) culturing the blood vessel organoid and the brain organoid without agitation for about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days, or any number of days within a range defined by any two of the aforementioned days; and b) culturing the organoids of step a) with agitation for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, or 70 days, or any number of days within a range defined by any two of the aforementioned days. In some embodiments, the blood vessel organoid and the brain organoid are cultured: without agitation for about 4-10 days; and subsequently with agitation for about 30-70 days. In some embodiments, the blood vessel organoid and the brain organoid are cultured: without agitation for about 4-10 days; and subsequently with

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agitation for about 1-40 days. In some embodiments, the organoids of step a) and step b) are cultured in a medium comprising growth factors that promote neuronal growth and/or growth factors that promote vascular growth. In some embodiments, the blood vessel organoid and the brain organoid are cultured without agitation in step a) for 7 days. In some embodiments, the organoids of step a) are cultured with agitation in step b) for at least 30 days. In some embodiments, the agitation comprises shaking. In some embodiments, the growth factors that promote neuronal growth comprise a cAMP pathway activator, ascorbic acid, brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), or any combination thereof. In some embodiments, the growth factors that promote vascular growth comprise FBS, vascular endothelial growth factor (VEGF) pathway activator, a fibroblast growth factor (FGF) activator (e.g., FGF2), or any combination thereof. In some embodiments, the blood vessel organoid and the brain organoid are cultured in a basement membrane matrix or component thereof, for example, Matrigel.

[0381] In some embodiments of any of the methods disclosed herein, the cAMP pathway activator is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, or 150 μM , or any concentration within a range defined by any two of the aforementioned concentrations, for example, 10-150 μM , 10-50 μM , 50-150 μM , or 20-100 μM . In some embodiments, the cAMP pathway activator is provided at 50 μM or about 50 μM . In some embodiments, the cAMP pathway activator is cAMP.

[0382] In some embodiments of any of the methods disclosed herein, the ascorbic acid is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, or 300 μM , or any concentration within a range defined by any two of the aforementioned concentrations, for example, 50-300 μM , 50-200 μM , 200-300 μM , or 150-250 μM . In some embodiments, the ascorbic acid is provided at 200 μM or about 200 μM .

[0383] In some embodiments of any of the methods disclosed herein, the BDNF is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 ng/mL, or any concentration within a range defined by any two of the aforementioned

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concentrations, for example, 1-30 ng/mL, 10-20 ng/mL, 20-30 ng/mL, or 15-25 ng/mL. In some embodiments, the BDNF is provided at 20 ng/mL or about 20 ng/mL.

[0384] In some embodiments of any of the methods disclosed herein, the GDNF is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 ng/mL, or any concentration within a range defined by any two of the aforementioned concentrations, for example, 1-30 ng/mL, 10-20 ng/mL, 20-30 ng/mL, or 15-25 ng/mL. In some embodiments, the GDNF is provided at 20 ng/mL or about 20 ng/mL.

[0385] In some embodiments of any of the methods disclosed herein, the growth serum is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20%, or any concentration within a range defined by any two of the aforementioned concentrations, for example, 0.5%-20%, 0.5%-5%, 1%-15%, 10-15%, 15-20%, or 12-18%. In some embodiments, the growth serum is provided at 15% or about 15%. In some embodiments, the growth serum is provided at 1% or about 1%. In some embodiments, the growth serum is fetal bovine serum (FBS).

[0386] In some embodiments of any of the methods disclosed herein, the VEGF pathway activator is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, or 150 ng/mL, or any concentration within a range defined by any two of the aforementioned concentrations, for example 10-150 ng/mL, 10-100 ng/mL, 100-150 ng/mL, or 80-120 ng/mL. In some embodiments, the VEGF is provided at 100 ng/mL or about 100 ng/mL. In some embodiments, the VEGF pathway activator is VEGF.

[0387] In some embodiments of any of the methods disclosed herein, the FGF pathway activator is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, or 150 ng/mL, or any concentration within a range defined by any two of the aforementioned concentrations, for example 10-150 ng/mL, 10-100 ng/mL, 100-150 ng/mL, or 80-120 ng/mL. In some embodiments, the FGF pathway activator is provided at 100 ng/mL or about 100 ng/mL. In some embodiments, the FGF pathway activator is FGF2.

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[0388] In some embodiments of any of the methods disclosed herein, the blood vessel organoid and/or the brain organoid are derived from pluripotent stem cells. In some embodiments, the pluripotent stem cells are embryonic stem cells or induced pluripotent stem cells. In some embodiments, the blood vessel organoid and/or the brain organoid are human. In some embodiments, the blood vessel organoid and/or the brain organoid have been derived from a subject, such as a human subject. In some embodiments, the subject comprises a cerebrovascular disease or a disease associated with blood-brain barrier dysfunction, for example, cerebral cavernous malformation, Alzheimer's disease, or amyotrophic lateral sclerosis.

[0389] In some embodiments the blood vessel organoid has been produced according to a method comprising: causing, for a first period of time, an angiogenic sprout to activate an FGF pathway, a VEGF pathway, and, optionally, a Wnt pathway of the angiogenic sprout, while the angiogenic sprout is in growth serum. In some embodiments, the first period of time is between about 1-30 days. In some embodiments, the first period of time is between about 10-30 days. In some embodiments, the first period of time is between about 5-25 days.

[0390] In some embodiments, the angiogenic sprout has been produced according to a method comprising: a) causing, for a second period of time, the pluripotent stem cells to activate a Wnt pathway and a BMP pathway of the pluripotent stem cells to form vascular lineage cells; and b) causing, for a third period of time, the vascular lineage cells to activate a VEGF pathway and a second cAMP pathway of the vascular lineage cells to form the angiogenic sprout. In some embodiments, the second period of time is between about 1-5 days. In some embodiments, the second period of time is about 3 days. In some embodiment, the third period of time is between about 1-4 days. In some embodiments, the third period of time is about 2 days. In some embodiments, the BMP pathway is activated via a BMP pathway activator. For example, the BMP pathway activator may be BMP4 or one or more BMP pathway activators disclosed herein. In some embodiments, the BMP pathway activator is provided at a concentration that is between about 10-100 ng/mL. In some embodiments, the BMP pathway activator is provided at a concentration that is between about 20-70 ng/mL. In some embodiments, the Wnt pathway is activated via a Wnt pathway activator, wherein the Wnt pathway activator is CHIR99021. In some embodiments, the Wnt pathway is activated via a Wnt pathway activator provided at a concentration that is between about 1-20 μ M. In some embodiments, the Wnt pathway is activated via a Wnt pathway activator provided at a concentration that is between about 1-12 μ M. In some

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embodiments, the Wnt pathway is activated via a Wnt pathway activator provided at a concentration that is between about 4-20 μ M. In some embodiments, the second cAMP pathway is activated via a cAMP pathway activator comprising forskolin. In some embodiments, the second cAMP pathway is activated via a second cAMP pathway activator provided at a concentration that is between about 0.5 - 4 μ M. In some embodiments, the second cAMP pathway is activated via a second cAMP pathway activator provided at a concentration that is between about 1-3 μ M. In some embodiments, the blood vessel organoid differs from a blood vessel organoid that has been produced without causing the angiogenic sprout to activate the Wnt pathway by having increased expression of blood-brain barrier-specific endothelial markers, optionally GLUT-1 and ZO-1.

[0391] In some embodiments, the brain organoid has been contacted with LIF and growth serum to induce astrocyte formation in the brain organoid.

Vascular Brain Organoids

[0392] Also disclosed herein are the vascularized brain organoids produced by any of the methods disclosed herein. In some embodiments, a vascularized brain organoid comprises endothelial cells linked with tight junctions, astrocytes, and pericytes. In some embodiments, the blood-brain barrier comprises endothelial cells linked with tight junctions, astrocytes, pericytes, and/or smooth muscle cells. In some embodiments, the endothelial cells express CD31, GLUT-1 and PDGFR- β ; the tight junctions comprise claudin-5, ZO-1 and cadherin 5; the astrocytes express S100B, GFAP, and AQP4; the pericytes express PDGFR- β , α SMA and NG2; and/or the smooth muscle cells express SMA. In some embodiments, the endothelial cells form a continuous basement membrane and express collagen IV. In some embodiments, the vascularized brain organoid comprises cells selected from the group consisting of neural progenitors, proliferative astrocytes, GABAergic neurons, glutamatergic neurons, proliferative cells, brain vascular endothelial cells, vascular leptomeningeal cells, perivascular adipocytes, and tendon cells. In some embodiments, the vascularized brain organoid comprises cells selected from the group consisting of neural progenitor cells, GABAergic neurons, glutamatergic neurons, proliferative astrocytes, proliferative GABAergic neurons, mesenchymal stem cells, endothelial cells, pericytes, vascular smooth muscle cells, fibroblast, and proliferative cells. In some embodiments, the cells are identified by cell-type specific gene expression markers. In some embodiments, the blood vessels

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comprise capillaries. In some embodiments, the capillaries are ensheathed by pericytes and end-feet of the astrocytes.

[0393] In some embodiments, the brain organoid has been produced according to a method comprising: causing, for a first period of time, pluripotent stem cells to inhibit a BMP pathway, a TGF-beta pathway, and a Wnt pathway of the pluripotent stem cells to form neuroectoderm cells; causing, for a second period of time, the neuroectoderm cells to inhibit a second TGF-beta pathway and activate a Wnt pathway of the neuroectoderm cells to form neuroepithelium cells; contacting the neuroepithelium cells with insulin for a third period of time to form cerebral tissue organoids; and for a fourth period of time: contacting the cerebral tissue organoid with GDNF, BDNF, and ascorbic acid, and activating a cAMP pathway activator of the cerebral tissue organoid to form the brain organoid.

[0394] In some embodiments, the brain organoid has been produced according to a method further comprising: contacting the cerebral tissue organoid with LIF and growth serum for a portion of the fourth period of time to induce astrocyte proliferation in the brain organoid. In some embodiments, the first period of time is between about 1-14 days. In some embodiments, the first period of time is between about 5-10 days. In some embodiments, the second period of time is between about 1-14 days. In some embodiments, the second period of time is between about 5-10 days. In some embodiments, the third period of time is between about 20-70 days. In some embodiments, the third period of time is between about 50-70 days. In some embodiments, the fourth period of time is between about 7-70 days. In some embodiments, the fourth period of time is between about 20-60 days. In some embodiments, the portion of the fourth period of time is between about 7-21 days.

[0395] In some embodiments, the BMP pathway is inhibited via a BMP pathway inhibitor (e.g., such as but not limited to LDN-193189). In some embodiments, the BMP pathway is inhibited via a BMP pathway inhibitor provided at a concentration that is between about 0.1-2 μM . In some embodiments, the BMP pathway is inhibited via a BMP pathway inhibitor provided at a concentration that is between about 0.5-1.5 μM .

[0396] In some embodiments, the TGF-beta pathway is inhibited via a TGF-beta pathway inhibitor and the second TGF-beta pathway is inhibited via a second TGF-beta pathway inhibitor. In some embodiments, the TGF-beta pathway is inhibited via a TGF-beta inhibitor (such as but not limited to A83-01). In some embodiments, the TGF-beta pathway is inhibited via a TGF-beta

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pathway inhibitor provided at a concentration that is between about 0.5-4 μM . In some embodiments, the TGF-beta pathway is inhibited via a TGF-beta pathway inhibitor provided at a concentration that is between about 1-3 μM . In some embodiments, the second TGF-beta pathway is inhibited via a TGF-beta pathway inhibitor comprising SB-431542. In some embodiments, the second TGF-beta pathway is inhibited via a second TGF-beta pathway inhibitor provided at a concentration that is between about 0.1-2 μM . In some embodiments, the second TGF-beta pathway is inhibited via a second TGF-beta pathway inhibitor provided at a concentration that is between about 0.5-1.5 μM .

[0397] In some embodiments, the Wnt pathway is inhibited via a Wnt pathway inhibitor (such as but not limited to IWR-1). In some embodiments, the Wnt pathway is inhibited via a Wnt pathway inhibitor provided at a concentration that is between about 0.5-5 μM . In some embodiments, the Wnt pathway is inhibited via a Wnt pathway inhibitor provided at a concentration that is between about 2-4 μM .

[0398] In some embodiments, the Wnt pathway is activated via a Wnt pathway activator (e.g., such as, but not limited to, CHIR99021). In some embodiments, the Wnt pathway is activated via a Wnt pathway activator provided at a concentration that is between about 0.1-2 μM . In some embodiments, the Wnt pathway is activated via a Wnt pathway activator provided at a concentration that is between about 0.5-1.5 μM . In some embodiments, the insulin is provided at a concentration that is between about 0.5-5 $\mu\text{g/mL}$. In some embodiments, the insulin is provided at a concentration that is between about 1-3 $\mu\text{g/mL}$. In some embodiments, the LIF is provided at a concentration that is between about 1-20 mg/mL . In some embodiments, the LIF is provided at a concentration that is between about 5-15 mg/mL .

[0399] In some embodiments, including any of the embodiments disclosed above and elsewhere herein, the blood vessel organoid and/or the brain organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM. In some embodiments, the blood vessel organoid but not the brain organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM. In some embodiments, the brain organoid but not the blood vessel organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM. In some embodiments, both the blood vessel organoid and the brain organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM. In some

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embodiments, the blood vessel organoid and/or the brain organoid are derived from pluripotent stem cells having a genetic mutation associated with CCM and/or from a subject suffering from CCM. In some embodiments, the genetic mutation associated with CCM is a deletion or loss of function mutation of one or more of KRIT1, CCM2 and/or PDCD10. In some embodiments, the genetic mutation associated with CCM is induced into the cell by a genetic modification technique, optionally using CRISPR.

[0400] In some embodiments, including any of the embodiments disclosed above and elsewhere herein, the method produces a vascularized brain organoid comprising a cerebral cavernous malformation (CCM)-like feature. In some embodiments, the CCM-like feature is one or more of the following, as compared to normal vascularized brain organoids: clusters of enlarged endothelial channels, optionally arranged back-to-back; upregulation of one or more lesion-marker genes, optionally in endothelial cells, optionally not in neuronal cells; upregulation of tip cell and/or tumor tip cell markers, optionally in endothelial cells, optionally not in neuronal cells; longer and/or wider tip cells when evaluated in an angiogenesis assay; expression of VEGFA and/or IGF2 in GABAergic neurons; decrease in or elimination of vascular smooth muscle cells (vSMCs); increase in the number of astrocytes; reduction in the expression of smooth muscle actin (SMA) protein, optionally measured by immunostaining, a reduction in expression of tight junction proteins; and/or disassembled basement membranes. In some embodiments, the CCM-like feature is a disruption, as compared to normal vascularized brain organoids, of the blood-brain barrier that is formed between the brain organoid and all or a portion of the blood vessels of the blood vessel organoid that have infiltrated the brain organoid.

[0401] An aspect of the present disclosure is a vascularized brain organoid produced by the method of any of the embodiments disclosed above, and elsewhere herein. An aspect of the present disclosure is a vascularized brain organoid comprising a CCM-like feature and endothelial cells linked with tight junctions, astrocytes, and pericytes. In some embodiments, the blood-brain barrier comprises endothelial cells linked with tight junctions, astrocytes, pericytes, and/or smooth muscle cells. In some embodiments, the endothelial cells express CD31, GLUT-1 and PDGFR- β ; the tight junctions comprise claudin-5, ZO-1 and cadherin 5; the astrocytes express S100B, GFAP, and AQP4; the pericytes express PDGFR- β , α SMA and NG2; and/or the smooth muscle cells express SMA. In some embodiments, the endothelial cells form a continuous basement membrane and express collagen IV. In some embodiments, the vascularized brain organoid comprises cells

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selected from the group consisting of neural progenitors, proliferative astrocytes, GABAergic neurons, glutamatergic neurons, proliferative cells, brain vascular endothelial cells, vascular leptomeningeal cells, perivascular adipocytes, and tendon cells. In some embodiments, the vascularized brain organoid comprises cells selected from the group consisting of neural progenitor cells, GABAergic neurons, glutamatergic neurons, proliferative astrocytes, proliferative GABAergic neurons, mesenchymal stem cells, endothelial cells, pericytes, vascular smooth muscle cells, fibroblast, and proliferative cells. In some embodiments, the cells are identified by cell-type specific gene expression markers. In some embodiments, the blood vessels comprise capillaries. In some embodiments, the capillaries are ensheathed by pericytes and end-feet of the astrocytes. In some embodiments, the vascularized brain organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM. In some embodiments, the vascularized brain organoid is derived from pluripotent stem cells having a genetic mutation associated with CCM and/or from a subject suffering from CCM. In some embodiments, the genetic mutation associated with CCM is a deletion or loss of function mutation of one or more of KRIT1, CCM2 and/or PDCD10. In some embodiments, the genetic mutation associated with CCM is induced into the cell by a genetic modification technique, optionally using CRISPR. In some embodiments, the vascularized brain organoid is human. In some embodiments, the vascularized brain organoid comprises a CCM-like feature, wherein the CCM-like feature is one or more of the following, as compared to normal vascularized brain organoids and/or normal brain tissue: clusters of enlarged endothelial channels, optionally arranged back-to-back; upregulation of one or more lesion-marker genes, optionally in endothelial cells, optionally not in neuronal cells; upregulation of tip cell and/or tumor tip cell markers, optionally in endothelial cells, optionally not in neuronal cells; longer and/or wider tip cells when evaluated in an angiogenesis assay; expression of VEGFA and/or IGF2 in GABAergic neurons; decrease in or elimination of vascular smooth muscle cells (vSMCs); increase in the number of astrocytes; reduction in the expression of smooth muscle actin (SMA) protein, optionally measured by immunostaining; a reduction in expression of tight junction proteins; and/or disassembled basement membranes. In some embodiments, the CCM-like feature is a disruption of the blood-brain barrier that is formed between the brain organoid and all or a portion of the blood vessels of the blood vessel organoid that have infiltrated the brain organoid, as compared to normal vascularized brain organoids and/or normal brain tissue.

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[0402] Aspects of the present disclosure also include vascular brain organoids. In some embodiments, a vascularized brain organoid comprises a brain organoid and a blood vessel organoid, wherein at least a portion of the blood vessels of the blood vessel organoid have infiltrated the brain organoid, and neurons of the brain organoid innervate at least a portion of the infiltrating blood vessels. In some embodiments, the vascularized brain organoid has disease features, optionally wherein the disease features are CCM-like features. In some embodiments, a blood-brain barrier that is formed between the brain organoid and all or a portion of the blood vessels that have infiltrated the brain organoid. In some embodiments, the vascular brain organoid includes, comprises, and/or exhibits gene expression markers indicative of excitatory neurons, inhibitory neurons, neural progenitors (NPs), astrocytes, endothelial cells (ECs), pericytes, mesenchymal stem cells (MSCs), smooth muscle cells (SMCs), and fibroblasts. In some embodiments, the vascularized brain organoid comprises endothelial cells linked with tight junctions, astrocytes, pericytes, and smooth muscle cells. The endothelial cells express CD31, GLUT-1 and PDGFR- β . The tight junctions comprise or express claudin-5, ZO-1 and cadherin 5. The astrocytes express S100B, GFAP, and AQP4. The pericytes express PDGFR- β , α SMA and NG2. The smooth muscle cells express SMA. In some embodiments, the endothelial cells form a continuous basement membrane and express collagen IV.

[0403] In some embodiments, the vascular brain organoid comprises cells selected from the group consisting of neural progenitors, proliferative astrocytes, GABAergic neurons, glutamatergic neurons, proliferative cells, brain vascular endothelial cells, vascular leptomeningeal cells, perivascular adipocytes, and tendon cells. In some embodiments, the cells are identified by cell-type specific gene expression markers. In some embodiments, the blood vessels of the vascularized brain organoid comprise capillaries. In some embodiments, the capillaries are ensheathed by pericytes and end-feet of the astrocytes. In some embodiments, the brain organoid is a forebrain organoid, a midbrain organoid, a hypothalamus organoid, a hippocampus organoid, a spinal cord organoid, and/or a striatal brain organoid. In some embodiments, the blood vessel organoid and the brain organoid were contacted and/or cultured in a basement membrane matrix or component thereof, optionally Matrigel.

[0404] In some embodiments, the vascularized brain organoid comprises cells having one or more genetic mutations and/or one or more phenotypes associated with neurodegenerative disorders, prion disease, Huntington disease, Alzheimer's disease, Fragile X syndrome,

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Parkinson's disease, amyotrophic lateral sclerosis (ALS), dementia, multiple sclerosis, encephalitis, epilepsy, infections, anxiety, bipolar disorder, depression, post-traumatic stress disorder, schizophrenia, attention deficit disorder, autism spectrum disorder, dyslexia, stroke, seizure, viral infection, bacterial infection, parasitic infection, traumatic brain injury, and/or cancer. In some embodiments, the vascularized brain organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM. In some embodiments, the vascularized brain organoid is derived from pluripotent stem cells having a genetic mutation associated with CCM and/or from a subject suffering from CCM. In some embodiments, the genetic mutation associated with CCM is a deletion or loss of function mutation of one or more of KRIT1, CCM2 and/or PDCD10. In some embodiments, the genetic mutation associated with CCM is induced into the cell by a genetic modification technique, optionally using CRISPR.

[0405] In some embodiments, the vascularized brain organoid comprises one or more CCM-like features. The CCM-like feature is one or more of the following, as compared to a normal vascularized brain organoids and/or a normal brain tissue: a cluster of enlarged endothelial channels, optionally arranged back-to-back; an upregulation of one or more lesion-marker genes, optionally in endothelial cells, optionally not in neuronal cells; an upregulation of tip cell and/or tumor tip cell markers, optionally in endothelial cells, optionally not in neuronal cells; a longer and/or a wider tip cell when evaluated in an angiogenesis assay; an expression of VEGFA and/or IGF2 in GABAergic neurons; a decrease in or an elimination of vascular smooth muscle cells (vSMCs); an increase in the number of astrocytes; a reduction in the expression of smooth muscle actin (SMA) protein, optionally measured by immunostaining; a reduction in expression of a tight junction protein; and/or a disassembled basement membrane. In some embodiments, the CCM-like feature is a disruption of the blood-brain barrier that is formed between the brain organoid and all or a portion of the blood vessels of the blood vessel organoid that have infiltrated the brain organoid, as compared to normal vascularized brain organoids and/or normal brain tissue.

[0406] In some embodiments, the vascularized brain organoid exhibits one or more of the following features, as compared to the blood vessel organoids prior to culturing the blood vessel organoid and the brain organoid: reduced endothelial fenestration; increased expression of drug pumps; and/or reduced expression of an immune cell adhesion molecule marker. In some embodiments, the reduced endothelial fenestration comprises reduced expression or non-

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expression of PLVAP. In some embodiments, the increased expression of drug pumps comprises increased expression of glucose transporter 1 (GLUT1), P-glycoprotein (P-gp), and/or one or more tight junction proteins. In some embodiments, the one or more tight junction proteins comprises one or more of Claudin-5 or ZO-1. In some embodiments, the reduced expression of immune cell adhesion molecule marker comprises the reduced expression or non-expression of immune cell adhesion molecule 1 (ICAM-1). In some embodiments, the vascularized brain organoid exhibits the following features, as compared to the blood vessel organoids priori to culturing the blood vessel organoid and the brain organoid: reduced permeability; and increased trans-endothelial resistance. In some embodiments, the increased trans-endothelial resistance comprises an increased transepithelial/trans-endothelial electrical resistance (TEER) value. In some embodiments, an increase in TEER value of the vascularized brain organoid, as compared to a TEER value of the blood vessel organoids priori to culturing the blood vessel organoid and the brain organoid, is between about 850-1200 $\Omega \cdot \text{cm}^2$. In some embodiments, an increase in TEER value of the vascularized brain organoid, as compared to a TEER value of the blood vessel organoids priori to culturing the blood vessel organoid and the brain organoid, is between about 950-1100 $\Omega \cdot \text{cm}^2$. In some embodiments, the vascularized brain organoid exhibits the following features, as compared to the brain organoid priori to culturing the blood vessel organoid and the brain organoid: increased numbers of mature astrocytes; more mature astrocytes; increased numbers of mature endothelial cells; and/or more mature endothelial cells.

CCM Primary Tissue Organoids

[0407] An aspect of the disclosure is a method of making a CCM primary tissue organoid. In some embodiments, the method comprises culturing primary cavernomas tissue in culture medium until CCM primary tissue organoids form. In some embodiments, the culturing primary cavernomas tissue in culture medium until organoids form is for a period of time about 1-2 weeks. In some embodiments, the method further comprises expanding the CCM primary tissue organoids by dissecting the CCM primary tissue organoids into pieces about 0.5 mm in diameter, and culturing the about 0.5 mm in diameter pieces until CCM primary tissue organoids form. In some embodiments, the method optionally comprises: a) incubating primary cavernomas tissue pieces about 0.5 mm diameter in red blood cell lysis buffer, optionally neutralizing the lysis buffer at the end of the incubation; b) culturing in ultra-low attachment plates with agitation primary

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cavernomas tissue pieces about 0.5 mm diameter following incubation in red blood cell lysis buffer, optionally following neutralization of the lysis buffer; c) culturing at about 37°C, 5% CO₂ and 95% air; and/or d) changing the culture medium about every 3 days.

[0408] In some embodiments, the culture media comprises about 10 ng/mL BDNF, about 10 ng/mL of GDNF, about 7.5% FBS, about 50 ng/mL VEGF-A, and about 50 ng/mL FGF-2, optionally wherein the culture media is half M4 complete media and half StemPro-34 SFM complete media, optionally further comprising about 0.025 mM cAMP, and about 0.1 mM of Ascorbic acid. In some embodiments, the cavernomas tissue is human. In some embodiments, the method produces a CCM primary tissue organoid comprising a CCM-like feature. In some embodiments, the CCM-like feature is one or more of the following, as compared to normal brain tissue: clusters of enlarged endothelial channels, optionally arranged back-to-back; upregulation of one or more lesion-marker genes, optionally in endothelial cells, optionally not in neuronal cells; upregulation of tip cell and/or tumor tip cell markers, optionally in endothelial cells, optionally not in neuronal cells; longer and/or wider tip cells when evaluated in an angiogenesis assay; expression of VEGFA and/or IGF2 in GABAergic neurons; decrease in or elimination of vascular smooth muscle cells (vSMCs); increase in the number of astrocytes; reduction in the expression of smooth muscle actin (SMA) protein, optionally measured by immunostaining; a reduction in expression of tight junction proteins; and/or disassembled basement membranes. In some embodiments, the CCM-like feature is a disruption of the blood-brain barrier as compared to normal brain tissue

[0409] An aspect of the disclosure is a CCM primary tissue organoid made by the method of any of the embodiments above, and disclosed elsewhere herein. An aspect of the invention is a CCM primary tissue organoid comprising a CCM-like feature. In some embodiments, the CCM-like feature is one or more of the following, as compared to normal brain tissue: clusters of enlarged endothelial channels, optionally arranged back-to-back; upregulation of one or more lesion-marker genes, optionally in endothelial cells, optionally not in neuronal cells; upregulation of tip cell and/or tumor tip cell markers, optionally in endothelial cells, optionally not in neuronal cells; longer and/or wider tip cells when evaluated in an angiogenesis assay; expression of VEGFA and/or IGF2 in GABAergic neurons; decrease in or elimination of vascular smooth muscle cells (vSMCs); increase in the number of astrocytes; reduction in the expression of smooth muscle actin (SMA) protein, optionally measured by immunostaining; a reduction in expression of tight

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junction proteins; and/or disassembled basement membranes. In some embodiments, the CCM-like feature is a disruption of the blood-brain barrier as compared to normal brain tissue.

Methods of use

[0410] Also disclosed herein are methods of treating a cerebrovascular disease or a disease associated with blood-brain barrier dysfunction in a subject in need thereof. In some embodiments, the methods comprise administering any of the vascularized brain organoids disclosed herein, or a portion or fragment thereof, to the subject

[0411] Also disclosed herein are methods of screening. In some embodiments, the methods comprise contacting any of the vascularized brain organoids disclosed herein, or portions thereof, with a candidate compound or composition, and assessing the effects of the candidate compound or composition on the vascularized brain organoid or portions thereof. In some embodiments, the effect comprises transport of the candidate compound or composition across the blood-brain barrier of the organoid or portions thereof. In some embodiments, the vascularized brain organoid is a model for a cerebrovascular disease or a disease associated with blood-brain barrier dysfunction, and assessing the effects of the candidate compound or composition on the vascularized organoid comprises assessing the effects of the candidate compound or composition on the cerebrovascular disease or the disease associated with blood-brain barrier dysfunction. In some embodiments, the vascularized brain organoid has been produced from cells derived from a subject. In some embodiments, the cells derived from the subject are pluripotent stem cells. In some embodiments, the subject has or is disposed to develop the cerebrovascular disease or the disease associated with blood-brain barrier dysfunction.

[0412] Disclosed herein is a method of treating a cerebrovascular disease or a disease associated with blood-brain barrier dysfunction in a subject in need thereof, comprising administering to the subject a vascularized brain organoid comprising a CCM-like feature, or a portion or fragment thereof, or a CCM primary tissue organoid, or a portion or fragment thereof.

[0413] Disclosed herein is a method of screening, comprising contacting a vascularized brain organoid comprising a CCM-like feature, or a CCM primary tissue organoid, or portions or fragments thereof, with a candidate compound or composition, and assessing the effects of the candidate compound or composition on a vascularized brain organoid comprising a CCM-like feature, a CCM primary tissue organoid, or portions thereof. In some embodiments, the effects

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comprises transport of the candidate compound or composition across the blood-brain barrier of a vascularized brain organoid comprising a CCM-like feature, a CCM primary tissue organoid, or portions thereof. In some embodiments, wherein a vascularized brain organoid comprising a CCM-like feature and/or CCM primary tissue organoid is a model for a cerebrovascular disease or a disease associated with blood-brain barrier dysfunction, and assessing the effects of the candidate compound or composition on the vascularized organoid and/or CCM primary tissue organoid comprises assessing the effects of the candidate compound or composition on the cerebrovascular disease or the disease associated with blood-brain barrier dysfunction. In some embodiments, a vascularized brain organoid comprising a CCM-like feature and/or CCM primary tissue organoid has been produced from cells derived from a subject, optionally wherein the cells derived from the subject are induced pluripotent stem cells. In some embodiments, the subject has or is disposed to develop the cerebrovascular disease or the disease associated with blood-brain barrier dysfunction. In some embodiments, the cerebrovascular disease or the disease associated with blood-brain barrier dysfunction is CCM. In some embodiments, the cerebrovascular disease or the disease associated with blood-brain barrier dysfunction is a cerebrovascular disease or a disease associated with blood-brain barrier dysfunction in addition to CCM.

[0414] Aspects of the present disclosure also relate to the use any of the methods, the vascularized brain organoids, the CCM primary tissue organoids, or cell culture media described herein, as a medicament, means for treatment and/or prevention of a disease, means for diagnosis, and/or medical research tool.

Compositions, Cell Culture Media, and Kits

[0415] Aspects of the present disclosure also relate to cell culture media for generating vascularized brain organoids, blood vessel organoids, and/or brain organoids, as well as compositions comprising the cell culture media and the vascularized brain organoids. In some embodiments, a cell culture media comprises a first media component that promotes neuronal growth, and a second media component that promotes vascular growth. In some embodiments, the first and/or second media component comprise added growth factors that promote neuronal growth and/or promote vascular growth. In some embodiments, the added growth factors that promote neuronal growth comprise, consist essentially of, or consist of a cAMP pathway activator, ascorbic acid, BDNF, and/or GDNF. In some embodiments, the added growth factors that promote vascular growth comprise, consist essentially of, or consist of growth serum, a VEGF pathway activator,

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and/or an FGF pathway activator. In some embodiments, the cell culture media further comprise a basement membrane matrix or component thereof.

[0416] In some embodiments, the added cAMP pathway activator is cAMP. In some embodiments, the added cAMP pathway activator is at a concentration that is between about 10-150 μ M. In some embodiments, the added cAMP pathway activator is at a concentration that is between about 20-100 μ M. In some embodiments, the added ascorbic acid is at a concentration that is between about 50-300 μ M. In some embodiments, the ascorbic acid is provided at a concentration that is between about 150 - 250 μ M. In some embodiments, the added BDNF is at a concentration that is between about 1-30 ng/mL. In some embodiments, the BDNF is provided at a concentration that is between about 15 - 25 ng/mL. In some embodiments, the added GDNF is at a concentration that is between about 1-30 ng/mL. In some embodiments, the GDNF is provided at a concentration that is between about 15 - 25 ng/mL. In some embodiments, the added growth factors are not xenogeneic to human cells, and/or are of good manufacturing practices (GMP) grade. In some embodiments, the added growth serum is fetal bovine serum (FBS). In some embodiments, the added growth serum is at a concentration that is between about 0.5%-20%. In some embodiments, the growth serum is provided at a concentration that is between about 12%-18%. In some embodiments, wherein the VEGF pathway activator is VEGF. In some embodiments, the added VEGF pathway activator is at a concentration that is between about 10-150 ng/mL. In some embodiments, the VEGF pathway activator is provided at a concentration that is between about 80-120 ng/mL. In some embodiments, the FGF pathway activator is FGF2. In some embodiments, the added FGF pathway activator is at a concentration that is between about 10-150 ng/mL. In some embodiments, the FGF pathway activator is provided at a concentration between about 80-120 ng/mL.

[0417] In some embodiments, the cell culture media comprises about 10 ng/mL BDNF, about 10 ng/mL of GDNF, about 7.5% FBS, about 50 ng/mL VEGF-A, and about 50 ng/mL FGF-2, optionally wherein the culture media is, or is about, half M4 complete media and is, or is about, half StemPro-34 SFM complete media, optionally further comprising about 0.025 mM cAMP, and about 0.1 mM of Ascorbic acid. In some embodiments, the cell culture media comprises a combination of half M4 complete medium and half StemPro-34 SFM complete medium, wherein the M4 complete medium comprises 0.05 mM of cAMP, 0.2 mM of Ascorbic acid, 20 ng/mL of BDNF, and 20 ng/mL of GDNF and M4 base medium, and wherein the StemPro-34 SFM complete

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medium comprises the StemPro-34 SFM base medium, supplemented with 15% FBS, 100 ng/mL VEGF-A, and 100 ng/mL FGF-2. In some embodiments, the cell culture media further comprises blood vessel organoids and/or brain organoids, optionally wherein the blood vessel organoids and/or brain vessel organoids are derived from pluripotent stem cells, optionally embryonic stem cells or induced pluripotent stem cells.

[0418] Aspects of the present disclosure also relate to another cell culture media for generating a vascularized brain organoid. In some embodiments, the cell culture media comprises: a base endothelial cell (EC) media; a vascular endothelial growth factor A (VEGF-A); and an endothelial cell growth supplement (ECGS). In some embodiments, the base EC media comprises FBS. In some embodiments, the base EC media comprises FBS at a concentration of about 0.5% to about 20% of the cell culture media. In some embodiments, the ECGS comprises acidic FGF. In some embodiments, the ECGS comprises acidic FGF at a concentration of about 10 to 500 ng/ml. In some embodiments, the VEGF-A is at a concentration of about 10 to 500 ng/ml of cell culture media. In some embodiments, the VEGF-A is at a concentration of about 50 to 200 ng/ml of cell culture media. In some embodiments, the cell culture media further comprises Heparin. In some embodiments, the Heparin has a concentration of about 0 to 20 µg/ml of the cell culture media. In some embodiments, the Heparin has a concentration of about 5 to 15 µg/ml of the cell culture media. In some embodiments, the base EC media comprises DMEM/F12.

[0419] Aspects of the present disclosure also relate to compositions. In some embodiments, a Composition is disclosed that comprises cell culture media disclosed herein and further comprise a vascularized brain organoid disclosed herein. In some embodiments, the vascularized brain organoid is produced by any of the methods described herein. In some embodiments, the composition further comprises blood vessel organoids and/or brain organoids, optionally wherein the blood vessel organoids and/or brain vessel organoids are derived from pluripotent stem cells, optionally embryonic stem cells or induced pluripotent stem cells.

[0420] In some embodiments, the vascularized brain organoid comprises cells having one or more genetic mutations and/or one or more phenotypes associated with Fragile X syndrome. In some embodiments, the cells having the one or more phenotypes associated with Fragile X syndrome results in enlarged capillary perimeters and/or diameters in the vascularized organoid, compared to cells not having one or more genetic mutations and/or one or more phenotypes associated with Fragile X syndrome. In some embodiments, the cells having the one or more

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genetic mutations associated with Fragile X syndrome comprises cells having an upregulation of an angiogenic growth factor, compared to cells not having the one or more genetic mutations and/or one or more phenotypes associated with Fragile X syndrome. In some embodiments, the cells having the one or more genetic mutations associated with Fragile X syndrome comprises cells having reduction of an Claudin-5, glucose transporter 1 (GLUT-1), and P-glycoprotein (P-gp), compared to cells not having the one or more genetic mutations and/or one or more phenotypes associated with Fragile X syndrome. In some embodiments, the cells having the one or more genetic mutations associated with Fragile X syndrome comprises cells with a hyperactivation of mTOR signaling pathway, compared to cells not having the one or more genetic mutations and/or one or more phenotypes associated with Fragile X syndrome. In some embodiments, the cells having the one or more genetic mutations associated with Fragile X syndrome comprises cells with a downregulation of Wnt signaling pathway, compared to cells not having the one or more genetic mutations and/or one or more phenotypes associated with Fragile X syndrome.

[0421] In some embodiments, the vascularized brain organoid comprises cells having one or more genetic mutations and/or one or more phenotypes associated with Alzheimer's Disease. In some embodiments, the cells having the one or more genetic mutations associated with Alzheimer's Disease comprises cells having reduction of an Claudin-5, glucose transporter 1 (GLUT-1), and P-glycoprotein (P-gp), compared to cells not having the one or more genetic mutations and/or one or more phenotypes associated with Alzheimer's Disease.

[0422] In some embodiments, the composition further comprises a viral vector. The vascularized brain organoid exhibits one or more phenotypes associated with infection by the viral vector. In some embodiments, the one or more phenotypes associated with infection by the viral vector comprises: infected neurons; infected neural progenitors; and/or infected astrocytes.

[0423] Aspects of the present disclosure also relate to a kit comprising means for performing any of the methods disclosed herein. Aspects of the present disclosure also relate to a kit comprising any one of the vascularized brain organoid or CCM primary tissue organoid, or means for generating any one of the vascularized brain organoid or CM primary tissue organoid described herein.

[0424] Aspects of the present disclosure also relate to a kit comprising any of the cell culture media, or means for generating the cell culture media described herein.

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EXAMPLES

Example 1. Generation of blood vessel (vascular) organoids

[0425] Exemplary methods for producing blood vessel (vascular) organoids from pluripotent stem cells may be found in Wimmer et al. Generation of blood vessel organoids from human pluripotent stem cells. *Nature Protocols* (2019) 14(11):3082-3100 and Wimmer et al. Human blood vessel organoids as a model of diabetic vasculopathy. *Nature* (2019) 565(7740):505-510, each of which is hereby expressly incorporated by reference in its entirety. A schematic for an improved method for producing blood vessel organoids from pluripotent stem cells is depicted in **FIG. 1J**.

[0426] Preparation of feeder-free iPSC culture:

[0427] Matrigel was thawed on ice for up to 1 hour. 100 μ L of thawed Matrigel was diluted in 6 mL of cold DMEM/F12 (for a 6 well plate). The Matrigel dilution was mixed well and used to coat a 6 well plate with 1 mL of Matrigel mixture per well. The plate was placed in a 37°C incubator for up to 2 hours or overnight. iPSCs grown to 60-80% confluence were washed with approximately 2 mL of room temperature phosphate buffered saline (PBS). 1 mL of ReleSR dissociation reagent (StemCell Technologies) was added per well containing iPSCs and after 1 minute, most of the ReleSR was aspirated out, leaving some to keep iPSCs covered. The iPSCs were incubated in the ReleSR reagent for 6-8 minutes at room temperature. The sides of the iPSC plate were tapped to detach the iPSCs. 1 mL of mTeSR Plus medium (StemCell Technologies) was added per well to neutralize the ReleSR dissociation reagent. The plate was shaken gently to wash the iPSCs. The dissociated iPSCs were transferred to a 15 mL tube and dissociated to single cells by pipetting. The Matrigel mixture was aspirated from the coated 6 well plate and 2 mL of mTeSR Plus per well was added to the Matrigel plate. iPSCs were added to the Matrigel-coated plate at the desired density (1:20 – 1:50 dilution, or 10,000 – 30,000 cells per well). The plate was shaken briefly and returned to a 37°C incubator. The growth medium was changed every day until the iPSCs reached approximately 80% confluence (5-7 days). These iPSCs can be used downstream for blood vessel organoid differentiation or passaged again for later use.

[0428] Preparation of blood vessel organoids:

[0429] One day prior to initiating the blood vessel organoid differentiation process (Day -1), iPSCs were seeded onto an AggreWell 400 (StemCell Technologies) 24-well plate at 1.2×10^6 cells per plate in aggregation medium (KnockOut DMEM/F12, 99 μ M β -mercaptoethanol, Knockout Serum Replacement, 1x Glutamax, 1x non-essential amino acids (NEAA), 1x penicillin-

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streptomycin) supplemented with 50 μ M Y-27632 (ROCK inhibitor), to form uniform stem cell aggregates.

[0430] On the first day (Day 0), the iPSCs were induced to differentiate into mesoderm by culturing the aggregates in N2B27 medium (50% DMEM/F12, 50% neurobasal medium, 99 μ M β -mercaptoethanol, 1x Glutamax, 1x penicillin-streptomycin, 1x B27 supplement, 1x N2 supplement) supplemented with 12 μ M CHIR99021 and 30 ng/mL BMP4.

[0431] After four days of mesoderm induction (Day 3), the differentiated mesoderm cells were induced to differentiate into a vascular lineage by culturing the mesoderm cells in N2B27 medium supplemented with 100 ng/mL VEGF and 2 μ M forskolin.

[0432] After 2 days of vascular induction (Day 5), the differentiated vascular lineage cells were further cultured to develop formation of blood vessels.

[0433] For one 12 well plate, 5 mL of a 2 mg/mL collagen I solution was prepared by mixing 300 μ L of 0.1 N NaOH, 450 μ L of ddH₂O, 313 μ L of 10x DMEM, 63 μ L of HEPES, 49 μ L of 7.5% sodium bicarbonate, 31 μ L of Glutamax, 460 μ L of Ham's F-12, and 3.33 mL of a 3 mg/mL collagen stock solution (PureCol; Advanced Biomatrix). The pH of this collagen I solution should be 7.4. Subsequently, a 4:1 collagen I solution to Matrigel mixture was prepared by mixing 4.5 mL of the collagen I solution with 1.5 mL of growth factor reduced Matrigel on ice.

[0434] 0.5 mL of the collagen I/Matrigel mixture was used to coat the wells of a 12 well plate and incubated at 37°C for 2 hours to solidify the mixture. The vascular lineage cells were resuspended in another fresh batch of collagen I/Matrigel mixture (unsolidified), and 0.5 mL of the cell suspension in collagen I/Matrigel was used to seed each well of the collagen I/Matrigel coated plates. The plate was returned to a 37°C incubator for 2 hours to solidify the collagen I/Matrigel mixture containing vascular lineage cells. Subsequently, complete StemPro-34 serum free medium (Thermo Fisher) supplemented with 15% fetal bovine serum (FBS), 4 μ M CHIR99021, 100 ng/mL VEGF, and 100 ng/mL FGF2 was added to the vascular lineage cells.

[0435] Over the course of 5 days of culturing in StemPro-34 media containing 15% FBS, 4 μ M CHIR99021, 100 ng/mL VEGF, and 100 ng/mL FGF2, the vascular lineage cells mature to form blood vessel organoids comprising vascular networks. These blood vessel organoids may be used for optional downstream studies, such as isolating the blood vessels from the organoids, or transplant of the organoids *in vivo*.

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[0436] In FIG. 1K, stem cells engineered to express GFP were differentiated to blood vessel organoids according to the method provided herein. The cells of the blood vessel organoids were organized in a vascular network. As shown in FIGS. 1D-1E, the cells of the blood vessel organoids expressed platelet endothelial cell adhesion molecule (PECAM-1; CD31) and platelet-derived growth factor receptor beta (PDGFR- β), which are markers for early endothelial cells. PDGFR- β is also expressed by pericyte progenitor cells, which give rise to pericytes that are involved in the blood-brain barrier. In FIG. 1E, endothelial tube structures with a lumen were present in the blood vessel organoids.

Example 2. Generation of cortical organoids

[0437] Exemplary methods for producing blood vessel organoids from pluripotent stem cells may be found in Qian et al. Brain-Region-Specific Organoids Using Mini-bioreactors for Modeling ZIKV Exposure. Cell (2016) 165(5):1238-1254 and Qian et al. Generation of human brain region-specific organoids using a miniaturized spinning bioreactor. Nature Protocols (2018) 13(3):565-580, each of which is expressly incorporated by reference in its entirety. A schematic for an exemplary method for producing dorsal forebrain organoids, which are a type of cortical organoid, is provided in FIG. 8A. These methods can be adapted to produce cortical organoids of alternative types, such as midbrain, striatal brain, hypothalamus, hippocampal, and spinal cord organoids.

[0438] On the first day (Day 0), iPSCs were seeded on to Aggrewell 800 24-well plates (StemCell Technologies) at a final cell density of approximately 4×10^6 cells per Aggrewell plate in 2 mL of Essential 8 medium (Thermo Fisher) supplemented with 10 μ M Y-27632 (ROCK inhibitor). The Aggrewell was centrifuged at 100xg for 3 minutes at 4°C to collect the stem cells at the bottom of the Aggrewell microwells. The Aggrewell may be first washed with Anti-Adherence Rinsing solution (StemCell Technologies) and washed with Essential 8 medium prior to adding the cells. The use of the Aggrewell plate is optional, but the use of this plate helps attain greater final numbers of organoids with more uniform size. A standard low attachment plate may also be used, which will result in larger organoids.

[0439] After 1 day of culture (Day 1) (or 2 days if the embryoid bodies are not large enough), the Aggrewell culture were agitated with a pipette to resuspend the embryoid bodies (EBs), and the resuspension was transferred to 15 mL tube. The EBs were washed with fresh DMEM/F12, resuspended in H1 medium (77% DMEM/F12, 20% KnockOut Serum Replacement medium, 1x

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Glutamax, 1x NEAA, 1x β -mercaptoethanol, 1x penicillin-streptomycin supplemented with 1 μ M LDN-193189, 2 μ M A83-01, and 3 μ M IWR-1), and seeded onto a ultra-low attachment 6 well plate. The EBs were allowed to settle, the medium was aspirated away, and 3 mL of H1 medium supplemented with 10 μ M Y-27632 was added to each well. The plate was incubated with shaking (120 rpm) at 37°C for 48 hours. After the 48 hours, the medium was replaced with 3 mL of fresh H1 medium (without the ROCK inhibitor). After another 24 hours (Day 3 of culture), the medium was again changed with fresh H1 medium (without the ROCK inhibitor).

[0440] After 4 days of culture in H1 medium (Day 4), the medium was changed out for H1 medium without IWR-1 and cultured for an additional 2 days with shaking (120 rpm) at 37°C.

[0441] After 2 days (Day 7), the resultant neuroectoderm cells were transferred to a 1.5 mL tube and allowed to settle. The supernatant was removed and the EBs were washed twice with 1 mL of F2 medium (DMEM/F12, 1x N2, 1x Glutamax, 1x NEAA, 1x β -mercaptoethanol, 1x penicillin-streptomycin supplemented with 1 μ M SB-431542 and 1 μ M CHIR99021). 67 μ L of the resuspended EB in F12 medium (containing less than 60 EBs) were transferred to a fresh tube and combined with 100 μ L of Matrigel. The EB/Matrigel mixture was added to a plate and solidified in a 37°C incubator for 30 minutes. 3 mL of F2 medium was carefully added to the wells containing the EB/Matrigel droplets and returned to a 37°C incubator without shaking for 48 hours. Every two days (Days 9, 11, 13) for a total of 6 days, the medium was replaced with fresh F2 medium.

[0442] At Day 14, the resultant neuroepithelium cells were released from the Matrigel and resuspended in 1-3 mL of H3 medium (50% DMEM/F12, 50% neurobasal medium, 1x N2, 1x B27, 1x Glutamax, 1x NEAA, 1x β -mercaptoethanol, 1x penicillin-streptomycin supplemented with 2.5 μ g/mL insulin). The EB resuspension in H3 medium was added to a regular 6 well plate, and an additional 4.5 mL of H3 medium was added to each well. The cells were incubated with shaking (120 rpm) at 37°C.

[0443] Over the course of 54 days (Days 16-70), the medium is changed for fresh H3 medium every 2 days.

[0444] When the cerebral tissue organoids grow to a diameter of more than 1 mm (after Day 70), the medium is changed out to 3 mL F4 medium (neurobasal medium, 1x B27, 1x Glutamax, 1x NEAA, 1x β -mercaptoethanol, 1x penicillin-streptomycin supplemented with 0.05 mM

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cAMP, 0.2 mM ascorbic acid, 20 ng/mL BDNF, and 20 ng/mL GDNF) to differentiate the cerebral tissue to cortical forebrain organoids. The medium is changed for fresh F4 medium every 2 days.

[0445] For astrocyte induction, the F4 medium used any time after Day 70 is supplemented with 1% FBS (which can be increased up to 15%) and 10 mg/mL leukemia inhibitory factor (LIF) for a span of 2 weeks.

[0446] As shown in **FIG. 1B**, the forebrain organoid produced according to the method herein expressed the neuronal cell marker class III beta-tubulin (Tuj1) and neural stem cell marker SRY-Box transcription factor (Sox2) as detected at Day 26 of culture, and the neural cell markers B-cell lymphoma/leukemia 11B (BCL11B; Ctjp1) and T-Box brain transcription factor 1 (Tbr1) as detected at Day 61 of culture.

[0447] **FIGS. 1C, 8C and 8D** show images of a forebrain organoid further cultured with 1% FBS and 10 mg/mL LIF for 2 weeks to induce astrocyte formation. Astrocyte presence was confirmed by the detection of astrocyte markers S100 calcium binding protein B (S100B), glial fibrillary acidic protein (GFAP), and aquaporin 4 (AQP4).

Example 3. Generation of fused vascularized cortical organoids

[0448] Blood vessel organoids and cortical organoids are produced according to methods provided herein (e.g., in the Examples), or otherwise generally known in the art. To generate vascularized cortical organoids modeling an intact blood-brain barrier, cortical organoids cultured to contain astrocytes were used. A schematic for an approach for forming fused vascularized cortical organoids is provided in **FIGS. 1A and 1F**.

[0449] A Matrigel mixture of 100 μ L Matrigel and 60 μ L of ice cold brain vascularization medium (a 50%/50% mixture of a) StemPro-34 medium with 15% FBS, 100 ng/mL VEGF, and 100 ng/mL FGF2 and b) F4 medium (neurobasal medium, 1x B27, 1x Glutamax, 1x NEAA, 1x 1x β -mercaptoethanol, 1x penicillin-streptomycin supplemented with 0.05 mM cAMP, 0.2 mM ascorbic acid, 20 ng/mL BDNF, and 20 ng/mL GDNF)) was prepared. This preparation volume is sufficient for making approximately 5 vascularized cortical organoids using 30 μ L of the mixture for each.

[0450] A single blood vessel organoid and a single cortical organoid was placed in a 1.5 mL tube and any medium in the tube carried over from transferring the organoids was removed. In this process, it was ensured that the two organoids are in direct contact with each other. 30 μ L of the Matrigel/brain vascularization medium mixture was added to the organoids, and the tube was

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placed in a 37°C incubator to solidify the Matrigel mixture. The tube can be gently centrifuged prior to Matrigel solidification if needed to settle smaller sized organoids. Additional brain vascularization medium was added to the tube, and the tube was then incubated at 37°C for a day. After a day, half of the liquid medium was changed with fresh brain vascularization medium. The tube should be opened for approximately 30-60 minutes each day in a sterile hood to allow for gas exchange. After another day, the Matrigel droplet was transferred to a low attachment 6 well plate containing brain vascularization medium and cultured for an additional 3 days. After the 3 days, the plate was incubated at 37°C with low speed shaking (100 rpm) for 3 days. Subsequently, the plate was incubated at 37°C with higher speed shaking (120 rpm). After a total of 20 days from the initial blood vessel organoid and cortical organoid contacting in Matrigel, the fused organoid was harvested for use.

[0451] In an alternative approach, a single blood vessel organoid and a single cortical organoid was placed onto a sterile surface (e.g., piece of sterile plastic). A droplet of the Matrigel/brain vascularization medium mixture was placed on the sterile surface, and the two organoids were manipulated to be in direct contact with each other in the center of the Matrigel/brain vascularization medium mixture. The sterile surface holding the organoids were placed in a 37°C incubator to solidify the Matrigel mixture. Subsequently, the solidified Matrigel droplet containing the organoids was transferred to a suitable tissue culture plate containing brain vascularization medium and incubated at 37°C for 4 days. After the 4 days, the plate was incubated at 37°C with low speed shaking (100 rpm) for 3 days. Subsequently, the plate was incubated at 37°C with higher speed shaking (120 rpm). After a total of 20 days from the initial blood vessel organoid and cortical organoid contacting in Matrigel, the fused organoid was harvested for use.

[0452] As shown in **FIGS. 1F and 1G**, the direct contact of blood vessel organoids and forebrain organoid led to their fusion, and infiltration of endothelial cells from the blood vessel organoids into the forebrain organoid. The formation of brain capillaries within the forebrain organoid was confirmed by detection of endothelial cell markers CD31 and cadherin 5 (CDH5) within the forebrain organoid, with neural cells closely associated with the endothelial cells. **FIGS. 2F and 2H** show the presence of GFAP-positive astrocytes and PDGFR- β -positive pericyte progenitor cells, which are additional cell types innately involved in the blood-brain barrier. Importantly, **FIG. 2C** shows the expression of claudin-5, which represents the tight junctions that

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are critical for blood-brain barrier function, and which has been observed to be absent or weakly present in prior models of the BBB.

[0453] Further by day 21, the majority of endothelial cells expressed BBB-specific markers such as glucose transporter 1 (Glut-1) and tight junction proteins such as Claudin-5 (FIGS. 3G-H) and (ZO-1 (not shown), indicating that the endothelial cells were differentiating towards a BBB-specific fate. Day 21 vascularized brain organoids immunostained for GFP, CD31, and Collagen IV shows that the brain endothelium (CD31) was covered by a continuous basement membrane, an important structure for regulating angiogenesis and maintaining the BBB, which was defined by the molecular marker Collagen IV (FIG. 3J). It is also noteworthy that astrocytic processes labeled by GFAP were well-aligned with endothelial tubes (FIGS. 2F, 9I) and that human astrocytes extended their end-feet labeled by AQP-4 to wrap up the abluminal capillary surface (FIG. 2G). The newly form capillaries were not only ensheathed by astrocytic processes but also by human pericytes (stained for PDGFR- β , FIG. 2H), indicative of the resemblance of in vivo human BBB-like structure with endothelial cells of the capillary wall connected through tight junctions, astrocytic end-feet and pericytes ensheathing, as well as neuron innervation (FIG. 2I and FIG. 9J).

[0454] **FIG. 9H** depicts electron micrographs of the fused vascularized forebrain organoid showing the presence of microvesicles (MV) protruding into the lumen of the brain capillary structure, tight junctions (TJ) and adherens junctions (AJ). This demonstrates that human brain microvascular endothelial cells (BMECs) formed capillaries through tight junctions.

Example 4. Single cell transcriptomic profiling of fused vascularized cortical organoids

[0455] The fused vascularized forebrain organoids were analyzed by single cell RNA transcriptomic sequencing. A total of 9342 cells were analyzed after quality control. Cells with mitochondrial gene ratios greater than 10% and less than 200 genes express were excluded. Clustering resolution was set to 0.5.

[0456] As shown in **FIG. 15A**, the vascularized organoids contain a multitude of cell types that are representative of the blood-brain barrier, including neurons, astrocytes, and endothelial cells. **FIG. 15B** shows that among the endothelial cell cluster, there exist sub-clusters, suggesting that the organoid contain diverse populations of cell types. **FIGS. 15C and 15D** show the relative expression of various cell markers in the different cell types identified in the single cell RNA sequencing. **FIG. 15E-15G** show maps of interactions between different known protein receptors

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and ligands expressed by cells associated with communication from 1) vascular cell types to vascular cell types, 2) neural cell types to vascular cell types, and 3) vascular cell types to neural cell types, respectively.

Example 5: Additional single-cell transcriptomic profiling of vascularized brain organoids

[0457] Additional single-cell RNA sequencing (scRNA-seq) was performed on Day 30 vascularized brain organoids derived from H9 embryonic stem cells with three replicate cultures to comprehensively decipher the cell populations present in vascularized brain organoids at the transcriptomic level. The sequencing data was aligned and quantified using Cell Ranger (10x Genomics) to obtain raw count data. The R package Seurat (version 4) was used to normalize the raw count data and DoubletFinder was applied to remove doublet cells in the scRNA-seq data. After doublet cell removal, a total of 28,062 cells were extracted for further analysis (n = three cultures). FindClusters was then applied to identify differentially expressed gene markers for each cell cluster. **FIG. 6A** shows clusters of excitatory neurons, inhibitory neurons, neural progenitors (NPs), astrocytes, endothelial cells (ECs), pericytes, mesenchymal stem cells (MSCs), smooth muscle cells (SMCs), and fibroblasts identified by gene expression markers. The presence of these cell types suggests that vascularized brain organoids can resemble a complete neurovascular unit. The single-cell transcriptomics analysis showed a similar cell population between biological replicas, indicative of the reliability of the protocol. Gene expression data from endothelial cells in vascularized brain organoids was compared with gene expression data from organ-specific endothelial cells generated by the Tabula Muris Consortium. **FIG. 2L** shows that endothelial cells in vascularized brain organoids presented an identical gene expression pattern with brain microvascular endothelial cells (BMECs) but not with other organ-specific endothelial cells, indicative of the endothelial acquisition of brain-specific transcriptomic signatures in vascularized brain organoids.

Example 6: Materials and Methods**Generation and characterization of healthy control and CCM patient iPSCs**

[0458] The B-Lymphocyte samples derived from two CCM patients were obtained from Coriell Institute (ND39588 and ND39589), and the healthy fibroblast samples were collected from three healthy subjects also from Coriell Institute (GM23815, GM09503, and GM00409). Supplemental Table 1 provides detailed epidemiological data for each subject.

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[0459] The Lymphoblastoid cell lines (LCLs) and the fibroblasts were reprogrammed to induced pluripotent stem cells (iPSCs) using the ThermoFisher Scientific Cytotune™-iPS 2.0 Sendai Reprogramming Kit. 200,000 cells were plated into two wells of 6-well plate in fibroblast medium for two days. Then, the cells were transduced using the Cytotune™-iPS 2.0 Sendai reprogramming vectors at an appropriate MOI for overnight, changing the fibroblast medium daily. After seven days, the cells were transferred into vitronectin-coated plate with Essential 8 medium. After approximately 18 days, once the colonies had reached a sufficient size, the iPSCs were expanded onto fresh vitronectin-coated plate with Essential 8 medium, passaged with ReleSR™ every 3–4 days and cryopreserved in stem cell freezing medium. All iPSCs generated from this project has be carefully characterized in terms of their karyotype, pluripotency, and expression of stemness markers.

Supplemental Table 1. B-Lymphocyte and Fibroblast for iPSCs generation.

Research ID	Coriell institute ID	Age	Sex	Sample	Status of Health
UHC4	GM23815	22 years	Male	fibroblast from skin, unspecified	Healthy
UHC3	GM09503	10 years	Male	fibroblast from skin, abdomen	Healthy
UHC1	GM00409	7 years	Male	fibroblast from skin, unspecified	Healthy
CCM588	ND39588	22 years	Male	Lymphoblastoid cell lines from B-Lymphocyte	Cerebral cavernous malformations
CCM589	ND39589	9 years	Female	Lymphoblastoid cell lines from B-Lymphocyte	Cerebral cavernous malformations

Primary cavernomas tissue and postmortem brain tissue collection

[0460] Patient cerebral cavernomas tissue was collected at Cincinnati Children's Hospital after obtaining informed patient consent under a protocol approved by the Cincinnati Children's Hospital Medical Center Institutional Review Board. All patient samples were de-identified before processing. The present study included a total of four patient cases, from both male and female subjects aged between 16 months to 21 years old. The postmortem brain tissue derived from two

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unaffected healthy controls were obtained from the NIH NeuroBiobank. Supplemental Table 2 provides detailed epidemiological data for each subject.

Supplemental Table 2. Collection of primary cavernomas tissue and postmortem brain tissue.

Research ID	Age	Sex	Tissue	Status of Health
CCM2022-0363-003	21 years	Male	Cavernomas	Cerebral cavernous malformations
CCM2022-0363-006	4 years	Female	Cavernomas	Cerebral cavernous malformations
CCM2022-0363-007	16 months	Female	Cavernomas	Cerebral cavernous malformations
CCM2022-0363-008	19 years	Male	Cavernomas	Cerebral cavernous malformations
936	49 years	Female	Human brain	Healthy
418	53 years	Male	Human brain	Healthy

Cerebral organoid differentiation and culture

[0461] Human embryonic stem cells (hESCs), H9 and iPSCs derived from healthy controls and CCM patients, were cultured on Matrigel-coated 6-well plates with Essential 8 flex medium at 37°C in a 5% CO₂/95% air incubator. On day 0, human pluripotent stem cells (hPSCs) including hECSs and iPSCs colonies were dissociated to single cells using ReleSR™ for 5 minutes at room temperature and then washed with DMEM/F12 medium. Approximately 4x10⁶ single stem cells were then resuspended in Essential 8 flex medium with 20μM Y-27632 and plated into one well of an AggreWell 800. The AggreWell was rinsed with 1mL of anti-adherence rinsing solution in different directions before cell plating.

[0462] On day 1, embryonic bodies (EBs) were transferred to ultra-low attachment surface-6 well plates containing H1 medium with 1μM LDN, 1μM SB, 2μg/mL Heparin, and plus 20μM Y-27632. The EBs were incubated with gentle shaking (100 rpm). For the next six days, the EBs were maintained with H1 medium without Y-27632. On day 7, the EBs were embedded in Matrigel in ultra-low attachment surface-6 well plates. Healthy EBs should have round, smooth surfaces with bright edges and were washed with F2 complete medium mixed with 1μM of SB and 1μM of CHIR99021. The entire matrix, including 100 μL of cold Matrigel mixed with 67 μL of F2 complete medium containing 60 aggregates, was placed in a well of the ultra-low attachment surface-6 well plates. The aggregates were then distributed in the Matrigel matrix using a 1μL tip pipette. The contents were incubated for 30 minutes to solidify the gel. Afterward, 3mL of F2

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complete medium was gently added to each well from the side. The medium was changed every other day.

[0463] After 7 days, the cerebral organoids were separated from the Matrigel matrix. The embedded organoids were dissociated from Matrigel using a cutting 0.1% BSA pre-coated 1mL tip with a fast up and slow down pipette technique, repeating the process 2 to 5 times. The embedded organoids were then washed with 5-7 mL of PBS, and the supernatant was removed. The organoids were then resuspended with 3 mL of H3 medium containing 2.5 µg/mL of Insulin and placed in the ultra-low attachment surface-6 well plates. The cerebral organoids were changed every other day and maintained in H3 complete medium.

[0464] On day 60, the cortical organoids were astrocyte-induced with M4 complete medium plus 1% FBS and 10 ng/mL of LIF. The M4 complete medium included 0.05 mM of cAMP, 0.2 mM of Ascorbic acid, 20 ng/mL of BDNF, and 20 ng/mL of GDNF. After two weeks of astrocyte induction, the cerebral organoids were maintained with M4 complete medium without FBS or LIF.

Blood vessel organoid differentiation and culture

[0465] Blood vessel organoids from hESCs and iPSCs were generated and marked with GFP via lentivirus induction using an optimized protocol. The hESCs and iPSCs were cultured on vitronectin-coated 6-well plates with Essential 8 flex medium in a 37°C, 5% CO₂, and 95% air sterile incubator.

[0466] On day -1, the hESCs or iPSCs colonies were dissociated into single cells using ReleSR™ for 5 minutes at room temperature, followed by a wash with DMEM/F12 medium. Approximately 1.2×10^6 single stem cells were resuspended in Essential 8 flex medium with 20µM Y-27632 and plated into a well of an AggreWell 400. The AggreWell was rinsed with 1mL anti-adherence rinsing solution in different directions before cell plating.

[0467] On day 0, the EBs were transferred to ultra-low attachment surface-6 well plates with mesoderm induction medium, including N2B27 medium, 12µM CHIR99021, and 30 ng/mL BMP-4, plus 20µM Y-27632. The EBs were incubated with gentle shaking (100 rpm). On the next two days, the EBs were maintained with the mesoderm induction medium without Y-27632.

[0468] On days 3 and 4, the cultured medium was switched to vascular induction medium consisting of a N2B27 medium with 100 ng/mL VEGF-A and 2 µM Forskolin. On day 5, the EBs were embedded in a 3D collagen I-Matrigel matrix in 12-well plates. The density of the embedding is 40-60 aggregates per well, depending on the size of the EBs. The matrix contains 2 layers. The

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first layer consists of 0.5 mL of cold collagen I-Matrigel solution per well distributed equally by swirling the plate followed by incubation at 37°C in 5% CO₂ / 95% air for 2 hours for solidification. The second layer consists of 0.5 mL of cold collagen I-Matrigel solution with approximately 40-60 aggregates added after the first gel layer solidified. The aggregates were distributed well by rocking the plate back and forth for less than 1 minute. The matrix was incubated for 2 hours for gel solidification. After 2 hours, 2 mL of pre-warmed (37°C) StemPro-34 SFM complete medium was added to the collagen I-Matrigel matrix, which included 15% FBS, 100 ng/mL VEGF-A, and 100 ng/mL FGF-2. The medium was changed every other day. After 5 days of sprouting (day 10), the vascular networks were extracted into single blood vessel organoids. The collagen I-Matrigel matrix was transferred to a 10 cm dish by a sterile lab spoon containing 3 mL of StemPro-34 SFM complete medium. The single blood vessel networks, including 5-8 sprouting vessels, were extracted from the whole collagen I-Matrigel matrix by two sterile 25-gauge needles under a microscope. The singularized networks could be plated in low-attachment 96-well U-bottom plates for 5-7 days with a daily medium change.

[0469] After 5 days of culturing, the individual blood vessel networks self-assembled, resulting in the formation of round blood vessel organoids. These organoids could then be transferred to ultra-low attachment surface-6 well plates for further maintenance. The round blood vessel organoids were sustained with StemPro-34 SFM complete medium supplemented with 15% FBS, 100 ng/mL VEGF-A, and 100 ng/mL FGF-2. Accordingly, the blood vessel organoids were considered ready for fusion from Day 15.

Cerebral-blood vessel assembly and blood-brain barrier assembloids culture

[0470] The astrocyte-induced cerebral organoids (Day 75 and older) and the blood vessel organoids (Day 15 and older) were prepared using the procedures as described above. To create a Matrigel mixture for four cerebral-blood vessel assembly, 100 µL of ice-cold Matrigel was mixed with 60 µL of cold BBB medium with half M4 complete and half StemPro-34 SFM complete medium. Four 0.5 cm x 0.5 cm parafilm sheets were then fixed onto the wells of the sterile ultra-low attachment surface-6 well plates.

[0471] Next, using a 0.1% BSA pre-coated 1mL tip, an astrocyte-induced cerebral organoid and a blood vessel organoid were placed onto a parafilm sheet. The remaining medium in the sheet was aspirated using a 200 µL pipette tip without touching the organoids. Then, 30 µL of the ice-cold Matrigel mixture was added to each parafilm sheet. The cortical and vessel organoids were

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positioned closely to the center of the Matrigel drop by dipping a 1 μ L micropipette tip in 0.1% BSA. The mixture was left to solidify for 30 minutes before 3mL of pre-warmed BBB medium was gently added to each well from the side.

[0472] The parafilm sheets were then carefully removed using a sharp tweezer without touching the cerebral-blood vessel assembly. The ultra-low attachment surface-6 well plates were placed in a 5% CO₂ / 95% air incubator at 37°C, and the medium was changed after 2 days and subsequently every other day. After 5-6 days of fusion, the ultra-low attachment surface-6 well plates were placed on a low-speed shaker (37°C, 100rpm). The cerebral-blood vessel assembly was continuously cultured with BBB medium for up to 2 months. During this time, the vascularized cerebral organoids matured and developed into more complex structures with a faithful blood- BBB formation, also referred to herein as BBB assembloids. BBB assembloids were prepared for further analysis through immunofluorescence for confocal imaging, single-cell dissociation for single cell RNA sequencing, bulk RNA sequencing, spatial transcriptomics analysis, transmission electron microscope, etc.

Processing primary cavernomas tissues and CCM organoid culture

[0473] The fresh resected primary cavernomas tissues were removed from the patients by the neurosurgeon and immediately placed in Hibernate medium, kept in an ice bucket, and transferred to the lab. To increase the reliability of CCM organoid generation, the tissues were processed immediately after surgical removal. In the lab, the tissues were transferred to a sterile glass dish containing H+GPSA medium, which consisted of Hibernate A, 1x GlutaMax, 1x Penicillin-streptomycin, and 1x Amphotericin B. The tissues were then dissected under a microscope within a laminar flow biosafety cabinet. The resected tissues were minced into pieces of around 0.5 mm diameter using a fine dissection knife and washed with H+GPSA medium afterward. Any necrosis and surrounding brain tissues were also removed from the desired pieces. The minced pieces were then placed in a 15 mL tube containing 1X red blood cell (RBC) lysis buffer under gentle agitation for 10 minutes at room temperature. The CCM pieces were washed twice with the H+GPSA medium to neutralize the RBC lysis buffer. Finally, the minced primary cavernomas tissues were transferred to an ultra-low attachment 6-well plate, cultured with BBB medium, and placed on a low-speed shaker (100 rpm) within a sterile incubator at 37°C, 5% CO₂, and 95% air. The culture medium was changed every three days. Within 2 weeks, the CCM organoids gradually formed and were ready for downstream analysis. To further expand the CCM organoids, they can be micro-

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dissected into pieces of approximately 0.5 mm in diameter and cultured using the procedure mentioned above. This process can be repeated twice to generate larger quantities of CCM organoids for downstream applications such as immunofluorescence, single-cell dissociation for single-cell RNA sequencing, bulk RNA sequencing, spatial transcriptomics analysis, transmission electron microscopy, etc.

Cryogenic tissue processing and immunostaining

[0474] The desired organoids and assembloids were fixed with 4% paraformaldehyde (4% PFA) in PBS at 4°C overnight. The following day, the fixed organoids were dehydrated in 30% sucrose at 4°C for 48 hours. The dehydrated organoids were then embedded in O.C.T compound and snap-frozen in a dry ice/ethanol bath before being transferred to a -80°C freezer for long-term storage. The frozen organoids were sectioned with a Microm HM550 cryostat at -20°C, with an optimal sectioning thickness of 20 µm, and stored at -20°C.

[0475] For the first step of immunofluorescence, cryosections were pretreated with PBS-T and incubated in a blocking solution containing 3% donkey serum and 0.1% Triton X-100 in PBS-T at room temperature in a humidifier chamber for 2 hours. After blocking, the cryosections were incubated with primary antibodies diluted in blocking buffer (see Table 3 for antibody information) at 4°C in a humidifier chamber overnight, followed by incubation with secondary antibodies diluted in a blocking buffer (see Table 3 for antibody information) at room temperature in a humidifier chamber for 2 hours. The cryosections were then embedded in PermaFluor™, protected by a coverslip, sealed with clear nail polish, and stored at 2-8°C until imaging.

[0476] Immunofluorescence images were acquired using a Nikon A1R inverted confocal laser scanning microscope with 4x, 10x, 20x, and 40x objectives. NIS Elements version 5.41 software was used for image analysis.

Single-cell dissociation

[0477] To dissociate the BBB assembloids into single cell suspensions, a papain dissociation system protocol was used (Worthington Biochemical, cat. no. LK003153). The desired assembloids were cut into small pieces and placed in Earle's Balanced Salt Solution containing 20 units/mL papain, 1 mM L-cysteine, 0.5 mM EDTA, and 0.005% DNase. The mixture was

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incubated for 25 minutes at 37°C with constant agitation, as per the manufacturer's protocol. Single cells were then suspended in DMEM/F12 with 10% FBS at a concentration of 1 million cells/mL for single cell gene expression assay or in 25 mM HEPES pH 7 at a concentration of 10 million cells/mL for flow cytometry analysis.

Cell sorting and flow cytometry

[0478] To ensure efficient cell sorting by flow cytometry, the single-cell concentration in the 25mM HEPES buffer should be within the range of 10 to 20 x 10⁶ cells/mL. To reduce DNA-induced cellular aggregation and clumping, 10U/mL DNase was added to the cell sorting buffer. Single cells were filtered immediately before sorting using a 35-µm cell strainer tube. The collection tubes used were 15 mL conical tubes that were pre-coated with 1% BSA in PBS to prevent sorted cells from sticking to the sides of the tubes. To maintain sample integrity, all solutions and samples were kept on ice during the sorting process.

[0479] The Sonny SH800S flow cytometer was used in this experiment. Laser measurements were taken at 525/50 nm. Cells were sorted based on four parameters, which included the peak height, area of forward scatter (FSC height and FSC area), and side scatter (BSC height and BSC area). The following cell sorting settings were applied: flow chip diameter, 100 µm; sheath pressure, 5 – 8 PSI; droplet frequency, 30 – 31 kHz; droplet drive, 15 – 30%; droplet delay, 30 – 32 drops; sort phase, 20 – 100 degrees; charge, 50%. After sorting, the collected sample was placed in cold BBB medium and filled up to one-third of the collection tube. This ensured that the sorted cells remained viable and that their phenotypes were not altered during the collection process.

Transmission electron microscope

[0480] For transmission electron microscopy (TEM), the selected BBB assembloids were first fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.4 at 4°C for 2 hours. Next, the specimens were fixed in 1% osmium tetroxide in 0.2 M sodium cacodylate buffer for 1 hour at 4°C. Subsequently, they were processed through a series of graded alcohols, infiltrated, and embedded in LX-112 resin. After polymerization at 60°C for three days, ultrathin sections (100 nm) were cut using a Leica EM UC7 microtome. The sections were then counterstained in 2% aqueous uranyl acetate and Reynolds's lead citrate. Finally, images were captured using a Hitachi H-6750 transmission electron microscope (TEM) equipped with a digital camera (AMT 2kx2K tem CCD) at 80 kV.

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Single-cell RNA sequencing pipeline and analysis

[0481] To prepare the scRNA-seq libraries, the Chromium 3'v3 GEM Kit (10x Genomics, CG000183RevC) and the CCHMC Gene Expression Core were utilized. Approximately 16,000 cells were loaded, aiming to capture 10,000 cells per sample for sequencing. The sequencing was performed on an Illumina NovaSeq 6000 platform with an S4 flow cell to generate approximately 400 million reads per sample. The raw scRNA-seq data was converted to FASTQ files, and Cell Ranger (10x Genomics) was used to align and quantify the sequencing data, obtaining raw count data. The R package Seurat (version 4) was then used to normalize the raw count data, and DoubletFinder was applied to remove any doublet cells in the scRNA-seq data. Cells expressing less than 50 percent mitochondrial-related genes were included. The cell cycle effect was also regressed out using established methods in Seurat. After identifying and removing doublets from each dataset, the newly filtered datasets were used for all downstream analysis. To identify differentially expressed gene markers for each cell cluster, FindClusters, which utilizes the Wilcoxon rank-sum test, was applied. In order to compare the results with those of the Tabula Muris Consortium, gene expression in ECs from BBB assembloids were analyzed and compared with that of organ-specific ECs generated by the Consortium.

[0482] To decipher the gene regulatory programs underlying cerebral cavernous malformation (CCM) pathology in a cell-type specific manner, gene expression data for each cell cluster of blood-brain barrier (BBB) assembloids were merged and a differential analysis was performed using the Wilcoxon rank-sum test with Bonferroni correction, comparing CCM to control BBB assembloids. Initially, the previously reported lesion-marker genes (detailed in the supplemental table) were checked within each cell cluster. Additionally, a set of tip cell and tumor tip cell markers were examined in vascular clusters, including MSCs 1, MSCs 2, ECs 1, ECs 2, ECs 3, Peri 1, Peri 2, vSMCs, and Fib.

[0483] To investigate the molecular basis of neuro-vascular cross-talk, a ligand-receptor mediated cell-cell communication analysis was conducted using single-cell transcriptomics data obtained from both control and CCM BBB assembloids. To score ligand-receptor interactions, the average receptor and ligand expression in the respective cell types were calculated, as previously described. Additionally, the probability score, which represents the statistical significance of each interaction score, was assessed using a one-sided Wilcoxon rank-sum test with Benjamini-Hochberg multiple hypothesis correction.

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[0484] The pseudotime analysis was conducted using the default parameters of either STREAM from Scanpy. For STREAM, the visualization of subway plots was achieved using `st.plot_stream` with log-normalized gene expression, which were colored by clusters that were initially identified via Seurat. In general, starting with a single-cell gene expression matrix, STREAM performs three main steps: selection of informative genes, dimensionality reduction, and simultaneous tree structure learning and fitting by ElPiGraph. The optimal structure is selected based on the elastic energy minimization among a set of candidate structures that are constructed after a tree node is added. The final tree is interpreted as a set of connected curves representing different trajectories. For subway map plot, after selecting an initial state in the flat tree plot, the tree is re-ordered to facilitate visualization.

Bulk RNA sequencing pipeline and analysis

[0485] Total cellular RNA was isolated using the Invitrogen mirVana kit in accordance with the manufacturer's instructions. For RNA sequencing, libraries were generated from 1 µg of total RNA from three biological replicates of sorted Day 30 BBB assembloids with GFP-positive and negative portions, as well as unassembled blood vessel and brain organoids using the NEBNext® Poly(A) mRNA Magnetic Isolation Module (NEB), as per the manufacturer's protocol. The quality and quantity of the libraries were assessed using an Agilent 2100 BioAnalyzer and DNA1000 kit and a KAPA Biosystems qPCR-based KAPA library quantification kit, respectively. Illumina HiSeq2500 was used to perform 100-cycle SR sequencing. Preprocessing of RNA-seq reads was conducted using the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), including adapter clipping, filtering of poor quality reads, and collapsing of identical reads. Preprocessed reads were then aligned to the human genome (build GRCh37/hg19) using Tophat2 with the default settings. Raw gene counts were obtained using `htseq-count` from the HTSeq library, and differential gene expression analysis was conducted using the R statistical package DESeq2 with an FDR of 0.05 and a fold change threshold of $\log_2(\text{fold change}) > \pm 1$ (Supplementary Table). Gene ontology (GO) analyses on biological processes were performed using the Database for Annotation, Visualization, and Integrated Discovery v6.8, while KEGG pathway analyses were conducted using the WEB-based Gene Set Analysis Toolkit (WebGestalt) update 2017. Plots were generated using R version 3.4.3 with the `ggplot2` package. The size of individual circles were determined by the percentage of genes within each ontology/pathway group present in the query gene list, and the color were determined by the P value for gene enrichment for each ontology/pathway term.

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HiFi-slide sequencing pipeline and spatial transcriptomics analysis

[0486] There are two libraries, (1) the HiFi-Slide library and (2) the recycled flow cell library. The HiFi-Slide library is a paired-end library where R1 is the spatial end and R2 is the RNA end. The recycled flow cell library is a single-end library containing the sequences of spatial barcodes (associated with a spatial location on the flow cell). The spatial end of the HiFi-Slide library is used to match with the spatial barcodes and assign a spatial location to the corresponding RNA end.

[0487] First, the HiFi-Slide R2 reads were pre-processed. These reads could mistakenly include a portion of R1 reads in the recycled flow cell. To identify such cases, overlap between R1 and R2 was searched for, and such R2 reads were filtered out (PEAR software). In addition, Illumina adapters, polyG and polyX tails were trimmed, and reads that are too short were filtered out (e.g., using FASTP software). Next, the pre-processed R2 reads were aligned to the human genome (STAR aligner). Uniquely mapped reads were then selected (Samtools) and eventually labeled with annotated genes (gencode.v41).

[0488] Then, in the recycled flow cell library, spatially redundant barcodes—the barcodes with the same sequence but different spatial locations -- were labeled (barcode deduplication step). Then, HiFi-Slide R1 reads were aligned to the spatial barcodes (BWA aligner). To reduce both storage occupation and computational time, HiFi-Slide R1 reads were subset aligned to select only those whose R2 ends are mapped over the genome. Next, HiFi read pairs were selected whose R1 ends align with the highest score to the spatial barcodes. If a HiFi R1 read aligned with the same score to multiple barcodes, \ these alignments were considered and kept track of by their number (N). At this point, HiFi-Slide read pairs were spatially resolved by exploiting the spatial information from the barcodes and the HiFi-Slide R1 aligned to them. Each HiFi-Slide read pair were assigned with a tile, a pair of spatial coordinates within the tile, and a weight (N) which signifies the number of different spatial locations for this read pair. If $N > 1$, all these “read-location” pairs were listed.

[0489] Leveraging the HiFi-Slide read ID, outputs from HiFi-Slide R1 and R2 processing were merged to obtain an output file with both spatial and gene information for each HiFi-Slide read pair. This tab-separated file contained unique “read-gene” pairs in each line. The next step involved transitioning from “read-gene” pairs to “spot-gene” pairs to obtain a final output file with the expression level of genes in each spot. The gene expression level was calculated as a “weighted

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read count,” which considered the potentially multiple locations of the read pairs. Thus, the flow cell grid was manually overlapped to the tissue microscope image to select the tiles under a smooth tissue region (Region of Interest, ROI), and the data was subsetted by selecting spots within the ROI, which go into the data analysis steps.

[0490] To assign cell types to spots a set of 59 marker genes was leveraged. If a spot expressed one or more markers of a cell type, it was assigned to that cell type. If a spot expressed marker genes associated with several cell types, it was assigned to the cell type corresponding to the marker with the highest expression level. If a spot expressed marker genes associated with several cell types and two or more marker genes have the same highest expression level, then it was not assigned.

[0491] To perform spatial clustering, the software Spaceflow (<https://github.com/hongleir/SpaceFlow>) was used. Spaceflow was run on the 2,380 spots (centroids) using the 54 cell type marker genes that were expressed using default parameters. 12 spatial domains (clusters) were identified in total. To test for the association between spatial domains and cell types across spots, a chi-square test was performed for each “cell type-domain” pair using a contingency table where rows were “in spatial domain” and “not in spatial domain”, and columns were “in cell type” and “not in cell type”. In each entry was the number of spots. Next, a “cell type-by-domain” matrix was generated with the odds ratios (Supplemental Table 4) and the corresponding p-values (Supplemental Table 5). Then significant entries whose odds ratio > 1 and p-value < 0.015 were selected (Supplemental Table 6). This allowed for statistically associating each cluster with a group of cell types. Finally, spatial clusters were shaded such that the spatial clusters enriched with any vascular cell type-associated cell type were shaded from pink to red while other brain cell types with shades of blue. Specifically, five spatial clusters were associated with vascular cell types were detected: cluster 0 and 1 were associated with EC.1; cluster 2 with EC.2 and GABA; cluster 3 with SMC and GluN; cluster 4 with Fib and NP; cluster 5 with MSC and PC. Also, six clusters associated with brain cell types were detected: cluster 6 with Ast; cluster 7 with Ast and NP; cluster 8 and 9 with GluN; cluster 10 with NP; cluster 11 with PC.

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Reagent set-upMEF/ Fibroblast medium

[0492] To prepare a volume (e.g., about 100 mL) of MEF/fibroblast medium, an amount of DMEM (e.g., about 79-95 mL, about mL about 80-100 mL, or about 85-95 mL of DMEM), an amount of FBS (e.g., about 0-20 mL, about 5-15 mL, or about 8-12 mL of FBS), an amount of a concentration of MEM Non-essential Amino Acids solution (e.g., about 0-5 mL of about 5-20 mM, about 0-5 mL of about 5-15 mM, or about 0-2 mL of about 8-12 mM MEM Non-essential Amino Acids solution), and an amount of a concentration of β -mercaptoethanol (e.g., about 20-200 μ L of about 10-100 mM, about 50-150 μ L of about 25-100 mM, or about 80-120 μ L of 40-60 mM β -mercaptoethanol) is mixed together. The medium can be prepared fresh and stored at a cool temperature (e.g., about 2-6°C) for up to about a week.

N2B27 medium, 50 mL

[0493] In some embodiments, to prepare the medium, an amount of Neurobasal medium (Gibco, cat. no. 21103049) (e.g., about 5-75 mL, 15-50 mL, or 15-35 mL of Neurobasal medium) and an amount of DMEM/F12 medium (Gibco, cat. no. 11330032) (e.g., about 5-75 mL, 15-50 mL, or 15-35 mL of DMEM/F12 medium) can be mixed together. Then, an amount of 50x B27 supplement (Gibco, cat. no. 12587010) (e.g., about 0-5 mL, 0-2 mL, or 1-2 mL of 50x B27 supplement), an amount of 100x N2 supplement (Gibco, cat. no. 17502048) (about 0-5 mL, 0-2 mL, or 0.5-1 mL of 100x N2 supplement), an amount of Glutamax (Gibco, cat. no. 35050061) (about 0-2, 0-1, or 0-0.5 mL of Glutamax), an amount of a concentration of β -mercaptoethanol (Gibco, cat. no. 21985-023) (e.g., about 10-200 μ L of 10-100 mM, 50-150 μ L of 25-100 mM, or 80-100 μ L of 40-70 mM β -mercaptoethanol), and an amount of 100x penicillin-streptomycin (Gibco, cat. no. 15140122) (e.g., about 0-5 mL, 0-2 mL, or 0.2-1 mL of 100x penicillin-streptomycin) were added to the mixture. The medium may be prepared fresh and stored at a cool temperature (e.g., about 4°C) for up to about 2 weeks.

Three-dimensional collagen I- Matrigel matrix

[0494] To set up the collagen I-Matrigel solution, it is preferable to keep solutions chilled. For a multi-well (e.g., 12-well) plate, the three-dimensional collagen I-Matrigel solution may be prepared by preparing an amount of a concentration of collagen I solution (e.g., about 1-20 mL of about 0-8.0 mg/mL, 2-10 mL of 0-5.0 mg/mL, or about 4-6 mL of about 1.0-3.0 mg/mL collagen

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I solution) by combining an amount of about 0.1 N Sodium hydroxide solution (NaOH; 1.0 N; Sigma, cat. no. S2770) (e.g., about 200-400 μ L, 250-350 μ L, or 275-325 mL) of 0.1N Sodium hydroxide solution), an amount of ddH₂O (e.g., about 350-550 μ L, 400-500 μ L, or 425-475 μ L of ddH₂O), an amount of 10x Dulbecco's Modified Eagle's Medium (DMEM; Sigma, cat. no. D5648-10L) (e.g., about 250-375 μ L, 275-325 μ L, or 310-315 μ L of 10x DMEM), an amount of 1M HEPES (Gibco, cat. no. 15630080) (e.g., about 40-80 μ L, 50-70 μ L, or 60-65 μ L of 1M HEPES), about 40-55 μ L of 7.5% sodium bicarbonate (Gibco, cat. no. 5080094), about 25-35 μ L of Glutamax, and about 420-500 μ L of Ham's F-12 (Gibco, cat. no. 11765054). In some embodiments, after mixing the solution, an amount of PureCol (Advanced BioMatrix, cat. no. 5005) may be added to the solution and subsequently mixed. In some embodiments, the resulting 3D collagen I-Matrigel solution can be diluted (e.g., to a 4:1 ratio). For example, to prepare about 6 mL of collagen I-Matrigel solution, about 4.5 mL of a 2.0 mg/mL collagen I solution and about 1.5 mL of a growth factor-reduced Matrigel (Corning, cat. no. 356231) can be combined (e.g., on ice or a cooled surface) and mixed gently. It may be preferable to prepare solutions fresh at the time of embedding embryoid bodies (EBs) and to keep it chilled until use.

StemPro-34 SFM base medium

[0495] In some embodiments, the StemPro-34 nutrient supplement can be dissolved overnight at a cool temperature (e.g., about 4°C) and divided into aliquots. These aliquots can be stored at subzero (e.g., about -20°C) for up to about one year. In some embodiments, the StemPro-34 SFM base medium may be prepared by mixing StemPro-34 SFM medium mix with an aliquot (e.g., about 1.3 mL) of the StemPro-34 nutrient supplement, an amount (e.g., about 0.5 mL) of Glutamax, and an amount (e.g., about 0.5 mL) of 100x penicillin-streptomycin. The medium may be prepared fresh and stored at a cool temperature (e.g., about 4°C) for up to about 2 weeks.

H1 base medium

[0496] The H1 medium may be prepared by mixing an amount of DMEM/F12 medium (e.g., about 300-500 mL, 350-450 mL, 380-390 mL of DMEM/F12 medium) with an amount of KOSR (20%) (e.g., about 50-150 mL, 75-125 mL, or 90-105 mL of KOSR (20%)), an amount of GlutaMAX (e.g., about 0-10 mL, 2-8 mL, 4-6 mL of GlutaMAX), an amount of MEM-NEAA (e.g., about 0-12 mL, 1-10 mL, or 4-6 mL of MEM-NEAA), an amount of a concentration of β -mercaptoethanol (e.g., about 800-1200 μ L of 10-100 mM, 500-1500 μ L of 25-100 mM, or 800-

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1000 μ L of 40-70 mM β -mercaptoethanol), and 100x penicillin–streptomycin (e.g., about 0-10 mL, 2-8 mL, or 4-6 mL of 100x penicillin–streptomycin).

F2 base medium

[0497] The F2 medium may be composed of an amount of DMEM/F12 medium (e.g., about 400-600 mL, 450-550 mL, and 450-500 mL of DMEM/F12 medium) mixed with an amount of 100x N2 supplement (e.g., about 0-10 mL, 2-8 mL, or 4-6 mL of 100x N2 supplement), an amount of GlutaMAX (e.g., about 0-10 mL, 2-8 mL, or 4-6 mL of GlutaMAX), an amount of MEM-NEAA (e.g., about 0-10 mL, 2-8 mL, or 4-6 mL of MEM-NEAA), an amount of a concentration of β -mercaptoethanol (e.g., about 800-1200 μ L of 10-100 mM, 500-1500 μ L of 25-100 mM, or 800-1000 μ L of 40-70 mM β -mercaptoethanol), and 5 mL of 100x penicillin–streptomycin (e.g., about 0-10 mL, 2-8 mL, or 4-6 mL of 100x penicillin–streptomycin).

H3 base medium

[0498] The F2 medium may be composed of an amount of DMEM/F12 medium (e.g., about 175-250 mL, 200-250 mL, or 225-245 mL of DMEM/F12 medium) mixed with an amount of Neurobasal medium (e.g., about 175-250 mL, 200-250 mL, or 225-245 mL of Neurobasal medium), an amount of 100x N2 supplement (e.g., about 0-15 mL, 1-10 mL, or 4-6 mL of 100x N2 supplement), an amount of 50x B27 supplement (e.g., about 1-20 mL, 5-15 mL, 9-11 mL of 50x B27 supplement), an amount of GlutaMAX (e.g., about 0-10 mL, 2-8 mL, or 4-6 mL of GlutaMAX), an amount of MEM-NEAA (e.g., about 0-10 mL, 2-8 mL, or 4-6 mL of MEM-NEAA), an amount of 55 mM β -mercaptoethanol (e.g., about 800-1200 μ L of 10-100 mM, 500-1500 μ L of 25-100 mM, or 800-1000 μ L of 40-70 mM β -mercaptoethanol), and an amount of 100x penicillin–streptomycin (e.g., about 0-10 mL, 2-8 mL, or 4-6 mL of 100x penicillin–streptomycin).

M4 base medium

[0499] The F4 medium may be composed of an amount of Neurobasal medium (e.g., about 375-550 mL, 450-500 mL, or 465-495 mL of Neurobasal medium), an amount of 50x B27 supplement (e.g., about 1-20 mL, 5-15 mL, 9-11 mL of 50x B27 supplement), an amount of GlutaMAX (e.g., about 0-10 mL, 2-8 mL, or 4-6 mL of GlutaMAX), an amount of MEM-NEAA (e.g., about 0-10 mL, 2-8 mL, or 4-6 mL of MEM-NEAA), an amount of a concentration of β -mercaptoethanol (e.g., about 800-1200 μ L of 10-100 mM, 500-1500 μ L of 25-100 mM, or 800-

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1000 μ L of 40-70 mM β -mercaptoethanol), and an amount of 100x penicillin–streptomycin (e.g., about 0-10 mL, 2-8 mL, or 4-6 mL of 100x penicillin-streptomycin).

BBB medium.

[0500] The BBB medium may be a combination of half M4 complete medium and half StemPro-34 SFM complete medium. In some embodiments, the M4 complete medium may be prepared with about 0.01 mM – 0.1 mM of cAMP (e.g., about 0.04 mM – 0.06 mM, or about 0.05 mM of cAMP), about 0.0 mM – 0.5 mM of Ascorbic acid (e.g., about 0.1 mM – 0.3 mM, or about 0.2 mM of Ascorbic acid), about 1-100 ng/mL of BDNF (e.g., about 1-50 ng/mL or about 20 ng/mL of BDNF), and about 1-100 ng/mL of GDNF (e.g., about 1-50 ng/mL or about 20 ng/mL of GDNF) added to the M4 base medium. The StemPro-34 SFM complete medium may include the StemPro-34 SFM base medium, supplemented with about 15% FBS, about 50-200 ng/mL VEGF-A (e.g., about 80-150 ng/mL or about 100 ng/mL VEGF-A), and about 50-150 ng/mL FGF-2 (e.g., about 75-125 ng/mL or about 100 ng/mL FGF-2).

0.1% BSA

[0501] In some embodiments, to prepare an amount (e.g., about 30 mL) of 0.1% BSA solution, about 40 μ L of 7.5% BSA solution may be diluted in about 30 mL of D-PBS.

4% Paraformaldehyde

[0502] In some embodiments, to prepare about 40 mL of 4% paraformaldehyde, about 10 mL of 16% paraformaldehyde solution may be diluted with about 30 mL of D-PBS.

PBS-T

[0503] In some embodiments, to prepare about 1 liter of 0.1% PBS-T, 1 mL of Tween-20 may be added to about 1 liter of D-PBS and mixed thoroughly.

30% Sucrose

[0504] In some embodiments, to prepare about a liter of 30% sucrose solution, about 300 g of sucrose may be added to D-PBS and mixed thoroughly until the sucrose is fully dissolved.

H+GPSA medium

[0505] In some embodiments, the medium may be composed of Hibernate A medium supplemented with 1X Glutamax, 1X Penicillin-Streptomycin, and 1X amphotericin B.

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Supplemental Table 3. Medium and reagent

Reagent	Company	Catalog #
Essential 8 Flex Medium	Gibco	A2858501
ReleSR™	StemCell Technologies	05872
DMEM/F12 Medium	Gibco	11330032
DMEM	Gibco	11960044
Rock Inhibitor Y-27632	StemCell Technologies	72304
Anti-adherence Rinsing Solution	StemCell Technologies	1000102227
Neurobasal Medium	Gibco	21103049
B27 Supplement	Gibco	12587010
N2 Supplement	Gibco	17502048
Glutamax	Gibco	35050061
β-mercaptoethanol	Gibco	21985-023
Penicillin–streptomycin	Gibco	15140122
CHIR99021	Tocris Bioscience	4423
Human BMP-4	Peprtech	120-05ET-100UG
VEGF-A	Peprtech	100-20-250UG
Forskolin	Sigma	F3917
0.1N Sodium Hydroxide Solution (NaOH)	Sigma	S2770
10x Dulbecco's Modified Eagle's Medium	Sigma	D5648-10L
1M HEPES	Gibco	15630080
7.5% Sodium Bicarbonate (NaHCO ₃)	Gibco	5080094
Ham's F-12	Gibco	11765054
PureCol	Advanced BioMatrix	5005
Growth Factor Reduced Matrigel	Corning	356231
StemPro-34 SFM Medium	Gibco	10639011
MEM-NEAA	Gibco	11140-050
LDN 193189	Peprtech	1066208
SB-431542	Cayman Chemical	13031
Heparin	Millipore Sigma	H3149-1MU
KOSR	Thermo Fisher Scientific	A3181502
Recombinant Human Insulin	Peprtech	10-365
FBS	VWR	89510-186
Dibutyl cAMP Sodium Salt	Sigma	D0627
L-ascorbic Acid (Vitamin C)	Sigma	A5960
Human/Murine/Rat BDNF	Peprtech	450-02-250UG
Human GDNF	Peprtech	450-10-250UG
7.5 % BSA	Sigma	A8412-100ML
Matrigel	Corning	356235
PBS	Gibco	2025-11
Tween	Sigma	P1379-1L

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Reagent	Company	Catalog #
Paraformaldehyde	Electron Microscopy Sciences	15710
100% Ethanol	Sigma	E7023-500ML
Donkey Serum	Sigma	D9663-10ML
Triton X-100	Sigma	X100-1L
PermaFluor™	Abcam	Ab104135
Papain Dissociation System	Worthington Biochemical	LK003153
Glutaraldehyde	Electron Microscopy sciences	16310
Sodium Cacodylate	Electron Microscopy Sciences	11653
HEPES	Gibco	15630-080
DAPI	Millipore Sigma	D9542-1MG
Hibernate A Medium	BrainBits	HA
RBC Lysis Buffer	Invitrogen	00-4333
Amphotericin B	Thermo Fisher Scientific	15290026
CytoTune-iPs 2.0 Sendai kit	Thermo Fisher Scientific	A16571
Stem Cell Freezing Medium	ATCC	ACS-3020-3
MEM Non-Essential Amino Acids Solution (10 nM)	Thermo Fisher Scientific	11140-050

Supplemental Table 4. Supplies

Reagent	Supplier	Catalog no.
6-well plates	Gibco	A31804
AggreWell 400	Stemcell technologies	344110
12-well Plates	BD Falcon	353043
35 x 10 mm Tissue Culture Dish	BD Falcon	353001
25-gauge needle	Becton Dickinson	305127
Prime Surface 96U Plate	Sumitomo Bakelite	MS-9096UZ
Ultra-low Attachment Surface-6 Well Plate	Corning	3471
AggreWell 800	Stemcell technologies	34811
Parafin	Bermis	PM-996
O.C.T.	Tissue-Tek	4583
Cryomold	Tissue-Tek	4565
Coverslip	ASI	SM2222
Microscope Slides	ASI	SM2576
35-µm Cell Strainer Tube	Falcon	352235

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Supplemental Table 5. Antibody

Antigen	Supplier	Catalog #	Host Specificity	Dilution Factor
Primary antibodies				
GFP	Aves lab	GFP-1010	Chicken	1:2000
CD31	R&D	AF806	Sheep	1:100
VE-cadherin (CDH5)	Santa Cruz	Sc9989	Mouse	1:50
ZO1	Invitrogen	339100	Mouse	1:100
GLUT-1	invitrogen	MA5-11315	Mouse	1:100
Claudin 5	Thermofisher	35-2500	Mouse	1:200
Occludin	Thermofisher	33-1500	Mouse	1:200
AQP4	Millipore Sigma	ABN910-100UG	Rabbit	1:1000
PDGF receptor β	Cell signaling	3169S	Rabbit	1:200
GFAP	Dako		Rabbit	1:1000
S-100B	SYSY		Genius pig	1:500
Collagen type IV	Millipore	AB769	Goat	1:200
SMA	Sigma	2547	Mouse	1:100
IBA1	SYSY	234004	Genius Pig	1:1500
Ki67	DSHB	AFFN-KI67-3E6	Rabbit	1:50
NG2	ThermoFisher	14-6504-82	Mouse	1:100
MRP1	Santa cruz	sc-18835	Mouse	1:500
MDR1(P-pg)	Santa cruz	sc-13131	Mouse	1:500
KRIT1 (CCM1)	Santa cruz	Sc-514371	Mouse	1:500
Fluorescent second antibodies				
Cy2-anti-Chicken IgG (H+L)	Jackson Immuno Research	703-225-155	Donkey	1:1000
Alexa Flour 594-anti-sheep IgG (H+L)	Jackson Immuno Research	713-585-147	Donkey	1:1000
Cy3-anti-mouse IgG (H+L)	Jackson Immuno Research	715-165-150	Donkey	1:1000
Cy3-anti-rabbit IgG (H+L)	Jackson Immuno Research	711-165-152	Donkey	1:1000

Improved Cell Culture Medium

[0506] The following new cell culture media can also be used as a replacement of the aforementioned StemPro™-34 SFM for the culturing of blood vessel organoids and BBB assembloids. The cell culture media comprised a base EC medium and an endothelial cell growth supplement (ECGS). The base endothelial cell (EC) medium can be composed of an amount (e.g., 30-50 mL) of DMEM/F12 mixed with an amount (e.g., 5-10 (e.g., about 7.5 mL)) of a growth serum (e.g., FBS) or replacement serum. In some aspects, the base EC medium may further include

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an amount (e.g., about 0.1 – 1 mL) of 100x Glutamax and/or an amount (e.g., 0.5 mL) of 100x Penicillin-streptomycin.

[0507] In some embodiments, the ECGS may be in the cell culture media at a concentration of about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 ug/mL of the cell culture media. In some embodiments, the ECGS may be in the cell culture media at a concentration that is at a range between any two of the aforementioned concentrations, for example, about 10-100 ug/mL, 20-70 ug/mL, or 40-60 ug/mL of the cell culture media. In some embodiments, the ECGS may be in the cell culture media at a concentration of about 50 ug/mL of the cell culture media.

[0508] In some embodiments, the cell culture media may further comprise Heparin. In some embodiments, the Heparin may be in the concentration of about 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 30, or 40 ug/ mL of the cell culture media. In some embodiments, the Heparin may be in the cell culture media at a concentration that is at a range between any two of the aforementioned concentrations, for example, about 0-40 ug/mL, 2-16 ug/mL, or 8-12 ug/mL of the cell culture media. In some embodiments, the Heparin may be in cell culture media at a concentration that is about 10 ug/ mL of the cell culture media.

[0509] In some embodiments, the cell culture media may further comprise VEGF-A. In some embodiments, the VEGF-A may have a concentration of 10, 30, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 300, or 500 ng/ mL the cell culture media. In some embodiments, VEGF-A may be in the cell culture media at a concentration that is at a range between any two of the aforementioned concentrations, for example, about 10-500 ng/mL, 50-150 ng/mL, 70-130 ng/mL, or 90-110 ng/mL of the cell culture media. In some embodiments, the VEGF-A may have a concentration of about 100 ng/ mL the cell culture media.

[0510] In some embodiments, the cell culture media may include, as primary components: a serum (e.g., FBS) or serum replacement (ranging from about 0.5% to 20% (e.g., about 2% - 15%, about 5%-15%)), VEGF-A (ranging from about 10 to 500 ng/ml (e.g., 50-150 ng/mL, 70-130 ng/mL, or 90-110 ug/mL)), and an endothelial cell growth supplement. In some embodiments, a primary component of the endothelial cell growth supplement may comprise an activator of the FGF signaling pathway, such as but not limited to acidic FGF, at a concentration range that is about 10 to 500 ng/ml (e.g., 50-150 ng/mL, 70-130 ng/mL, or 90-110 ng/mL).

[0511] An example embodiment of the improved cell culture media is shown in the table below:

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Reagent	Company	Catalog #
DMEM/F12 Medium	Gibco	11330032
Glutamax	Gibco	35050061
Penicillin–streptomycin	Gibco	15140122
FBS	VWR	89510-186
Endothelial cell growth supplement	Corning	354006
Heparin	Millipore Sigma	H3149-1MU
VEGF-A	Peptrotech	100-20-250UG

Use and Modifications Of The Vascularized Organoids For Disease Modeling

[0512] Embodiments of the present disclosure also relate to the use or modifications of the vascularized brain organoids described herein to model or recapitulate various diseases (e.g., Fragile X Syndrome, Alzheimer’s Disease, viral infections). Such embodiments are based on the following experiments and studies conducted for the present disclosure.

1. Disease Modeling Fragile X Syndrome (FXS)

[0513] FXS primarily results from expanded trinucleotide (CGG) repeats in the fragile X mental retardation syndrome 1 gene (FMR1) gene, leading to transcriptional silencing and loss of Fragile X mental retardation protein (FMRP). A study of BBB assembloids derived from FXS patients revealed several key phenotypic and mechanistic findings, including but not limited to: hypervascularization of the blood vessels in the BBB assembloids, BBB defects, mTOR signaling pathway hyperactivation, and Wnt signaling pathway inhibition.

[0514] The study utilized a diverse set of iPSCs from individuals with FXS, alongside gender- and age matched controls, to comprehensively investigate brain vascular and BBB abnormalities in FXS, considering potential sex differences. In the study, CRISPR-Cas9 technology was used to generate CGG repeat-erasing isogenic controls using the vascularized brain organoid described herein, to gain mechanistic insights into FMRP deficiency-dependent brain vascular and BBB defects in the context of FXS.

[0515] To comprehensively model FXS, 12 iPSC lines from six FXS patients and six age- and gender-matched healthy controls were obtained. Rigorous characterization confirmed

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pluripotency, stemness, and karyotype integrity. CGG repeat analysis, verified by Asuragen testing, revealed that all healthy controls had 30 or fewer CGG repeats, while patients exhibited over 200 CGG repeats in the FMR1 gene's 5' untranslated region, confirming their full FXS mutations.

[0516] Based on the methods described herein, cerebral cortical organoids and blood vessel organoids (vessel cells labeled with GFP) were generated, then assembled in a gel matrix to create the BBB assembloids. At Day 30, BBB assembloid derived from an FXS subject exhibited an extensive vessel network, with the majority of endothelial cells ($91.2 \pm 1.5 \%$, $n = 4$ cultures) expressing the BBB specific marker, glucose transporter 1 (GLUT-1), indicating endothelial acquisition of brain-specific signatures (e.g., as shown in FIG. 17A). FMRP is typically expressed in all neurovascular cells with low cell type specificity. Western blot analysis showed a loss of FMRP expression in BBB assembloids derived from FXS patients, in contrast to those derived from healthy controls (e.g., as shown in FIG. 17B). Intriguingly, FXS BBB assembloids exhibited enlarged capillary perimeters, diameters, and area coverage fractions compared to controls (FIG. 17C), indicating increased angiogenesis and a hypervascularization phenotype. This finding can be compared to *Fmr1* knockout (*Fmr1* KO) mice (Fig. 17D), suggesting that FMRP deficiency leads to disease-associated hypervascularization, a characteristic typically found in FXS patients. To examine BBB integrity, tight junction protein expression was assessed, specifically the expression of the tight junction protein, Claudin-5. A significant reduction of Claudin-5 expression was observed in the endothelium of both FXS BBB assembloids and *Fmr1* KO mice (e.g., as shown in FIGS. 18A-18B) compared to controls and wild type (WT) mice. Beyond decreased protein expression, junction like structures were also diminished, indicating BBB breakdown (e.g., as shown in FIGS. 18A-18B (see arrows)). Next, BBB-specific transporter expression was assessed, such as the expression of glucose transporter 1 (GLUT-1), which is crucial for BBB function. A reduction of GLUT-1 expression was observed not only in FXS BBB assembloids but also in *Fmr1* knockout mice and FXS postmortem brain tissues (e.g., as shown in FIGS. 18C-18F). Quantitatively, Western blot analysis showed a significant reduction in the protein expression of BBB-specific tight junction (e.g., Occludin) and transporters (e.g., GLUT-1 and P-glycoprotein, abbreviated as P-gp) in FXS BBB assembloids, indicating FXS-associated BBB defects.

[0517] To identify the cell type(s) responsible for hypervascularization and BBB dysfunction, single cell RNA sequencing analysis was performed on Day 30 BBB assembloids. In total, 18,573

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cells were analyzed from two FXS BBB assembloids and 17,540 cells from two healthy controls. Unsupervised clustering identified nine cell types: astrocytes (Ast), endothelial cells (EC), GABAergic neurons (GABA), glutamatergic neurons (GluN), neural progenitors (NP), pericytes (Peri), vascular smooth muscle cells (vSMC), fibroblasts (Fib), and mesenchymal stem cell (MSC) (e.g., as shown in FIG. 19A).

[0518] Both control and FXS BBB assembloids were observed to be capable of producing most neurovascular cell types, validating robustness of the protocol. Cell-type marker expression profiles are shown in FIG. 19B. To uncover the gene regulatory programs underlying FXS-associated hypervascularization and BBB defects in a cell-type-specific manner, gene expression data for each cell cluster were merged and a differential analysis was performed using the Wilcoxon rank-sum test with Bonferroni correction, comparing FXS to control BBB assembloids. Interestingly, genes related to angiogenic growth factors and receptors and the mTOR signaling pathway were upregulated in many neurovascular cells, while genes related to BBB function and the Wnt signaling pathway were downregulated exclusively in endothelial cells (e.g., as shown in FIG. 19C). This highlights the molecular mechanisms underlying FXS-associated vascular and BBB abnormalities.

[0519] A study was conducted to confirm and model mTOR hyperactivation in FXS, evidenced by increased levels of mTOR pathway downstream effectors, including p110 β , phospho-S6 (pS6), and phospho-Akt (pAkt) (e.g., as shown in FIGS. 20A-20B). Since mTOR pathways can be activated by angiogenic growth factors like vascular endothelial growth factor (VEGF), mTOR hyperactivation was modeled in endothelial cells of FXS BBB assembloids and compared to controls. The comparison revealed increased VEGF receptor 2 (VEGFR2) expression (e.g., as shown in FIGS. 20C-20D). Conversely, a reduction in T-cell factor/lymphoid enhancer factor (TCF/LEF) expression was observed in the FXS BBB assembloids. Since TCF/LEF is a downstream transcriptional factor of Wnt signaling pathways, the reduction in TCF/LEF expression indicated Wnt inhibition in FXS (e.g., as shown in FIGS. 20E-20F).

[0520] Using the vascularized brain organoids, the study also demonstrated that FMRP target mRNAs encoding mTOR and Wnt signaling components. To identify mRNAs encoding mTOR and Wnt signaling components as FMRP targets, an anti-flag pulldown experiment was performed. HEK293T cells were transfected with flag-mCherry-FMRP and flag-mCherry plasmids, followed by pulldown using anti-flag M2 affinity gel (e.g., as shown in FIG. 21A). Significant enrichment

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of PIK3CB (p110 β) mRNA was observed in anti-flag pulldowns with flag-tagged mCherry–FMRP but not with flag-tagged mCherry (e.g., as shown in FIG. 21B). There was no specific enrichment detected for β -actin mRNA, indicating p110 β mRNA as an FMRP target. Similarly, CTNNB1 (β -catenin) mRNA showed significant enrichment, indicating it was also an FMRP target. To investigate whether FMRP regulates p110 β translation, p110 β mRNA distribution was analyzed in polysomal fractions of sucrose gradients. The association of p110 β mRNA with polysomes was sensitive to puromycin treatment, indicating active translation (e.g., as shown in FIG. 21C). In *Fmr1* KO mice, the association of p110 β and PSD95 (positive control) mRNAs with heavy polysomes was significantly increased compared to WT (e.g., as shown in FIG. 21D), suggesting enhanced basal translation of p110 β mRNA in the absence of FMRP. Meanwhile, the stability of CTNNB1 mRNA was assessed, showing faster decay of CTNNB1 mRNA when FMR1 was knocked down by siRNAs in HEK293T cells (e.g., as shown in FIG. 21E), indicating FMRP's role in regulating the stability of mRNAs encoding Wnt signaling components.

[0521] Based on the above information obtained using the vascularized brain organoids as a model, the study demonstrated that FMRP deficiency, which is a characteristic of FXS, leads to disease-associated hypervascularization and BBB abnormalities through posttranscriptional hyperactivation of the mTOR pathway and inhibition of Wnt pathway, respectively (e.g., as shown in FIG. 22). Furthermore, the study demonstrated that inhibitors of the mTOR signaling pathway may prevent diseases of the BBB (e.g., FXS, CCM, etc.) caused by mTOR pathway hyperactivation.

2. Modeling Treatment of BBB Diseases

[0522] In particular, it was found that Rapamycin treatment (e.g., about 10 nM for 2 weeks) administered on Day 30 BBB assembloids derived from CCM patients ameliorated disease-related hypervascularization and reduce VEGFR expression in these assembloids. For example, as shown in FIG. 23, rapamycin attenuated phenotypic symptoms of CCM, such as by significantly reducing vessel width and VEGFR expression in CCM BBB assembloids.

3. Alzheimer's Disease (AD) Modeling in Vascularized Brain Organoids

[0523] The present disclosure also relates to the use and modification of the vascularized brain organoids described herein to model and understand AD pathology. AD is characterized by an

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accumulation of abnormal neuritic plaques and neurofibrillary tangles in the brain, resulting in a loss of neurons and neuronal communications. A study was conducted by comparing marker expression of vascularized brain organoid models based on BBB assembloids derived from familial and sporadic AD patients to a control sample of BBB assembloids derived from normal patients. The comparison revealed several key AD-related effects on the BBB.

[0524] As shown in FIG. 24, the vascularized brain organoids based on the AD BBB assembloids were found to exhibit reduced expression of tight junction proteins (e.g., Claudin-5) and glucose transporters (e.g., GLUT-1). Additionally, a single-cell sequencing on AD BBB assembloids was performed (e.g., as shown in FIG. 25). Using the CellChat R package, altered cell-cell communications were predicted and a global decrease in the number and strength of these communications was observed (e.g., as shown in FIG. 26). This indicated disconnections among neurovascular cells underlying AD pathology.

4. Modeling Infection By Viral Vectors in Vascularized Brain Organoids

[0525] The present disclosure also relates to the use and modification of the vascularized brain organoids described herein to model and understand infection by viral vectors. A study was conducted to assess viral vectors across the human BBB. For the initial test, AAV vectors (rAAV9 and rAAV.eB) were microinjected into the vessels of BBB assembloids developed using methods described herein. Viral infection of these BBB assembloids were assessed two weeks later. As shown in FIGS. 27A-27C, it was found that both rAAV9 and rAAV.eB, labeled by GFP, can cross the human BBB and infect human neurons (labeled by DCX, as shown in FIG. 27A), human neural progenitors (labeled by Sox2, as shown in FIG. 27B), and human astrocytes (labeled by S100B, as shown in FIG. 27C). Notably, a significant increase in the infection rate of human neurons, astrocytes, and neural progenitors by rAAV.eB compared to rAAV9 was observed, indicating higher translational potential for rAAV.eB.

Quantification and statistics analysis

[0526] Individual assembloids and organoids were considered biological replicates unless stated otherwise in the Figure Legends. Data are presented as mean $\pm$ S.E.M. or mean $\pm$ S.D., as specified in the Figure Legends. Statistical analyses were conducted using the Student's t-test in Excel or Prism software. Statistically significant results possessed a P value less than or equal to

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0.05. Organoid samples were randomly selected from the culture for experiments and analyses, and sample sizes were determined empirically to account for variability between organoids and human iPSC cell lines while adhering to current standards in human organoid-related studies. Other statistical details of experiments can be found in the Figure Legends. Data analyses comparing control and disease individual-derived assembloids and organoids were performed blindly, and no data was excluded.

[0527] In at least some of the previously described embodiments, one or more elements used in an embodiment can interchangeably be used in another embodiment unless such a replacement is not technically feasible. It will be appreciated by those skilled in the art that various other omissions, additions and modifications may be made to the methods and structures described herein without departing from the scope of the claimed subject matter. All such modifications and changes are intended to fall within the scope of the subject matter, as defined by the appended claims.

[0528] With respect to the use of substantially any plural and/or singular terms herein, those having skill in the art can translate from the plural to the singular and/or from the singular to the plural as is appropriate to the context and/or application. The various singular/plural permutations may be expressly set forth herein for sake of clarity.

[0529] It will be understood by those within the art that, in general, terms used herein, and especially in the appended claims (e.g., bodies of the appended claims) are generally intended as “open” terms (e.g., the term “including” should be interpreted as “including but not limited to,” the term “having” should be interpreted as “having at least,” the term “includes” should be interpreted as “includes but is not limited to,” etc.). It will be further understood by those within the art that if a specific number of an introduced claim recitation is intended, such an intent will be explicitly recited in the claim, and in the absence of such recitation no such intent is present. For example, as an aid to understanding, the following appended claims may contain usage of the introductory phrases “at least one” and “one or more” to introduce claim recitations. However, the use of such phrases should not be construed to imply that the introduction of a claim recitation by the indefinite articles “a” or “an” limits any particular claim containing such introduced claim recitation to embodiments containing only one such recitation, even when the same claim includes the introductory phrases “one or more” or “at least one” and indefinite articles such as “a” or “an”

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(e.g., “a” and/or “an” should be interpreted to mean “at least one” or “one or more”); the same holds true for the use of definite articles used to introduce claim recitations. In addition, even if a specific number of an introduced claim recitation is explicitly recited, those skilled in the art will recognize that such recitation should be interpreted to mean at least the recited number (e.g., the bare recitation of “two recitations,” without other modifiers, means at least two recitations, or two or more recitations). Furthermore, in those instances where a convention analogous to “at least one of A, B, and C, etc.” is used, in general such a construction is intended in the sense one having skill in the art would understand the convention (e.g., “a system having at least one of A, B, and C” would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together, etc.). In those instances where a convention analogous to “at least one of A, B, or C, etc.” is used, in general such a construction is intended in the sense one having skill in the art would understand the convention (e.g., “a system having at least one of A, B, or C” would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together, etc.). It will be further understood by those within the art that virtually any disjunctive word and/or phrase presenting two or more alternative terms, whether in the description, claims, or drawings, should be understood to contemplate the possibilities of including one of the terms, either of the terms, or both terms. For example, the phrase “A or B” will be understood to include the possibilities of “A” or “B” or “A and B.”

[0530] In addition, where features or aspects of the disclosure are described in terms of Markush groups, those skilled in the art will recognize that the disclosure is also thereby described in terms of any individual member or subgroup of members of the Markush group.

[0531] As will be understood by one skilled in the art, for any and all purposes, such as in terms of providing a written description, all ranges disclosed herein also encompass any and all possible sub-ranges and combinations of sub-ranges thereof. Any listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, tenths, etc. As a non-limiting example, each range discussed herein can be readily broken down into a lower third, middle third and upper third, etc. As will also be understood by one skilled in the art all language such as “up to,” “at least,” “greater than,” “less than,” and the like include the number recited and refer to ranges which can be subsequently broken down into sub-ranges as discussed herein. Finally, as will be understood by one skilled in

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the art, a range includes each individual member. Thus, for example, a group having 1-3 articles refers to groups having 1, 2, or 3 articles. Similarly, a group having 1-5 articles refers to groups having 1, 2, 3, 4, or 5 articles, and so forth.

[0532] While various aspects and embodiments have been disclosed herein, other aspects and embodiments will be apparent to those skilled in the art. The various aspects and embodiments disclosed herein are for purposes of illustration and are not intended to be limiting, with the true scope and spirit being indicated by the following claims.

[0533] All references cited herein, including but not limited to published and unpublished applications, patents, and literature references, are incorporated herein by reference in their entirety and are hereby made a part of this specification. To the extent publications and patents or patent applications incorporated by reference contradict the disclosure contained in the specification, the specification is intended to supersede and/or take precedence over any such contradictory material.

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WHAT IS CLAIMED IS:

1. A method for producing a vascularized brain organoid, comprising:
culturing a blood vessel organoid and a brain organoid for a period of time until the blood vessel organoid and the brain organoid fuse together and blood vessels of the blood vessel organoid infiltrate the brain organoid;
wherein neurons of the brain organoid innervate the blood vessels of the blood vessel organoid that have infiltrated the organoid, thereby forming the vascularized brain organoid; and
wherein the vascularized brain organoid comprises a blood-brain barrier that is formed between the brain organoid and all or a portion of the blood vessels of the blood vessel organoid that have infiltrated the brain organoid.
2. The method of claim 1, wherein the blood-brain barrier comprises endothelial cells linked with tight junctions, astrocytes, pericytes, and/or smooth muscle cells.
3. The method of claim 2, wherein the endothelial cells express CD31, GLUT-1 and PDGFR- β ; the tight junctions comprise claudin-5, ZO-1 and cadherin 5; the astrocytes express S100B, GFAP, and AQP4; the pericytes express PDGFR- β , α SMA and NG2, and/or the smooth muscle cells express SMA.
4. The method of claim 2 or 3, wherein the endothelial cells form a continuous basement membrane and express collagen IV.
5. The method of any one of the preceding claims, wherein the vascularized brain organoid comprises cells selected from the group consisting of neural progenitors, proliferative astrocytes, GABAergic neurons, glutamatergic neurons, proliferative cells, brain vascular endothelial cells, vascular leptomeningeal cells, perivascular adipocytes, and tendon cells.
6. The method of any one of the preceding claims, wherein the cells of the vascular brain organoid are identified by cell-type specific gene expression markers.

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7. The method of any one of the preceding claims, wherein the blood vessels comprise capillaries.
8. The method of claim 7, wherein the capillaries are ensheathed by pericytes and end-feet of astrocytes.
9. The method of any one of the preceding claims, wherein the brain organoid is a forebrain organoid, a midbrain organoid, a hypothalamus organoid, a hippocampus organoid, a spinal cord organoid, or a striatal brain organoid.
10. The method of any one of the preceding claims, wherein the blood vessel organoid and the brain organoid are contacted and/or cultured in a basement membrane matrix or component thereof, optionally Matrigel.
11. The method of any one of the preceding claims, wherein the blood vessel organoid and the brain organoid are cultured for a period of time that is between about 1- 70 days.
12. The method of any one of the preceding claims, wherein the blood vessel organoid and the brain organoid are cultured for a period of time that is about 30-70 days.
13. The method of any one of the preceding claims, wherein the blood vessel organoid and the brain organoid are cultured for a period of time that is about 30-60.
14. The method of any one of the preceding claims, wherein the blood vessel organoid and the brain organoid are cultured with agitation, optionally shaking, for at least a portion of the period of time.
15. The method of any one of the preceding claims, wherein the blood vessel organoid and the brain organoid are cultured:
without agitation for about 1-14 days; and subsequently

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with agitation for about 1-70 days.

16. The method of any one of the preceding claims, wherein the blood vessel organoid and the brain organoid are cultured:

without agitation for about 4-10 days; and subsequently
with agitation for about 30-70 days.

17. The method of any one of the preceding claims, wherein the blood vessel organoid and the brain organoid are cultured:

without agitation for about 4-10 days; and subsequently
with agitation for about 1-40 days.

18. The method of any one of the preceding claims, wherein the blood vessel organoid and the brain organoid are cultured in a medium that promotes neuronal growth and/or vascular growth.

19. The method of any one of the preceding claims, wherein the blood vessel organoid and the brain organoid are cultured in a medium that comprises growth factors that promote neuronal growth and/or growth factors that promote vascular growth.

20. The method of claim 19, wherein the growth factors that promote neuronal growth comprise a cAMP pathway activator, ascorbic acid, brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), or any combination thereof.

21. The method of claim 19 or 20, wherein the growth factors that promote vascular growth comprise growth serum, a vascular endothelial growth factor (VEGF) pathway activator, a fibroblast growth factor (FGF) pathway activator, or any combination thereof.

22. The method of any one of the preceding claims, wherein culturing the blood vessel organoid and the brain organoid comprises:

culturing the blood vessel organoid and the brain organoid without agitation for about 4-10 days; and subsequently

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culturing the organoids of with agitation for about 1-40 days;
wherein the organoids of are cultured with and without agitation in a medium comprising growth factors that promote neuronal growth and/or growth factors that promote vascular growth; optionally wherein the agitation comprises shaking;
optionally wherein the growth factors that promote neuronal growth comprise a cAMP pathway activator, ascorbic acid, BDNF, GDNF, or any combination thereof;
optionally wherein the growth factors that promote vascular growth comprise growth serum, a VEGF pathway activator, an FGF pathway activator, or any combination thereof;
optionally wherein the blood vessel organoid and the brain organoid are cultured in a basement membrane matrix or component thereof, optionally Matrigel.

23. The method of claim 22, wherein the cAMP pathway activator is cAMP.
24. The method of claim 22 or 23, wherein the cAMP pathway activator is provided at a concentration that is between about 10-150 μM .
25. The method of any one of claims 22-24, wherein the cAMP pathway activator is provided at a concentration that is between about 20-100 μM .
26. The method of any one of claims 22-25, wherein the ascorbic acid is provided at a concentration that is between about 50 - 300 μM .
27. The method of any one of claims 22-26, wherein the ascorbic acid is provided at a concentration that is between about 50 - 300 μM .
28. The method of any one of claims 22-27, wherein the ascorbic acid is provided at a concentration that is between about 150 - 250 μM .
29. The method of any one of claims 22-28, wherein the BDNF is provided at a concentration that is between about 1 -30 ng/mL.

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30. The method of any one of claims 22-29, wherein the BDNF is provided at a concentration that is between about 15 - 25 ng/mL.
31. The method of any one of claims 22-30, wherein the GDNF is provided at a concentration that is between about 1-30 ng/mL.
32. The method of any one of claims 22-31, wherein the GDNF is provided at a concentration that is between about 15 - 25 ng/mL.
33. The method of any one of claims 22-32, wherein the growth serum is fetal bovine serum (FBS).
34. The method of any one of claims 22-33, wherein the growth serum is provided at a concentration that is between about 0.5%-20%.
35. The method of any one of claims 22-34, wherein the growth serum is provided at a concentration that is between about 12%-18%.
36. The method of any one of claims 22-35, wherein the VEGF pathway activator is VEGF.
37. The method of any one of claims 22-36, wherein the VEGF pathway activator is provided at a concentration that is between about 10-150 ng/mL.
38. The method of any one of claims 22-37, wherein the VEGF pathway activator is provided at a concentration that is between about 80-120 ng/mL.
39. The method of any one of claims 22-38, wherein the FGF pathway activator is FGF2.

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40. The method of any one of claims 22-39, wherein the FGF pathway activator is provided at a concentration between about 10-150 ng/mL.
41. The method of any one of claims 22-40, wherein the FGF pathway activator is provided at a concentration between about 80-120 ng/mL.
42. The method of any one of the preceding claims, wherein the blood vessel organoid and/or the brain organoid are derived from pluripotent stem cells, optionally embryonic stem cells or induced pluripotent stem cells.
43. The method of any one of the preceding claims, wherein the blood vessel organoid has been produced according to a method comprising:
causing, for a first period of time, an angiogenic sprout to activate an FGF pathway, a VEGF pathway, and, optionally, a Wnt pathway of the angiogenic sprout, while the angiogenic sprout is in growth serum.
44. The method of claim 43, wherein the first period of time is between about 1-30 days.
45. The method of claim 43 or 44, wherein the first period of time is between about 10-30 days.
46. The method of any one of claims 43-45, wherein the first period of time is between about 5-25 days.
47. The method of any one of claims 43-46, wherein the angiogenic sprout has been produced according to a method comprising:
a) causing, for a second period of time, the pluripotent stem cells to activate a Wnt pathway and a BMP pathway of the pluripotent stem cells to form vascular lineage cells; and
b) causing, for a third period of time, the vascular lineage cells to activate a VEGF pathway and a second cAMP pathway of the vascular lineage cells to form the angiogenic sprout.
48. The method of claim 47, wherein the second period of time is between about 1-5 days.

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49. The method of claim 47 or 48, wherein the second period of time is about 3 days.
50. The method of any one of claims 47-49, wherein the third period of time is between about 1-4 days.
51. The method of any one of claims 47-50, wherein the third period of time is about 2 days.
52. The method of any one of claims 47-51, wherein the BMP pathway is activated via a BMP pathway activator, wherein the BMP pathway activator is BMP4.
53. The method of any one of claims 47-52, wherein the BMP pathway activator is provided at a concentration that is between about 10-100 ng/mL.
54. The method of any one of claims 47-53, wherein the BMP pathway activator is provided at a concentration that is between about 20-70 ng/mL.
55. The method of any one of claims 47-54, wherein the Wnt pathway is activated via a Wnt pathway activator, wherein the Wnt pathway activator is CHIR99021.
56. The method of any one of claims 47-55, wherein the Wnt pathway is activated via a Wnt pathway activator provided at a concentration that is between about 1-20 μ M.
57. The method of any one of claims 47-56, wherein the Wnt pathway is activated via a Wnt pathway activator provided at a concentration that is between about 1-12 μ M.
58. The method of any one of claims 47-57, wherein the Wnt pathway is activated via a Wnt pathway activator provided at a concentration that is between about 4-20 μ M.
59. The method of any one of claims 47-58, wherein the second cAMP pathway is activated via a cAMP pathway activator comprising forskolin.

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60. The method of any one of claims 47-59, wherein the second cAMP pathway is activated via a second cAMP pathway activator provided at a concentration that is between about 0.5 - 4 μ M.

61. The method of any one of claims 47-60, wherein the second cAMP pathway is activated via a second cAMP pathway activator provided at a concentration that is between about 1-3 μ M.

62. The method of any one of claims 43-61, wherein the blood vessel organoid differs from a blood vessel organoid that has been produced without causing the angiogenic sprout to activate the Wnt pathway by having increased expression of blood-brain barrier-specific endothelial markers, optionally GLUT-1 and ZO-1.

63. The method of any one of the preceding claims, wherein the brain organoid has been contacted with LIF and growth serum to induce astrocyte formation in the brain organoid.

64. The method of any one of the preceding claims, wherein the brain organoid has been produced according to a method comprising:

causing, for a first period of time, pluripotent stem cells to inhibit a BMP pathway, a TGF-beta pathway, and a Wnt pathway of the pluripotent stem cells to form neuroectoderm cells;

causing, for a second period of time, the neuroectoderm cells to inhibit a second TGF-beta pathway and activate a Wnt pathway of the neuroectoderm cells to form neuroepithelium cells;

contacting the neuroepithelium cells with insulin for a third period of time to form cerebral tissue organoids; and

for a fourth period of time:

contacting the cerebral tissue organoid with GDNF, BDNF, and ascorbic acid, and

activating a cAMP pathway activator of the cerebral tissue organoid to form the brain organoid.

65. The method of claim 64, wherein the brain organoid has been produced according to a method further comprising:

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contacting the cerebral tissue organoid with LIF and growth serum for a portion of the fourth period of time to induce astrocyte proliferation in the brain organoid.

66. The method of claim 64 or 65, wherein the first period of time is between about 1-14 days.

67. The method of any one of claims 64-66, wherein the first period of time is between about 5-10 days.

68. The method of any one of claims 64-67, wherein the second period of time is between about 1-14 days.

69. The method of any one of claims 64-68, wherein the second period of time is between about 5-10 days.

70. The method of any one of claims 64-69, wherein the third period of time is between about 20-70 days.

71. The method of any one of claims 64-70, wherein the third period of time is between about 50-70 days.

72. The method of any one of claims 64-71, wherein the fourth period of time is between about 7-70 days.

73. The method of any one of claims 64-72, wherein the fourth period of time is between about 20-60 days.

74. The method of any one of claims 65-73, wherein the portion of the fourth period of time is between about 7-21 days.

75. The method of any one of claims 64-74, wherein the BMP pathway is inhibited via a BMP pathway inhibitor comprising LDN-193189.

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76. The method of any one of claims 64-75, wherein the BMP pathway is inhibited via a BMP pathway inhibitor provided at a concentration that is between about 0.1-2 μM .
77. The method of any one of claims 64-76, wherein the BMP pathway is inhibited via a BMP pathway inhibitor provided at a concentration that is between about 0.5-1.5 μM .
78. The method of any one of claims 64-77, wherein the TGF-beta pathway is inhibited via a TGF-beta pathway inhibitor and the second TGF-beta pathway is inhibited via a second TGF-beta pathway inhibitor.
79. The method of any one of claims 64-78, wherein the TGF-beta pathway is inhibited via a TGF-beta inhibitor comprising A83-01.
80. The method of any one of claims 64-79, wherein the TGF-beta pathway is inhibited via a TGF-beta pathway inhibitor provided at a concentration that is between about 0.5-4 μM .
81. The method of any one of claims 64-80, wherein the TGF-beta pathway is inhibited via a TGF-beta pathway inhibitor provided at a concentration that is between about 1-3 μM .
82. The method of any one of claims 64-81, wherein the second TGF-beta pathway is inhibited via a TGF-beta pathway inhibitor comprising SB-431542.
83. The method of any one of claims 64-82, wherein the second TGF-beta pathway is inhibited via a second TGF-beta pathway inhibitor provided at a concentration that is between about 0.1-2 μM .
84. The method of any one of claims 64-83, wherein the second TGF-beta pathway is inhibited via a second TGF-beta pathway inhibitor provided at a concentration that is between about 0.5-1.5 μM .

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85. The method of any one of claims 64-84, wherein the Wnt pathway is inhibited via a Wnt pathway inhibitor comprising IWR-1.
86. The method of any one of claims 64-85, wherein the Wnt pathway is inhibited via a Wnt pathway inhibitor provided at a concentration that is between about 0.5-5 μ M.
87. The method of any one of claims 64-86, wherein the Wnt pathway is inhibited via a Wnt pathway inhibitor provided at a concentration that is between about 2-4 μ M.
88. The method of any one of claims 64-87, wherein the Wnt pathway is activated via a Wnt pathway activator comprising CHIR99021.
89. The method of any one of claims 64-88, wherein the Wnt pathway is activated via a Wnt pathway activator provided at a concentration that is between about 0.1-2 μ M.
90. The method of any one of claims 64-89, wherein the Wnt pathway is activated via a Wnt pathway activator provided at a concentration that is between about 0.5-1.5 μ M.
91. The method of any one of claims 64-90, wherein the insulin is provided at a concentration that is between about 0.5-5 μ g/mL.
92. The method of any one of claims 64-91, wherein the insulin is provided at a concentration that is between about 1-3 μ g/mL.
93. The method of any one of claims 65-92, wherein the LIF is provided at a concentration that is between about 1-20 mg/mL.
94. The method of any one of claims 65-93, wherein the LIF is provided at a concentration that is between about 5-15 mg/mL.

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95. The method of any one of claims 64-94, wherein the brain organoid comprises astrocytes that express S100B, GFAP, and AQP4.
96. The method of any one of the preceding claims, wherein the blood vessel organoid and/or the brain organoid are human.
97. The method of any one of the preceding claims, wherein the blood vessel organoid and/or the brain organoid have been derived from a subject, optionally a human subject.
98. The method of claim 97, wherein the subject is afflicted by a cerebrovascular disease or a disease associated with blood-brain barrier dysfunction, optionally wherein the cerebrovascular disease or disease associated with blood-brain barrier dysfunction comprises cerebral cavernous malformation, Alzheimer's disease, or amyotrophic lateral sclerosis.
99. The method of any one of the preceding claims, wherein the blood vessel organoid and/or the brain organoid comprises cells having a genetic mutation associated with cerebral cavernous malformation (CCM) and/or are from a subject suffering from CCM; optionally wherein the blood vessel organoid but not the brain organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM; optionally wherein the brain organoid but not the blood vessel organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM; optionally wherein both the blood vessel organoid and the brain organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM.
100. The method of any one of the preceding claims, wherein the blood vessel organoid and/or the brain organoid are derived from pluripotent stem cells having a genetic mutation associated with CCM and/or from a subject suffering from CCM.
101. The method of claim 99 or 100, wherein the genetic mutation associated with CCM is a deletion or loss of function mutation of one or more of KRIT1, CCM2 and/or PDCD10.

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102. The method of claim 101, wherein the genetic mutation associated with CCM is induced into the cell by a genetic modification technique, optionally using CRISPR.

103. A vascularized brain organoid produced by the method of any one of the preceding claims.

104. The vascularized brain organoid of claim 103, comprising a CCM-like feature, wherein the CCM-like feature is one or more of the following, as compared to a normal vascularized brain organoid:

- a cluster of enlarged endothelial channels, optionally arranged back-to-back;
- an upregulation of one or more lesion-marker genes, optionally in endothelial cells, optionally not in neuronal cells;
- an upregulation of tip cell and/or tumor tip cell markers, optionally in endothelial cells, optionally not in neuronal cells;
- a longer and/or a wider tip cell when evaluated in an angiogenesis assay;
- an expression of VEGFA and/or IGF2 in GABAergic neurons;
- a decrease in or an elimination of vascular smooth muscle cells (vSMCs);
- an increase in the number of astrocytes;
- a reduction in the expression of smooth muscle actin (SMA) protein, optionally measured by immunostaining;
- a reduction in expression of a tight junction protein; and/or
- a disassembled basement membrane.

105. The vascularized brain organoid of claim 104, wherein the CCM-like feature is a disruption, as compared to a normal vascularized brain organoid, of the blood-brain barrier that is formed between the brain organoid and all or a portion of the blood vessels of the blood vessel organoid that have infiltrated the brain organoid.

106. A vascularized brain organoid comprising a CCM-like feature and endothelial cells linked with tight junctions, astrocytes, and pericytes, optionally wherein the vascularized brain organoid

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is produced by the method of any one of claims 1-103, and optionally wherein the vascularized brain organoid having the CCM-like feature is any one of claims 104 or 105.

107. A vascularized brain organoid comprising a brain organoid and a blood vessel organoid, wherein at least a portion of the blood vessels of the blood vessel organoid have infiltrated the brain organoid, and neurons of the brain organoid innervate at least a portion of the infiltrating blood vessels.

108. The vascularized brain organoid of any one of claims 103-107, wherein the vascularized brain organoid has disease features, optionally wherein the disease features are CCM-like features.

109. The vascularized brain organoid of any one of claims 103-108, comprising a blood-brain barrier that is formed between the brain organoid and all or a portion of the blood vessels that have infiltrated the brain organoid.

110. The vascularized brain organoid of any one of claims 103-109, comprising gene expression markers indicative of excitatory neurons, inhibitory neurons, neural progenitors (NPs), astrocytes, endothelial cells (ECs), pericytes, mesenchymal stem cells (MSCs), smooth muscle cells (SMCs), and fibroblasts.

111. The vascularized brain organoid of any one of claims 103-110, comprising endothelial cells linked with tight junctions, astrocytes, pericytes, and smooth muscle cells, wherein the endothelial cells express CD31, GLUT-1 and PDGFR- β ; the tight junctions express claudin-5, ZO-1 and cadherin 5; the astrocytes express S100B, GFAP, and AQP4; the pericytes express PDGFR- β , α SMA and NG2; and/or the smooth muscle cells express SMA.

112. The vascularized brain organoid of any one of claims 103-111, wherein the endothelial cells form a continuous basement membrane and express collagen IV.

113. The vascularized brain organoid of any of claims 103-112 comprising cells selected from the group consisting of neural progenitors, proliferative astrocytes, GABAergic neurons,

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glutamatergic neurons, proliferative cells, brain vascular endothelial cells, vascular leptomeningeal cells, perivascular adipocytes, and tendon cells.

114. The vascularized brain organoid of claim 113, wherein the cells are identified by cell-type specific gene expression markers.

115. The vascularized brain organoid of any of claims 103-114, wherein the blood vessels comprise capillaries.

116. The vascularized brain organoid of any of claims 103-115, wherein the capillaries are ensheathed by pericytes and end-feet of the astrocytes.

117. The vascularized brain organoid of any of claims 103-116, wherein the brain organoid is a forebrain organoid, a midbrain organoid, a hypothalamus organoid, a hippocampus organoid, a spinal cord organoid, and/or a striatal brain organoid.

118. The vascularized brain organoid of any of claims 103-117, wherein the blood vessel organoid and the brain organoid were contacted and/or cultured in a basement membrane matrix or component thereof, optionally Matrigel.

119. The vascularized brain organoid of any of claims 103-118, wherein the vascularized brain organoid comprises cells having one or more genetic mutations and/or one or more phenotypes associated with neurodegenerative disorders, prion disease, Huntington disease, Alzheimer's disease, Fragile X syndrome, Parkinson's disease, amyotrophic lateral sclerosis (ALS), dementia, multiple sclerosis, encephalitis, epilepsy, infections, anxiety, bipolar disorder, depression, post-traumatic stress disorder, schizophrenia, attention deficit disorder, autism spectrum disorder, dyslexia, stroke, seizure, viral infection, bacterial infection, parasitic infection, traumatic brain injury, and/or cancer.

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120. The vascularized brain organoid of any of claims 103-119, wherein the vascularized brain organoid comprises cells having a genetic mutation associated with CCM and/or are from a subject suffering from CCM.

121. The vascularized brain organoid of any of claims 103-120, wherein the vascularized brain organoid is derived from pluripotent stem cells having a genetic mutation associated with CCM and/or from a subject suffering from CCM.

122. The vascularized brain organoid of claim 121, wherein the genetic mutation associated with CCM is a deletion or loss of function mutation of one or more of KRIT1, CCM2 and/or PDCD10.

123. The vascularized brain organoid of claim 121 or 122, wherein the genetic mutation associated with CCM is induced into the cell by a genetic modification technique, optionally using CRISPR.

124. The vascularized brain organoid of any one of claims 105-123 comprising one or more CCM-like features, wherein the CCM-like feature is one or more of the following, as compared to a normal vascularized brain organoids and/or a normal brain tissue:

- a cluster of enlarged endothelial channels, optionally arranged back-to-back;
- an upregulation of one or more lesion-marker genes, optionally in endothelial cells, optionally not in neuronal cells;
- an upregulation of tip cell and/or tumor tip cell markers, optionally in endothelial cells, optionally not in neuronal cells;
- a longer and/or a wider tip cell when evaluated in an angiogenesis assay;
- an expression of VEGFA and/or IGF2 in GABAergic neurons;
- a decrease in or an elimination of vascular smooth muscle cells (vSMCs);
- an increase in the number of astrocytes;
- a reduction in the expression of smooth muscle actin (SMA) protein, optionally measured by immunostaining;
- a reduction in expression of a tight junction protein; and/or

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a disassembled basement membrane.

125. The vascularized brain organoid of any one of the preceding claims, comprising one or more CCM-like feature, wherein the CCM-like feature is a disruption of the blood-brain barrier that is formed between the brain organoid and all or a portion of the blood vessels of the blood vessel organoid that have infiltrated the brain organoid, as compared to normal vascularized brain organoids and/or normal brain tissue.

126. The vascularized brain organoid of any one of the preceding claims, wherein the vascularized brain organoid exhibits one or more of the following features, as compared to the blood vessel organoids prior to culturing the blood vessel organoid and the brain organoid:

- reduced endothelial fenestration;
- increased expression of drug pumps; and/or
- reduced expression of an immune cell adhesion molecule marker.

127. The vascularized brain organoid of claim 126, wherein the reduced endothelial fenestration comprises reduced expression or non-expression of PLVAP.

128. The vascularized brain organoid of any one of claims 126 or 127, wherein the increased expression of drug pumps comprises increased expression of glucose transporter 1 (GLUT1), P-glycoprotein (P-gp), and/or one or more tight junction proteins.

129. The vascularized brain organoid of claim 128, wherein the one or more tight junction proteins comprises one or more of Claudin-5 or ZO-1.

130. The vascularized brain organoid of any one of claims 126-129, wherein the reduced expression of immune cell adhesion molecule marker comprises the reduced expression or non-expression of immune cell adhesion molecule 1 (ICAM-1).

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131. The vascularized brain organoid of any one of the preceding claims, wherein the vascularized brain organoid exhibits the following features, as compared to the blood vessel organoids prior to culturing the blood vessel organoid and the brain organoid:

reduced permeability; and
increased trans-endothelial resistance.

132. The vascularized brain organoid of claim 131, wherein the increased trans-endothelial resistance comprises an increased transepithelial/trans-endothelial electrical resistance (TEER) value.

133. The vascularized brain organoid of claim 132, wherein an increase in TEER value of the vascularized brain organoid, as compared to a TEER value of the blood vessel organoids prior to culturing the blood vessel organoid and the brain organoid, is between about 850-1200 $\Omega \cdot \text{cm}^2$.

134. The vascularized brain organoid of claim 133, wherein an increase in TEER value of the vascularized brain organoid, as compared to a TEER value of the blood vessel organoids prior to culturing the blood vessel organoid and the brain organoid, is between about 950-1100 $\Omega \cdot \text{cm}^2$.

135. The vascularized brain organoid of any one of the preceding claims, wherein the vascularized brain organoid exhibits the following features, as compared to the brain organoid prior to culturing the blood vessel organoid and the brain organoid:

increased numbers of mature astrocytes;
more mature astrocytes;
increased numbers of mature endothelial cells; and/or
more mature endothelial cells.

136. A method of making a CCM primary tissue organoid, the method comprising:
culturing primary cavernomas tissue in a culture medium and on ultra-low attachment plates with agitation, until CCM primary tissue organoids form.

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137. The method of claim 136, wherein said culturing primary cavernomas tissue in culture medium until organoids form is for a period of time of about 1-2 weeks.

138. The method of any one of claims 136-137, wherein the method further comprises expanding the CCM primary tissue organoids by dissecting the CCM primary tissue organoids into pieces about 0.5 mm in diameter, and culturing the about 0.5 mm in diameter pieces until CCM primary tissue organoids form.

139. The method of any one of claims 136-138, wherein the method optionally comprises:

- a) incubating primary cavernomas tissue, as pieces having a diameter of about 0.5 mm, in red blood cell lysis buffer, optionally neutralizing the red blood cell lysis buffer at the end of the incubation;
- b) culturing, in ultra-low attachment plates with agitation, the primary cavernomas tissue pieces following the incubation in red blood cell lysis buffer, optionally following the neutralization of the red blood cell lysis buffer;
- c) culturing at about 37°C, 5% CO₂ and/or 95% air; and/or
- d) changing the culture medium about every 3 days.

140. The method of any one of claims 136-139, wherein the culture media comprises about 10 ng/mL BDNF, about 10 ng/mL of GDNF, about 7.5% FBS, about 50 ng/mL VEGF-A, and about 50 ng/mL FGF-2, optionally wherein the culture media is half M4 complete media and half StemPro-34 SFM complete media, optionally further comprising about 0.025 mM cAMP, and about 0.1 mM of Ascorbic acid.

141. The method of any one of claims 136-140, wherein the cavernomas tissue is human.

142. The method of any one of claims 136-141, wherein the CCM-like feature is one or more of the following, as compared to a normal brain tissue:

- a cluster of enlarged endothelial channels, optionally arranged back-to-back;
- an upregulation of one or more lesion-marker genes, optionally in endothelial cells, optionally not in neuronal cells;

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an upregulation of tip cell and/or tumor tip cell markers, optionally in endothelial cells, optionally not in neuronal cells;

a longer and/or a wider tip cell when evaluated in an angiogenesis assay;

an expression of VEGFA and/or IGF2 in GABAergic neurons;

a decrease in or an elimination of vascular smooth muscle cells (vSMCs);

an increase in the number of astrocytes;

a reduction in the expression of smooth muscle actin (SMA) protein, optionally measured by immunostaining;

a reduction in expression of a tight junction protein; and/or

a disassembled basement membrane.

143. A CCM primary tissue organoid made by the method of any one of claims 136-142.

144. A CCM primary tissue organoid comprising a CCM-like feature, optionally made by the method of any one of claims 136-143.

145. The CCM primary tissue organoid of any one of the preceding claims, wherein the CCM-like feature is one or more of the following, as compared to a normal brain tissue:

a cluster of enlarged endothelial channels, optionally arranged back-to-back;

an upregulation of one or more lesion-marker genes, optionally in endothelial cells, optionally not in neuronal cells;

an upregulation of tip cell and/or tumor tip cell markers, optionally in endothelial cells, optionally not in neuronal cells;

a longer and/or a wider tip cell when evaluated in an angiogenesis assay;

an expression of VEGFA and/or IGF2 in GABAergic neurons;

a decrease in or an elimination of vascular smooth muscle cells (vSMCs);

an increase in the number of astrocytes;

a reduction in the expression of smooth muscle actin (SMA) protein, optionally measured by immunostaining;

a reduction in expression of a tight junction protein; and/or

a disassembled basement membrane.

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146. The CCM primary tissue organoid of any one of the preceding claims, wherein the CCM-like feature is a disruption of the blood-brain barrier as compared to normal brain tissue.

147. A method of treating a cerebrovascular disease or a disease associated with blood-brain barrier dysfunction in a subject in need thereof, comprising administering to the subject the vascularized brain organoid of any one of claims 105-135, or a portion or fragment thereof, or the CCM primary tissue organoid of any one of claims 144-146, or a portion or fragment thereof.

148. A method of screening, comprising contacting the vascularized brain organoid of any of claims 105-135, or the CCM primary tissue organoid of any one of claims 144-146, or portions or fragments thereof, with a candidate compound or composition, and assessing the effects of the candidate compound or composition on the vascularized brain organoid, the CCM primary tissue organoid, or portions thereof.

149. The method of claim 148, wherein the effects comprises transport of the candidate compound or composition across the blood-brain barrier of the vascularized brain organoid, the CCM primary tissue organoid, or portions thereof.

150. The method of claim 148 or 149, wherein the vascularized brain organoid and/or CCM primary tissue organoid is a model for a cerebrovascular disease or a disease associated with blood-brain barrier dysfunction, and assessing the effects of the candidate compound or composition on the vascularized organoid and/or CCM primary tissue organoid comprises assessing the effects of the candidate compound or composition on the cerebrovascular disease or the disease associated with blood-brain barrier dysfunction.

151. The method of any one of claims 148-150, wherein the vascularized brain organoid and/or CCM primary tissue organoid has been produced from cells derived from a subject, optionally wherein the cells derived from the subject are induced pluripotent stem cells.

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152. The method of claim 151, wherein the subject has or is disposed to develop the cerebrovascular disease or the disease associated with blood-brain barrier dysfunction.

153. The method of any one of claims 148-152, wherein the cerebrovascular disease or the disease associated with blood-brain barrier dysfunction is CCM.

154. The method of any one of claims 148-153, the cerebrovascular disease or the disease associated with blood-brain barrier dysfunction is a cerebrovascular disease or a disease associated with blood-brain barrier dysfunction in addition to CCM.

155. A cell culture media comprising, a first media component that promotes neuronal growth, and a second media component that promotes vascular growth.

156. The cell culture media of claim 155, wherein the first and/or second media component comprise added growth factors that promote neuronal growth and/or promote vascular growth.

157. The cell culture media of claim 155 or 156, wherein the added growth factors that promote neuronal growth comprise, consist essentially of, or consist of a cAMP pathway activator, ascorbic acid, BDNF, and/or GDNF.

158. The cell culture media of any one of claims 155-157, wherein the added growth factors that promote vascular growth comprise, consist essentially of, or consist of growth serum, a VEGF pathway activator, and/or an FGF pathway activator.

159. The cell culture media of any one of claims 155-158, further comprising a basement membrane matrix or component thereof.

160. The cell culture media of any one of claims 155-159, wherein the added cAMP pathway activator is cAMP.

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161. The cell culture media of any one of claims 155-160, wherein the added cAMP pathway activator is at a concentration that is between about 10-150 μ M.

162. The cell culture media of any one of claims 155-161, wherein the added cAMP pathway activator is at a concentration that is between about 20-100 μ M.

163. The cell culture media of any one of claims 155-162, wherein the added ascorbic acid is at a concentration that is between about 50-300 μ M.

164. The cell culture media of any one of claims 155-163, wherein the ascorbic acid is provided at a concentration that is between about 150 - 250 μ M.

165. The cell culture media of any one of claims 155-164, wherein the added BDNF is at a concentration that is between about 1-30 ng/mL.

166. The cell culture media of any one of claims 155-165, wherein the BDNF is provided at a concentration that is between about 15 - 25 ng/mL.

167. The cell culture media of any one of claims 155-166, wherein the added GDNF is at a concentration that is between about 1-30 ng/mL.

168. The cell culture media of any one of claims 155-167, wherein the GDNF is provided at a concentration that is between about 15 - 25 ng/mL.

169. The cell culture media of any one of claims 155-168, wherein the added growth factors are not xenogeneic to human cells, and/or are of good manufacturing practices (GMP) grade.

170. The cell culture media of any one of claims 155-169, wherein the added growth serum is fetal bovine serum (FBS).

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171. The cell culture media of any one of claims 155-170, wherein the added growth serum is at a concentration that is between about 0.5%-20%.

172. The cell culture media of any one of claims 155-171, wherein the growth serum is provided at a concentration that is between about 12%-18%.

173. The cell culture media of any one of claims 155-172, wherein the VEGF pathway activator is VEGF.

174. The cell culture media of any one of claims 155-173, wherein the added VEGF pathway activator is at a concentration that is between about 10-150 ng/mL.

175. The cell culture media of any one of claims 155-174, wherein the VEGF pathway activator is provided at a concentration that is between about 80-120 ng/mL.

176. The cell culture media of any one of claims 155-175, wherein the FGF pathway activator is FGF2.

177. The cell culture media of any one of claims 155-176, wherein the added FGF pathway activator is at a concentration that is between about 10-150 ng/mL.

178. The cell culture media of any one of claims 155-177, wherein the FGF pathway activator is provided at a concentration between about 80-120 ng/mL.

179. The cell culture media of any one of claims 155-178, comprising about 10 ng/mL BDNF, about 10 ng/mL of GDNF, about 7.5% FBS, about 50 ng/mL VEGF-A, and about 50 ng/mL FGF-2, optionally wherein the culture media is, or is about, half M4 complete media and is, or is about, half StemPro-34 SFM complete media, optionally further comprising about 0.025 mM cAMP, and about 0.1 mM of Ascorbic acid.

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180. The cell culture media of any one of claims 155-179, comprising a combination of half M4 complete medium and half StemPro-34 SFM complete medium, wherein the M4 complete medium comprises 0.05 mM of cAMP, 0.2 mM of Ascorbic acid, 20 ng/mL of BDNF, and 20 ng/mL of GDNF and M4 base medium, and wherein the StemPro-34 SFM complete medium comprises the StemPro-34 SFM base medium, supplemented with 15% FBS, 100 ng/mL VEGF-A, and 100 ng/mL FGF-2.

181. The cell culture media of any one of claims 155-180, further comprising blood vessel organoids and/or brain organoids, optionally wherein the blood vessel organoids and/or brain vessel organoids are derived from pluripotent stem cells, optionally embryonic stem cells or induced pluripotent stem cells.

182. A cell culture media for generating a vascularized brain organoid, the cell culture media comprising:

- a base endothelial cell (EC) media;
- a vascular endothelial growth factor A (VEGF-A); and
- an endothelial cell growth supplement (ECGS).

183. The cell culture media of claim 182, wherein the base EC media comprises FBS.

184. The cell culture media of claim 182 or 183, wherein the base EC media comprises FBS at a concentration of about 0.5% to about 20% of the cell culture media.

185. The cell culture media of any one of claims 181-184, wherein the ECGS comprises acidic FGF.

186. The cell culture media of any one of claims 181-185, wherein the ECGS comprises acidic FGF at a concentration of about 10 to 500 ng/ml.

187. The cell culture media of any one of claims 181-186, wherein the VEGF-A is at a concentration of about 10 to 500 ng/ml of cell culture media.

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188. The cell culture media of any one of claims 181-187, wherein the VEGF-A is at a concentration of about 50 to 200 ng/ml of cell culture media.

189. The cell culture media of any one of claims any one of claims 181-188, further comprising: Heparin.

190. The cell culture media of claim 189, wherein the Heparin has a concentration of about 0 to 20 µg/ml of the cell culture media.

191. The cell culture media of claim 189 or 190, wherein the Heparin has a concentration of about 5 to 15 µg/ml of the cell culture media.

192. The cell culture media of any one of claims 182-191, wherein the base EC media comprises DMEM/F12.

193. A composition comprising the cell culture media of any one of claims 181-192, and further comprising:
a vascularized brain organoid.

194. The composition of claims 193, wherein the vascularized brain organoid is produced by any one of claims 1-102.

195. The composition of claim 193 or 194, further comprising blood vessel organoids and/or brain organoids, optionally wherein the blood vessel organoids and/or brain vessel organoids are derived from pluripotent stem cells, optionally embryonic stem cells or induced pluripotent stem cells.

196. The composition of any one of claims 193-195, wherein the vascularized brain organoid comprises cells having one or more genetic mutations and/or one or more phenotypes associated with Fragile X syndrome.

197. The composition of claim 196, wherein the cells having the one or more phenotypes associated with Fragile X syndrome results in enlarged capillary perimeters and/or diameters in the vascularized organoid, compared to cells not having one or more genetic mutations and/or one or more phenotypes associated with Fragile X syndrome.

198. The composition of claim 196 or 197, wherein the cells having the one or more genetic mutations associated with Fragile X syndrome comprises cells having an upregulation of an angiogenic growth factor, compared to cells not having the one or more genetic mutations and/or one or more phenotypes associated with Fragile X syndrome.

199. The composition of any one of claims 196-198, wherein the cells having the one or more genetic mutations associated with Fragile X syndrome comprises cells having reduction of an Claudin-5, glucose transporter 1 (GLUT-1), and P-glycoprotein (P-gp), compared to cells not having the one or more genetic mutations and/or one or more phenotypes associated with Fragile X syndrome.

200. The composition of any one of claims 196-199, wherein the cells having the one or more genetic mutations associated with Fragile X syndrome comprises cells with a hyperactivation of mTOR signaling pathway, compared to cells not having the one or more genetic mutations and/or one or more phenotypes associated with Fragile X syndrome.

201. The composition of any one of claims 196-200, wherein the cells having the one or more genetic mutations associated with Fragile X syndrome comprises cells with a downregulation of Wnt signaling pathway, compared to cells not having the one or more genetic mutations and/or one or more phenotypes associated with Fragile X syndrome.

202. The composition of any one of claims 193-201, wherein the vascularized brain organoid comprises cells having one or more genetic mutations and/or one or more phenotypes associated with Alzheimer's Disease.

203. The composition of any one of claims 193-202, wherein the cells having the one or more genetic mutations associated with Alzheimer's Disease comprises cells having reduction of an Claudin-5, glucose transporter 1 (GLUT-1), and P-glycoprotein (P-gp), compared to cells not having the one or more genetic mutations and/or one or more phenotypes associated with Alzheimer's Disease.

204. The composition of any one of claims 193-203, further comprising a viral vector, wherein the vascularized brain organoid exhibits one or more phenotypes associated with infection by the viral vector.

205. The composition of claim 204, wherein the one or more phenotypes associated with infection by the viral vector comprises:

- infected neurons;
- infected neural progenitors; and/or
- infected astrocytes.

206. A kit comprising means for performing the method according to any one of claims 1-102 and 148-154.

207. A kit comprising the vascularized brain organoid or CCM primary tissue organoid, or means for generating the vascularized brain organoid or CM primary tissue organoid of any one of claims 103-135.

208. A kit comprising the cell culture media, or means for generating the cell culture media, of any one of claims 155-192.

209. Use of the method, the vascularized brain organoid, the CCM primary tissue organoid, or cell culture media of any one of the preceding claims as a medicament, means for treatment and/or prevention of a disease, means for diagnosis, and/or medical research tool.

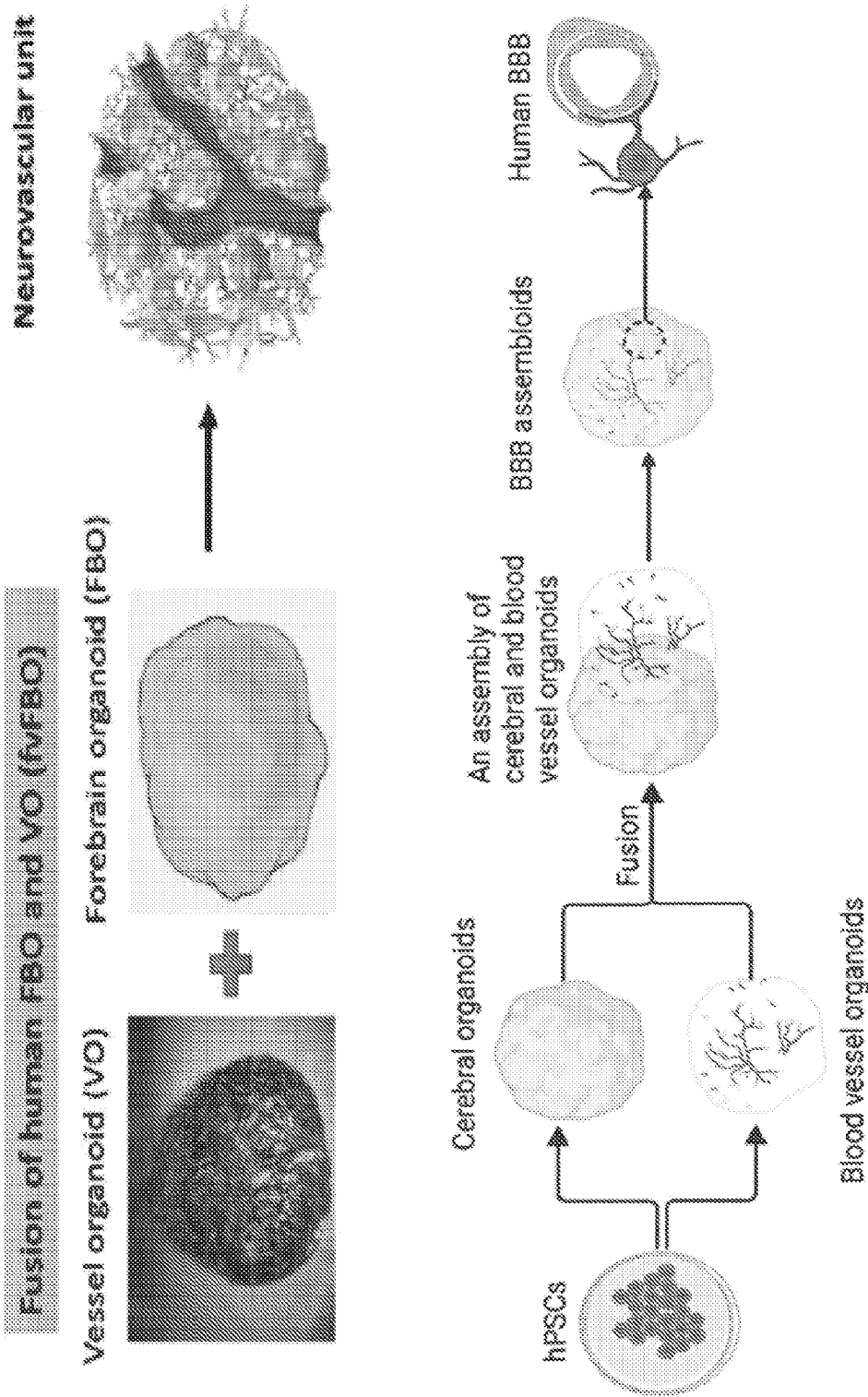


FIG. 1A

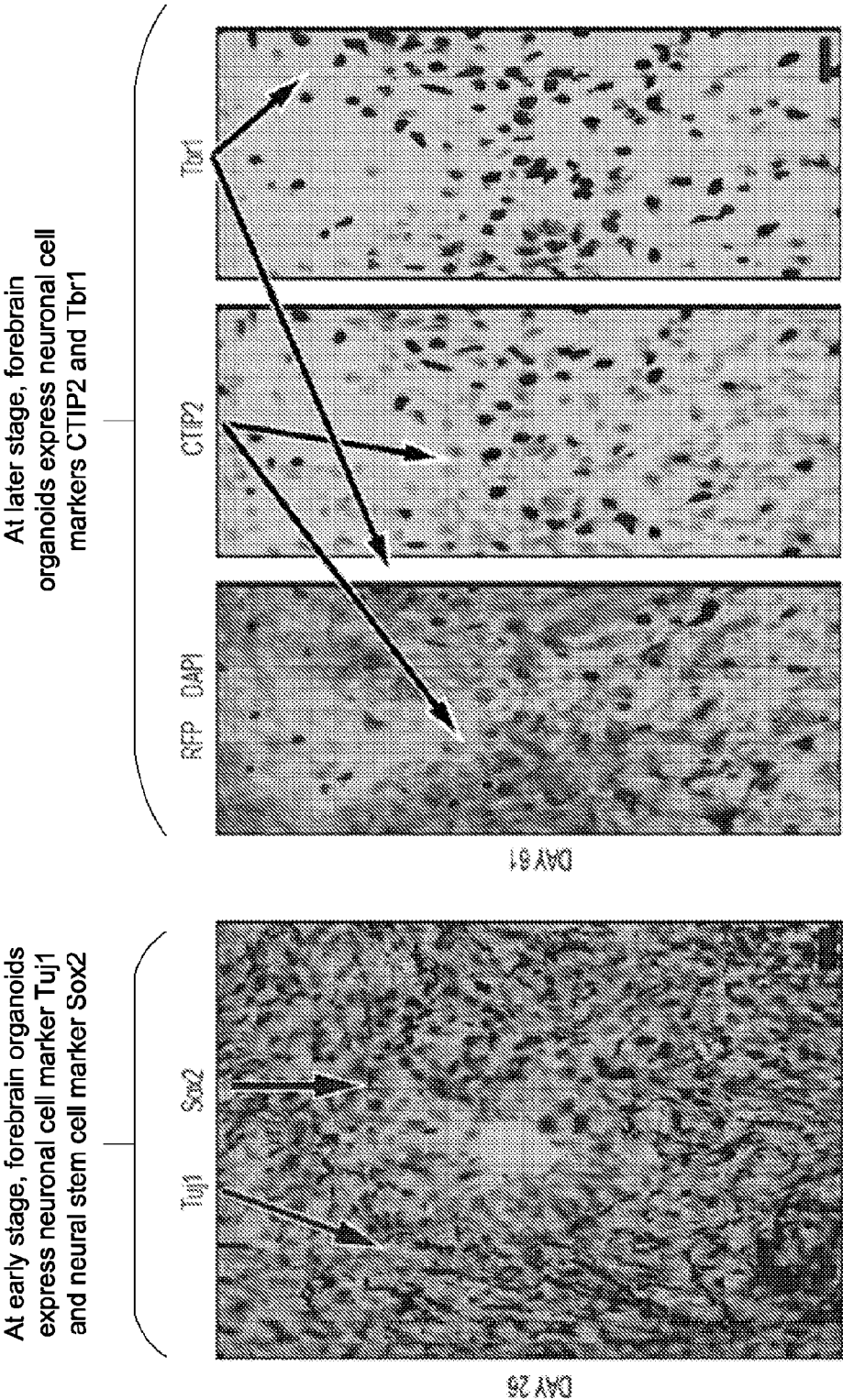
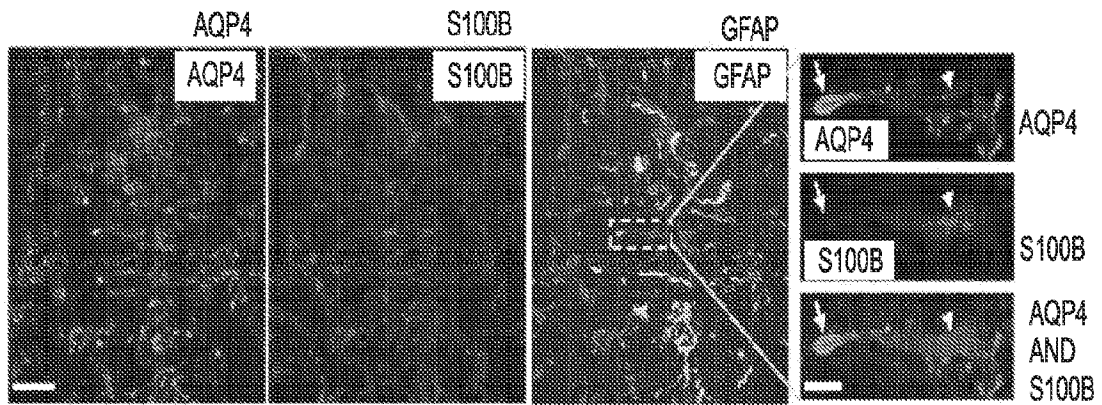
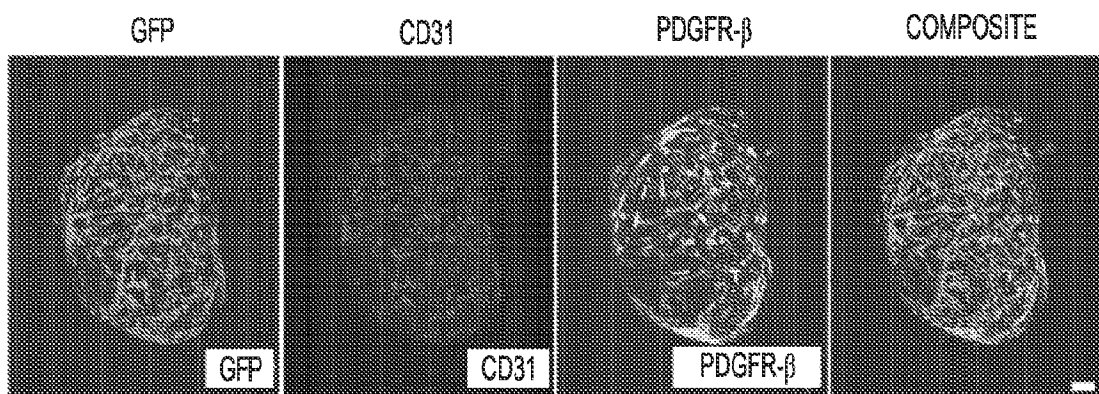
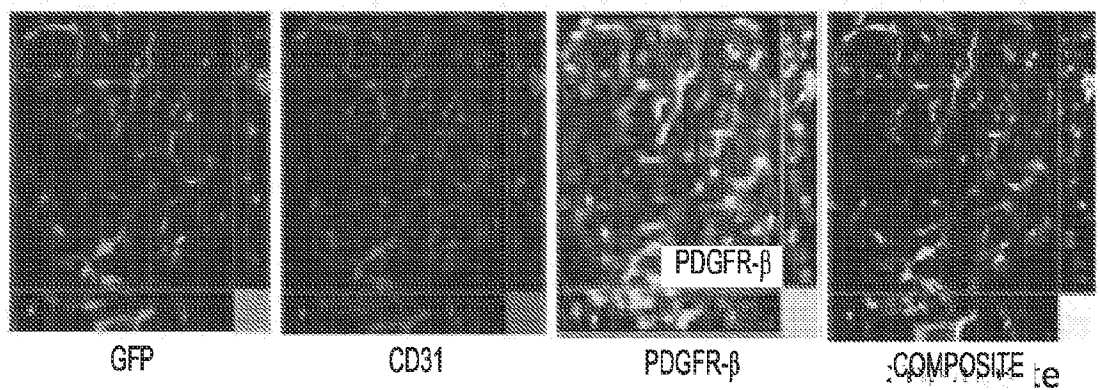
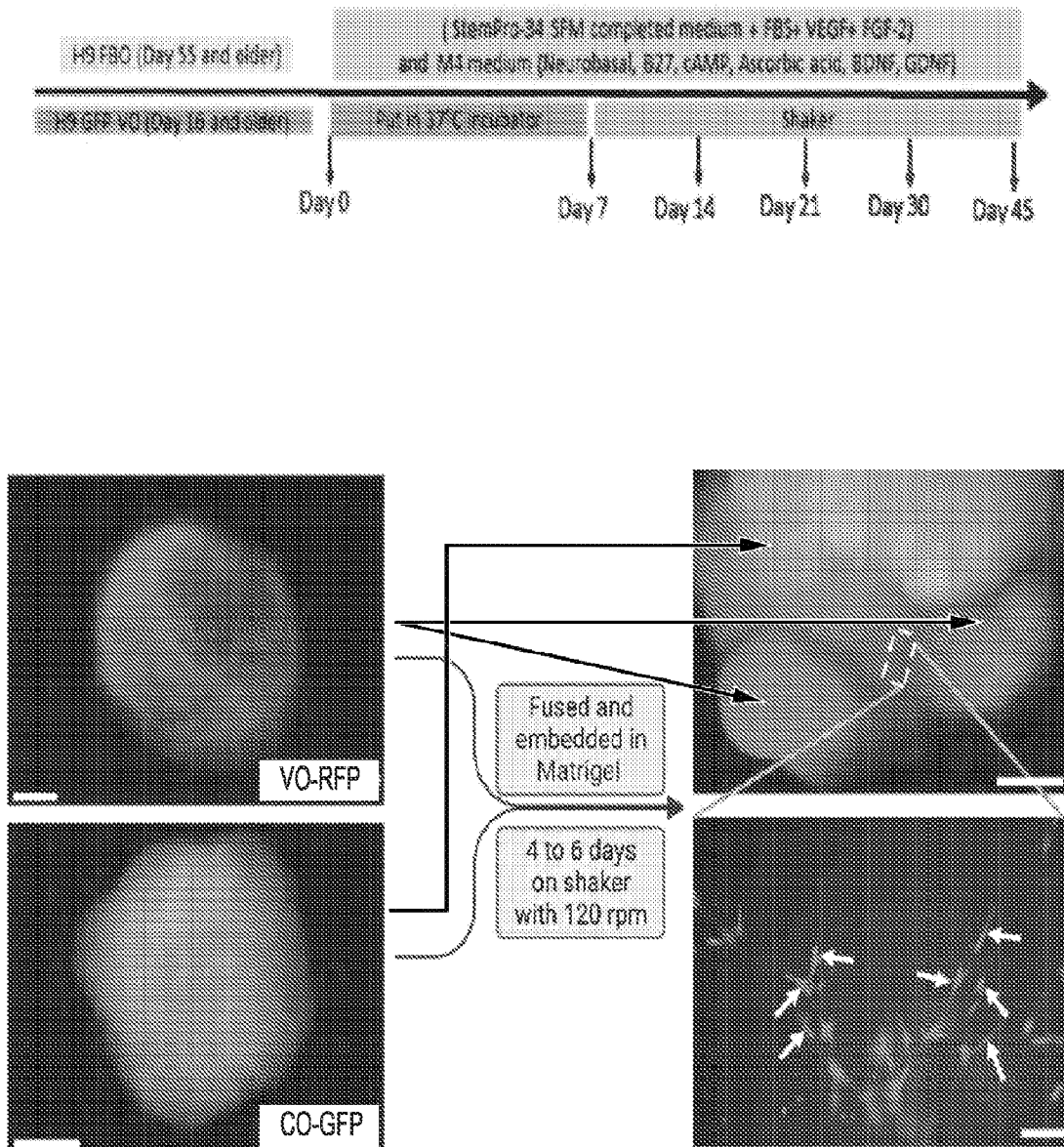
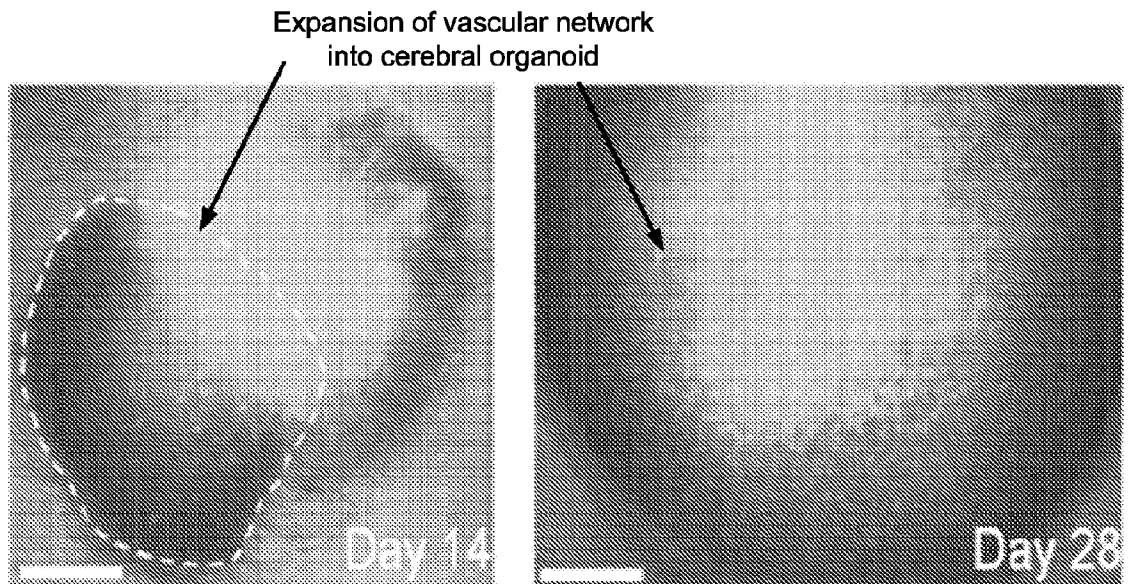
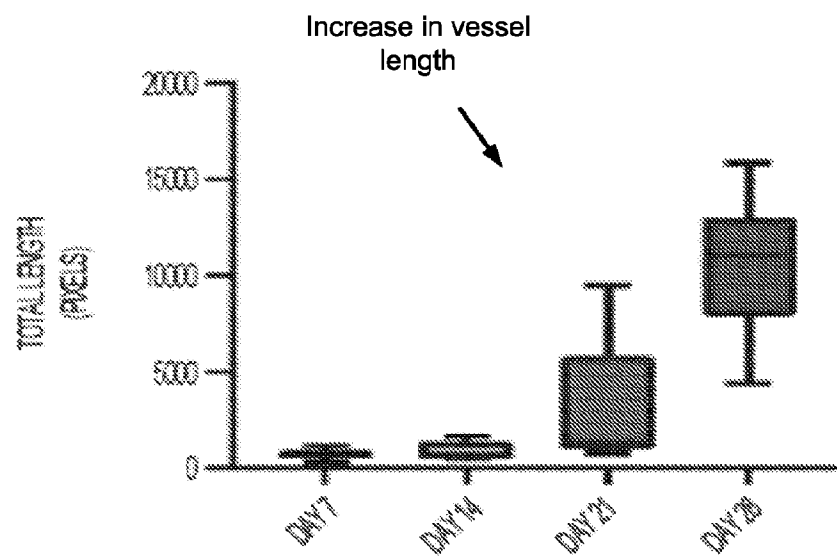


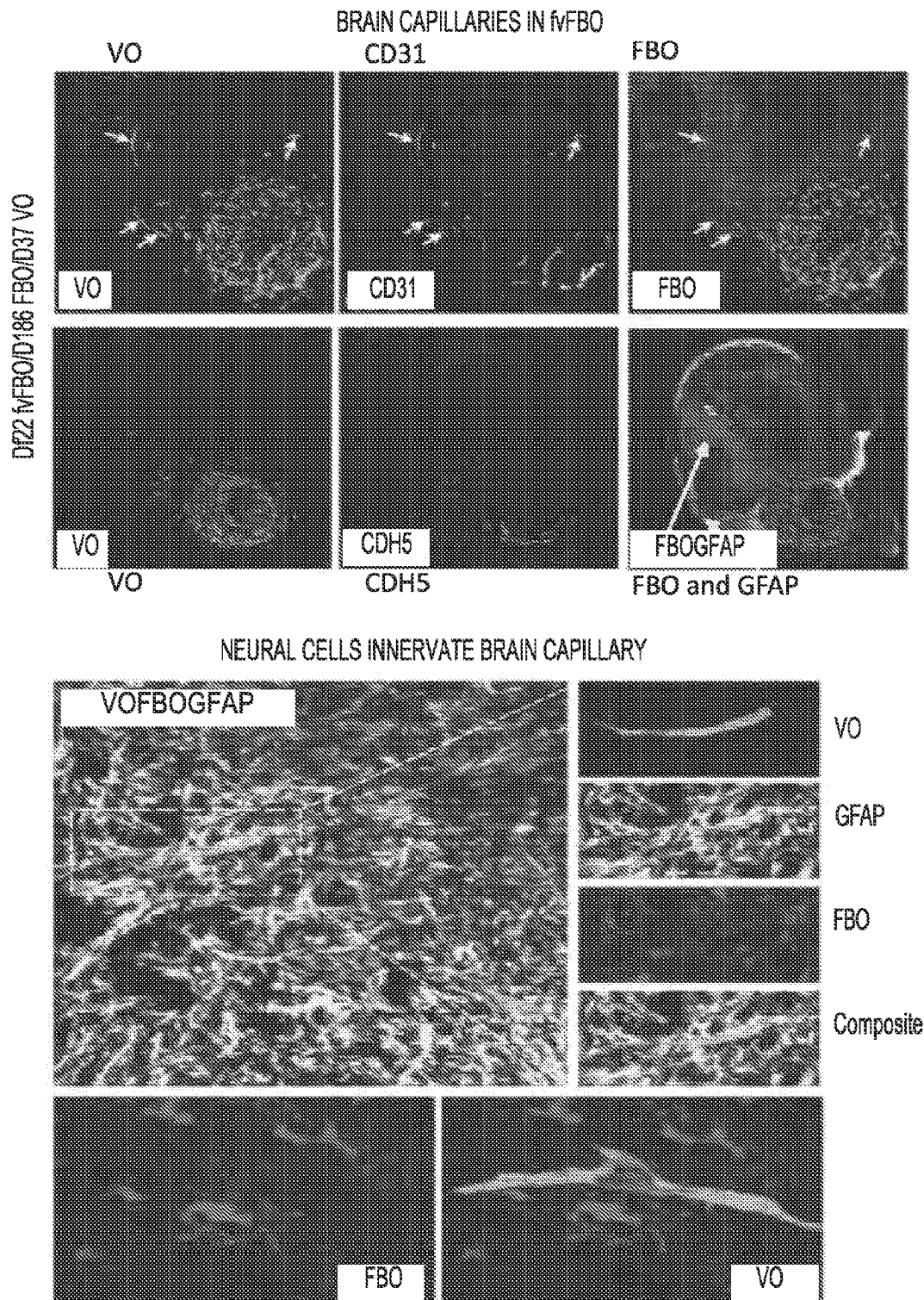
FIG. 1B

**FIG. 1C****FIG. 1D****FIG. 1E**

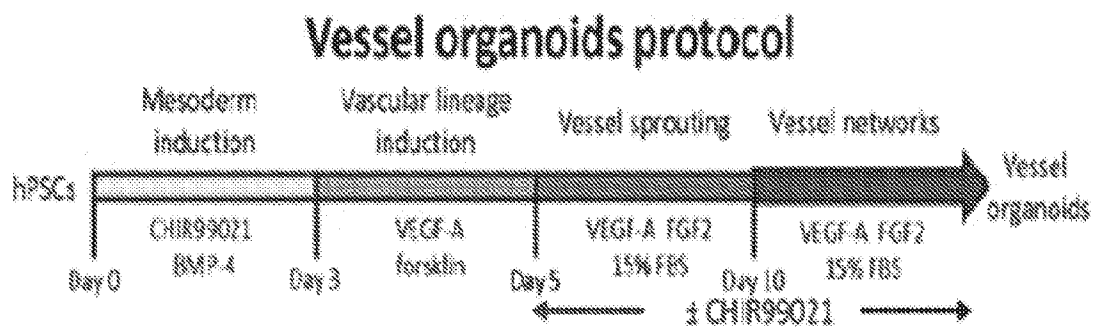
**FIG. 1F**

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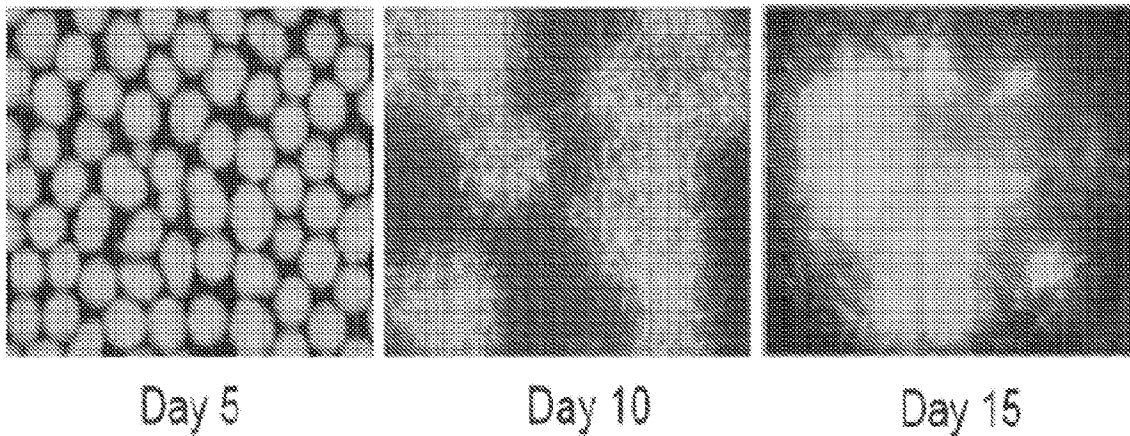
**FIG. 1G****FIG. 1H**

**FIG. 11**

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**FIG. 1J**

Blood vessel organoids differentiating from hPSCs, and expressing GFP

**FIG. 1K**

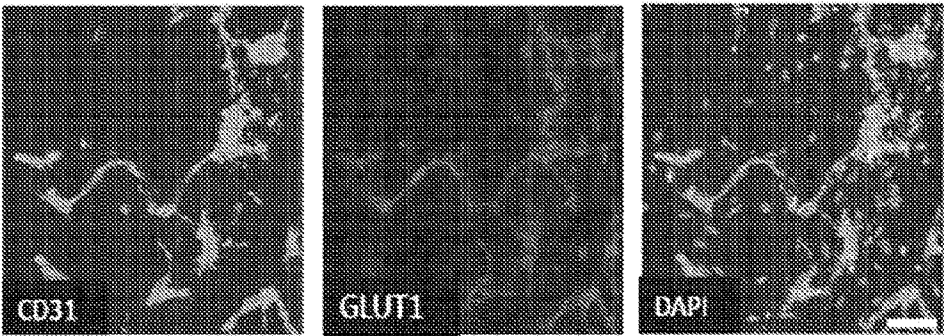


FIG. 2A

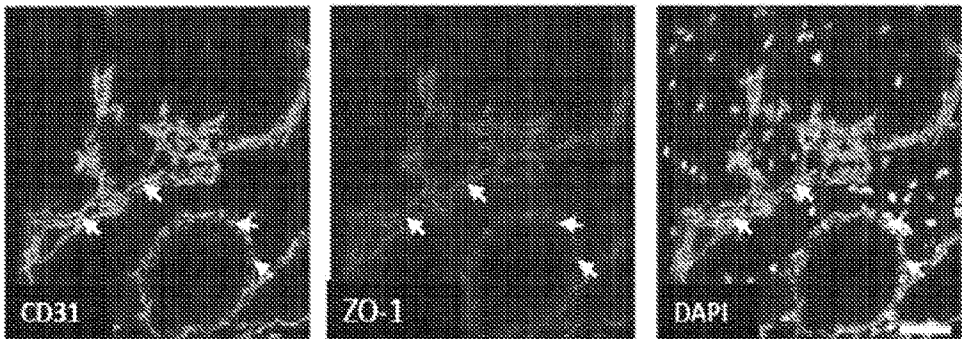


FIG. 2B



FIG. 2C

BBB
Assembloids
were found
to express
GLUT1, ZO-
1, and
Claudin-5

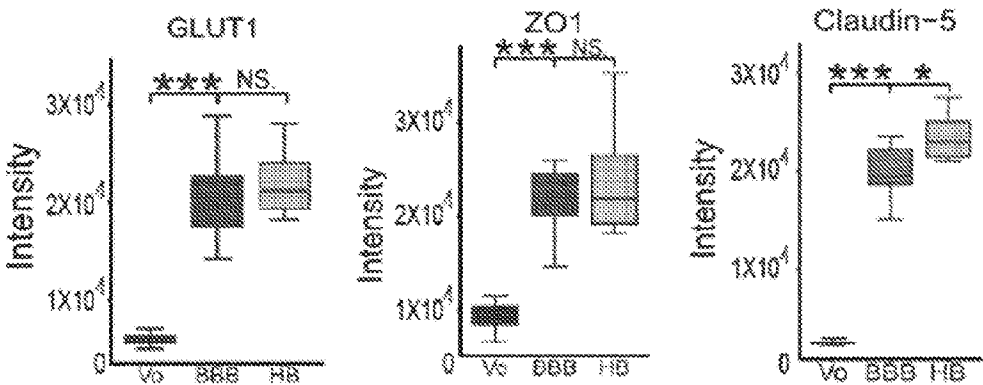
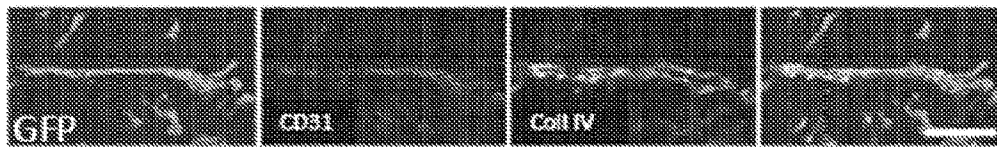


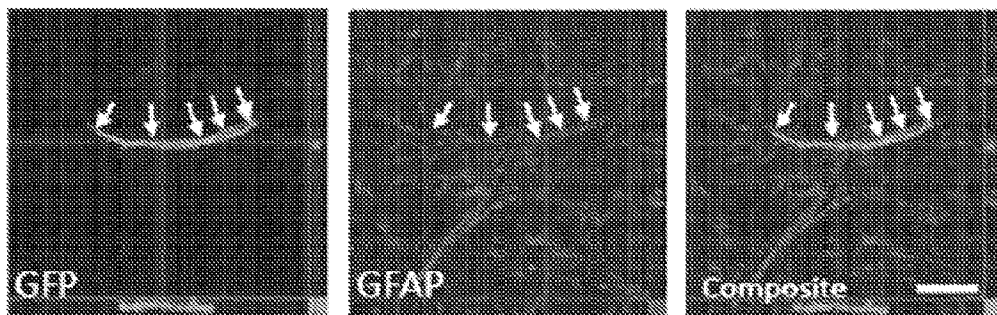
FIG. 2D

BBB
Assembloids
were found
to express
these
markers at
similar levels
to In Vivo
Human BBB

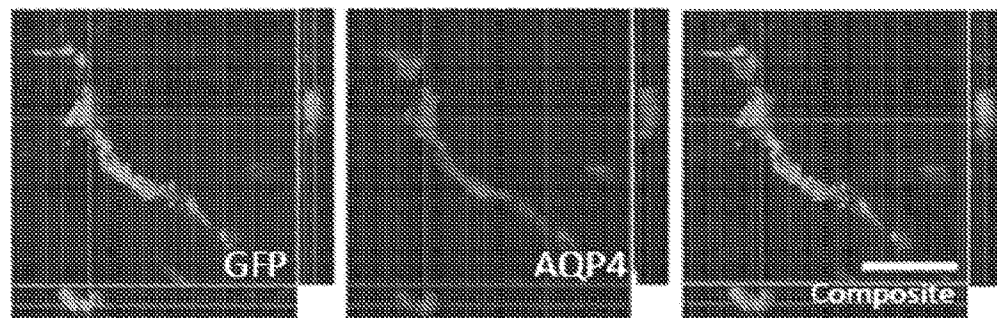
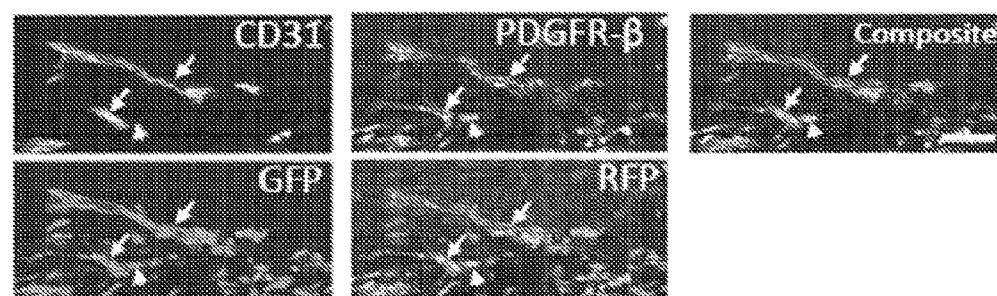
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**FIG. 2E**

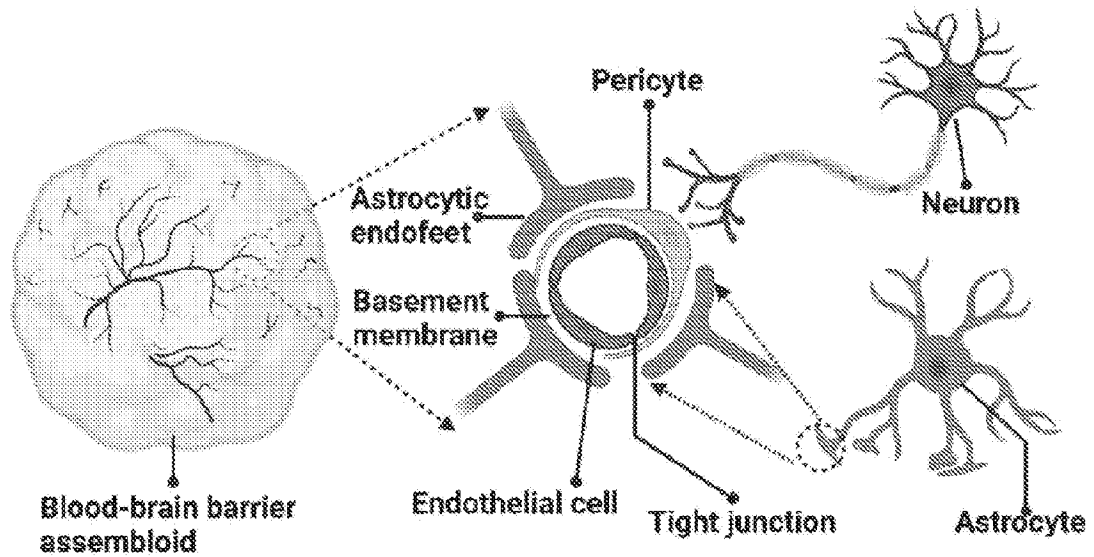
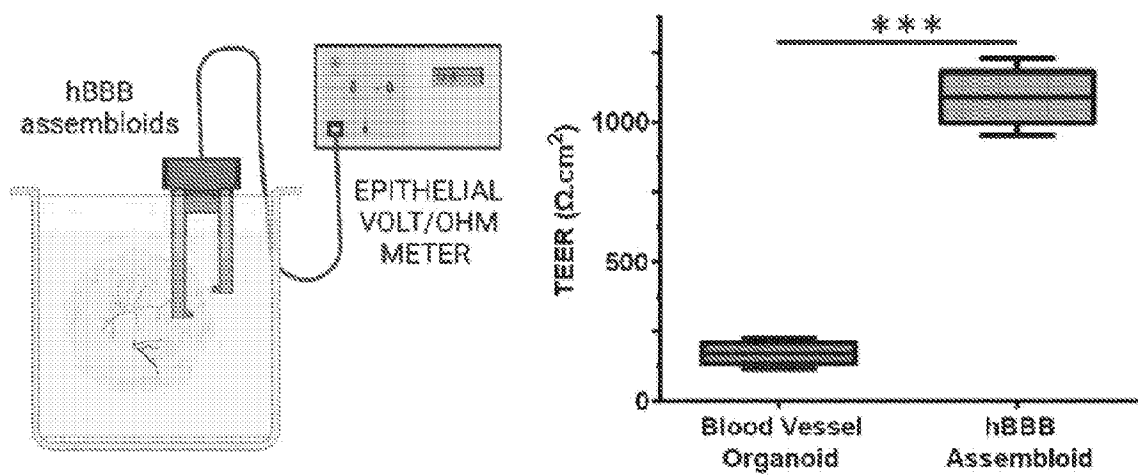
BBB
assembloid
endothelium
covered by
collagen IV
basement
membrane

**FIG. 2F**

BBB
assembloids
exhibit
astrocytic
processes
and
astrocytic
end-feet
ensheathing
capillary
walls

**FIG. 2G****FIG. 2H**

BBB
assembloids
show
innervation
between
capillaries
and pericyte
processes

**FIG. 2I****FIG. 2J**

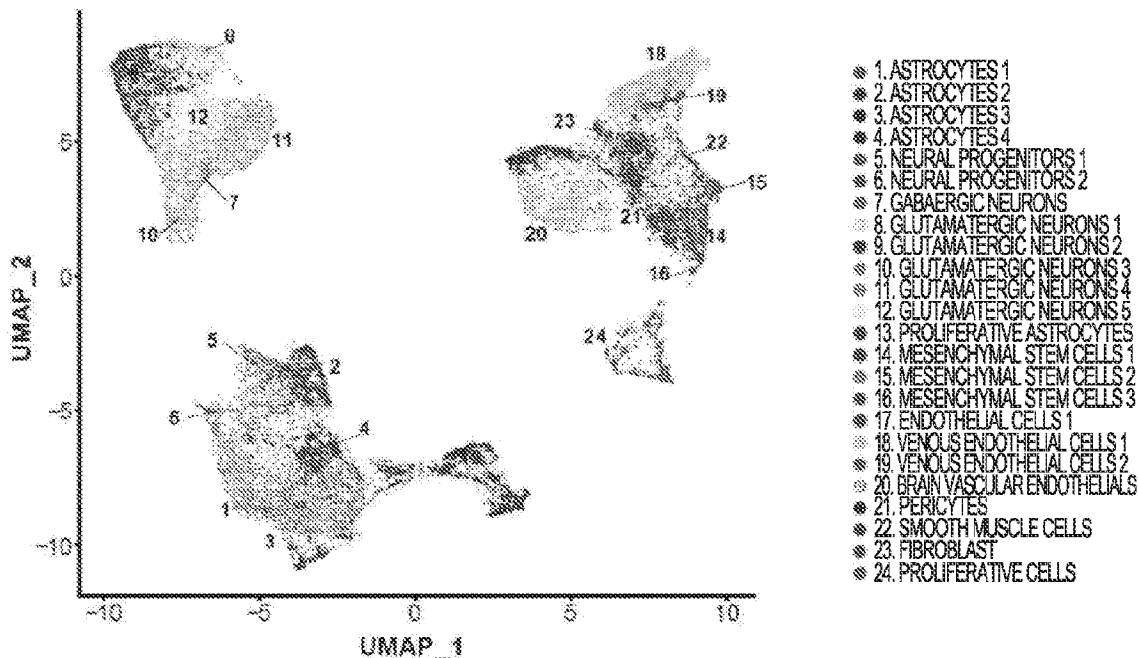


FIG. 2K

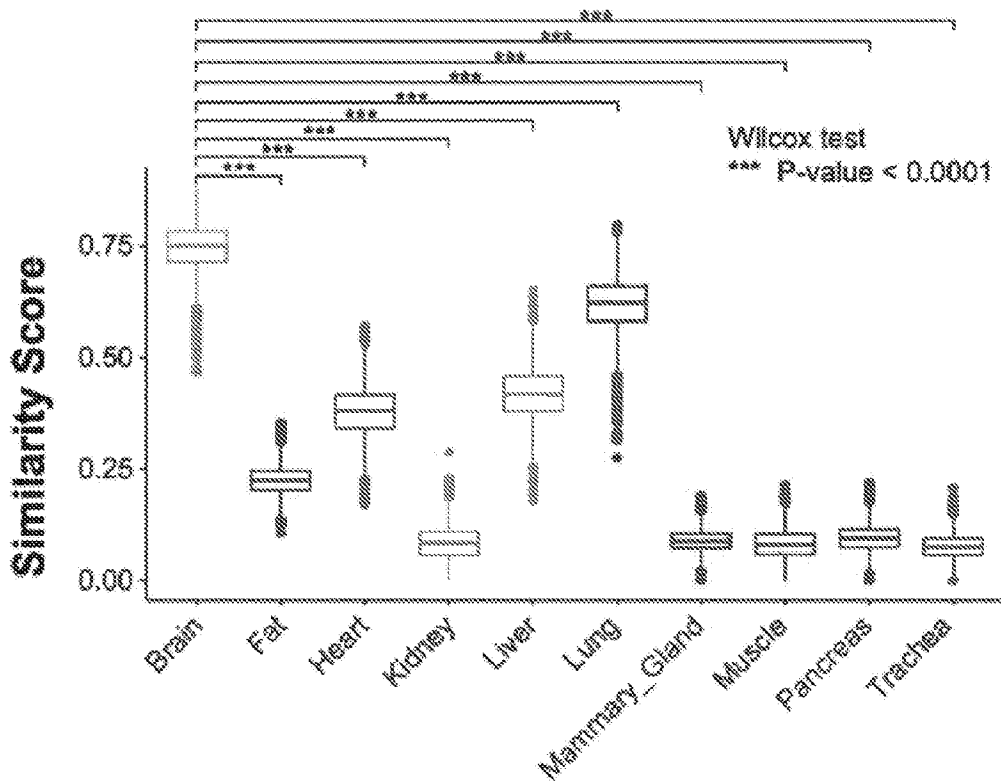
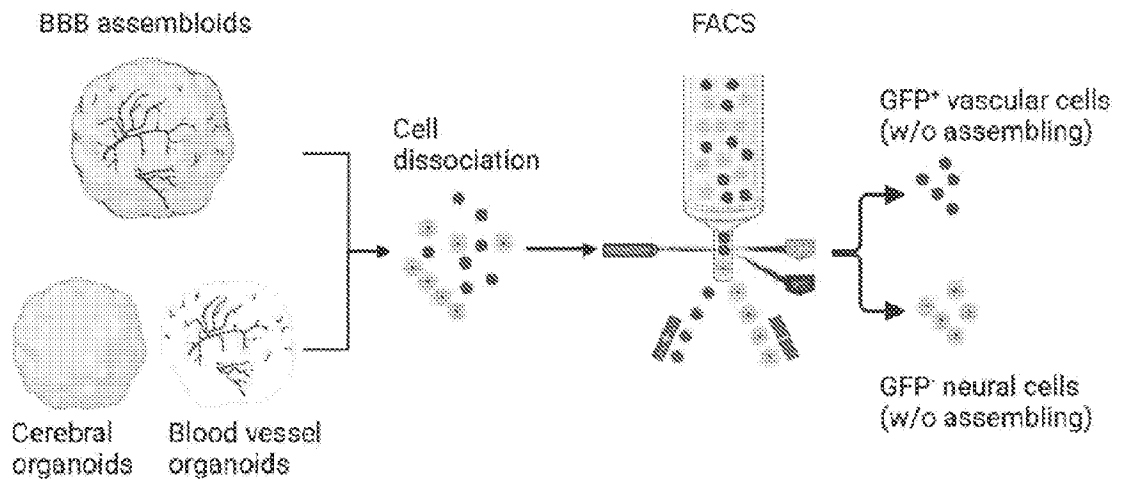
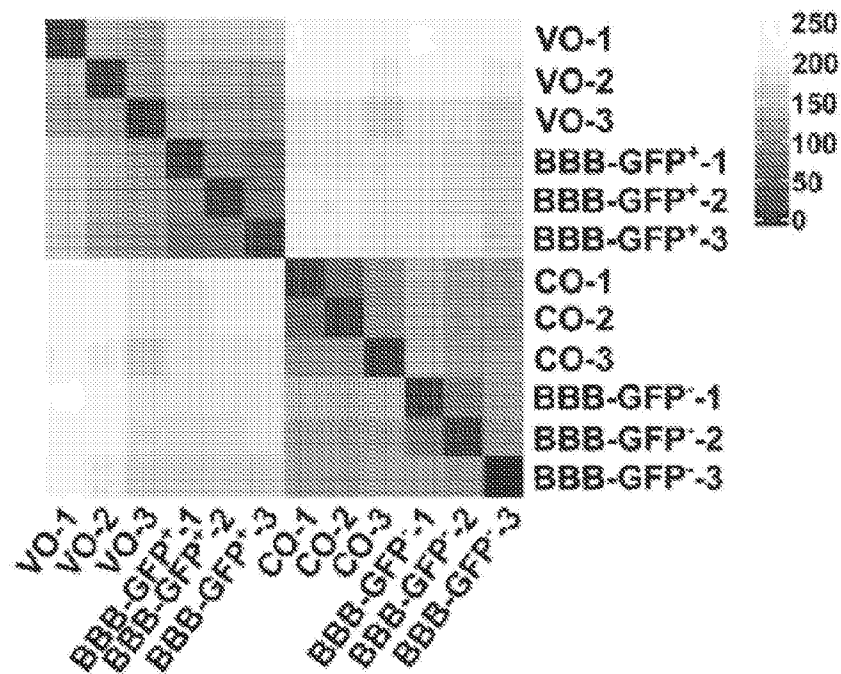


FIG. 2L

**FIG. 3A****FIG. 3B**

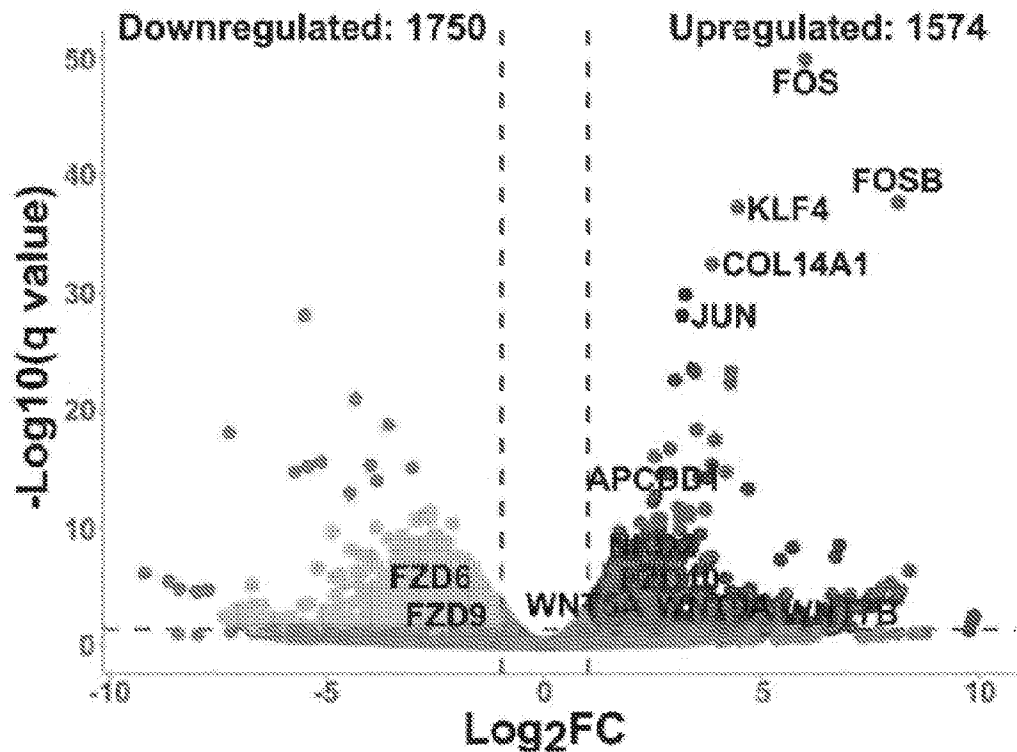


FIG. 3C

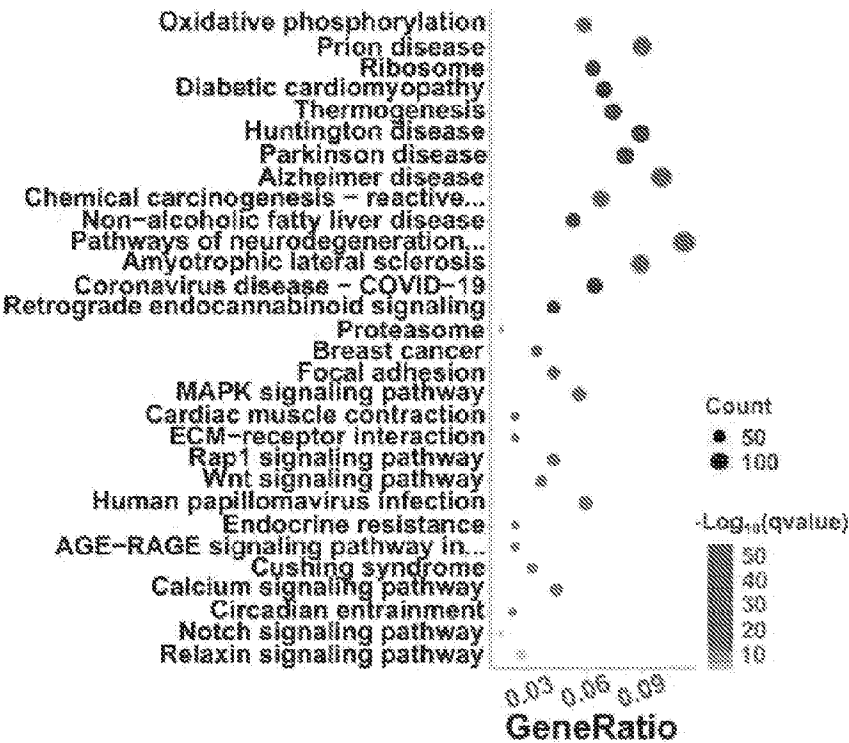


FIG. 3D

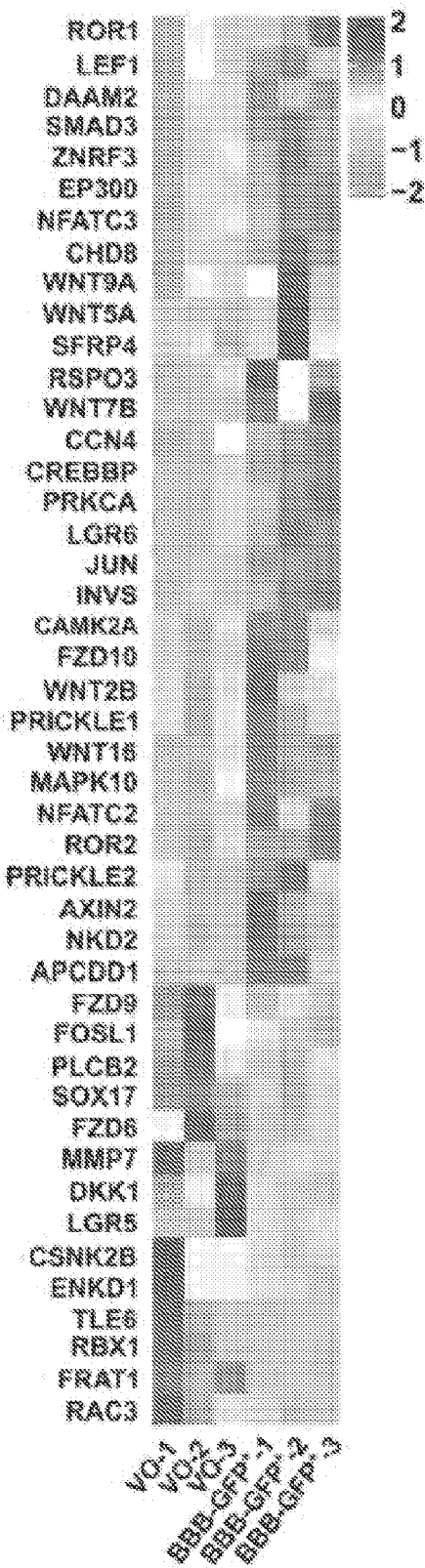


FIG. 3E

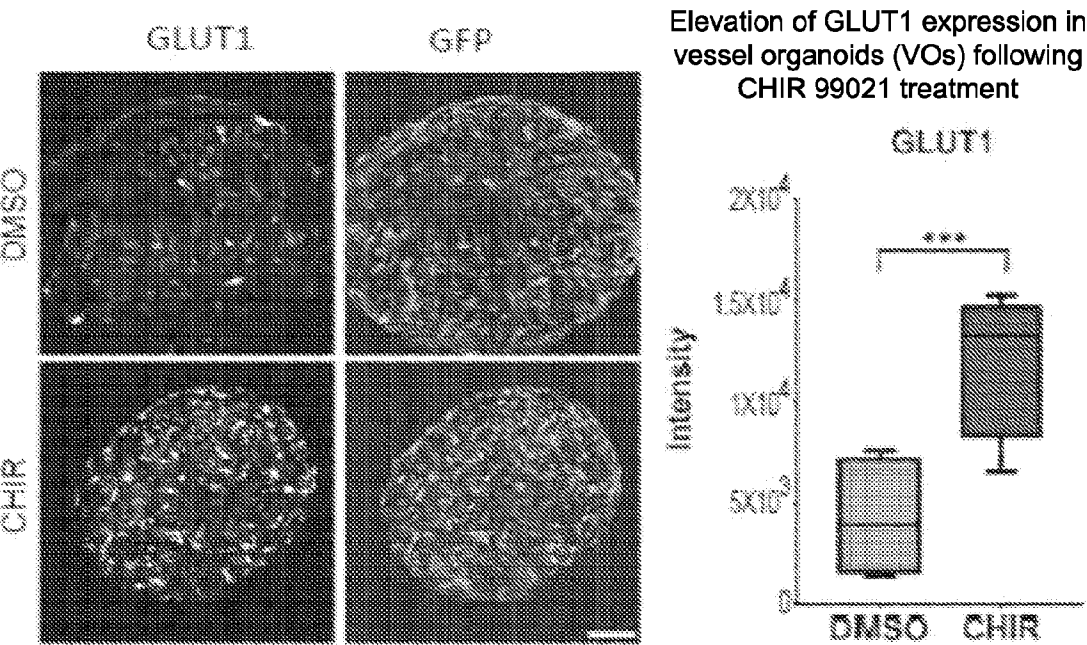


FIG. 3F

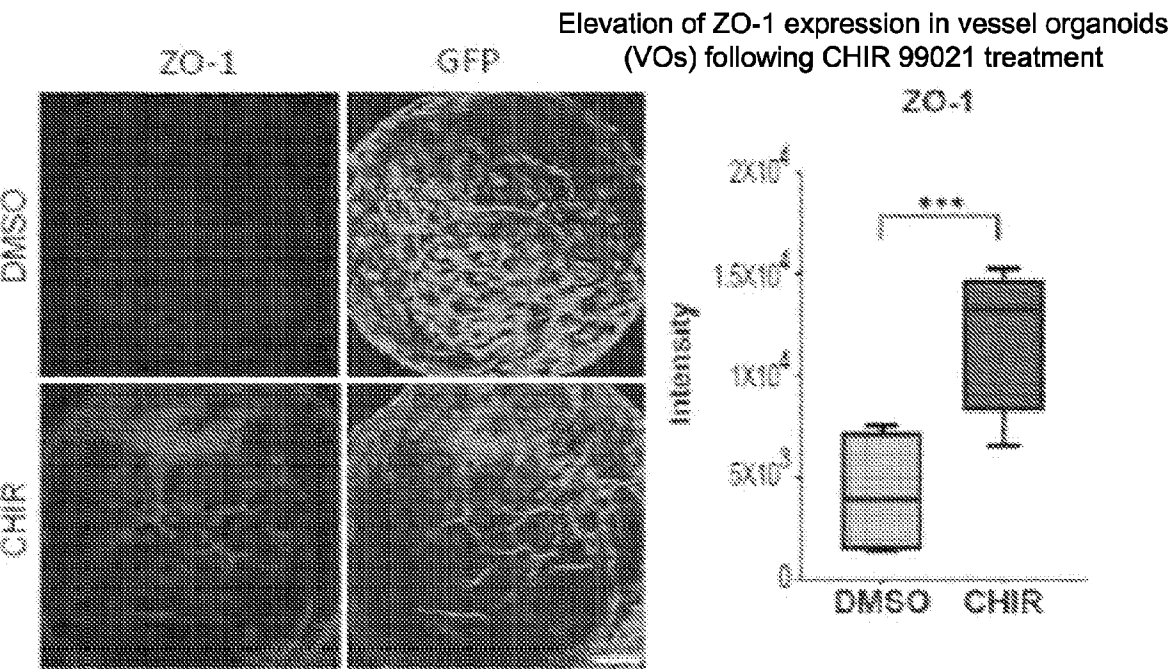
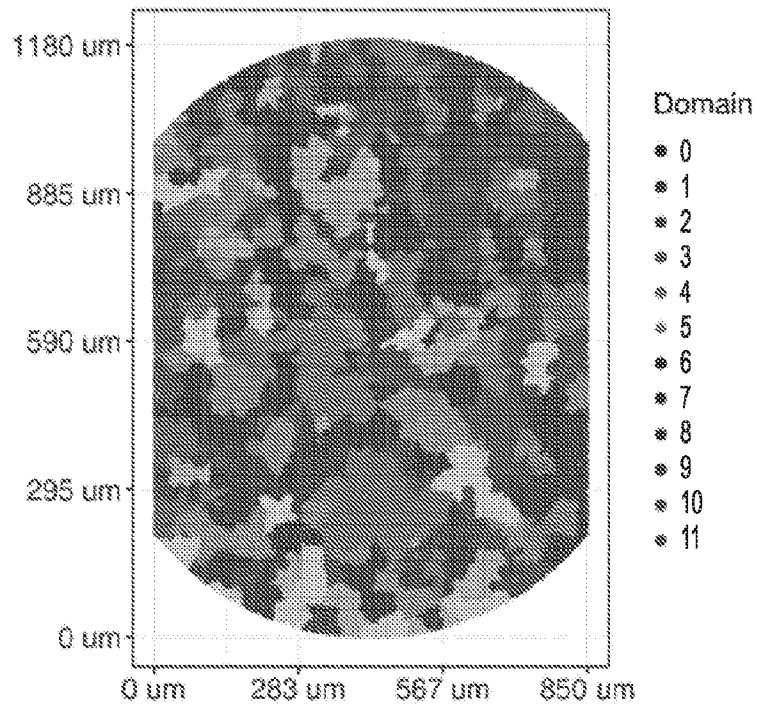
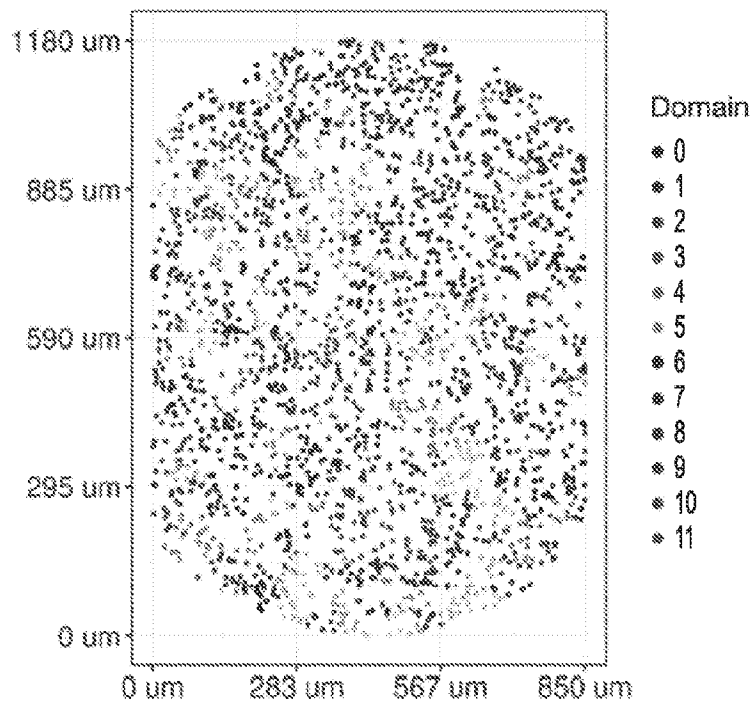


FIG. 3G

**FIG. 4A****FIG. 4B**

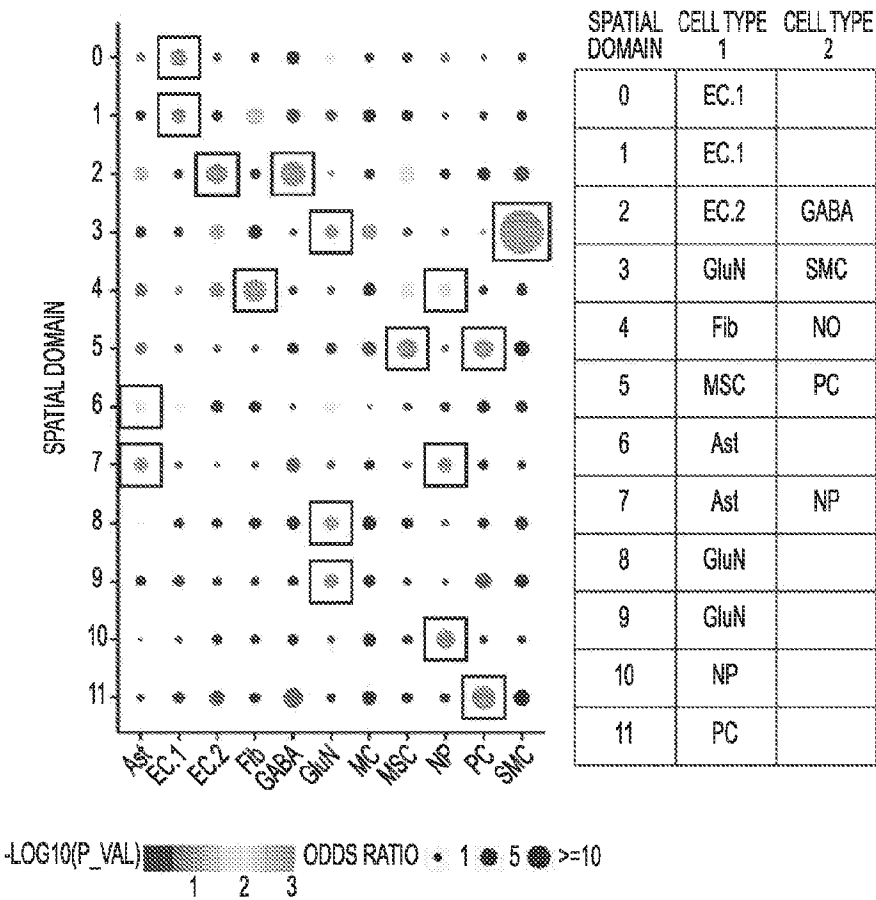


FIG. 4C

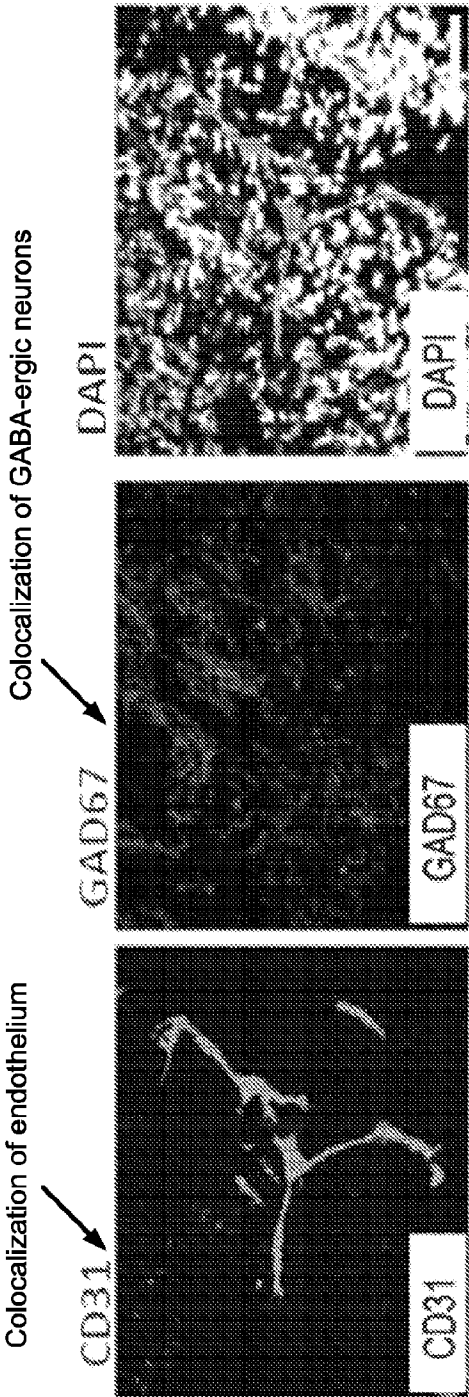


FIG. 4D

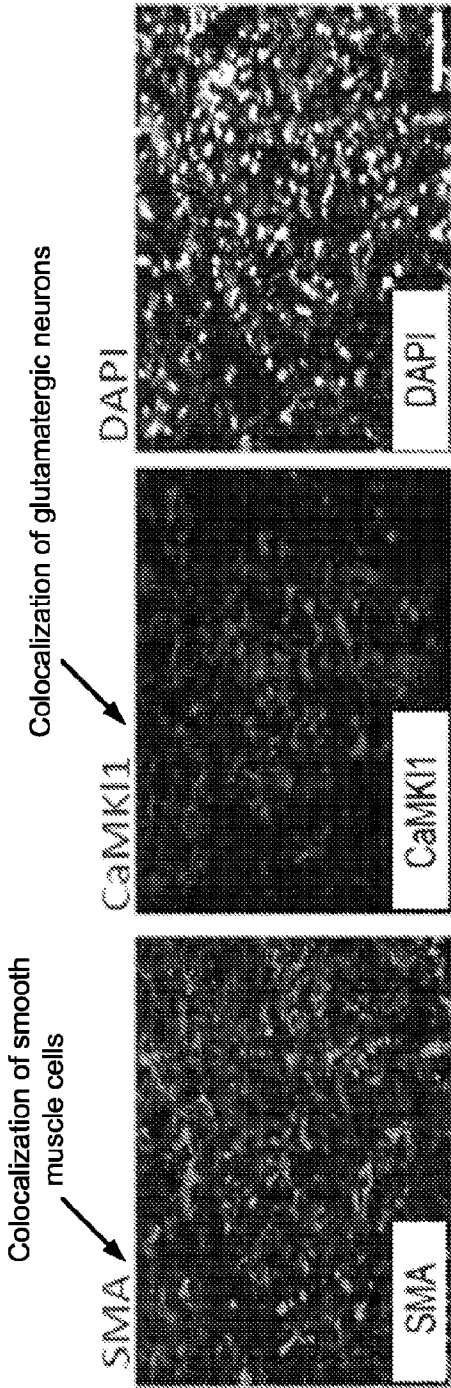
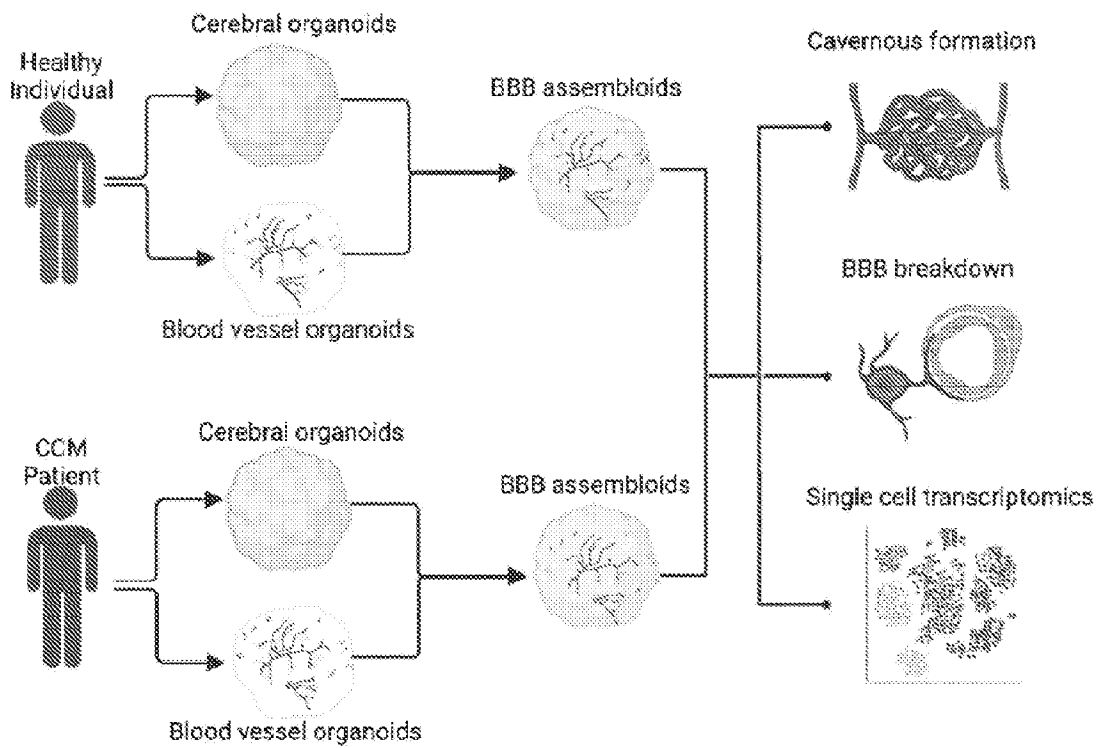
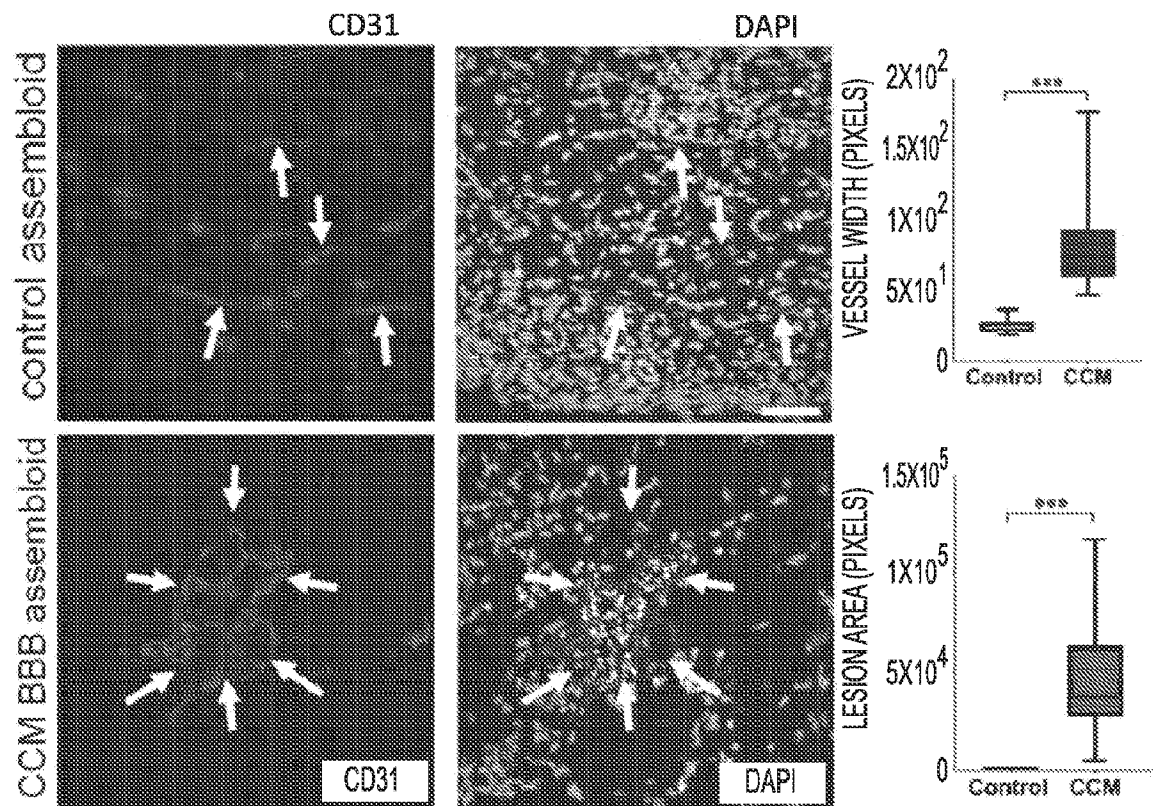


FIG. 4E

**FIG. 5A****FIG. 5B**

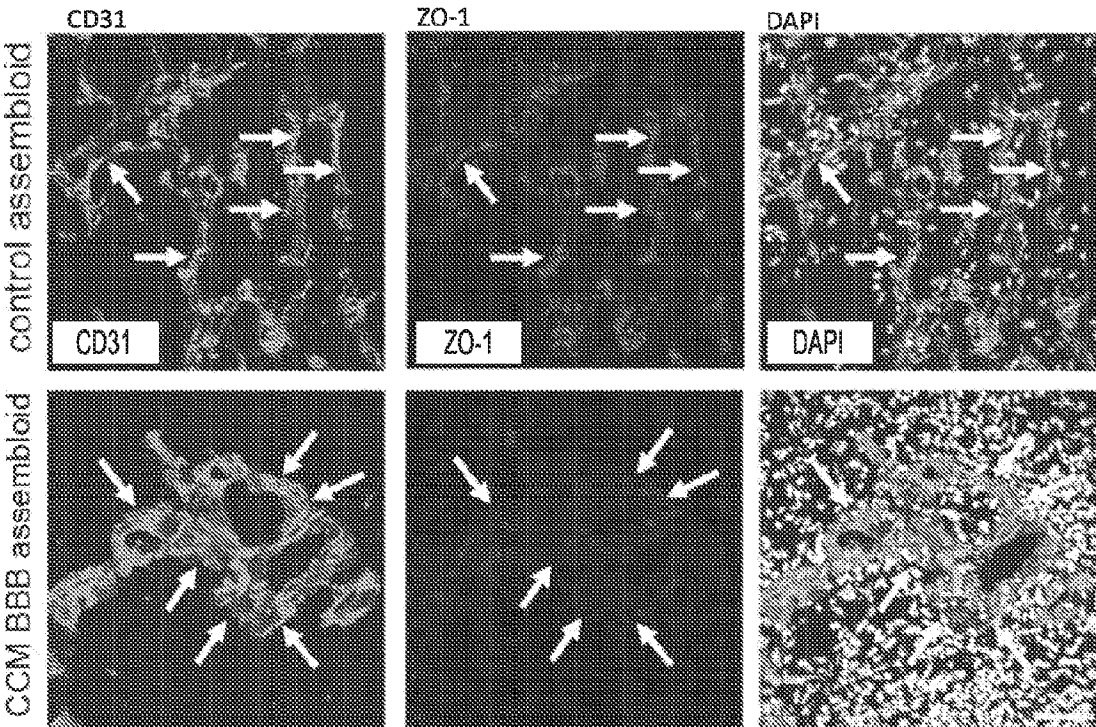


FIG. 5C

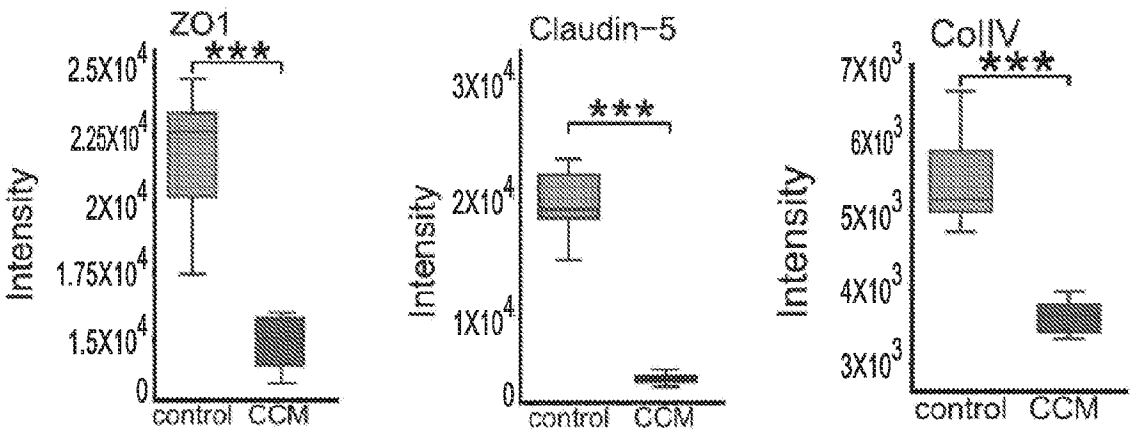
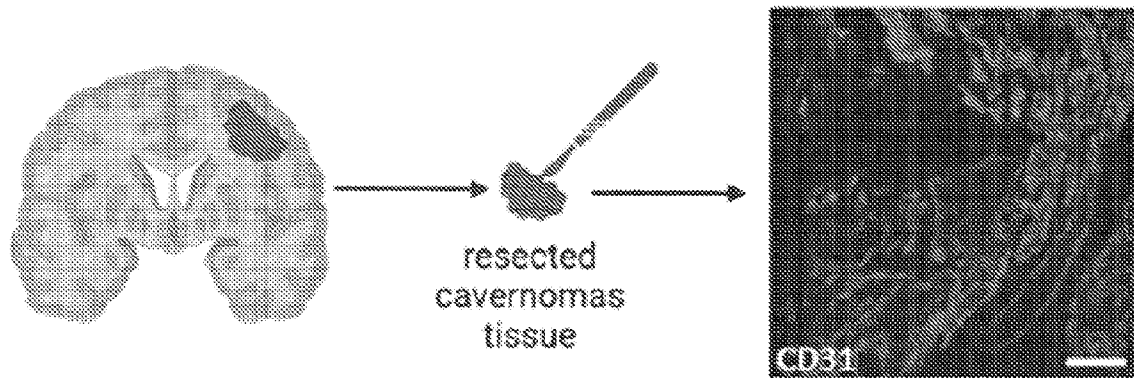
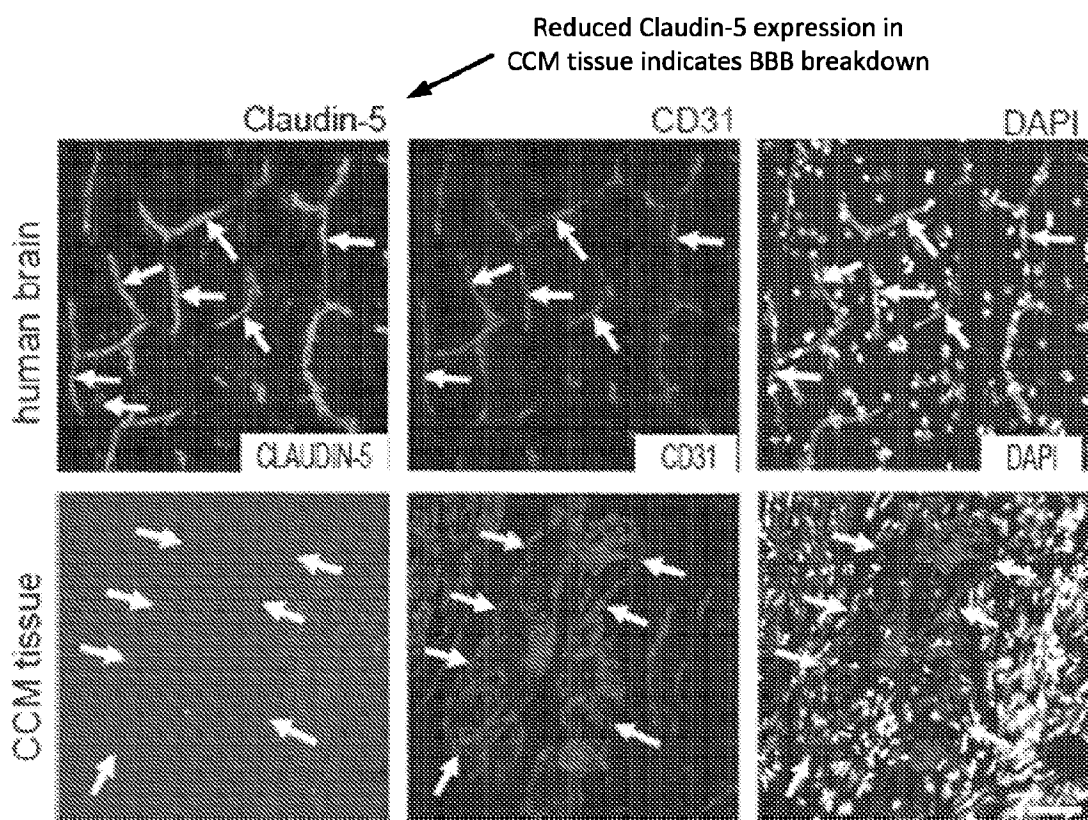


FIG. 5D

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**FIG. 5E****FIG. 5F**

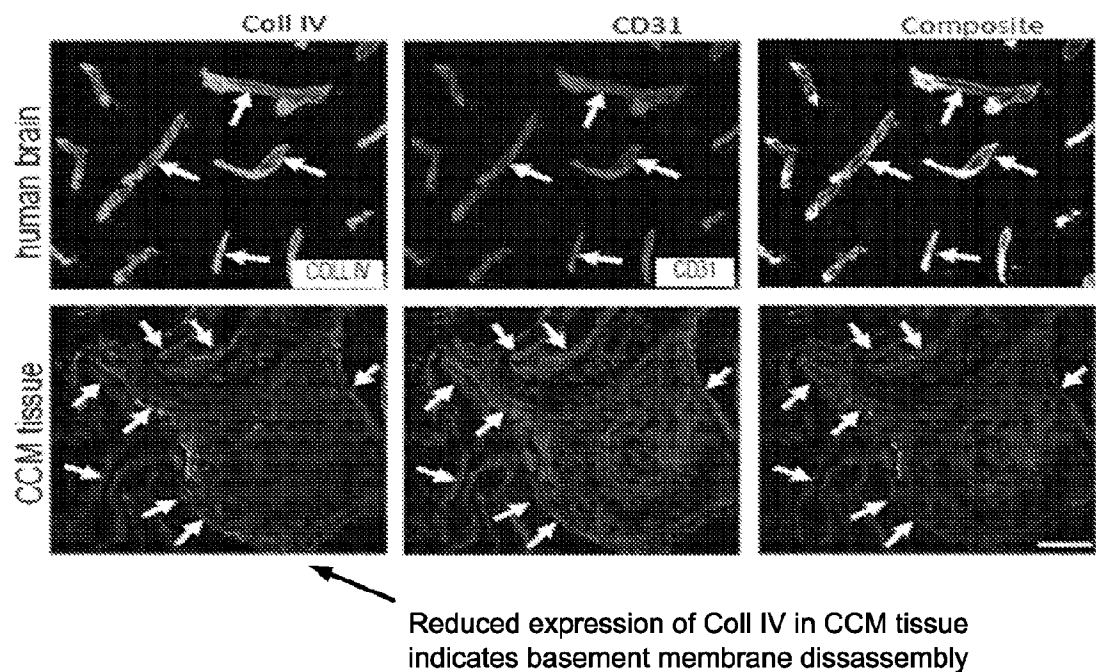


FIG. 5G

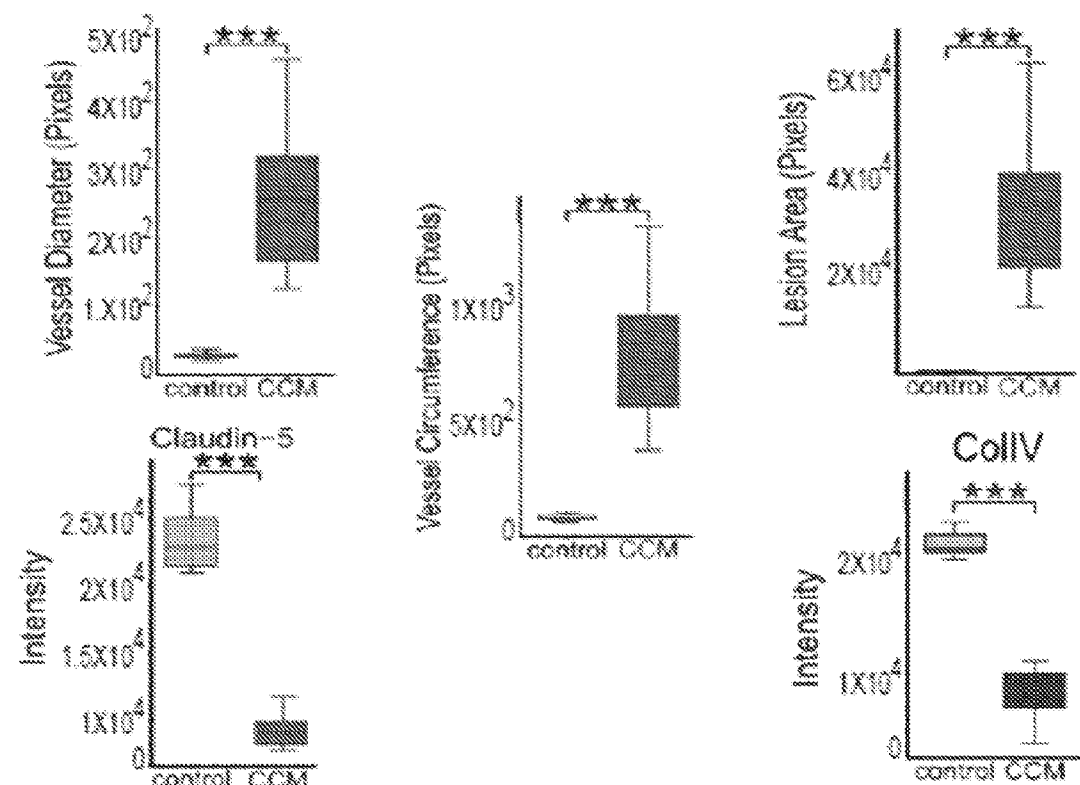


FIG. 5H

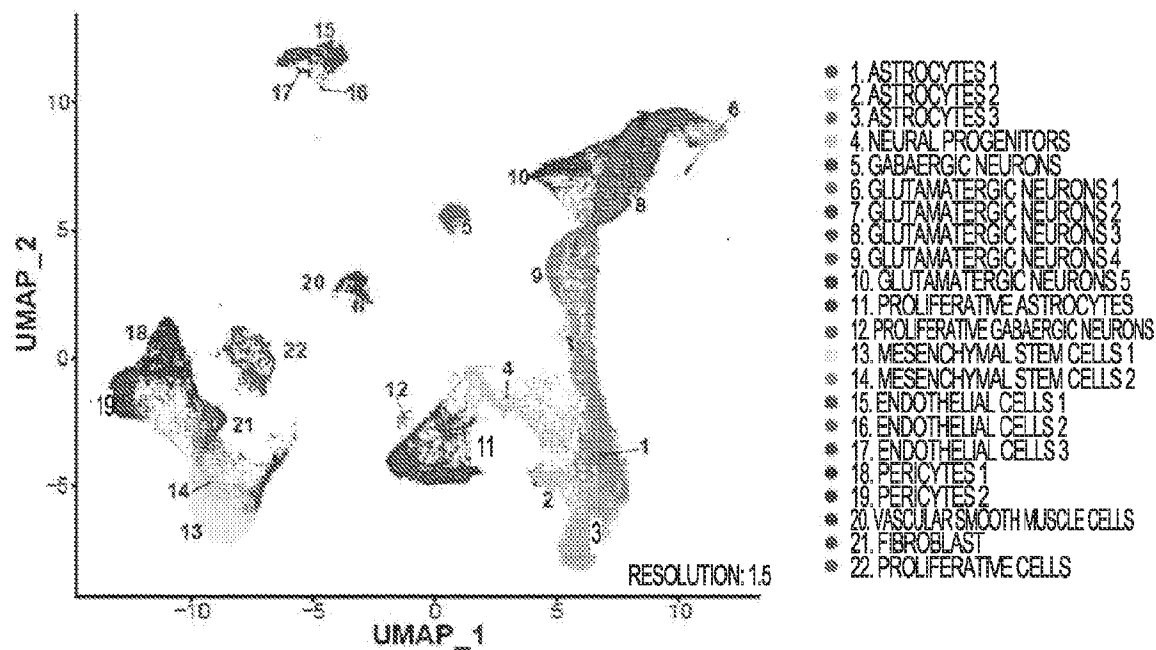


FIG. 6A

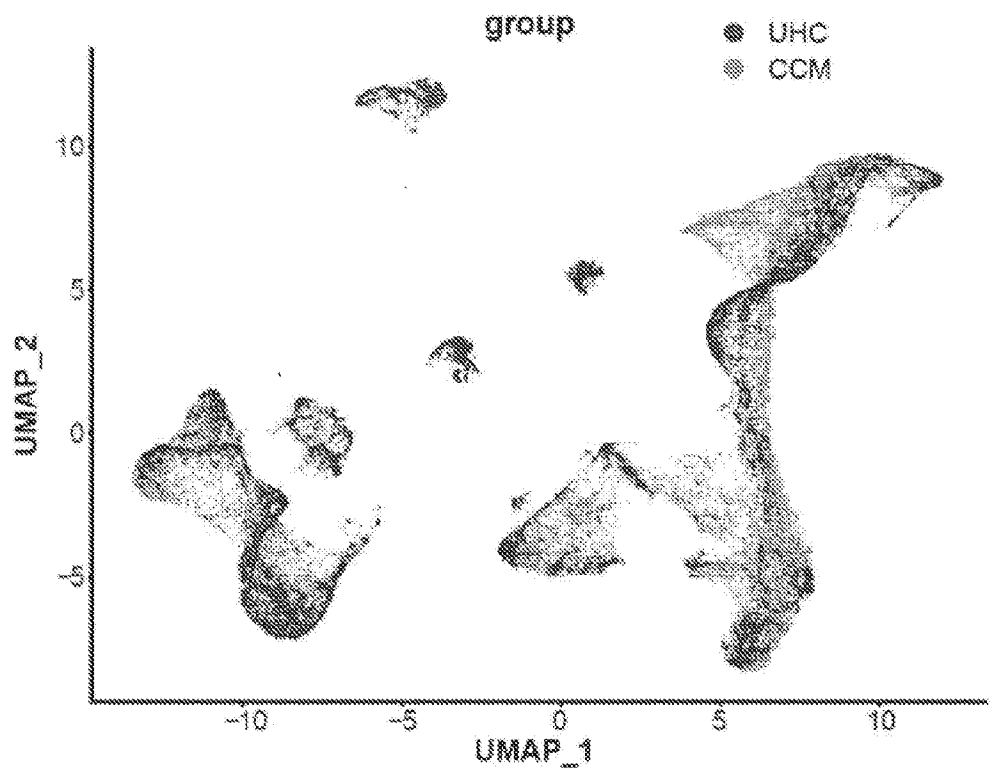


FIG. 6B

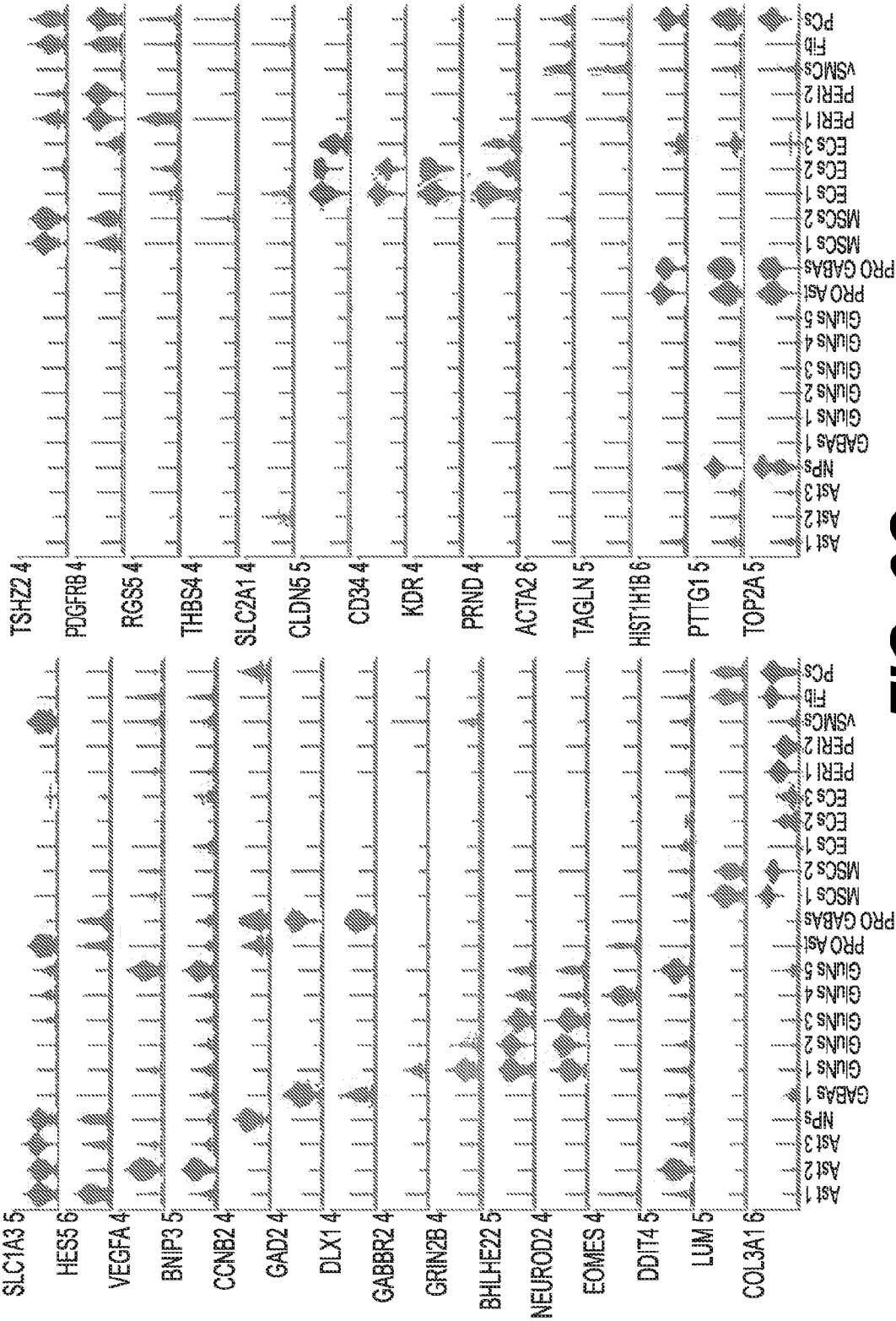


FIG. 6C

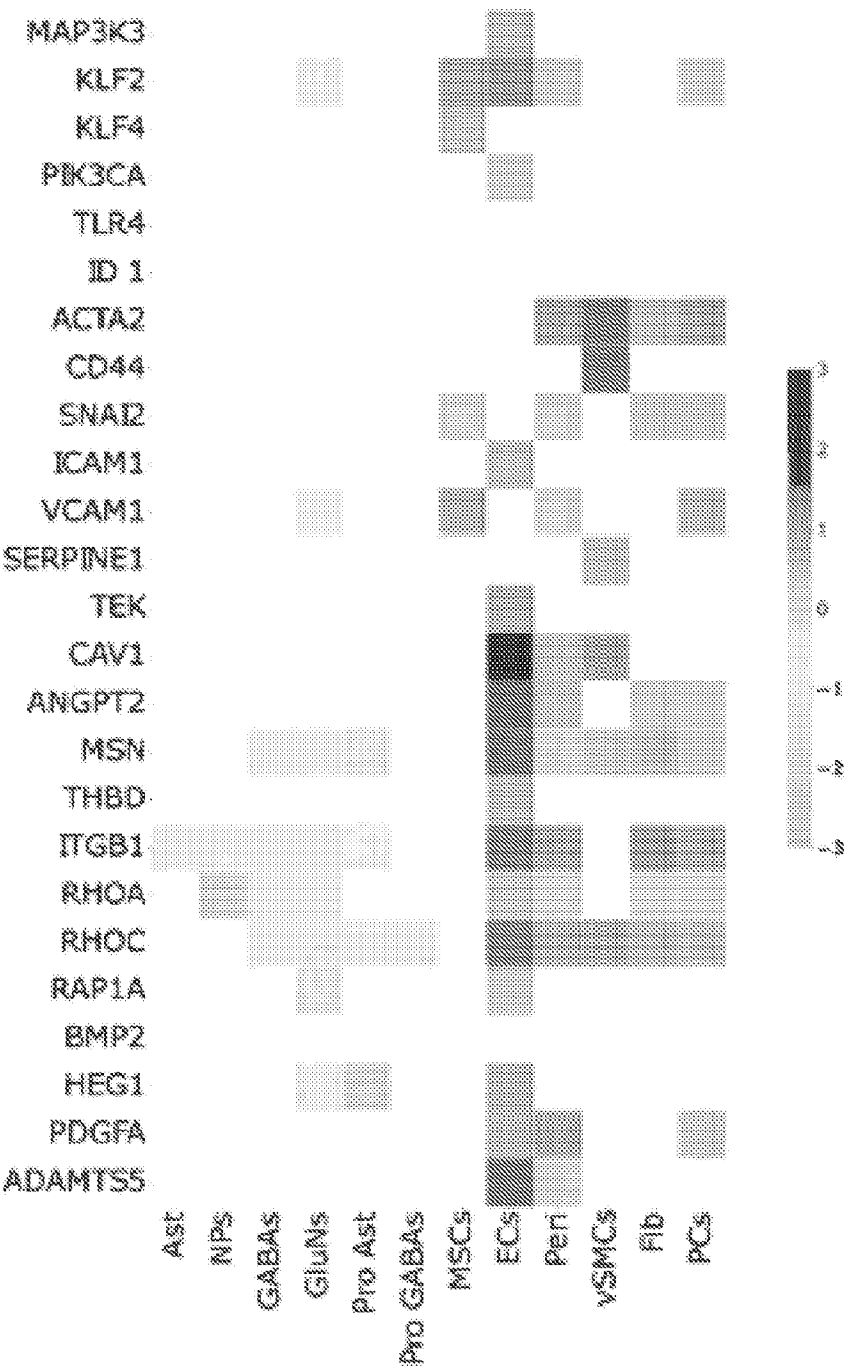


FIG. 6D

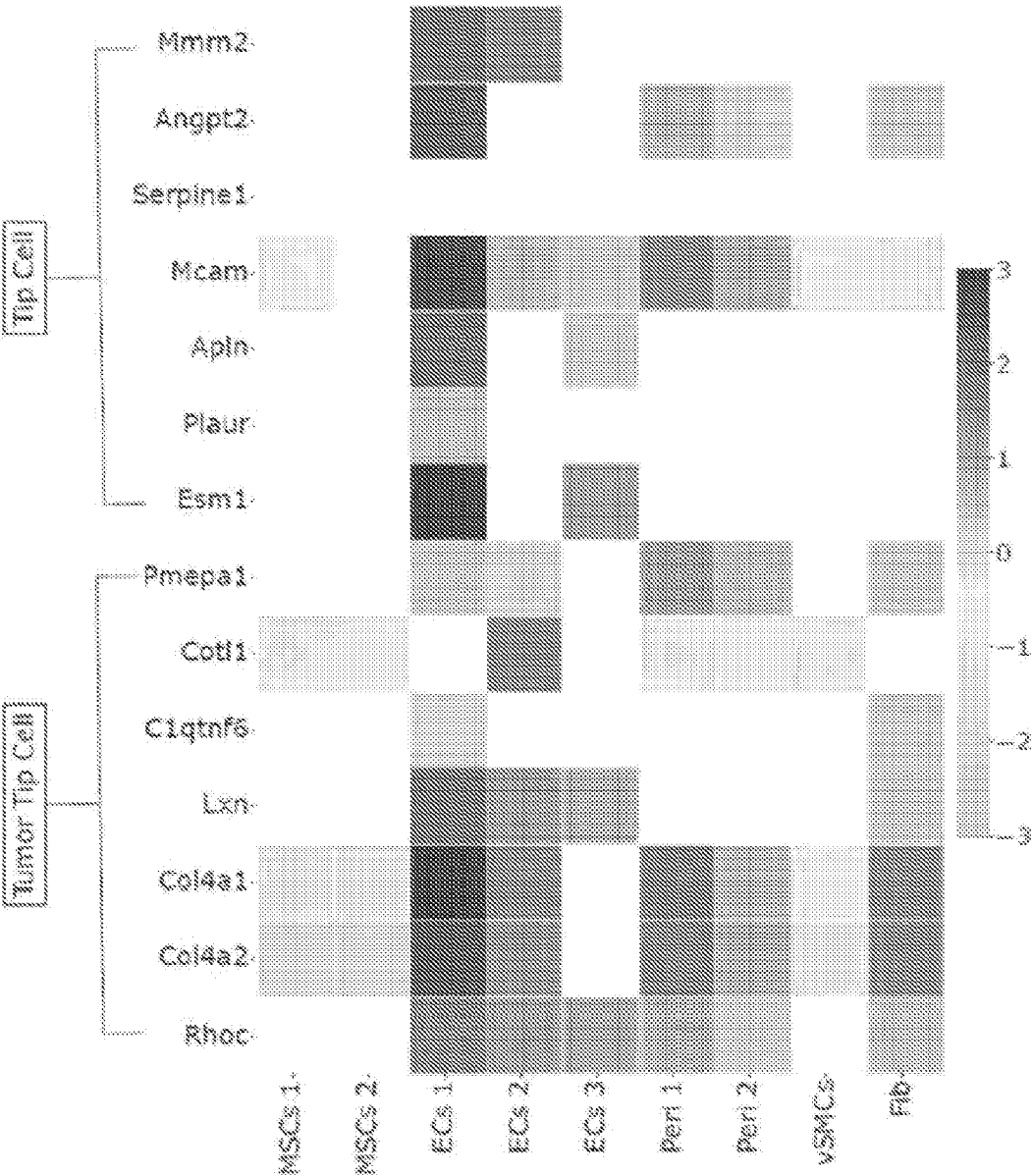
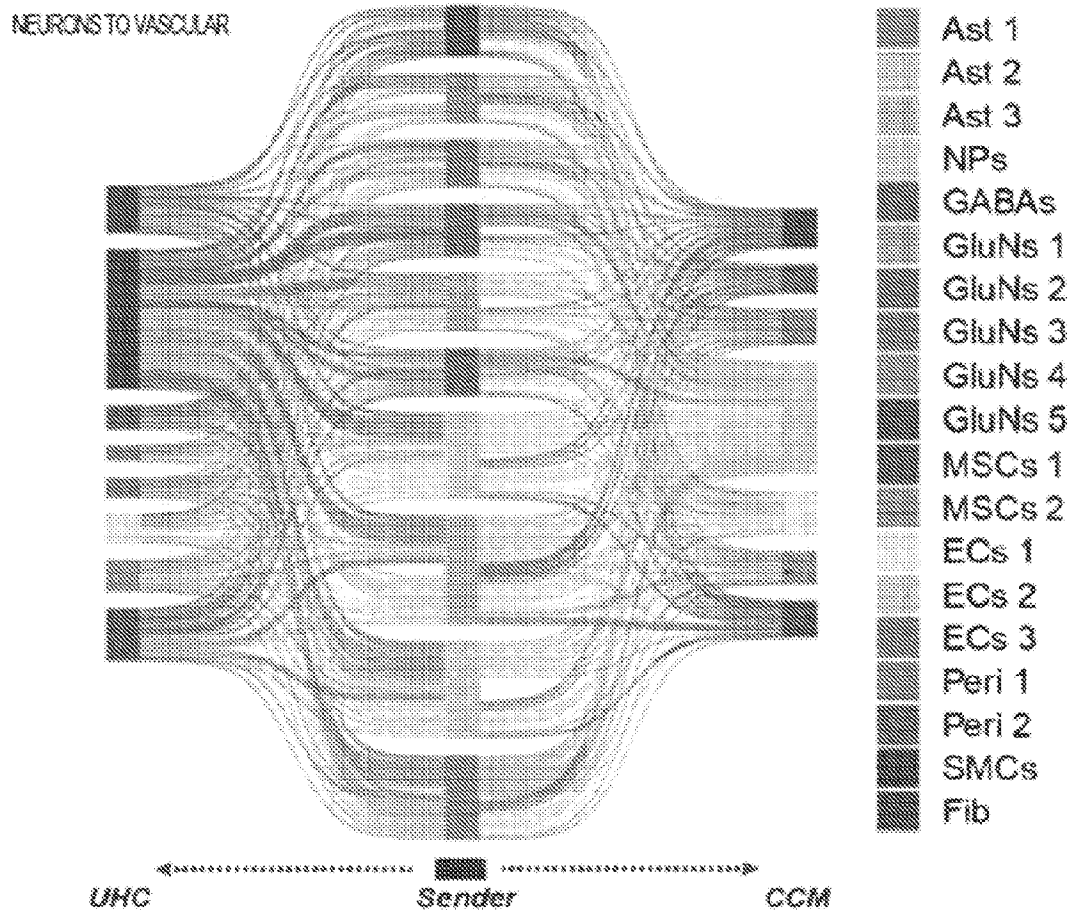


FIG. 6E

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Note the significant shift of ligand-receptor expression and top responsive vascular cells in CCM BBB assembloids compared with controls (UHC)

**FIG. 6F**

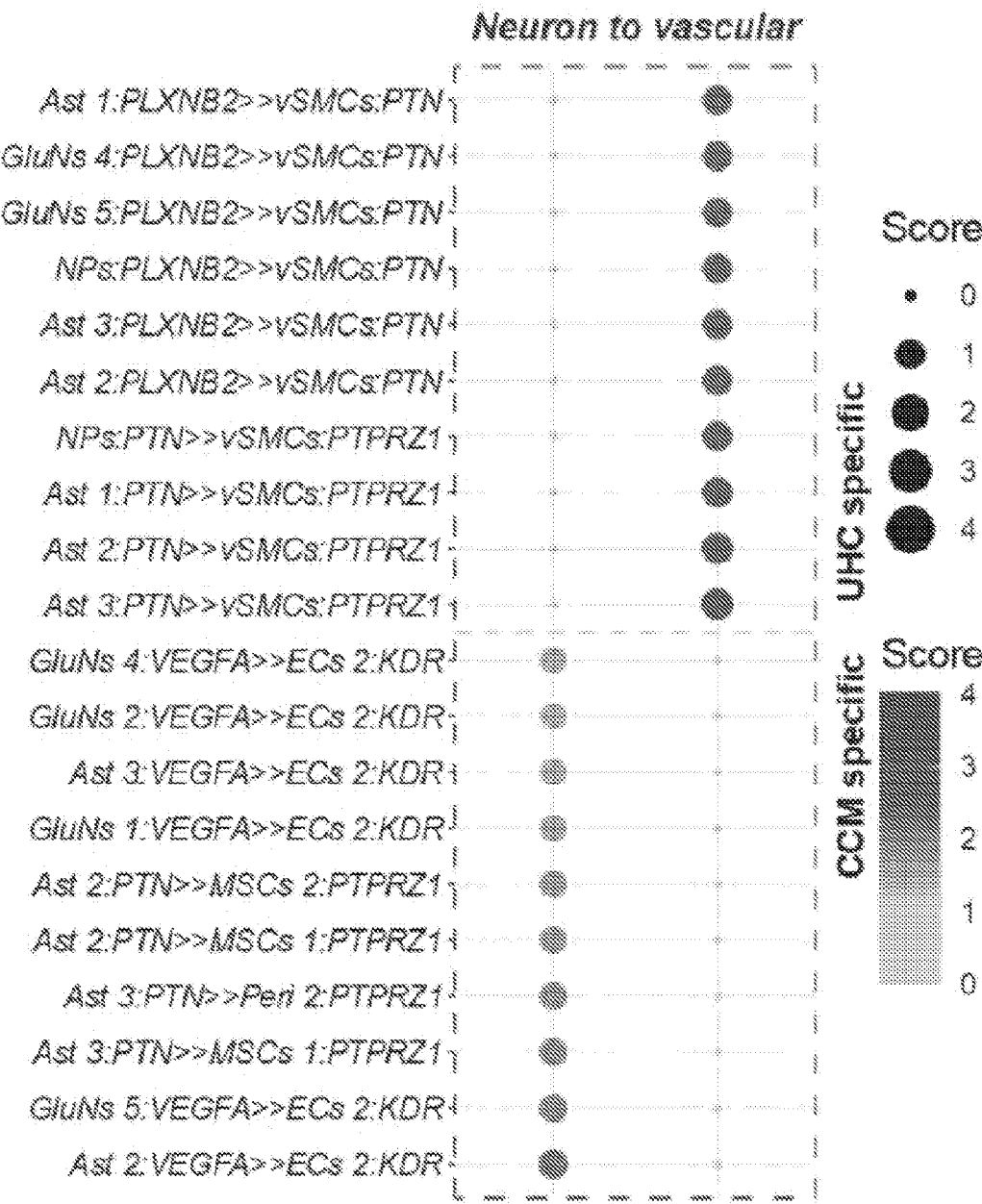


FIG. 6G



FIG. 7A

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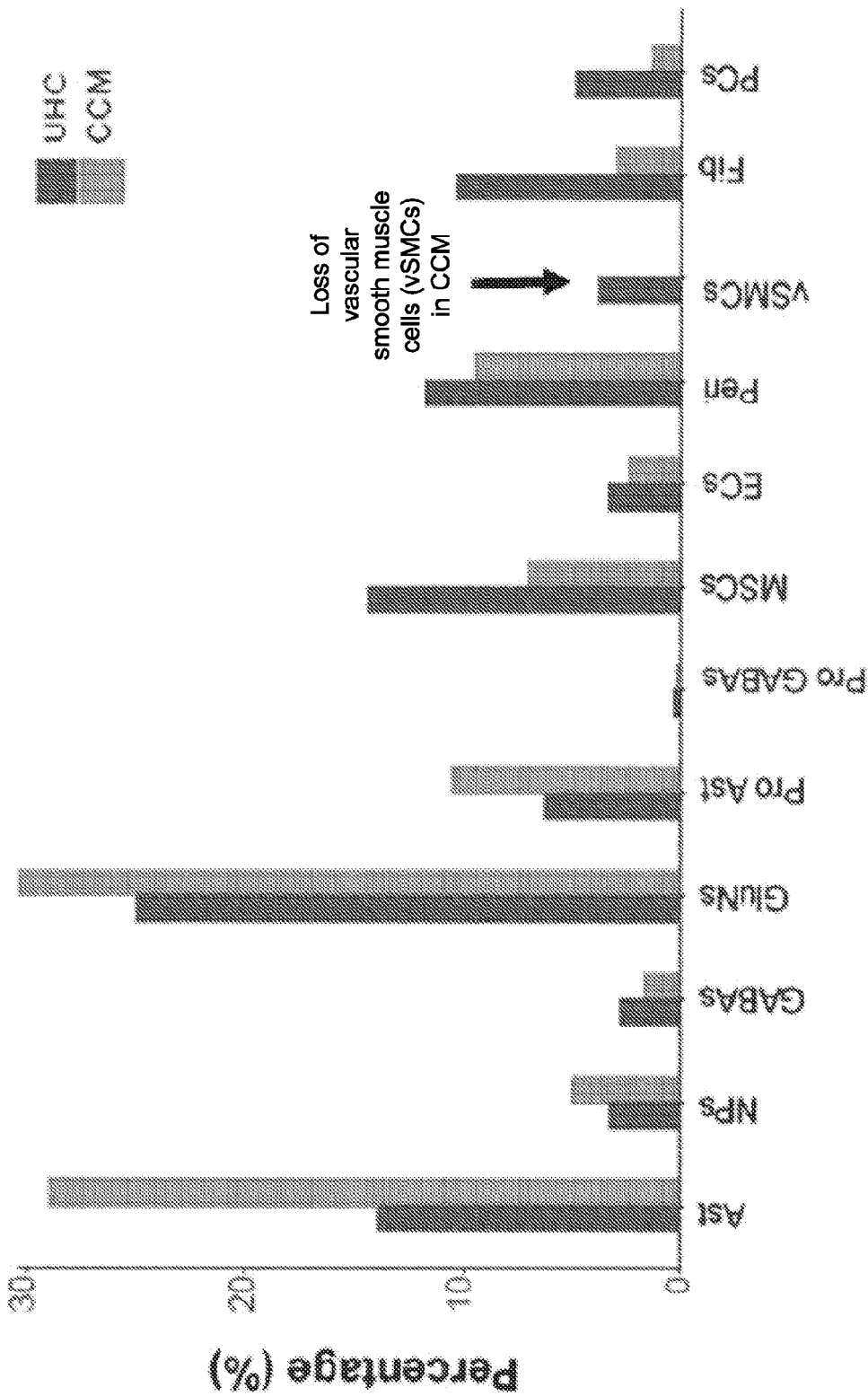


FIG. 7B

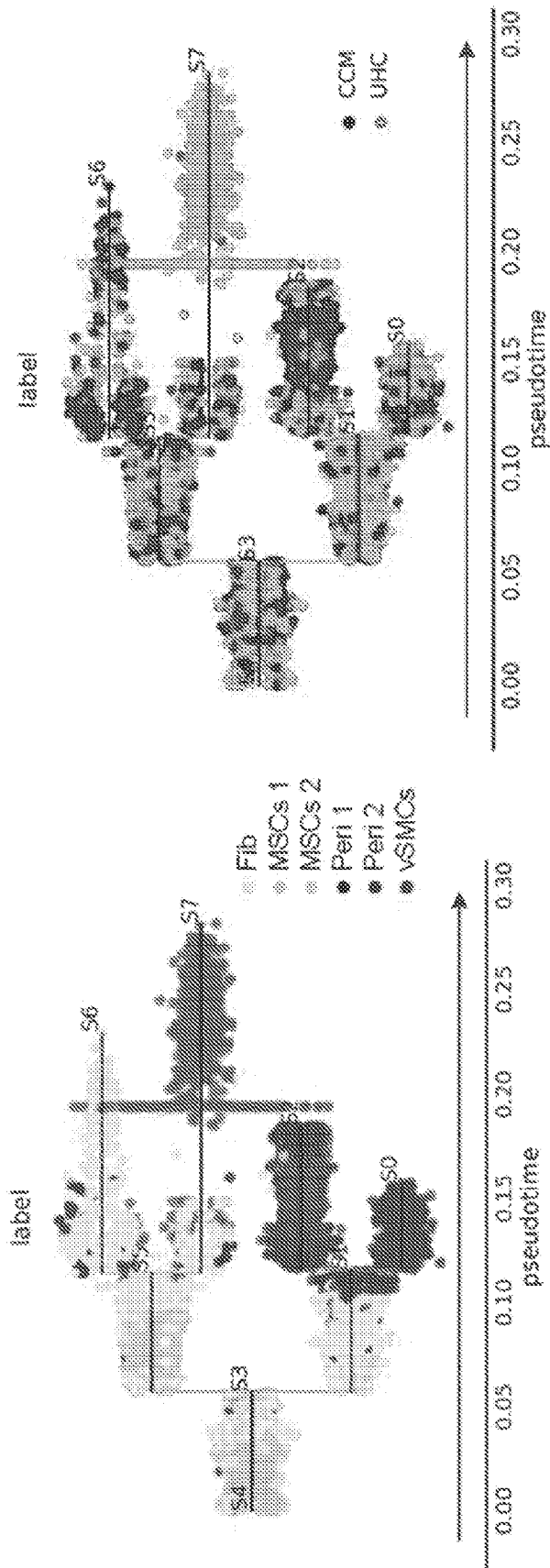


FIG. 7C

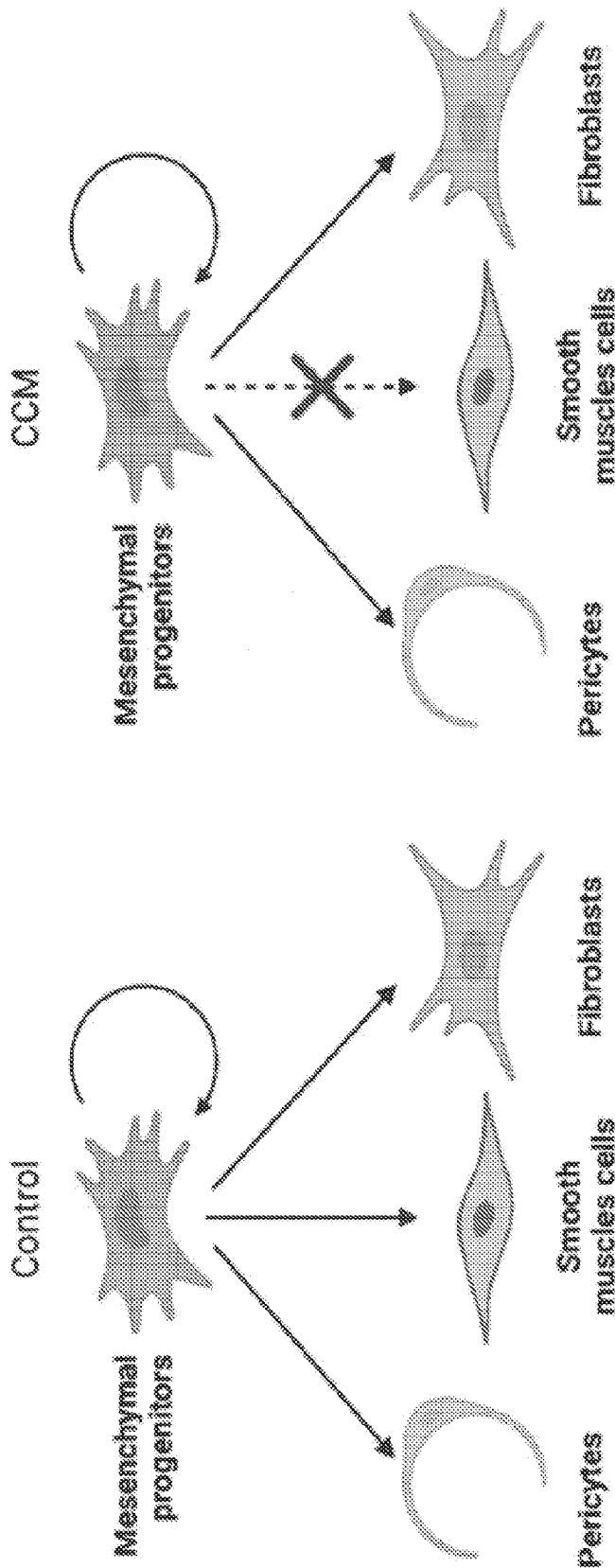


FIG. 7D

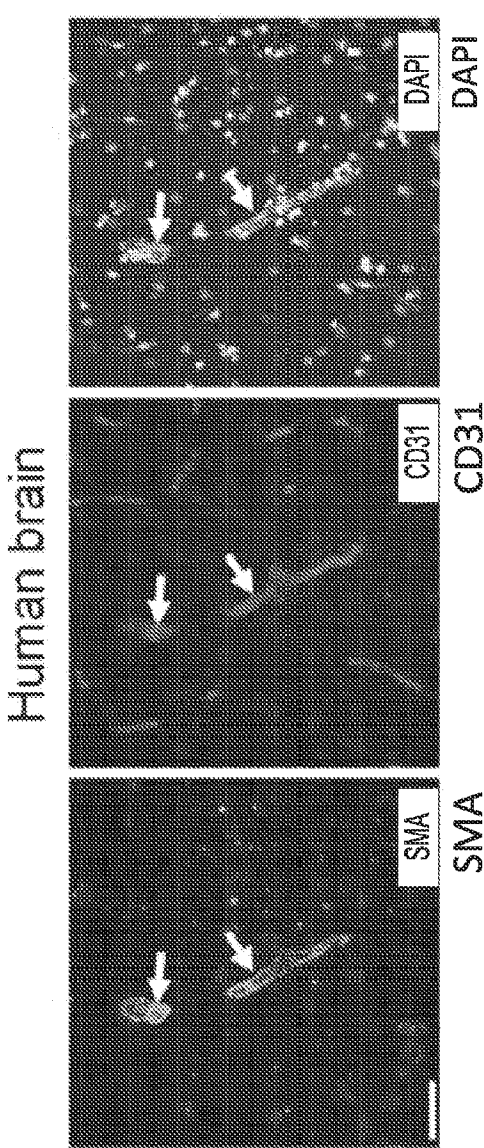


FIG. 7E

CCM tissue

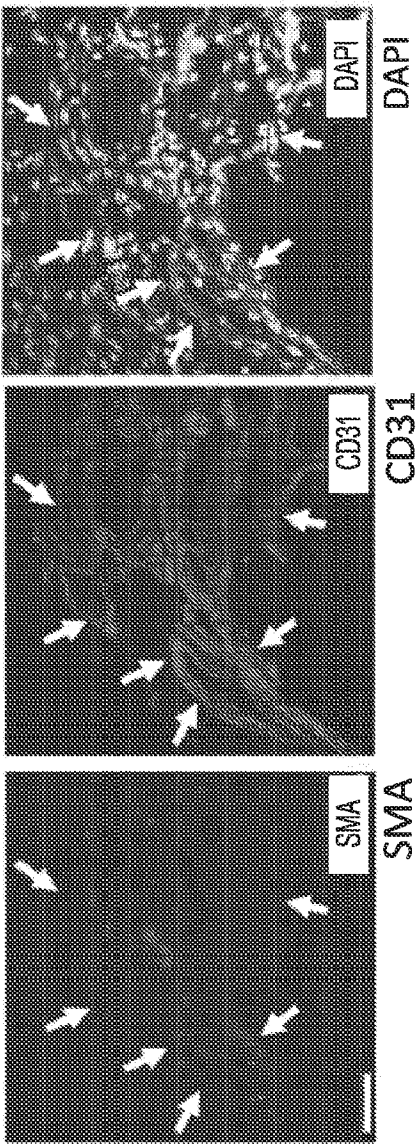


FIG. 7F

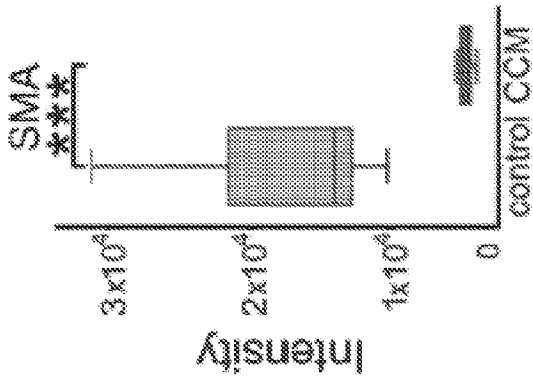


FIG. 7G

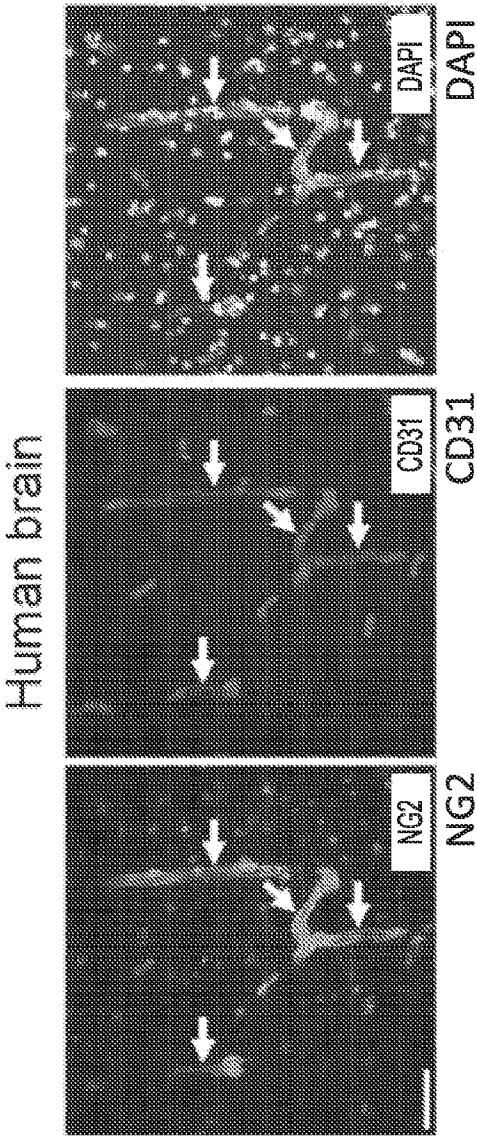


FIG. 7H

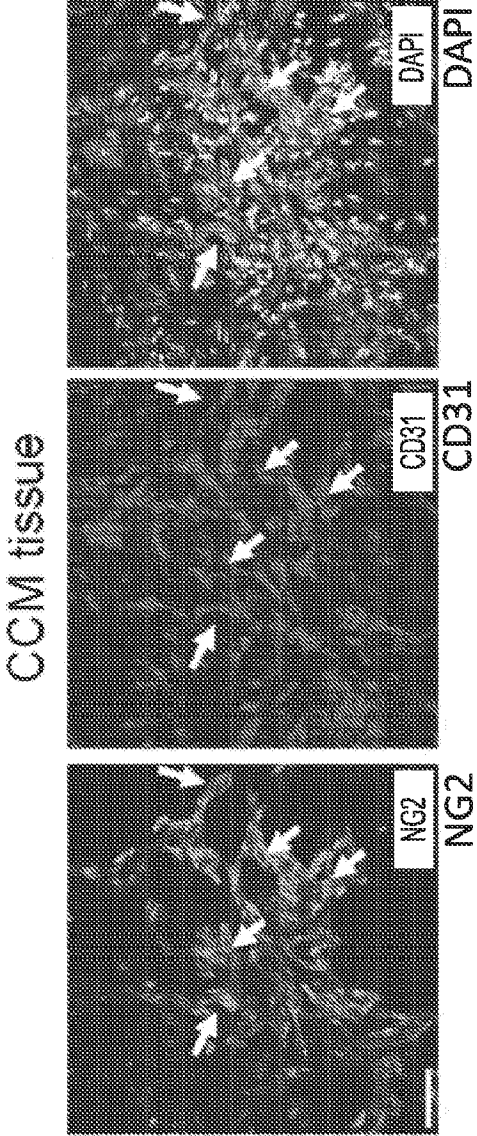


FIG. 7I

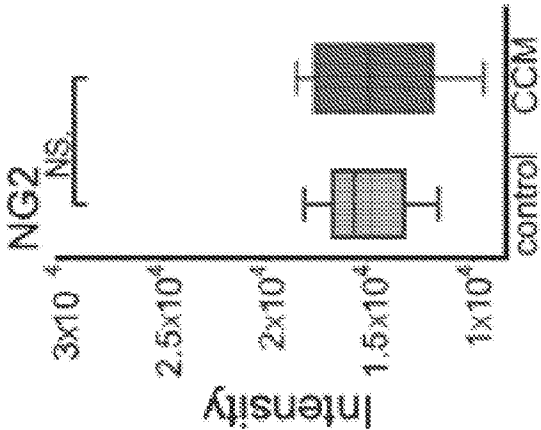


FIG. 7J

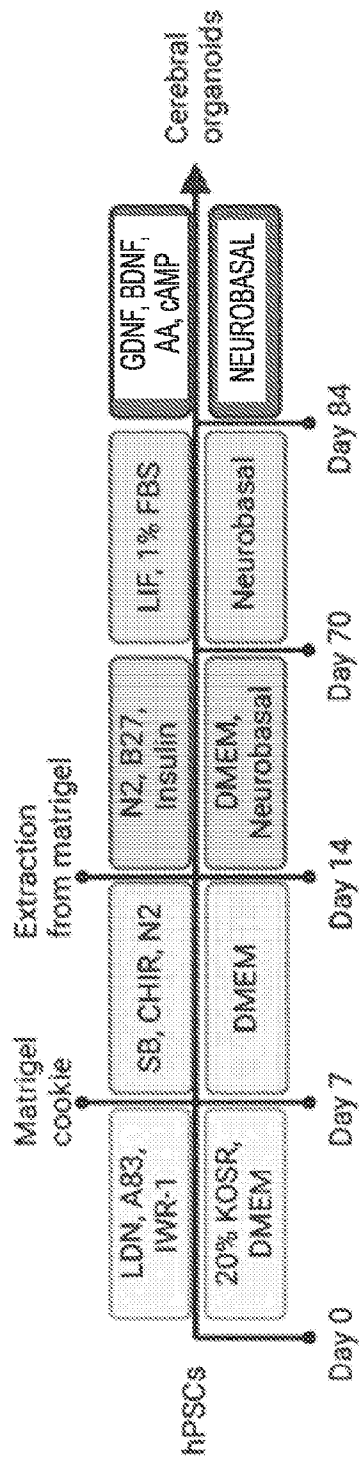


FIG. 8A

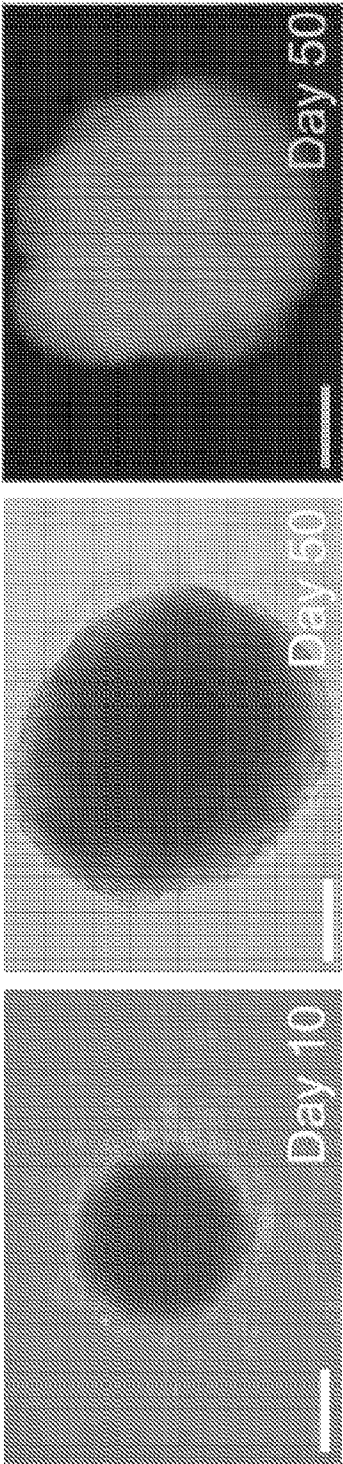


FIG. 8B

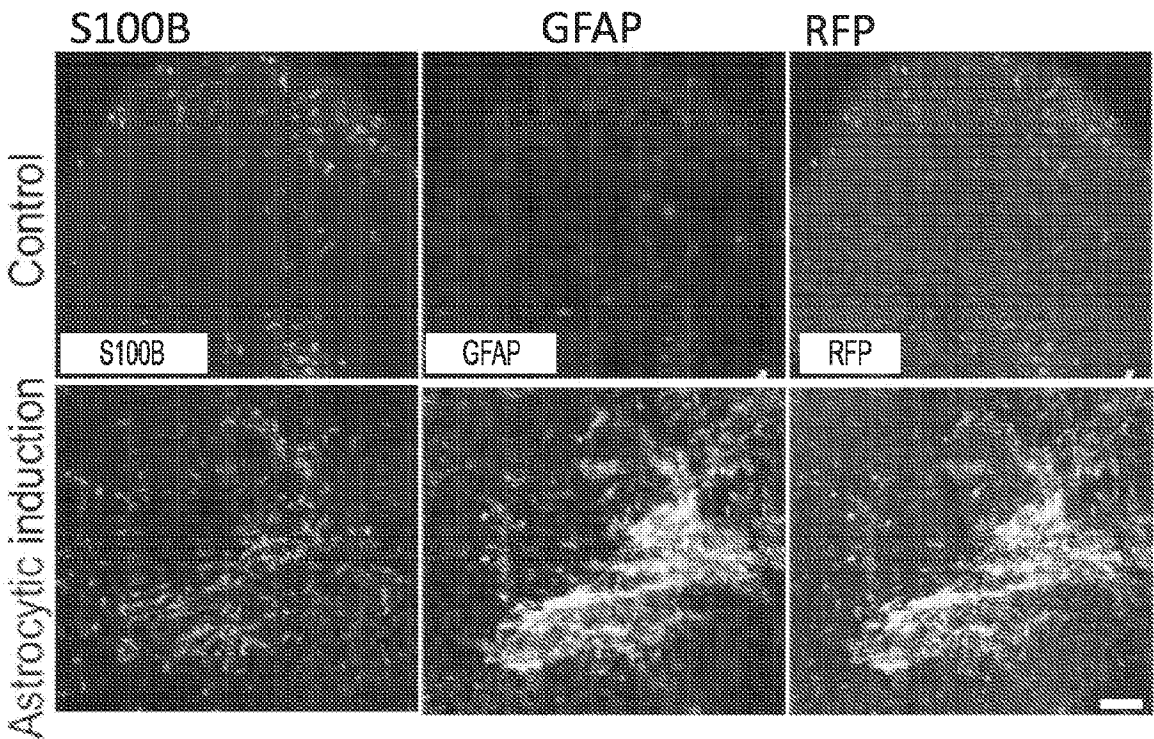


FIG. 8C

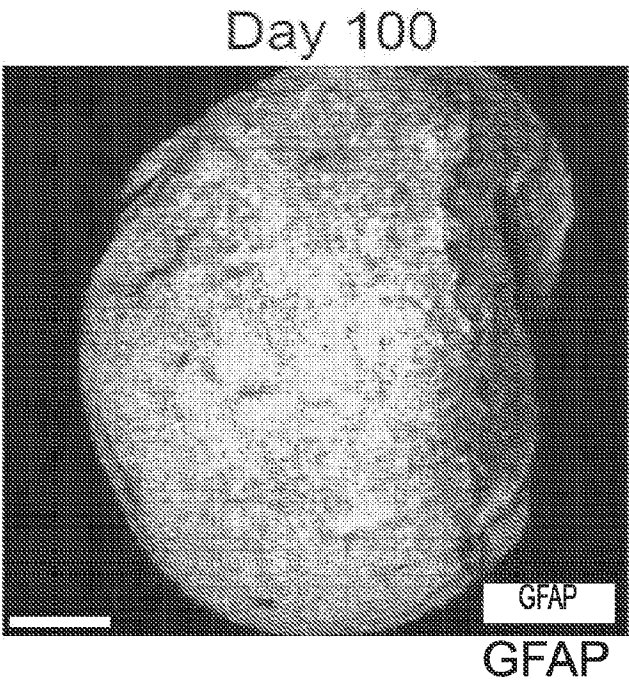


FIG. 8D

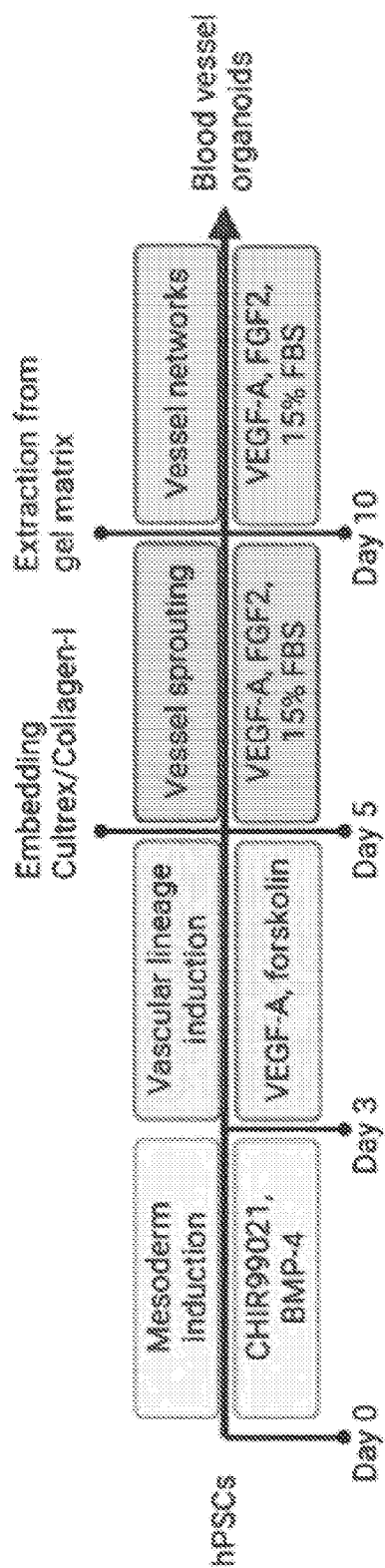


FIG. 8E

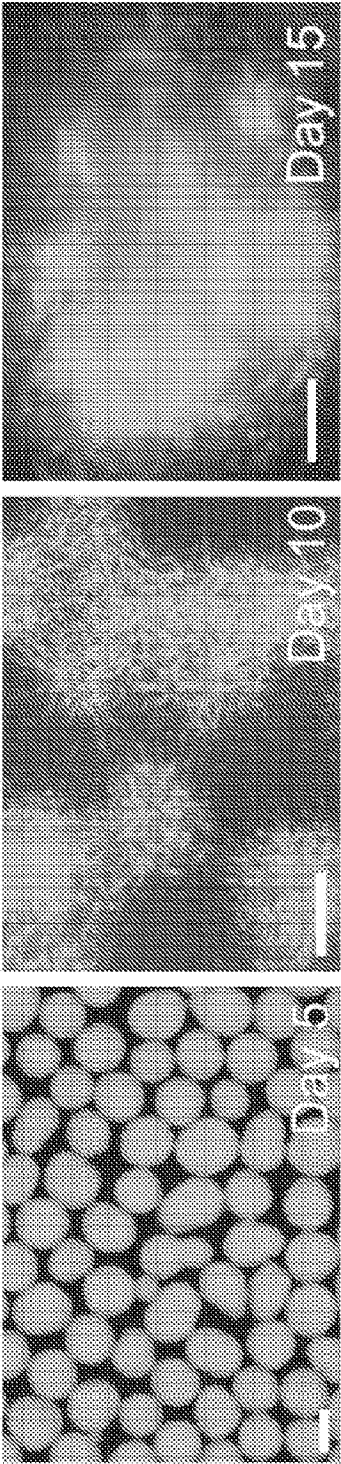


FIG. 8F

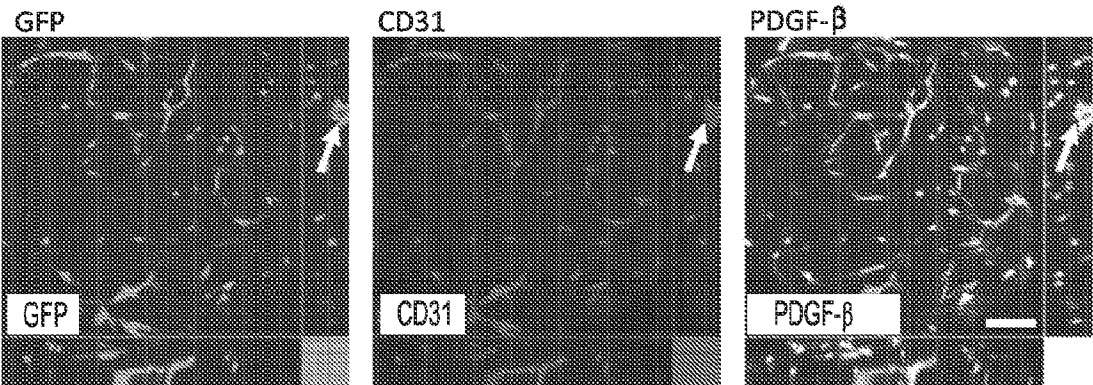


FIG. 8G

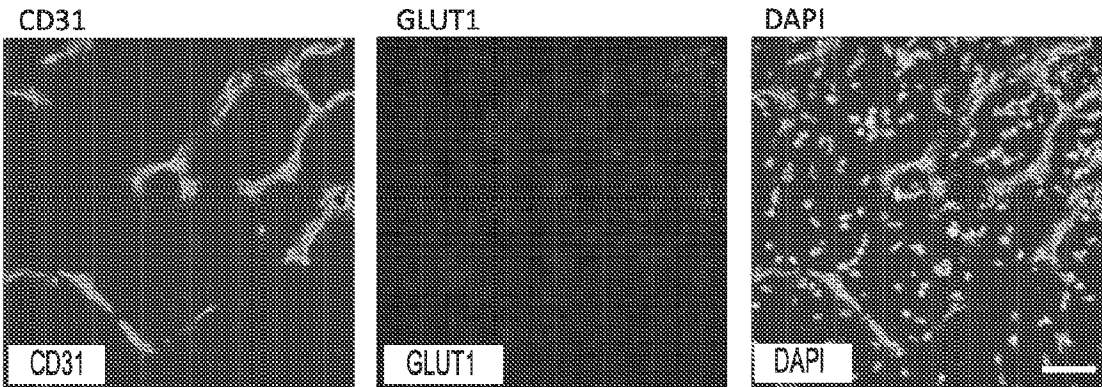


FIG. 8H

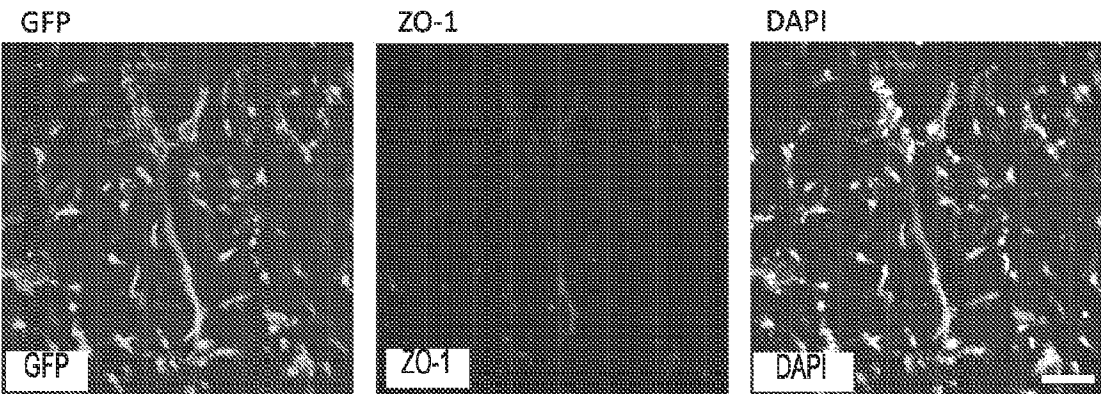


FIG. 8I

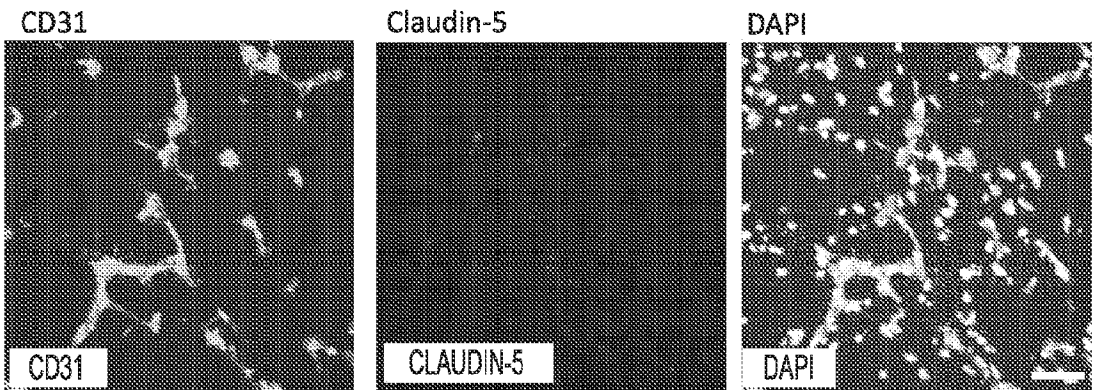


FIG. 8J

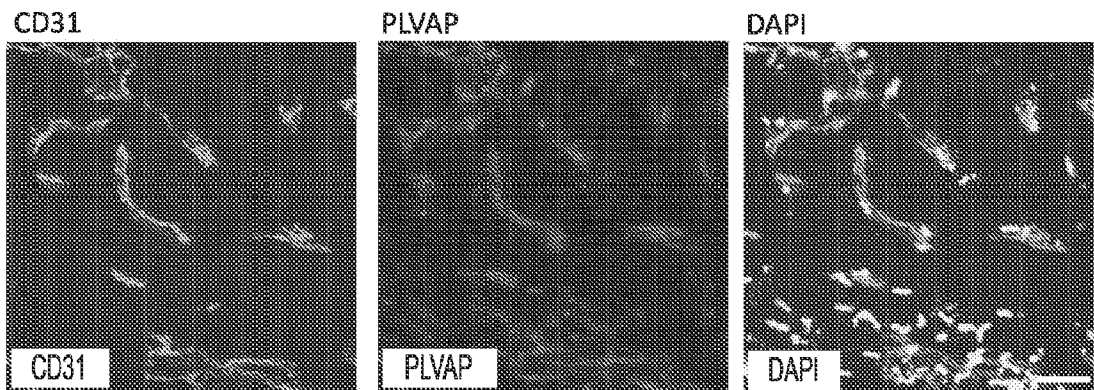


FIG. 8K

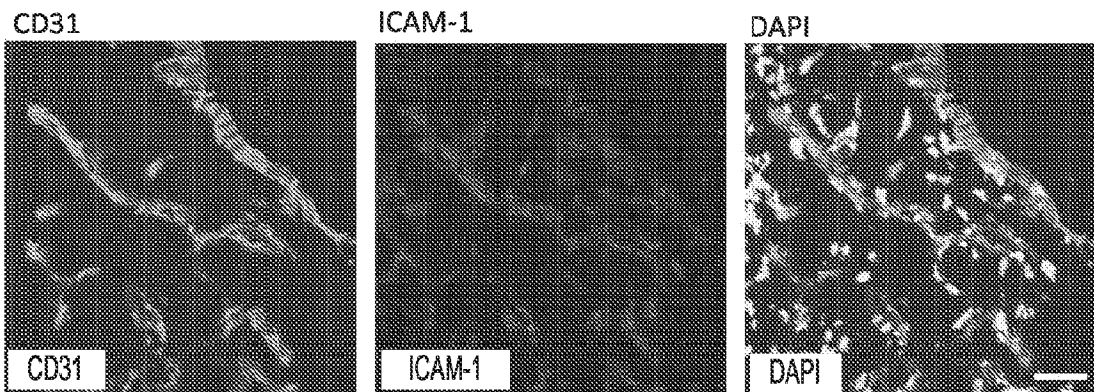


FIG. 8L

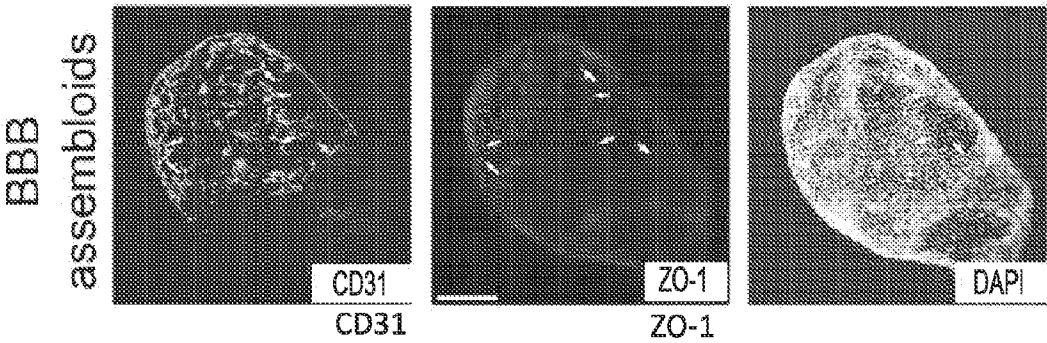


FIG. 9A

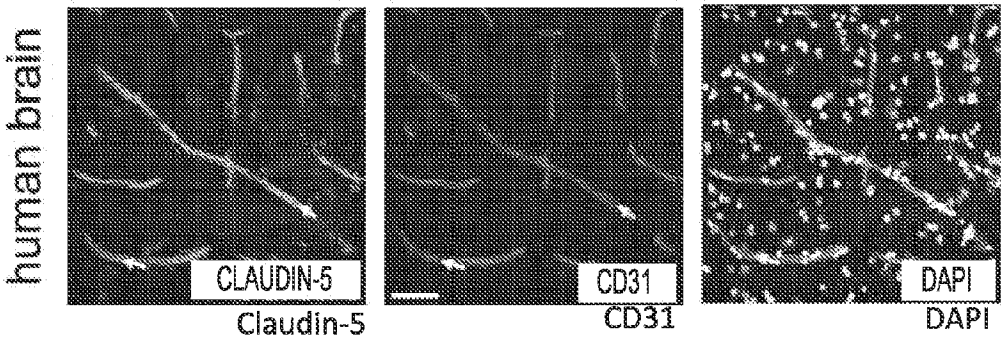


FIG. 9B

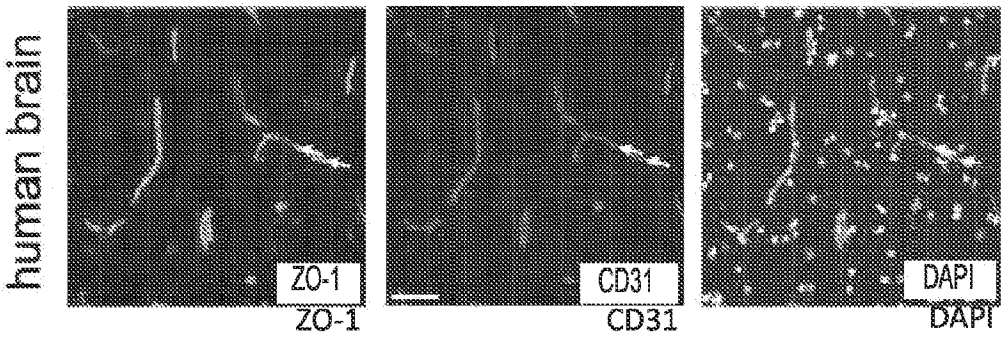


FIG. 9C

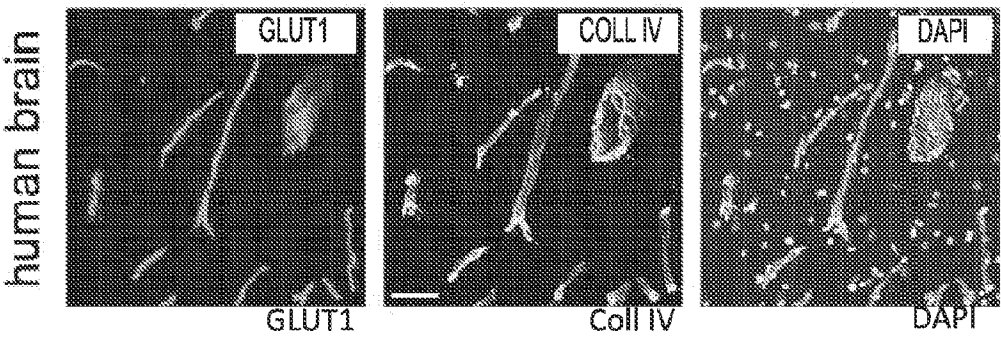


FIG. 9D

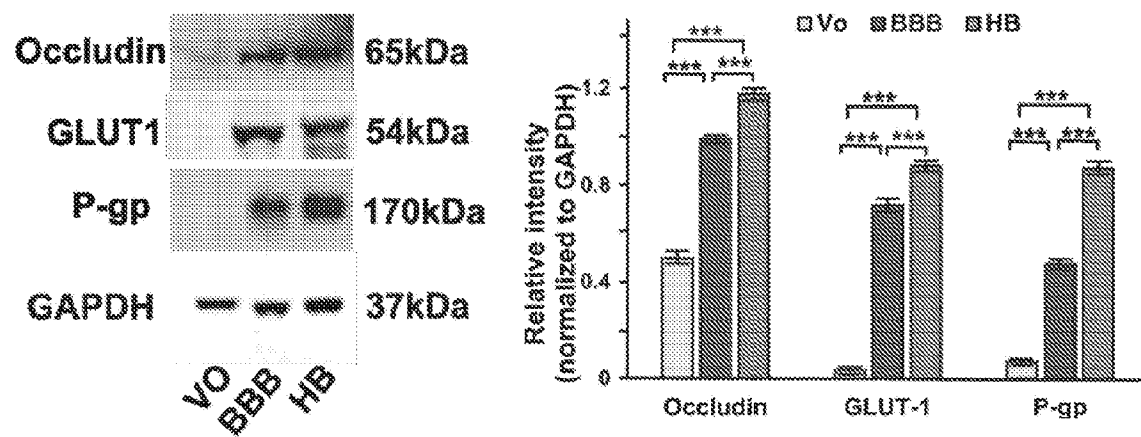


FIG. 9E

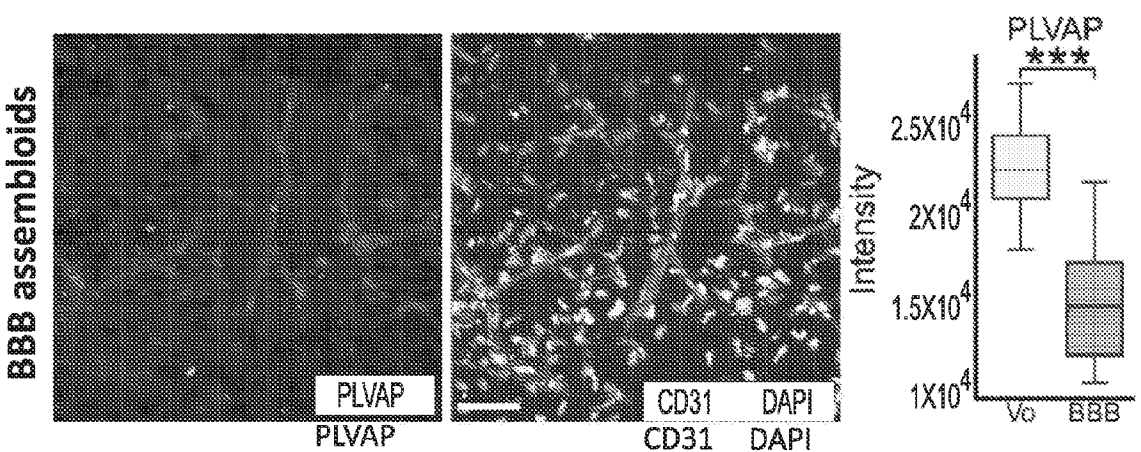


FIG. 9F

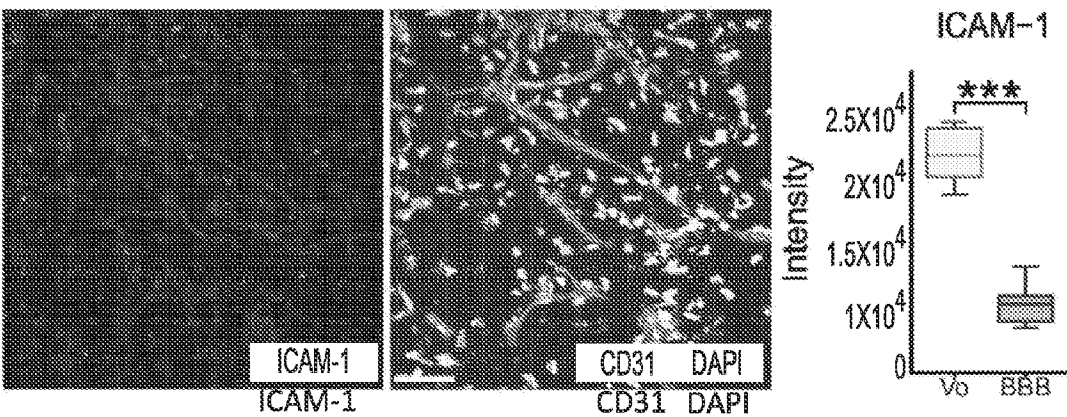


FIG. 9G

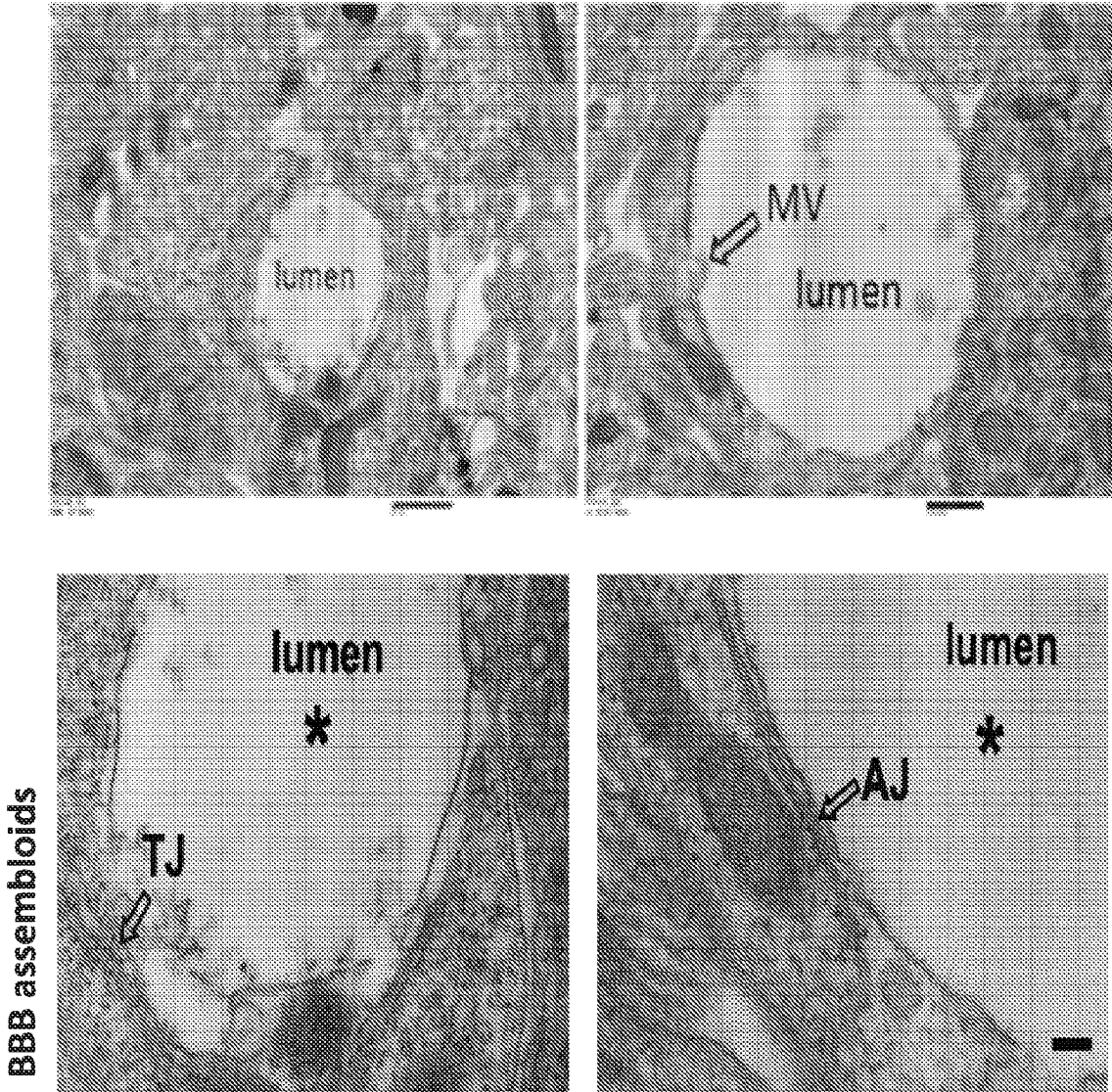


FIG. 9H

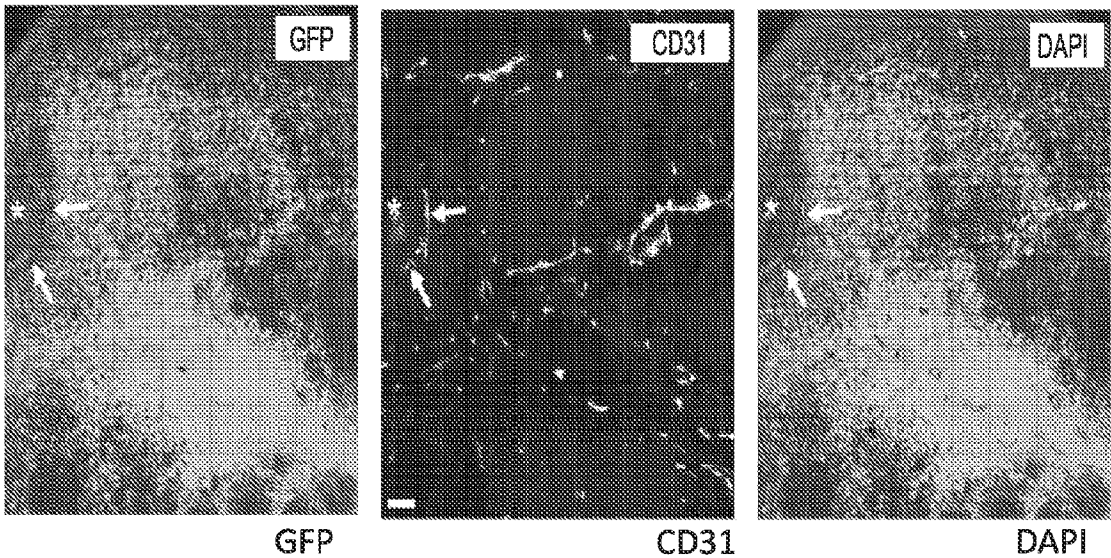


FIG. 9I

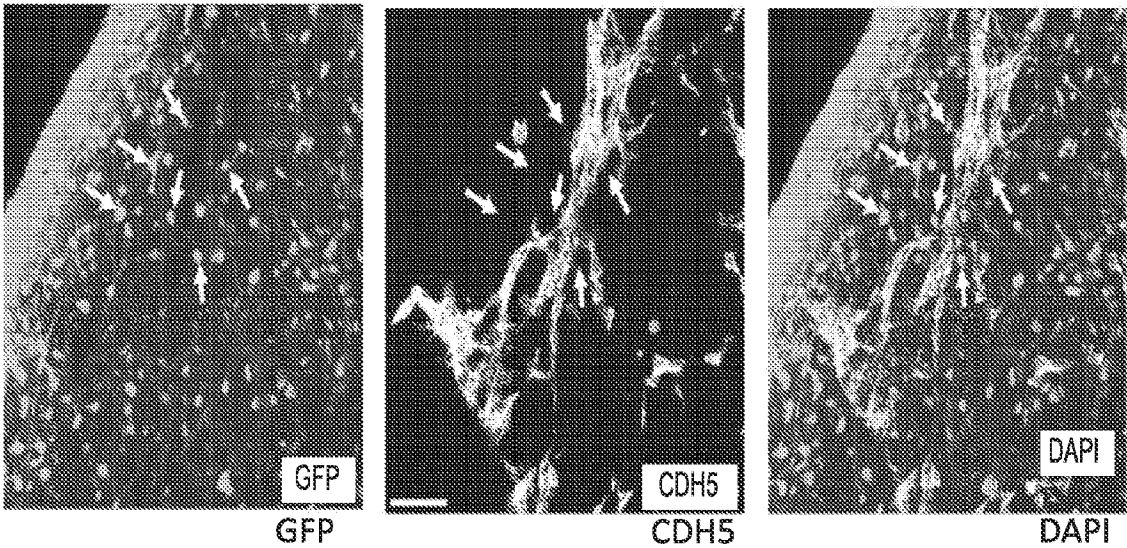
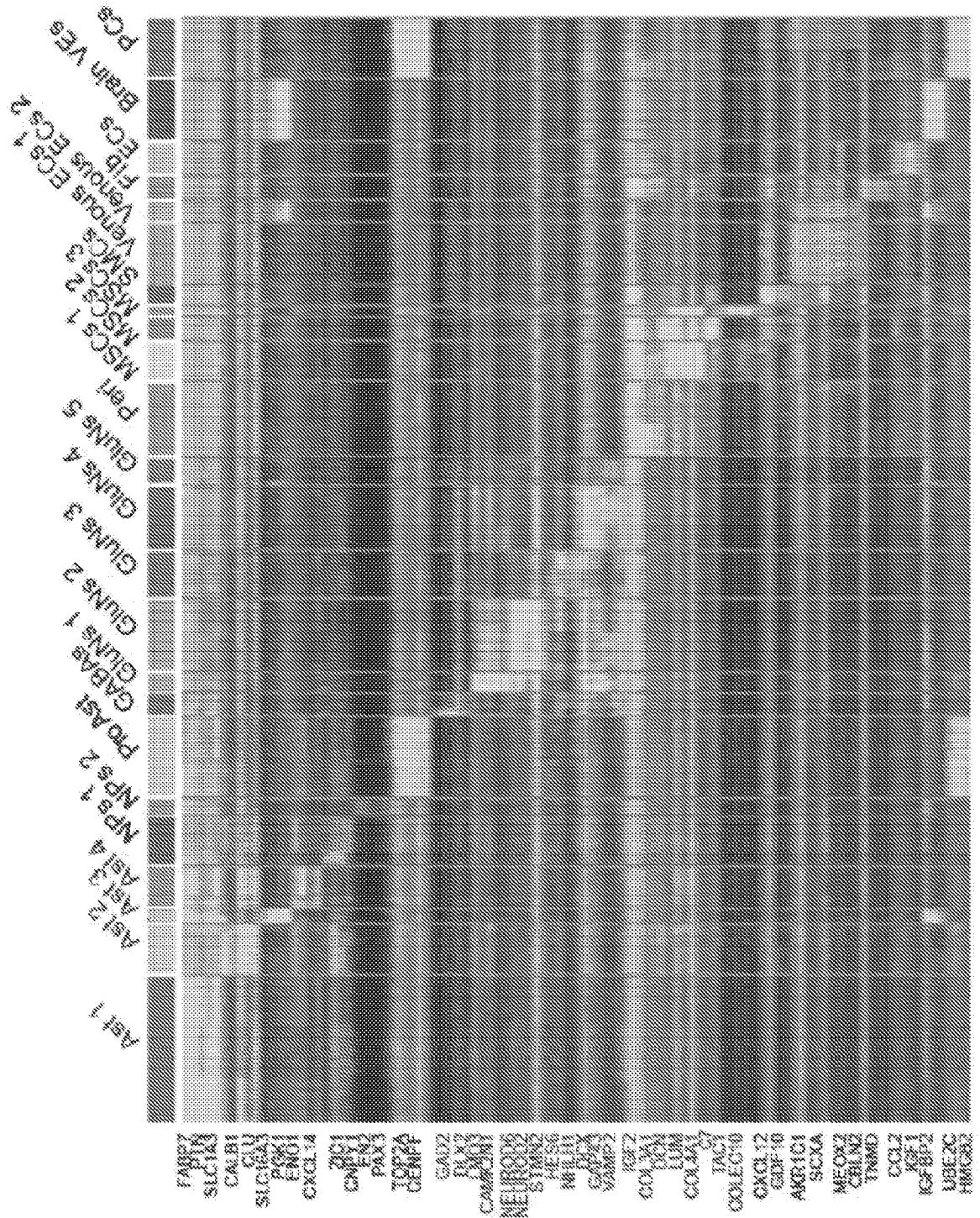


FIG. 9J

FIG. 9K



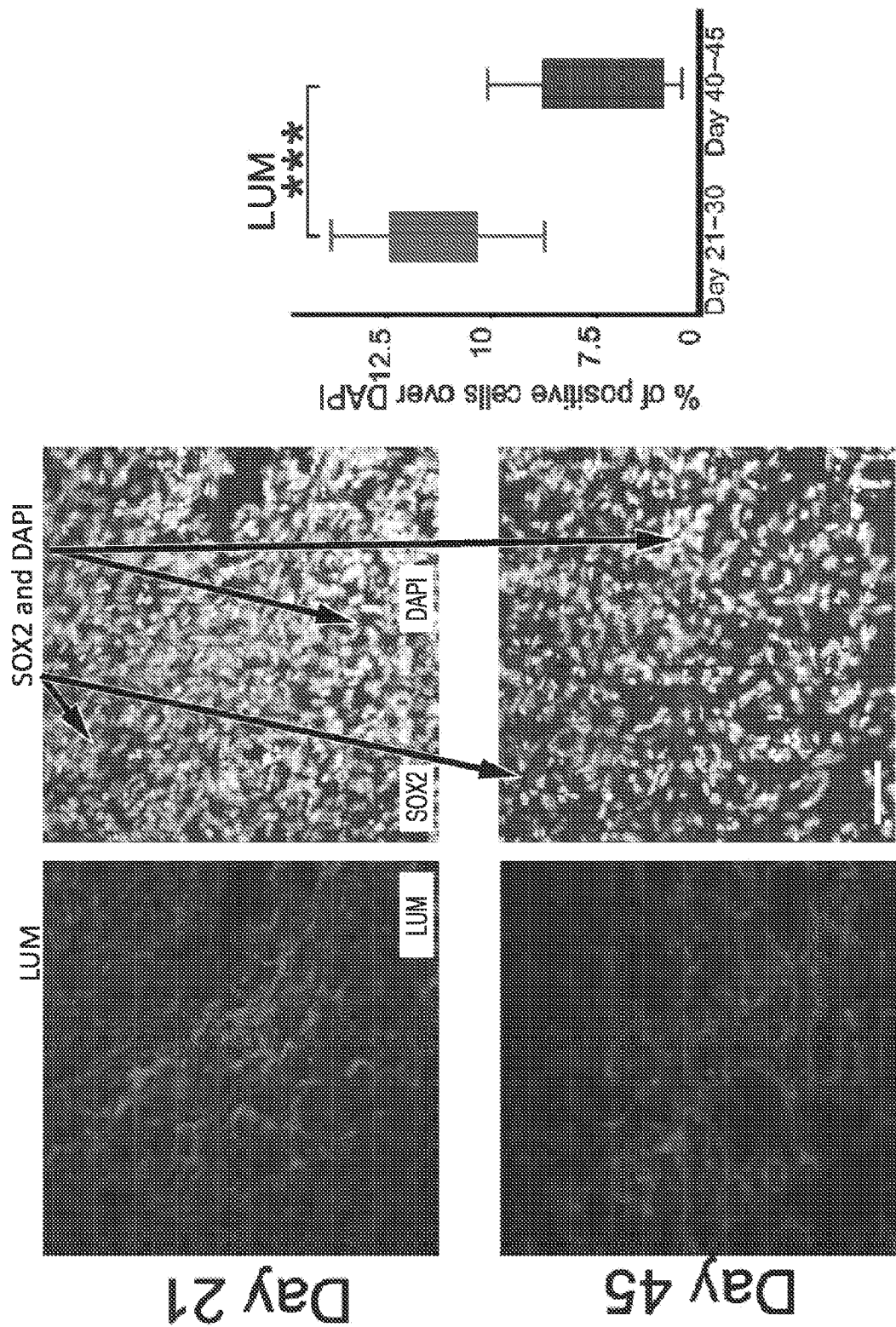


FIG. 9L

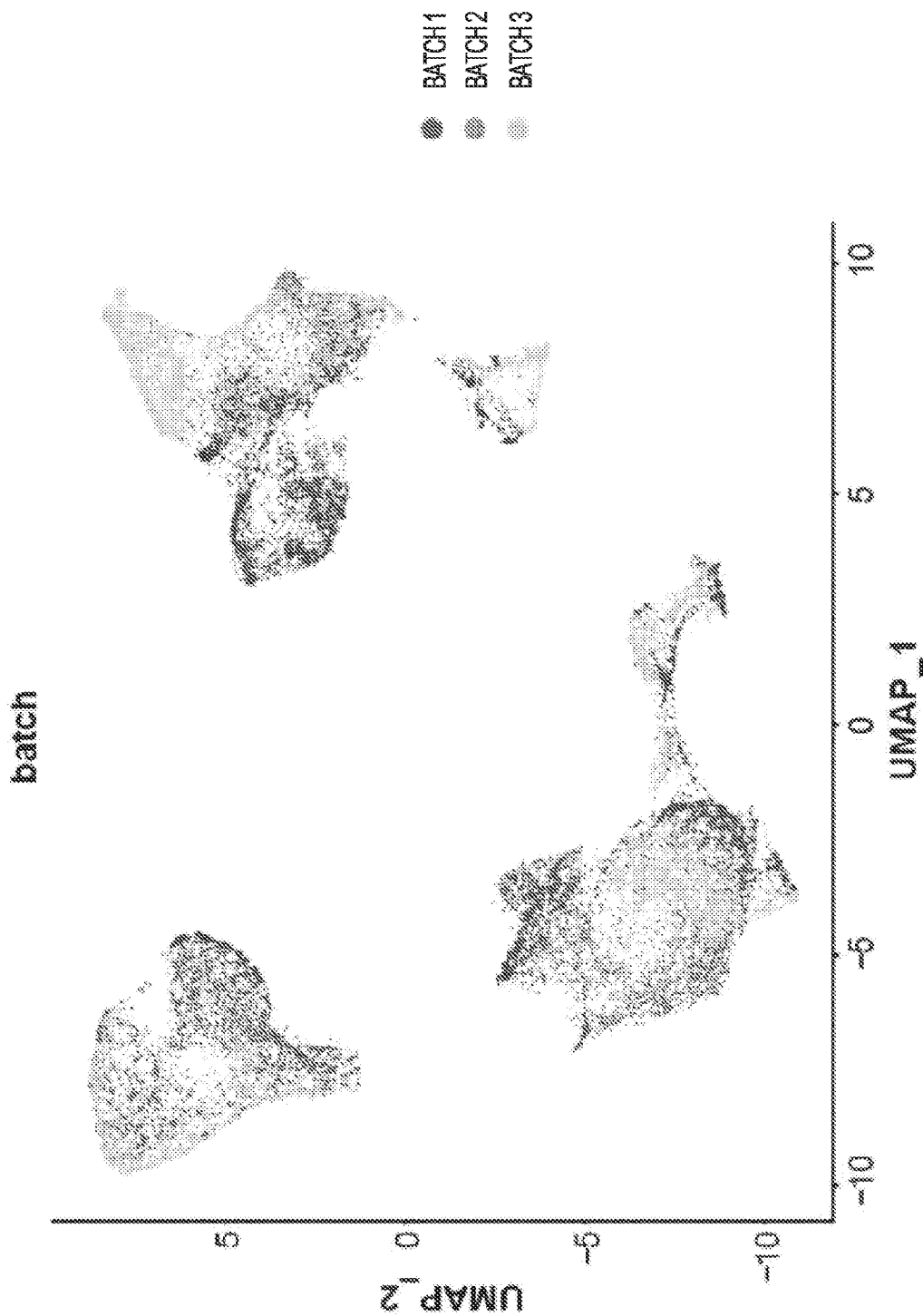


FIG. 9M

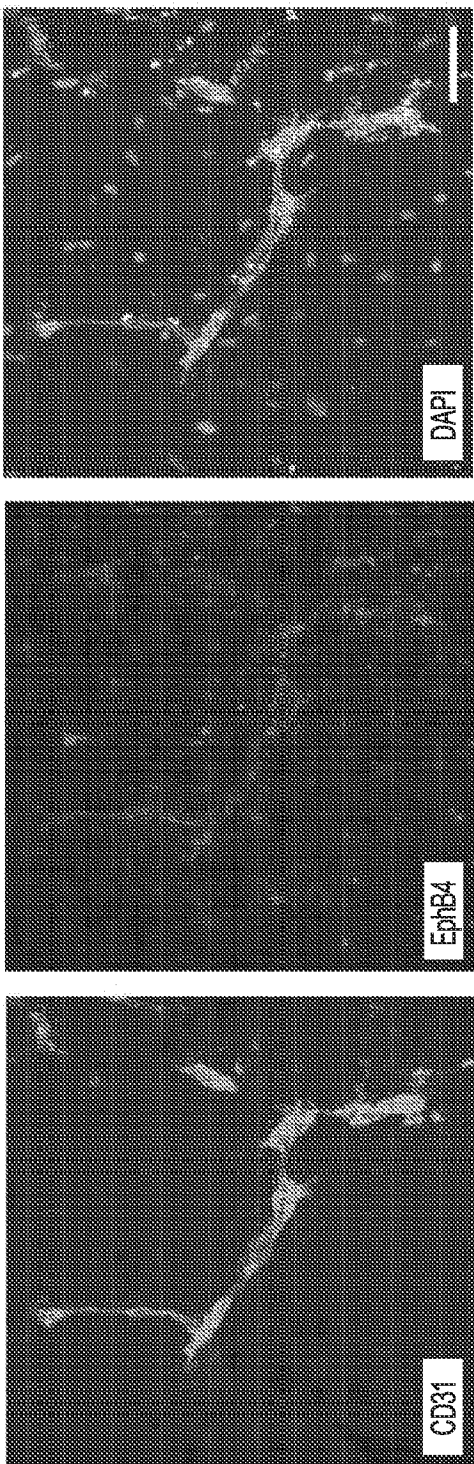


FIG. 9N

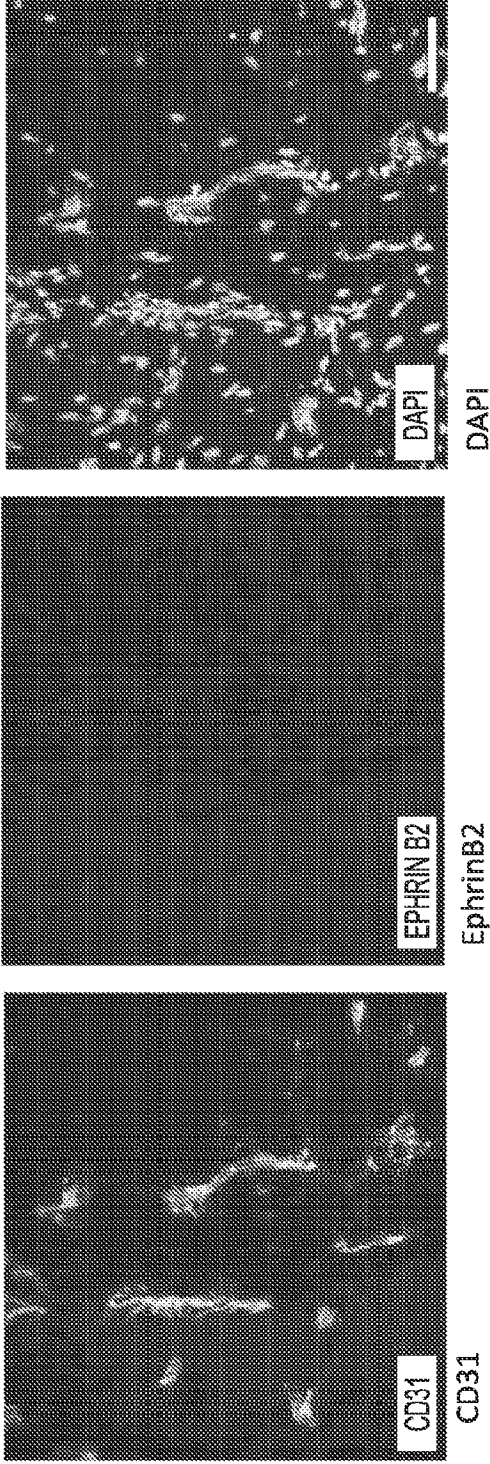
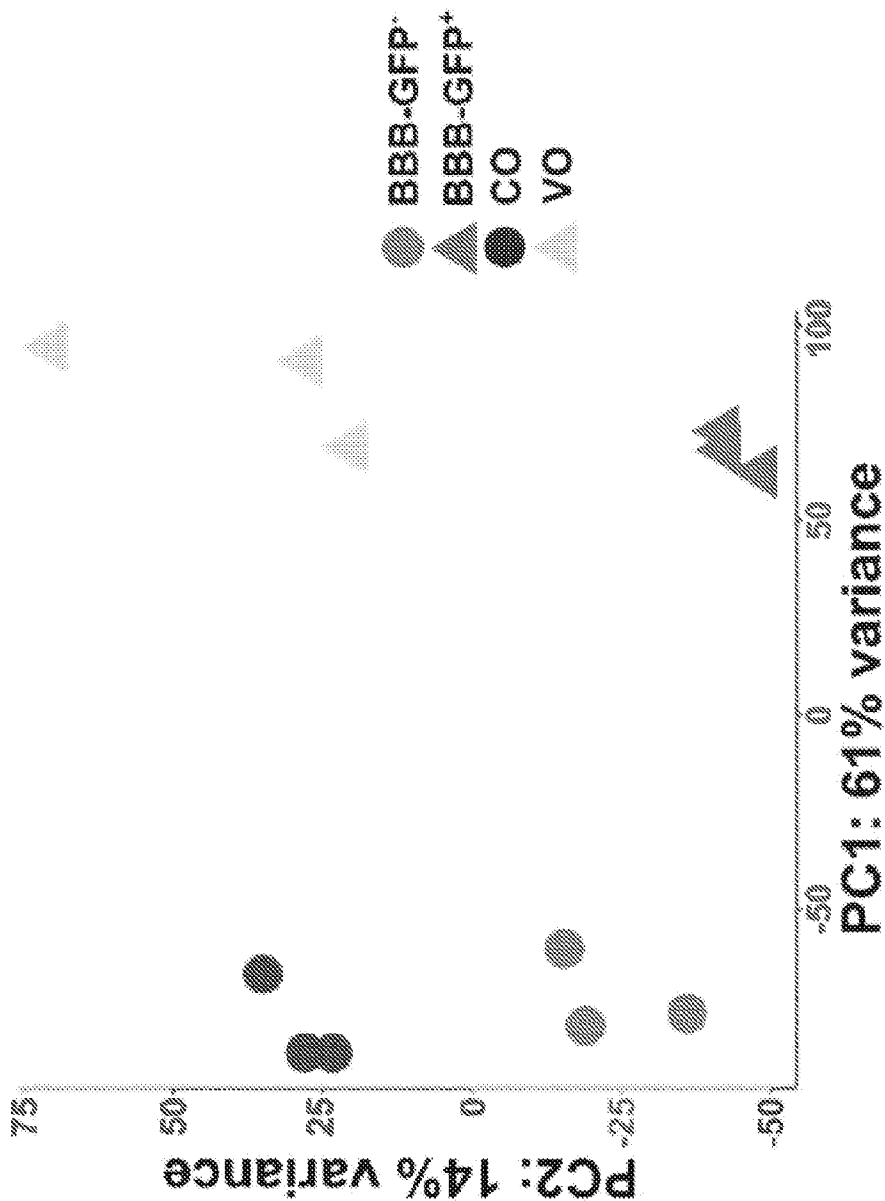


FIG. 9O



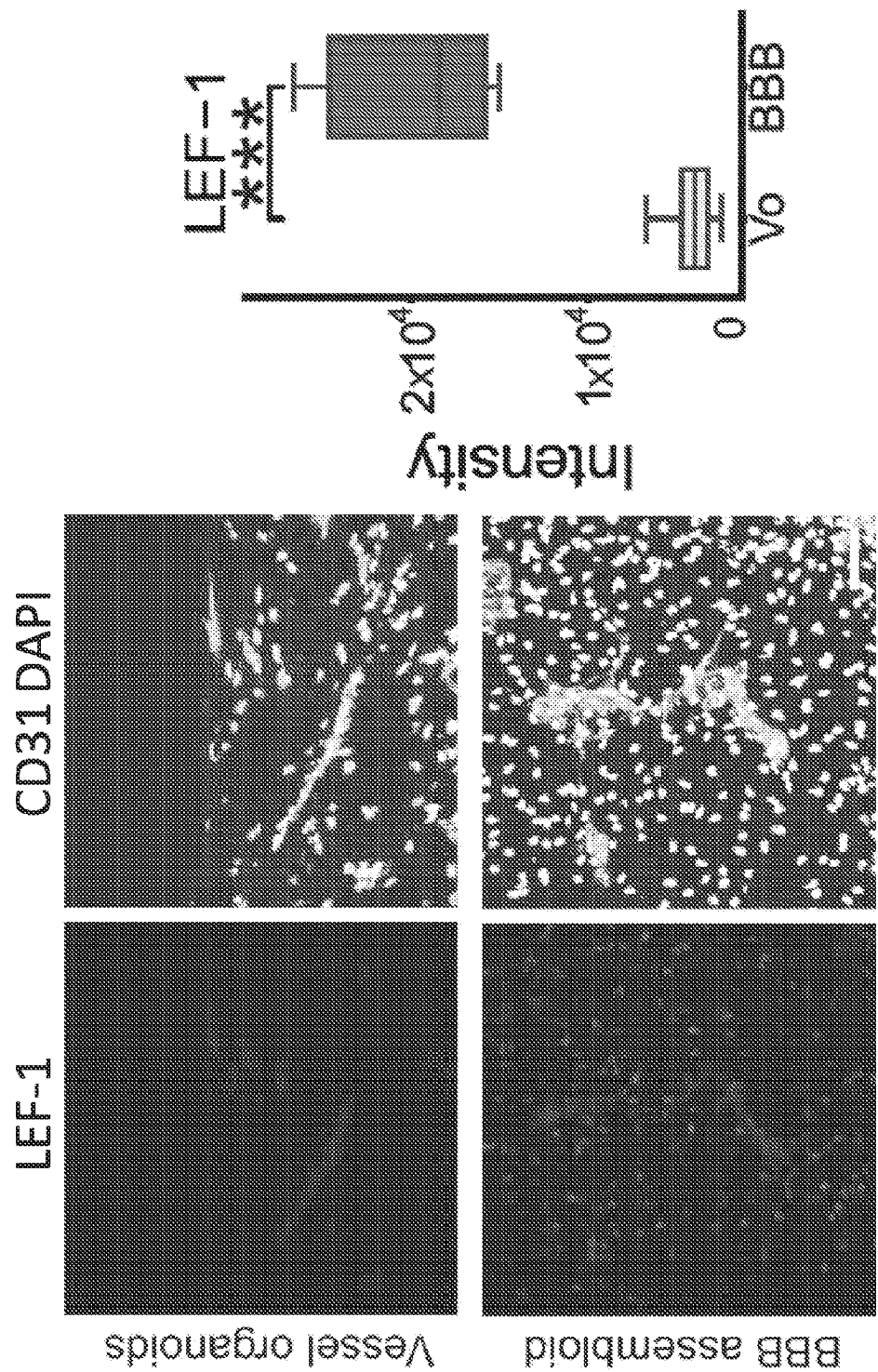


FIG. 10B

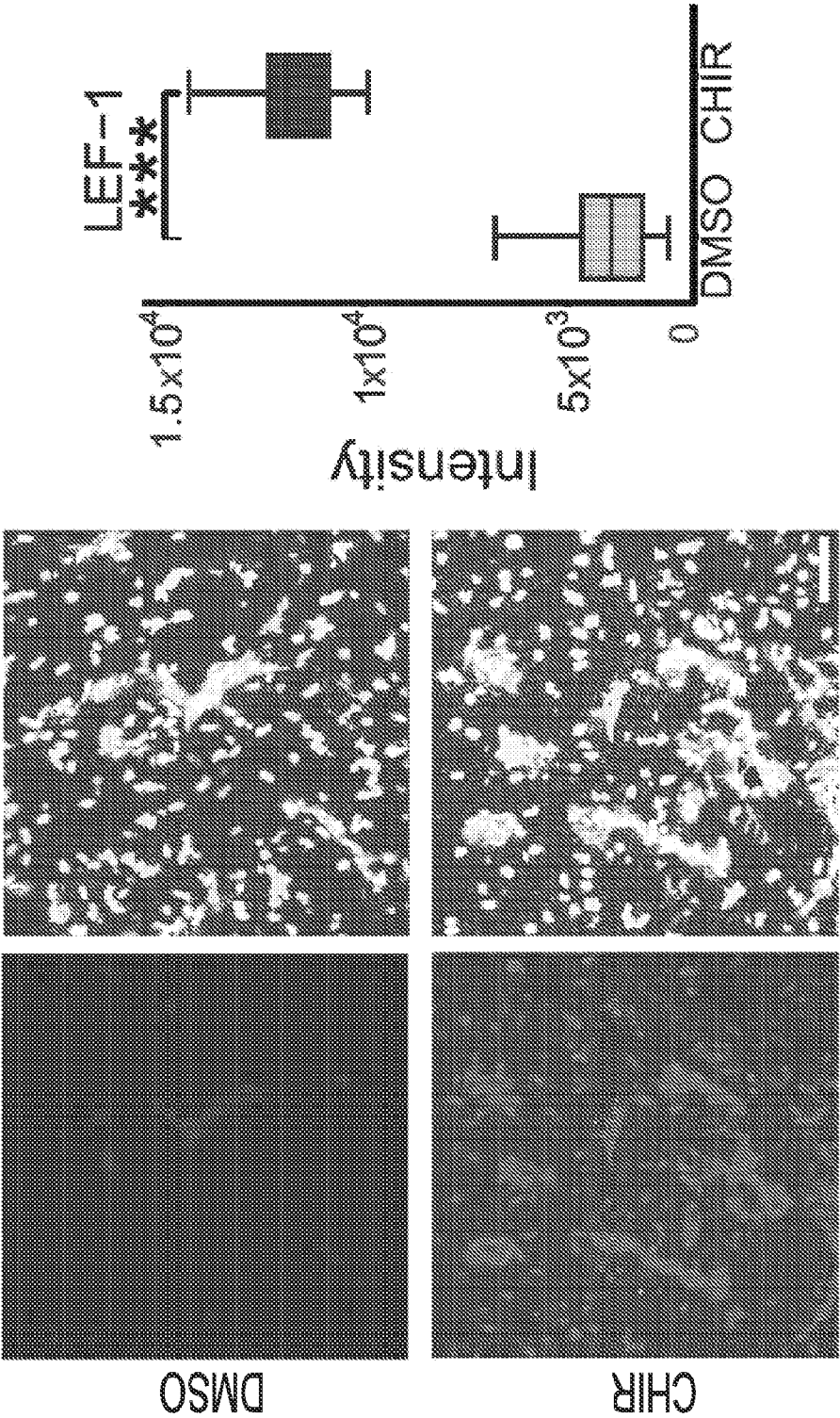


FIG. 10C

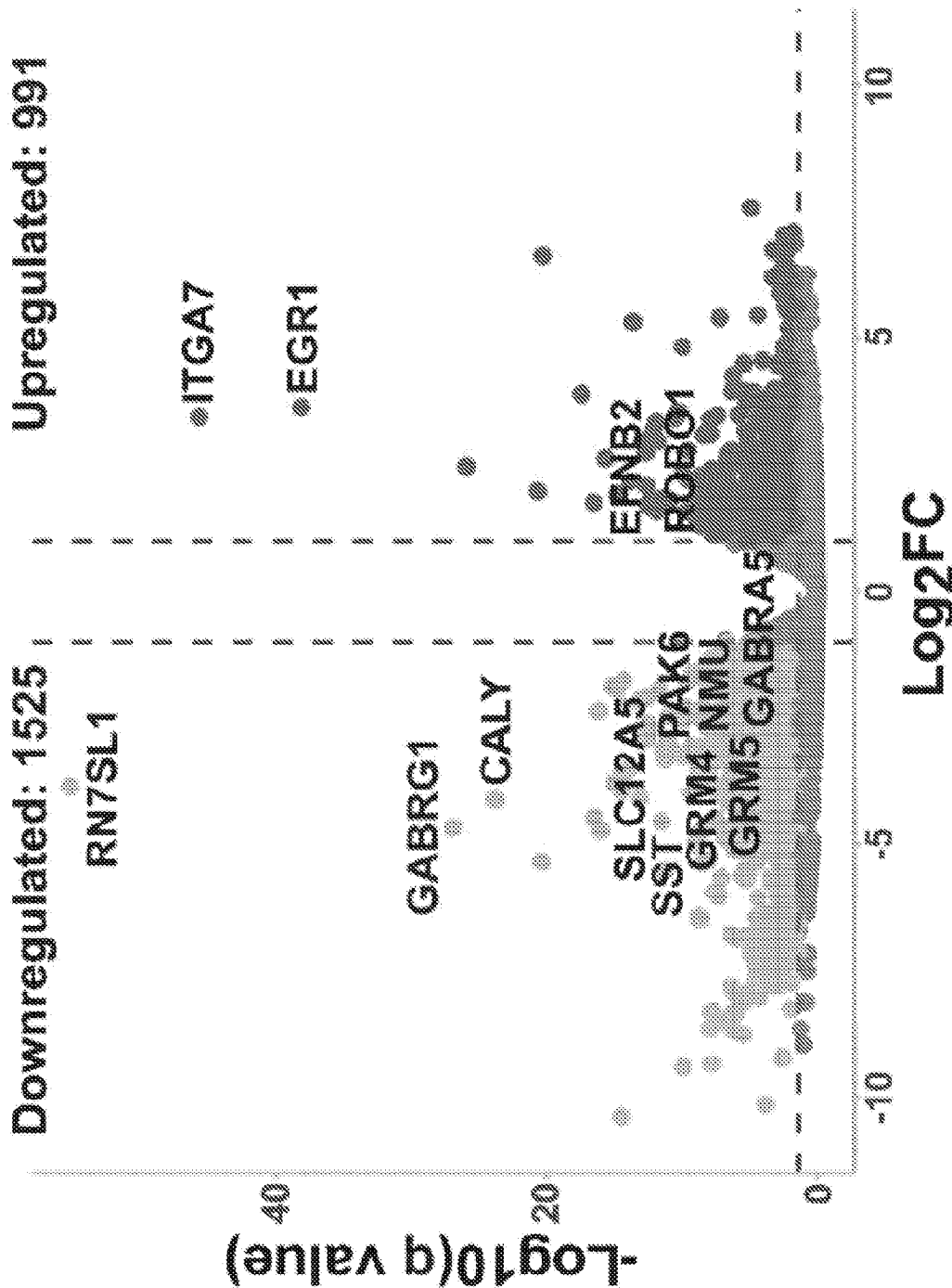


FIG. 10D

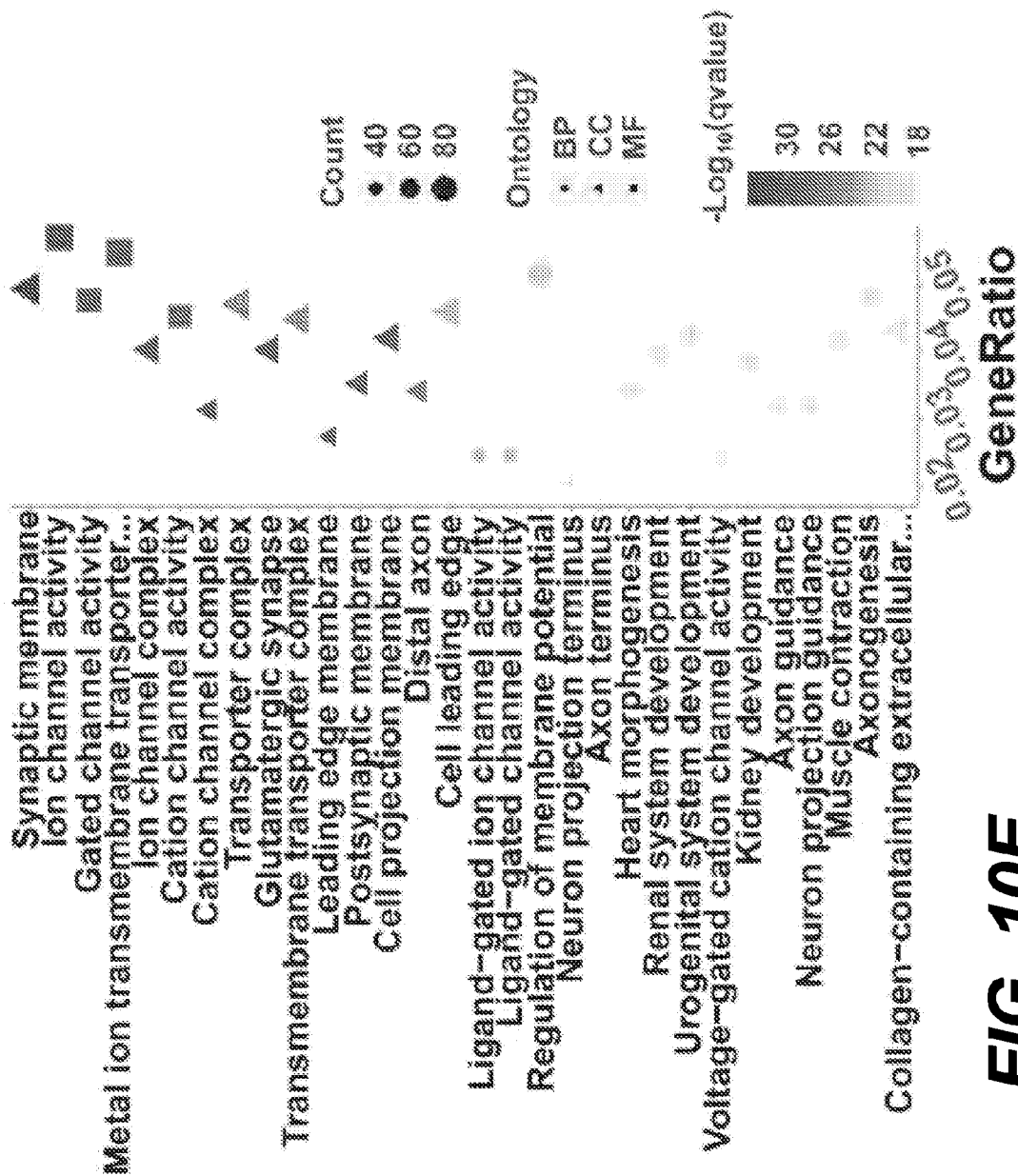


FIG. 10E

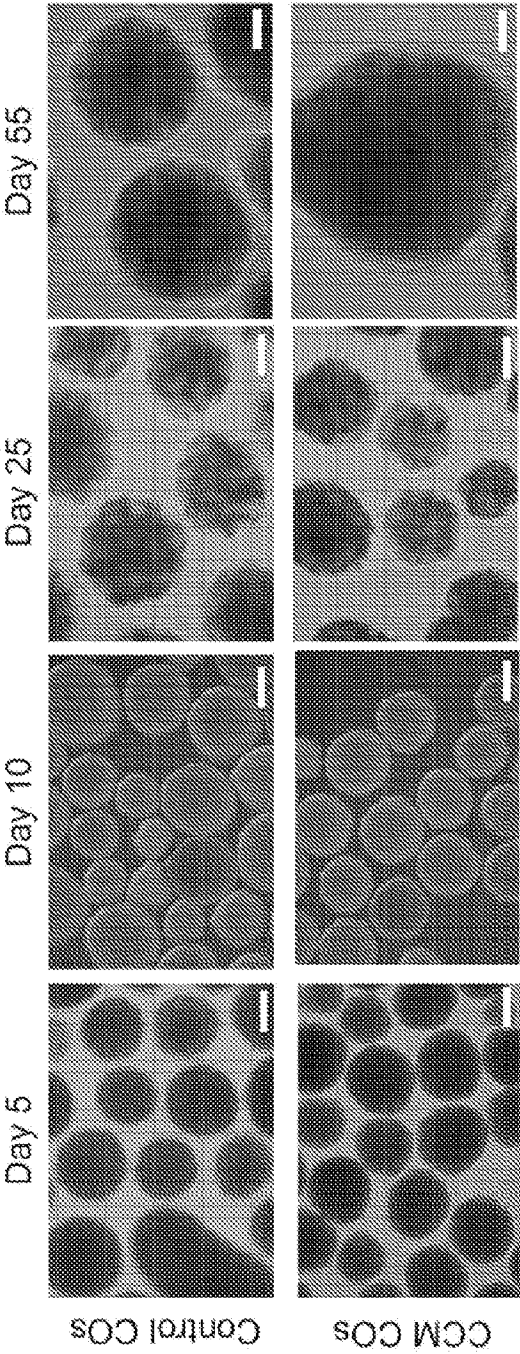


FIG. 11A

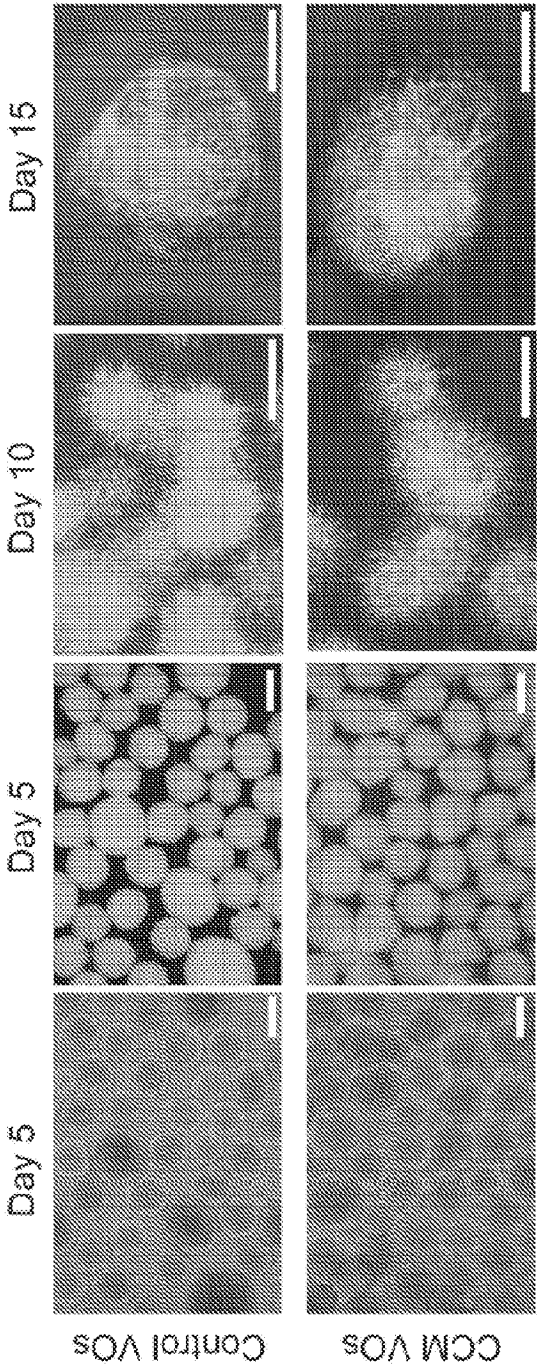


FIG. 11B

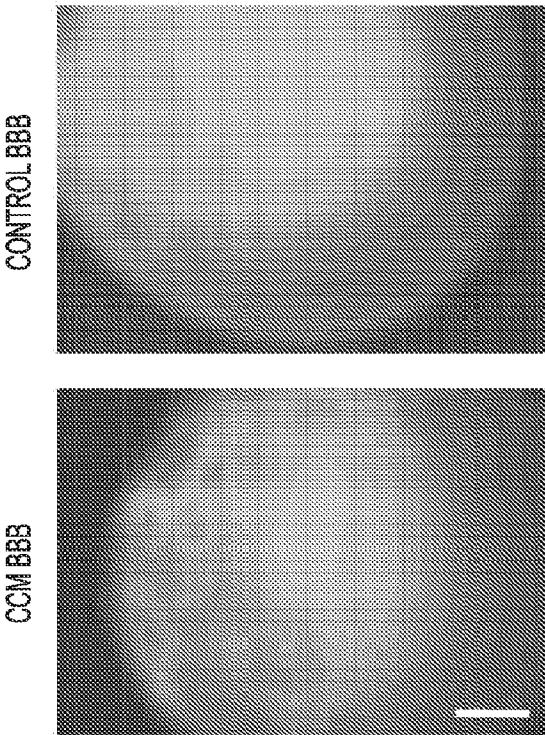


FIG. 11C

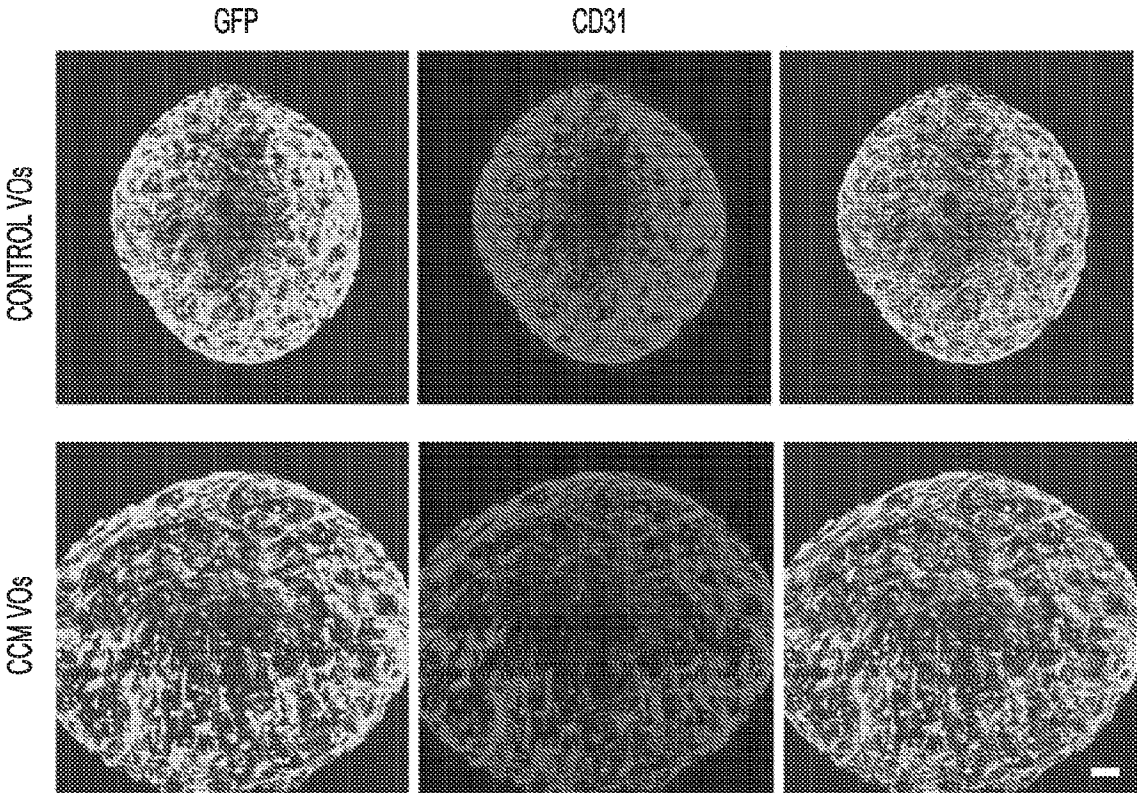


FIG. 11D

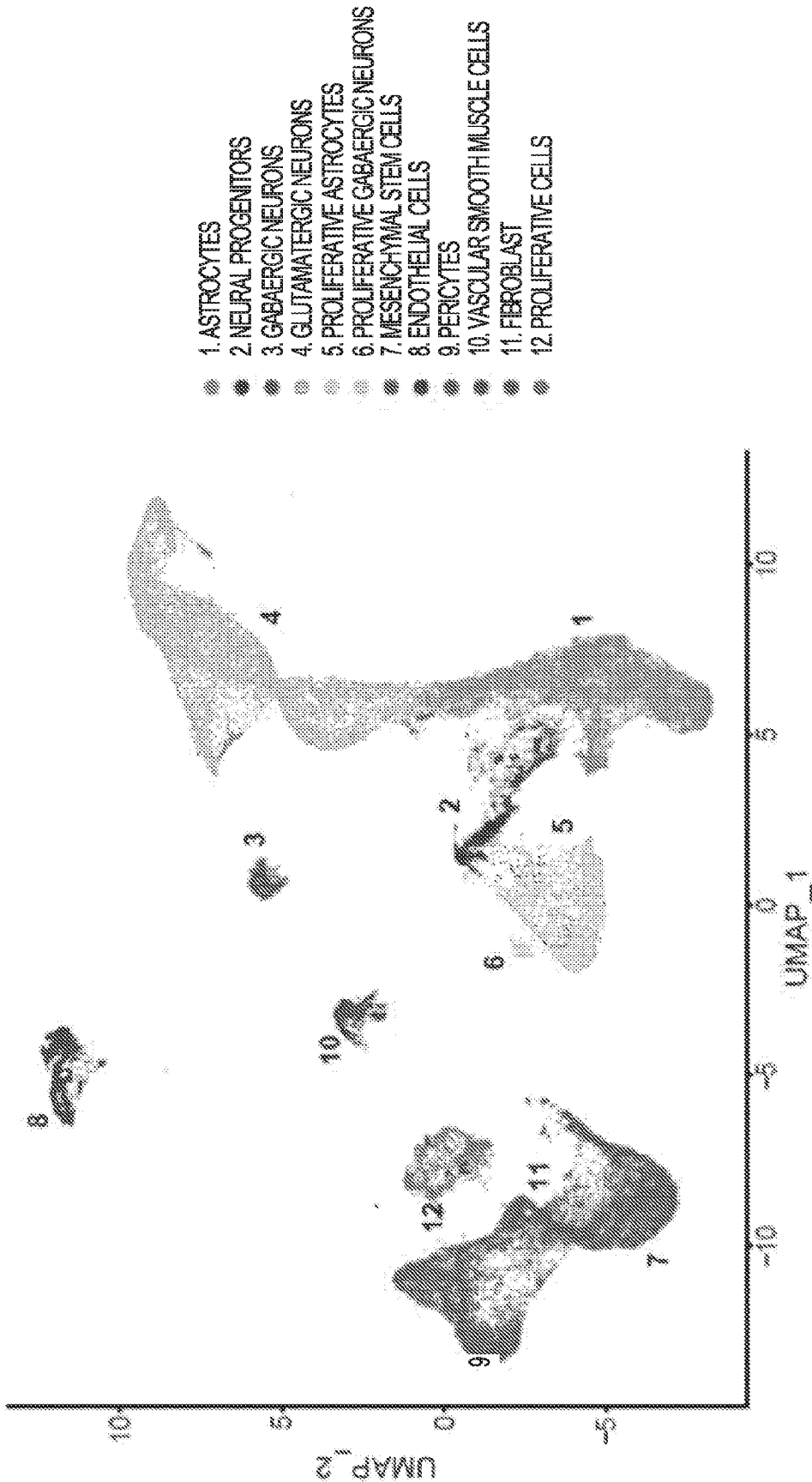


FIG. 12A

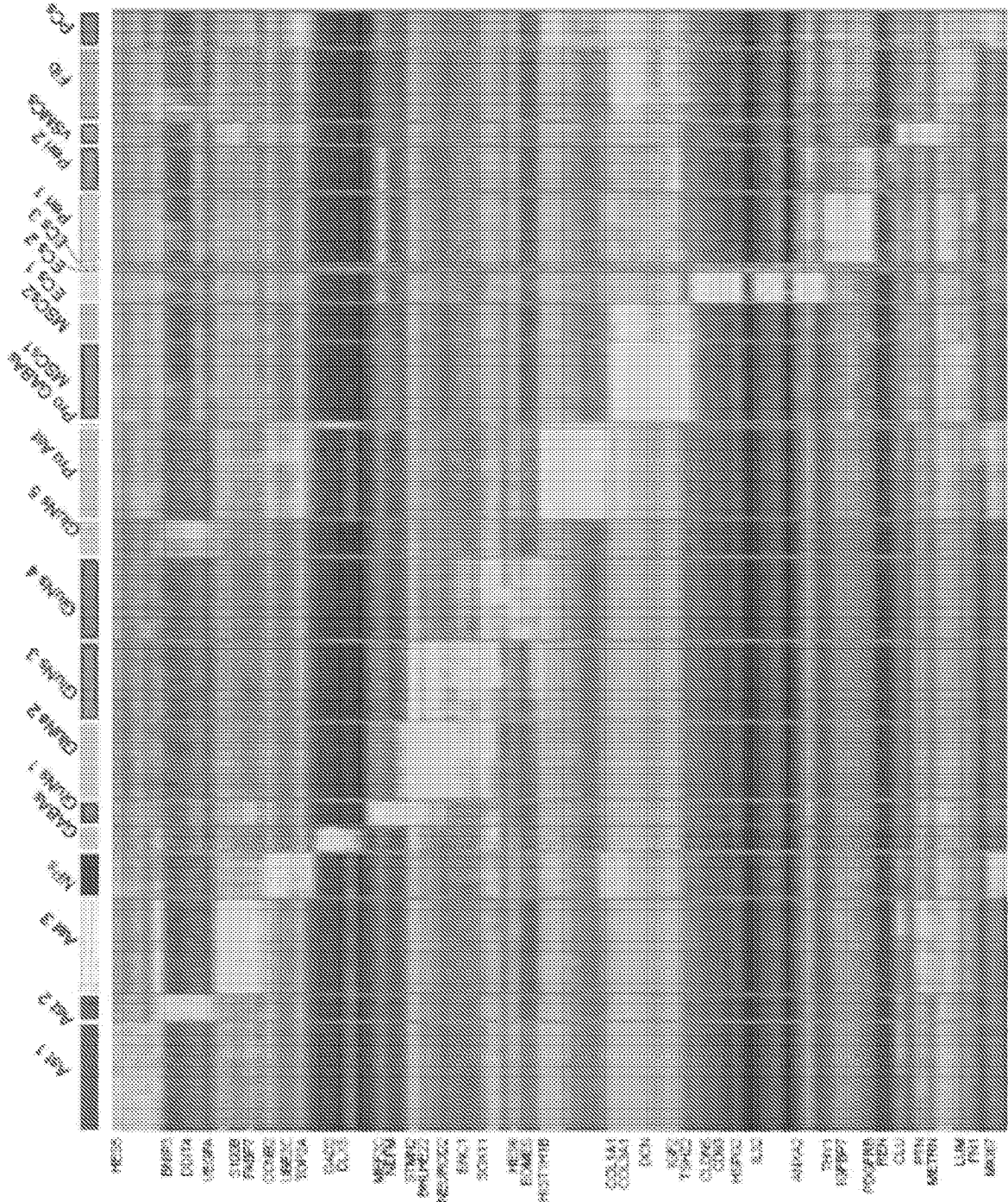
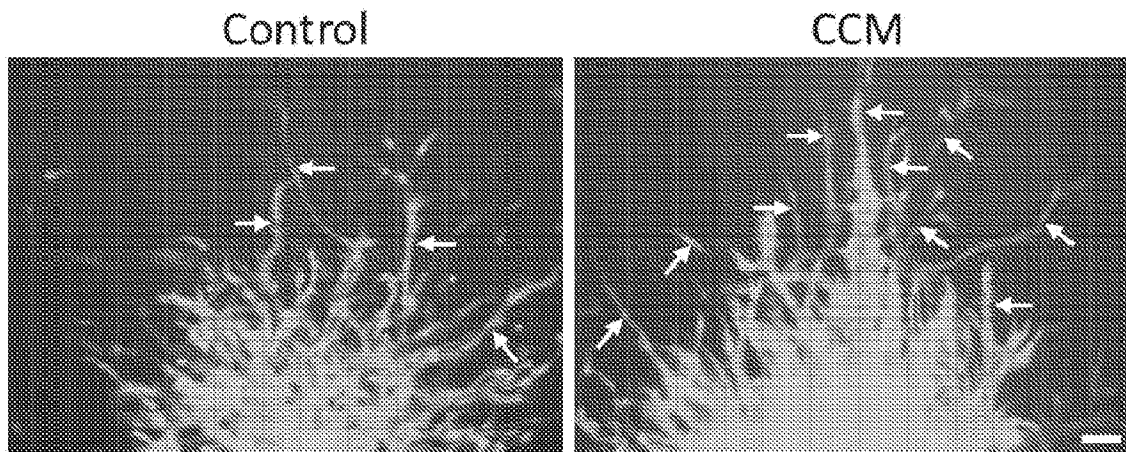
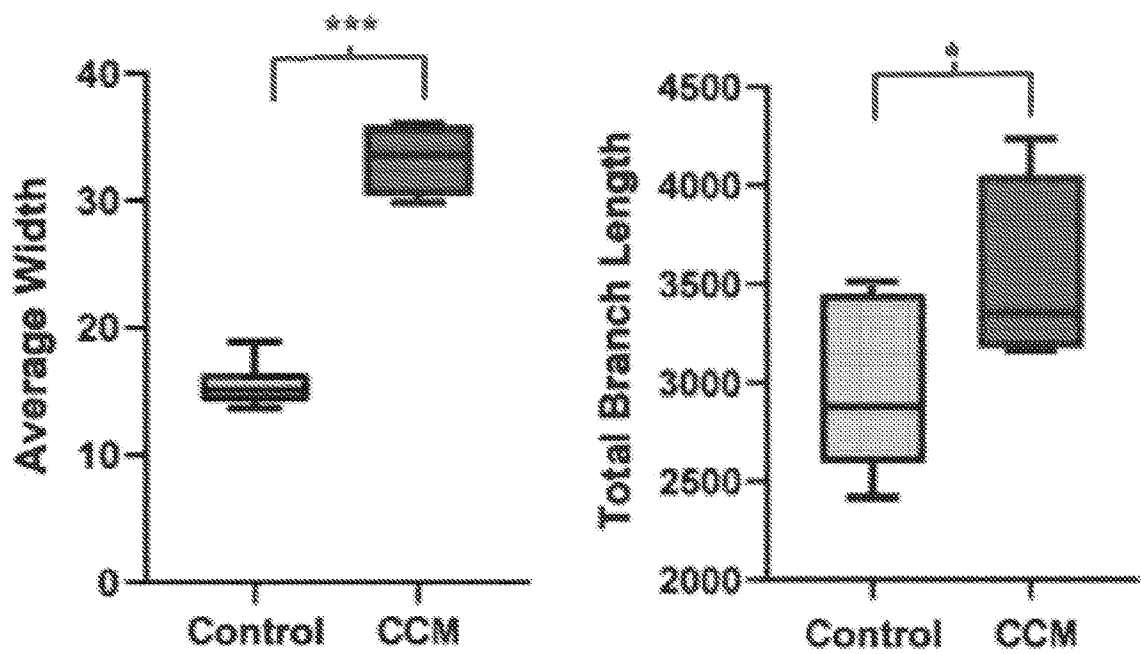


FIG. 12B

**FIG. 12C****FIG. 12D**

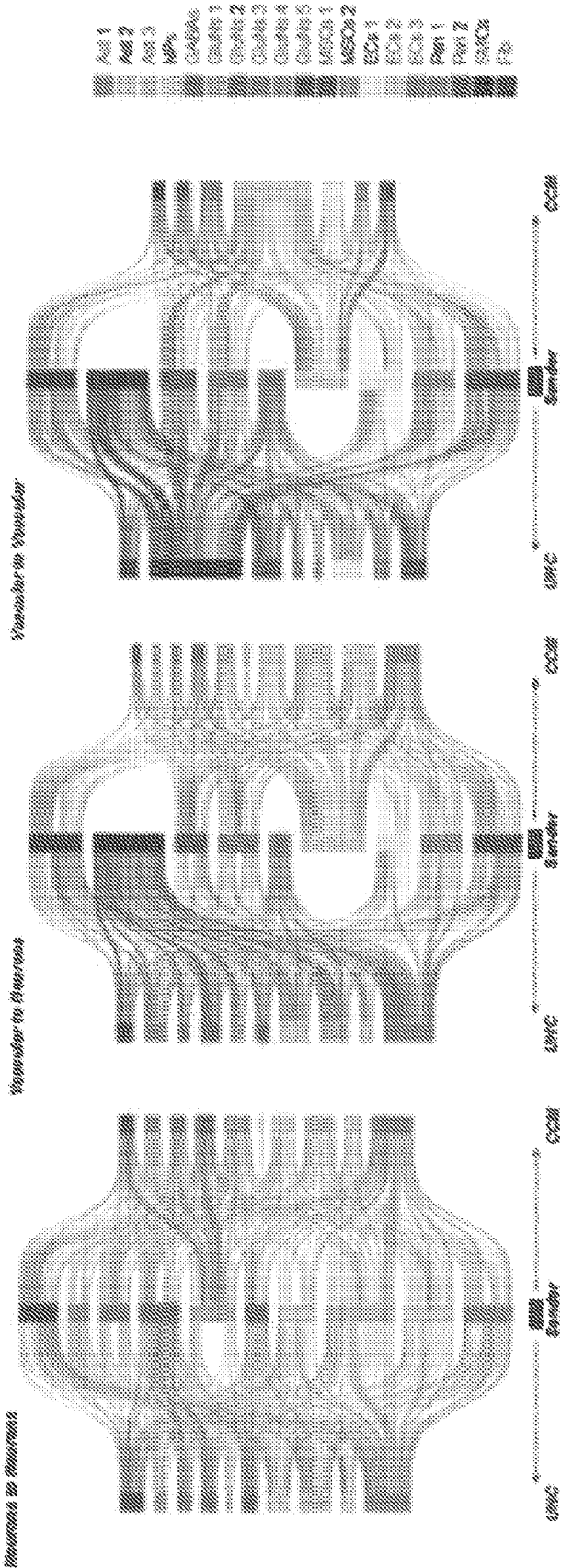


FIG. 13A

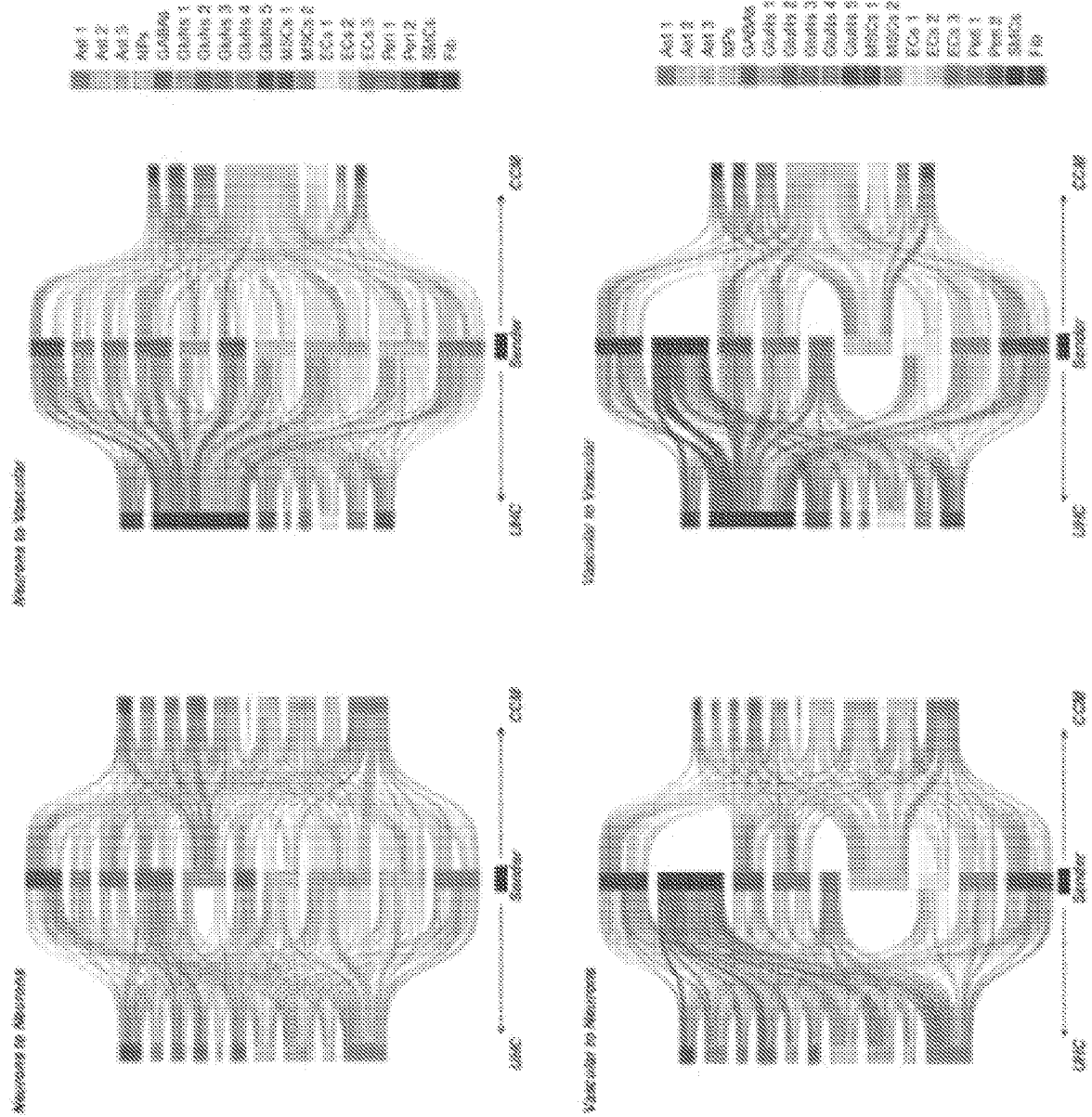


FIG. 13B

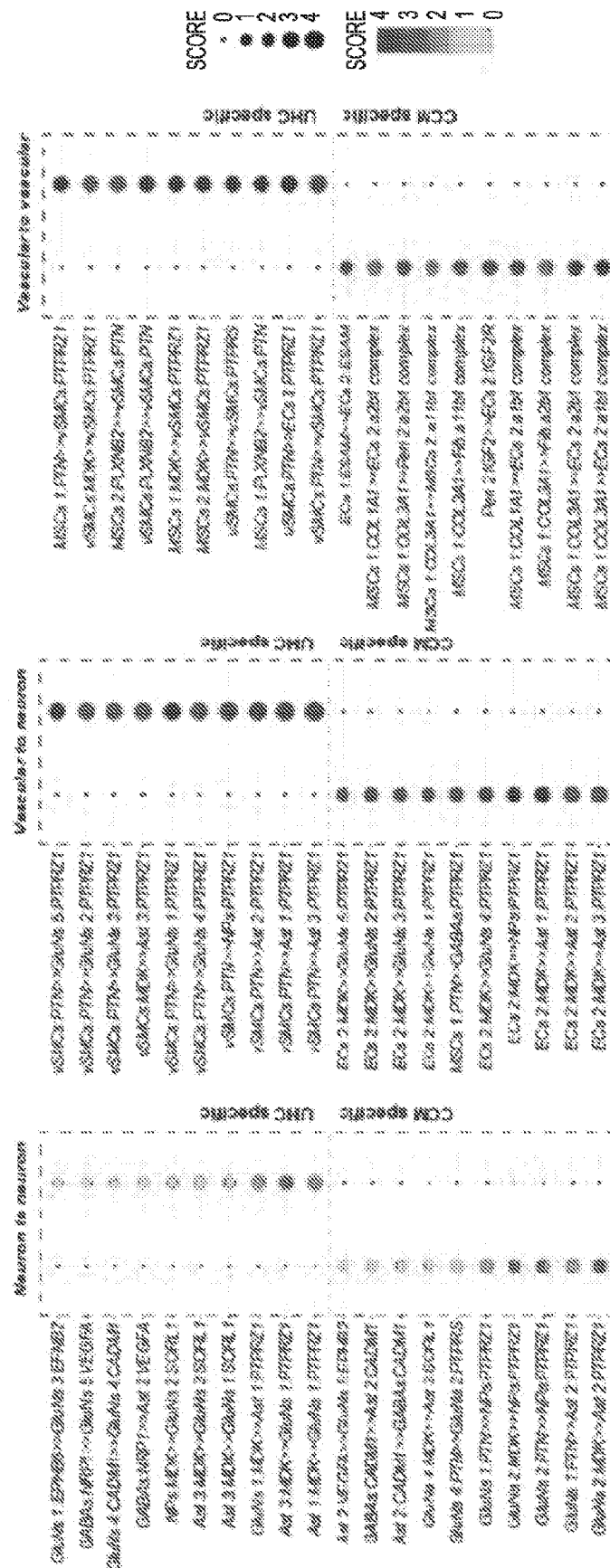


FIG. 13C

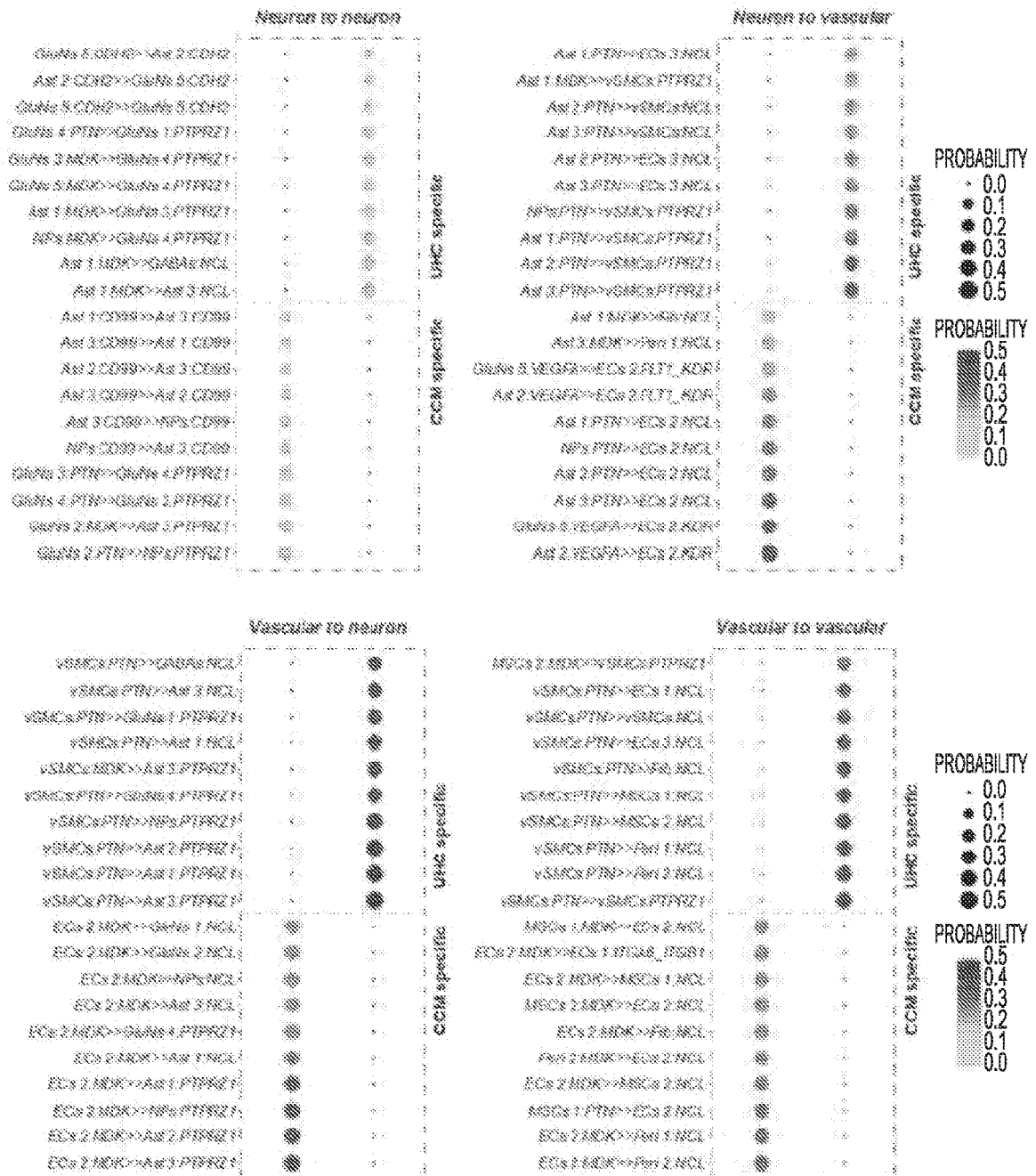


FIG. 13D

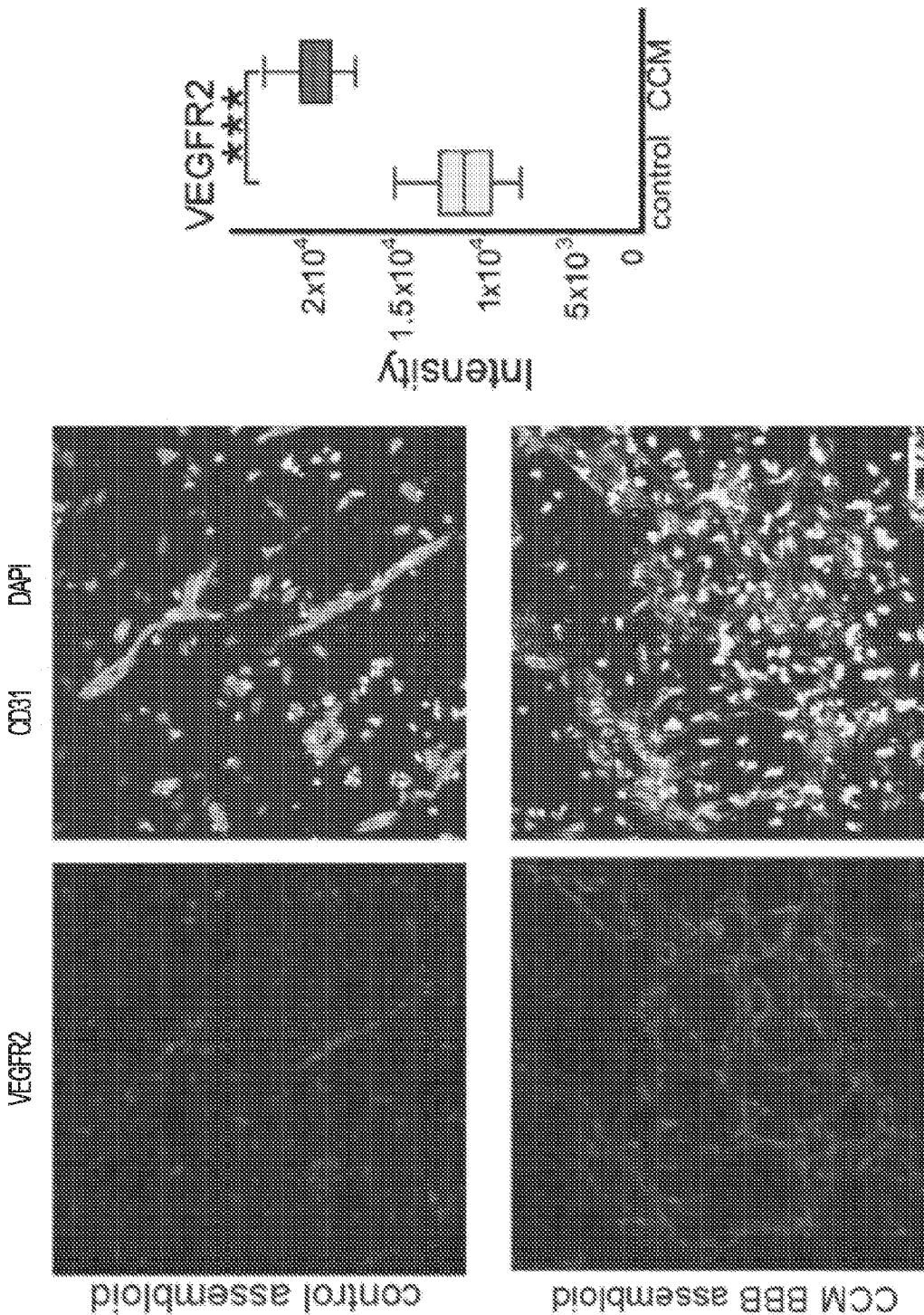


FIG. 13E

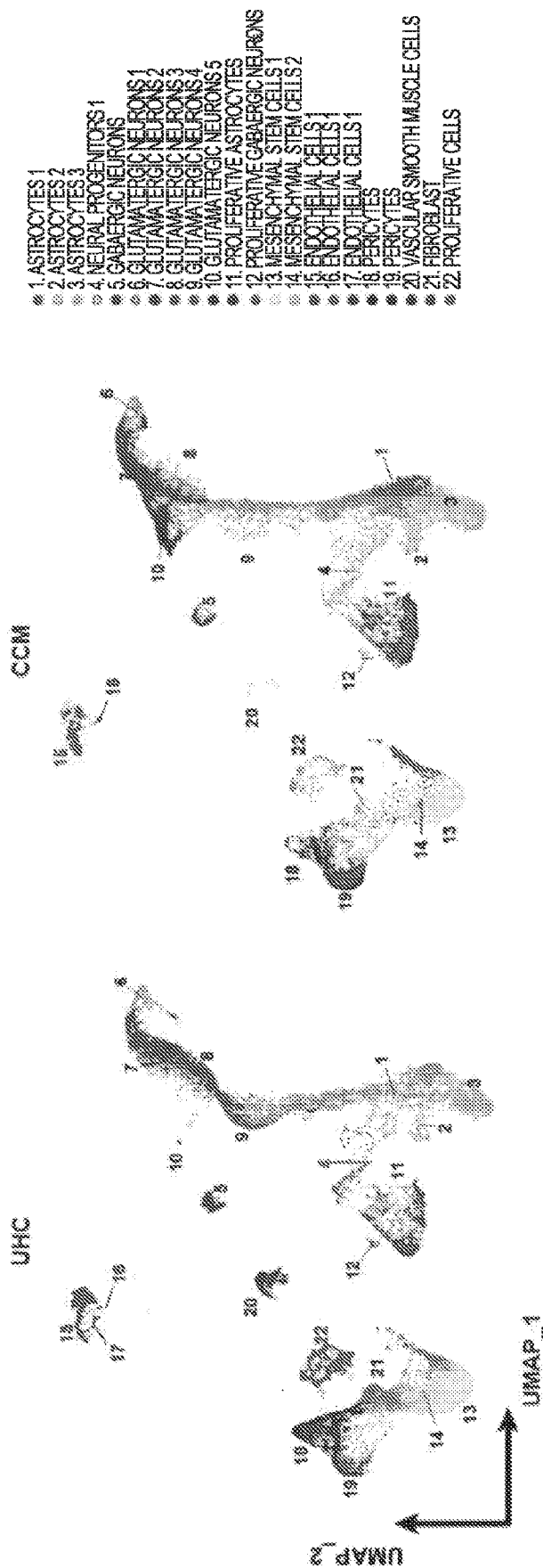
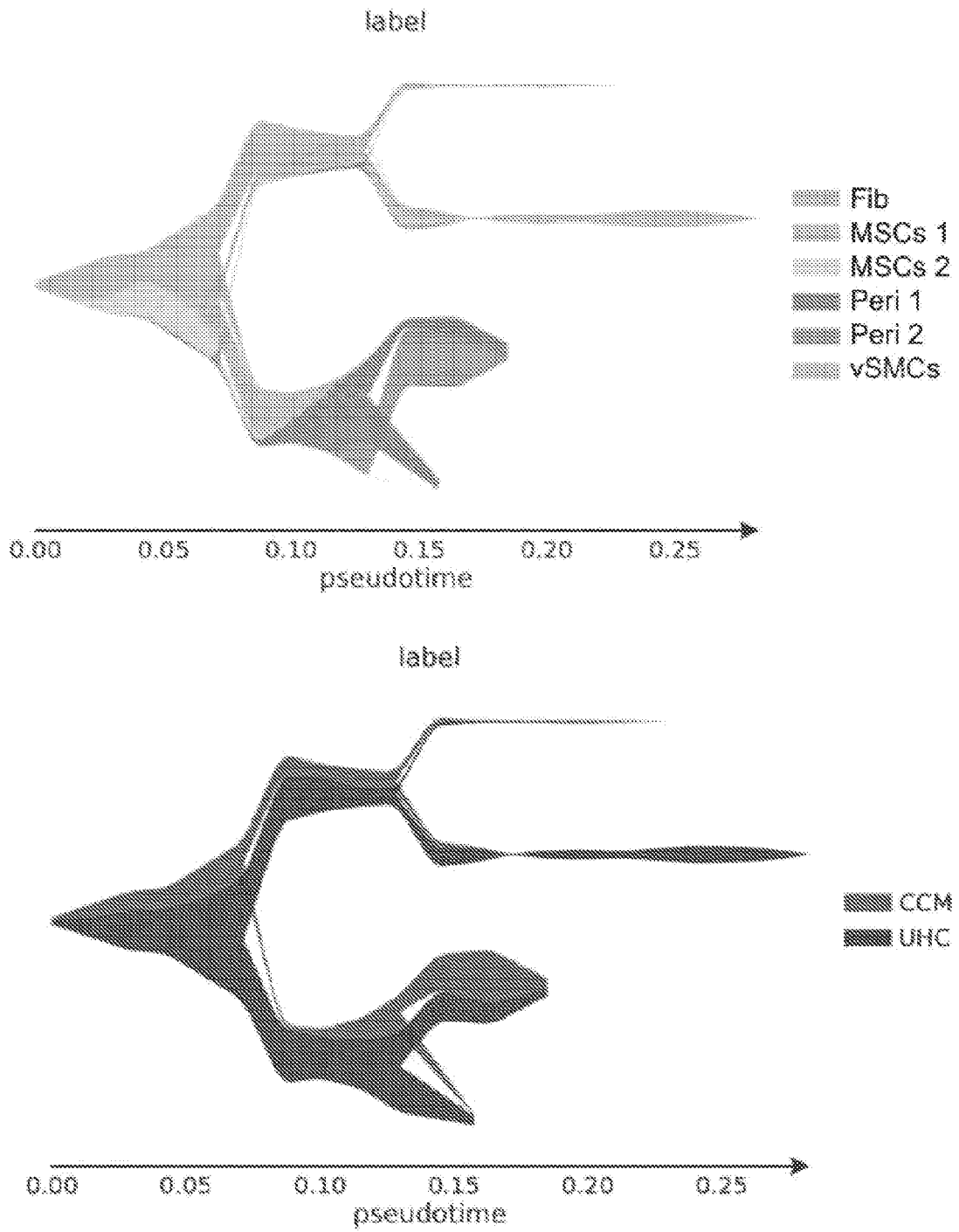


FIG. 14A

**FIG. 14B**

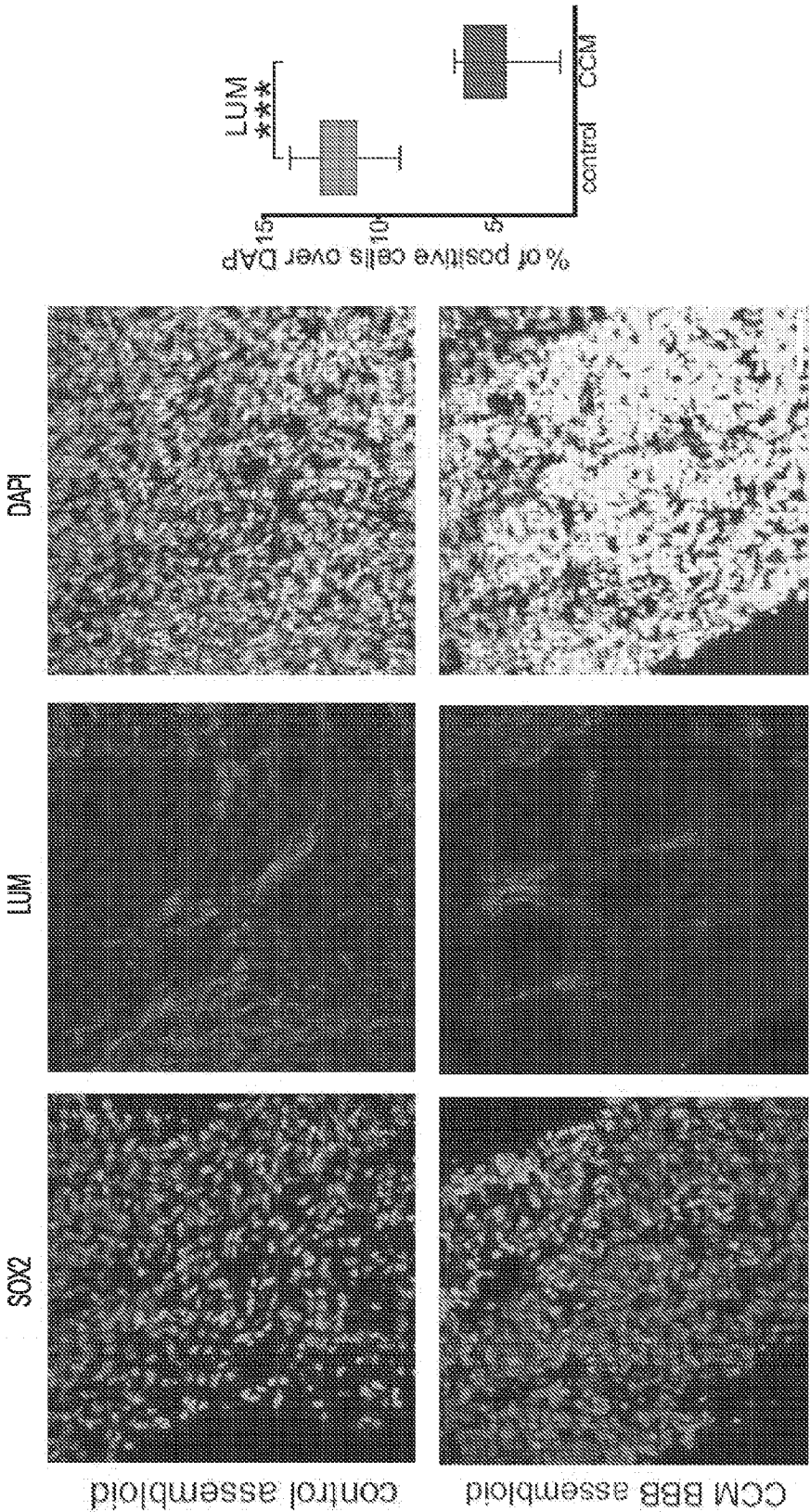


FIG. 14C

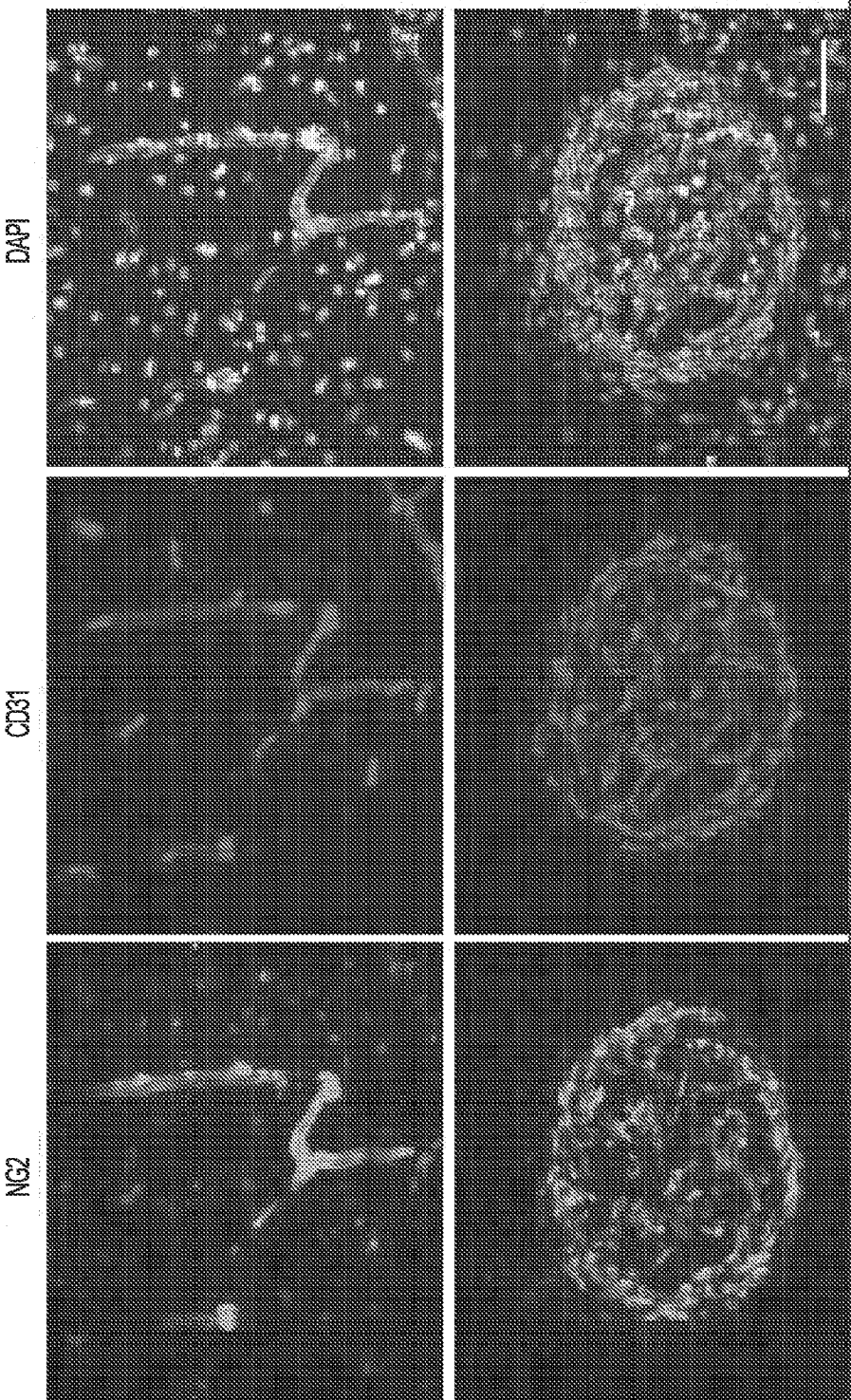


FIG. 14D

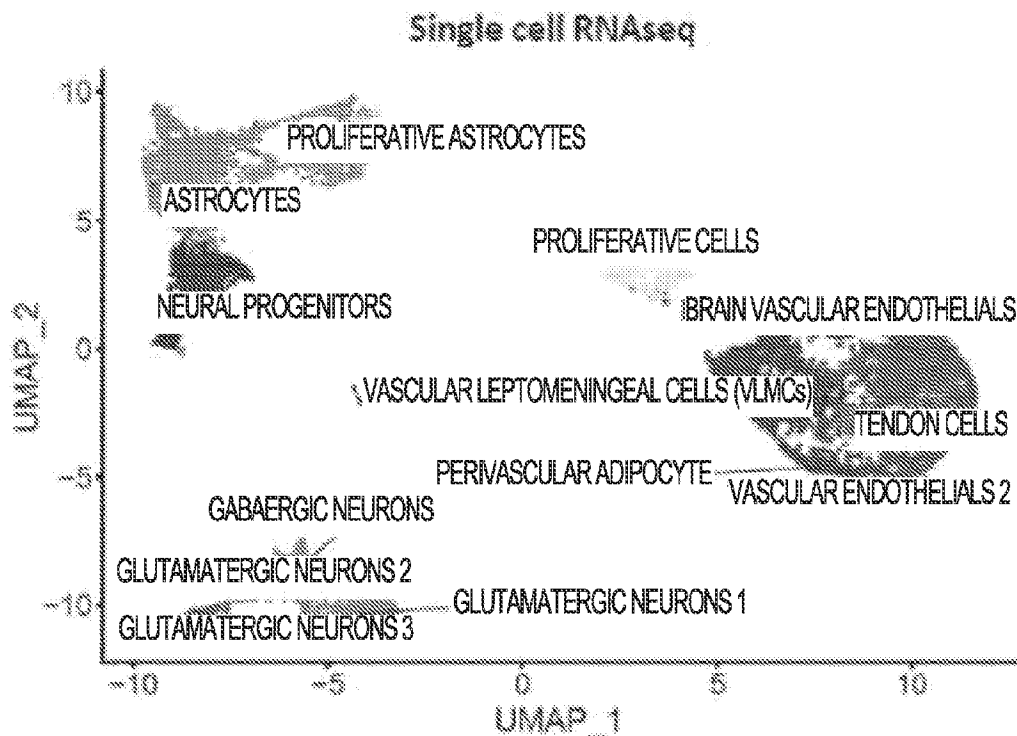


FIG. 15A

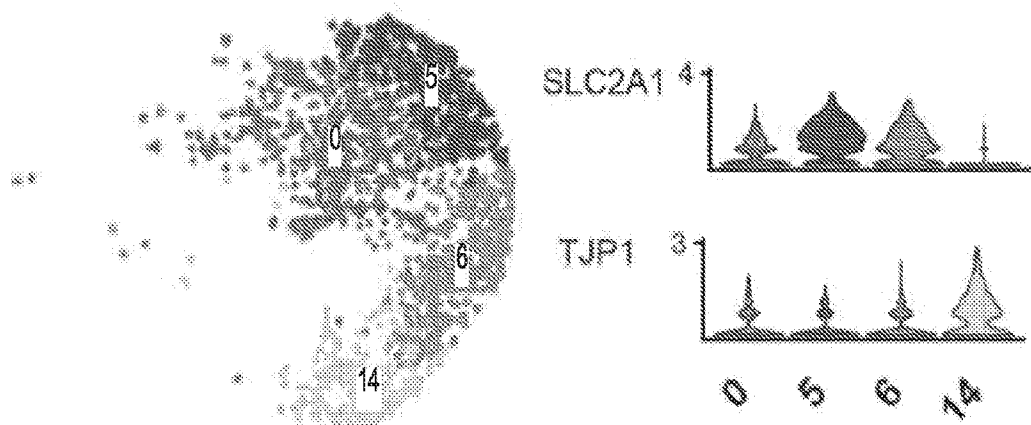


FIG. 15B

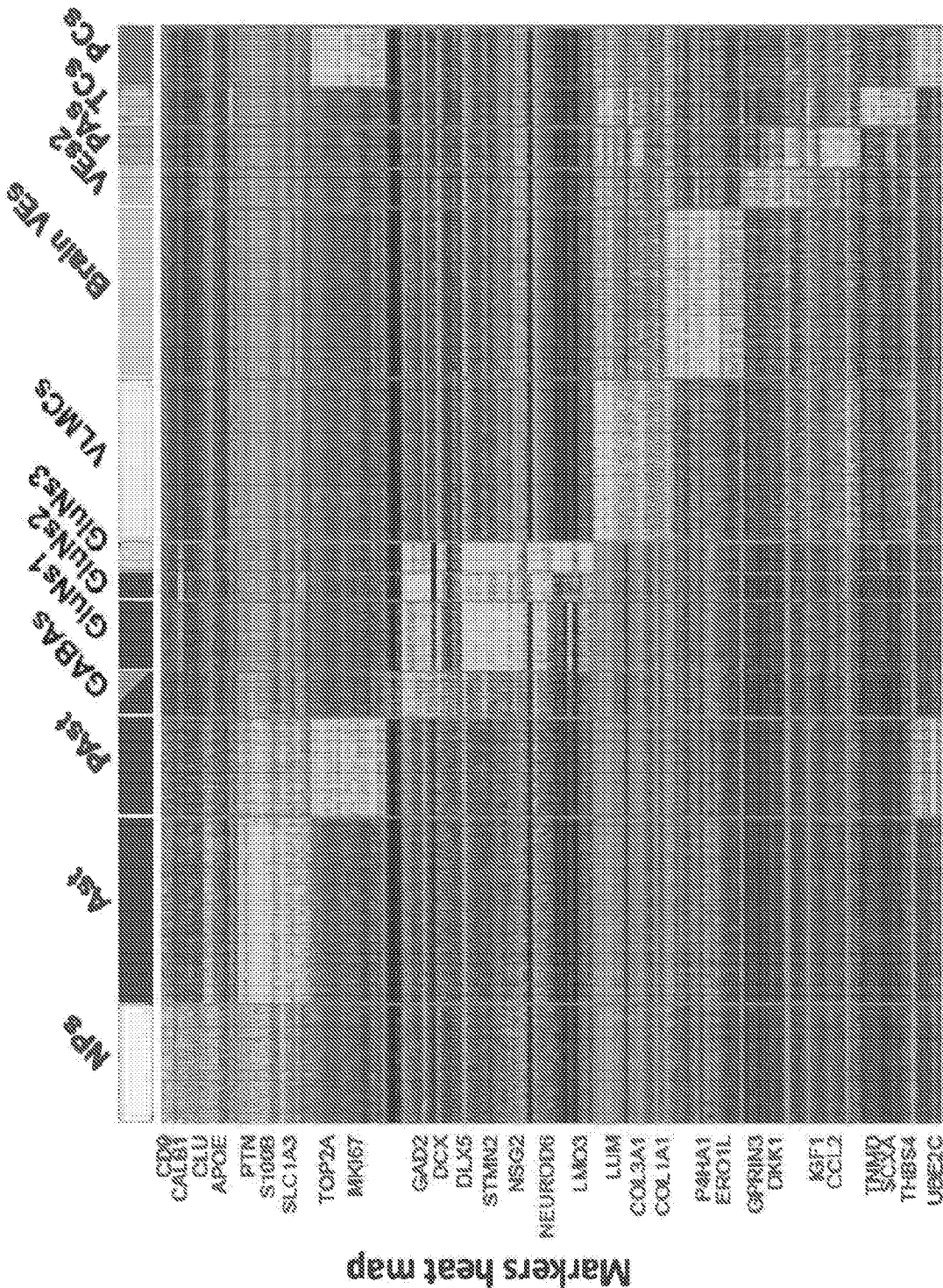


FIG. 15C

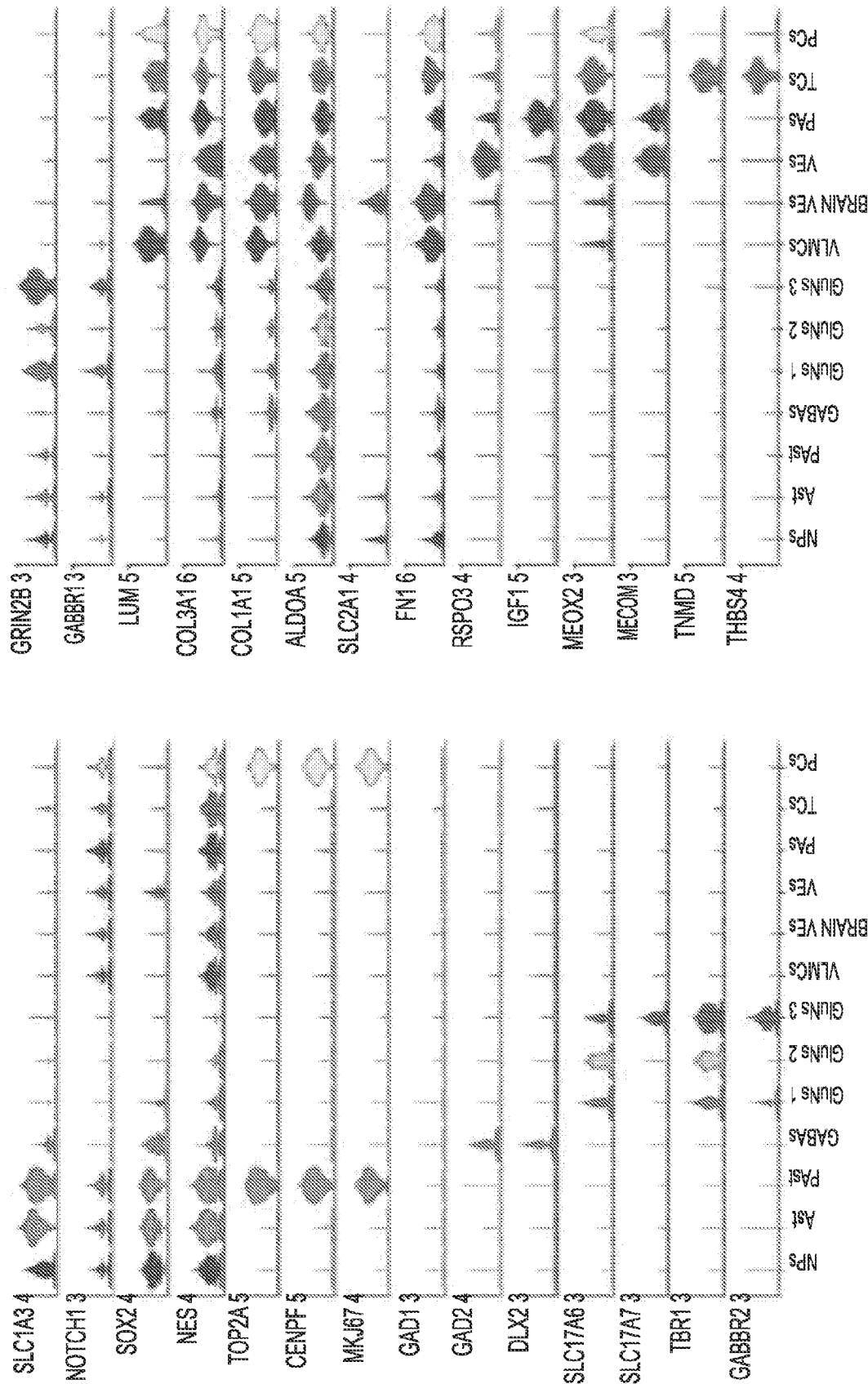
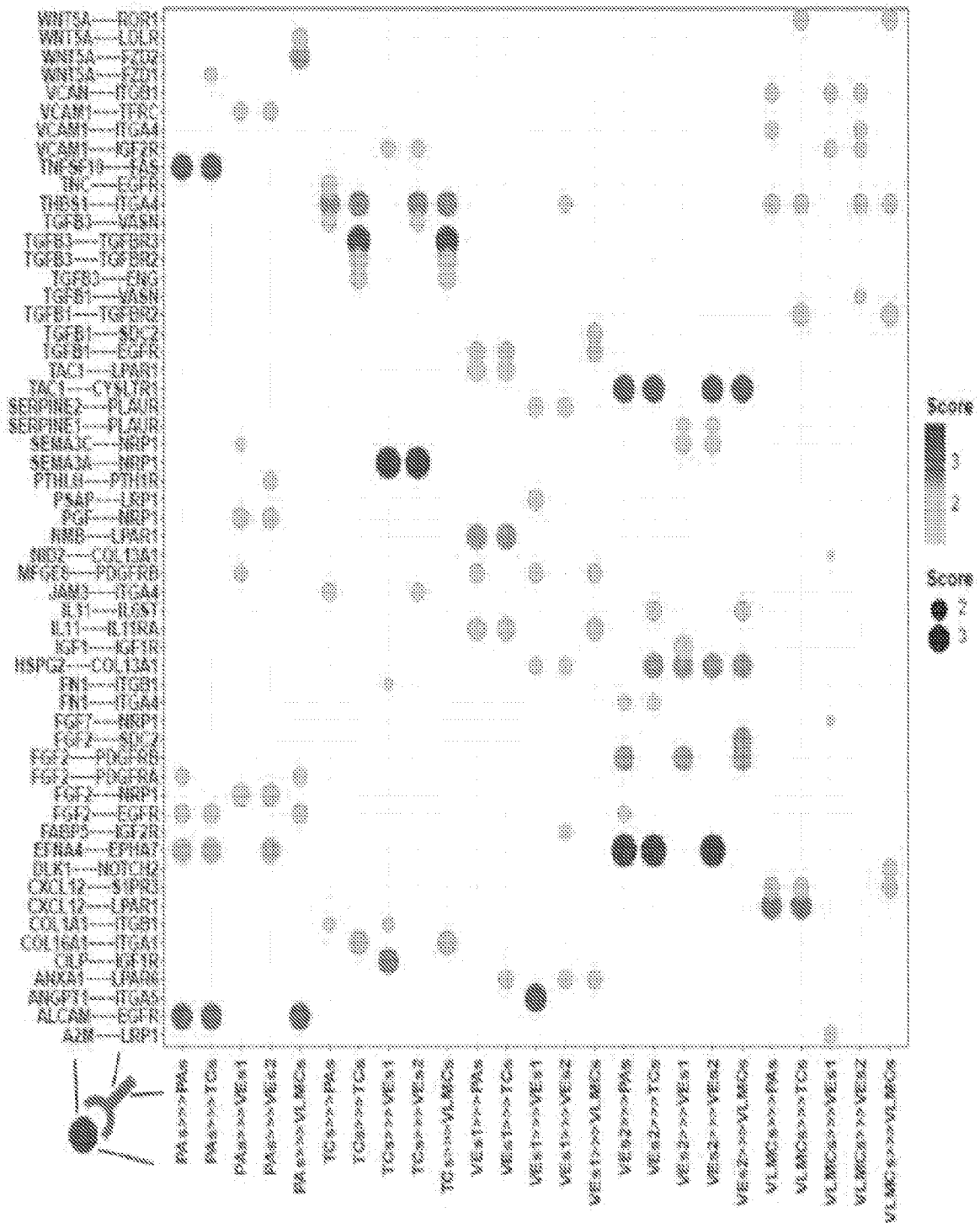
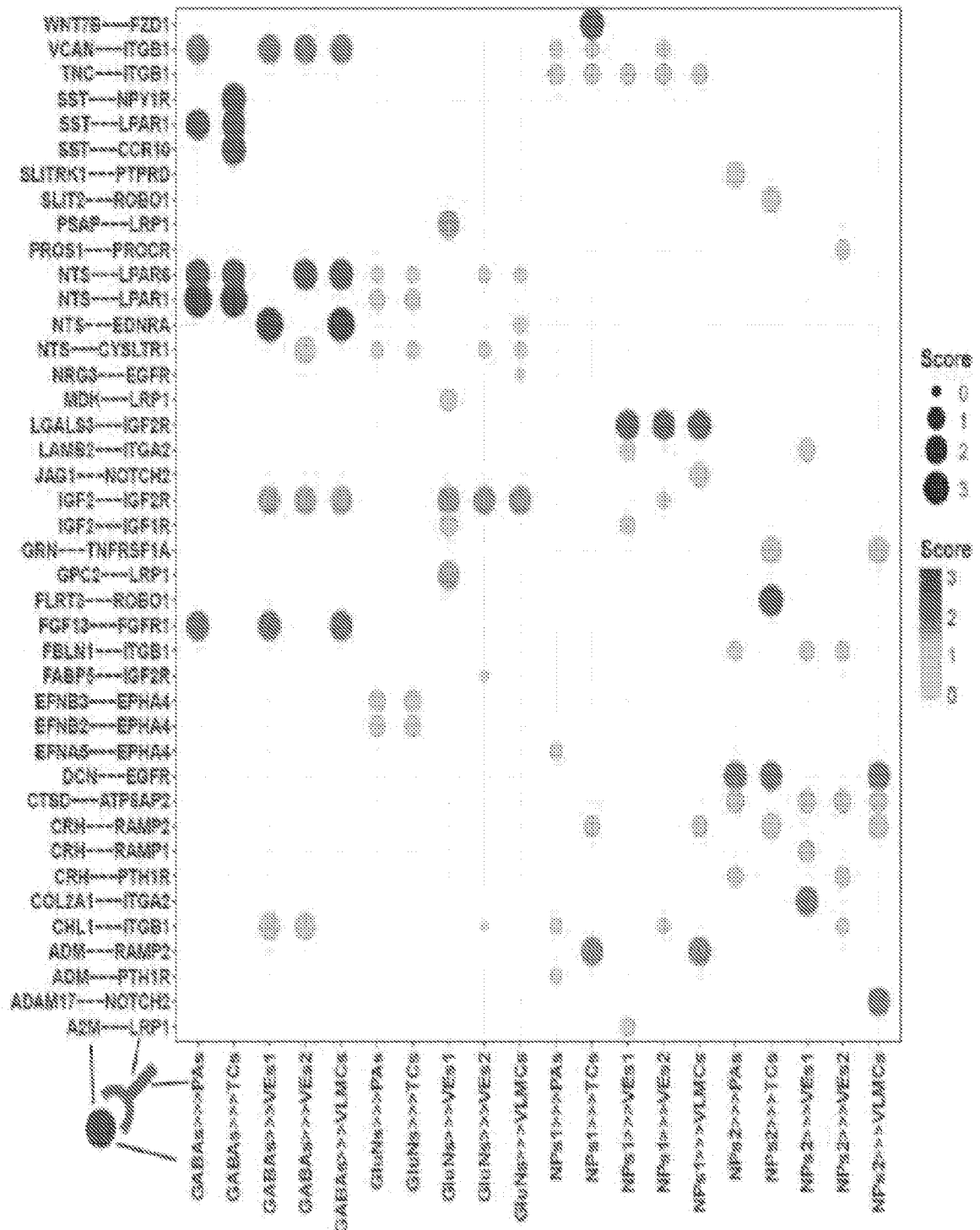
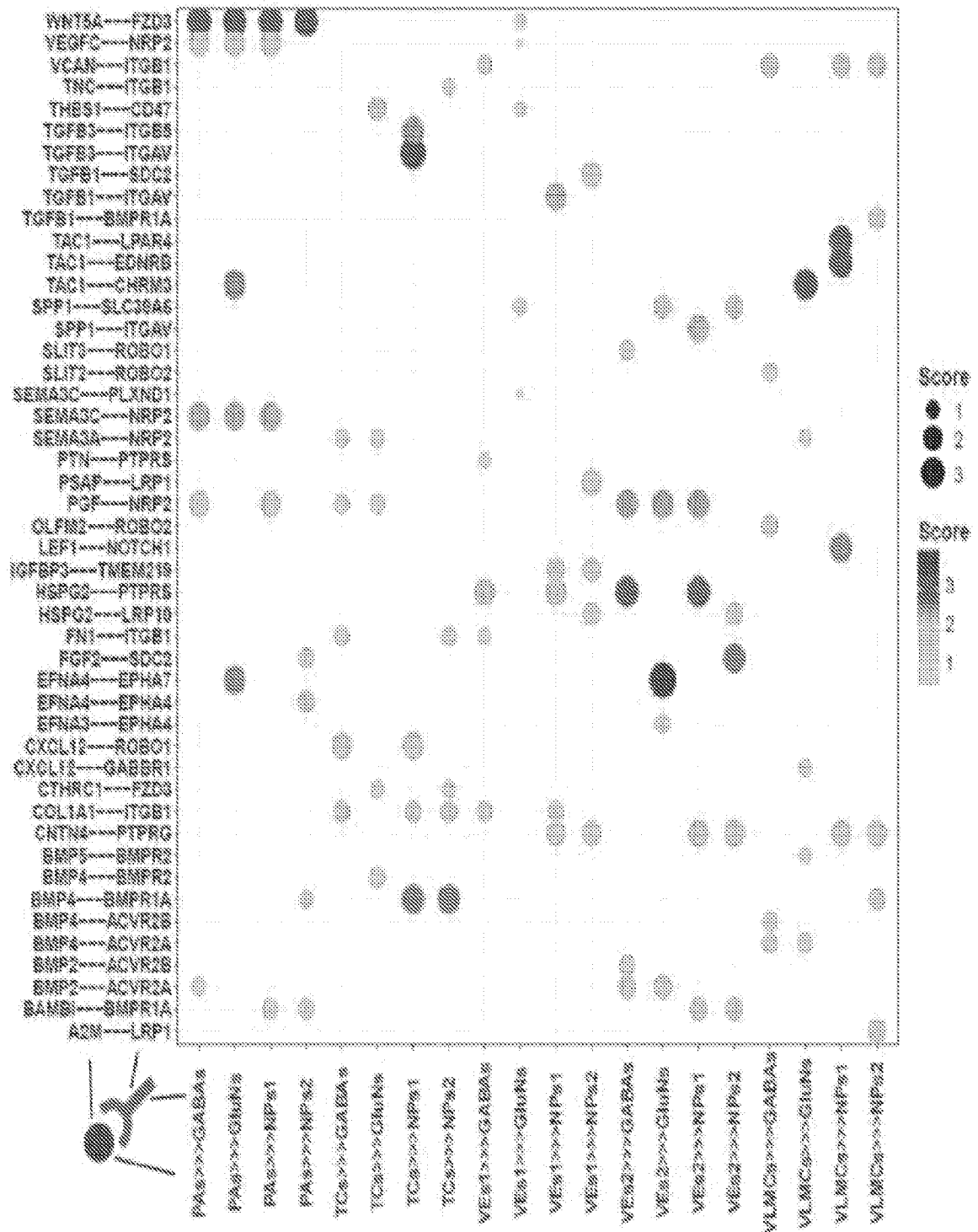
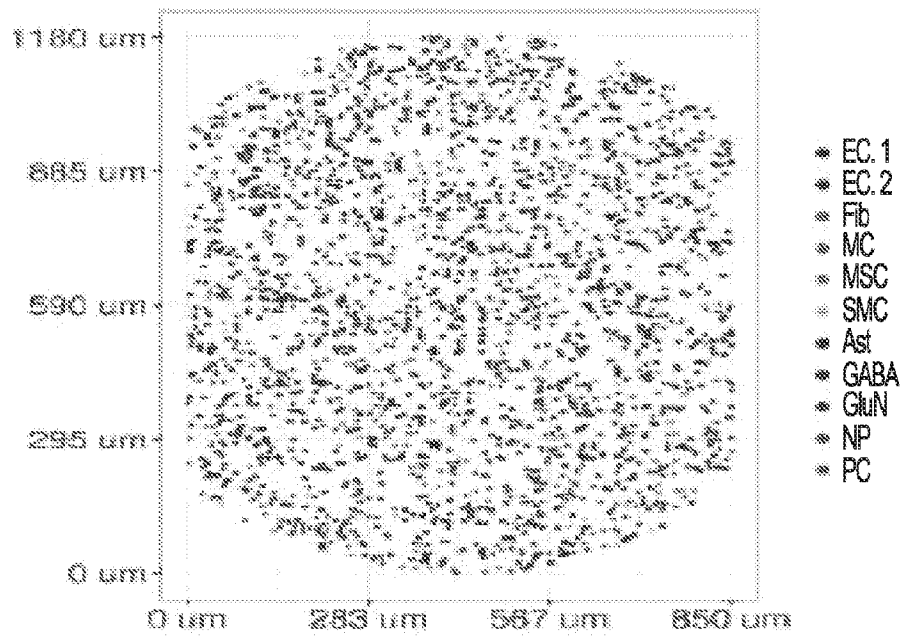
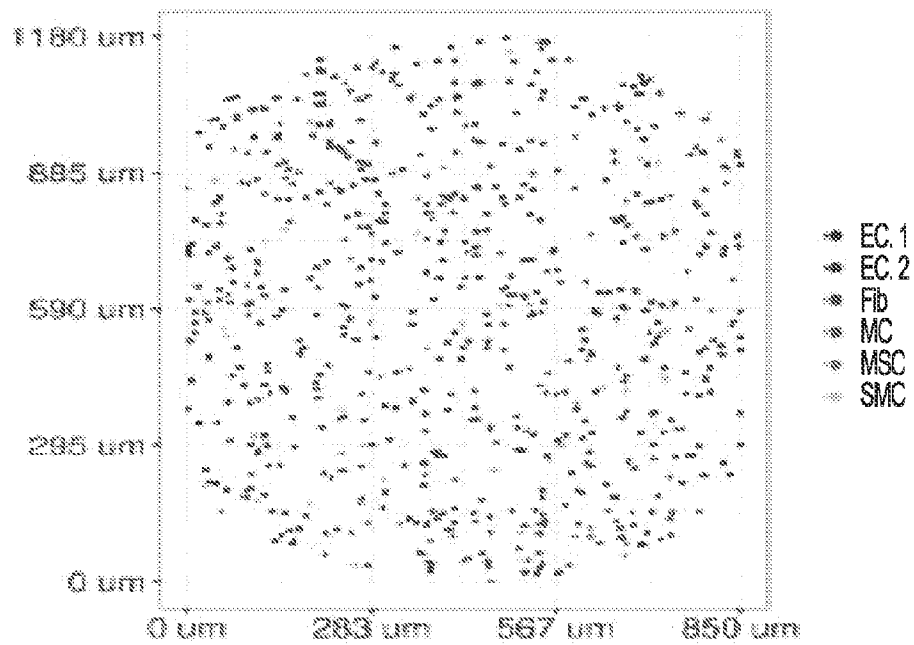


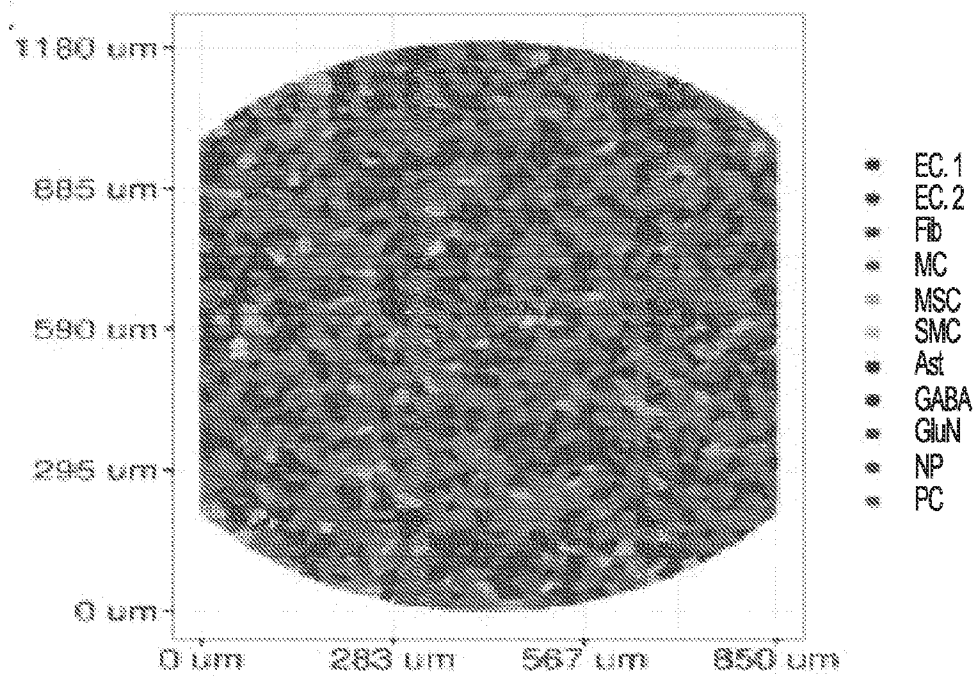
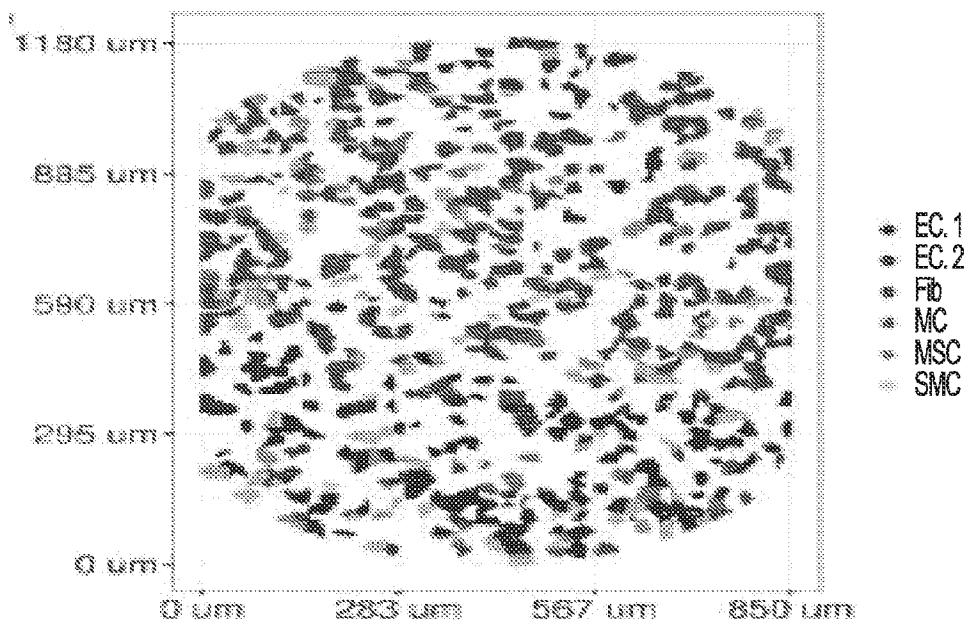
FIG. 15D



**FIG. 15F**

**FIG. 15G**

**FIG. 16A****FIG. 16B**

**FIG. 16C****FIG. 16D**

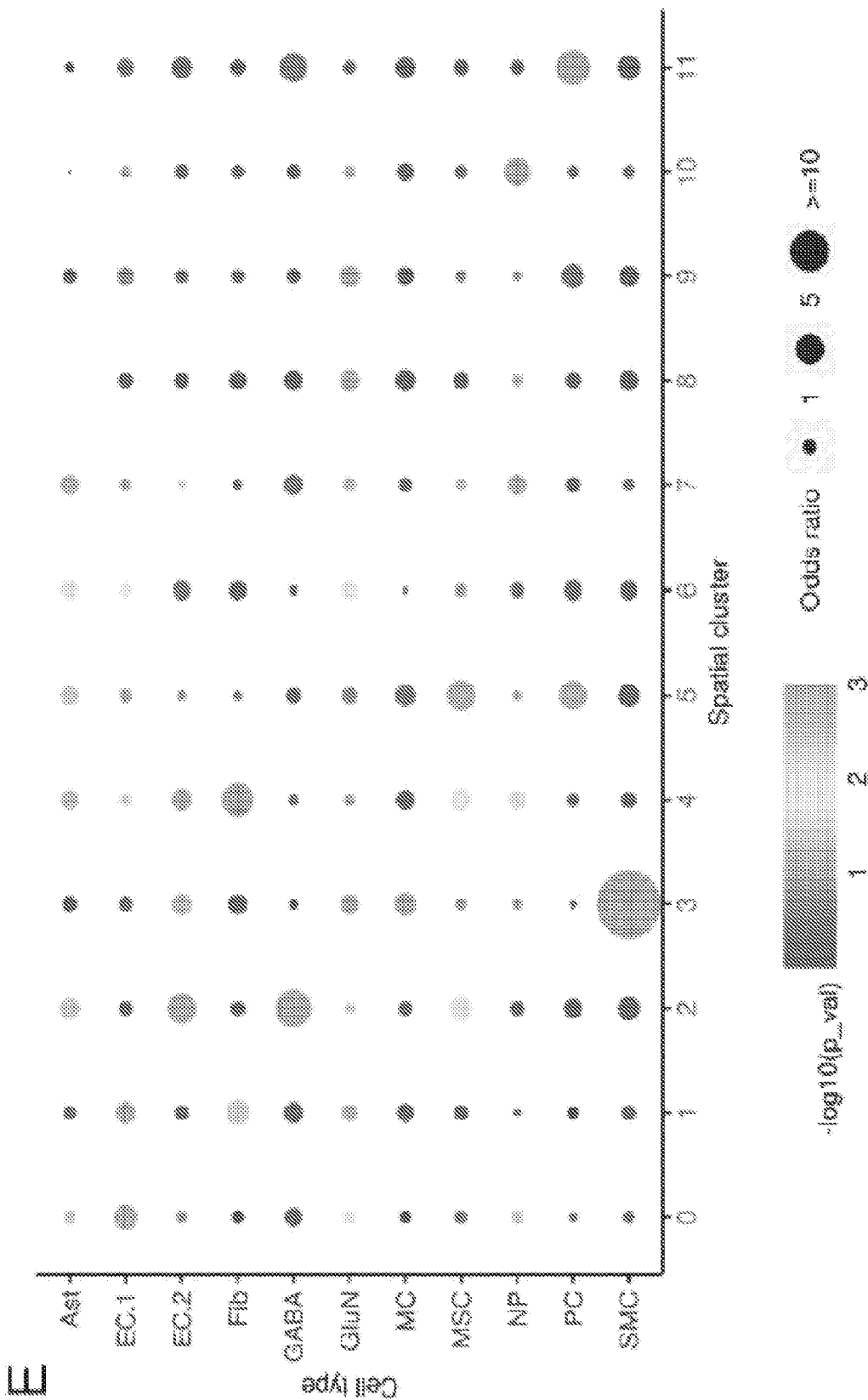
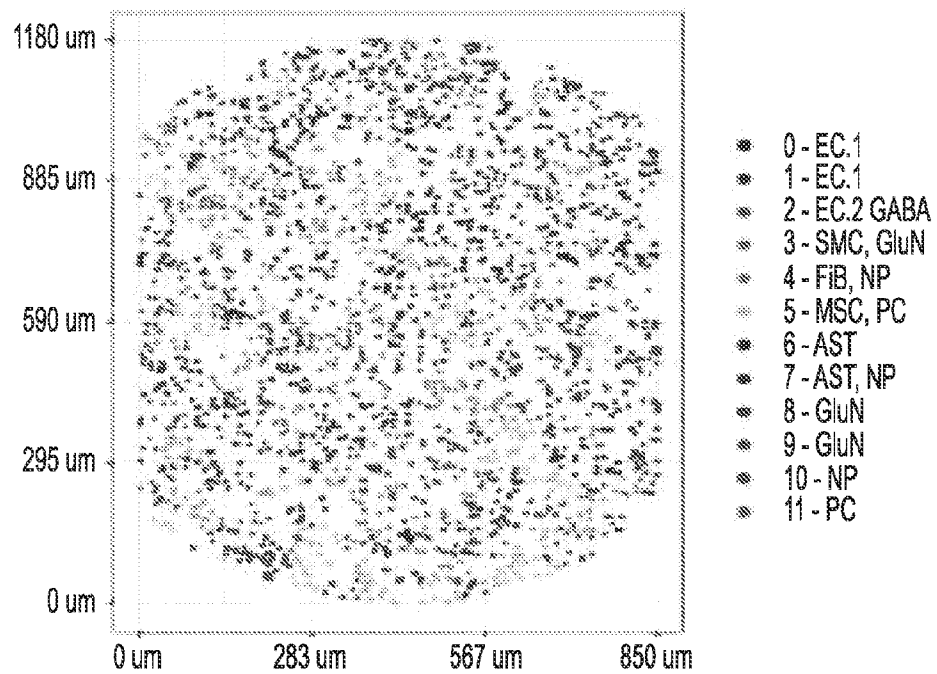
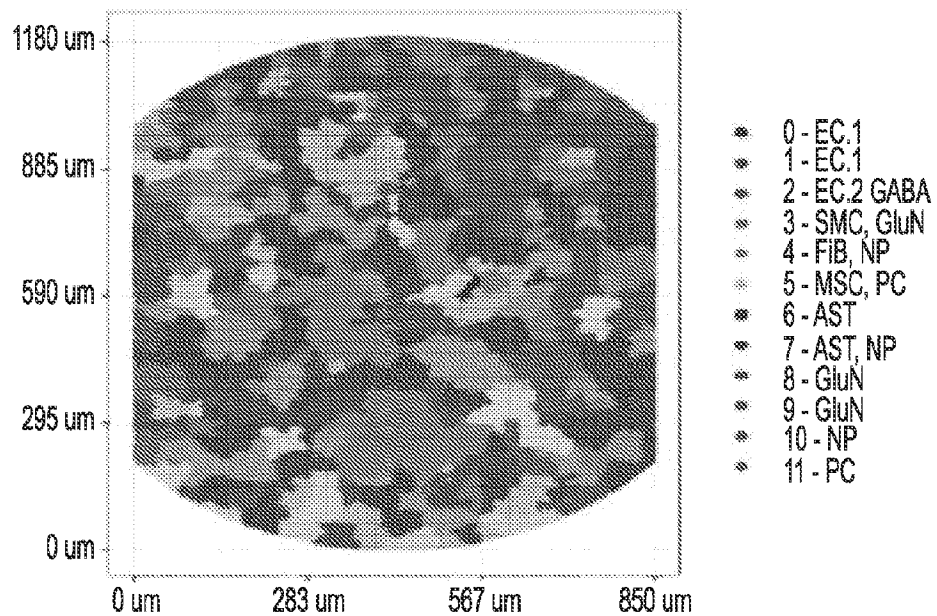


FIG. 16E

**FIG. 16F****FIG. 16G**

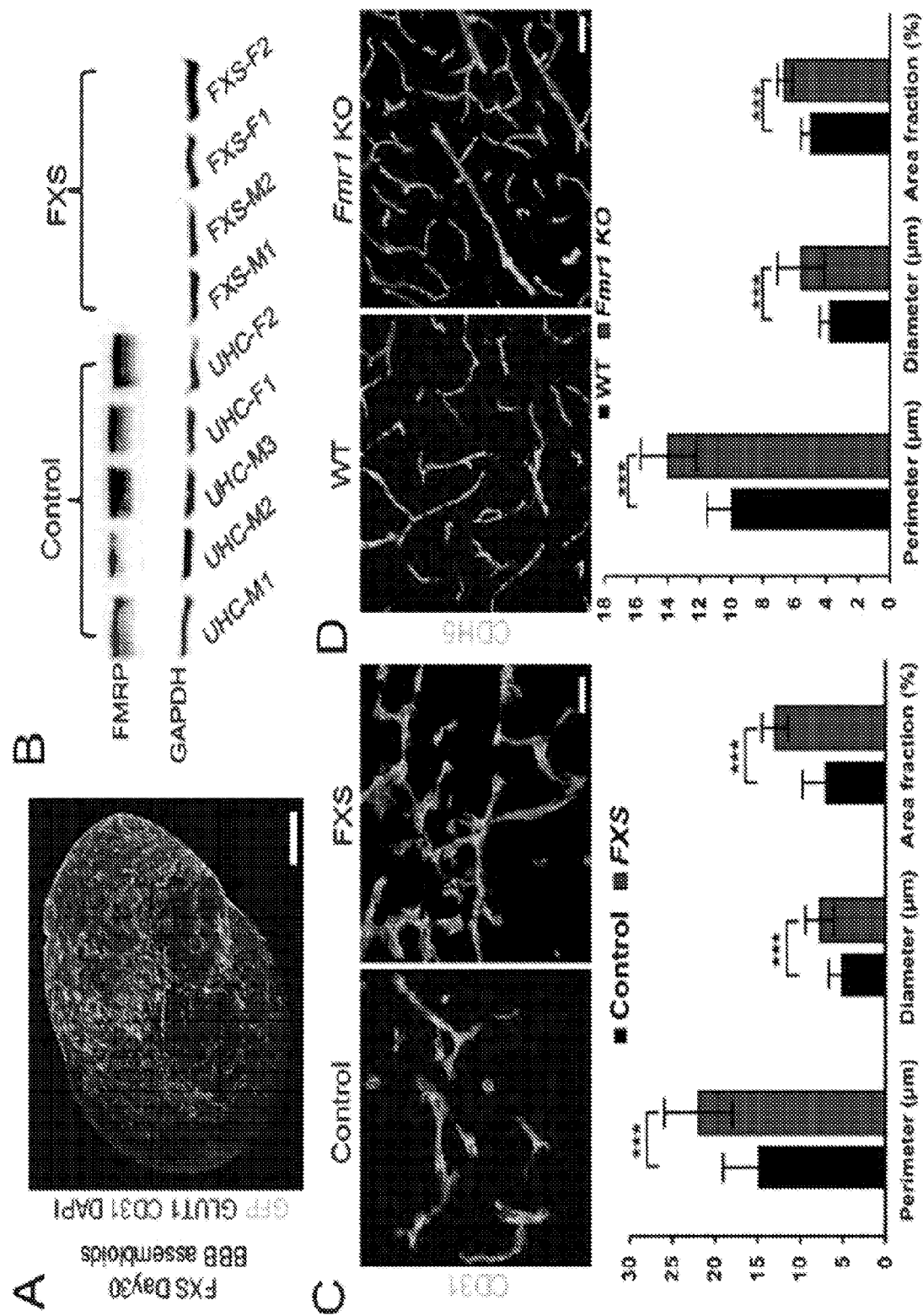


FIG. 17

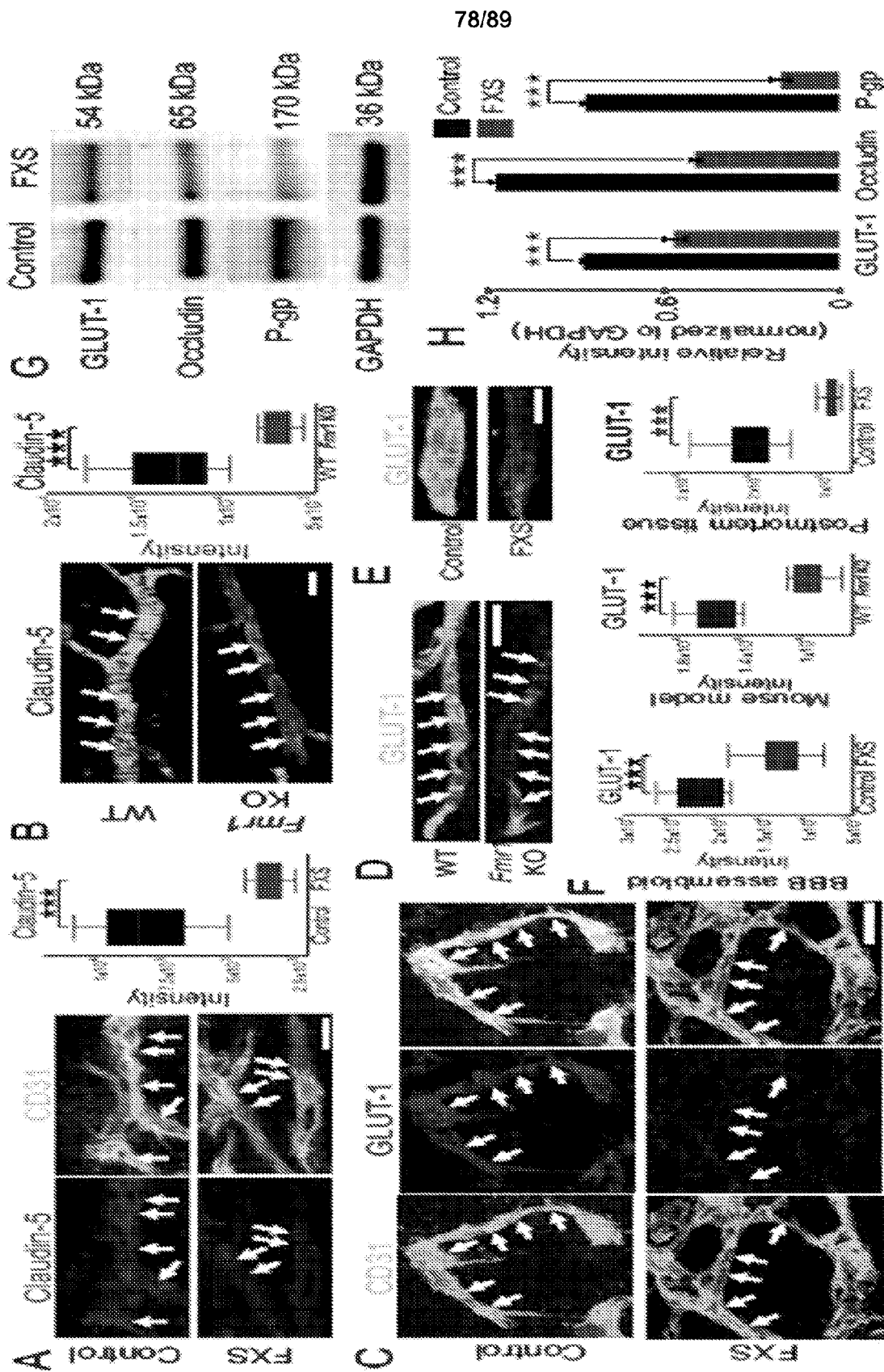


FIG. 18

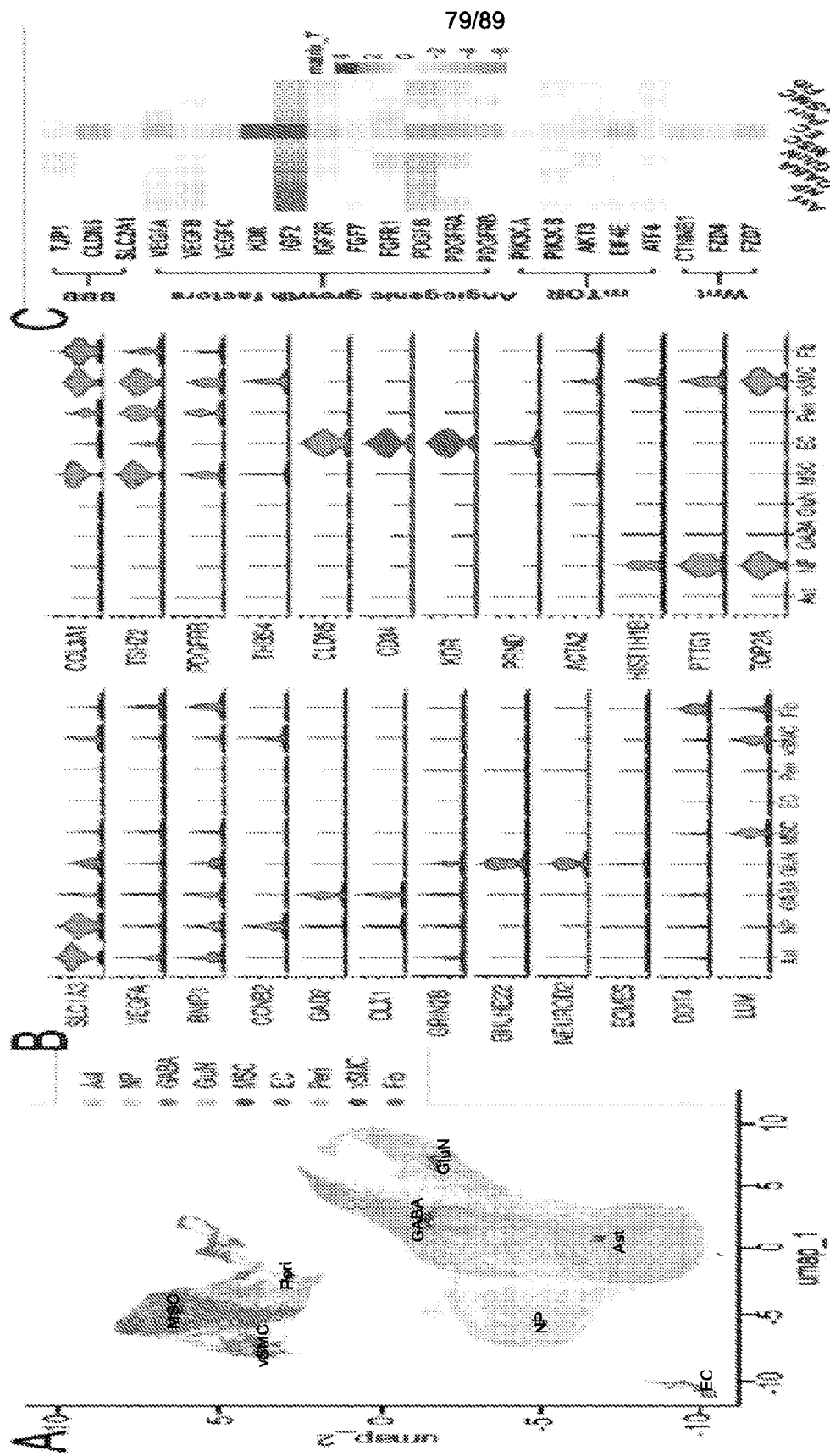


FIG. 19

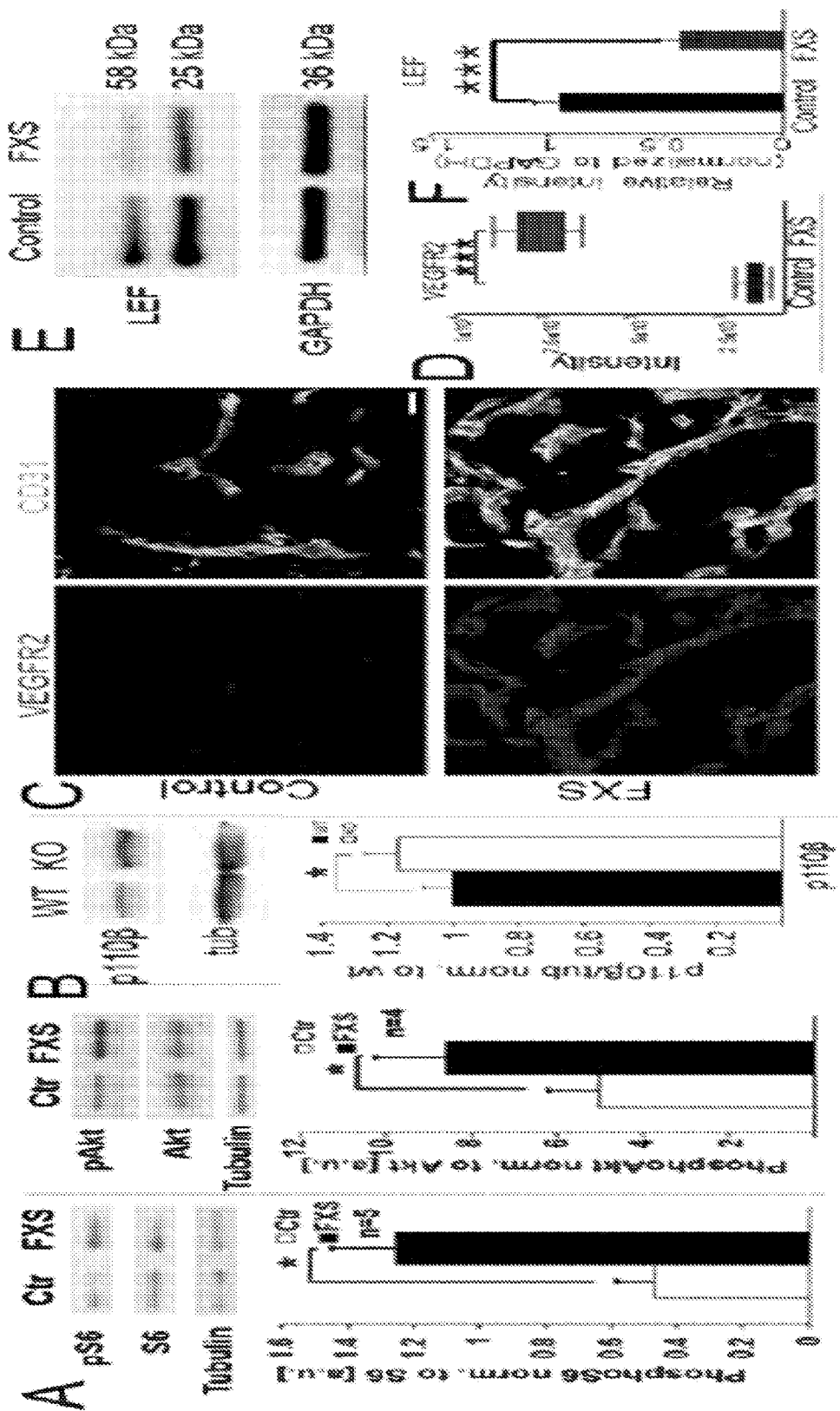
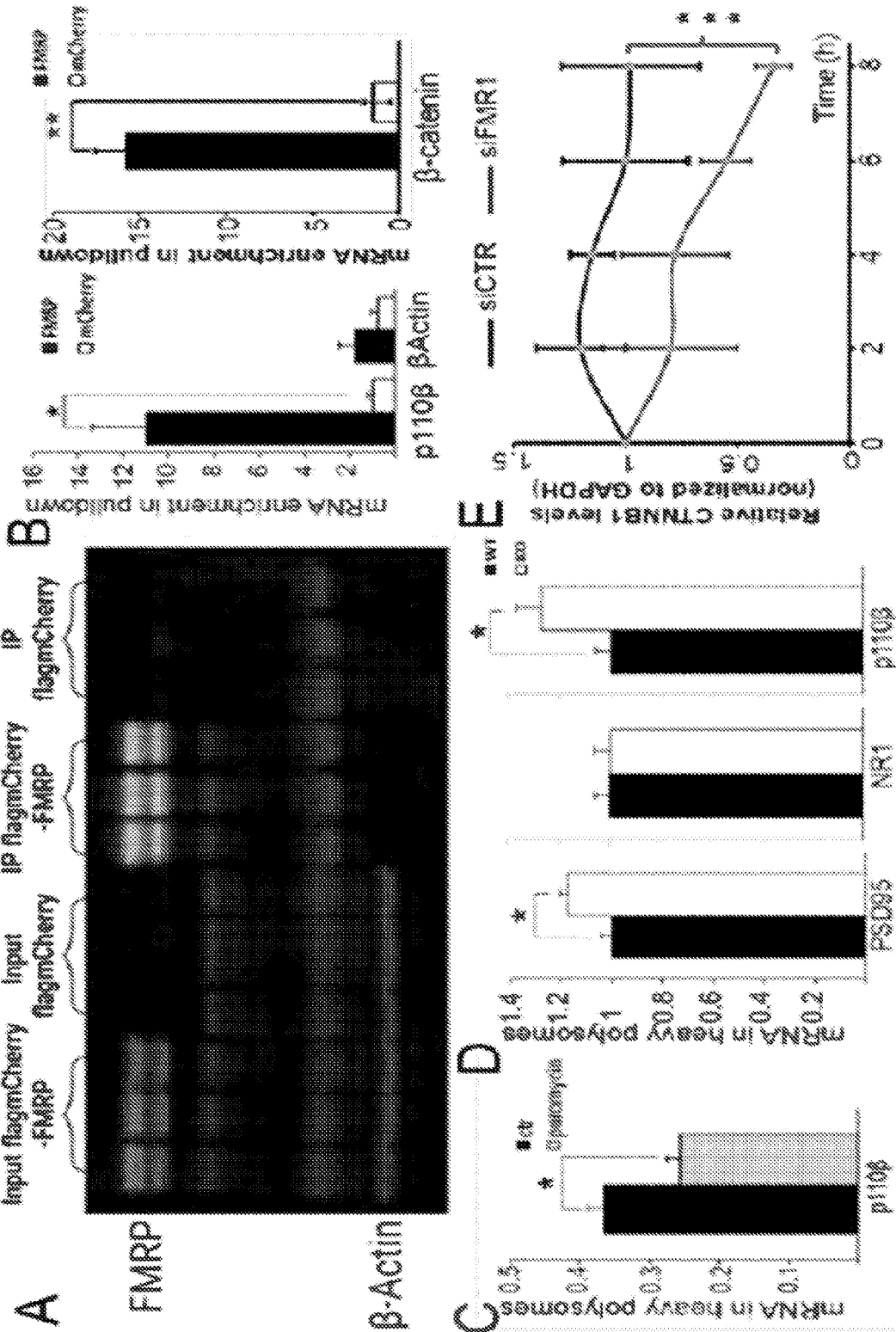


FIG. 20



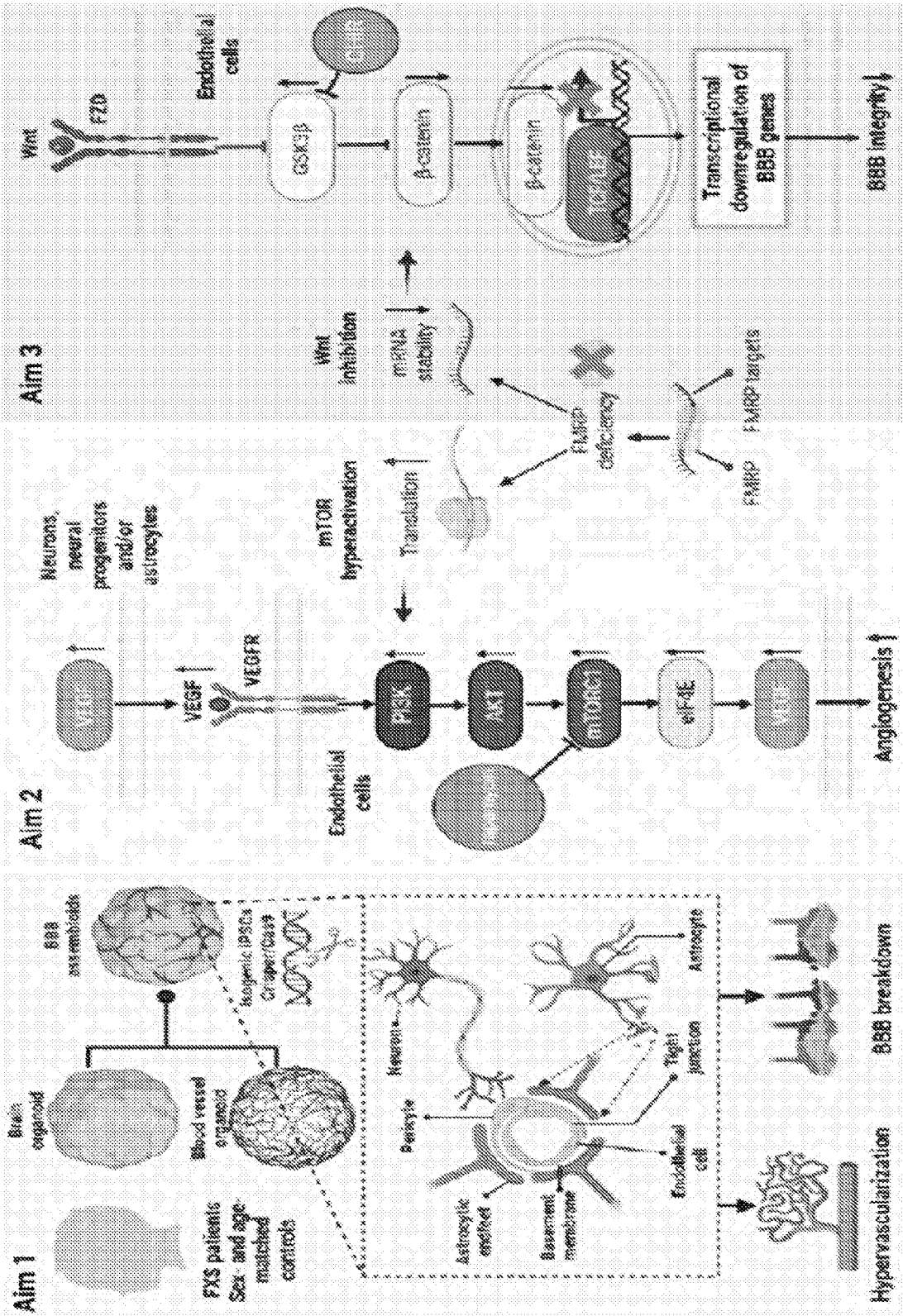
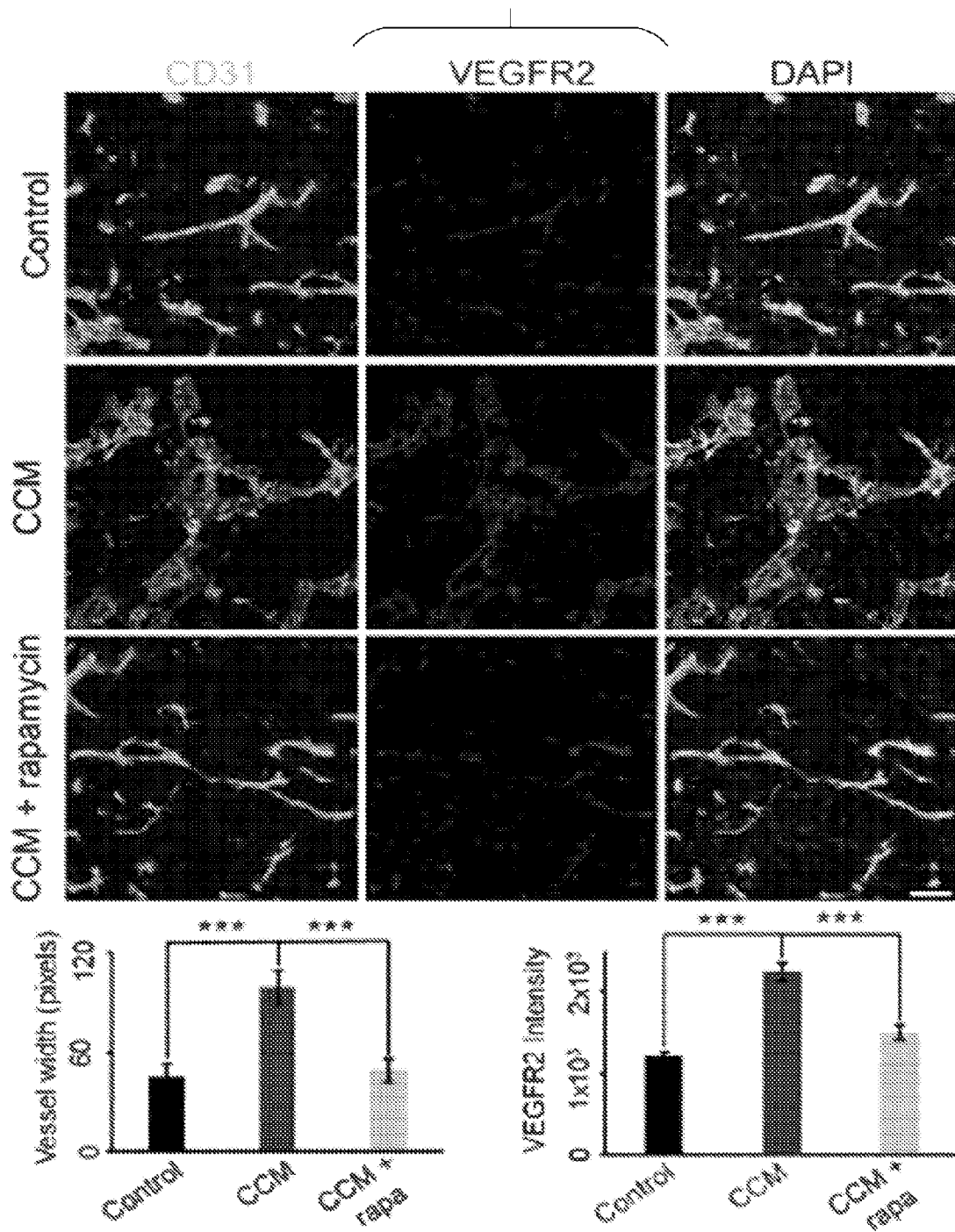


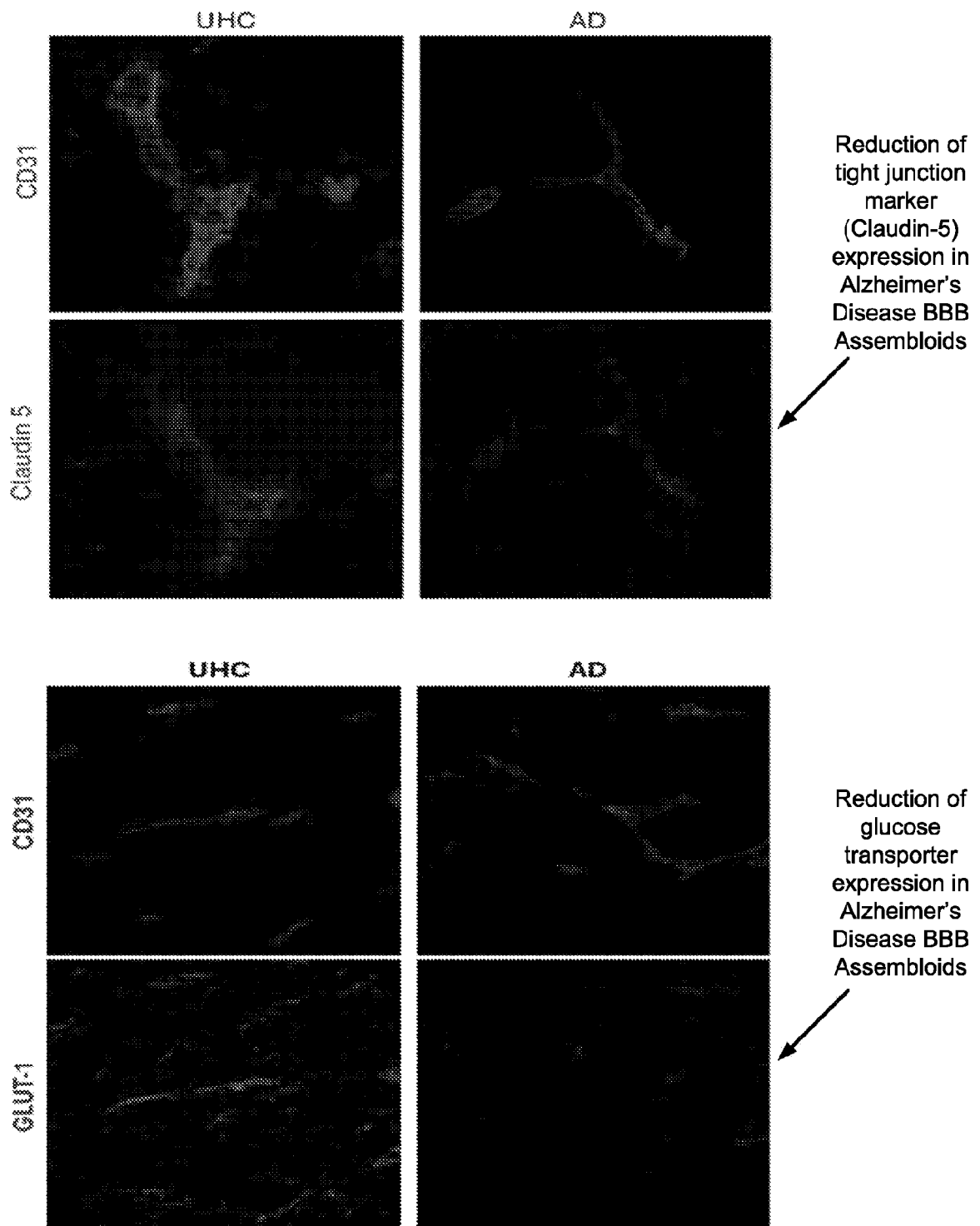
FIG. 22

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Rapamycin can weaken CCM, as
shown by lower VEGFR2 expression

**FIG. 23**

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**FIG. 24**

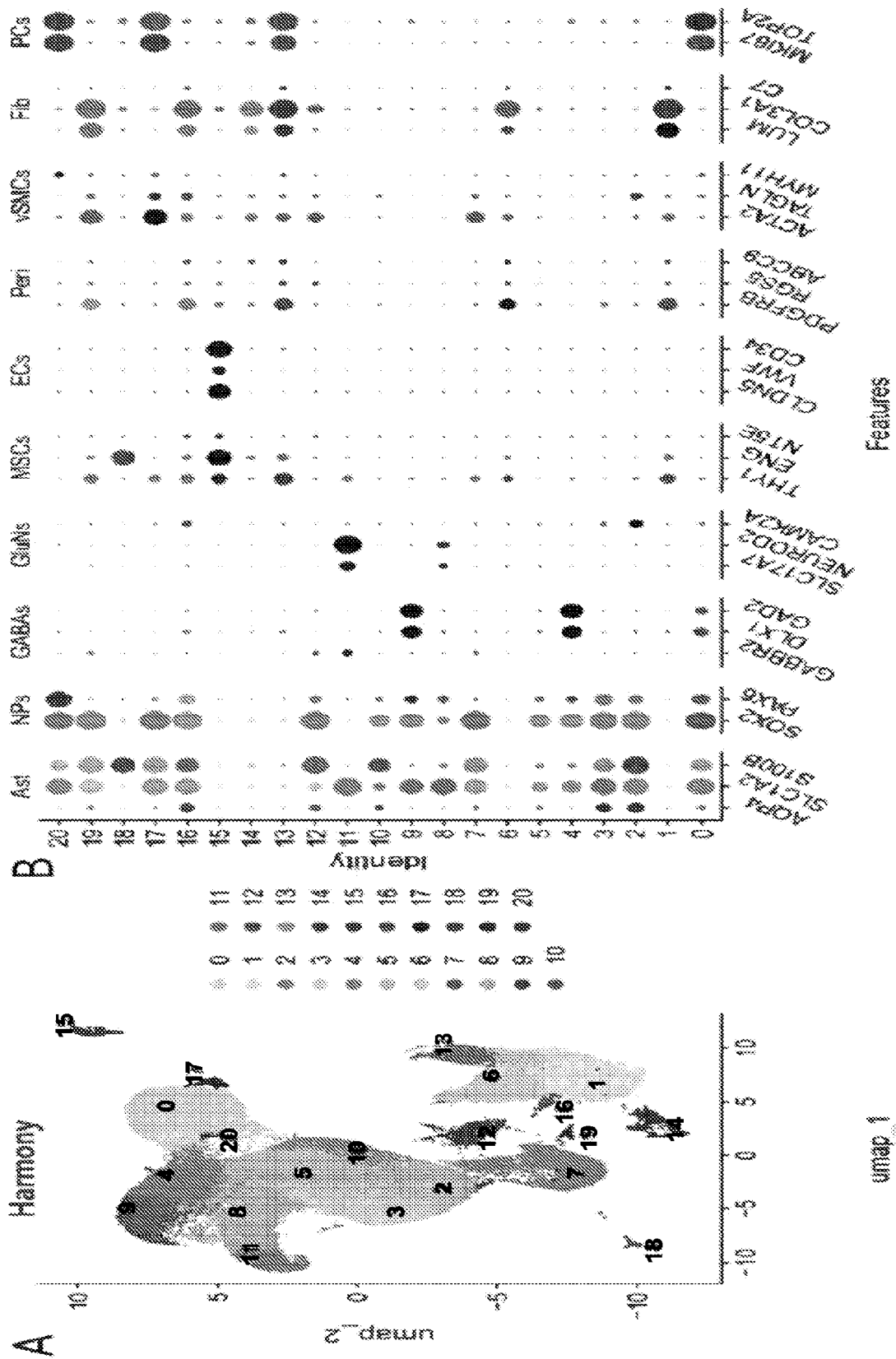


FIG. 25

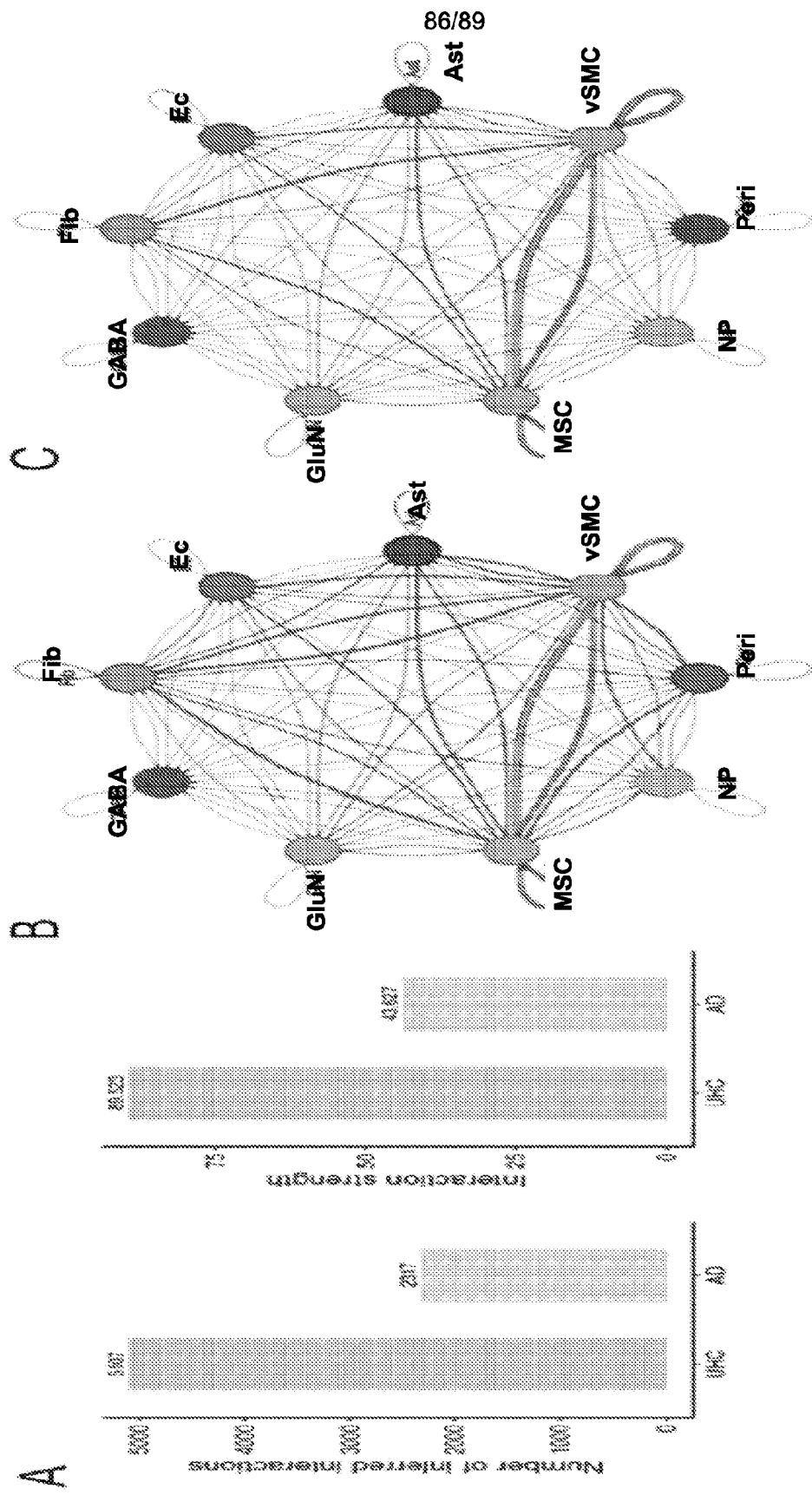
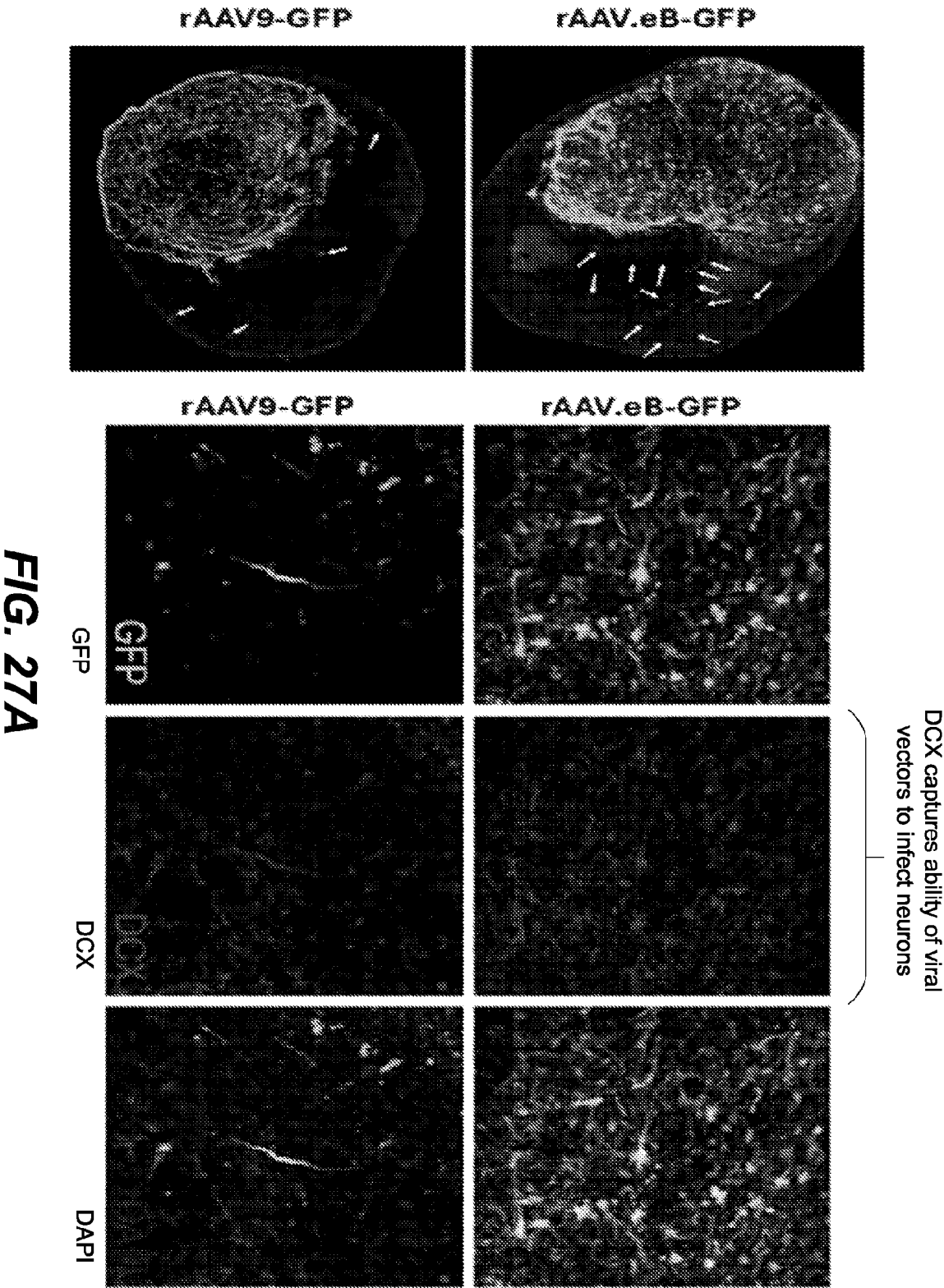


FIG. 26



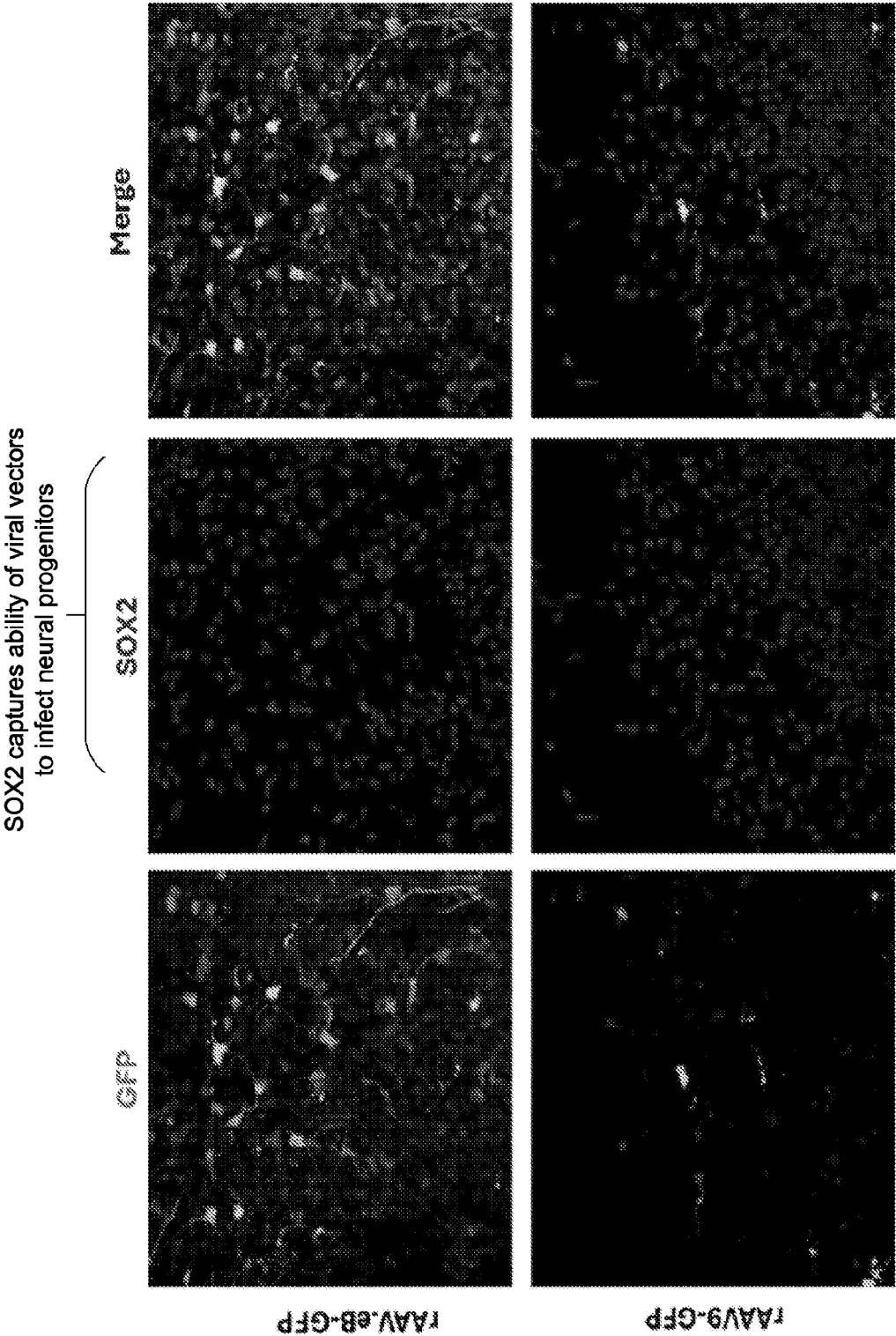


FIG. 27B

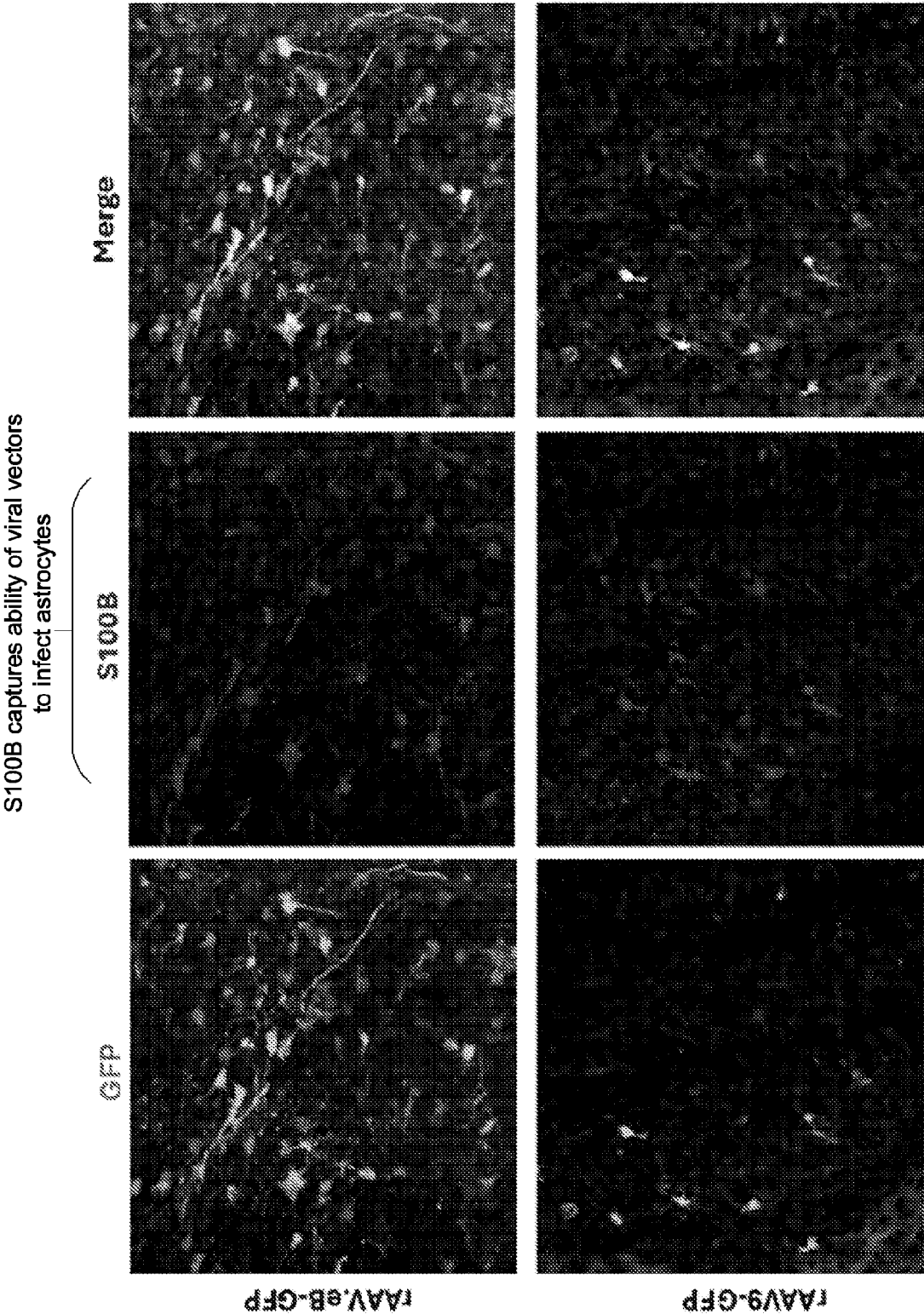


FIG. 27C