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(54) Title: METHODS OF PROMOTING LYMPHANGIOGENESIS

(57) Abstract: A method of promoting lymphatic endothelial cell (LEC) specification is disclosed. The method comprising upregulating the activity of Wnt canonical pathway in stem cells and/or vascular progenitor cells and/or angioblasts, thereby promoting LEC specification. Methods of inducing lymphangiogenesis are also disclosed. Pharmaceutical compositions and methods of treatment are also disclosed.



WO 2016/185457 A1

METHODS OF PROMOTING LYMPHANGIOGENESIS

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods of promoting lymphangiogenesis and, more particularly, but not exclusively, to use of the Wnt pathway therefore.

The lymphatic system plays a crucial role in normal and pathological conditions. It is essential for maintaining fluid homeostasis, for immune responses and for dietary lipid absorption, and is exploited by tumors to metastasize. Close to a century ago, two models describing the origins of the lymphatic system were proposed. While Florence Sabin [Sabin F.R., *American Journal of Anatomy* (1902) 1: 367-389] suggested a venous origin for the lymphatic endothelium, the second model, put forward by Huntington and McClure [Huntington G. and McClure C., *Am. J. Anat.* (1910) 10: 177-311], postulated that lymphatic vessels form by concrescence of discontinuous and independent lymph vesicles, and that mesenchymal-derived cells constitute the walls of the lymphatic vessels. Studies performed during the last decade, involving *in vivo* imaging in zebrafish, and lineage tracing in mice have extensively confirmed Sabin's hypothesis. Nevertheless, the presence of mesenchymal lymphangioblast-derived lymphatic vessels has been described in *Xenopus*, and chick embryos. At present, the embryonic origins of the lymphatic endothelium still remain controversial.

During the past years, specific markers of the lymphatic endothelium have been identified, which provided new insights into the mechanisms controlling lymphatic specification and growth. Assembly of the lymphatic vascular network is considered a stepwise process, which begins at approximately gestational week E9.5 when the expression of Prox1, a master regulator of lymphatic differentiation and maintenance is first detected in a subpopulation of endothelial cells (ECs) within the cardinal vein (CV). Two additional transcription factors- Sox18 and COUPTFII/Nr2f2 - were shown to be required for induction of Prox1 expression. The newly specified lymphatic progenitors then bud from the CV in response to Vascular endothelial growth factor C (VEGF-C) signaling and form primitive lymph sacs, which eventually give rise to the entire lymphatic vasculature. Most recently, an important role for BMP2 and the RAF1/MEK/ERK signaling cascade in the specification of lymphatic fate has also been established. Nevertheless, as none of these factors appears to be asymmetrically expressed,

the question of how only a subset of cells within the CV is initially specified towards a lymphatic fate, as opposed to cells that will maintain a venous identity, remains unanswered.

The zebrafish was recently shown to possess a lymphatic system that shares many similarities with lymphatic vessels found in higher vertebrates. *In vivo* imaging of 2-4 days-post-fertilization (dpf) zebrafish embryos demonstrated that the parachordal cells (PACs), which form at approximately 2 dpf along the embryo's midline and serve as building-blocks for the lymphatic system later on, are derived from the posterior cardinal vein (PCV). Starting at approximately 2.5 dpf PACs migrate ventrally to generate the main lymphatic vessel, the thoracic duct (TD).

Wnt (wingless-type MMTV integration site family) protein family is a diverse family of 19 secreted cysteine-rich glycoproteins that operate via three pathways: the canonical Wnt pathway, the non-canonical planar cell polarity (PCP) pathway, and the non-canonical Wnt/calcium pathway. These pathways control cell differentiation, proliferation and motility. Wnt proteins are not intrinsically canonical or non-canonical, pathway decisions are determined by distinct sets of receptors and co-receptors. Thus, pathway decisions may be cell type-specific and may depend on receptor and co-receptor availability. Dysregulation of WNT signaling pathways has been observed under pathological conditions, e.g., in cancer, rheumatoid arthritis and Alzheimer's disease.

Recently Buttler et al. [Buttler K. et al. *Developmental Biology* (2013) 381(2): 365–376] reported a link between Wnt5a, acting via a non-canonical pathway, and the morphogenesis of dermal lymphatic capillaries. Specifically, Buttler et al. teach that Wnt5a as well as other non-canonical pathway components are expressed in skin-derived lymphatic endothelial cells (LECs) and play important roles in the morphogenesis of dermal lymphatic capillaries (i.e. Wnt5a deficiency results in a significantly lower number of dermal lymphatic capillaries, while the mean size of individual lymphatics and the LEC number per vessel are greater). The study was performed in Wnt5a knockout mice. No ectopic effect of Wnt5a on stem cells was studied.

Additional background art includes

PCT Publication No. WO2012113779 teaches that Wnt-1 polynucleotides and/or Wnt-1 polypeptides reduce VEGF-C expression, lymphangiogenesis and cancer metastasis.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a method of promoting lymphatic endothelial cell (LEC) specification, the method comprising upregulating the activity of Wnt canonical pathway in stem cells and/or vascular progenitor cells and/or angioblasts, thereby promoting LEC specification.

According to an aspect of some embodiments of the present invention there is provided a method of inducing lymphangiogenesis, the method comprising: (a) promoting LEC specification in stem cells and/or vascular progenitor cells and/or angioblasts so as to obtain a LEC committed cell according to the method of some embodiments of the invention; and (b) subjecting the LEC committed cell to a signal selected from the group consisting of a LEC morphogenesis signal, a LEC proliferation signal and a LEC survival signal, thereby inducing lymphangiogenesis.

According to an aspect of some embodiments of the present invention there is provided an isolated population of cells generated according to the method of some embodiments of the invention.

According to an aspect of some embodiments of the present invention there is provided a pharmaceutical composition comprising the isolated population of cells of some embodiments of the invention and a pharmaceutically acceptable carrier or diluent.

According to an aspect of some embodiments of the present invention there is provided a use of the isolated population of cells of some embodiments of the invention for the manufacture of a medicament identified for treating or preventing a disease or disorder related to lymphangiogenesis.

According to an aspect of some embodiments of the present invention there is provided a use of an agent capable of upregulating the activity of Wnt canonical pathway for the manufacture of a medicament identified for treating or preventing a disease or disorder related to lymphangiogenesis.

According to an aspect of some embodiments of the present invention there is provided a method of treating or preventing a disease or disorder related to lymphangiogenesis in a subject in need thereof, the method comprising administering to the subject the isolated population of cells of some embodiments of the invention, thereby treating or preventing the disease or disorder related to lymphangiogenesis.

According to an aspect of some embodiments of the present invention there is provided a method of treating or preventing a disease or disorder related to lymphangiogenesis in a subject in need thereof, the method comprising administering to the subject an agent capable of upregulating the activity of Wnt canonical pathway, thereby treating or preventing the disease or disorder related to lymphangiogenesis.

According to an aspect of some embodiments of the present invention there is provided a method of transplanting lymphatic endothelial cells (LECs) into a subject in need thereof, the method comprising: (a) generating LECs according to the method of some embodiments of the invention; and (b) transplanting the LECs into the subject, thereby transplanting the LECs.

According to an aspect of some embodiments of the present invention there is provided a method of screening for an agent capable of upregulating the activity of Wnt canonical pathway for the formation of LECs, the method comprising: (a) contacting the agent with stem cells and/or vascular progenitor cells and/or angioblasts; (b) analyzing an expression of a lymphatic marker on the stem cells and/or vascular progenitor cells and/or angioblasts; and (c) comparing the expression of the marker following the contacting to prior to the contacting, wherein an expression of the marker is indicative of an agent suitable for formation of LECs.

According to some embodiments of the invention, the method further comprises differentiating the stem cells into endothelial precursor cells or angioblasts prior to the promoting the LEC specification.

According to some embodiments of the invention, the method further comprises the step of selecting LECs from the stem cells and/or vascular progenitor cells.

According to some embodiments of the invention, selecting is effected by analyzing expression of a marker selected from the group consisting of prox1, lymphatic vessel endothelial hyaluronan receptor-1 (lyve1), flt4, sox18, nr2f2, podoplanin (Pdpn), CD31 and GP38.

According to some embodiments of the invention, the LEC morphogenesis signal comprises upregulating the activity of Wnt non-canonical pathway in the LEC.

According to some embodiments of the invention, the LEC morphogenesis signal comprises an endothelial growth factor.

According to some embodiments of the invention, the method is effected *ex-vivo*.

According to some embodiments of the invention, the upregulating the activity of Wnt canonical pathway is effected by contacting the stem cells and/or vascular progenitor cells and/or angioblasts with an inhibitor of a Wnt canonical pathway member selected from the group consisting of an Axin, a GSK3, an adenomatosis polyposis coli (APC) and a sFRP.

According to some embodiments of the invention, the inhibitor of a Wnt canonical pathway factor is a small molecule.

According to some embodiments of the invention, the upregulating the activity of Wnt canonical pathway is effected by contacting the stem cells and/or vascular progenitor cells and/or angioblasts with an activator of a Wnt canonical pathway member selected from the group consisting of a Wnt5b, a Wnt3a, a Wnt7a, a Wnt7b, a LRP5/6, a β -catenin, Dishevelled (Dsh), PP2A, ARFGAP1, and TCF.

According to some embodiments of the invention, the Wnt5b is selected from the group consisting of a Wnt5b polypeptide, a Wnt5b analogue and a Wnt5b polynucleotide.

According to some embodiments of the invention, the upregulating the activity of Wnt non-canonical pathway is effected by contacting the LEC with an activator of a Wnt non-canonical pathway member selected from the group consisting of a Wnt5a, Wnt4, Wnt11, Ror2, Ryk, G protein, Dishevelled (Dsh) and Dishevelled-associated activator of morphogenesis 1 (DAAM1).

According to some embodiments of the invention, the stem cells and/or vascular progenitor cells are derived from a source selected from the group consisting of hematopoietic stem cells, mesodermal progenitor cells, endothelial progenitor cells, angioblasts, induced pluripotent stem cells (iPS) and human embryonic stem cells (hESCs).

According to some embodiments of the invention, the use further comprises an agent capable of upregulating the activity of Wnt non-canonical pathway.

According to some embodiments of the invention, the method further comprises a administering to the subject an agent capable of upregulating the activity of Wnt non-canonical pathway.

According to some embodiments of the invention, the disease or disorder related to lymphangiogenesis is selected from the group consisting of cancer, melanoma, solid tumor, breast cancer, lung cancer, kidney cancer, pancreatic cancer, hematopoietic tumor,

graft rejection, autoimmune disorder, rheumatoid arthritis, psoriasis, oedema, chronic wound, lymphedema, lymphatic fistula, lymphorrhea and lymphocele/seroma formation.

According to some embodiments of the invention, the LECs are syngeneic or allogeneic with respect to the subject.

5 According to some embodiments of the invention, the subject is a human subject.

According to some embodiments of the invention, the lymphatic marker is selected from the group consisting prox1, lyve1, flt4, sox18, nr2f2, Pdpn, CD31 and GP38.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those
10 described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

15 BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the
20 description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIGs. 1A-G show that lymphatic progenitors originate in the vPCV. Figures 1A-D are snapshots from a time-lapse sequence of a *Tg(fli1:EGFP)* embryo, showing the origin
25 of a PAC cell (green) in the vPCV ($n_{\text{PACs}}=16$; $N_{\text{imaged-embryos}}=13$); Figures 1E-G illustrate photoswitching of ventral (Figure 1E) and dorsal (Figure 1F) PCV in *Tg(fli1:gal4;uasKaede)* embryos at 24 hpf (light-blue arrowheads), and Figure 1G shows the percentage of red PACs (white arrows) at 48 hpf ($n_{\text{vPCV-photoswitched-embryos}}=10$, $n_{\text{dPCV-photoswitched-embryos}}=8$ * $P=2.66e^{-10}$). Scale bar 30 μm . Error bars mean \pm s.e.m.

30 FIGs. 1H-T show that lymphatic progenitors originate in the vPCV. Figures 1H-K are snapshots from a time-lapse sequence of a *Tg(fli1:nEGFP)^{y7}* zebrafish embryo,

showing the origin of a PAC cell (yellow) in the vPCV; Figures 1L-M are snapshots of dPCV (1M)-, and vPCV (1L)- Kaede photoconverted cells at 48 hpf; Figures 1N-O are snapshots of Kaede-photoswitched “medial” (1N)-, and “early lateral” (1O)- angioblasts; Figures 1P-S are snapshots from a time-lapse sequence of a *plcg1* mutant, showing the origin of a PAC cell (green) in the vPCV ($n_{\text{imaged embryos}}=3$); and Figure 1T is a quantification of symmetric and asymmetric division events in the vPCV and dPCV of double *Tg(flt1_9a_cFos:GFP; lyve1:dsRed2^{nz101})* embryos ($n_{\text{imaged embryos}}=6$). Scale bar 30 μm .

FIGs. 2A-Q show that vPCV cells are specialized angioblasts. Figures 2A-D are snapshots from a time-lapse movie of a *Tg(fli1:gal4;uasKaede)* embryo showing photoswitched-vPCV cell (light-blue arrowhead), which generates PACs (white arrowhead) through asymmetric division; Figures 2E-G show a single photoswitched-vPCV cell in *Tg(fli1:gal4;uasKaede)* embryo at 24 hpf (light-blue arrowhead), whose progeny populates the SIA (white arrows) and SIV (yellow arrows) at 56 hpf; Figures 2H-I show the location of vPCV-, and dPCV- progeny at 56 hpf, following photoswitching at different stages ($n_{\text{photoswitched-vPCV cells}}=73$, $n_{\text{photoswitched-dPCV cells}}=45$); Figures 2J-M show photoswitching of medial, and early-lateral angioblasts at 17-, and 20 hpf (light-blue arrowheads) respectively in *Tg(kdrl:Kaede)^{wz3}* embryos; Figure 2N shows the percentage of red PACs at 48 hpf ($n_{\text{medial-angioblasts}}=16$, $n_{\text{early lateral-angioblasts}}=16$ * $P=2.1e^{-5}$); *Tg(flt1_9a_cFos:GFP;fli1:dsRed)* embryos show *flt1_9a:GFP⁺* ECs in the SIA, ISA, DA (Figure 2O), and vPCV (Figure 2P, green, orange arrowheads); and Figure 2Q shows selected genes enriched in vPCV cells. Scale bar 30 μm . Error bars mean \pm s.e.m.

FIGs. 3A-K show cell proliferation in the dPCV, vPCV and DA. Figures 3A-H show a phospho-histone H3 staining illustrating no difference in the number of proliferative ECs among the DA, dPCV and vPCV ($n_{24\text{hpf-embryos}}=17$, $n_{26\text{hpf-embryos}}=16$, $n_{28\text{hpf-embryos}}=16$, $n_{30\text{hpf-embryos}}=16$); and Figures 3I-K shown that ectopic induction of Wnt5b in *Tg(hsp70l:wnt5b;fli1:EGFP)* does not result in enhanced proliferation of ECs (26 hpf $n_{\text{control-embryos}}=15$ $n_{\text{hsp70:wnt5b-embryos}}=8$, 28 hpf $n_{\text{control-embryos}}=14$ $n_{\text{hsp70:wnt5b-embryos}}=8$, 30 hpf $n_{\text{control-embryos}}=14$ $n_{\text{hsp70:wnt5b-embryos}}=10$). Scale bar 60 μm . Error bars, mean \pm s.e.m.

FIGs. 4A-H show additional fates of vPCV cells. Figure 4A is a schematic representation of the Subintestinal plexus at 72 hpf. Subintestinal Vein (SIV, green), interconnecting SI vessels (purple), Supraintestinal Artery (SIA, pink), Posterior Cardinal

Vein (PCV, blue), Dorsal Aorta (DA, red); Figures 4B-C show quantification of the number of Intersegmental Arteries (ISA) and Intersegmental Veins (ISV) in the first four segments of *Tg(flt1_9a_cFos:GFP; lyve1:dsRed2)* double transgenic embryos ($n_{\text{embryos}}=41$). IS# denotes the position of Intersegmental Vessel; Figures 4D-E are confocal images of *Tg(lyve1:dsRed2)* (4D) and *Tg(flt1_9a_cFos:GFP; lyve1:dsRed2)* (4E) embryos showing *lyve1:dsred2*⁺ ECs in PACs, venous ISVs, PCV and SIV and *flt1_9a:GFP*⁺ ECs in the SIA; and Figures 4F-H are photographs illustrating *flt1_9a:GFP*⁺ vPCV angioblast (light-blue arrowhead), which divides asymmetrically (curved arrow) to generate cells that populate the SIV (31.5 hpf, white arrowhead), and the SIA (53.5 hpf, white arrowhead). Scale bar 30 μm . Error bars mean \pm s.e.m.

FIGs. 5A-D show global expression profiling via RNASeq. Figure 5A is a schematic illustration of an experimental setup used for RNASeq analysis of FACS isolated vPCV and dPCV cells; Figure 5B shows FACS isolation of green versus red (photoconverted) ECs from *Tg(fli1:gal4;uasKaede)* embryos following photoswitching of dorsal or ventral PCV ($n_{\text{independent-experiments}}=4$); Figure 5C is a qRT-PCR analysis of selected candidates showing enrichment in ventral-, versus dorsal- PCV cells ($n_{\text{independent-experiments}}=2$); and Figure 5D shows Gene Ontology enrichment in vPCV versus dPCV cells (results represent 2 out of 4 independent biological repeats). Error bars, geometrical mean \pm s.e.m.

FIGs. 6A-H show that LEC specification is induced in the vPCV angioblasts. Figures 6A-E show that a *(prox1a:TagRFP;fli1:EGFP)*⁺ cell in the vPCV (yellow) generates a daughter cell that translocates dorsally, buds from the PCV and forms PACs (white arrowheads); Figure 6F shows a quantification of *Tg(fli1:EGFP;prox1a:KalTA-UAS:uncTagRFP)* embryos with 1-2, or more than 2, *(prox1a:TagRFP; fli1:EGFP)*⁺ cells in vPCV and dPCV at 22-24 and 26-30 hpf ($n_{22-24\text{hpf}}=11$; $n_{26-28\text{hpf}}=24$); Figure 6G shows Prox1 immunostaining at 24 hpf illustrating expression in vPCV cell (light-blue arrowheads); and Figure 6H shows a quantification of *(flt1_9a:GFP;prox1a:TagRFP)*⁺ cells in vPCV versus dPCV at 22-24 and 26-28 hpf ($n_{22-24\text{hpf}}=18$ $n_{26-28\text{hpf}}=13$ * $P=0.01$). Scale bar Figures 6A-E 30 μm and Figure 6G 60 μm . Error bars mean \pm s.e.m.

FIGs. 7A-L show that *Wnt5b* is necessary and sufficient for LEC specification. Figures 7A-B are histological sections at 22 and 24 hpf, depicting the position of the vPCV angioblasts (light-blue arrows) and the endoderm (depicted by the letter 'e'); Figure

7C is a schematic illustration of the Posterior Cardinal Vein (PCV), the ventral side of the PCV (vPCV) and the endoderm (depicted by the letter 'e'); Figure 7D is a graph illustrating the number of PAC-containing segments in WT and *cas* mutants ($n_{cas}=22$, $n_{WT}=15$ * $P=3e^{-12}$); Figure 7E shows an *in situ* hybridization at 18 hpf illustrating expression of *wnt5b* mRNA in the endoderm; Figure 7F is a graph illustrating the number of PAC-containing segments in *wnt5b* morphants ($n_{UI}=63$, $n_{wnt5bMO}=57$ * $P=1.83e^{-30}$); Figures 7G-I show that Wnt5b overexpression in *Tg(hsp70l:wnt5b-GFP;lyve1:dsRed2)* embryos induces ectopic lymphangiogenesis ($n_{WT}=19$, $n_{hsp70l:Wnt5b-GFP}=22$ * $P=9.47e^{-12}$); and Figures 7J-L show the number of PAC-containing segments in *sox32*-MO injected *Tg(hsp70l:wnt5b-GFP;lyve1:dsRed2)* embryos ($n_{sox32MO}=18$, $n_{sox32MO;hsp70:wnt5b}=18$ * $P=0.0005$). Scale bar Figures 7A-B 20 μ m, Figures 7G, 7H, 7J and 7K 60 μ m. Error bars mean \pm s.e.m.

FIGs. 8A-M show that Wnt5b is required during lymphatic development. Figures 8A-B show PAC-containing segments in WT (arrowhead) and *cas* mutants (asterisks); Figures 8C-D show *in situ* hybridization at 20 hpf illustrating expression of *wnt5b* mRNA (blue arrowhead) in the endoderm of WT embryos; Figures 8E-F show PAC-containing segments in UI (arrowhead) and *wnt5b* MO-injected embryos (asterisks); Figure 8G is a graph illustrating *ppt* mutants injected with *wnt5b* MO (subdose) which display significant reduction in PAC number ($n_{UI-embryos}=38$, $n_{wnt5b-MO-embryos\ sub}=38$, $n_{ppt-UI\ embryos}=34$, $n_{ppt, wnt5b\ MO\ sub-embryos}=34$ * $P=1.2e^{-5}$); Figures 8H-J show *wnt5b* morphants which exhibit marked reduction in the number of TD-containing segments (asterisks) as compared to uninjected (UI) siblings ($n_{UI-embryos}=38$, $n_{wnt5b\ MO-embryos}=32$ * $P=4.5e^{-30}$); and Figures 8K-M show that the number of *flt1*⁺ vPCV progenitors is not affected in *wnt5b* morphants ($n_{UI-embryos}=31$, $n_{wnt5b\ MO-embryos}=31$). Scale bars Figures 8A-B and 8E-F 60 μ m, Figures 8C-D, 8H-I and 8K-L 30 μ m. Error bars mean \pm s.e.m.

FIGs. 9A-H show specificity of Wnt5b role in lymphatic specification. Figures 9A-C show phenotypic analysis of Wnt5b overexpression in *Tg(hsp70l:wnt5b-GFP;lyve1:dsRed2)* embryos, following 25-30 minutes heat-shock (HS), at 23, 25, and 27 hpf (23 hpf embryos $n_{HS-25\ min}=18$ $n_{HS-30\ min}=14$ $n_{HS-40\ min}=15$, 25 hpf embryos $n_{HS-25\ min}=14$ $n_{HS-30\ min}=17$ $n_{HS-40\ min}=20$, 27 hpf embryos $n_{HS-25\ min}=19$ $n_{HS-30\ min}=17$ $n_{HS-40\ min}=10$); Figures 9D-F show that the number of vISVs versus aISVs is unaltered in *wnt5b* morphants as compared to Control MO-injected siblings ($n_{Control\ MO-embryos}=43$, $n_{wnt5b\ MO-embryos}=43$).

embryos=41); and Figures 9G-H show that *flt1_9a*⁺ vPCV cells are detected in the Supraintestinal Artery (SIA) and Subintestinal Vein (SIV) of *wnt5b* MO injected embryos ($n_{\text{Ctrl MO}}=16$, $n_{\text{wnt5b MO}}=16$). Figures 9D-E and 9G-H 60 μm . Error bars mean \pm s.e.m.

FIGs. 10A-U show that loss of Wnt5b specifically affects lymphatic specification. Figures 10A-D show selected frames from a time-lapse sequence of a *Tg(fli1:gal4;uasKaede)* embryo injected with *wnt5b* MO. Photoconverted vPCV cell (white arrow) divides normally (arrows at 48 hpf point to 2 daughter cells), but does not engage in dorsal migration to generate PACs; Figures 10E-L show *in situ* hybridization of Ctrl MO-, and *wnt5b* MO-injected zebrafish at 30 hpf, with *lyve1*, *sox18*, *nr2f2* and *cdh5* probes, showing specific decrease in lymphatic marker expression in the floor of the PCV (white arrowheads) of *wnt5b* morphants. The pan-endothelial marker- *cdh5*-, as well as the arterial expression of *sox18*, remain unchanged in *wnt5b* morphants; Figures 10M-R show that *vegfc* and *ccbe1* mRNA levels remain unaltered in *sox32*-, and *wnt5b*- morphants; Figures 10S-T show an immunostaining of Prox1 illustrating significant increase in protein levels following ectopic activation of Wnt5b in *Tg(hsp70l:wnt5b; fli1:EGFP)* embryos (co-localization channel is shown in yellow, white arrowheads); and Figure 10U is a graph showing qRT-PCR analysis of *FLT4* and *CDH5* in hESCs treated with WNT5B ($n_{\text{independent-experiments}}=3$ *P=0.03 by one sample t-test). Scale bar 60 μm . Error bars, geometrical mean \pm s.e.m.

FIGs. 11A-H show that Wnt5b induces lymphatic specification in zebrafish and hESCs. Figures 11A-D show quantification of *Prox1a*⁺ vPCV cells (light-blue arrowheads) in *Tg(fli1:EGFP;prox1a:Kalt4-UAS:uncTagRFP)* embryos following Wnt5b- induction, and downregulation ($n_{\text{wnt5bMO}}=7$, $n_{\text{hsp70:wnt5b}}=9$, $n_{\text{WT}}=7$ *P=0.05, **P=0.001); Figure 11E shows *prox1a* mRNA levels in 24 hpf *Tg(fli1:EGFP;prox1a:Kalt4-UAS:uncTagRFP;hsp70l:wnt5b-GFP)* embryos following heat-shock at 21 hpf ($n_{\text{independent-experiments}}=4$); Figures 11F-G show fraction of LYVE1⁺ cells; and Figure 11H shows *PROX1* mRNA levels in hESC-derived angioblasts treated with WNT5B ($n_{\text{independent-experiments}}=3$). Scale bar 60 μm . Figures 11A-C Error bars, mean \pm s.e.m. Figures 11E, 11H Error bars, geometrical mean \pm s.e.m.

FIGs. 12A-H show that Wnt5b controls lymphatic specification through activation of the canonical pathway. Figures 12A-C show PAC-containing segments in *wnt5b* MO-injected *mbl* mutants ($n_{\text{wnt5bMO}}=42$, $n_{\text{mbl:wnt5bMO}}=52$ *P=3.4e⁻¹⁰); Figures 12D-F show *apc*

mutants ($n_{WT}=18$, $n_{apc}=19$ * $P=0.0006$); Figure 12G show *axin1* mRNA-injected embryos ($n_{UI}=33$, $n_{axin-mRNA}=46$ * $P=1.73e^{-14}$); and Figure 12H show IWR-1 treated embryos ($n_{DMSO}=55$, $n_{IWR}=54$ * $P=1.05e^{-21}$). Scale bar 60 μ m. Error bars, mean \pm s.e.m.

FIGs. 13A-J show that Wnt5b controls lymphatic specification through activation
 5 of the canonical pathway. Figure 13A shows that PAC number remains unchanged in TNP-470 treated *Tg(fli1:EGFP)* embryos as compared to DMSO (control) ($n_{DMSO}=19$, $n_{TNP-470}=38$); Figures 13B-F show quantification of PAC-containing segments in the trunk of UI, *pcf7*, *lef1* and *pcf4* MO-injected embryos ($n_{UI-embryos}=59$, $n_{pcf7-MO-embryos}=33$, $n_{lef1-MO-embryos}=16$, $n_{pcf4-MO-embryos}=25$ * $P=4.53e^{-25}$ ** $P=9.62e^{-08}$ *** $P=9.12e^{-09}$); and Figures
 10 13G-J show photoswitching of vPCV cells in *pcf7* MO-injected *Tg(fli1:gal4;uasKaede)* embryos (white arrowheads) at 24 hpf. At 48 hpf photoconverted-, red vPCV cells (arrowheads) remain in the PCV and do not generate PACs. Scale bar 30 μ m. Error bars, mean \pm s.e.m.

FIGs. 14A-G show that Wnt5b induces LEC specification through activation of the
 15 canonical pathway. Figures 14A-B show *Tg(7xTCF-Xla.Siam:nlsMCherry;fli1:EGFP)* embryo illustrating TCF activity in vPCV cells at 24 hpf (light-blue arrowheads), and PACs at 48 hpf (white arrows); Figure 14C shows *Tg(7xTCF-Xla.Siam:nlsMCherry;flt1_9a_cFos:GFP)* embryo illustrating TCF activity in *flt1_9a*⁺ vPCV angioblasts at 24 hpf (light-blue arrowheads); Figure 14D shows *KalT4* mRNA levels in *Tg(prox1a:KalT4-UAS:uncTagRFP;hsp70l:wnt5b-GFP)* 24 hpf embryos,
 20 following heat-shock at 21 hpf ($n_{independent-experiments}=3$); and Figures 14E-G is a schematic model of LEC specification and formation of first lymphatic vessels in the zebrafish trunk. Scale bar Figure 14A-C 30 μ m. Figure 14D Error bars, geometrical mean \pm s.e.m.

FIGs. 15A-H show specific activation of TCF in lymphatic progenitors. Figures
 25 15A-E show selected frames from a time-lapse sequence showing β -catenin/TCF activity in a single vPCV angioblast (light-blue arrowhead), which generates PACs (white arrowhead) through asymmetric cell division ($n=2$); and Figures 15F-H show confocal images of the trunks of *Tg(7xTCFXla.Siam:nlsMCherry;fli1:EGFP)* double transgenic zebrafish injected with *wnt5b* MO, showing decreased β -catenin/TCF activation in vPCV cells (quantified in c) ($n_{UI-embryos}=18$, $n_{wnt5b-embryos}=17$ * $P=4e^{-5}$). Purple signal depicts co-localization of cytoplasmic EGFP and nuclear mCherry. Scale bar 30 μ m. Error bars, mean
 30 \pm s.e.m.

FIGs. 16A-C are schematical illustrations of the Wnt canonical and non-canonical pathways. Canonical pathway (Figure 16A), non-canonical PCP pathway (Figure 16B) and non-canonical Wnt/calcium pathway (Figure 16C) are illustrated.

5 DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods of promoting lymphangiogenesis and, more particularly, but not exclusively, to use of the Wnt pathway therefore.

The principles and operation of the present invention may be better understood
10 with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also,
15 it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

A fundamental question in developmental and regenerative biology is how cells acquire their fate. Multipotent progenitors undergo cell-fate restriction in response to cues from the microenvironment, the nature of which is poorly understood. In the case of the
20 lymphatic system, venous cells from the cardinal vein (CV) are thought to generate lymphatic vessels, through trans-differentiation.

While reducing the present invention to practice, the present inventors have uncovered that the canonical and non-canonical Wnt pathways are involved in different stages of lymphangiogenesis. Specifically, the present inventors have uncovered that the
25 Wnt canonical pathway is involved in lymphatic endothelial cell (LEC) specification while the non-canonical Wnt pathway is involved in additional steps of lymphatic morphogenesis and remodeling. This elucidation paves the way towards the generation of lymphatic vessels *in vitro* and for the treatment of lymphatic disorders or diseases *in vivo*.

As is illustrated hereinbelow and in the Examples section which follows, the
30 present inventors have uncovered that lymphatic progenitors arise from a previously uncharacterized niche of specialized angioblasts within the floor of the Posterior Cardinal Vein (PCV), which bears the potential to also generate arterial and venous fates (see

results in zebrafish embryos, Examples 1 and 2 of the Examples section which follows). Anatomically, these cells develop in close proximity to the endoderm, which serves as source of Wnt5b - a novel inductive signal promoting the angioblast-to-lymphatic transition (see Example 3 of the Examples section which follows). The present inventors
5 have further identified Wnt5b as a lymphatic inductive signal that promotes the “angioblast-to-lymphatic” transition in human embryonic stem cells (see Example 3 of the Examples section which follows). The present results also established that Wnt5b induces LEC specification through activation of Wnt canonical pathway (see Example 4 of the Examples section which follows). Taken together, these results substantiate the value of
10 upregulating the Wnt signaling pathways for promoting lymphangiogenesis.

Thus, according to one aspect of the present invention there is provided a method of promoting lymphatic endothelial cell (LEC) specification, the method comprising upregulating the activity of Wnt canonical pathway in stem cells and/or vascular progenitor cells and/or angioblasts, thereby promoting LEC specification.

15 The term “LEC specification” as used herein is interchangeably used with the terms “LEC differentiation” or “determination” and refers to the process by which stem cells and/or vascular progenitor cells and/or angioblasts change into lymphatic endothelial cells (LECs). Change into LECs may be indicated, for example, by changes in marker expression, gene expression, biochemistry, structure and/or function of the cells, e.g. LECs
20 typically express remarkably high levels of specific transcription factors and receptors, such as, but not limited to, prox1, lyve1, podoplanin and vegfr3. Markers of LECs are described in detail below.

The term “lymphatic endothelial cell” or “LEC” as used herein refers to a cell which makes up the lymphatic endothelium (LE) that lines the lymphatic vascular system,
25 and expresses typical LEC markers including, but not limited to, the intracellular expression of Prox1, lymphatic vessel endothelial hyaluronan receptor-1 (lyve1), VEGFR-3, flt4, sox18, nr2f2, neuropilin 2 (Nrp2), forkhead box C2 (Foxc2), podoplanin (Pdpn), angiopoietin 2 (Ang2), ephrin B2 (Efnb2), cxcr4, activin receptor-like kinase 1 (ALK-1), activin receptor-like kinase 2 (ALK-2), protein phosphatase 1 regulatory (inhibitor)
30 subunit 13B (Aspp1) and/or core 1 synthase glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 (T-synthase); or the cell membrane expression of CD31, CD34 and/or GP38. LECs typically also produce chemokines, including but not limited to,

CCL21. According to a specific embodiment, LECs express *lyve1*, *prox1*, *Pdpm* and/or VEGFR-3. Determination of LECs may be carried out using any method known in the art, e.g. by FACS analysis, by PCR or by ELISA, selecting cells expressing the LEC markers as described in detail above.

5 A “LEC committed cell” is a cell, which has been specified particularly into a LEC fate.

As used herein, the phrase “stem cells” refers to cells which are capable of remaining in an undifferentiated state (*e.g.*, totipotent, pluripotent or multipotent stem cells) for extended periods of time in culture until induced to differentiate into other cell
10 types having a particular, specialized function (*e.g.*, fully differentiated cells). Totipotent cells, such as embryonic cells within the first couple of cell divisions after fertilization are the only cells that can differentiate into embryonic and extra-embryonic cells and are able to develop into a viable human being. Preferably, the phrase “pluripotent stem cells” refers to cells which can differentiate into all three embryonic germ layers, *i.e.*, ectoderm,
15 endoderm and mesoderm or remaining in an undifferentiated state. The pluripotent stem cells include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPS). The multipotent stem cells include adult stem cells and hematopoietic stem cells.

The phrase “embryonic stem cells” refers to embryonic cells which are capable of differentiating into cells of all three embryonic germ layers (*i.e.*, endoderm, ectoderm and
20 mesoderm), or remaining in an undifferentiated state. The phrase “embryonic stem cells” may comprise cells which are obtained from the embryonic tissue formed after gestation (*e.g.*, blastocyst) before implantation of the embryo (*i.e.*, a pre-implantation blastocyst), extended blastocyst cells (EBCs) which are obtained from a post-implantation/pre-gastrulation stage blastocyst (see WO2006/040763), embryonic germ (EG) cells which are
25 obtained from the genital tissue of a fetus any time during gestation, preferably before 10 weeks of gestation, and cells originating from an unfertilized ova which are stimulated by parthenogenesis (parthenotes).

Induced pluripotent stem cells (iPS; embryonic-like stem cells), are cells obtained by de-differentiation of adult somatic cells which are endowed with pluripotency (*i.e.*,
30 being capable of differentiating into the three embryonic germ cell layers, *i.e.*, endoderm, ectoderm and mesoderm). According to some embodiments of the invention, such cells are obtained from a differentiated tissue (*e.g.*, a somatic tissue such as skin) and undergo

de-differentiation by genetic manipulation, which re-program the cell to acquire embryonic stem cells characteristics. According to some embodiments of the invention, the induced pluripotent stem cells are formed by inducing the expression of Oct-4, Sox2, Klf4 and c-Myc in a somatic stem cell.

5 The phrase “adult stem cells” (also called “tissue stem cells” or a stem cell from a somatic tissue) refers to any stem cell derived from a somatic tissue [of either a postnatal or prenatal animal (especially the human)]. The adult stem cell is generally thought to be a multipotent stem cell, capable of differentiation into multiple cell types. Adult stem cells can be derived from any adult, neonatal or fetal tissue such as adipose tissue, skin,
10 kidney, liver, prostate, pancreas, intestine, bone marrow and placenta.

Hematopoietic stem cells, which may also referred to as adult tissue stem cells, include stem cells obtained from blood or bone marrow tissue of an individual at any age or from cord blood of a newborn individual. Preferred stem cells according to this aspect of some embodiments of the invention are embryonic stem cells, preferably of a human or
15 primate (e.g., monkey) origin. Human hematopoietic stem cells (HSCs) are typically positive for the expression of the markers CD34, CD59 and/or Thy1/CD90 and may be positive for expression of CD38 and/or C-kit/CD117.

Placental and cord blood stem cells may also be referred to as “young stem cells”.

The embryonic stem cells of some embodiments of the invention can be obtained
20 using well-known cell-culture methods. For example, human embryonic stem cells can be isolated from human blastocysts. Human blastocysts are typically obtained from human *in vivo* preimplantation embryos or from *in vitro* fertilized (IVF) embryos. Alternatively, a single cell human embryo can be expanded to the blastocyst stage. For the isolation of human ES cells the zona pellucida is removed from the blastocyst and the inner cell mass
25 (ICM) is isolated by immunosurgery, in which the trophoctoderm cells are lysed and removed from the intact ICM by gentle pipetting. The ICM is then plated in a tissue culture flask containing the appropriate medium which enables its outgrowth. Following 9 to 15 days, the ICM derived outgrowth is dissociated into clumps either by a mechanical dissociation or by an enzymatic degradation and the cells are then re-plated on a fresh
30 tissue culture medium. Colonies demonstrating undifferentiated morphology are individually selected by micropipette, mechanically dissociated into clumps, and re-plated. Resulting ES cells are then routinely split every 4-7 days. For further details on methods

of preparation human ES cells see Thomson et al., [U.S. Pat. No. 5,843,780; Science 282: 1145, 1998; Curr. Top. Dev. Biol. 38: 133, 1998; Proc. Natl. Acad. Sci. USA 92: 7844, 1995]; Bongso et al., [Hum Reprod 4: 706, 1989]; and Gardner et al., [Fertil. Steril. 69: 84, 1998].

5 It will be appreciated that commercially available stem cells can also be used according to some embodiments of the invention. Human ES cells can be purchased from the NIH human embryonic stem cells registry [Hypertext Transfer Protocol://grants (dot) nih (dot) gov/stem_cells/registry/current (dot) htm]. Non-limiting examples of commercially available embryonic stem cell lines are BG01, BG02, BG03, BG04, CY12,
10 CY30, CY92, CY10, TE03, TE32, CHB-4, CHB-5, CHB-6, CHB-8, CHB-9, CHB-10, CHB-11, CHB-12, HUES 1, HUES 2, HUES 3, HUES 4, HUES 5, HUES 6, HUES 7, HUES 8, HUES 9, HUES 10, HUES 11, HUES 12, HUES 13, HUES 14, HUES 15, HUES 16, HUES 17, HUES 18, HUES 19, HUES 20, HUES 21, HUES 22, HUES 23, HUES 24, HUES 25, HUES 26, HUES 27, HUES 28, CyT49, RUES3, WA01, UCSF4,
15 NYUES1, NYUES2, NYUES3, NYUES4, NYUES5, NYUES6, NYUES7, UCLA 1, UCLA 2, UCLA 3, WA077 (H7), WA09 (H9), WA13 (H13), WA14 (H14), HUES 62, HUES 63, HUES 64, CT1, CT2, CT3, CT4, MA135, Eneavour-2, WIBR1, WIBR2, WIBR3, WIBR4, WIBR5, WIBR6, HUES 45, Shef 3, Shef 6, BJNhem19, BJNhem20, SA001, SA001.

20 In addition, ES cells can be obtained from other species as well, including mouse (Mills and Bradley, 2001), golden hamster [Doetschman et al., 1988, Dev Biol. 127: 224-7], rat [Iannaccone et al., 1994, Dev Biol. 163: 288-92] rabbit [Giles et al. 1993, Mol Reprod Dev. 36: 130-8; Graves & Moreadith, 1993, Mol Reprod Dev. 1993, 36: 424-33], several domestic animal species [Notarianni et al., 1991, J Reprod Fertil Suppl. 43: 255-
25 60; Wheeler 1994, Reprod Fertil Dev. 6: 563-8; Mitalipova et al., 2001, Cloning. 3: 59-67] and non-human primate species (Rhesus monkey and marmoset) [Thomson et al., 1995, Proc Natl Acad Sci U S A. 92: 7844-8; Thomson et al., 1996, Biol Reprod. 55: 254-9].

Extended blastocyst cells (EBCs) can be obtained from a blastocyst of at least nine days post fertilization at a stage prior to gastrulation. Prior to culturing the blastocyst, the
30 zona pellucida is digested [for example by Tyrode's acidic solution (Sigma Aldrich, St Louis, MO, USA)] so as to expose the inner cell mass. The blastocysts are then cultured as whole embryos for at least nine and no more than fourteen days post fertilization (*i.e.*,

prior to the gastrulation event) *in vitro* using standard embryonic stem cell culturing methods.

Another method for preparing ES cells is described in Chung et al., Cell Stem Cell, Volume 2, Issue 2, 113-117, 7 February 2008. This method comprises removing a single
5 cell from an embryo during an *in vitro* fertilization process. The embryo is not destroyed in this process.

EG cells are prepared from the primordial germ cells obtained from fetuses of about 8-11 weeks of gestation (in the case of a human fetus) using laboratory techniques known to anyone skilled in the arts. The genital ridges are dissociated and cut into small
10 chunks which are thereafter disaggregated into cells by mechanical dissociation. The EG cells are then grown in tissue culture flasks with the appropriate medium. The cells are cultured with daily replacement of medium until a cell morphology consistent with EG cells is observed, typically after 7-30 days or 1-4 passages. For additional details on methods of preparation human EG cells see Shambloott et al., [Proc. Natl. Acad. Sci. USA
15 95: 13726, 1998] and U.S. Pat. No. 6,090,622.

Embryonic stem cells (e.g., human ESCs) originating from an unfertilized ova stimulated by parthenogenesis (parthenotes) are known in the art (e.g., Zhenyu Lu et al., 2010. J. Assist Reprod. Genet. 27:285-291; "Derivation and long-term culture of human parthenogenetic embryonic stem cells using human foreskin feeders", which is fully
20 incorporated herein by reference). Parthenogenesis refers to the initiation of cell division by activation of ova in the absence of sperm cells, for example using electrical or chemical stimulation. The activated ovum (parthenote) is capable of developing into a primitive embryonic structure (called a blastocyst) but cannot develop to term as the cells are pluripotent, meaning that they cannot develop the necessary extra-embryonic tissues (such
25 as amniotic fluid) needed for a viable human fetus.

Induced pluripotent stem cells (iPS) (embryonic-like stem cells) can be generated from somatic cells by genetic manipulation of somatic cells, e.g., by retroviral transduction of somatic cells such as fibroblasts, hepatocytes, gastric epithelial cells with transcription factors such as Oct-3/4, Sox2, c-Myc, and KLF4 [Yamanaka S, Cell Stem
30 Cell. 2007, 1(1):39-49; Aoi T, et al., Generation of Pluripotent Stem Cells from Adult Mouse Liver and Stomach Cells. Science. 2008 Feb 14. (Epub ahead of print); IH Park, Zhao R, West JA, et al. Reprogramming of human somatic cells to pluripotency with

defined factors. Nature 2008;451:141-146; K Takahashi, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861-872]. Other embryonic-like stem cells can be generated by nuclear transfer to oocytes, fusion with embryonic stem cells or nuclear transfer into zygotes if the recipient cells are arrested in mitosis.

Adult tissue stem cells can be isolated using various methods known in the art such as those disclosed by Alison, M.R. [J Pathol. 2003 200(5): 547-50], Cai, J. et al., [Blood Cells Mol Dis. 2003 31(1): 18-27], Collins, A.T. et al., [J Cell Sci. 2001; 114(Pt 21): 3865-72], Potten, C. S. and Morris, R. J. [Epithelial stem cells *in vivo*. 1988. J. Cell Sci. Suppl. 10, 45-62], Dominici, M et al., [J. Biol. Regul. Homeost. Agents. 2001, 15: 28-37], Caplan and Haynesworth [U.S. Pat. No. 5,486,359] Jones E.A. et al., [Arthritis Rheum. 2002, 46(12): 3349-60]. Fetal stem cells can be isolated using various methods known in the art such as those disclosed by Eventov-Friedman S, et al., PLoS Med. 2006, 3: e215; Eventov-Friedman S, et al., Proc Natl Acad Sci U S A. 2005, 102: 2928-33; Dekel B, et al., 2003, Nat Med. 9: 53-60; and Dekel B, et al., 2002, J. Am. Soc. Nephrol. 13: 977-90. Hematopoietic stem cells can be isolated using various methods known in the arts such as those disclosed by "Handbook of Stem Cells" edit by Robert Lanze, Elsevier Academic Press, 2004, Chapter 54, pp609-614, isolation and characterization of hematopoietic stem cells, by Gerald J Spangrude and William B Stayton.

Generally, isolation of adult tissue stem cells is based on the discrete location (or niche) of each cell type included in the adult tissue, *i.e.*, the stem cells, the transit amplifying cells and the terminally differentiated cells [Potten, C. S. and Morris, R. J. (1988). Epithelial stem cells *in vivo*. J. Cell Sci. Suppl. 10, 45-62]. Thus, an adult tissue such as, for example, prostate tissue is digested with Collagenase and subjected to repeated unit gravity centrifugation to separate the epithelial structures of the prostate (e.g., organoids, acini and ducts) from the stromal cells. Organoids are then disaggregated into single cell suspensions by incubation with Trypsin/EDTA (Life Technologies, Paisley, UK) and the basal, CD44-positive, stem cells are isolated from the luminal, CD57-positive, terminally differentiated secretory cells, using anti-human CD44 antibody (clone G44-26; Pharmingen, Becton Dickinson, Oxford, UK) labeling and incubation with MACS (Miltenyi Biotec Ltd, Surrey, UK) goat anti-mouse IgG microbeads. The cell suspension is then applied to a MACS column and the basal cells are eluted and re-

suspended in WAJC 404 complete medium [Robinson, E.J. et al. (1998). Basal cells are progenitors of luminal cells in primary cultures of differentiating human prostatic epithelium Prostate 37, 149-160].

Since basal stem cells can adhere to basement membrane proteins more rapidly
5 than other basal cells [Jones, P.H. et al. (1995). Stem cell patterning and fate in human epidermis. Cell 60, 83-93; Shinohara, T., et al. (1999). β 1- and α 6-integrin are surface markers on mouse spermatogonial stem cells. Proc. Natl. Acad. Sci. USA 96, 5504-5509] the CD44 positive basal cells are plated onto tissue culture dishes coated with either type I collagen (52 μ g/ml), type IV collagen (88 μ g/ml) or laminin 1 (100 μ g/ml; Biocoat®,
10 Becton Dickinson) previously blocked with 0.3 % bovine serum albumin (fraction V, Sigma-Aldrich, Poole, UK) in Dulbecco's phosphate buffered saline (PBS; Oxoid Ltd, Basingstoke, UK). Following 5 minutes, the tissue culture dishes are washed with PBS and adherent cells, containing the prostate tissue basal stem cells are harvested with trypsin-EDTA.

15 ***BM-derived stem cell, mesenchymal stem cells***

According to one embodiment, the stem cells utilized by some embodiments of the invention are BM-derived stem cells including hematopoietic, stromal or mesenchymal stem cells (Dominici, M et al., 2001. Bone marrow mesenchymal cells: biological properties and clinical applications. J. Biol. Regul. Homeost. Agents. 15: 28-37). BM-
20 derived stem cells may be obtained from iliac crest, femora, tibiae, spine, rib or other medullar spaces.

Of the above described BM-derived stem cells, mesenchymal stem cells are the formative pluripotent blast cells, and as such are preferred for use with some embodiments of the invention. Mesenchymal stem cells give rise to one or more mesenchymal tissues
25 (e.g., adipose, osseous, cartilaginous, elastic and fibrous connective tissues, myoblasts) as well as to tissues other than those originating in the embryonic mesoderm (e.g., neural cells) depending upon various influences from bioactive factors such as cytokines. Although such cells can be isolated from embryonic yolk sac, placenta, umbilical cord, fetal and adolescent skin, blood and other tissues, their abundance in the BM far exceeds
30 their abundance in other tissues and as such isolation from BM is presently preferred.

Methods of isolating, purifying and expanding mesenchymal stem cells (MSCs) are known in the arts and include, for example, those disclosed by Caplan and

Haynesworth in U.S. Pat. No. 5,486,359 and Jones E.A. et al., 2002, Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells, *Arthritis Rheum.* 46(12): 3349-60.

Preferably, mesenchymal stem cell cultures are generated by diluting BM aspirates (usually 20 ml) with equal volumes of Hank's balanced salt solution (HBSS; GIBCO Laboratories, Grand Island, NY, USA) and layering the diluted cells over about 10 ml of a Ficoll column (Ficoll-Paque; Pharmacia, Piscataway, NJ, USA). Following 30 minutes of centrifugation at 2,500 x g, the mononuclear cell layer is removed from the interface and suspended in HBSS. Cells are then centrifuged at 1,500 x g for 15 minutes and resuspended in a complete medium (MEM, α medium without deoxyribonucleotides or ribonucleotides; GIBCO); 20 % fetal calf serum (FCS) derived from a lot selected for rapid growth of MSCs (Atlanta Biologicals, Norcross, GA); 100 units/ml penicillin (GIBCO), 100 μ g/ml streptomycin (GIBCO); and 2 mM L-glutamine (GIBCO). Resuspended cells are plated in about 25 ml of medium in a 10 cm culture dish (Corning Glass Works, Corning, NY) and incubated at 37 °C with 5 % humidified CO₂. Following 24 hours in culture, nonadherent cells are discarded, and the adherent cells are thoroughly washed twice with phosphate buffered saline (PBS). The medium is replaced with a fresh complete medium every 3 or 4 days for about 14 days. Adherent cells are then harvested with 0.25 % trypsin and 1 mM EDTA (Trypsin/EDTA, GIBCO) for 5 min at 37 °C, replated in a 6-cm plate and cultured for another 14 days. Cells are then trypsinized and counted using a cell counting device such as for example, a hemocytometer (Hausser Scientific, Horsham, PA). Cultured cells are recovered by centrifugation and resuspended with 5 % DMSO and 30 % FCS at a concentration of 1 to 2 X 10⁶ cells per ml. Aliquots of about 1 ml each are slowly frozen and stored in liquid nitrogen.

To expand the mesenchymal stem cell fraction, frozen cells are thawed at 37 °C, diluted with a complete medium and recovered by centrifugation to remove the DMSO. Cells are resuspended in a complete medium and plated at a concentration of about 5,000 cells/cm². Following 24 hours in culture, nonadherent cells are removed and the adherent cells are harvested using Trypsin/EDTA, dissociated by passage through a narrowed Pasteur pipette, and preferably replated at a density of about 1.5 to about 3.0 cells/cm². Under these conditions, MSC cultures can grow for about 50 population doublings and be expanded for about 2000 fold [Colter DC., et al. Rapid expansion of recycling stem cells

in cultures of plastic-adherent cells from human bone marrow. Proc Natl Acad Sci USA. 97: 3213-3218, 2000].

MSC cultures utilized by some embodiments of the invention preferably include three groups of cells which are defined by their morphological features: small and agranular cells (referred to as RS-1, hereinbelow), small and granular cells (referred to as RS-2, hereinbelow) and large and moderately granular cells (referred to as mature MSCs, hereinbelow). The presence and concentration of such cells in culture can be assayed by identifying a presence or absence of various cell surface markers, by using, for example, immunofluorescence, *in situ* hybridization, and activity assays.

When MSCs are cultured under the culturing conditions of some embodiments of the invention they exhibit negative staining for the hematopoietic stem cell markers CD34, CD11B, CD43 and CD45. A small fraction of cells (less than 10 %) are dimly positive for CD31 and/or CD38 markers. In addition, mature MSCs are dimly positive for the hematopoietic stem cell marker, CD117 (c-Kit), moderately positive for the osteogenic MSCs marker, Stro-1 [Simmons, P. J. & Torok-Storb, B. (1991). Blood 78, 5562] and positive for the thymocytes and peripheral T lymphocytes marker, CD90 (Thy-1). On the other hand, the RS-1 cells are negative for the CD117 and Stro1 markers and are dimly positive for the CD90 marker, and the RS-2 cells are negative for all of these markers.

It will be appreciated that undifferentiated stem cells are of a distinct morphology, which is clearly distinguishable from differentiated cells of embryo or adult origin by the skilled in the art. Typically, undifferentiated stem cells have high nuclear/cytoplasmic ratios, prominent nucleoli and compact colony formation with poorly discernable cell junctions. Additional features of undifferentiated stem cells are further described hereinunder.

Additional cells that can be used with the present teachings are as follows:

The phrase “mesodermal progenitor cells” refers to the population of cells that exhibit both mesenchymal and endothelial differentiation potential. According to one embodiment, mesodermal progenitor cells are capable of differentiating into mesodermal angioblasts and endothelial cells (EC) under specific conditions. Mesodermal progenitor cells (MPCs) can be selected by the phenotype CD105⁺SSEA-4⁺CD90^{neg} and typically lack the markers, MSCA-1, CD166, CD271, W5B5, and CD146. MPCs typically express the pluripotency-associated marker SSEA-4 and the nuclear factors *Oct-3/4* and *Nanog*.

Mesodermal progenitor cells may be obtained, generated and/or selected by any method known to one of skill in the art, for example, those disclosed in U.S. Patent Application No. 20100150887, incorporated herein by reference. Another protocol to harvest mesodermal progenitor cells (MPCs) e.g. from human bone marrow mono-nucleate cells (BMMNCs) supplemented with autologous serum can be found in Trombi L. et al. [Trombi L. et al. (2009) Selective culture of mesodermal progenitor cells. *Stem Cells Dev* 18(8): 1227–1234 and Petrini M. et al., *Stem Cells and Development* (2009) 18(6): 857-866, both incorporated herein by reference].

As used herein, the phrase “vascular progenitor cells” refers to adult stem cells which are capable of differentiating into endothelial lineage. Vascular progenitor cells can be induced to differentiate into endothelial progenitor cells and endothelial cells (EC) under specific conditions. Vascular progenitor cells can be selected by expression of characteristic markers, such as but not limited to, CD34, CD31 (PECAM-1), CD144, CD146, CD105, PDGFR-beta, KDR and/or ETV2. Typically, vascular progenitor cells do not express CD133.

The phrases “endothelial progenitor cells”, “endothelial precursor cells” or “EPCs” refer to cells of the endothelial lineage capable of differentiating into mature endothelial cells, for example, into blood vessel endothelial cells. Exemplary EPCs include monocytic EPCs and hemangioblastic EPCs.

Endothelial progenitor cells may be obtained by any method known to one of skill in the art, for example, those disclosed in U.S. Patent Application No. 20130224116, incorporated herein by reference. According to one embodiment, EPCs may be selected for expression of any of sphingosine kinase 1 (SK-1), CD34, CD14, CD133, CD45, CD31 and/or VEGFR2. Alternatively or additionally, EPCs may not express significant or above background levels of CD144 and/or vWF and/or eNOS and/or Tie2. Alternatively or additionally, EPCs may produce pro-angiogenic factors, e.g., hepatocyte growth factor and/or insulin-like growth factor-1 and/or basic fibroblast growth factor and/or VEGF. According to one embodiment, EPCs do not adhere to tissue culture plastic-ware, optionally plastic-ware coated with extracellular matrix or a component thereof (e.g., fibronectin). Thus, according to one example, EPCs may be isolated from 4-7 day cultured non-adherent CD133 expressing mononuclear cells.

The phrase “angioblasts” refers to cells of the embryonic mesenchymal tissue committed to endothelial lineage differentiation. Angioblasts are capable of differentiating into endothelial progenitor cells and endothelial cells (EC) under specific conditions. Typically, angioblasts can be selected by expression of VEGFR2, CD31, CD133/1 and/or CD117. They may also express the transcription factor GATA-2 and/or ETV2.

According to one embodiment, the term angioblasts refers to multipotent angioblasts (e.g. meso-angioblasts or mesodermal angioblasts, i.e. multipotent, self-renewing cells that differentiate into most mesodermal tissues). Such cells can be identified by expression of the marker Flk-1, and optionally ETV2.

Angioblasts can be obtained by any method known in the art, for example, angioblasts may be obtained from stem cells in the bone marrow. According to one embodiment, the angioblasts are bone marrow derived angioblasts, as described by Kocher A et al., *Nat Med.* (2001) 7(4):430-6.

According to one embodiment, the angioblasts are angioblast-like progenitor cells derived from fibroblasts as taught by Kurian L. et al. *Nature Methods* 10, 77–83 (2013), incorporated herein by reference. Other methods of obtaining angioblasts are described in Yamashita, J. et al. *Nature* 408: 92-96 (2000) and in Kusuma, S. et al., *Proceedings of the National Academy of Sciences of the United States of America* 110: 12601-12606 (2013), both incorporated herein by reference.

According to one aspect of the invention, promoting LEC specification in stem cells or vascular progenitor cells and/or angioblasts so as to obtain a LEC committed cell is effected by upregulating the activity of Wnt canonical pathway in the stem cells or vascular progenitor cells and/or angioblasts.

As used herein the phrase “Wnt canonical pathway” refers to the Wnt signaling pathway which activates transcription of a TCF regulated gene (i.e., a gene comprising a Tcf/LEF consensus binding site in its promoter), including TCF1, TCF2, TCF3, LEF1 and LEF2. In some embodiments, activation of the Wnt signaling pathway results in the activation of transcription of a Wnt target gene. Wnt target genes are described in further detail below.

In some embodiments, the pathway involves signaling from Wnt receptors (e.g. Frizzleds) to β -catenin. In some embodiments, the activation of the Wnt canonical signaling pathway results in an increase in activity of β -catenin, such as an accumulation

of active β -catenin in the cytoplasm, such as an increase in the amount of phosphorylated β -catenin in the cytoplasm.

The Wnt signaling pathway is described in detail in [www\(dot\)stanford\(dot\)edu/about\(dot\)rnusse/wntwindow\(dot\)html](http://www.stanford.edu/about/rnusse/wntwindow.html), as well as in Thorstensen et al (2003), Atlas Genet Cytogenet Oncol Haematol. April 2003 (5 [www\(dot\)infobiogen\(dot\)fr/services/chromcancer/Deep/WntSignPathID20042\(dot\)html](http://www.infobiogen.fr/services/chromcancer/Deep/WntSignPathID20042(dot)html)). Detailed reviews of Wnt signaling and action are set out in Logan and Nusse (2004), Annu. Rev. Cell Dev. Biol. 20, 781-810 and Wodarz and Nusse (1998), Annu. Rev. Cell Dev. Biol. 14, 59-88, all incorporated herein by reference. The Wnt canonical pathway is also illustrated in Figure 16A. (10

Generally, the canonical pathway functions through the activation of the Wnt receptor to deactivate the destruction complex-GSK3, Axin, APC and CK1 α and to stabilize β -catenin so as to mediate its function in transcription.

In short, Wnt signaling begins when one of the Wnt proteins binds to a Frizzled (Fz) family receptor, a G-protein coupled receptors (GPCRs). To facilitate Wnt signaling, co-receptors may also be required alongside the interaction between the Wnt protein and Fz receptor. Such co-receptors include, for example, the lipoprotein receptor-related protein (LRP)-5/6, the receptor tyrosine kinase (Ryk), or ROR2. Upon binding and activation of the receptor, a signal is sent to the phosphoprotein Dishevelled (Dsh), which (20 is located in the cytoplasm. This signal is transmitted via a direct interaction between Fz and Dsh. Dsh then becomes phosphorylation and activated and inhibits the activity of GSK3 of the destruction complex (described in detail below). This allows β -catenin to accumulate and localize to the nucleus and subsequently induce a cellular response via gene transduction alongside the TCF/LEF (T-cell factor/lymphoid enhancing factor) transcription factors. Without Wnt signaling, β -catenin does not accumulate in the cytoplasm since the destruction complex degrades it. This destruction complex includes the following proteins: Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GSK3, e.g. GSK3 β) and casein kinase 1 α (CK1 α). It degrades β -catenin by targeting it for ubiquitination, which subsequently sends it to the proteasome to be digested. However, as soon as Wnt binds Fz and LRP-5/6, the destruction complex function becomes disrupted. This is due to Wnt causing the translocation of the negative Wnt regulator, Axin, and the destruction complex to the (30

plasma membrane. Phosphorylation by other proteins in the destruction complex subsequently binds Axin to the cytoplasmic tail of LRP-5/6. Axin becomes de-phosphorylated and its stability and levels are decreased.

Thus, any composition which acts to upregulate activity or expression of β -catenin
5 is contemplated herein.

In some embodiments, the activity of Wnt canonical signaling in the stem cells or vascular progenitor cells or angioblasts may be increased by 10 %, 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 % or 100 % or more, as compared to stem cells or vascular progenitor cells or angioblasts not treated by a composition which acts to upregulate Wnt canonical
10 signaling activity, to effect differentiation of the stem cells or vascular progenitor cells or angioblasts towards LEC. In some embodiments, the activity or activation of the Wnt canonical signaling pathway may be assayed using any method known to one of skill in the art, such as described below.

According to one embodiment, upregulating the activity of Wnt canonical pathway
15 is effected by contacting the stem cells and/or vascular progenitor cells and/or angioblasts with an activator of a Wnt canonical pathway member, including but not limited to, Wnt5b, a Wnt3a, a Wnt7a, a Wnt7b, a LRP5/6, a β -catenin, Dishevelled (Dsh), PP2A, ARFGAP1, and TCF.

Upregulation of the Wnt canonical pathway can be effected at the protein or
20 nucleic acid level, as summarized infra.

According to another embodiment, upregulating the activity of Wnt canonical pathway is effected by down-regulation of any antagonist or negative regulator or component of the Wnt canonical pathway. Exemplary antagonist or negative regulator or component of the Wnt pathway include, but are not limited to, Axin, glycogen synthase
25 kinase 3 (GSK3, e.g. GSK3 β), APC and sFRP (Secreted frizzled-related proteins).

Down-regulation can be effected on the genomic and/or the transcript level as summarized in further detail hereinbelow.

As used herein, the phrase “Wnt non-canonical pathway” refers to the β -catenin-independent signaling pathway. Typically this pathway can be divided into two distinct
30 branches, the Planar Cell Polarity pathway (or PCP pathway) and the Wnt/Ca²⁺ pathway. The Wnt non-canonical pathway is also illustrated in Figures 16B-C (PCP and Wnt/calcium pathways, respectively).

In both pathways signaling is transduced through Fzd independent of LRP5/6 leading to the activation of Dsh. In the PCP pathway, Dsh through Daam1 mediates activation of Rho which in turn activates Rho kinase (ROCK). Daam1 also mediates actin polymerization through the actin binding protein Profilin. Dsh also mediates activation of Rac, which in turn activates JNK.

Any means for increasing Wnt non-canonical signaling activity may be used, including both direct and indirect modulation. These may include for example, modulating the expression of any endogenous gene for any member of the Wnt non-canonical signaling pathway (described in further detail below) at the transcriptional, translational or post-translational level, such as modulating the persistence or breakdown of messenger RNA for the member of the Wnt non-canonical signaling pathway, modulating the persistence or breakdown of protein, etc. They may also include modulation of the activity of a member of the Wnt non-canonical signaling pathway, such as by use of agonists thereof. Furthermore, the expression and/or activity of activators of any member of the Wnt non-canonical signaling pathway may be modulated to upregulate Wnt non-canonical signaling activity. These are described in further detail below.

In some embodiments, the activity of Wnt non-canonical signaling in LEC committed cells may be increased by 10 %, 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 % or 100 % or more, as compared to LEC committed cells not effected by a LEC morphogenesis signal, to effect morphogenesis of the LEC committed cell. In some embodiments, the activity or activation of the Wnt non-canonical signaling pathway may be assayed using any method known to one of skill in the art, such as described below.

According to one embodiment, upregulating the activity of Wnt non-canonical pathway is effected by contacting a LEC with an activator of a Wnt non-canonical pathway member, including but not limited to, Wnt5a, Wnt4, Wnt11, Ror2, Ryk, G protein, Dishevelled (Dsh) and Dishevelled-associated activator of morphogenesis 1 (DAAM1).

Upregulation of the Wnt non-canonical pathway can be effected at the protein or nucleic acid level, as summarized infra.

Upregulation of the activity of Wnt canonical or non-canonical pathways can be effected at the genomic level (e.g., polynucleotide sequences encoding the expression product of interest and/or activation of transcription via promoters, enhancers, regulatory

elements), at the transcript level (*i.e.*, correct splicing, polyadenylation, activation of translation, cDNA introduction) or at the protein level e.g., administering the protein of interest (e.g., soluble protein, e.g., secreted).

Following is a list of agents capable of upregulating the expression level and/or
5 activity of the Wnt canonical or non-canonical pathways.

An agent capable of upregulating expression of a Wnt canonical or non-canonical pathway component may be an exogenous polynucleotide sequence designed and constructed to express at least a functional portion of a Wnt canonical or non-canonical pathway component [e.g. for Wnt canonical pathway: a Frizzled receptor, an LRP co-
10 receptor, a Wnt ligand, a Dishevelled (Dsh), PP2A, ARFGAP1, dominant stable mutated β -catenin (e.g. mutated in exon3-GSK-3b phosphorylation sites as described in cancerres(dot)aacrjournals(dot)org/content/58/5/1021(dot)full(dot)pdf, and in www(dot)ncbi(dot)nlm(dot)nih(dot)gov/pubmed/10545105, both incorporated herein by reference; e.g. for Wnt non-canonical pathway: Dsh, DAAM1, Wnt4, Wnt5a or Wnt11].
15 Accordingly, the exogenous polynucleotide sequence may be a DNA or RNA sequence encoding a molecule capable of upregulating the Wnt canonical or non-canonical pathway component (e.g. for Wnt canonical pathway: a Frizzled receptor, a Wnt ligand, a Dishevelled (Dsh), PP2A or ARFGAP1; e.g. for Wnt non-canonical pathway: Dsh, DAAM1, Wnt4, Wnt5a or Wnt11).

20 The phrase “functional portion” as used herein refers to part of the protein (*i.e.*, a polypeptide) which exhibits functional properties of the protein such as binding to a substrate. According to preferred embodiments of some embodiments of the invention the functional portion of a Wnt canonical or non-canonical pathway component (e.g. for Wnt canonical pathway: a Frizzled receptor, a Wnt ligand, a Dishevelled (Dsh), PP2A or
25 ARFGAP1; e.g. for Wnt non-canonical pathway: Dsh, DAAM1, Wnt4, Wnt5a or Wnt11) is a polypeptide sequence.

Wnt canonical or non-canonical pathway components (e.g. for Wnt canonical pathway: a Frizzled receptor, a Wnt ligand, a Dishevelled (Dsh), PP2A, ARFGAP1, etc.; e.g. for Wnt non-canonical pathway: Dsh, DAAM1, Wnt4, Wnt5a or Wnt11) have been
30 cloned from human, rat and mouse sources. Accordingly, coding sequences information for Wnt canonical or non-canonical pathway component (e.g. for Wnt canonical pathway: a Frizzled receptor, a Wnt ligand, a Dishevelled (Dsh), PP2A or ARFGAP1; e.g. for Wnt

non-canonical pathway: Dsh, DAAM1, Wnt4, Wnt5a or Wnt11) is available from several databases including the GenBank database available at the National Center for Biotechnology Information at [www\(dot\)ncbi\(dot\)nlim\(dot\)nih\(dot\)gov/](http://www.ncbi.nlm.nih.gov/).

To express an exogenous Wnt canonical or non-canonical pathway component (e.g. for Wnt canonical pathway: a Frizzled receptor, a Wnt ligand, a Dishevelled (Dsh), PP2A or ARFGAP1; e.g. for Wnt non-canonical pathway: Dsh, DAAM1, Wnt4, Wnt5a or Wnt11) in mammalian cells, a polynucleotide sequence encoding the Wnt canonical or non-canonical pathway component (e.g. a Frizzled receptor, a Wnt ligand, a Dishevelled (Dsh), PP2A, ARFGAP1, DAAM1, exemplary GenBank Accession numbers are provided hereinbelow) is preferably ligated into a nucleic acid construct suitable for mammalian cell expression. Such a nucleic acid construct includes a promoter sequence for directing transcription of the polynucleotide sequence in the cell in a constitutive or inducible manner.

It will be appreciated that the nucleic acid construct of some embodiments of the invention can also utilize homologues which exhibit the desired activity (*i.e.*, promote LEC specification).

It will be appreciated that the nucleic acid construct of some embodiments of the invention can also utilize homologues which exhibit the desired activity (*i.e.*, promote LEC specification). Such homologues can be, for example, at least 80 %, at least 81 %, at least 82 %, at least 83 %, at least 84 %, at least 85 %, at least 86 %, at least 87 %, at least 88 %, at least 89 %, at least 90 %, at least 91 %, at least 92 %, at least 93 %, at least 94 %, at least 95 %, at least 96 %, at least 97 %, at least 98 %, at least 99 % or 100 % identical to the desired sequences, as determined using the BestFit software of the Wisconsin sequence analysis package, utilizing the Smith and Waterman algorithm, where gap weight equals 50, length weight equals 3, average match equals 10 and average mismatch equals -9.

Constitutive promoters suitable for use with some embodiments of the invention are promoter sequences which are active under most environmental conditions and most types of cells such as the cytomegalovirus (CMV) and Rous sarcoma virus (RSV). Inducible promoters suitable for use with some embodiments of the invention include for example the tetracycline-inducible promoter (Zabala M, et al., Cancer Res. 2004, 64(8): 2799-804).

The nucleic acid construct (also referred to herein as an "expression vector") of some embodiments of the invention includes additional sequences which render this vector

suitable for replication and integration in prokaryotes, eukaryotes, or preferably both (e.g., shuttle vectors). In addition, typical cloning vectors may also contain a transcription and translation initiation sequence, transcription and translation terminator and a polyadenylation signal. By way of example, such constructs will typically include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second-strand DNA synthesis, and a 3' LTR or a portion thereof.

The nucleic acid construct of some embodiments of the invention typically includes a signal sequence for secretion of the peptide from a host cell in which it is placed. Preferably the signal sequence for this purpose is a mammalian signal sequence or the signal sequence of the polypeptide variants of some embodiments of the invention.

Eukaryotic promoters typically contain two types of recognition sequences, the TATA box and upstream promoter elements. The TATA box, located 25-30 base pairs upstream of the transcription initiation site, is thought to be involved in directing RNA polymerase to begin RNA synthesis. The other upstream promoter elements determine the rate at which transcription is initiated.

Preferably, the promoter utilized by the nucleic acid construct of some embodiments of the invention is active in the specific cell population transformed. Examples of cell type-specific and/or tissue-specific promoters include promoters such as albumin that is liver specific [Pinkert et al., (1987) *Genes Dev.* 1:268-277], lymphoid specific promoters [Calame et al., (1988) *Adv. Immunol.* 43:235-275]; in particular promoters of T-cell receptors [Winoto et al., (1989) *EMBO J.* 8:729-733] and immunoglobulins; [Banerji et al. (1983) *Cell* 33729-740], neuron-specific promoters such as the neurofilament promoter [Byrne et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477], pancreas-specific promoters [Edlunch et al. (1985) *Science* 230:912-916] or mammary gland-specific promoters such as the milk whey promoter (U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166).

Enhancer elements can stimulate transcription up to 1,000 fold from linked homologous or heterologous promoters. Enhancers are active when placed downstream or upstream from the transcription initiation site. Many enhancer elements derived from viruses have a broad host range and are active in a variety of tissues. For example, the SV40 early gene enhancer is suitable for many cell types. Other enhancer/promoter combinations that are suitable for some embodiments of the invention include those

derived from polyoma virus, human or murine cytomegalovirus (CMV), the long term repeat from various retroviruses such as murine leukemia virus, murine or Rous sarcoma virus and HIV. See, *Enhancers and Eukaryotic Expression*, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. 1983, which is incorporated herein by reference.

5 In the construction of the expression vector, the promoter is preferably positioned approximately the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

 Polyadenylation sequences can also be added to the expression vector in order to
10 increase the efficiency of mRNA translation. Two distinct sequence elements are required for accurate and efficient polyadenylation: GU or U rich sequences located downstream from the polyadenylation site and a highly conserved sequence of six nucleotides, AAUAAA, located 11-30 nucleotides upstream. Termination and polyadenylation signals that are suitable for some embodiments of the invention include those derived from SV40.

15 In addition to the elements already described, the expression vector of some embodiments of the invention may typically contain other specialized elements intended to increase the level of expression of cloned nucleic acids or to facilitate the identification of cells that carry the recombinant DNA. For example, a number of animal viruses contain DNA sequences that promote the extra chromosomal replication of the viral genome in
20 permissive cell types. Plasmids bearing these viral replicons are replicated episomally as long as the appropriate factors are provided by genes either carried on the plasmid or with the genome of the host cell.

 The vector may or may not include a eukaryotic replicon. If a eukaryotic replicon is present, then the vector is amplifiable in eukaryotic cells using the appropriate
25 selectable marker. If the vector does not comprise a eukaryotic replicon, no episomal amplification is possible. Instead, the recombinant DNA integrates into the genome of the engineered cell, where the promoter directs expression of the desired nucleic acid.

 The expression vector of some embodiments of the invention can further include additional polynucleotide sequences that allow, for example, the translation of several
30 proteins from a single mRNA such as an internal ribosome entry site (IRES) and sequences for genomic integration of the promoter-chimeric polypeptide.

It will be appreciated that the individual elements comprised in the expression vector can be arranged in a variety of configurations. For example, enhancer elements, promoters and the like, and even the polynucleotide sequence(s) encoding a Wnt canonical or non-canonical pathway component (e.g. a Frizzled receptor, a Wnt ligand, a Dishevelled (Dsh), PP2A, ARFGAP1 or DAAM1) can be arranged in a "head-to-tail" configuration, may be present as an inverted complement, or in a complementary configuration, as an anti-parallel strand. While such variety of configuration is more likely to occur with non-coding elements of the expression vector, alternative configurations of the coding sequence within the expression vector are also envisioned.

Examples for mammalian expression vectors include, but are not limited to, pcDNA3, pcDNA3.1(+/-), pGL3, pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pSinRep5, DH26S, DHBB, pNMT1, pNMT41, pNMT81, which are available from Invitrogen, pCI which is available from Promega, pMbac, pPbac, pBK-RSV and pBK-CMV which are available from Strategene, pTRES which is available from Clontech, and their derivatives.

Expression vectors containing regulatory elements from eukaryotic viruses such as retroviruses can be also used. SV40 vectors include pSVT7 and pMT2. Vectors derived from bovine papilloma virus include pBV-1MTHA, and vectors derived from Epstein Bar virus include pHEBO, and p2O5. Other exemplary vectors include pMSG, pAV009/A⁺, pMTO10/A⁺, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV-40 early promoter, SV-40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

As described above, viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of viral vectors utilizes its natural specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected cell. Thus, the type of vector used by some embodiments of the invention will depend on the cell type transformed. The ability to select suitable vectors according to the cell type transformed is well within the capabilities of the ordinary skilled artisan and as such no general description of selection consideration

is provided herein. For example, bone marrow cells can be targeted using the human T cell leukemia virus type I (HTLV-I) and kidney cells may be targeted using the heterologous promoter present in the baculovirus *Autographa californica* nucleopolyhedrovirus (AcMNPV) as described in Liang CY et al., 2004 (Arch Virol. 149: 51-60).

Recombinant viral vectors are useful for *in vivo* expression of a Wnt canonical or non-canonical pathway component (e.g. a Frizzled receptor, a Wnt ligand, a Dishevelled (Dsh), PP2A, ARFGAP1, or DAAM1) since they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

Various methods can be used to introduce the expression vector of some embodiments of the invention into stem cells or vascular progenitor cells or angioblasts. Such methods are generally described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Md. (1989), Chang et al., *Somatic Gene Therapy*, CRC Press, Ann Arbor, Mich. (1995), Vega et al., *Gene Targeting*, CRC Press, Ann Arbor Mich. (1995), *Vectors: A Survey of Molecular Cloning Vectors and Their Uses*, Butterworths, Boston Mass. (1988) and Gilboa et al. [*Biotechniques* 4 (6): 504-512, 1986] and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see U.S. Pat. Nos. 5,464,764 and 5,487,992 for positive-negative selection methods.

Introduction of nucleic acids by viral infection offers several advantages over other methods such as lipofection and electroporation, since higher transfection efficiency can be obtained due to the infectious nature of viruses.

Currently preferred *in vivo* nucleic acid transfer techniques include transfection with viral or non-viral constructs, such as adenovirus, lentivirus, Herpes simplex I virus, or adeno-associated virus (AAV) and lipid-based systems. Useful lipids for lipid-mediated transfer of the gene are, for example, DOTMA, DOPE, and DC-Chol [Tonkinson et al., 5 Cancer Investigation, 14(1): 54-65 (1996)]. The most preferred constructs for use in gene therapy are viruses, most preferably adenoviruses, AAV, lentiviruses, or retroviruses. A viral construct such as a retroviral construct includes at least one transcriptional promoter/enhancer or locus-defining element(s), or other elements that control gene expression by other means such as alternate splicing, nuclear RNA export, or post-10 translational modification of messenger. Such vector constructs also include a packaging signal, long terminal repeats (LTRs) or portions thereof, and positive and negative strand primer binding sites appropriate to the virus used, unless it is already present in the viral construct. In addition, such a construct typically includes a signal sequence for secretion of the peptide from a host cell in which it is placed. Preferably the signal sequence for this 15 purpose is a mammalian signal sequence or the signal sequence of the polypeptide variants of some embodiments of the invention. Optionally, the construct may also include a signal that directs polyadenylation, as well as one or more restriction sites and a translation termination sequence. By way of example, such constructs will typically include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second-strand DNA synthesis, and a 20 3' LTR or a portion thereof. Other vectors can be used that are non-viral, such as cationic lipids, polylysine, and dendrimers.

Administration of the a Wnt canonical or non-canonical pathway component expressing cells of some embodiments of the invention can be effected using any suitable route such as intravenous, intra peritoneal, intra kidney, intra gastrointestinal track, 25 subcutaneous, transcutaneous, intramuscular, intracutaneous, intrathecal, epidural and rectal. According to presently preferred embodiments, the Wnt canonical or non-canonical pathway component expressing cells of some embodiments of the invention are introduced to the individual using intravenous, intra kidney, intra gastrointestinal track and/or intra peritoneal administrations.

30 Wnt canonical or non-canonical pathway component (e.g. a Frizzled receptor, a Wnt ligand, a Dishevelled (Dsh), PP2A, ARFGAP1, DAAM1) expressing cells of some embodiments of the invention can be derived from either autologous sources such as self

bone marrow cells or from allogeneic sources such as bone marrow or other cells derived from non-autologous sources. Since non-autologous cells are likely to induce an immune reaction when administered to the body several approaches have been developed to reduce the likelihood of rejection of non-autologous cells. These include either suppressing the recipient immune system or encapsulating the non-autologous cells or tissues in immunoisolating, semipermeable membranes before transplantation.

Encapsulation techniques are generally classified as microencapsulation, involving small spherical vehicles and macroencapsulation, involving larger flat-sheet and hollow-fiber membranes (Uludag, H. et al. Technology of mammalian cell encapsulation. Adv Drug Deliv Rev. 2000; 42: 29-64).

Methods of preparing microcapsules are known in the arts and include for example those disclosed by Lu MZ, et al., Cell encapsulation with alginate and alpha-phenoxycinnamylidene-acetylated poly(allylamine). Biotechnol Bioeng. 2000, 70: 479-83, Chang TM and Prakash S. Procedures for microencapsulation of enzymes, cells and genetically engineered microorganisms. Mol Biotechnol. 2001, 17: 249-60, and Lu MZ, et al., A novel cell encapsulation method using photosensitive poly(allylamine alpha-cyanocinnamylideneacetate). J Microencapsul. 2000, 17: 245-51.

For example, microcapsules are prepared by complexing modified collagen with a ter-polymer shell of 2-hydroxyethyl methacrylate (HEMA), methacrylic acid (MAA) and methyl methacrylate (MMA), resulting in a capsule thickness of 2-5 μm . Such microcapsules can be further encapsulated with additional 2-5 μm ter-polymer shells in order to impart a negatively charged smooth surface and to minimize plasma protein absorption (Chia, S.M. et al. Multi-layered microcapsules for cell encapsulation Biomaterials. 2002 23: 849-56).

Other microcapsules are based on alginate, a marine polysaccharide (Sambanis, A. Encapsulated islets in diabetes treatment. Diabetes Technol. Ther. 2003, 5: 665-8) or its derivatives. For example, microcapsules can be prepared by the polyelectrolyte complexation between the polyanions sodium alginate and sodium cellulose sulphate with the polycation poly(methylene-co-guanidine) hydrochloride in the presence of calcium chloride.

It will be appreciated that cell encapsulation is improved when smaller capsules are used. Thus, the quality control, mechanical stability, diffusion properties, and *in vitro*

activities of encapsulated cells improved when the capsule size was reduced from 1 mm to 400 μm (Canaple L. et al., Improving cell encapsulation through size control. *J Biomater Sci Polym Ed.* 2002;13: 783-96). Moreover, nanoporous biocapsules with well-controlled pore size as small as 7 nm, tailored surface chemistries and precise microarchitectures were found to successfully immunoisolate microenvironments for cells (Williams D. Small is beautiful: microparticle and nanoparticle technology in medical devices. *Med Device Technol.* 1999, 10: 6-9; Desai, T.A. Microfabrication technology for pancreatic cell encapsulation. *Expert Opin Biol Ther.* 2002, 2: 633-46).

An agent capable of upregulating a Wnt canonical or non-canonical pathway component (e.g. a Frizzled receptor, a Wnt ligand, a Dishevelled (Dsh), PP2A, ARFGAP1 or DAAM1) may also be any compound which is capable of increasing the transcription and/or translation of an endogenous DNA or mRNA encoding the Wnt canonical or non-canonical pathway component and thus increasing endogenous Wnt pathway activity.

An agent capable of upregulating a Wnt canonical or non-canonical pathway component (e.g. a Frizzled receptor, a Wnt ligand, a Dishevelled (Dsh), PP2A, ARFGAP1 or DAAM1) may also be an exogenous polypeptide including at least a functional portion (as described hereinabove) of the a Wnt canonical or non-canonical pathway component (e.g. a Frizzled receptor, a Wnt ligand, a Dishevelled (Dsh), PP2A, ARFGAP1 or DAAM1).

Upregulation of a Wnt canonical or non-canonical pathway component (e.g. a Frizzled receptor, a Wnt ligand, a Dishevelled (Dsh), PP2A, ARFGAP1 or DAAM1) can be also achieved by introducing at least one substrate of a Wnt canonical or non-canonical pathway component.

Thus, any means for increasing Wnt canonical or non-canonical signaling activity may be used, including both direct and indirect modulation. These may include for example, modulating the expression of any endogenous gene for any member of the Wnt canonical or non-canonical signaling pathway (described in further detail below) at the transcriptional, translational or post-translational level, such as degradation of messenger RNA for the member of the Wnt canonical or non-canonical signaling pathway, modulating the persistence or degradation of protein, etc. They may also include modulation of the activity of a member of the Wnt canonical or non-canonical signaling pathway, such as by use of agonists thereof. Furthermore, the expression and/or activity of

activators of any member of the Wnt canonical or non-canonical signaling pathway may be modulated to upregulate Wnt canonical or non-canonical signaling activity. These are described in further detail below.

According to one embodiment, Wnt canonical signaling pathway is activated by
5 activating any of the receptors for Wnt canonical signaling, i.e., a Wnt receptor. For example, any of the Frizzled receptors may be up-regulated to activate the Wnt canonical signaling pathway. Of note, in order to activate β -catenin the receptor has to bind to its ligand, alternatively, a constitutively active receptor may be used (as further detailed hereinbelow). Exemplary Frizzled receptors include, but are not limited to, FZD1
10 (frizzled-1, accession nos. NM_003505.1, NP_003496.1), FZD2 (frizzled-2, accession nos. NM_001466.3 and NP_001457.1), FZD3 (frizzled-3, accession nos. NM_017412.3 and NP_059108.1), FZD4 (frizzled-4, accession nos. NM_012193.3 and NP_036325.2), FZD5 (frizzled-5, accession nos. NM_003468.3 and NP_003459.2), FZD6 (frizzled-6, accession nos. NM_001164615.1 and NP_001158087.1), FZD7 (frizzled-7, accession nos.
15 NM_003507.1 and NP_003498.1), FZD8 (frizzled-8, accession nos. NM_031866.2 and NP_114072.1), FZD9 (frizzled-9, accession nos. NM_003508.2 and NP_003499.1) and/or FZD10 (frizzled-10, accession nos. NM_007197.3 and NP_009128.1).

Receptor activation may be achieved in a number of ways, for example, by upregulating the expression of the receptor. This may be achieved, for example, by
20 ligating a polynucleotide sequence encoding the receptor into a suitable expression vector and introducing same into the stem cell or vascular progenitor cell or angioblast. Furthermore, receptor activation may be achieved by introduction of a constitutively active Frizzled receptor to the stem cell or vascular progenitor cell or angioblast, for example by ligating a polynucleotide sequence encoding the constitutively active receptor
25 into a suitable expression vector and introducing same into the stem cell or vascular progenitor cell or angioblast. An exemplary constitutively active Frizzled receptor is described in Holmen et al., *Journal of Biological Chemistry* (2002) 277(38): 34727–34735, incorporated herein by reference.

Additionally or alternatively, the Norrin ligand which binds to Frizzled with high
30 affinity [described in detail in Xu et al., (2004) *Cell* 116(6):883-95], may be used to activate the Wnt canonical signaling pathway. The R-spondin2 protein which also binds to the Frizzled receptors [described in detail in Kazanskaya et al (2004) *Dev Cell*. 7(4):525-

34 and Kim et al., (2005) Science 309(5738):1256-9] may also be used in accordance with the present teachings.

Receptors for Wnt, e.g. Frizzled receptors, may also be activated by binding of Wnt ligand. Thus, the Wnt canonical signaling pathway may be activated by increasing the activity or expression of Wnt ligand, or by decreasing the activity or expression of antagonists of Wnt or Frizzled.

Exemplary Wnt ligands include, but are not limited to, Wnt1 (wingless-type MMTV integration site family, member 1, accession nos. NM_005430.3 and NP_005421.1), Wnt2 (wingless-type MMTV integration site family, member 2, accession nos. NM_003391.2 and NP_003382.1), Wnt2b (wingless-type MMTV integration site family, member 2b, accession nos. NM_001291880.1 and NP_001278809.1), Wnt3 (wingless-type MMTV integration site family, member 3, accession nos. NM_030753.4 and NP_110380.1), Wnt3a (wingless-type MMTV integration site family, member 3a, accession nos. NM_033131.3 and NP_149122.1), Wnt5b (wingless-type MMTV integration site family, member 5b, accession nos. NM_030775.2, NM_032642.2, NP_110402.2 and NP_116031.1), Wnt6 (wingless-type MMTV integration site family, member 6, accession nos. NM_006522.3 and NP_006513.1), Wnt7a (wingless-type MMTV integration site family, member 7a, accession nos. NM_004625.3 and NP_004616.2), Wnt7b (wingless-type MMTV integration site family, member 7b, accession nos. NM_058238.2 and NP_478679.1), Wnt8a (wingless-type MMTV integration site family, member 8a, accession nos. NM_001300938.1, NM_001300939.1, NM_058244.3, NP_001287867.1, NP_001287868.1 and NP_490645.1), Wnt8b (wingless-type MMTV integration site family, member 8b, accession nos. NM_003393.3 and NP_003384.2), Wnt9a (wingless-type MMTV integration site family, member 9a, accession nos. NM_003395.2 and NP_003386.1), Wnt9b (wingless-type MMTV integration site family, member 9b, accession nos. NM_003396.1 and NP_003387.1), Wnt10a (wingless-type MMTV integration site family, member 10a, accession nos. NM_025216.2 and NP_079492.2), Wnt10b (wingless-type MMTV integration site family, member 10b, accession nos. NM_003394.3 and NP_003385.2) and/or Wnt16 (wingless-type MMTV integration site family, member 16, accession nos. NM_016087.2, NM_057168.1, NP_057171.2 and NP_476509.1).

According to a specific embodiment, the Wnt ligand comprises Wnt5b.

According to one embodiment, the invention contemplates using a Wnt that signals through the non-canonical Wnt signaling pathway. Exemplary Wnts that signal through the non-canonical pathway include, but are not limited to, Wnt4 (wingless-type MMTV integration site family, member 4, accession nos. NM_030761.4 and NP_110388.2),
5 Wnt5a (wingless-type MMTV integration site family, member 5a, accession nos. NM_001256105.1, NM_003392.4, NP_001243034.1 and NP_003383.2) and/or Wnt11 (wingless-type MMTV integration site family, member 11, accession nos. NM_004626.2 and NP_004617.2). In a specific embodiment, the Wnt comprises a polypeptide, an analogue or a polynucleotide (as discussed in detail above).

10 Thus, the LEC committed cell of some embodiments of the invention may be exposed to any of the known Wnt non-canonical pathway ligands, additionally or alternatively, upregulating the activity of Wnt non-canonical pathway may be effected by contacting the LEC with an activator of any Wnt non-canonical pathway member. Exemplary Wnt non-canonical pathway members include, but are not limited to, Ror2
15 (receptor tyrosine kinase-like orphan receptor 2, accession nos. NM_004560.3 and NP_004551.2), Ryk (receptor-like tyrosine kinase, accession nos. NM_001005861.2, NM_002958.3, NP_001005861.1 and NP_002949.2), G protein (homo sapiens guanine nucleotide binding protein (G protein), alpha 11 (Gq class) (GNA11), accession nos. NM_002067.4 and NP_002058.2), Dishevelled e.g. Dvl1 (dishevelled segment polarity
20 protein 1, accession nos. NM_004421.2 and NP_004412.2), Dvl2 (dishevelled segment polarity protein 2, accession nos. NM_004422.2 and NP_004413.1) or Dvl3 (dishevelled segment polarity protein 3, accession nos. NM_004423.3 and NP_004414.3), and Dishevelled-associated activator of morphogenesis e.g. DAAM1 (dishevelled associated
25 activator of morphogenesis 1, accession nos. NM_001270520.1, NM_014992.2, NP_001257449.1 and NP_055807.1) or DAAM2 (dishevelled associated activator of morphogenesis 2, accession nos. NM_001201427.1, NM_015345.3, NP_001188356.1 and NP_056160.2).

Any of the proteinaceous members of the signaling pathways described herein may be provided in the form of the polynucleotide (especially in the case of transmembrane
30 proteins), polypeptides or analogues as further defined hereinbelow.

According to one embodiment, the Wnt ligand comprises a polypeptide, an analogue or a polynucleotide.

As used herein, the term “polypeptide” encompasses a naturally occurring polypeptide which is comprised solely of natural amino acid residues or synthetically prepared polypeptides, comprised of a mixture of natural and modified (non-natural) amino acid residues.

5 The term "analogue" is used herein to extend to any amino acid derivative of the polypeptides described herein, as long as their function e.g., promoting LEC specification, is maintained.

As used herein, the term “polynucleotide” refers to a single or double stranded nucleic acid sequence which is isolated and provided in the form of an RNA sequence, a
10 complementary polynucleotide sequence (cDNA), a genomic polynucleotide sequence and/or a composite polynucleotide sequences (e.g., a combination of the above).

Thus, the stem cell and/or vascular progenitor cell and/or angioblast may be exposed to any of the known Wnt ligands, such as purified polypeptides. Wnt ligands are available commercially from R&D Systems (Minnesota, USA), from PeproTech, Inc
15 (New Jersey, USA) and from Calbiochem, and may also be made by recombinant means, such as by expression of an expression vector comprising a Wnt nucleic acid in a suitable host cell.

According to one embodiment, the Wnt canonical or non-canonical signaling pathway is activated by increasing the expression of Wnt ligands. For example, activation
20 of the Wnt signaling pathway by Wnt over-expression is described in detail in U.S. application no. 2004/0014209, incorporated herein by reference. The teachings of U.S. 2004/0014209 may be used to prepare mRNAs from expression constructs and plasmids comprising Wnt sequences, for injection into stem cells or vascular progenitor cells or angioblasts to promote LEC specification. Furthermore, expression vectors may be
25 transiently or permanently transfected into stem cells or vascular progenitor cells or angioblasts to achieve the same purpose (as described in detail hereinabove).

Additionally or alternatively, the Wnt canonical or non-canonical signaling pathway may be activated *ex vivo* by exposing the stem cell or vascular progenitor cell or angioblast to medium containing the Wnt ligand. An example of such a medium is a
30 medium in which Wnt secreting cells, such as cells transfected with Wnt expression vectors, is growing. The presence of the appropriate Wnt ligand in the conditioned

medium may be established through known means, such as by Western blots. Cells producing Active Wnt include, but are not limited to, Mouse Wnt3A (ATCC CRL-2647).

According to one embodiment, the Wnt canonical signaling pathway may be activated by upregulating the activity or Dishevelled (Dsh) (e.g. DVL1, DVL2, DVL3, and Dvl1L1), β -catenin, LDL-receptor related proteins LRP5 or LRP6 (LRP5/6), PP2A, ADP-ribosylation factor 1 (ARFGAP1) and/or T-cell factor (TCF)/Lymphoid enhancing factor (LEF) family of proteins.

According to one embodiment, serine/threonine phosphatase PP2A activity may be upregulated using the small molecule IQ-1, commercially available e.g. from Tocris Bioscience or from Santa Cruz Biotech.

According to one embodiment, ARFGAP1 activity may be upregulated using the small molecule QS11, commercially available e.g. from Tocris Bioscience or from Abcam.

According to one embodiment, β -catenin activity may be upregulated using the small molecule deoxycholic acid (DCA).

It will be appreciated that when the factors are soluble they can be provided as a polypeptide or polypeptide analogue. In other cases small molecules (activators or inhibitors dependent on the target) may be used, essentially as described herein. Each of the above can be used *in vivo* or *in vitro*.

As mentioned, the Wnt canonical signaling pathway may be activated by decreasing the activity or expression of antagonists of the Wnt pathway (e.g. of Wnt or Frizzled).

Thus, according to one embodiment, activation of a Wnt canonical signaling pathway is achieved by down-regulation of any antagonist or negative regulator or component of the Wnt canonical pathway. Exemplary antagonist or negative regulator or component of the Wnt pathway include, but are not limited to, Axin, glycogen synthase kinase 3 (GSK3, e.g. GSK3 β), APC and sFRP (Secreted frizzled-related proteins).

Down-regulation can be effected on the genomic and/or the transcript level using a variety of molecules which interfere with transcription and/or translation [e.g., RNA silencing agents (e.g., antisense, siRNA, shRNA, micro-RNA), Ribozyme DNAzyme and a CRISPR system (e.g. CRISPR/Cas)], or on the protein level using e.g., and small molecules, antagonists, antibodies, enzymes that cleave the polypeptide or inhibit

functionality of the peptide and the like. Thus, down-regulation may be achieved by inhibiting enzymatic activity or lowering protein concentration.

Following is a list of agents capable of downregulating expression level and/or activity of an antagonist or negative regulator or component of the Wnt pathway (e.g. Axin, GSK3, APC and sFRP).

One example, of an agent capable of downregulating an antagonist or negative regulator or component of the Wnt pathway (e.g. Axin, GSK3, APC and sFRP) is an antibody or antibody fragment capable of specifically binding to the antagonist or negative regulator or component of the Wnt pathway (e.g. Axin, GSK3, APC and sFRP). Preferably, the antibody specifically binds at least one epitope of the antagonist or negative regulator or component of the Wnt pathway. As used herein, the term "epitope" refers to any antigenic determinant on an antigen to which the paratope of an antibody binds.

Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or carbohydrate side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

The term "antibody" as used in this invention includes intact molecules as well as functional fragments thereof, such as Fab, F(ab')₂, and Fv that are capable of binding to macrophages. These functional antibody fragments are defined as follows: (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) Fab', the fragment of an antibody molecule that can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule; (3) (Fab')₂, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab')₂ is a dimer of two Fab' fragments held together by two disulfide bonds; (4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and (5) Single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

Methods of producing polyclonal and monoclonal antibodies as well as fragments thereof are well known in the art (See for example, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference).

5 Antibody fragments according to some embodiments of the invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* or mammalian cells (e.g. Chinese hamster ovary cell culture or other protein expression systems) of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody
10 fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')₂. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc
15 fragment directly. These methods are described, for example, by Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647, and references contained therein, which patents are hereby incorporated by reference in their entirety. See also Porter, R. R. [*Biochem. J.* 73: 119-126 (1959)]. Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other
20 enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

Fv fragments comprise an association of VH and VL chains. This association may be noncovalent, as described in Inbar et al. [*Proc. Nat'l Acad. Sci. USA* 69:2659-62 (1972)]. Alternatively, the variable chains can be linked by an intermolecular disulfide
25 bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv fragments comprise VH and VL chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the VH and VL domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into
30 a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by [Whitlow and Filpula, *Methods* 2: 97-105 (1991); Bird et al.,

Science 242:423-426 (1988); Pack et al., Bio/Technology 11:1271-77 (1993); and U.S. Pat. No. 4,946,778, which is hereby incorporated by reference in its entirety.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units")
5 can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick and Fry [Methods, 2: 106-10 (1991)].

Humanized forms of non-human (e.g., murine) antibodies are chimeric molecules
10 of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab').sub.2 or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-
15 human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody
20 will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a
25 human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often
30 referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature 332:323-327

(1988); Verhoeyen et al., *Science*, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the
5 corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, *J. Mol. Biol.*, 227:381 (1991);
10 Marks et al., *J. Mol. Biol.*, 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985) and Boerner et al., *J. Immunol.*, 147(1):86-95 (1991)]. Similarly, human antibodies can be made by introduction of human immunoglobulin loci into transgenic animals, e.g., mice in which
15 the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific
20 publications: Marks et al., *Bio/Technology* 10,: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368 812-13 (1994); Fishwild et al., *Nature Biotechnology* 14, 845-51 (1996); Neuberger, *Nature Biotechnology* 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.* 13, 65-93 (1995).

Downregulation of an antagonist or negative regulator or component of the Wnt
25 pathway (e.g. Axin, GSK3, APC and sFRP) can be also achieved by RNA silencing. As used herein, the phrase "RNA silencing" refers to a group of regulatory mechanisms [e.g. RNA interference (RNAi), transcriptional gene silencing (TGS), post-transcriptional gene silencing (PTGS), quelling, co-suppression, and translational repression] mediated by
30 RNA molecules which result in the inhibition or "silencing" of the expression of a corresponding protein-coding gene. RNA silencing has been observed in many types of organisms, including animals, and fungi.

As used herein, the term "RNA silencing agent" refers to an RNA which is capable of specifically inhibiting or "silencing" the expression of a target gene. In certain embodiments, the RNA silencing agent is capable of preventing complete processing (e.g, the full translation and/or expression) of an mRNA molecule through a post-transcriptional silencing mechanism. RNA silencing agents include noncoding RNA molecules, for example RNA duplexes comprising paired strands, as well as precursor RNAs from which such small non-coding RNAs can be generated. Exemplary RNA silencing agents include dsRNAs such as siRNAs, miRNAs and shRNAs. In one embodiment, the RNA silencing agent is capable of inducing RNA interference. In another embodiment, the RNA silencing agent is capable of mediating translational repression.

According to an embodiment of the invention, the RNA silencing agent is specific to the target RNA (e.g., an antagonist or negative regulator or component of the Wnt pathway e.g. Axin, GSK3, APC and sFRP) and does not cross inhibit or silence a gene or a splice variant which exhibits 99% or less global homology to the target gene, e.g., less than 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81% global homology to the target gene.

RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes and is commonly shared by diverse flora and phyla. Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or from the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs). Short interfering RNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes. The RNAi response also features an endonuclease

complex, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex.

5 Accordingly, some embodiments of the invention contemplates use of dsRNA to downregulate protein expression from mRNA.

According to one embodiment, the dsRNA is greater than 30 bp. The use of long dsRNAs (i.e. dsRNA greater than 30 bp) has been very limited owing to the belief that these longer regions of double stranded RNA will result in the induction of the interferon and PKR response. However, the use of long dsRNAs can provide numerous advantages in that the cell can select the optimal silencing sequence alleviating the need to test numerous siRNAs; long dsRNAs will allow for silencing libraries to have less complexity than would be necessary for siRNAs; and, perhaps most importantly, long dsRNA could prevent viral escape mutations when used as therapeutics.

15 Various studies demonstrate that long dsRNAs can be used to silence gene expression without inducing the stress response or causing significant off-target effects - see for example [Strat et al., *Nucleic Acids Research*, 2006, Vol. 34, No. 13 3803–3810; Bhargava A et al. *Brain Res. Protoc.* 2004;13:115–125; Diallo M., et al., *Oligonucleotides.* 2003;13:381–392; Paddison P.J., et al., *Proc. Natl Acad. Sci. USA.* 2002;99:1443–1448; Tran N., et al., *FEBS Lett.* 2004;573:127–134].

In particular, the invention according to some embodiments thereof contemplates introduction of long dsRNA (over 30 base transcripts) for gene silencing in cells where the interferon pathway is not activated (e.g. embryonic cells and oocytes) see for example Billy et al., *PNAS* 2001, Vol 98, pages 14428-14433. and Diallo et al, *Oligonucleotides*, 25 October 1, 2003, 13(5): 381-392. doi:10.1089/154545703322617069.

The invention according to some embodiments thereof also contemplates introduction of long dsRNA specifically designed not to induce the interferon and PKR pathways for down-regulating gene expression. For example, Shinagwa and Ishii [*Genes & Dev.* 17 (11): 1340-1345, 2003] have developed a vector, named pDECAP, to express 30 long double-strand RNA from an RNA polymerase II (Pol II) promoter. Because the transcripts from pDECAP lack both the 5'-cap structure and the 3'-poly(A) tail that

facilitate ds-RNA export to the cytoplasm, long ds-RNA from pDECAP does not induce the interferon response.

Another method of evading the interferon and PKR pathways in mammalian systems is by introduction of small inhibitory RNAs (siRNAs) either via transfection or
5 endogenous expression.

The term "siRNA" refers to small inhibitory RNA duplexes (generally between 18-30 basepairs) that induce the RNA interference (RNAi) pathway. Typically, siRNAs are chemically synthesized as 21mers with a central 19 bp duplex region and symmetric 2-base 3'-overhangs on the termini, although it has been recently described that chemically
10 synthesized RNA duplexes of 25-30 base length can have as much as a 100-fold increase in potency compared with 21mers at the same location. The observed increased potency obtained using longer RNAs in triggering RNAi is theorized to result from providing Dicer with a substrate (27mer) instead of a product (21mer) and that this improves the rate or efficiency of entry of the siRNA duplex into RISC.

15 It has been found that position of the 3'-overhang influences potency of an siRNA and asymmetric duplexes having a 3'-overhang on the antisense strand are generally more potent than those with the 3'-overhang on the sense strand (Rose et al., 2005). This can be attributed to asymmetrical strand loading into RISC, as the opposite efficacy patterns are observed when targeting the antisense transcript.

20 The strands of a double-stranded interfering RNA (e.g., an siRNA) may be connected to form a hairpin or stem-loop structure (e.g., an shRNA). Thus, as mentioned the RNA silencing agent of some embodiments of the invention may also be a short hairpin RNA (shRNA).

The term "shRNA", as used herein, refers to an RNA agent having a stem-loop
25 structure, comprising a first and second region of complementary sequence, the degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide analogs) within the loop region. The number of nucleotides in the loop is a number between and
30 including 3 to 23, or 5 to 15, or 7 to 13, or 4 to 9, or 9 to 11. Some of the nucleotides in the loop can be involved in base-pair interactions with other nucleotides in the loop. Examples of oligonucleotide sequences that can be used to form the loop include 5'-UUCAAGAGA-

3' (Brummelkamp, T. R. et al. (2002) Science 296: 550) and 5'-UUUGUGUAG-3' (Castanotto, D. et al. (2002) RNA 8:1454). It will be recognized by one of skill in the art that the resulting single chain oligonucleotide forms a stem-loop or hairpin structure comprising a double-stranded region capable of interacting with the RNAi machinery.

5 Synthesis of RNA silencing agents suitable for use with some embodiments of the invention can be effected as follows. First, the mRNA sequence of the antagonist or negative regulator or component of the Wnt pathway (e.g. Axin, GSK3, APC and sFRP) is scanned downstream of the AUG start codon for AA dinucleotide sequences. Occurrence of each AA and the 3' adjacent 19 nucleotides is recorded as potential siRNA target sites.
10 Preferably, siRNA target sites are selected from the open reading frame, as untranslated regions (UTRs) are richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA endonuclease complex [Tuschl ChemBiochem. 2:239-245]. It will be appreciated though, that siRNAs directed at untranslated regions may also be effective, as demonstrated for
15 GAPDH wherein siRNA directed at the 5' UTR mediated about 90 % decrease in cellular GAPDH mRNA and completely abolished protein level (www(dot)ambion(dot)com/techlib/tn/91/912(dot)html).

 Second, potential target sites are compared to an appropriate genomic database (e.g., human, mouse, rat etc.) using any sequence alignment software, such as the BLAST
20 software available from the NCBI server (www(dot)ncbi(dot)nml(dot)nih(dot)gov/BLAST/). Putative target sites which exhibit significant homology to other coding sequences are filtered out.

 Qualifying target sequences are selected as template for siRNA synthesis. Preferred sequences are those including low G/C content as these have proven to be more
25 effective in mediating gene silencing as compared to those with G/C content higher than 55 %. Several target sites are preferably selected along the length of the target gene for evaluation. For better evaluation of the selected siRNAs, a negative control is preferably used in conjunction. Negative control siRNA preferably include the same nucleotide composition as the siRNAs but lack significant homology to the genome. Thus, a
30 scrambled nucleotide sequence of the siRNA is preferably used, provided it does not display any significant homology to any other gene.

For example, a suitable siRNA can be an Axin siRNA commercially available from Santa Cruz Biotechnology or from OriGene, a GSK3 siRNA commercially available from SignalChem, an APC siRNA commercially available from Santa Cruz Biotechnology, and a sFRP siRNA commercially available from OriGene.

5 It will be appreciated that the RNA silencing agent of some embodiments of the invention need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides.

In some embodiments, the RNA silencing agent provided herein can be functionally associated with a cell-penetrating peptide." As used herein, a "cell-penetrating peptide" is a peptide that comprises a short (about 12-30 residues) amino acid sequence or
10 functional motif that confers the energy-independent (i.e., non-endocytotic) translocation properties associated with transport of the membrane-permeable complex across the plasma and/or nuclear membranes of a cell. The cell-penetrating peptide used in the membrane-permeable complex of some embodiments of the invention preferably
15 comprises at least one non-functional cysteine residue, which is either free or derivatized to form a disulfide link with a double-stranded ribonucleic acid that has been modified for such linkage. Representative amino acid motifs conferring such properties are listed in U.S. Pat. No. 6,348,185, the contents of which are expressly incorporated herein by reference. The cell-penetrating peptides of some embodiments of the invention preferably
20 include, but are not limited to, penetratin, transportan, pIsl, TAT(48-60), pVEC, MTS, and MAP.

According to another embodiment the RNA silencing agent may be a miRNA.

The term "microRNA", "miRNA", and "miR" are synonymous and refer to a collection of non-coding single-stranded RNA molecules of about 19-28 nucleotides in
25 length, which regulate gene expression. miRNAs are found in a wide range of organisms (viruses.fwdarw.humans) and have been shown to play a role in development, homeostasis, and disease etiology.

Below is a brief description of the mechanism of miRNA activity.

Genes coding for miRNAs are transcribed leading to production of a miRNA
30 precursor known as the pri-miRNA. The pri-miRNA is typically part of a polycistronic RNA comprising multiple pri-miRNAs. The pri-miRNA may form a hairpin with a stem and loop. The stem may comprise mismatched bases.

The hairpin structure of the pri-miRNA is recognized by Drosha, which is an RNase III endonuclease. Drosha typically recognizes terminal loops in the pri-miRNA and cleaves approximately two helical turns into the stem to produce a 60–70 nucleotide precursor known as the pre-miRNA. Drosha cleaves the pri-miRNA with a staggered cut
5 typical of RNase III endonucleases yielding a pre-miRNA stem loop with a 5' phosphate and ~2 nucleotide 3' overhang. It is estimated that approximately one helical turn of stem (~10 nucleotides) extending beyond the Drosha cleavage site is essential for efficient processing. The pre-miRNA is then actively transported from the nucleus to the cytoplasm by Ran-GTP and the export receptor Ex-portin-5.

10 The double-stranded stem of the pre-miRNA is then recognized by Dicer, which is also an RNase III endonuclease. Dicer may also recognize the 5' phosphate and 3' overhang at the base of the stem loop. Dicer then cleaves off the terminal loop two helical turns away from the base of the stem loop leaving an additional 5' phosphate and ~2 nucleotide 3' overhang. The resulting siRNA-like duplex, which may comprise
15 mismatches, comprises the mature miRNA and a similar-sized fragment known as the miRNA*. The miRNA and miRNA* may be derived from opposing arms of the pri-miRNA and pre-miRNA. MiRNA* sequences may be found in libraries of cloned miRNAs but typically at lower frequency than the miRNAs.

20 Although initially present as a double-stranded species with miRNA*, the miRNA eventually become incorporated as a single-stranded RNA into a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC). Various proteins can form the RISC, which can lead to variability in specificity for miRNA/miRNA* duplexes, binding site of the target gene, activity of miRNA (repress or activate), and which strand of the miRNA/miRNA* duplex is loaded in to the RISC.

25 When the miRNA strand of the miRNA:miRNA* duplex is loaded into the RISC, the miRNA* is removed and degraded. The strand of the miRNA:miRNA* duplex that is loaded into the RISC is the strand whose 5' end is less tightly paired. In cases where both ends of the miRNA:miRNA* have roughly equivalent 5' pairing, both miRNA and miRNA* may have gene silencing activity.

30 The RISC identifies target nucleic acids based on high levels of complementarity between the miRNA and the mRNA, especially by nucleotides 2-7 of the miRNA.

A number of studies have looked at the base-pairing requirement between miRNA and its mRNA target for achieving efficient inhibition of translation (reviewed by Bartel 2004, Cell 116-281). In mammalian cells, the first 8 nucleotides of the miRNA may be important (Doench & Sharp 2004 GenesDev 2004-504). However, other parts of the microRNA may also participate in mRNA binding. Moreover, sufficient base pairing at the 3' can compensate for insufficient pairing at the 5' (Brennecke et al, 2005 PLoS 3-e85). Computation studies, analyzing miRNA binding on whole genomes have suggested a specific role for bases 2-7 at the 5' of the miRNA in target binding but the role of the first nucleotide, found usually to be "A" was also recognized (Lewis et al 2005 Cell 120-15). Similarly, nucleotides 1-7 or 2-8 were used to identify and validate targets by Krek et al (2005, Nat Genet 37-495).

The target sites in the mRNA may be in the 5' UTR, the 3' UTR or in the coding region. Interestingly, multiple miRNAs may regulate the same mRNA target by recognizing the same or multiple sites. The presence of multiple miRNA binding sites in most genetically identified targets may indicate that the cooperative action of multiple RISCs provides the most efficient translational inhibition.

MiRNAs may direct the RISC to downregulate gene expression by either of two mechanisms: mRNA cleavage or translational repression. The miRNA may specify cleavage of the mRNA if the mRNA has a certain degree of complementarity to the miRNA. When a miRNA guides cleavage, the cut is typically between the nucleotides pairing to residues 10 and 11 of the miRNA. Alternatively, the miRNA may repress translation if the miRNA does not have the requisite degree of complementarity to the miRNA. Translational repression may be more prevalent in animals since animals may have a lower degree of complementarity between the miRNA and binding site.

It should be noted that there may be variability in the 5' and 3' ends of any pair of miRNA and miRNA*. This variability may be due to variability in the enzymatic processing of Drosha and Dicer with respect to the site of cleavage. Variability at the 5' and 3' ends of miRNA and miRNA* may also be due to mismatches in the stem structures of the pri-miRNA and pre-miRNA. The mismatches of the stem strands may lead to a population of different hairpin structures. Variability in the stem structures may also lead to variability in the products of cleavage by Drosha and Dicer.

The term "microRNA mimic" refers to synthetic non-coding RNAs that are capable of entering the RNAi pathway and regulating gene expression. miRNA mimics imitate the function of endogenous microRNAs (miRNAs) and can be designed as mature, double stranded molecules or mimic precursors (e.g., or pre-miRNAs). miRNA mimics can be comprised of modified or unmodified RNA, DNA, RNA-DNA hybrids, or alternative nucleic acid chemistries (e.g., LNAs or 2'-O,4'-C-ethylene-bridged nucleic acids (ENA)). For mature, double stranded miRNA mimics, the length of the duplex region can vary between 13-33, 18-24 or 21-23 nucleotides. The miRNA may also comprise a total of at least 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 or 40 nucleotides. The sequence of the miRNA may be the first 13-33 nucleotides of the pre-miRNA. The sequence of the miRNA may also be the last 13-33 nucleotides of the pre-miRNA.

For example, a suitable miRNA can be a miRNA targeting Axin commercially available from Genecopoeia, a miRNA targeting GSK3 commercially available from OriGene, a miRNA targeting APC commercially available from OriGene, and a miRNA targeting sFRP commercially available from OriGene.

It will be appreciated from the description provided herein above, that contacting stem cells or vascular progenitor cells or angioblasts with a miRNA may be affected in a number of ways:

1. Transiently transfecting the stem cells or vascular progenitor cells or angioblasts with the mature double stranded miRNA;
2. Stably, or transiently transfecting the stem cells or vascular progenitor cells or angioblasts with an expression vector which encodes the mature miRNA.
3. Stably, or transiently transfecting the stem cells or vascular progenitor cells or angioblasts with an expression vector which encodes the pre-miRNA. The pre-miRNA sequence may comprise from 45-90, 60-80 or 60-70 nucleotides. The sequence of the pre-miRNA may comprise a miRNA and a miRNA* as set forth herein. The sequence of the pre-miRNA may also be that of a pri-miRNA excluding from 0-160 nucleotides from the 5' and 3' ends of the pri-miRNA.
4. Stably, or transiently transfecting the stem cells or vascular progenitor cells or angioblasts with an expression vector which encodes the pri-miRNA. The pri-miRNA sequence may comprise from 45-30,000, 50-25,000, 100-20,000,

1,000-1,500 or 80-100 nucleotides. The sequence of the pri-miRNA may comprise a pre-miRNA, miRNA and miRNA*, as set forth herein, and variants thereof.

Preparation of miRNAs mimics can be effected by chemical synthesis methods or
5 by recombinant methods.

Another agent capable of downregulating an antagonist or negative regulator or component of the Wnt pathway (e.g. Axin, GSK3, APC and sFRP) is a DNAzyme molecule capable of specifically cleaving a mRNA transcript or DNA sequence of the antagonist or negative regulator or component of the Wnt pathway (e.g. Axin, GSK3, APC
10 and sFRP). DNAzymes are single-stranded polynucleotides which are capable of cleaving both single and double stranded target sequences (Breaker, R.R. and Joyce, G. Chemistry and Biology 1995;2:655; Santoro, S.W. & Joyce, G.F. Proc. Natl, Acad. Sci. USA 1997;943:4262) A general model (the "10-23" model) for the DNAzyme has been proposed. "10-23" DNAzymes have a catalytic domain of 15 deoxyribonucleotides,
15 flanked by two substrate-recognition domains of seven to nine deoxyribonucleotides each. This type of DNAzyme can effectively cleave its substrate RNA at purine:pyrimidine junctions (Santoro, S.W. & Joyce, G.F. Proc. Natl, Acad. Sci. USA 199; for rev of DNAzymes see Khachigian, LM [Curr Opin Mol Ther 4:119-21 (2002)].

Examples of construction and amplification of synthetic, engineered DNAzymes
20 recognizing single and double-stranded target cleavage sites have been disclosed in U.S. Pat. No. 6,326,174 to Joyce et al. DNAzymes of similar design directed against the human Urokinase receptor were recently observed to inhibit Urokinase receptor expression, and successfully inhibit colon cancer cell metastasis in vivo (Itoh et al., 20002, Abstract 409, Ann Meeting Am Soc Gen Ther [www\(dot\)asgt\(dot\)org](http://www.asgt.org)). In another
25 application, DNAzymes complementary to bcr-abl oncogenes were successful in inhibiting the oncogenes expression in leukemia cells, and lessening relapse rates in autologous bone marrow transplant in cases of CML and ALL.

Downregulation of an antagonist or negative regulator or component of the Wnt pathway (e.g. Axin, GSK3, APC and sFRP) can also be effected by using an antisense
30 polynucleotide capable of specifically hybridizing with an mRNA transcript encoding the antagonist or negative regulator or component of the Wnt pathway (e.g. Axin, GSK3, APC and sFRP).

Design of antisense molecules which can be used to efficiently downregulate an antagonist or negative regulator or component of the Wnt pathway (e.g. Axin, GSK3, APC and sFRP) must be effected while considering two aspects important to the antisense approach. The first aspect is delivery of the oligonucleotide into the cytoplasm of the appropriate cells, while the second aspect is design of an oligonucleotide which specifically binds the designated mRNA within cells in a way which inhibits translation thereof.

The prior art teaches of a number of delivery strategies which can be used to efficiently deliver oligonucleotides into a wide variety of cell types [see, for example, Luft J Mol Med 76: 75-6 (1998); Kronenwett et al. Blood 91: 852-62 (1998); Rajur et al. Bioconjug Chem 8: 935-40 (1997); Lavigne et al. Biochem Biophys Res Commun 237: 566-71 (1997) and Aoki et al. (1997) Biochem Biophys Res Commun 231: 540-5 (1997)].

In addition, algorithms for identifying those sequences with the highest predicted binding affinity for their target mRNA based on a thermodynamic cycle that accounts for the energetics of structural alterations in both the target mRNA and the oligonucleotide are also available [see, for example, Walton et al. Biotechnol Bioeng 65: 1-9 (1999)].

Such algorithms have been successfully used to implement an antisense approach in cells. For example, the algorithm developed by Walton et al. enabled scientists to successfully design antisense oligonucleotides for rabbit beta-globin (RBG) and mouse tumor necrosis factor-alpha (TNF alpha) transcripts. The same research group has more recently reported that the antisense activity of rationally selected oligonucleotides against three model target mRNAs (human lactate dehydrogenase A and B and rat gp130) in cell culture as evaluated by a kinetic PCR technique proved effective in almost all cases, including tests against three different targets in two cell types with phosphodiester and phosphorothioate oligonucleotide chemistries.

In addition, several approaches for designing and predicting efficiency of specific oligonucleotides using an in vitro system were also published (Matveeva et al., Nature Biotechnology 16: 1374 - 1375 (1998)].

For example, a suitable antisense oligonucleotides can be an antisense oligonucleotides targeted against GSK3 commercially available from Isis Pharmaceuticals.

Several clinical trials have demonstrated safety, feasibility and activity of antisense oligonucleotides. For example, antisense oligonucleotides suitable for the treatment of

cancer have been successfully used [Holmund et al., *Curr Opin Mol Ther* 1:372-85 (1999)], while treatment of hematological malignancies via antisense oligonucleotides targeting c-myc gene, p53 and Bcl-2 had entered clinical trials and had been shown to be tolerated by patients [Gerwitz *Curr Opin Mol Ther* 1:297-306 (1999)].

5 More recently, antisense-mediated suppression of human heparanase gene expression has been reported to inhibit pleural dissemination of human cancer cells in a mouse model [Uno et al., *Cancer Res* 61:7855-60 (2001)].

Thus, the current consensus is that recent developments in the field of antisense technology which, as described above, have led to the generation of highly accurate
10 antisense design algorithms and a wide variety of oligonucleotide delivery systems, enable an ordinarily skilled artisan to design and implement antisense approaches suitable for downregulating expression of known sequences without having to resort to undue trial and error experimentation.

Another agent capable of downregulating an antagonist or negative regulator or
15 component of the Wnt pathway (e.g. Axin, GSK3, APC and sFRP) is a ribozyme molecule capable of specifically cleaving an mRNA transcript encoding an antagonist or negative regulator or component of the Wnt pathway (e.g. Axin, GSK3, APC and sFRP). Ribozymes are being increasingly used for the sequence-specific inhibition of gene expression by the cleavage of mRNAs encoding proteins of interest [Welch et al., *Curr*
20 *Opin Biotechnol.* 9:486-96 (1998)]. The possibility of designing ribozymes to cleave any specific target RNA has rendered them valuable tools in both basic research and therapeutic applications. In the therapeutics area, ribozymes have been exploited to target viral RNAs in infectious diseases, dominant oncogenes in cancers and specific somatic mutations in genetic disorders [Welch et al., *Clin Diagn Virol.* 10:163-71 (1998)]. Most
25 notably, several ribozyme gene therapy protocols for HIV patients are already in Phase 1 trials. More recently, ribozymes have been used for transgenic animal research, gene target validation and pathway elucidation. Several ribozymes are in various stages of clinical trials. ANGIOZYME was the first chemically synthesized ribozyme to be studied in human clinical trials. ANGIOZYME specifically inhibits formation of the VEGF-r
30 (Vascular Endothelial Growth Factor receptor), a key component in the angiogenesis pathway. Ribozyme Pharmaceuticals, Inc., as well as other firms have demonstrated the importance of anti-angiogenesis therapeutics in animal models. HEPTAZYME, a

ribozyme designed to selectively destroy Hepatitis C Virus (HCV) RNA, was found effective in decreasing Hepatitis C viral RNA in cell culture assays (Ribozyme Pharmaceuticals, Incorporated - WEB home page).

Another agent capable of downregulating an antagonist or negative regulator or component of the Wnt pathway (e.g. Axin, GSK3, APC and sFRP) is a RNA-guided endonuclease technology e.g. CRISPR system.

As used herein, the term "CRISPR system" also known as Clustered Regularly Interspaced Short Palindromic Repeats refers collectively to transcripts and other elements involved in the expression of or directing the activity of CRISPR-associated genes, including sequences encoding a Cas gene (e.g. CRISPR-associated endonuclease 9), a tracr (trans-activating CRISPR) sequence (e.g. tracrRNA or an active partial tracrRNA), a tracr-mate sequence (encompassing a "direct repeat" and a tracrRNA-processed partial direct repeat) or a guide sequence (also referred to as a "spacer") including but not limited to a crRNA sequence (i.e. an endogenous bacterial RNA that confers target specificity yet requires tracrRNA to bind to Cas) or a sgRNA sequence (i.e. single guide RNA).

In some embodiments, one or more elements of a CRISPR system is derived from a type I, type II, or type III CRISPR system. In some embodiments, one or more elements of a CRISPR system (e.g. Cas) is derived from a particular organism comprising an endogenous CRISPR system, such as *Streptococcus pyogenes*, *Neisseria meningitides*, *Streptococcus thermophilus* or *Treponema denticola*.

In general, a CRISPR system is characterized by elements that promote the formation of a CRISPR complex at the site of a target sequence (also referred to as a protospacer in the context of an endogenous CRISPR system).

In the context of formation of a CRISPR complex, "target sequence" refers to a sequence to which a guide sequence (i.e. guide RNA e.g. sgRNA or crRNA) is designed to have complementarity, where hybridization between a target sequence and a guide sequence promotes the formation of a CRISPR complex. Full complementarity is not necessarily required, provided there is sufficient complementarity to cause hybridization and promote formation of a CRISPR complex. Thus, according to some embodiments, global homology to the target sequence may be of 50 %, 60 %, 70 %, 75 %, 80 %, 85 %, 90 %, 95 % or 99 %. A target sequence may comprise any polynucleotide, such as DNA

or RNA polynucleotides. In some embodiments, a target sequence is located in the nucleus or cytoplasm of a cell.

Thus, the CRISPR system comprises two distinct components, a guide RNA (gRNA) that hybridizes with the target sequence, and a nuclease (e.g. Type-II Cas9 protein), wherein the gRNA targets the target sequence and the nuclease (e.g. Cas9 protein) cleaves the target sequence. The guide RNA may comprise a combination of an endogenous bacterial crRNA and tracrRNA, i.e. the gRNA combines the targeting specificity of the crRNA with the scaffolding properties of the tracrRNA (required for Cas9 binding). Alternatively, the guide RNA may be a single guide RNA capable of directly binding Cas.

Typically, in the context of an endogenous CRISPR system, formation of a CRISPR complex (comprising a guide sequence hybridized to a target sequence and complexed with one or more Cas proteins) results in cleavage of one or both strands in or near (e.g. within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, or more base pairs from) the target sequence. Without wishing to be bound by theory, the tracr sequence, which may comprise or consist of all or a portion of a wild-type tracr sequence (e.g. about or more than about 20, 26, 32, 45, 48, 54, 63, 67, 85, or more nucleotides of a wild-type tracr sequence), may also form part of a CRISPR complex, such as by hybridization along at least a portion of the tracr sequence to all or a portion of a tracr mate sequence that is operably linked to the guide sequence.

In some embodiments, the tracr sequence has sufficient complementarity to a tracr mate sequence to hybridize and participate in formation of a CRISPR complex. As with the target sequence, a complete complementarity is not needed, provided there is sufficient to be functional. In some embodiments, the tracr sequence has at least 50 %, 60 %, 70 %, 80 %, 90 %, 95 % or 99 % of sequence complementarity along the length of the tracr mate sequence when optimally aligned.

Introducing CRISPR/Cas into a cell may be effected using one or more vectors driving expression of one or more elements of a CRISPR system such that expression of the elements of the CRISPR system direct formation of a CRISPR complex at one or more target sites. For example, a Cas enzyme, a guide sequence linked to a tracr-mate sequence, and a tracr sequence could each be operably linked to separate regulatory elements on separate vectors. Alternatively, two or more of the elements expressed from the same or

different regulatory elements, may be combined in a single vector, with one or more additional vectors providing any components of the CRISPR system not included in the first vector. CRISPR system elements that are combined in a single vector may be arranged in any suitable orientation, such as one element located 5' with respect to ("upstream" of) or 3' with respect to ("downstream" of) a second element. The coding sequence of one element may be located on the same or opposite strand of the coding sequence of a second element, and oriented in the same or opposite direction. A single promoter may drive expression of a transcript encoding a CRISPR enzyme and one or more of the guide sequence, tracr mate sequence (optionally operably linked to the guide sequence), and a tracr sequence embedded within one or more intron sequences (e.g. each in a different intron, two or more in at least one intron, or all in a single intron).

An additional method of regulating the expression of an antagonist or negative regulator or component of the Wnt pathway (e.g. Axin, GSK3, APC and sFRP) gene in cells is via triplex forming oligonucleotides (TFOs). Recent studies have shown that TFOs can be designed which can recognize and bind to polypurine/polypyrimidine regions in double-stranded helical DNA in a sequence-specific manner. These recognition rules are outlined by Maher III, L. J., et al., *Science*,1989;245:725-730; Moser, H. E., et al., *Science*,1987;238:645-630; Beal, P. A., et al, *Science*,1992;251:1360-1363; Cooney, M., et al., *Science*,1988;241:456-459; and Hogan, M. E., et al., EP Publication 375408. Modification of the oligonucleotides, such as the introduction of intercalators and backbone substitutions, and optimization of binding conditions (pH and cation concentration) have aided in overcoming inherent obstacles to TFO activity such as charge repulsion and instability, and it was recently shown that synthetic oligonucleotides can be targeted to specific sequences (for a recent review see Seidman and Glazer, *J Clin Invest* 2003;112:487-94).

In general, the triplex-forming oligonucleotide has the sequence correspondence:

oligo	3'--A	G	G	T
duplex	5'--A	G	C	T
duplex	3'--T	C	G	A

However, it has been shown that the A-AT and G-GC triplets have the greatest triple helical stability (Reither and Jeltsch, *BMC Biochem*, 2002, Sept12, Epub). The same authors have demonstrated that TFOs designed according to the A-AT and G-GC

rule do not form non-specific triplexes, indicating that the triplex formation is indeed sequence specific.

Thus for any given sequence in the antagonist or negative regulator or component of the Wnt pathway (e.g. Axin, GSK3, APC and sFRP) regulatory region a triplex forming sequence may be devised. Triplex-forming oligonucleotides preferably are at least 15, more preferably 25, still more preferably 30 or more nucleotides in length, up to 50 or 100 bp.

Transfection of cells (for example, via cationic liposomes) with TFOs, and formation of the triple helical structure with the target DNA induces steric and functional changes, blocking transcription initiation and elongation, allowing the introduction of desired sequence changes in the endogenous DNA and resulting in the specific downregulation of gene expression. Examples of such suppression of gene expression in cells treated with TFOs include knockout of episomal supFG1 and endogenous HPRT genes in mammalian cells (Vasquez et al., Nucl Acids Res. 1999;27:1176-81, and Puri, et al, J Biol Chem, 2001;276:28991-98), and the sequence- and target specific downregulation of expression of the Ets2 transcription factor, important in prostate cancer etiology (Carbone, et al, Nucl Acid Res. 2003;31:833-43), and the pro-inflammatory ICAM-1 gene (Besch et al, J Biol Chem, 2002;277:32473-79). In addition, Vuyisich and Beal have recently shown that sequence specific TFOs can bind to dsRNA, inhibiting activity of dsRNA-dependent enzymes such as RNA-dependent kinases (Vuyisich and Beal, Nuc. Acids Res 2000;28:2369-74).

Additionally, TFOs designed according to the abovementioned principles can induce directed mutagenesis capable of effecting DNA repair, thus providing both downregulation and upregulation of expression of endogenous genes (Seidman and Glazer, J Clin Invest 2003;112:487-94). Detailed description of the design, synthesis and administration of effective TFOs can be found in U.S. Patent Application Nos. 2003 017068 and 2003 0096980 to Froehler et al, and 2002 0128218 and 2002 0123476 to Emanuele et al, and U.S. Pat. No. 5,721,138 to Lawn.

Another agent capable of downregulating an antagonist or negative regulator or component of the Wnt pathway (e.g. Axin, GSK3, APC and sFRP) would be any molecule which binds to and/or cleaves the antagonist or negative regulator or component of the Wnt pathway (e.g. Axin, GSK3, APC and sFRP). Such molecules can be antagonists, or

inhibitory peptides.

It will be appreciated that a non-functional analogue of at least a catalytic or binding portion of an antagonist or negative regulator or component of the Wnt pathway (e.g. Axin, GSK3, APC and sFRP) can be also used as an agent which downregulates the antagonist or negative regulator or component of the Wnt pathway (e.g. Axin, GSK3, APC and sFRP).

Another agent which can be used along with some embodiments of the invention to downregulate an antagonist or negative regulator or component of the Wnt pathway (e.g. Axin, GSK3, APC and sFRP) is a molecule which prevents activation or substrate binding.

According to one embodiment, upregulation of the Wnt canonical pathway is achieved by inhibiting GSK3 kinase activity. Inhibiting GSK3 activity may be achieved by inhibiting the enzymatic activity of GSK3, e.g. by use of chemical inhibitors or antagonists, which may be competitive or non-competitive, as described below. Such inhibitors may include kinase inhibitors.

GSK3 activity may be down-regulated by down-regulating the expression of GSK3 protein, such as by use of antisense RNA, or RNAi, or siRNA or by inhibiting the conversion of inactive forms of GSK3 to active forms, or by increasing the rate of degradation of GSK-3. The methods and compositions described here may also employ loss of function and dominant negative mutations in GSK-3, described for example in Hedgepeth et al. (1997) *Dev Biol.* 185(1):82-91. Kinase deficient mutants of GSK3, for example as described in Crowder and Freeman, 2000, *J. Biol. Chem.* 275 (2000), pp. 34266-34271, incorporated herein by reference, may be transfected into stem cells and/or vascular progenitor cells or angioblasts to achieve LEC specification.

Fibroblast growth factor (FGF) may also be used as an activator of Wnt canonical signaling pathway as exposure to FGF activates Akt and thus inhibits GSK3, described in Hashimoto et al., 2002, *J. Biol. Chem.* 277 (2002), pp. 32985-32991, incorporated herein by reference.

Wnt canonical signaling pathway may also be activated via upregulation, for example, over-expression, of FRAT1, a negative regulator of GSK3, as described in Crowder and Freeman, 2000, *J. Biol. Chem.* 275 (2000), pp. 34266-34271 and Culbert, et al., 2001, *FEBS Lett.* 507 (2001), pp. 288-294, incorporated herein by reference.

In some embodiments, GSK-3 is down-regulated using a chemical inhibitor. A number of chemical inhibitors of GSK3 activity are known in the art, as described in for example U.S. Pat. No. 6,441,053, incorporated herein by reference.

In some embodiments, the chemical inhibitors of GSK3 comprise lithium and its salts, including lithium chloride (LiCl); indirubins, e.g., Tyrian purple indirubins; 6-bromoindirubin-3'-oxime (BIO); iGSK-3 and its variants, SB-216763 and SB-415286, (i.e. structurally distinct maleimides). Additional GSK3 inhibitors are commercially available, e.g. from Calbiochem (San Diego, USA) and include e.g. 1-Azakenpaullone, Alsterpaullone, FRATtide, GSK-3b Inhibitor VII, GSK-3b Inhibitor XI, GSK-3b Inhibitor I, GSK-3b Inhibitor II, GSK-3b Inhibitor III and GSK-3 Inhibitor IX.

According to one embodiment, upregulation of the Wnt canonical pathway is achieved by inhibiting Axin activity. Inhibiting Axin activity may be achieved using any method known in the art, e.g. by RNAi as described in detail above.

According to one embodiment, upregulation of the Wnt canonical pathway is achieved by inhibiting APC activity. Inhibiting APC activity may be achieved using any method known in the art, e.g. by RNAi as described in detail above.

According to one embodiment, upregulation of the Wnt canonical pathway is achieved by inhibiting sFRP (e.g. sFRP-1) activity. Inhibiting sFRP activity may be achieved using any method known in the art, e.g. by RNAi or using the small molecule inhibitor WAY-316606, commercially available e.g. from Tocris Bioscience and R&D systems. Further methods are as described in detail hereinabove.

In other embodiments, Wnt canonical signaling may be activated by use of the cyclin dependent kinase (CDK) inhibitors described in Leclerc, et al., (2001), *J. Biol. Chem.* 276 (2001), pp. 251-260, and Knockaert et al., (2002), *J. Biol. Chem.* 277 (2002), pp. 25493-25501, incorporated herein by reference.

As mentioned, any of the components of the Wnt canonical signaling pathway may be modulated (e.g. upregulated or downregulated dependent on the target) in order to activate the pathway. Activation of the Wnt canonical signaling pathway in stem cells or vascular progenitor cells or angioblasts may be assessed in a number of ways known in the art. In general, such an assay will seek to detect the modulation of the target component, or a component downstream of the component which is the target of activation.

In some embodiments, an assay for activation of the Wnt canonical signaling pathway may comprise detection of a reduced activity of GSK3. GSK3 activity may be assessed in a number of ways, for example, utilizing GSK3 Kinase Assays. Such an assay may be particularly suitable where GSK3 activity is targeted for inhibition as a means to
5 activate the Wnt canonical signaling pathway.

In some embodiments, an assay for activation of the Wnt canonical signaling pathway may comprise detecting accumulation of β -catenin in the cytoplasm, or the nucleus, or both, when an increase in the amount or quantity of β -catenin in either or both locations may be assessed as an indication of Wnt canonical signaling pathway activation.
10 This may be achieved by making nuclear or cytoplasmic extracts of the cells in question, e.g., stem cells and/or vascular progenitor cells and/or angioblasts, using means known in the art, and detection of β -catenin protein by antibody Western blots. Particularly useful assays include, for example, those assays which detect active forms of β -catenin, or non-phosphorylated forms of β -catenin, using antibodies specific for such forms.

15 In some embodiments, phosphorylation of Dishevelled, or phosphorylation of the LRP may also be detected as a means of gauging activation of Wnt canonical signaling pathway.

Similarly, activation of the Wnt non-canonical signaling pathway may be assessed using any method known to one of skill in the art.

20 In some embodiments, an assay for activation of the Wnt non-canonical signaling pathway may comprise detection of intracellular Ca^{2+} release.

In some embodiments, an assay for activation of the Wnt non-canonical signaling pathway may comprise detecting accumulation of Dsh, Daam1, Rho, Rho kinase (ROCK), Rac and/or JNK in the cytoplasm, or the nucleus, or both, when an increase in the amount
25 or phosphorylation of Dsh, Daam1, Rho, Rho kinase (ROCK), Rac and/or JNK in either or both locations may be assessed as an indication of Wnt non-canonical signaling pathway activation. This may be achieved by making nuclear or cytoplasmic extracts of the cells in question, e.g., LECs, using means known in the art, and detection of Dsh, Daam1, Rho, Rho kinase (ROCK), Rac and/or JNK protein by antibody Western blots. Particularly
30 useful assays include, for example, those assays which detect active forms of Dsh, Daam1, Rho, Rho kinase (ROCK), Rac and/or JNK, or non-phosphorylated forms thereof, using antibodies specific for such forms.

In order to obtain LEC specification, the Wnt canonical signaling pathway in the stem cells or vascular progenitor cells or angioblasts may be activated for 6 hours or more, 12 hours or more, 24 hours or more, 36 hours or more, 48 hours or more, 3 days or more, 4 days or more, 5 days or more, 6 days or more, 7 days or more, 8 days or more, 9 days or more, 10 days or more, 11 days or more, 12 days or more, 13 days or more, or 14 days or more. According to a specific embodiment, the Wnt canonical signaling pathway in the stem cells or vascular progenitor cells or angioblasts is activated for 6 hours to 14 days, for 1 to 14 days, for 2 to 14 days, for 4 to 14 days, for 6 to 14 days, for 8 to 14 days, for 4 to 10 days, for 4 to 8 days, for 6 to 8 days or 6 to 7 days. It will be appreciated that the Wnt canonical signaling pathway may be activated for as long as necessary, depending on the application, for example, the Wnt canonical signaling pathway may be activated for 1 week, 2 weeks, 3 weeks, 4 weeks, etc, as necessary. In such embodiments, the Wnt canonical signaling pathway may be activated continuously during that time.

According to one embodiment, the stem cells or vascular progenitor cells or angioblasts are first induced to differentiate into endothelial cells prior to promoting LEC specification. In such a case, the stem cells or vascular progenitor cells or angioblasts may be treated with a factor known to induce endothelial cell differentiation, e.g. Collagen (e.g. Collagen-IV), vascular endothelial growth factor (VEGF). The stem cells or vascular progenitor cells or angioblasts may be further treated with an agent which blocks differentiation into other cell types, e.g. into smooth muscle cells, such an agent may include e.g. SB431542.

Isolation and selection of ECs may be carried out using any method known in the art, e.g. by FACS analysis, selecting cells expressing the surface markers KDR, CD31 and/or or CD144 (VE-cadherin).

According to an exemplary embodiment, the stem cells or vascular progenitor cells or angioblasts are induced to differentiate towards the endothelial lineage by first being seeded as single cells on Collagen-IV coated plates at $1-20 \times 10^4$ cells/cm² (e.g. 5×10^4 cells/cm²) and cultured with MEM-alpha, 5-20 % FBS (e.g. 10 %) and 0.01-1 mM β -mercaptoethanol (e.g. 0.1 mM) for 3-10 days (e.g. 6 days). On the last day (e.g. day 6), cells are re-seeded on collagen-IV coated plates at $0.5-5 \times 10^4$ cells/cm² (e.g. 1.25×10^4 cells/cm²) and are cultured under ECGM, 10-40 % FBS (e.g. 20 %), 5-100 ng/ml VEGF-A (e.g. 50 ng/ml) and 1-50 μ M SB431542 (e.g. 10 μ M). To induce lymphatic specification

from the EC cells generated, the endothelial cells may be subjected to Wnt ligand, e.g. Wnt5b (e.g. 100 ng/ml) starting at day 3-10 (e.g. day 6) every other day for about 3-10 days (e.g. 6 days).

Isolation and selection of LECs may be carried out using any method known in the art, e.g. by FACS analysis, selecting cells expressing the LEC markers as described in detail above, e.g. *prox1*, *lyve1*, *flt4*, *sox18*, *nr2f2*, *CD31* and *GP38*.

According to one embodiment, activation of the Wnt canonical signaling pathway may be used to cause a stem cell or vascular progenitor cell or angioblast which is still pluripotent, but already committed to differentiation along a specific pathway (e.g., an arterial or venous pathway), to enter a lymphatic differentiation pathway.

It will be appreciated, that in order to promote LEC specification, the stem cells or vascular progenitor cells or angioblasts may be further subjected to additional factors that can enhance or promote LEC specification.

According to one embodiment, expression of the orphan nuclear receptor COUP transcription factor II (COUP-TFII), SRY-related HMG-domain transcription factor *Sox18*, ERK and/or homeobox transcription factor *Prox1* may be upregulated in the stem cells or vascular progenitor cells or angioblasts to promote LEC specification.

According to an aspect of the present invention there is provided a method of inducing lymphangiogenesis, the method comprising: (a) promoting LEC specification in stem cells or vascular progenitor cells or angioblasts so as to obtain a LEC committed cell according to the method of some embodiments of the invention; and (b) subjecting the LEC committed cell to a LEC morphogenesis signal, thereby inducing lymphangiogenesis.

The term "lymphangiogenesis" as used herein refers to the development, growth or formation of new lymphatic vessels e.g. capillaries, collecting vessels, and ducts.

In order to induce lymphangiogenesis (e.g. formation of lymphatic capillaries, collecting vessels, and ducts), the LEC committed cells are subjected to a LEC morphogenesis signal, a LEC proliferation signal or a LEC survival signal. As used herein, the phrase "LEC morphogenesis signal" refers to any agent which induces lymphangiogenesis in the LEC committed cells.

As used herein, the phrase "LEC proliferation signal" refers to any agent which induces cell division in the LEC committed cells.

As used herein, the phrase “LEC survival signal” refers to any agent which reduced cell death in the LEC committed cells.

According to one embodiment, the LEC morphogenesis, proliferation or survival signal comprises an endothelial growth factor.

5 According to one embodiment, the endothelial growth factor is a vascular endothelial growth factor (VEGF) or a receptor thereof. According to a specific embodiment, the VEGF is VEGF-C or VEGF-D, or their receptors VEGFR-2 or VEGFR-3. According to another embodiment, the factor is a collagen and calcium-binding EGF domain-containing protein 1 (ccbe1) previously shown to be required for embryonic
10 lymphangiogenesis and venous sprouting through upregulation of Vegfc (Hogan, 2009 or Le Guen, 2009).

According to one embodiment, the LEC committed cell may be exposed to endothelial growth factor (e.g. VEGF) *ex vivo* in cell medium comprising the endothelial growth factor.

15 According to another embodiment, the LEC morphogenesis signal comprises upregulating the activity of a Wnt non-canonical pathway in the LECs (as described in detail hereinabove).

It will be appreciated, that in order to promote lymphangiogenesis, the LEC committed cells may be further subjected to additional factors that can enhance or promote
20 lymphangiogenesis.

According to one embodiment, expression of VEGF, SH2 domain containing leukocyte protein of 76 kDa (Slp76), spleen tyrosine kinase (Syk) and phospholipase C, gamma 2 (Plcg2) may be upregulated in the LEC committed cells to promote lymphangiogenesis.

25 Selecting LECs that have undergone lymphangiogenesis can be effected using any method known in the art, e.g. by microscopy, isolating those cells that have formed any of the lymphatic vasculature (lymphatic capillaries, collecting vessels, and ducts) or by phenotypic cell sorting (e.g. FACS) selecting cells expressing LEC markers e.g. neuropilin 2 (Nrp2), forkhead box C2 (Foxc2), podoplanin (Pdpn), angiopoietin 2 (Ang2), ephrin B2
30 (Efnb2), protein phosphatase 1 regulatory (inhibitor) subunit 13B (Aspp1) and/or core 1 synthase glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 (T-synthase).

According to one embodiment, there is provided an isolated population of cells generated according to the above described methods.

For *ex-vivo* therapy, stem cells or vascular progenitor cells or angioblasts are preferably treated with the agent of the present invention (as detailed in further detail
5 hereinaabove), following which they are administered to the subject in need thereof.

Thus, according to one aspect of the present invention there is provided a method of treating or preventing a disease or disorder related to lymphangiogenesis in a subject in need thereof, the method comprising administering to the subject the isolated population of cells of some embodiments of the invention, thereby treating or preventing the disease
10 or disorder related to lymphangiogenesis.

The term "treating" refers to inhibiting, preventing or arresting the development of a pathology (disease, disorder or condition) and/or causing the reduction, remission, or regression of a pathology. Those of skill in the art will understand that various methodologies and assays can be used to assess the development of a pathology, and
15 similarly, various methodologies and assays may be used to assess the reduction, remission or regression of a pathology.

As used herein, the term "preventing" refers to keeping a disease, disorder or condition from occurring in a subject who may be at risk for the disease, but has not yet been diagnosed as having the disease.

As used herein, the term "subject" refers to an animal, preferably a mammal, or a human being, of any age or sex, who suffers from or is predisposed to a disease or disorder related to lymphangiogenesis.
20

Diseases or disorders related to lymphangiogenesis include any condition in which there is a deviation from or interruption of the normal structure or function of the lymph or
25 lymph vessels. Collectively, such diseases and disorders are referred to herein as "lymphatic disorders or diseases."

Lymphatic disorders or diseases include, but are not limited to, lymphedema e.g. lymphangiomatosis, lymphangioliomyomatosis, mixed vascular/lymphatic malformation syndromes or conditions, e.g. Turner-Weber and Klippel Trenaunay Syndrome, conditions
30 arising from filariasis, trauma, infection or surgeries e.g. of the breast, prostate, uterus, cervix, abdomen, orthopedic, cosmetic (liposuction), malignant melanoma, conditions arising from treatments used for malignant diseases, e.g. lymphoma (e.g. Hodgkin's and

non-Hodgkin's lymphoma), radiation therapy, sport injuries, tattooing, diabetes, obesity and any physical insult to the lymphatic pathways, the inability to control infections (e.g. such as that associated with HIV/AIDS), the inability to deliver antibiotic and anti-viral medication to infected tissues and organs, inflammatory and auto-immune diseases (e.g. such as but not limited to, rheumatoid arthritis and systemic lupus erythematosis, scleroderma, Wegener's granulomatosis), lymphatic insufficiency of the internal organs, impairment of lymphatic development in the intestines (e.g. which leads to malabsorption, ascites, underdevelopment from malnutrition, immune malfunction) and pulmonary lymphangiectasia, cystic hygromas and lymphangiomas that may lead to impaired vision, swallowing and breathing.

According to one embodiment, the lymphatic disorders or diseases include, but are not limited to, cancer, melanoma, solid tumor, breast cancer, lung cancer, kidney cancer, pancreatic cancer, hematopoietic tumor, graft rejection, autoimmune disorder, rheumatoid arthritis, psoriasis, oedema, chronic wound (e.g. chronic wound not healing because of lymphorrhea or lymph secretions in surgical wounds), lymphedema, lymphatic fistula, lymphorrhea and lymphocele/seroma formation.

According to one embodiment, there is provided a method of transplanting lymphatic endothelial cells (LECs) into a subject in need thereof, the method comprising generating LECs according to the method of some embodiments of the invention; and transplanting the LECs into the subject, thereby transplanting the LECs.

As used herein, "transplanting" refers to providing the LECs of the present invention, using any suitable route.

Administration of the *ex-vivo* treated cells of the present invention (e.g. LECs) can be effected using any suitable route of introduction, such as intravenous, intraperitoneal, intra-kidney, intra-gastrointestinal track, subcutaneous, transcutaneous, intramuscular, intracutaneous, intrathecal, epidural, and rectal. According to a specific embodiment, the *ex-vivo* treated cells of the present invention may be introduced to the individual using an intravenous mode of administration.

The stem cells and/or vascular progenitor cells or angioblasts may be obtained from any autologous or non-autologous (i.e., allogeneic or xenogeneic) donor. For example, cells may be isolated from a human cadaver or from a human hematopoietic cell

donor. Alternatively, cells may be obtained from any xenogeneic donor (e.g. porcine origin).

Stem cells and/or vascular progenitor cells or angioblasts of xenogeneic origin (e.g. porcine) are preferably obtained from a source which is known to be free of zoonoses, such as porcine endogenous retroviruses. Similarly, human-derived stem cells and/or vascular progenitor cells or angioblasts are preferably obtained from substantially pathogen-free sources.

Since non-autologous cells are likely to induce an immune reaction when administered to the body several approaches have been developed to reduce the likelihood of rejection of non-autologous cells. These include either suppressing the recipient immune system or encapsulating the non-autologous cells in immunoisolating, semipermeable membranes before transplantation. Alternatively, cells may be used which do not express xenogenic surface antigens, such as those developed in transgenic pigs.

Encapsulation techniques and methods of preparing microcapsules are described in detail hereinabove.

Examples of immunosuppressive agents which may be used in conjunction with the *ex-vivo* treatment include, but are not limited to, methotrexate, cyclophosphamide, cyclosporine, cyclosporin A, chloroquine, hydroxychloroquine, sulfasalazine (sulphasalazopyrine), gold salts, D-penicillamine, leflunomide, azathioprine, anakinra, infliximab (REMICADE.sup.R), etanercept, TNF.alpha. blockers, a biological agent that targets an inflammatory cytokine, and Non-Steroidal Anti-Inflammatory Drug (NSAIDs). Examples of NSAIDs include, but are not limited to acetyl salicylic acid, choline magnesium salicylate, diflunisal, magnesium salicylate, salsalate, sodium salicylate, diclofenac, etodolac, fenoprofen, flurbiprofen, indomethacin, ketoprofen, ketorolac, meclofenamate, naproxen, nabumetone, phenylbutazone, piroxicam, sulindac, tolmetin, acetaminophen, ibuprofen, Cox-2 inhibitors and tramadol.

According to another embodiment of the present invention, treating or preventing a disease or disorder related to lymphangiogenesis is effected by administering to the subject the agent *per se* capable of upregulating the activity of Wnt canonical pathway. The method may be further effected by administering to the subject an agent capable of inducing LEC morphogenesis signal (e.g. upregulating the activity of Wnt non-canonical pathway).

For *in-vivo* therapy, the agent (as detailed hereinabove) is administered to the subject as is or as part of a pharmaceutical composition.

Thus, the *ex-vivo* treated LECs or the agent capable of upregulating the activity of Wnt canonical and/or non-canonical pathway of the present invention can be administered
5 to the individual *per se* or as part of a pharmaceutical composition which also includes a physiologically acceptable carriers or excipients.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical
10 composition is to facilitate administration of a compound to an organism.

Herein the term "active ingredient" refers to the *ex-vivo* treated LECs or the agent capable of upregulating the activity of Wnt canonical and/or non-canonical pathway accountable for the biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically
15 acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical
20 composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in
25 "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct
30 intraventricular, intracardiac, e.g., into the right or left ventricular cavity, into the common coronary artery, intravenous, intraperitoneal, intranasal, or intraocular injections.

Conventional approaches for drug delivery to the central nervous system (CNS) include: neurosurgical strategies (e.g., intracerebral injection or intracerebroventricular

infusion); molecular manipulation of the agent (e.g., production of a chimeric fusion protein that comprises a transport peptide that has an affinity for an endothelial cell surface molecule in combination with an agent that is itself incapable of crossing the BBB) in an attempt to exploit one of the endogenous transport pathways of the BBB; 5 pharmacological strategies designed to increase the lipid solubility of an agent (e.g., conjugation of water-soluble agents to lipid or cholesterol carriers); and the transitory disruption of the integrity of the BBB by hyperosmotic disruption (resulting from the infusion of a mannitol solution into the carotid artery or the use of a biologically active agent such as an angiotensin peptide). However, each of these strategies has limitations, 10 such as the inherent risks associated with an invasive surgical procedure, a size limitation imposed by a limitation inherent in the endogenous transport systems, potentially undesirable biological side effects associated with the systemic administration of a chimeric molecule comprised of a carrier motif that could be active outside of the CNS, and the possible risk of brain damage within regions of the brain where the BBB is 15 disrupted, which renders it a suboptimal delivery method.

Alternately, one may administer the pharmaceutical composition in a local rather than systemic manner, for example, via injection of the pharmaceutical composition directly into a tissue region of a patient.

Pharmaceutical compositions of some embodiments of the invention may be 20 manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with some embodiments of the invention thus may be formulated in conventional manner using one or more 25 physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as 30 Hank's solution, Ringer's solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the pharmaceutical composition can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the pharmaceutical composition to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use according to some embodiments of the invention are conveniently delivered in the form of an aerosol

spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and
5 cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The pharmaceutical composition described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with
10 optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the
15 active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the
20 suspension may also contain suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use.

The pharmaceutical composition of some embodiments of the invention may also
25 be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of some embodiments of the invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically
30 effective amount means an amount of active ingredients (LECs or agent capable of upregulating the activity of Wnt canonical and/or non-canonical pathway) effective to

prevent, alleviate or ameliorate symptoms of a disorder (e.g., a disease or disorder related to lymphangiogenesis) or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

5 For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from in vitro and cell culture assays. For example, a dose can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

10 Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures in vitro, in cell cultures or experimental animals. The data obtained from these in vitro and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration
15 utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide ample levels of the active ingredient which are sufficient to induce or suppress the biological effect
20 (minimal effective concentration, MEC). The MEC will vary for each preparation, but can be estimated from in vitro data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing
25 can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the
30 judgment of the prescribing physician, etc.

Compositions of some embodiments of the invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or

more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

According to an aspect of some embodiments of the present invention there is provided a method of screening for an agent capable of upregulating the activity of Wnt canonical pathway for the formation of LECs, the method comprising: contacting the agent with stem cells and/or vascular progenitor cells or angioblasts; analyzing an expression of a lymphatic marker on the stem cells and/or vascular progenitor cells or angioblasts; and comparing the expression of the marker following the contacting to prior to the contacting, wherein an expression of the marker is indicative of an agent suitable for formation of LECs.

According to one embodiment, the lymphatic marker includes, but is not limited to, Prox1, lyve1, VEGFR-3, flt4, sox18, nr2f2, Foxc2, Pdpn, Ang2, Efnb2, Aspp1 and/or T-synthase.

Once such an agent is identified it is synthesized and qualified in other assays such as in vitro in hESC or in zebrafish models (e.g. as discussed in detail in the examples section which follows) for being used for promoting the formation of LECs.

As used herein the term "about" refers to $\pm 10\%$.

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of" means "including and limited to".

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional

ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at
5 least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be
10 considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of
15 the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and
20 are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known
25 manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity,
30 described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be

considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in

Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other
 5 general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

GENERAL MATERIALS AND EXPERIMENTAL PROCEDURES

10 *Zebrafish husbandry and transgenic lines*

Zebrafish were raised by standard methods previously described [Avraham-Davidi I. et al., *Nature medicine* (2012) 18: 967-973] and were handled according to the Weizmann Institute Animal Care and Use Committee. The following models were used: *plcgl^{y10}*, *Tg(fli1:EGFP)^{yl}*, *Tg(fli1:nEGFP)^{y7}*, *Tg(fli1:dsRed)^{um13}*,
 15 *Tg(fli1:gal4^{ubs3};uasKaede^{rk8})*, *Tg(hsp70l:wnt5b-GFP)^{w33}*, *Tg(7xTCF-Xla.Siam.nlsmCherry)^{ia5}*, *cas^{ta56}*, *pptⁱⁱ²⁶⁵*, *mbl^{tm213}*, *apc^{mcr}*, *Tg(lyve1:dsRed2)^{nz101}* and *TgBAC(prox1a:Kalt4-UAS:uncTagRFP)^{nim5}*. The *Tg(ftl1_9a_cFos:GFP)^{wz2}* reporter was generated by cloning the previously identified zebrafish *ftl1_9a* enhancer into pGW_cFosGFP as previously described [Fisher, S. et al., *Nat Protoc* (2006) 1: 1297-
 20 1305]. The *Tg(kdrl:Kaede)^{wz3}* was generated by cloning a Kaede fragment in a Tol2-compatible vector containing 2.5 kb from the *kdrl* promoter using the Gateway methodology [Villefranc J. A. et al., *Dev Dyn* (2007) 236: 3077-3087].

In situ hybridization and Immunofluorescence

1. Whole-mount *in situ* hybridization was carried out as previously described
 25 [Avraham-Davidi I. et al., *Nature medicine* (2012) supra] using *flt4*, *ccbe1* [Hogan B. M. et al., *Nature genetics* (2009) 41: 396-398], *sox18* [Cermenati, S. et al., *Blood* (2007) 111: 2657-2666] and *cdh5* [Avraham-Davidi I. et al., *Nature medicine* (2012) supra] antisense mRNA probes. The *lyve1* (5'-AGACGTGGGTGAAATCCAAG-3' and 5'-GATGATGTTGCTGCATGTCC-3', SEQ ID NOs: 1 and 2, respectively), *wnt5b* (5'-
 30 ATGGATGTGAGAATGAACCAAGGAC-3' and 5'-CTACTTGACACAACTGGTCTACG-3', SEQ ID NOs: 3 and 4, respectively), and *vegfc* (5'-CATCAGCACTTCATACATCAGC-3' and 5'-

GTCCAGTCTTCCCCAGTATG-3', SEQ ID NOs: 5 and 6, respectively) probes were amplified by PCR from 24 hpf cDNA. A fragment (1269 bp) flanking the (5'-GTACAAAAAAGCAGGCTCCGCGGCC-3'...5'-

TCATCAGGGATATGTTGCTGTCTCGGG-3', SEQ ID NOs: 7 and 8, respectively) sequence of the *nr2f2* gene was cloned into Pcs2 plasmid, and linearized using NotI. Embryos were imaged using a Leica M165 FC imaging system.

2. Phospho-histone staining was carried out as previously described [Shepard, J. L. et al., *Methods in cell biology* (2004) 76: 109-125] using p-histone H3 antibody (1:300) (Santa Cruz).

3. For detection of Prox1 protein, embryos were fixed in 4 % PFA O/N, washed in 100 % metOH, incubated 1 hour on ice in 3 % H₂O₂ in metOH, washed in 100 % metOH and stored at -20 °C. Embryos were then permeabilized in wash buffer (PBS/0.1 % tween/0.1 % Triton), blocked in 10 % goat serum/1 % BSA in wash buffer for 5 hrs at 4 °C, and incubated with Prox1 antibody (1:750) O/N. Samples were then washed 5 times with wash buffer, followed by washes with maleic buffer (150 mM Maleic acid/100 mM NaCl/0.001 % Tween20 pH 7.4 saturated with 10 N NaOH), blocking in maleic buffer containing 2 % blocking reagent (Roche), and incubation O/N at 4 °C with Goat anti Rabbit IgG-HRP (Jackson 1:500) for TSA signal amplification. Following washes with maleic buffer and PBS, samples were incubated for 3 hrs with TSA Plus Cyanine 3 reaction (Perkin Elmer) and washed with wash buffer several times through 1-2 days at room temperature.

Manipulation of zebrafish embryos

Heat-shock, TNP-470 and IWR1 treatments

24-26 hours post-fertilization (hpf) *Tg(hsp70l:wnt5b-GFP)* embryos were heat-shocked at 37 °C for 25 minutes and analyzed for parachordal cells (PAC) number at 56 hpf. For Prox1 immunostaining *Tg(hsp70l:wnt5b-GFP)* embryos were heat-shocked at 19-20 hpf, for 25 minutes and fixed as described above at 28 hpf. For qRT-PCR analyses *Tg(prox1:Kalt4-UAS:uncTagRFP;hsp70l:wnt5b-GFP)* embryos were heat-shocked at 21 hpf, for 25-30 minutes.

IWR1 (Sigma) and TNP-470 (Sigma) were dissolved in Dimethyl sulfoxide (DMSO) as previously described [Cirone, P. et al., *Angiogenesis* (2008) 11: 347-360]. Embryos were treated with 30 μM IWR1, for 2 days starting at 20 hpf. TNP-470 was

added at a concentration of 25 μ M, for 1 day starting at 23 hpf. PAC formation was assessed at 3 dpf.

Morpholino Injection

The following antisense morpholino oligonucleotides were used:

5 *sox32* [Griffin, K. J. and Kimelman, D., *Nat Cell Biol* (2002) 4: 821-825] (1 ng),
tcf7 [Nyholm, M. K. et al., *Development (Cambridge, England)* (2007) 134: 735-746] (8
 ng), *lef1* [Dorsky, R. I. et al., *Dev Biol* (2002) 241: 229-237] (3 ng), *wnt5b* [Lele, Z. et al.,
Genesis (2001) 30: 190-194] (7.5 ng or 4 ng for subdose), *vegfc* [Yaniv, K. et al. *Nature*
medicine (2006) 12: 711-716] (5 ng), *tcf4* (5'-CTGCGGCATTTTTCCCGAGGAGCGC-
 10 3', SEQ ID NO: 9) (8 ng), control MO (5'-CCTCTTACC TCAGTTACAATTTATA-3',
 SEQ ID NO: 10) (8 ng). MOs (Gene-tools) were resuspended and injected as previously
 described [Avraham-Davidi I. et al., *Nature medicine* (2012) supra].

DNA and mRNA injection

15 *axin* mRNA [Shimizu, T. et al. *Mech Dev* (2000) 91: 293-303] (260 pg) was
 injected at 1-cell stage. To generate the *Tg(flt1_9a_cFos:GFP)*, and *Tg(kdrl:Kaede)*
 transgenic lines, approximately 30 pg plasmid were injected along with 30 pg of
Tol2 transposase mRNA into 1-cell stage embryos.

quantitative real-time PCR (qRT-PCR)

20 qRT-PCR was carried out as previously described [Avraham-Davidi I. et al.,
Nature medicine (2012) supra] using the following primers:

zf-prox1a (5'-AATCCAAGAGGGGCTTTCGC-3' and 5'-
 TGCAGCGGTTAAACTTCACG- 3', SEQ ID NOs: 11 and 12, respectively), KaltA4 (5'-
 GACGCTGTGACAGACCGATT-3' and 5'-CAGCTGTCTCTGTCCCTTGT-3', SEQ ID
 NOs: 13 and 14, respectively), *zf-bactin2* [Avraham-Davidi I. et al., *Nature medicine*
 25 (2012) supra], *zf-etv2* (5'-TACCCAGGATCTGGACCCAT-3' and 5'-
 CAGCCATCACCAGTCCAAC-3', SEQ ID NOs: 15 and 16, respectively), *zf-frz7a* (5'-
 TGTCTCGTGCGGACTGTTAC-3' and 5'-CACTGTTCATGAGGCTCCGT-3', SEQ ID
 NOs: 17 and 18, respectively), *zf-nr2f2* (5'- ACAGAGTGGTCGCCTTTATGG-3' and
 5'-CCACACGCATCTGAAGTGAA-3', SEQ ID NOs: 19 and 20, respectively). h-Prox1
 30 (5'-CCACTGACCAGACAGAAGCA-3' and 5'-TGGGCTCTGAAATGGATAGG-3',
 SEQ ID NOs: 21 and 22, respectively), h-beta-actin (5'-TCCACCTTCCAGCAGATGTG-
 3' and 5'- GCATTTGCGGTGGACGAT-3', SEQ ID NOs: 23 and 24, respectively), h-

Flt4 (5'-AAGAAGTTCCACCACCAAACAT-3' and 5'-TGAAAATCCTGGCTCACAAGC-3', SEQ ID NOs: 25 and 26, respectively) and h-Cdh5 (5'-AACTTCCCCTTCTTCACCC-3' and 5'-AAAGGCTGCTGGAAAATG-3', SEQ ID NOs: 27 and 28, respectively).

5 **Scoring and Quantification of phenotypes**

To assess the contribution of dorsal versus ventral posterior cardinal vein (PCV) to different vascular beds, single EC, or pan-Kaede photoconversion was carried out in *Tg(fli1:gal4;uasKaede)* embryos. Photoswitching was performed using a 405 nm laser. To assess the contribution of medial versus early lateral angioblasts to PACs, ECs in 4
10 segments of *Tg(kdrl:Kaede)* embryos were photoswitched at 17-18 hpf and 20-21 hpf respectively. 28 hours later, embryos were imaged, and the number of green versus red PACs was counted in 6 segments over the photoswitched area.

For quantification of phenotypes, the average number of PACs/TD/cell number in 9-10 segments over the yolk extension was calculated. Embryos with no fluorescence or
15 with gross vascular morphological defects were excluded from quantification. For analysis, embryos that meet all the criteria above were randomly selected. For quantification of PAC related phenotypes in *ppt* mutants, a subdose of wnt5b MO (4 ng) was injected into *ppt* embryos to abolish maternal RNA contribution as previously described [Kim, H. J. et al., *BMC biology* (2005) 3: 23].

20 **Imaging**

Confocal imaging was performed using a Zeiss LSM 780 upright confocal microscope (Carl Zeiss, Jena, Germany) with a W-Plan Apochromat ×20 objective, NA 1.0. Fluorescent proteins were excited sequentially with single-photon lasers (488 nm, 563 nm). 2Photon imaging of eGFP was carried out at 920 nm. Time-lapse, *in-vivo* imaging
25 was performed as previously described [Ben Shoham, A. et al., *Development (Cambridge, England)* (2012) 139: 3859-3869] using a custom-built chamber for perfusion of embryos with temperature-controlled physiological medium. *z*-stacks were acquired at 2.5-3 μm increments, every 10-12 minutes.

Embryo dissociation, fluorescence activated cell sorting (FACS) and RNASeq

30 Following pan-Kaede photoconversion of dorsal, or ventral PCV in *Tg(fli1:gal4;uasKaede)* embryos at 24 hpf, 6 embryos per group were used for FACS isolation of Kaede photoconverted (red) ECs. Single-cell suspensions were prepared as

previously described [Avraham-Davidi I. et al., *Nature medicine* (2012) supra] with some modifications (the embryos were not chopped, and no Liberase was used). Sorting was performed at 4 °C in a FACSAria cell sorter using a 70-µm nozzle. Photoconverted (red) ECs were collected in 1 ml PBS, washed with PBS and centrifuged twice at 300 g, at 4 °C
5 for 5 minutes Total RNA was extracted using Tri@Reagent (Sigma) as previously described [Levin, M. et al., *Dev Cell* (2012) 22: 1101-1108], except that only GenElute-LPA (Sigma) was added to help precipitate the RNA. RNASeq was performed as previously described [Hashimshony, T. et al., *Cell reports* (2012) 2: 666-673] with the following modification – a new set of primers was used with a shorter barcode, and a 5
10 base UMI to enable transcript counting [described in Hashimshony, T. et al., (2012) supra].

RNASeq data analysis

CEL-Seq data was normalized by dividing the reads of each gene by the total reads of the sample and multiplying by 10,000 (transcript per 10,000). Genes without any
15 detected expression, or with expression detected only in one sample were filtered out. For identification of significant differentially expressed genes, fold change was calculated and a two-sample t-test was conducted.

Gene Ontology analysis was performed with Ontologizer 2.0 as previously described [Bauer, S. et al., *Bioinformatics* (2008) 24: 1650-1651] using the Topology-
20 Weighted algorithm on the set of genes with a change of at least 1.5 fold between the ventral and dorsal samples. The associations were taken from geneontology(dot)org, Version 1.4 from ZFIN.

Image processing

Images were processed off-line using ImageJ (NIH) and Imaris (Bitplane).
25 Selected datasets were deconvoluted with Autoquant X3 (Media Cybernetics). For colocalization analyses confocal images were first deconvoluted and then analyzed using the Imaris 'Colocalization Module'. This new channel was used to mark, and manually count cells that were labeled with both EGFP and mCherry/TagRFP fluorophores. Colocalization thresholds and nuclei quantification were set manually. Where necessary,
30 movies were registered with the "Linear Stack Alignment with SIFT" plugin of FIJI.

Histology

Tg(fli1:EGFP) embryos were fixed in 4 % PFA for 20 minutes at RT, embedded in Gelatin-Bovine albumin medium (0.35 % Gelatin, 21 % Bovine albumin) as previously described [Levin, M. *J Biochem Biophys Methods* (2004) 58: 85-96]. 50-100 μm cross-
5 sections were obtained using a Leica VT 1000s vibratome and stained in 1:200 dilution of TRITC-Phalloidin (Sigma) as previously described [Jin, S. W. et al., *Development (Cambridge, England)* (2005) 132: 5199-5209].

Human embryonic stem cells (hESCs)

Induction of differentiation towards the endothelial lineages has been previously
10 described [Kusuma, S. et al., *Proceedings of the National Academy of Sciences of the United States of America* (2013) 110: 12601-12606]. Briefly, H9 cells were seeded as single cells on Collagen-IV (Sigma) coated plates at 5×10^4 cells/cm² and cultured with MEM-alpha (Invitrogen), 10 % FBS (Hyclone) and 0.1 mM β -mercaptoethanol for 6 days. At day 6, cells were re-seeded on collagen-IV coated plates at 1.25×10^4 cells/cm² and
15 cultured under ECGM (Promocell) + 20 % FBS, 50 ng/ml VEGF-A (Biolegend Inc, San Diego CA) and 10 μM SB431542 (Sigma Aldrich).

To induce lymphatic differentiation, 100 ng/ml Wnt5b (R&D) was added to the cells starting at day 6 every other day. At day 12 RNA was extracted with Tri@Reagent (Sigma)/Chlorophorm, and cDNA was produced using the SuperscriptIII kit (Invitrogen).

20 H9 cells were obtained and handled by the Stem Cells Research Center at the Weizmann Institute (Israel), and were routinely checked for karyotype and for mycoplasma contamination.

For FACS analyses cells were harvested using non-enzymatic dissociation solution (Sigma), stained with an APC-conjugated Lyve1 Antibody (R&D systems, Minneapolis,
25 MN) for 30 minutes at RT, washed with PBS 3 % FCS, stained with Propidium Iodide (Sigma) and analyzed via FACSariaIII. Dead cells were excluded from analysis by gating out Propidium Iodide positive cells.

Statistical analyses

Data was analyzed using the unpaired two-tailed Student's t test assuming unequal
30 variance from at least two independent experiments, unless stated otherwise. In all cases normality was assumed and variance was comparable between groups. Sample size was selected empirically following previous experience in the assessment of experimental

variability. The investigators were not blinded to allocation during experiments and outcome assessment. The adequate tests were chosen according to the data distribution to fulfill test assumptions. Numerical data are the mean \pm s.e.m, unless stated otherwise.

For qRT-PCR experiments, standard error was computed for each fold-change. For genes with more than a single fold-change value, x_1, x_2, \dots, x_n , each with a standard error $\Delta x_1, \Delta x_2, \dots, \Delta x_n$, the mean fold-change was computed by taking geometrical average, $\bar{x} = \sqrt[n]{x_1 \cdot x_2 \cdot \dots \cdot x_n}$. Its standard error was computed using error propagation, $\Delta \bar{x} = \frac{\bar{x}}{n} \sqrt{\left(\frac{\Delta x_1}{x_1}\right)^2 + \left(\frac{\Delta x_2}{x_2}\right)^2 + \dots + \left(\frac{\Delta x_n}{x_n}\right)^2}$.

EXAMPLE 1

Lymphatic progenitors originate in the floor of the Posterior Cardinal Vein (PCV)

To characterize the initial events controlling lymphatic specification, the origins of lymphatic endothelial cells (LECs) within the Posterior Cardinal Vein (PCV) of zebrafish embryos were fate-mapped. *Tg(fli1:EGFP)^{y1}* (Figures 1A-D) and *Tg(fli1:nEGFP)^{y7}* (Figures 1H-K) embryos were imaged starting at 22-24 hpf and until 60 hpf, when PACs are fully discernible. Tracking of PAC-LECs back in time and space demonstrated that 81 % of these cells originated in the ventral side of the PCV (vPCV) as compared to 19 % that originated in the dorsal-PCV (dPCV). To corroborate these results, *Tg(fli1:gal4^{ubs3};uasKaede^{rk8})* embryos were used which express the photoconvertible molecule Kaede in ECs. Pan-Kaede photoconversion of vPCV cells at 24 hpf, rendered approximately 90 % red PACs, indicating that they originated in the floor of the PCV. In contrast, less than 10 % red PACs were observed following dPCV photoconversion (Figures 1E-G and Figures 1L-M). vPCV cells generated PACs also in *plcg1* mutants (Figures 1P-S), which lack arterial ISVs as well as blood flow, but develop venous sprouts and PACs, suggesting that the specification of lymphatic progenitors is not affected by nearby arteries or by blood circulation.

EXAMPLE 2

The ventral PCV harbors a niche of specialized angioblasts

Previous reports [Isogai, S. et al., *Development (Cambridge, England)* (2003) 130: 5281-5290] indicated that the budding of LEC progenitors from the PCV persists for approximately 24 hrs. The present inventors reasoned that a continuous exit of cells would

eventually result in disruption of the PCV wall, unless LECs arise from a population of specialized progenitors that repeatedly divide. Time-lapse sequences of *Tg(fli1:gal4^{ubs3};uasKaede^{rk8})* embryos revealed that vPCV cells undergo asymmetric division (Figures 2A-D) and generate progeny that contribute to the nascent PACs (data not shown). To further confirm these results, symmetric versus asymmetric division events were scored on each half of the PCV. Cell division was defined as asymmetric if (i) it generated a cell of different fate, and (ii) the plane of division was perpendicular to the PCV main axis. A significantly higher number of asymmetric divisions in the vPCV were found at 24-34 hpf (initial stages of LEC specification), with no changes in symmetric division events (Figure 1T). In addition, no differences in global cell proliferation were detected in the dPCV, vPCV and Dorsal Aorta (DA) (Figures 3A-H) suggesting that the specific arising of LECs from the floor of the PCV is not a result of this being a more proliferative area.

Unexpectedly, during the course of tracing photoconverted vPCV cells the present inventors noticed that in addition to generating PACs, these cells also migrated ventrally to incorporate into the Supraintestinal Artery (SIA), and the Subintestinal Vein (SIV) (Figures 2E-G and Figure 4A). Single-cell Kaede photoconversion, revealed the dynamics of specification of the vPCV progenitors (Figures 2H-I) - while at 23 hpf most of these cells give rise to either PACs or venous ISVs, at 27 hpf there is a shift towards population of the SIV and SIA. In contrast, dPCV cells generated mostly venous ISVs throughout all analyzed developmental stages. Altogether these results unveiled the presence of specialized cells within the floor of the PCV, which divide asymmetrically, and generate arterial, venous and lymphatic fates.

The present inventors then asked whether these cells represent in fact angioblasts that originate directly in the lateral plate mesoderm (LPM) and migrate medially to colonize the floor of the PCV. Alternatively, these cells could be of arterial origin, and sprout ventrally from the DA to reach the ventral PCV. To answer this question, a pan-Kaede photoconversion was performed of a population of LPM medial angioblasts that colonize the DA by 17 hpf, or of a population of ventral cells (LPM early-lateral angioblasts), detected in the trunk by approximately 19 hpf (Figures 2J-M and Figures 1N-O). Fate analysis of the photoconverted cells at 48 hpf demonstrated, that the vast majority of vPCV progenitors giving rise to PACs in the trunk did not originate in the DA, but migrated directly from the LPM to reach their final position in the vPCV (Figures 2J-N).

These results suggest that the vPCV cells are specialized angioblasts, which originate directly in the LPM and retain their multipotency throughout later stages of development.

To gain insight into the molecular identity of the newly identified vPCV angioblasts, *Tg(fli1:dsRed)^{um13}* zebrafish crossed to *Tg(flt1_9a_cFos:GFP)^{wz2}*- a *vegfr1* (*flt1*) enhancer, which specifically labels arterial ECs, were initially analyzed (Figures 4B-5 C). Green fluorescence was detected in well-established “arterial” structures including the DA, arterial ISVs, and SIA (Figure 2O). Surprisingly however, a few GFP⁺ cells were also detected within the PCV (Figure 2P). To understand whether the *flt1_9a:GFP⁺*-vPCV cells represent the population of multipotent angioblasts that give rise to LECs, 10 *Tg(flt1_9a_cFos:GFP; lyve1:dsRed2^{nz101 24})* double transgenic embryos, in which arterial ECs are GFP⁺, while venous and lymphatic ECs display red fluorescence, were imaged (Figures 4B-E). Time-lapse sequences revealed that 100 % of PACs traced (n=9), originated from *flt1_9a:GFP⁺* cells (data not shown), through a process of asymmetric cell division. Interestingly it was found that the vast majority of these progenitors were located 15 in the ventral-PCV (n=7). Nonetheless, the small number of dPCV cells that generate PACs (Figure 1G) was also labeled by *flt1_9a:GFP* (n=2), highlighting this angioblast population as the sole origin of LECs in the zebrafish trunk. Similar asymmetric division events were detected during SIV formation (Figures 4F-H). In this case *flt1_9a:GFP⁺*-vPCV cells generated progeny that populated the SI Vein and the SI Artery. Altogether 20 these results highlight the PCV as a highly heterogeneous tissue, containing “non-venous” cells competent to give rise to multiple fates, including LECs.

The fact that lymphatic vessels originate from a novel population of PCV angioblasts and not from fully differentiated venous ECs as previously postulated, prompted the present inventors to enquire into the molecular signature of these cells. 25 Global expression profiling via RNASeq (Figures 5A-B) revealed significant enrichment of well-established angioblast, lymphatic, and arterial markers in the vPCV versus dPCV cells (Figure 2Q and Figures 5C-D). It was then asked when these progenitors acquire a lymphatic fate. In mammals, the expression of the transcription factor Prox1 in certain cells of the CV marks the onset of lymphatic specification. To investigate whether this is 30 the case in zebrafish as well, *TgBAC(prox1a:Kalt4-UAS:uncTagRFP)^{nim5}* embryos were imaged. It was found that the first cells expressing *prox1a* are already visible at 22-24 hpf in the ventral-PCV (Figures 6A-E). Later on these cells divide, translocate to the dorsal

PCV, and bud from the PCV to generate PACs (Figures 6A-E). Approximately 80 % of *Tg(fli1:EGFP;prox1a:Kalt4-UAS:uncTagRFP)* embryos displayed 1-2 *prox1a*⁺ cells in the vPCV at 22-24 hpf, in contrast to approximately 20 % embryos displaying *prox1a*⁺ cells in the dPCV (Figure 6F). At later stages (26-30 hpf), an increased number of *prox1a*⁺ cells was detected in the dPCV, reflecting the proliferation and dorsal translocation of the newly specified LECs. Similar results were obtained when the distribution of the Prox1 protein was analyzed (Figure 6G). It was further found that most of the cells that expressed *prox1a* at 22-24 hpf were *flt1_9a:GFP*⁺ vPCV-angioblasts (Figure 6H). Taken together, the present results analyzing global gene expression and lymphatic-specific markers, demonstrate that lymphatic specification is induced in a restricted population of angioblasts in the vPCV. Furthermore these results confirm that LECs acquire a lymphatic fate prior to their budding from the PCV.

EXAMPLE 3

15 *Wnt5b induces LEC specification*

Having identified the floor of the PCV as the origin of lymphatic progenitors, the present inventors analyzed surrounding tissues in search for a source of spatially-restricted inductive signals. Histological sections of 22-, and 24 hpf *Tg(fli:EGFP)* embryos showed that vPCV cells develop close to the endoderm (Figures 7A-C). In addition, analysis of *cas*^{ia56} (*sox32*) mutants, which lack endoderm-derived tissues revealed that PACs do not develop in these mutants (Figure 7D and Figures 8A-B), suggesting that the signal(s) necessary for LEC specification comes from the endoderm.

Recently, the Wnt- β -catenin-TCF/LEF signaling pathway has been shown to directly activate *Nr2f2* and *Prox1*- members of the LEC specification cascade-, in the context of adipogenesis and neurogenesis [Okamura, M. et al., *Proceedings of the National Academy of Sciences of the United States of America* (2009) 106: 5819-5824; Karalay, O. et al., *Proceedings of the National Academy of Sciences of the United States of America* (2011) 108: 5807-5812]. The present inventors thus wondered whether endoderm-secreted Wnt(s), could serve as inducer(s) of lymphatic specification in the vPCV cells. *In situ* hybridization revealed clear expression of *wnt5b* mRNA in the endoderm of 18-20 hpf embryos (Figure 7E and Figures 8C-D). Analysis of *wnt5b* morphants and *ppt*^{ti265} (*wnt5b*) mutants indicated a significant reduction in the percentage

of PAC-, and thoracic duct (TD)- containing segments, with no changes in the initial number of *flt1_9a:GFP*⁺-vPCV angioblasts (Figure 7F and Figures 8E-M). In contrast, overexpression of Wnt5b in *Tg(hsp70l:wnt5b-GFP^{w33};lyve1:dsRed2)* double transgenic embryos at 23-24 hpf (Figures 9A-C), resulted in a strong pro-lymphangiogenic response reflected by the presence of ectopic PAC sprouts (Figures 7G-I). Finally, Wnt5b induction led to a significant recovery in the number of PACs in *sox32* MO-injected *Tg(hsp70l:wnt5b-GFP)* embryos (Figures 7J-L). Taken together these results highlight the endoderm-secreted Wnt5b as both necessary and sufficient for lymphatic formation during embryonic development.

To confirm that Wnt5b is specifically required for lymphatic specification, and not for general sprouting from the PCV, the number of venous versus arterial ISVs in *wnt5b*-, and Control MO-injected *Tg(flt1_9a_cFos:GFP;fli:dsRed)* embryos was assessed, and no differences were found (Figures 9D-F). Likewise, *flt1_9a:GFP*⁺-vPCV progenitors were normally found within the SIV plexus of *wnt5b* morphants (Figures 9G-H), confirming that Wnt5b does not inhibit PAC formation by unselectively impeding sprouting from the PCV, but rather by affecting LEC specification. To ascertain whether Wnt5b affects LEC proliferation, vPCV cells in *wnt5b* MO-injected- *Tg(fli1:gal4;uasKaede)* (Figures 10A-D) and *Tg(fli1:nEGFP; fli1:dsRed)* (data not shown) embryos were photoconverted and time-lapse imaged. While in control siblings approximately 30 % of the vPCV cells reached the PACs by 48 hpf (Figure 2H-I), they did not engage in dorsal migration to generate PACs in *wnt5b* morphants (Figures 10A-D). Interestingly, although the cells were viable and divided normally, the only asymmetric division events detected involved cells that migrated ventrally to populate the SIVs (data not shown). In addition, ectopic induction of Wnt5b did not result in enhanced EC proliferation (Figures 3I-K).

Conclusive evidence supporting a role for Wnt5b as an inducer of LEC specification was provided by the analysis of lymphatic marker expression following *wnt5b* downregulation and overexpression. *In situ* hybridization revealed a pronounced reduction in lymphatic-specific transcripts in the PCV of *wnt5b* morphants, whereas the expression of pan-endothelial genes remained unchanged (Figures 10E-L). This phenotype, indicative of a defect in lymphatic specification, was not reported following loss of VEGF-C, a signal specifically required for LEC budding from the PCV. In addition, the expression of *vegfc* and *ccbe1* remained unchanged in *sox32* and *wnt5b*

morphants (Figures 10M-R), ruling out the possibility that Wnt5b controls lymphatic specification through activation of these genes. Finally, the number of *prox1a*⁺ cells was reduced in *wnt5b* morphants, and increased following Wnt5b overexpression (Figures 11A-D). Moreover, Wnt5b activation induced upregulation of the *prox1a* transcript (Figure 11E), and the Prox1 protein (Figures 10S-T). Taken together these data indicate that Wnt5b is mainly required for lymphatic specification, and not migration or proliferation, of the vPCV angioblasts.

Recently, a divergence in the molecular mechanisms controlling lymphatic specification in zebrafish and mice was postulated [van Impel, A. et al. *Development (Cambridge, England)* (2014) 141: 1228-1238]. To ascertain whether the novel mechanism of LEC specification uncovered here is conserved among vertebrates, the ability of recombinant WNT5B to induce lymphatic specification in human embryonic stem cell (hESCs)-derived vascular progenitors was tested. As seen in Figures 11F-G, WNT5B induced a marked increase in the fraction of LYVE1⁺ cells detected in the culture, as well as in the levels of *PROX1* (Figure 11H) and *FLT4* (Figure 10U) mRNAs, indicating that the role of Wnt5b as potent inducer of LEC specification is evolutionarily conserved. Furthermore these findings suggest that Wnt5b acts directly on vascular progenitors to promote the “angioblast-to-lymphatic” specification.

EXAMPLE 4

Wnt5b induces LEC specification through activation of Wnt canonical pathway

The present inventors next characterized the downstream components of the Wnt pathway involved in lymphatic specification. Wnt5 is mostly referred to as a non-canonical Wnt ligand, which can also repress and/or activate the canonical pathway in different contexts. It is well-established that a key step in the activation of canonical-Wnt pathway is the inhibition of a destruction complex composed of APC, Axin, GSK3 β , and other proteins, which results in stabilization and nuclear translocation of cytoplasmic β -catenin. The present inventors therefore began by analyzing lymphatic development following manipulation of Axin and APC. Injection of *wnt5b* MOs into *mbl^{tm213} (axin1)* mutants did not affect PAC formation (Figures 12A-C), confirming the requirement of Axin downstream of Wnt5b. Likewise, *apc^{mer}* mutants displayed significantly increased PAC numbers (Figures 12D-F), resembling the Wnt5b overexpression phenotype (Figures

7G-I). Conversely, Axin overexpression (Figure 12G), as well as treatment with IWR1- a small molecule shown to lower the levels of β -catenin-, rendered a significant reduction in the number of PAC-containing segments (Figure 12H). In contrast to these results, the inhibitor of β -catenin-independent Wnt activation, TNP-470, did not cause any detectable lymphatic defects (Figure 13A). The role of the TCF/LEF transcription factors in early lymphangiogenesis was next analyzed. Downregulation of *pcf4*, *pcf7*, *lef1* and *pcf3b* (Figures 13B-F and data not shown), resulted in reduced number of PACs with an otherwise normal blood vasculature. In line with the phenotypes resulting from Wnt5b downregulation, arterious/venous differentiation was not impaired in these morphants (Figures 13B-E), and photoswitched vPCV cells did not migrate dorsally to generate PACs (Figures 13G-J). Altogether these results indicate that induction of lymphatic specification by Wnt5b occurs primarily through β -catenin/TCF activation.

The lymphatic defects derived from Wnt/ β -catenin inhibition could be secondary to Wnt5b-signaling depletion. Alternatively, they could reflect a cell-autonomous requirement for Wnt-signaling within prospective LEC progenitors. To distinguish between these two possibilities, β -catenin/TCF activity within vPCV-angioblasts was assessed using *Tg(fli1:EGFP;7xTCF-Xla.Siam:nlsMCherry)^{ia5}* double transgenic embryos. As seen in Figures 14A-B, TCF activity was detected in these cells at 24 hpf, and in PACs at 48 hpf. Furthermore, time-lapse imaging revealed that only vPCV angioblasts with active β -catenin/TCF undergo asymmetric cell division and generate PACs (Figures 15A-E). Moreover, these cells were also *fli1_9a:GFP⁺* (Figure 14C). The number of β -catenin/TCF⁺ vPCV-angioblasts was significantly reduced following *wnt5b* downregulation (Figures 15F-H), confirming that the β -catenin/TCF activity detected in these cells was Wnt5b-dependent. Altogether, the present results analyzing β -catenin/TCF activity in LEC progenitors *in vivo*, in combination with LEC specification in cultured hESCs, indicated that Wnt5b-dependent activation of β -catenin is cell-autonomously required within vascular progenitors for proper lymphatic specification, and highlight Prox1 as one of the major downstream targets of Wnt5b.

The changes in *prox1* mRNA levels observed in zebrafish and hESCs (Figures 11E and 11H) could result from either transcriptional regulation, or post-transcriptional modifications that alter RNA stability of the *prox1* transcript. To distinguish between these two possibilities the *TgBAC(prox1a:KalT4-UAS:uncTagRFP)* zebrafish reporter was

utilized (Figure 11E), in which the *KalT4* fragment recapitulates the transcriptional activation of the endogenous *prox1a* promoter, without being subjected to the post-transcriptional modifications of the *prox1a* gene (the *KalT4* cassette possess its own 3'UTR). The present inventors hypothesized that if Wnt5b transcriptionally regulates *prox1a* mRNA, overexpression of Wnt5b would result in a significant increase in the levels of *KalT4* mRNA. If in turn, *prox1a* upregulation involves alterations in its mRNA stability, the levels of *KalT4* mRNA would remain unchanged upon *hsp70:Wnt5b* activation. As seen in Figure 14D, overexpression of Wnt5b resulted in elevated levels of the *KalT4* transcript. Although the possibility that post-transcriptional modifications may also be involved in *prox1a* regulation cannot be excluded, the present results strongly support a mechanism involving transcriptional regulation of *prox1a* in response to Wnt5b. Whether this is a direct or indirect regulation remains to be elucidated.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

WHAT IS CLAIMED IS:

1. A method of promoting lymphatic endothelial cell (LEC) specification, the method comprising upregulating the activity of Wnt canonical pathway in stem cells and/or vascular progenitor cells and/or angioblasts, thereby promoting LEC specification.
2. The method of claim 1, further comprising differentiating said stem cells into endothelial precursor cells or angioblasts prior to said promoting said LEC specification.
3. The method of any one of claims 1 or 2, further comprising the step of selecting LECs from the stem cells and/or vascular progenitor cells.
4. The method of claim 3, wherein said selecting is effected by analyzing expression of a marker selected from the group consisting of prox1, lymphatic vessel endothelial hyaluronan receptor-1 (lyve1), flt4, sox18, nr2f2, podoplanin (Pdpn), CD31 and GP38.
5. A method of inducing lymphangiogenesis, the method comprising:
 - (a) promoting LEC specification in stem cells and/or vascular progenitor cells and/or angioblasts so as to obtain a LEC committed cell according to the method of any one of claims 1-4; and
 - (b) subjecting said LEC committed cell to a signal selected from the group consisting of a LEC morphogenesis signal, a LEC proliferation signal and a LEC survival signal, thereby inducing lymphangiogenesis.
6. The method of claim 5, wherein said LEC morphogenesis signal comprises upregulating the activity of Wnt non-canonical pathway in said LEC.
7. The method of claim 5, wherein said LEC morphogenesis signal comprises an endothelial growth factor.

8. The method of any one of claims 1-7, wherein said method is effected *ex-vivo*.

9. The method of any one of claims 1-8, wherein said upregulating the activity of Wnt canonical pathway is effected by contacting said stem cells and/or vascular progenitor cells and/or angioblasts with an inhibitor of a Wnt canonical pathway member selected from the group consisting of an Axin, a GSK3, an adenomatosis polyposis coli (APC) and a sFRP.

10. The method of claim 9, wherein said inhibitor of a Wnt canonical pathway factor is a small molecule.

11. The method of any one of claims 1-8, wherein said upregulating the activity of Wnt canonical pathway is effected by contacting said stem cells and/or vascular progenitor cells and/or angioblasts with an activator of a Wnt canonical pathway member selected from the group consisting of a Wnt5b, a Wnt3a, a Wnt7a, a Wnt7b, a LRP5/6, a β -catenin, Dishevelled (Dsh), PP2A, ARFGAP1, and TCF.

12. The method of claim 11, wherein said Wnt5b is selected from the group consisting of a Wnt5b polypeptide, a Wnt5b analogue and a Wnt5b polynucleotide.

13. The method of claim 6, wherein said upregulating said activity of Wnt non-canonical pathway is effected by contacting said LEC with an activator of a Wnt non-canonical pathway member selected from the group consisting of a Wnt5a, Wnt4, Wnt11, Ror2, Ryk, G protein, Dishevelled (Dsh) and Dishevelled-associated activator of morphogenesis 1 (DAAM1).

14. The method of any one of claims 1-13, wherein the stem cells and/or vascular progenitor cells are derived from a source selected from the group consisting of hematopoietic stem cells, mesodermal progenitor cells, endothelial progenitor cells, angioblasts, induced pluripotent stem cells (iPS) and human embryonic stem cells (hESCs).

15. An isolated population of cells generated according to the method of any one of claims 1-14.

16. A pharmaceutical composition comprising the isolated population of cells of claim 15 and a pharmaceutically acceptable carrier or diluent.

17. Use of the isolated population of cells of claim 15 for the manufacture of a medicament identified for treating or preventing a disease or disorder related to lymphangiogenesis.

18. Use of an agent capable of upregulating the activity of Wnt canonical pathway for the manufacture of a medicament identified for treating or preventing a disease or disorder related to lymphangiogenesis.

19. The use of claim 18, further comprising an agent capable of upregulating the activity of Wnt non-canonical pathway.

20. A method of treating or preventing a disease or disorder related to lymphangiogenesis in a subject in need thereof, the method comprising administering to the subject the isolated population of cells of claim 15, thereby treating or preventing the disease or disorder related to lymphangiogenesis.

21. A method of treating or preventing a disease or disorder related to lymphangiogenesis in a subject in need thereof, the method comprising administering to the subject an agent capable of upregulating the activity of Wnt canonical pathway, thereby treating or preventing the disease or disorder related to lymphangiogenesis.

22. The method of claim 21, further comprising administering to said subject an agent capable of upregulating the activity of Wnt non-canonical pathway.

23. The use of any one of claims 17-19, or method of any one of claims 20-22, wherein said disease or disorder related to lymphangiogenesis is selected from the group

consisting of cancer, melanoma, solid tumor, breast cancer, lung cancer, kidney cancer, pancreatic cancer, hematopoietic tumor, graft rejection, autoimmune disorder, rheumatoid arthritis, psoriasis, oedema, chronic wound, lymphedema, lymphatic fistula, lymphorrhea and lymphocele/seroma formation.

24. A method of transplanting lymphatic endothelial cells (LECs) into a subject in need thereof, the method comprising:

- (a) generating LECs according to the method of any one of claims 1-14; and
- (b) transplanting said LECs into the subject, thereby transplanting said LECs.

25. The method of claim 24, wherein said LECs are syngeneic or allogeneic with respect to the subject.

26. The method of any one of claims 20, 21, 22, 24 or 25, wherein said subject is a human subject.

27. A method of screening for an agent capable of upregulating the activity of Wnt canonical pathway for the formation of LECs, the method comprising:

- (a) contacting the agent with stem cells and/or vascular progenitor cells and/or angioblasts;
- (b) analyzing an expression of a lymphatic marker on said stem cells and/or vascular progenitor cells and/or angioblasts; and
- (c) comparing said expression of said marker following said contacting to prior to said contacting, wherein an expression of said marker is indicative of an agent suitable for formation of LECs.

28. The method of claim 27, wherein said lymphatic marker is selected from the group consisting prox1, lyve1, flt4, sox18, nr2f2, Pdpn, CD31 and GP38.

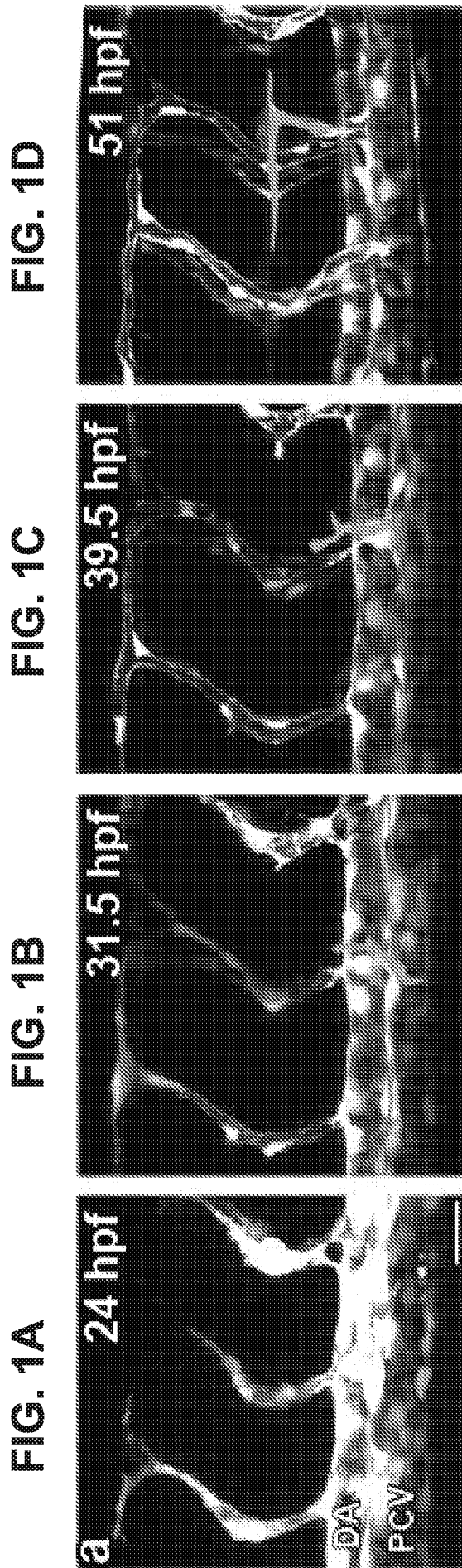


FIG. 1E

24 hpf

48 hpf

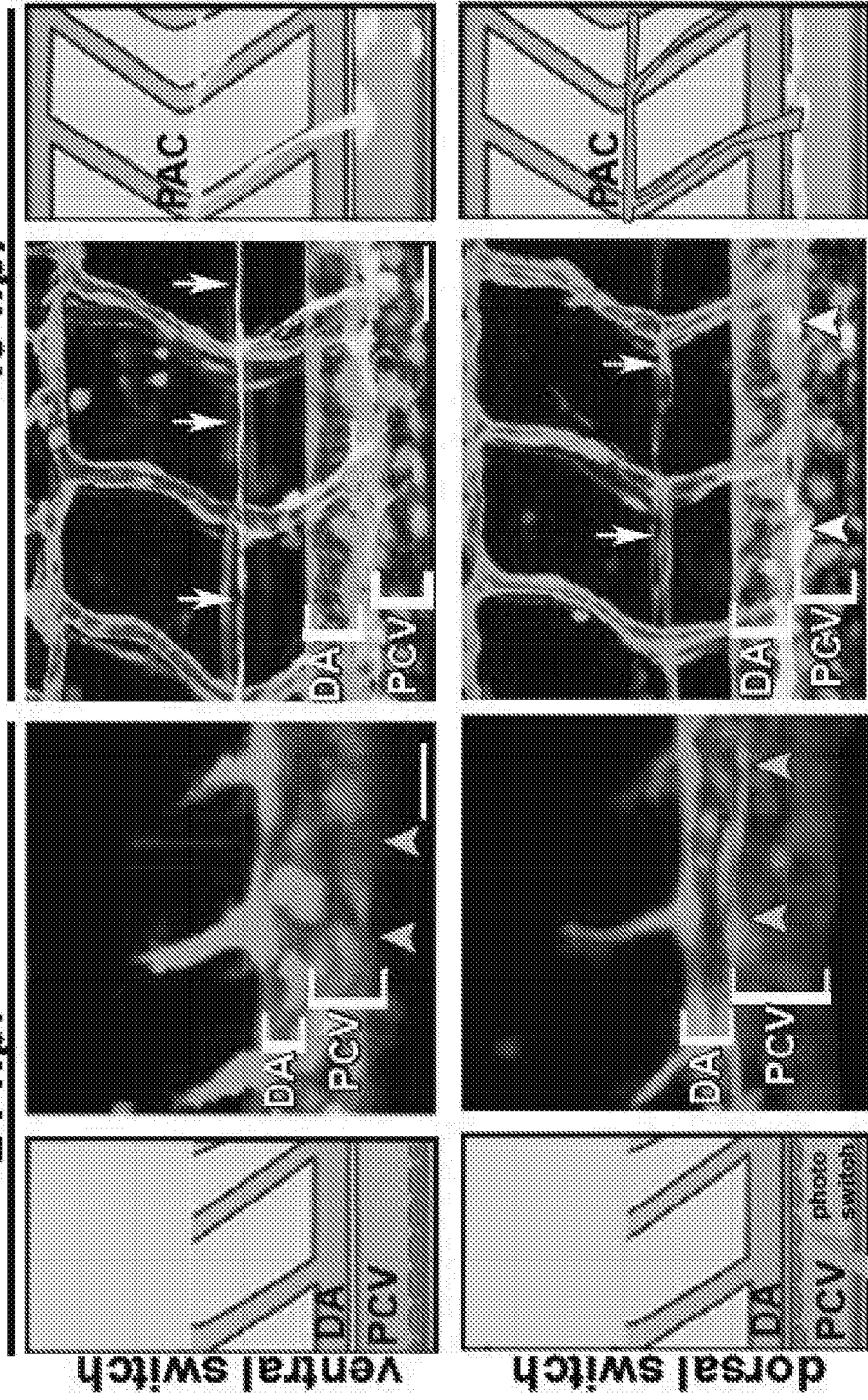


FIG. 1G

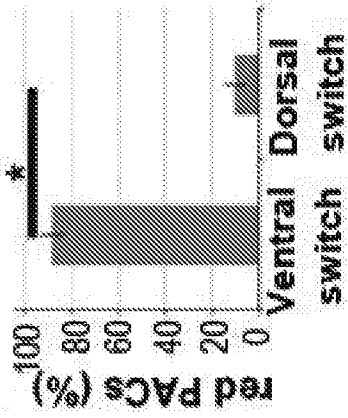


FIG. 1F

FIG. 1H

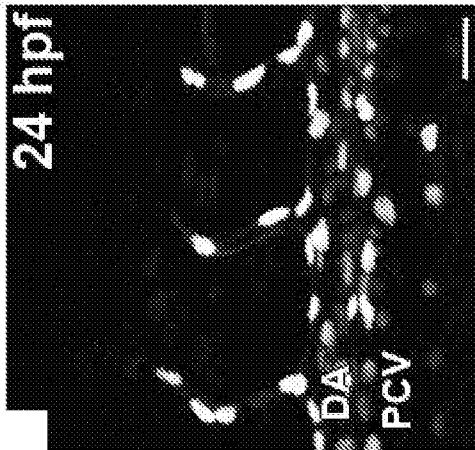


FIG. 1I

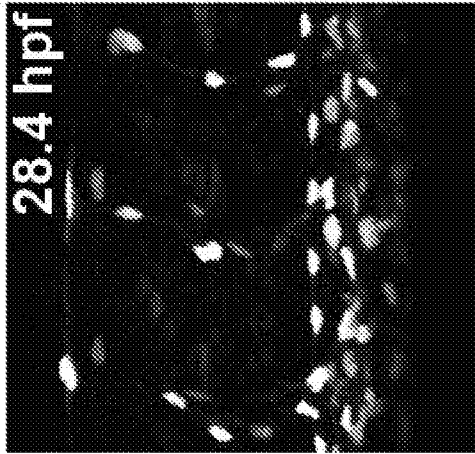


FIG. 1J

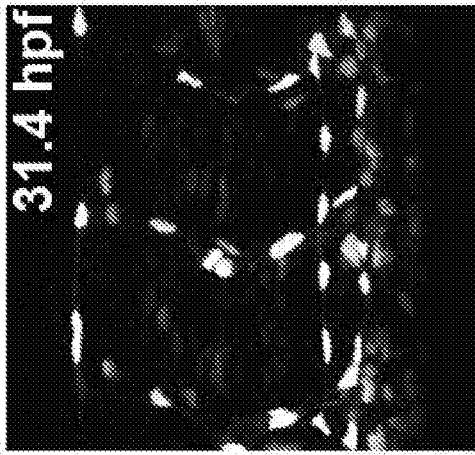
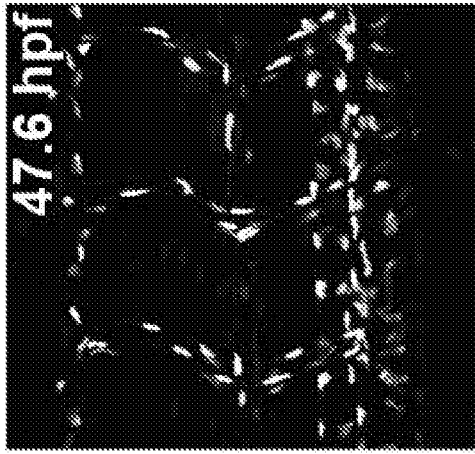
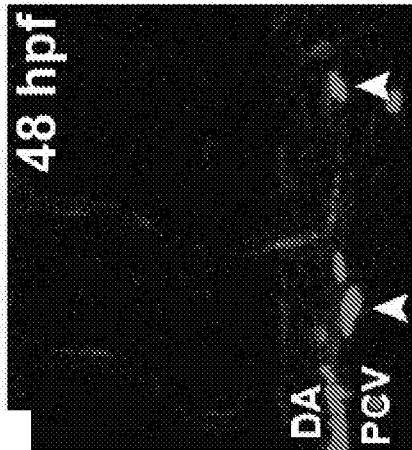


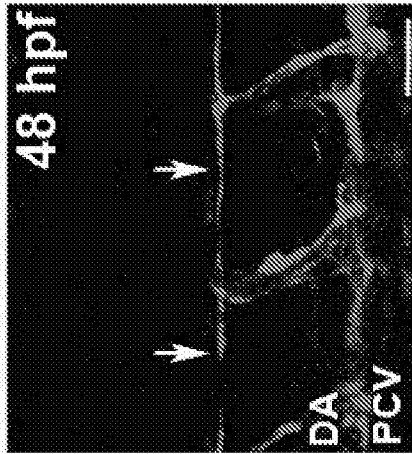
FIG. 1K



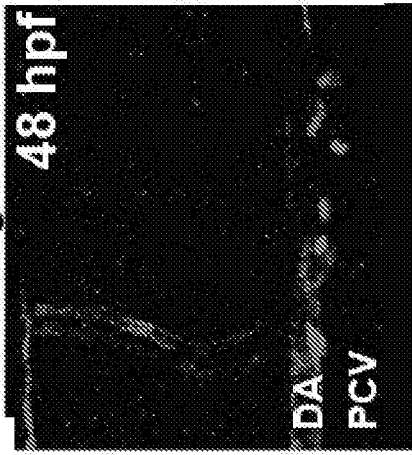
dorsal switch



ventral switch



medial angioblasts



early lateral angioblasts

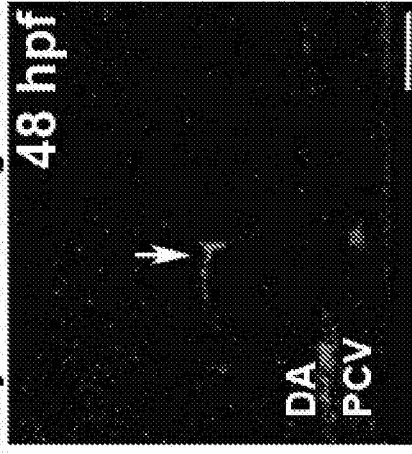


FIG. 1L

FIG. 1M

FIG. 1N

FIG. 1O

FIG. 1P

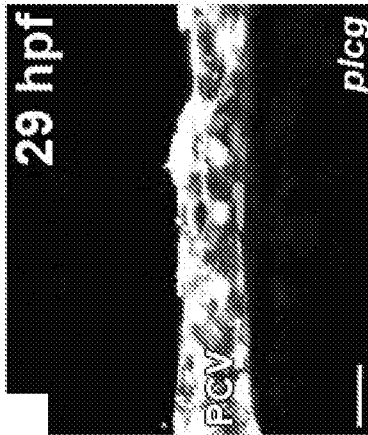


FIG. 1Q

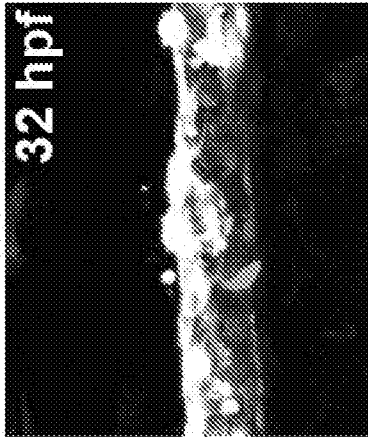
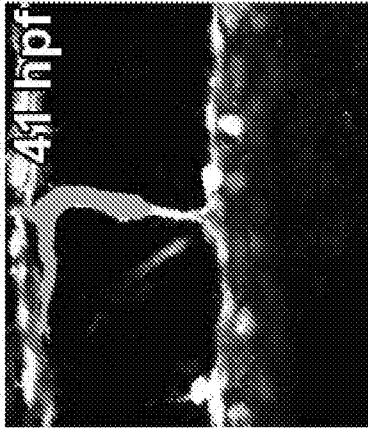


FIG. 1R



FIG. 1S



24-34 hpf

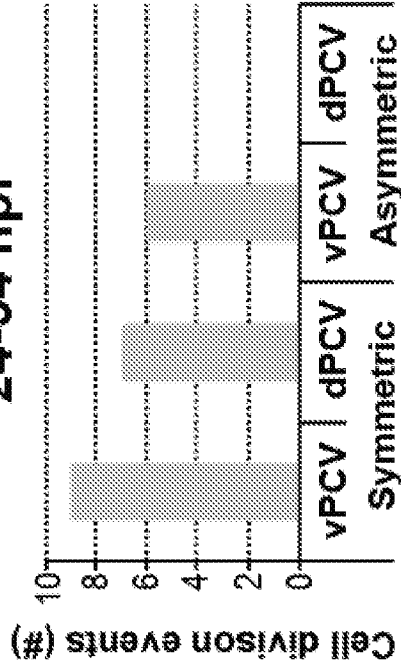


FIG. 1T

FIG. 2A

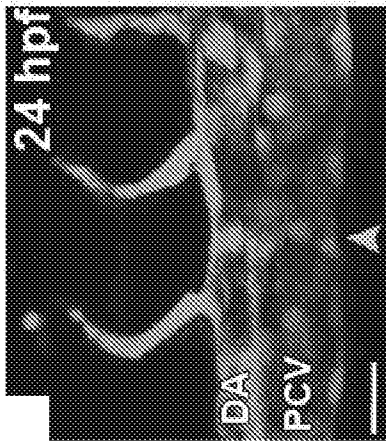


FIG. 2B

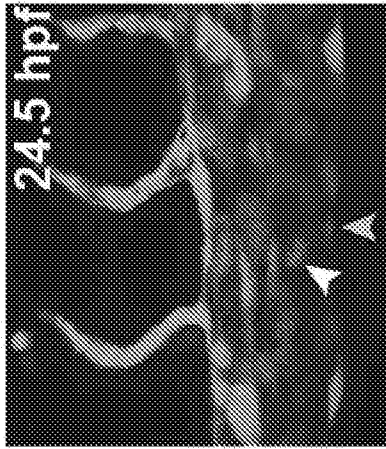


FIG. 2C

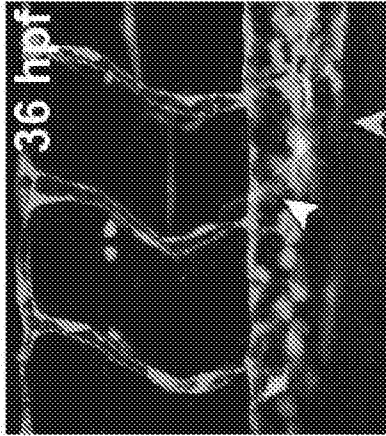
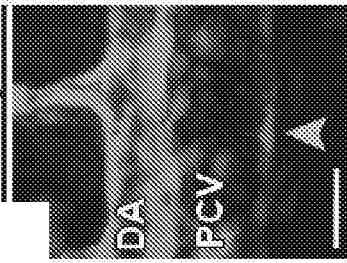


FIG. 2D



24 hpf



56 hpf

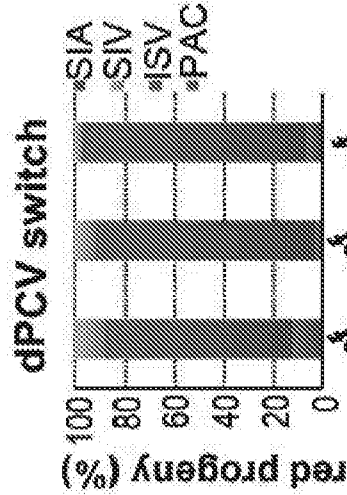
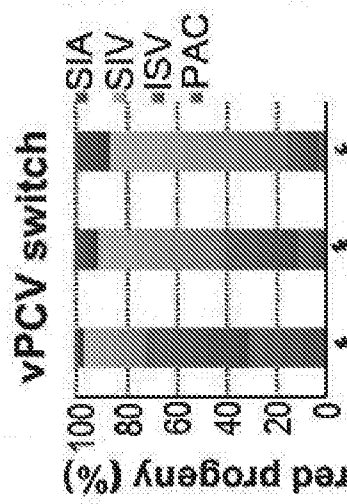
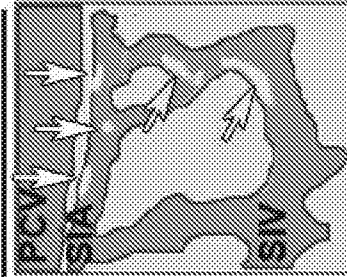


FIG. 2E

FIG. 2F

FIG. 2G

FIG. 2H

FIG. 2I

FIG. 2J

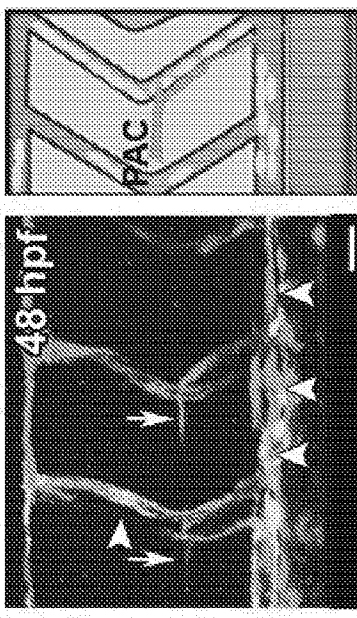
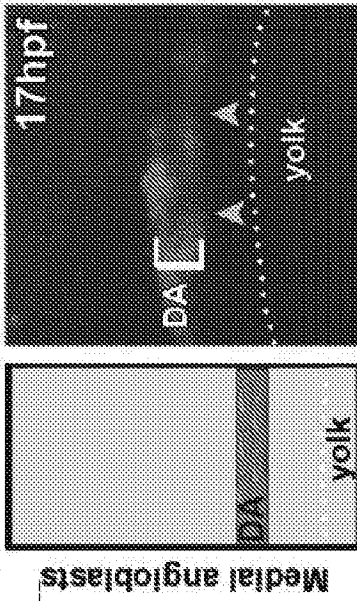


FIG. 2N

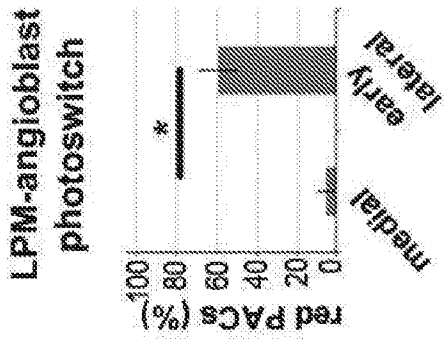


FIG. 2K

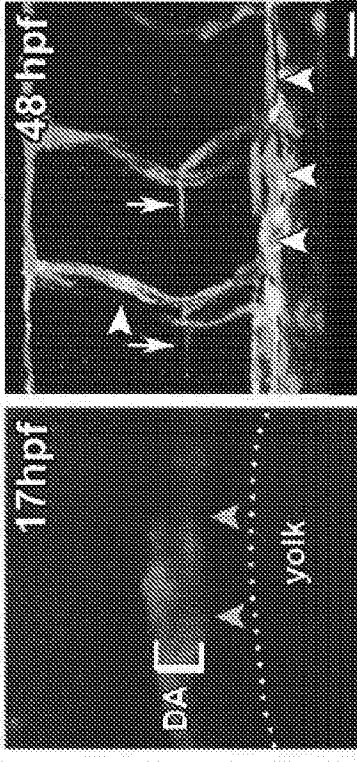
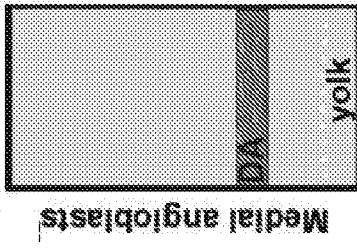


FIG. 2M

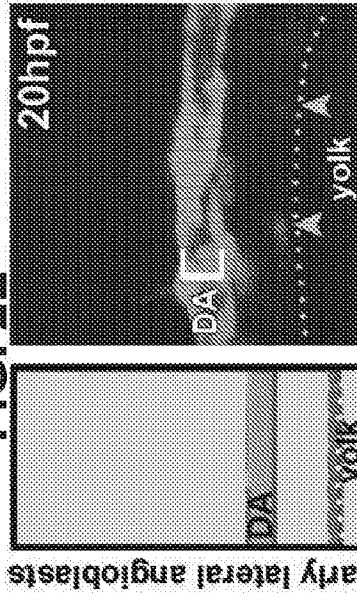


FIG. 2L

Early lateral angioblasts

m1-0a:GFP;M1:dsRed

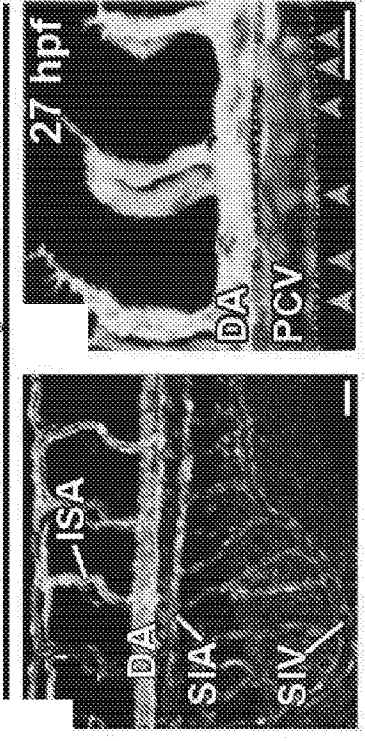


FIG. 2O

FIG. 2P

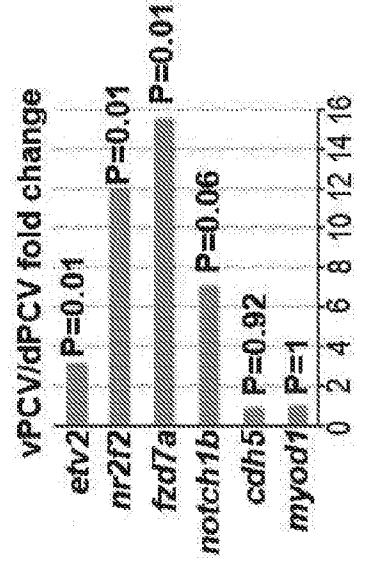


FIG. 2Q

FIG. 3A

22hpf

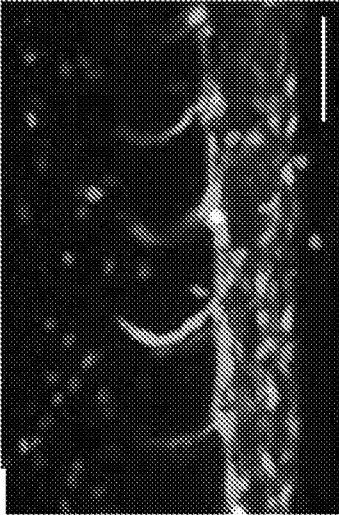


FIG. 3B

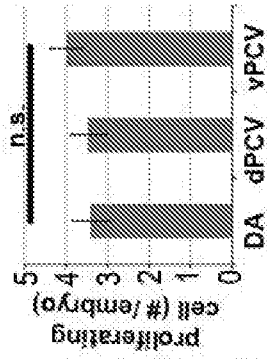


FIG. 3C

24hpf

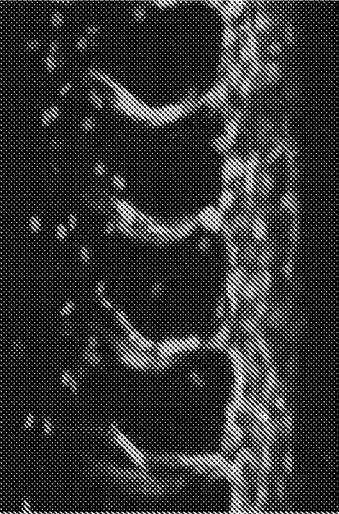
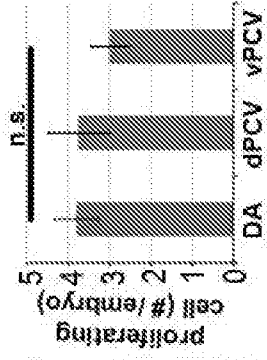


FIG. 3D



26hpf



28hpf

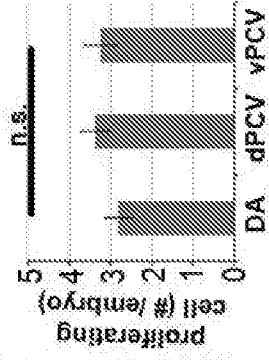
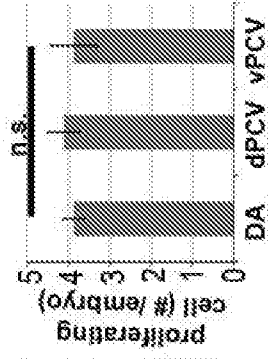
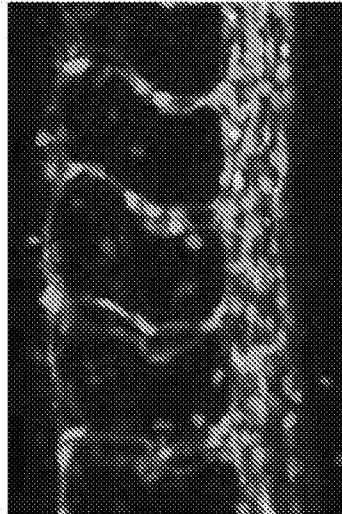


FIG. 3E

FIG. 3F

FIG. 3G

FIG. 3H

■ fl:EGFP
 ■ anti p-Histone H3
 ■ merged

FIG. 3K

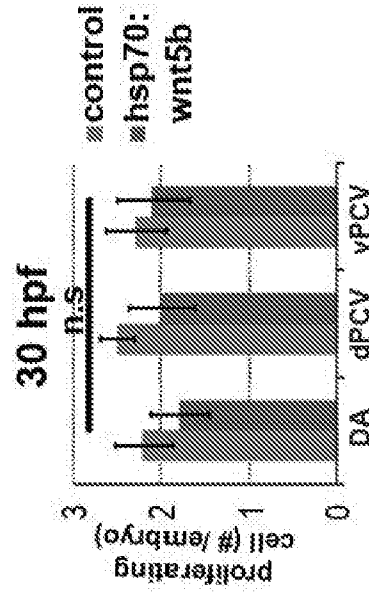


FIG. 3J

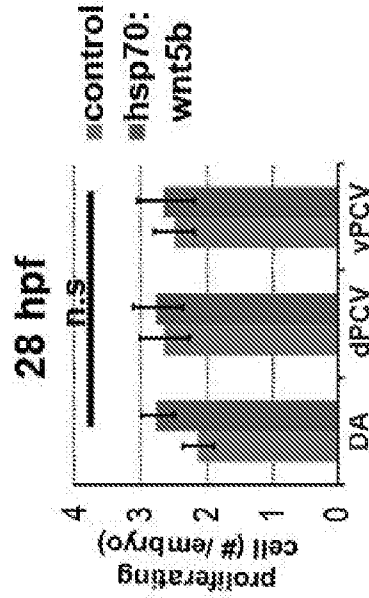


FIG. 3I

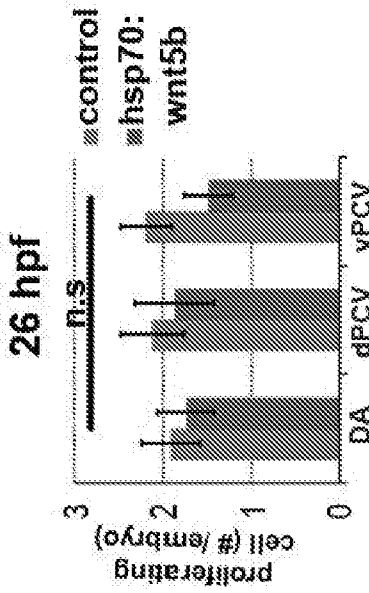


FIG. 4A

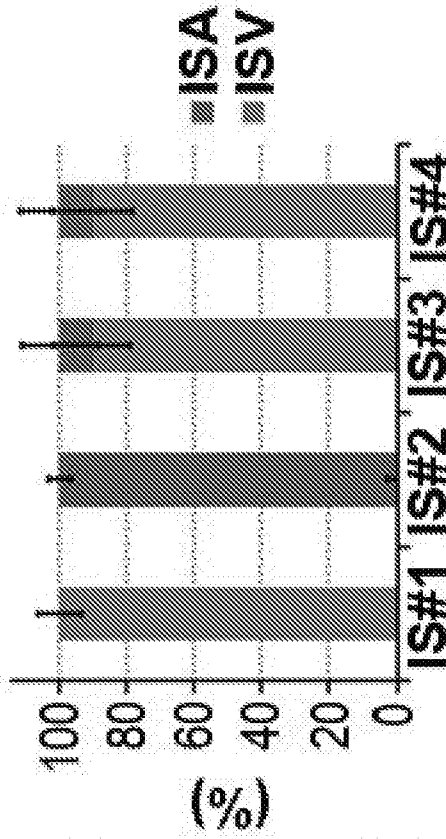
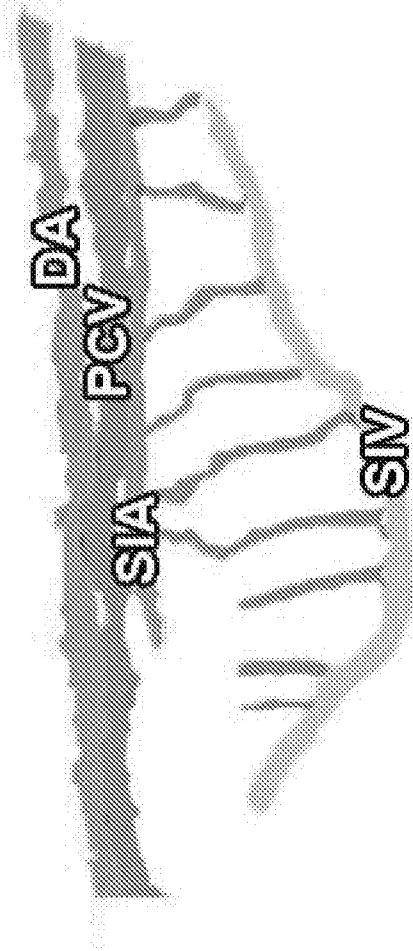


FIG. 4C

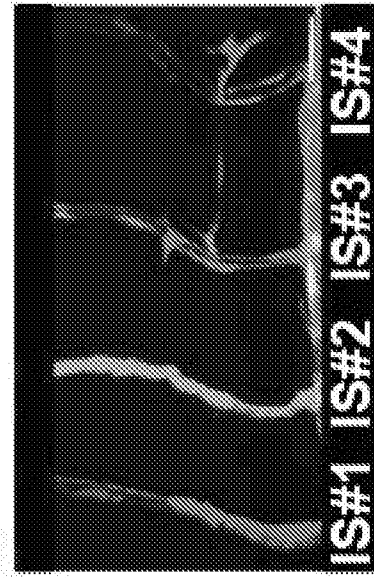


FIG. 4B

FIG. 4D FIG. 4E

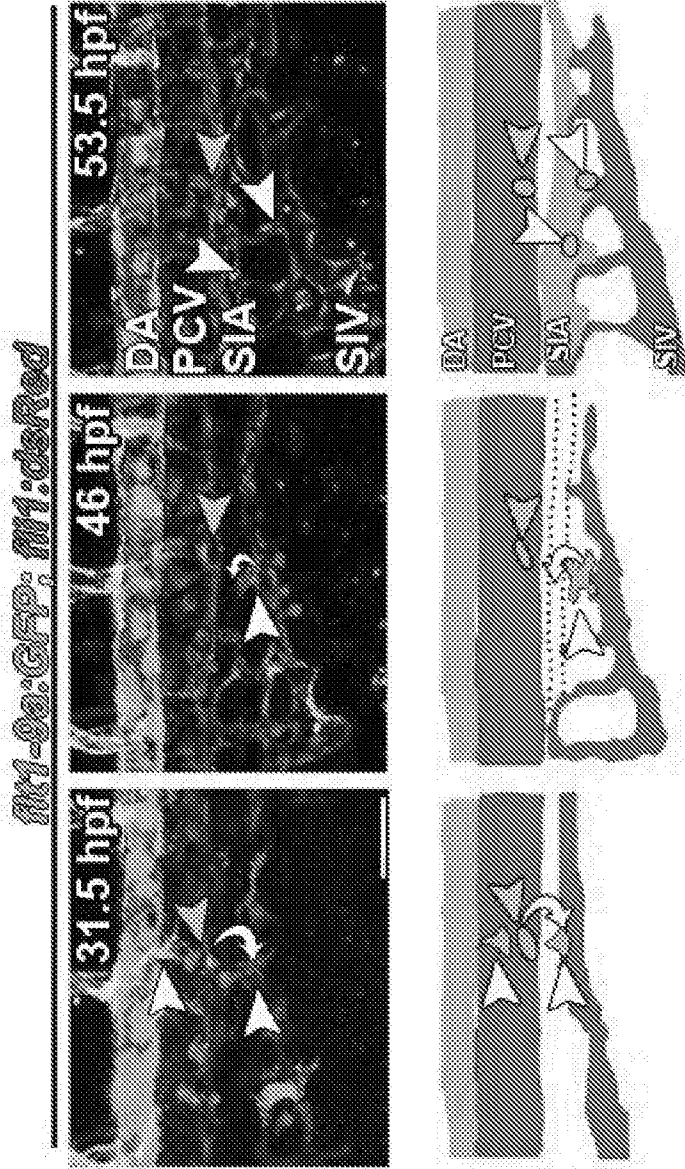
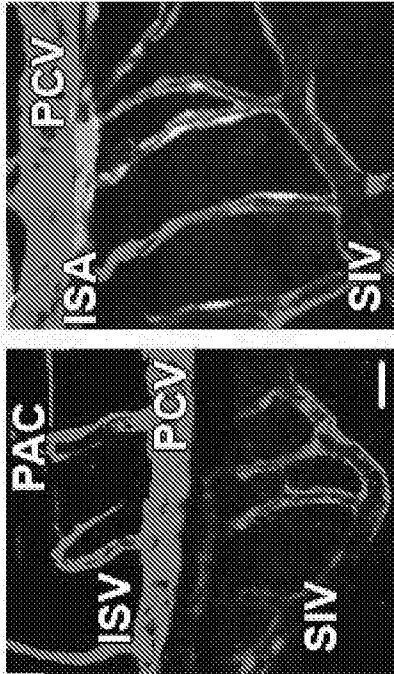


FIG. 4F FIG. 4G FIG. 4H

FIG. 5A

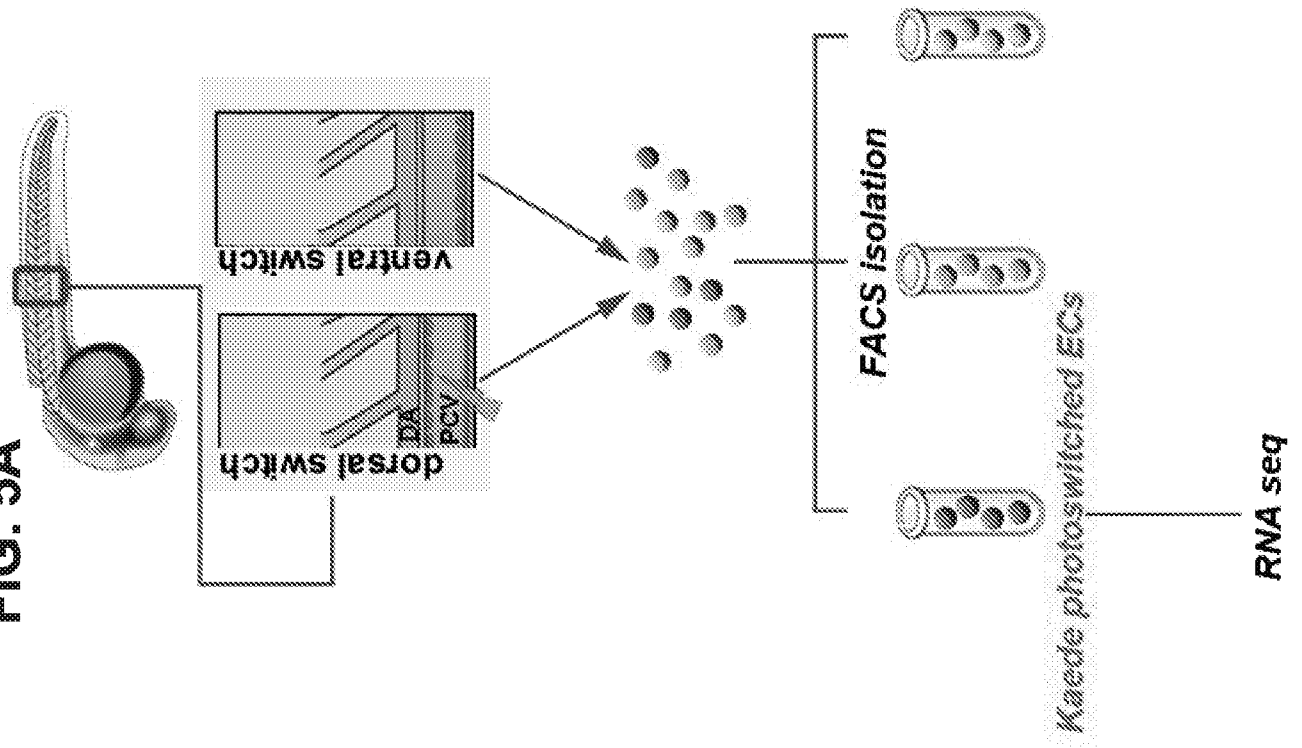


FIG. 5B

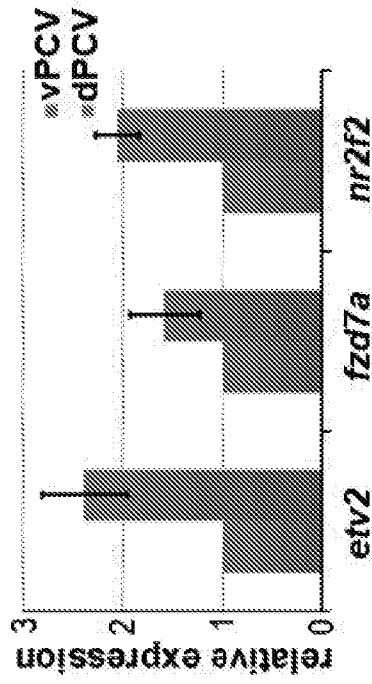
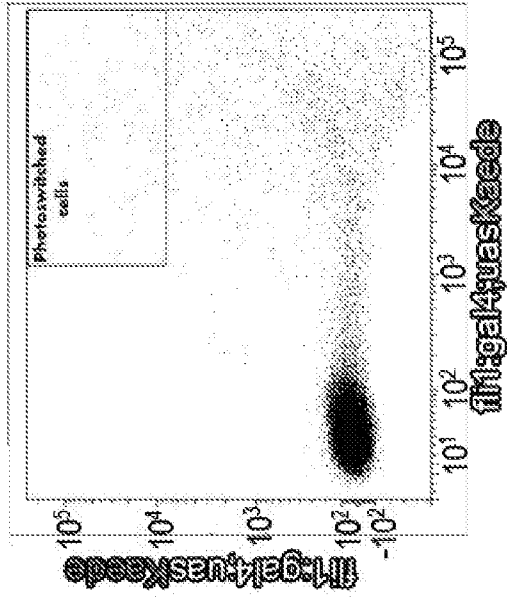


FIG. 5C

FIG. 5D

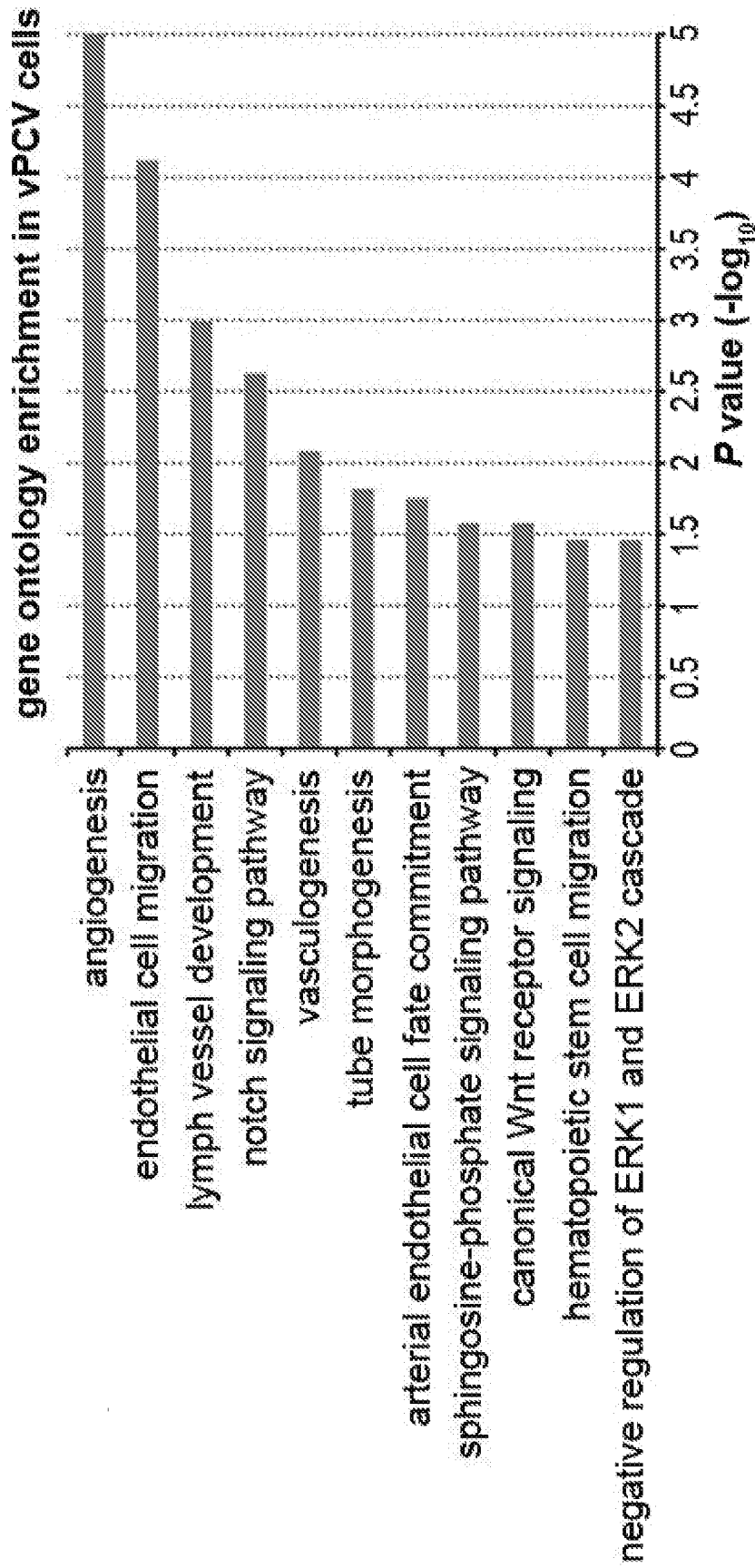


FIG. 6A FIG. 6B FIG. 6C FIG. 6D FIG. 6E

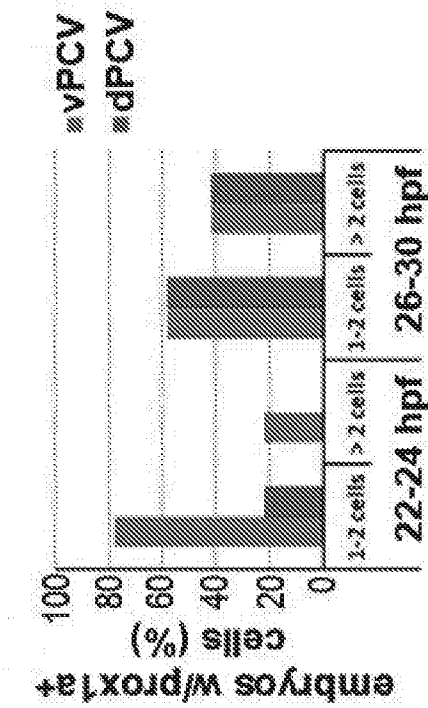
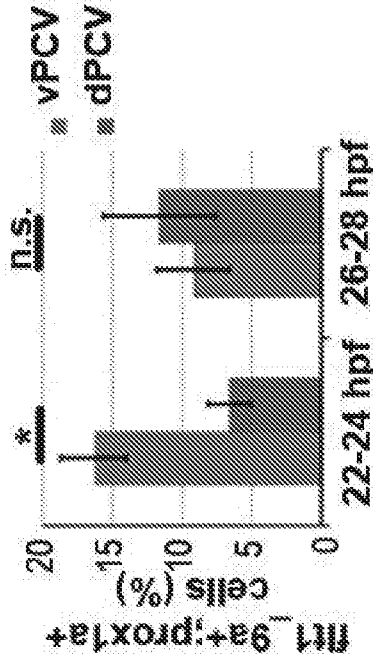
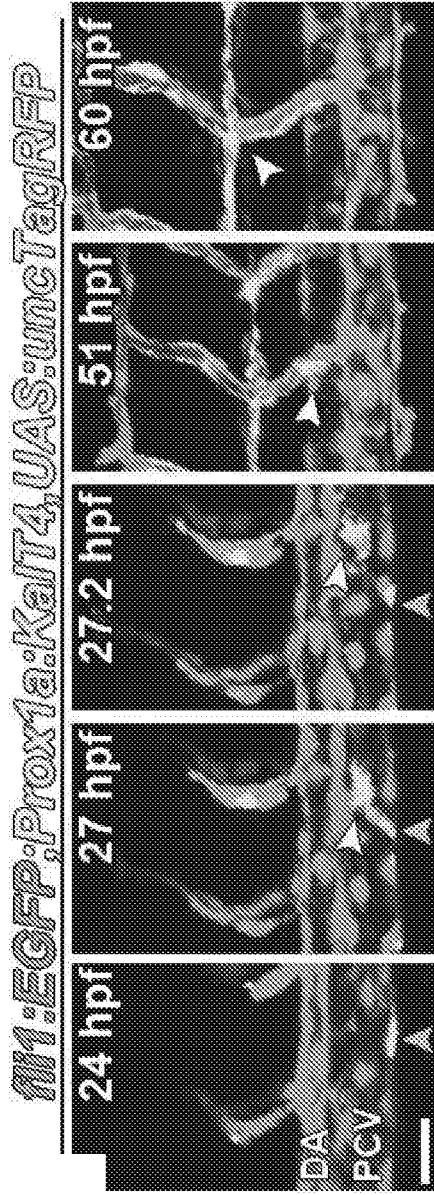


FIG. 6H

FIG. 6G

FIG. 6F

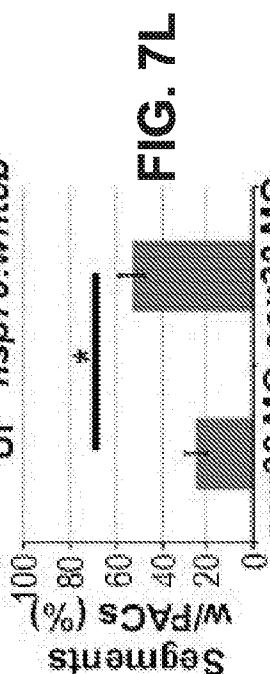
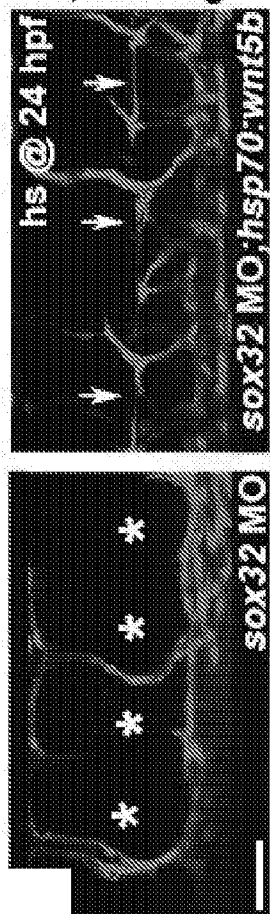
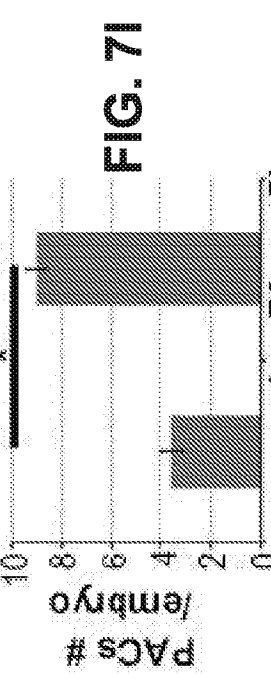
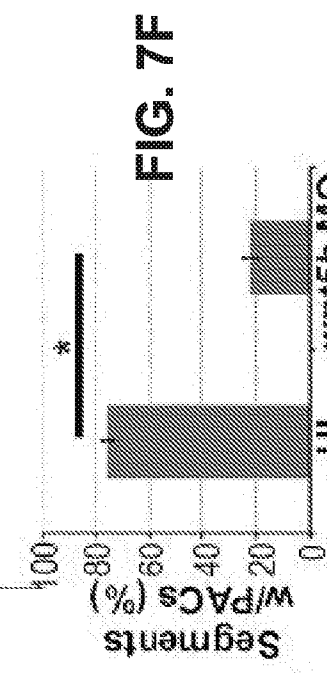
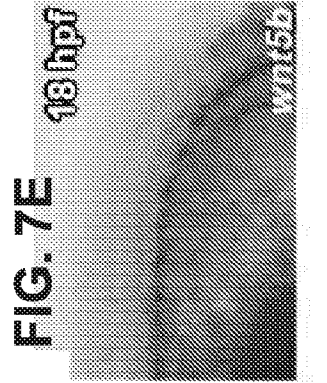
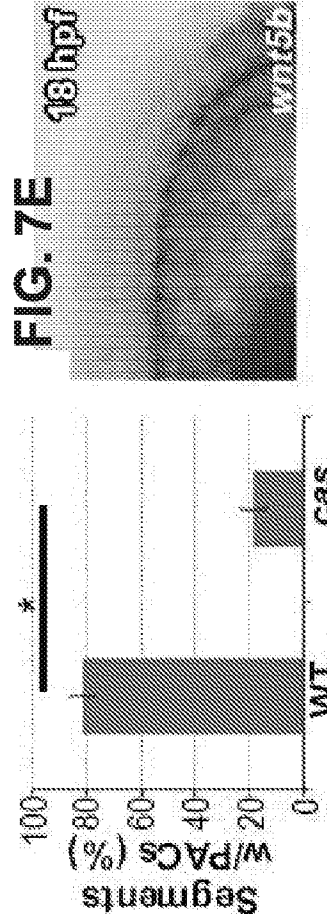
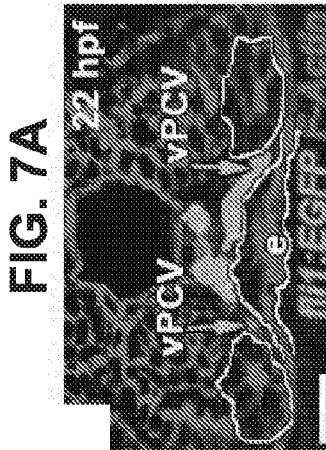
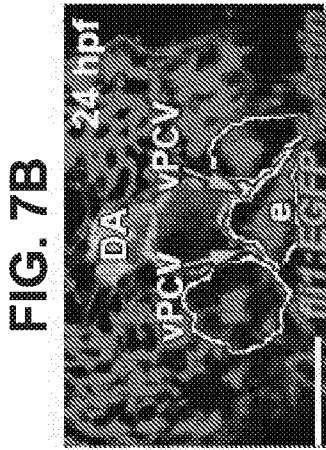
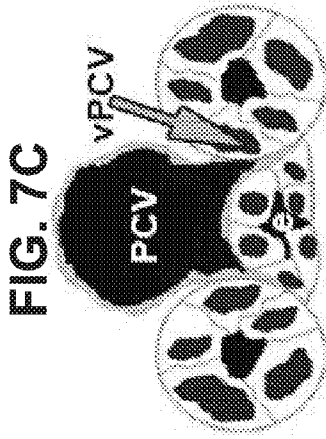


FIG. 7K

FIG. 8A

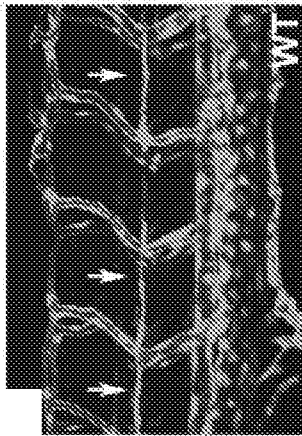


FIG. 8B

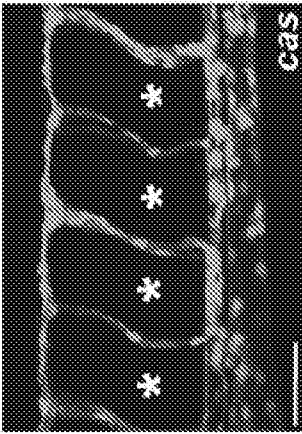


FIG. 8C

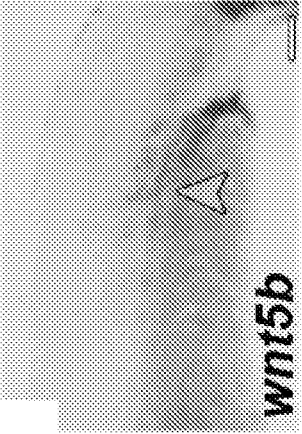


FIG. 8D

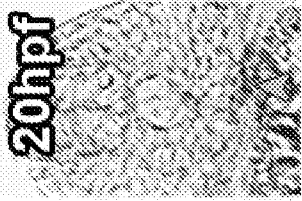


FIG. 8E

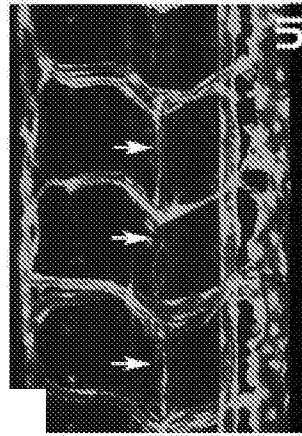


FIG. 8F

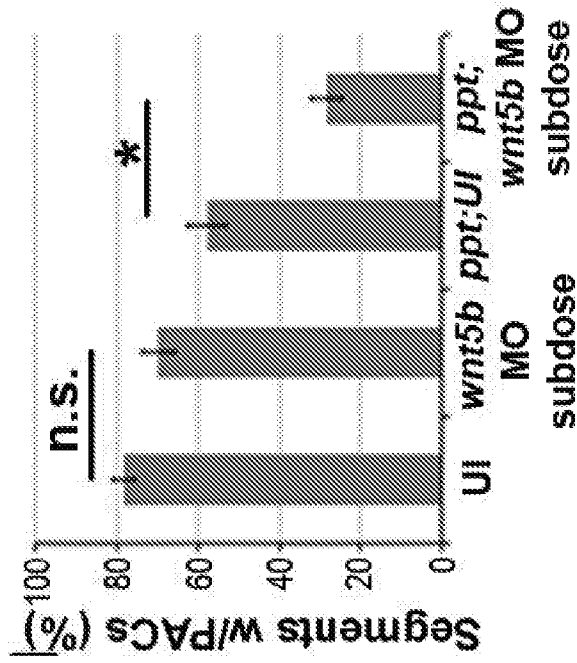
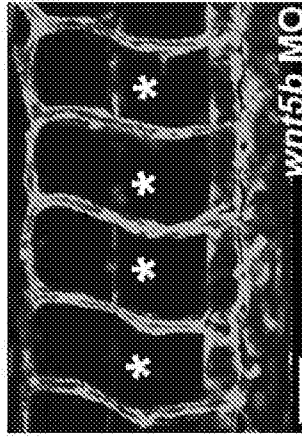


FIG. 8G

FIG. 8H

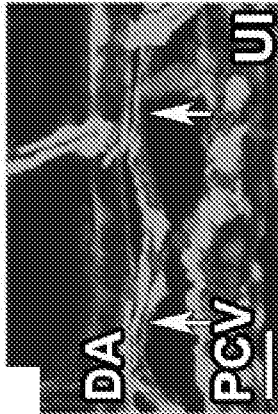


FIG. 8I

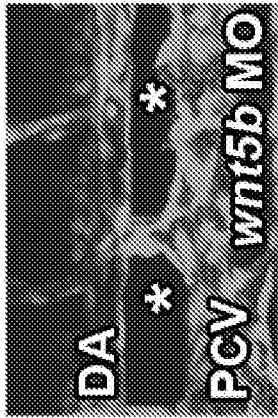


FIG. 8J

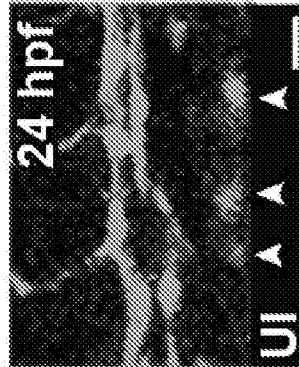
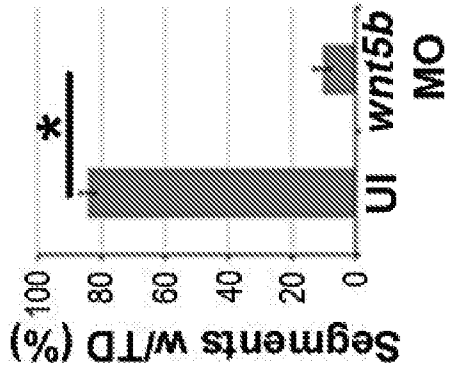


FIG. 8K

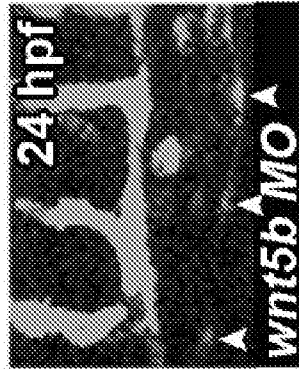


FIG. 8L

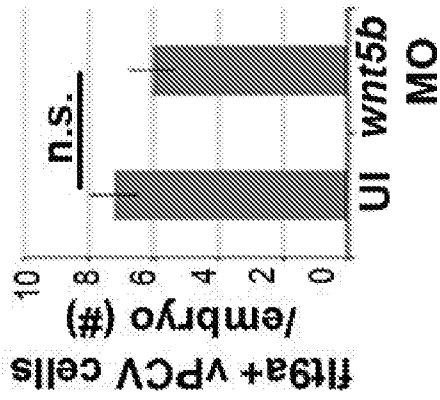


FIG. 8M

FIG. 9A

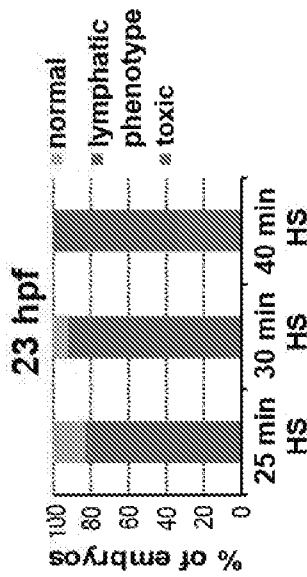


FIG. 9B

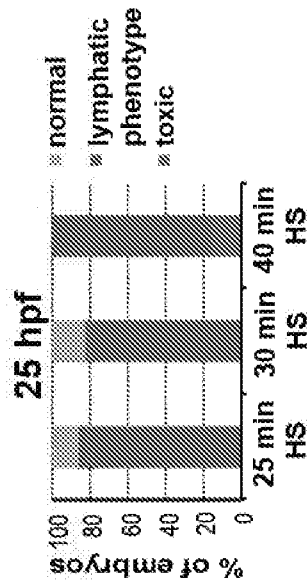
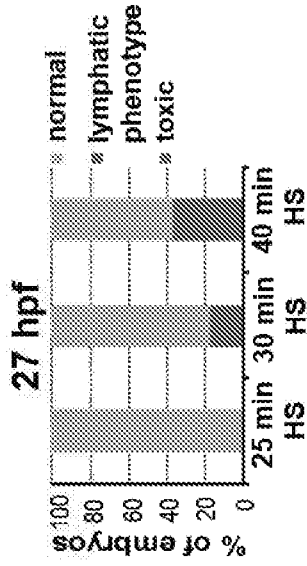
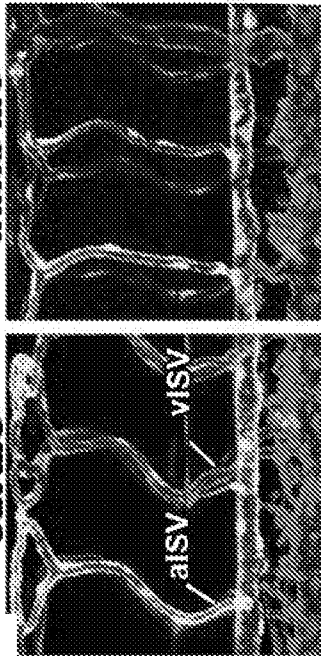


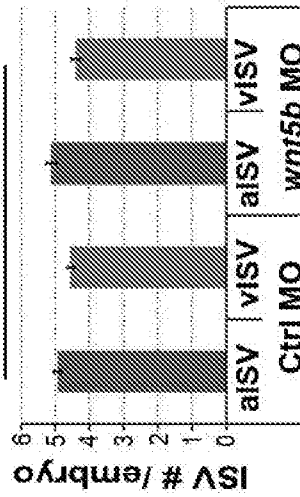
FIG. 9C



Ctrl MO



n.s.



Ctrl MO



wnt5b MO

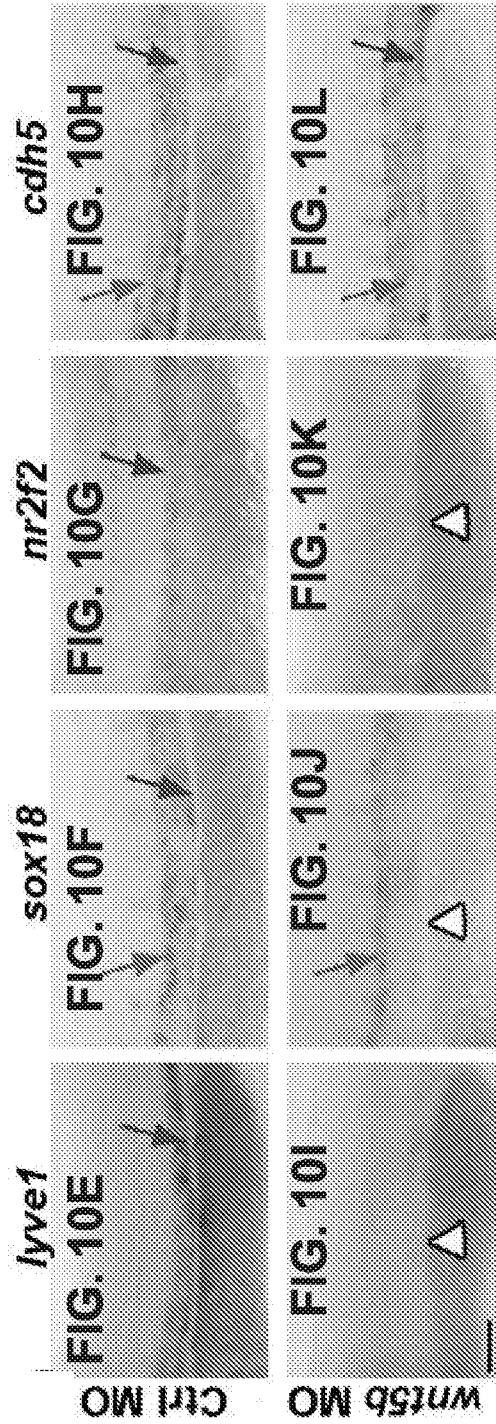
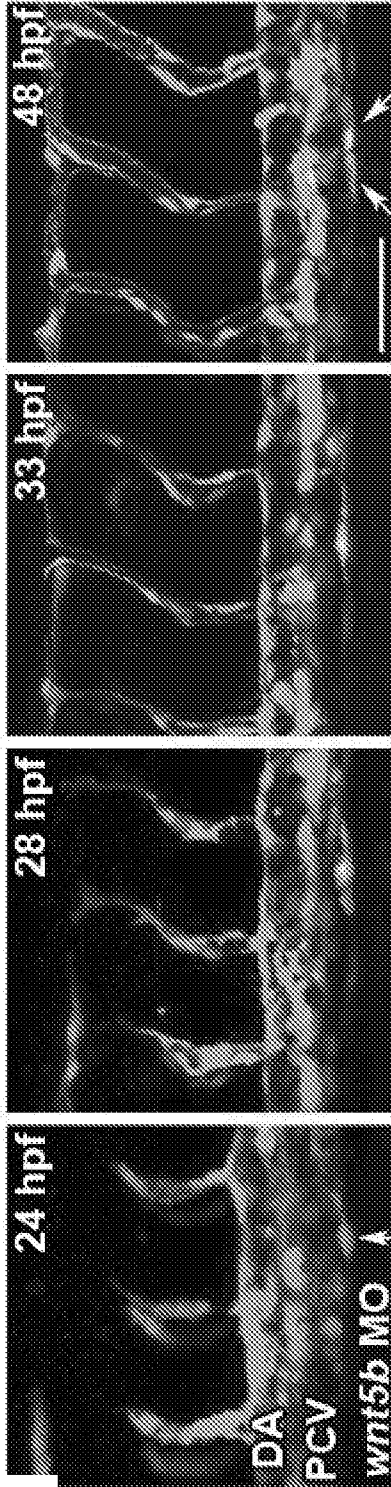
FIG. 9D

FIG. 9F

FIG. 9G

FIG. 9H

FIG. 10A FIG. 10B FIG. 10C FIG. 10D



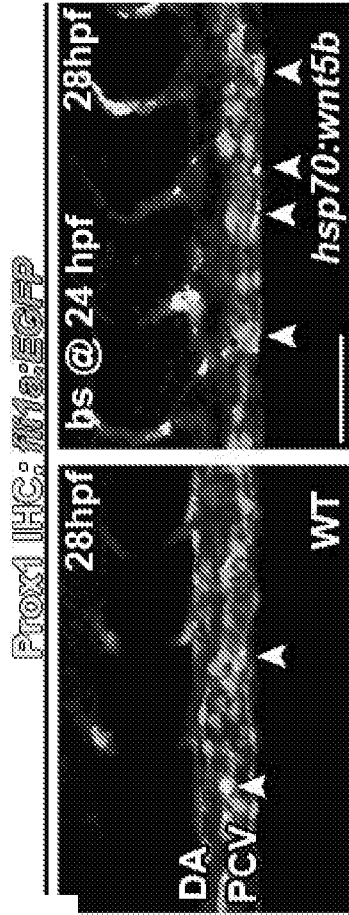
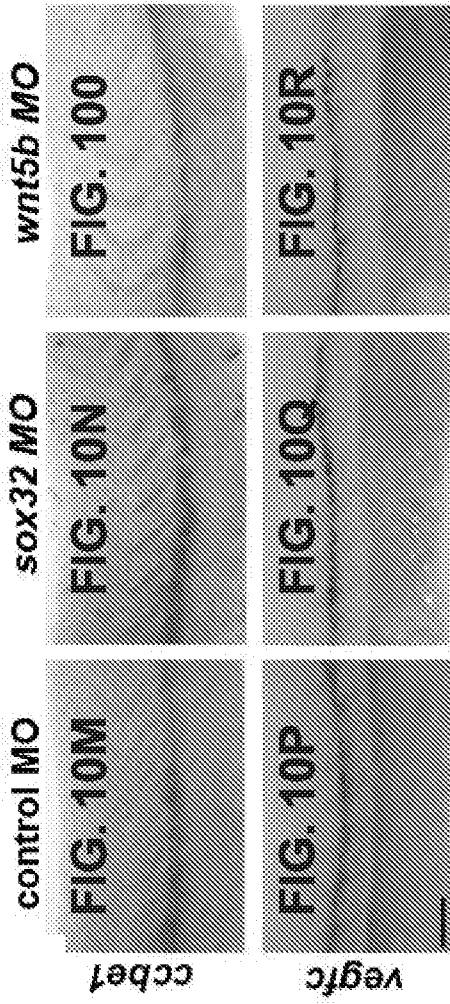


FIG. 10S

FIG. 10T

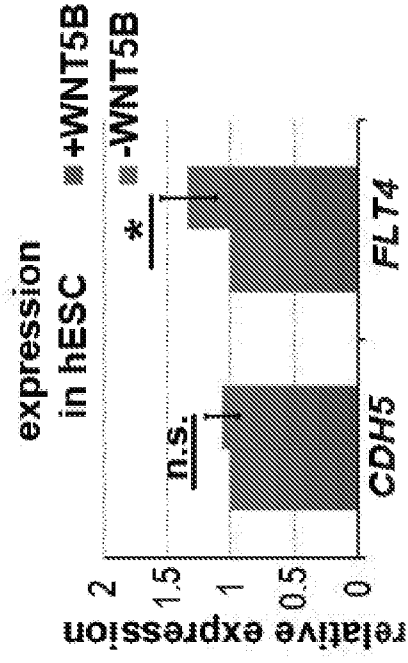


FIG. 10U

FIG. 11A FIG. 11B FIG. 11C

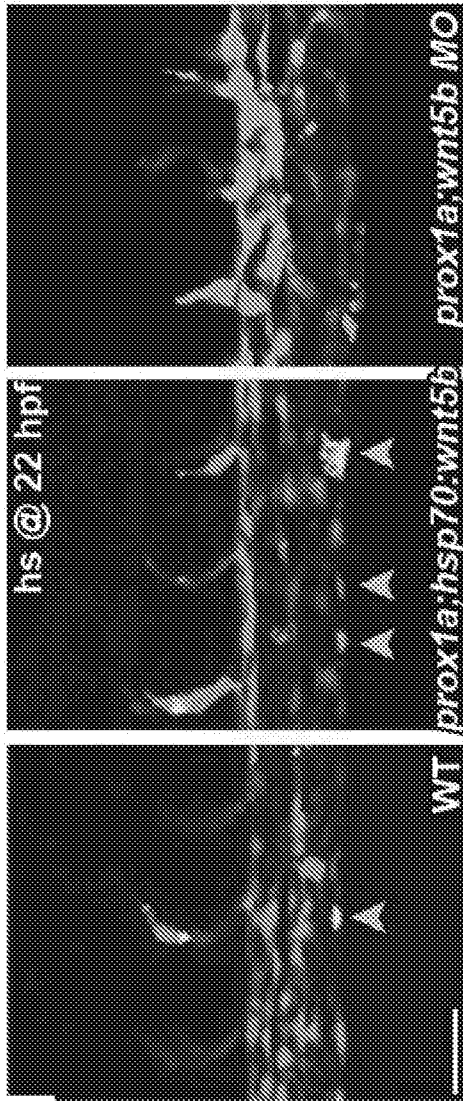
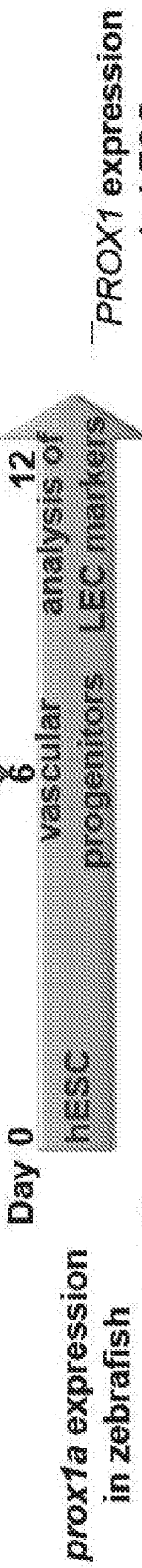
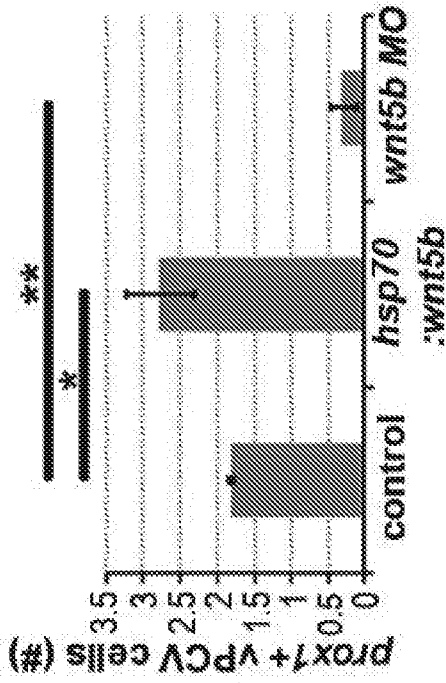


FIG. 11D



prox1a expression in zebrafish

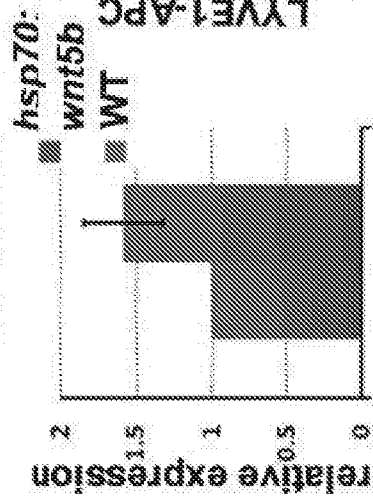


FIG. 11E

PROX1 expression in hESC

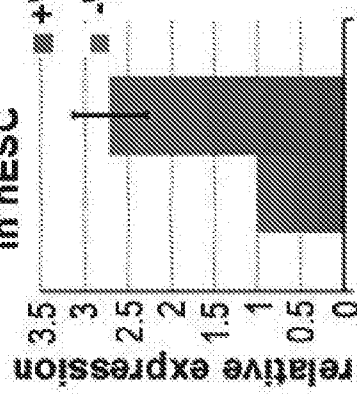


FIG. 11H

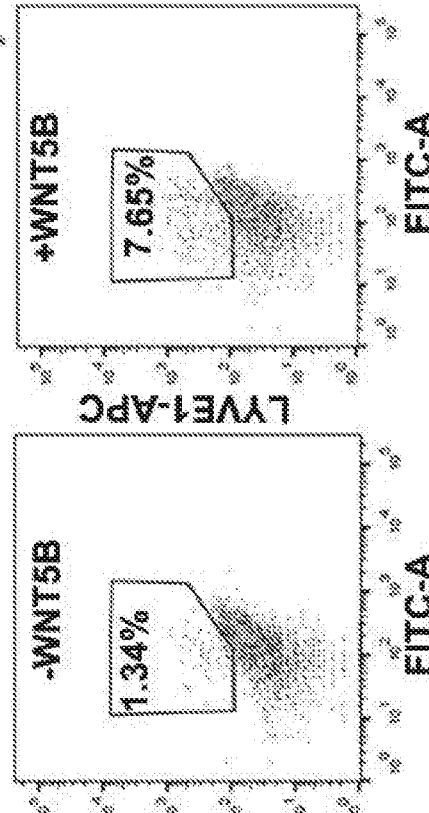


FIG. 11G

FIG. 12A

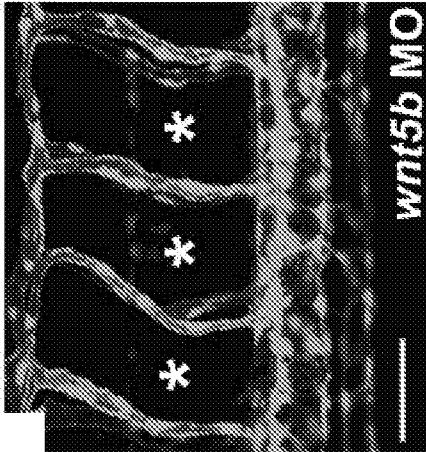


FIG. 12B

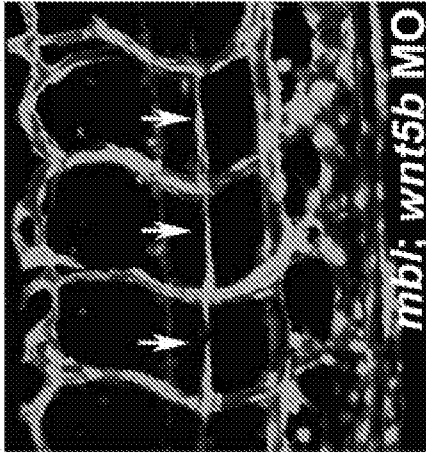


FIG. 12D

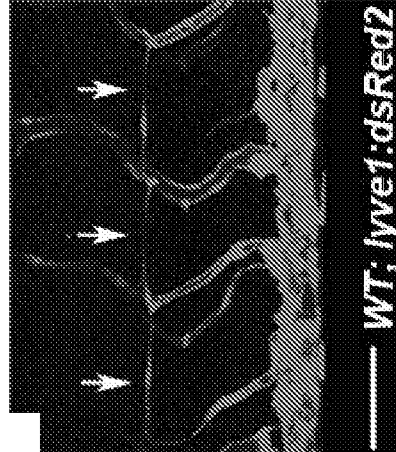


FIG. 12E

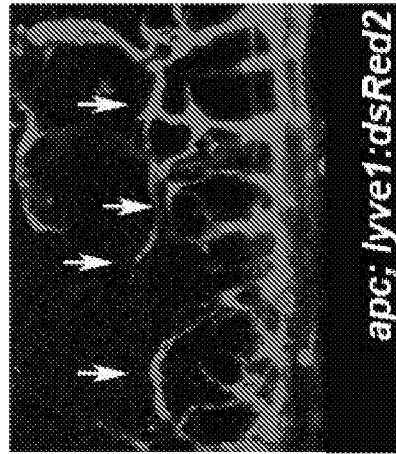


FIG. 12C

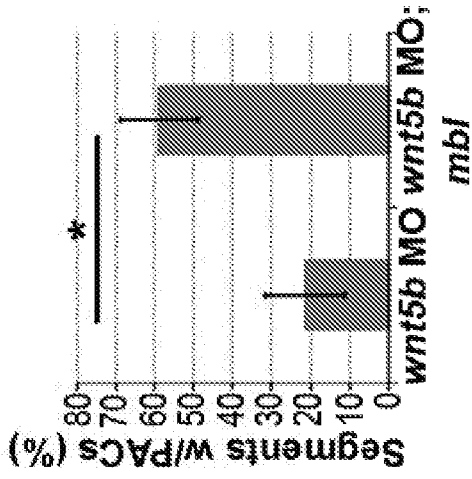


FIG. 12G

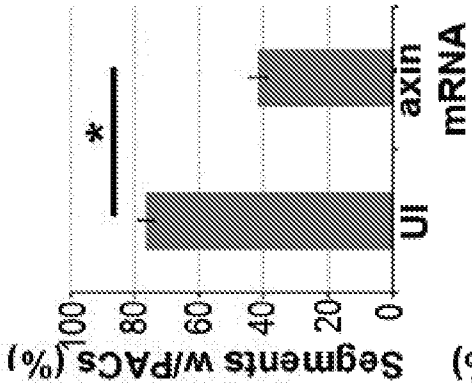


FIG. 12F

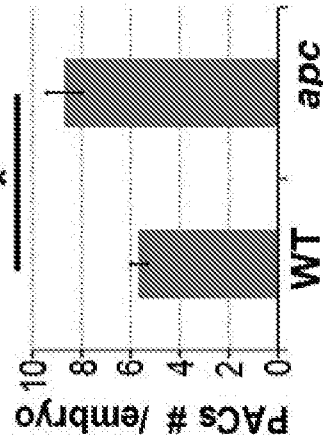


FIG. 12H

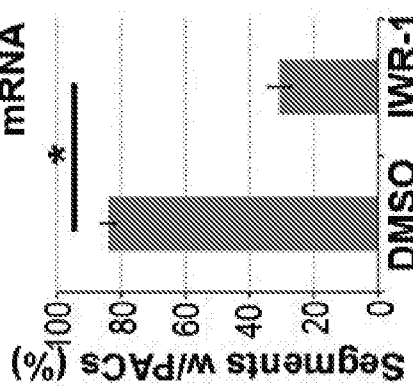


FIG. 13A

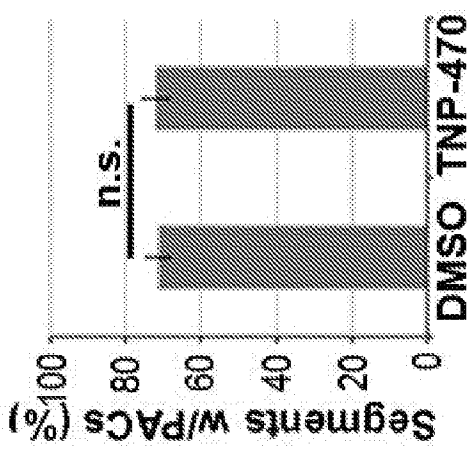


FIG. 13B

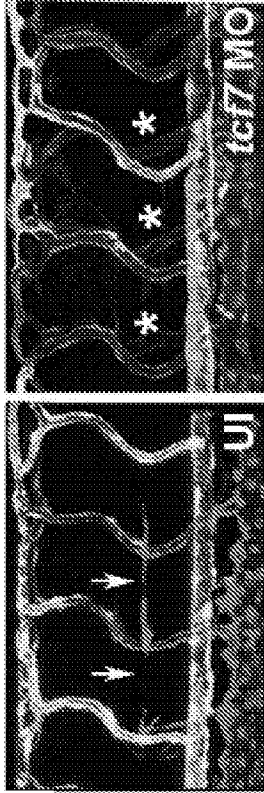


FIG. 13C

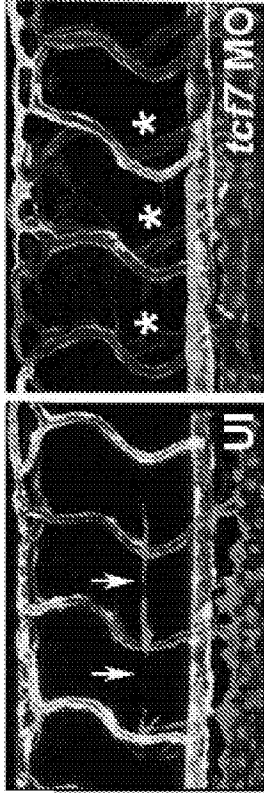


FIG. 13D

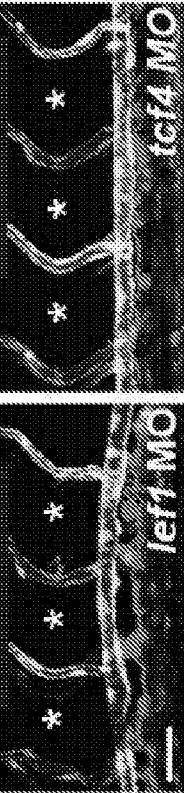


FIG. 13E

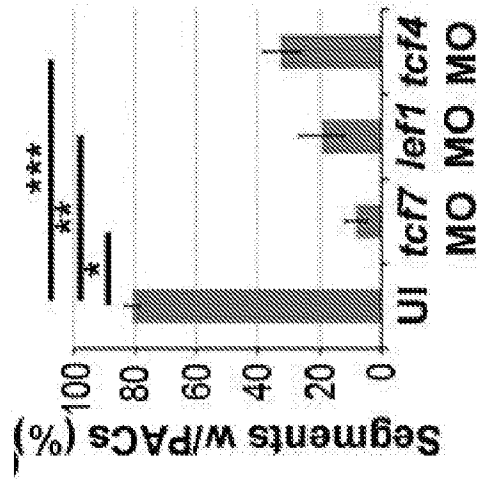
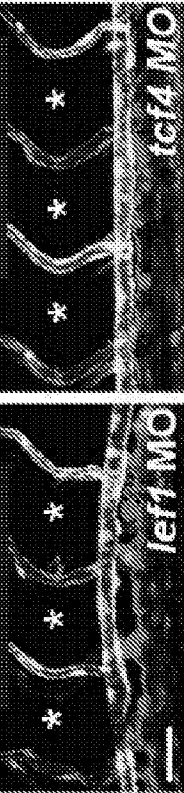


FIG. 13F

FIG. 13G 24 hpf 48 hpf FIG. 13H

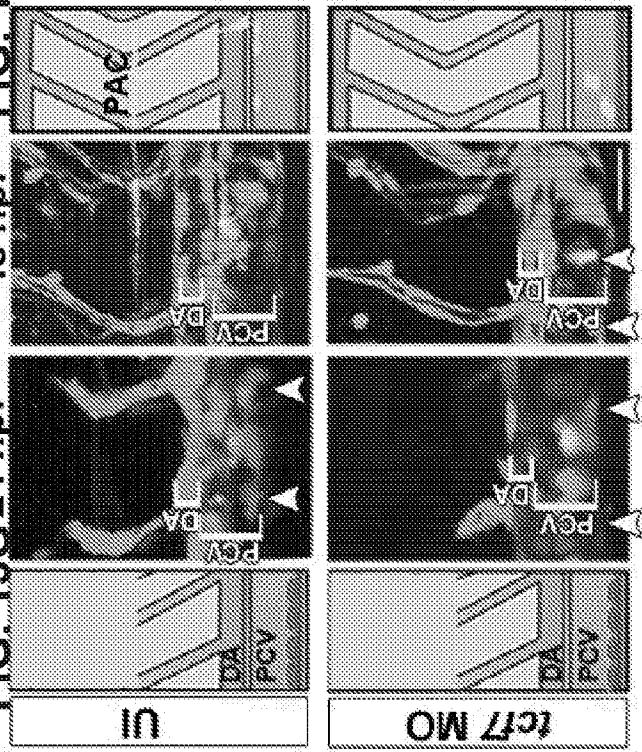


FIG. 13I

FIG. 13J

FIG. 14A

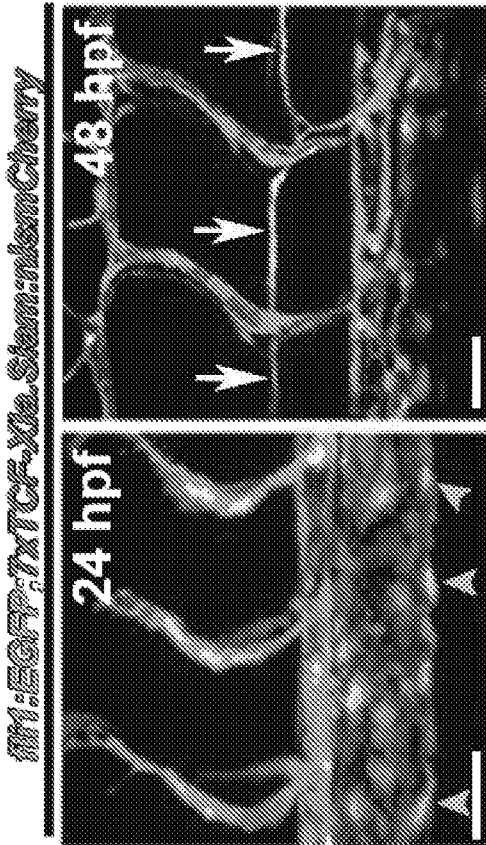


FIG. 14C

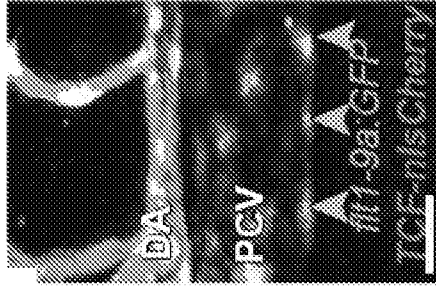
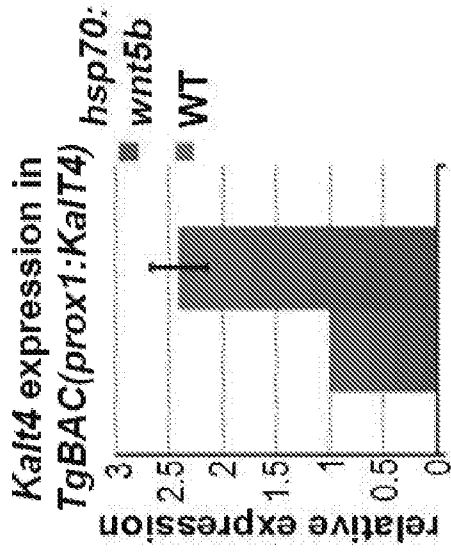
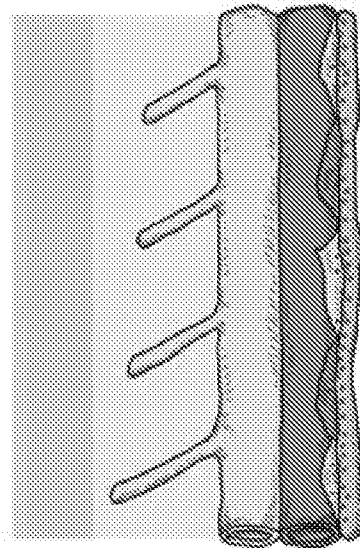


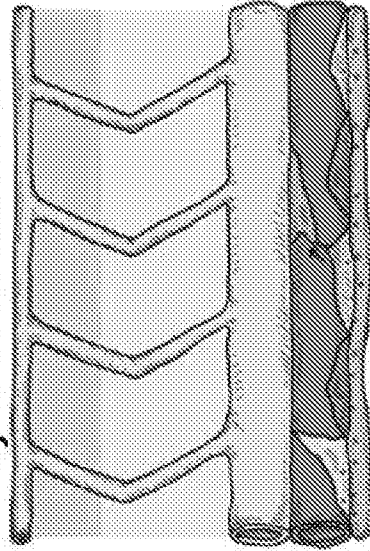
FIG. 14D



1. Specialized angioblasts in the CV niche



2. Induction of LEC specification by endodermal Wnt5b



3. LEC budding and assembly of primitive lymphatic vessels

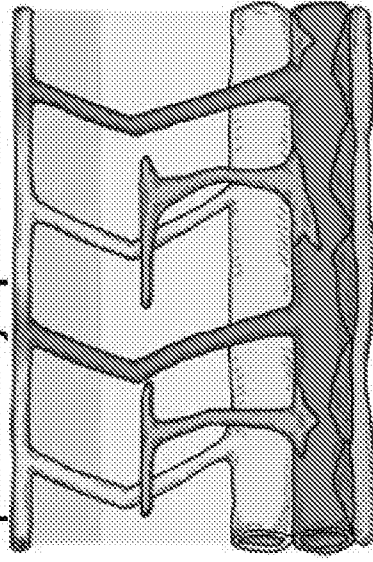


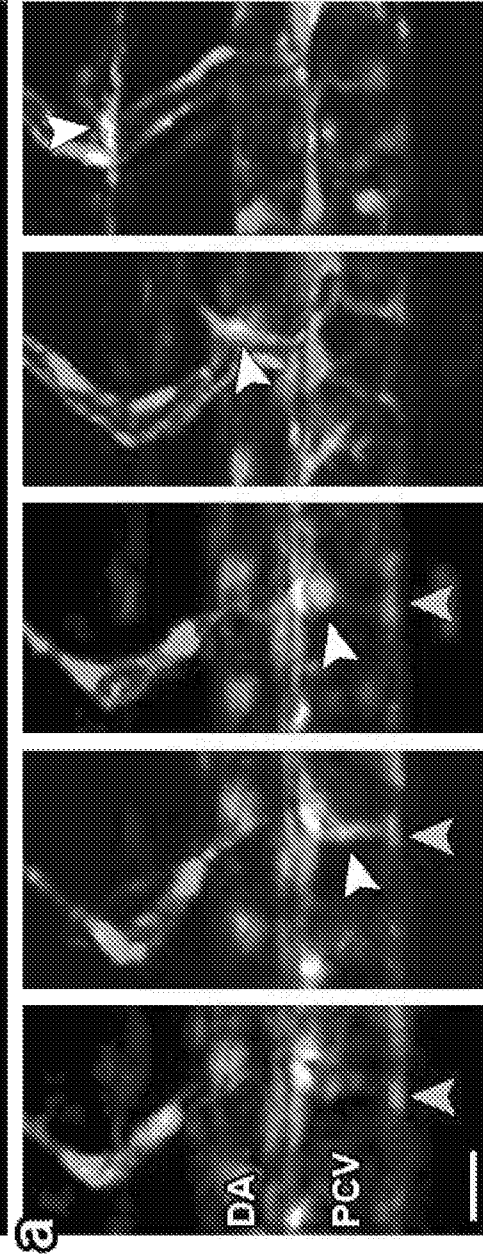
FIG. 14E

FIG. 14F

FIG. 14G

FIG. 15A FIG. 15B FIG. 15C FIG. 15D FIG. 15E

m1:EGFP;7xTCF-Xa-Stamm3bCherry



m1:EGFP;7xTCF-Xa-Stamm3bCherry

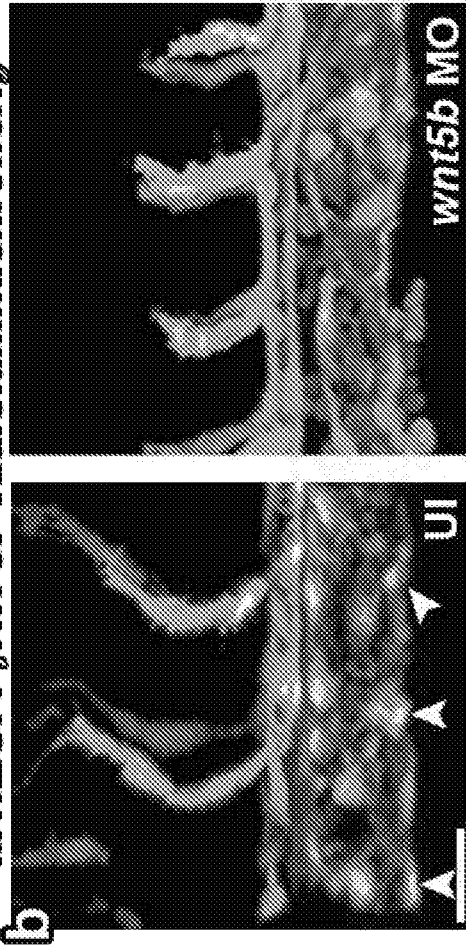


FIG. 15F

FIG. 15G

FIG. 15H

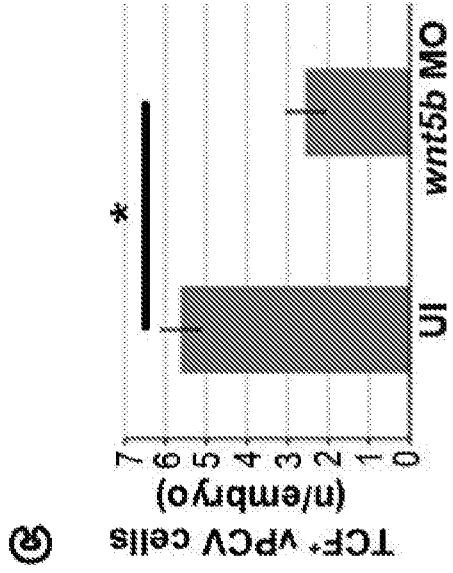


FIG. 16A

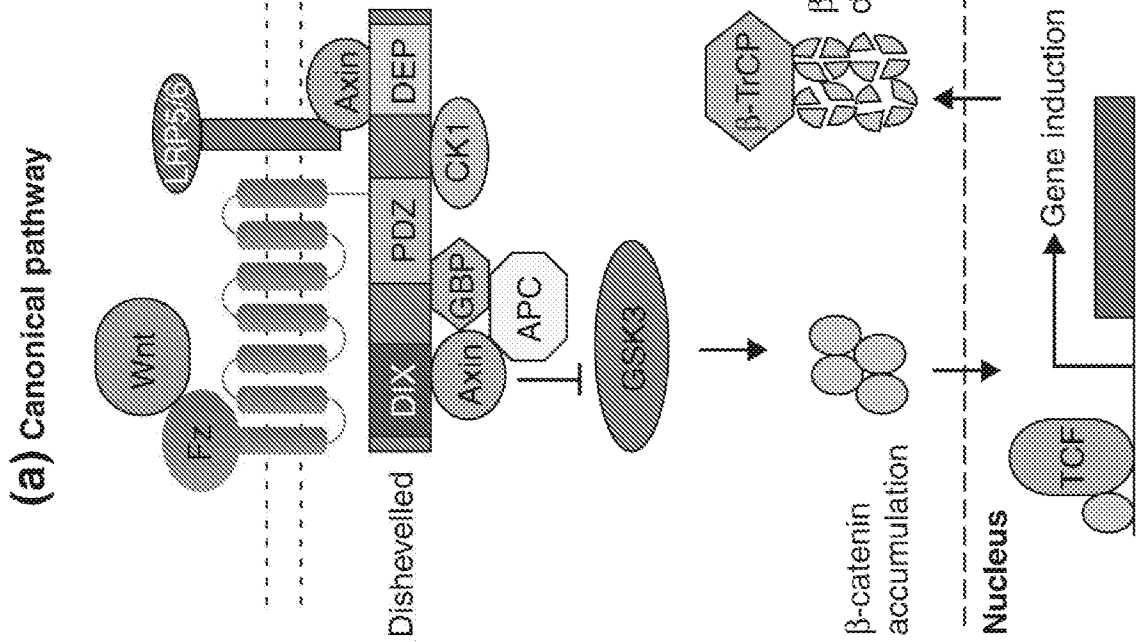


FIG. 16B

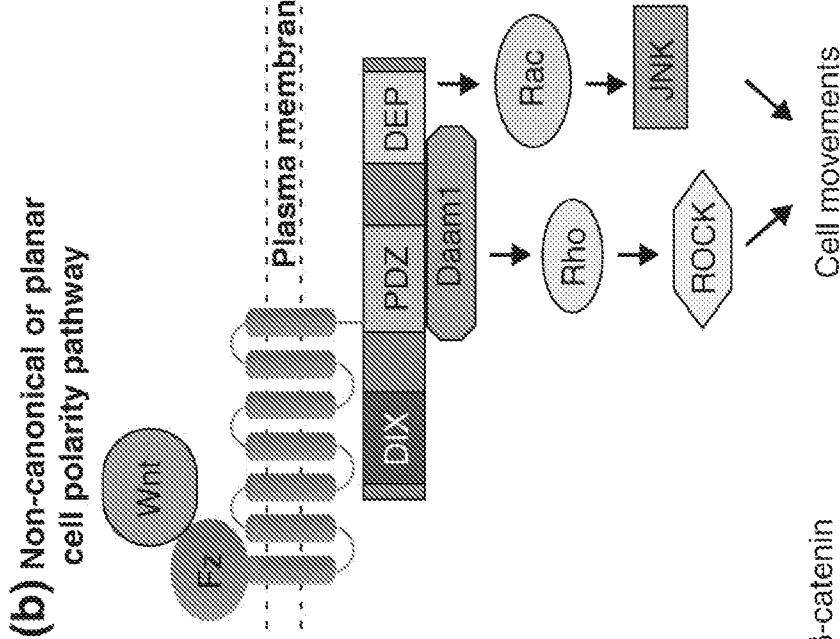
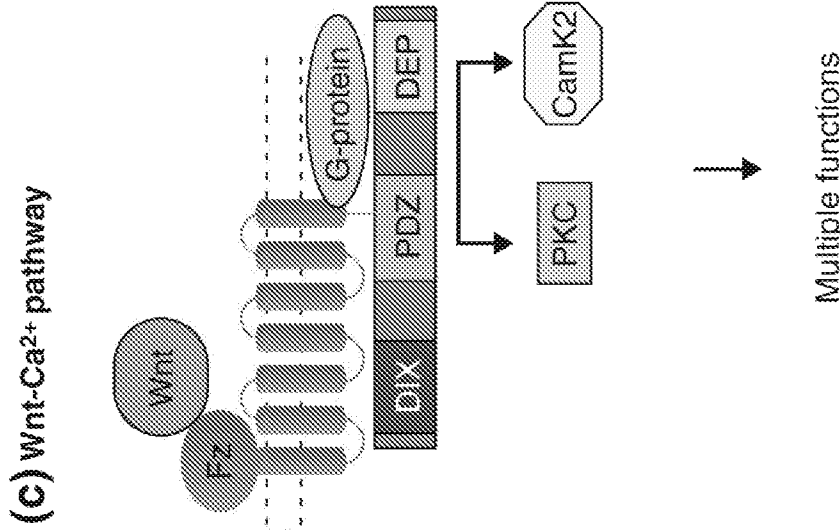


FIG. 16C



Incorporated from Habas and Dawid,
Journal of Biology 2005, 4:2

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2015/051030

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/071 A61K35/44 G01N33/50
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C12N A61K G01N
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	D.-H. YANG ET AL: "Wnt5a Is Required for Endothelial Differentiation of Embryonic Stem Cells and Vascularization via Pathways Involving Both Wnt/ -Catenin and Protein Kinase C", CIRCULATION RESEARCH., vol. 104, no. 3, 13 February 2009 (2009-02-13), pages 372-379, XP055246249, US ISSN: 0009-7330, DOI: 10.1161/CIRCRESAHA.108.185405 abstract figure 2 ----- -/--	1-28

Further documents are listed in the continuation of Box C.

See patent family annex.

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Date of the actual completion of the international search 1 February 2016	Date of mailing of the international search report 12/02/2016
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Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Loubradou-Bourges, N
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INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2015/051030

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	P. S. WOLL ET AL: "Wnt signaling promotes hematoendothelial cell development from human embryonic stem cells", BLOOD, vol. 111, no. 1, 1 January 2008 (2008-01-01), pages 122-131, XP055246264, US ISSN: 0006-4971, DOI: 10.1182/blood-2007-04-084186 abstract	1-28
X	----- XIAOJUN LIAN ET AL: "Efficient Differentiation of Human Pluripotent Stem Cells to Endothelial Progenitors via Small-Molecule Activation of WNT Signaling", STEM CELL REPORTS, vol. 3, no. 5, 1 November 2014 (2014-11-01), pages 804-816, XP055246300, United States ISSN: 2213-6711, DOI: 10.1016/j.stemcr.2014.09.005 abstract	1-28
X	----- FANG LIU ET AL: "Enhanced Hemangioblast Generation and Improved Vascular Repair and Regeneration from Embryonic Stem Cells by Defined Transcription Factors", STEM CELL REPORTS, vol. 1, no. 2, 1 August 2013 (2013-08-01), pages 166-182, XP055246315, United States ISSN: 2213-6711, DOI: 10.1016/j.stemcr.2013.06.005 abstract	1-28
X	----- US 2014/287498 A1 (PALECEK SEAN PAUL [US] ET AL) 25 September 2014 (2014-09-25) claims 1-16	1-28
X	----- BUTTLER KERSTIN ET AL: "Maldevelopment of dermal lymphatics in Wnt5a-knockout-mice", DEVELOPMENTAL BIOLOGY, vol. 381, no. 2, 11 July 2013 (2013-07-11) , pages 365-376, XP028698022, ISSN: 0012-1606, DOI: 10.1016/J.YDBIO.2013.06.028 cited in the application abstract	1-28
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INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2015/051030

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KARINA YANIV ET AL: "Live imaging of lymphatic development in the zebrafish", NATURE MEDICINE., vol. 12, no. 6, 28 May 2006 (2006-05-28), pages 711-716, XP055246318, US ISSN: 1078-8956, DOI: 10.1038/nm1427 the whole document	1-28
X	G. OLIVER ET AL: "Endothelial cell plasticity: how to become and remain a lymphatic endothelial cell", DEVELOPMENT, vol. 137, no. 3, 14 January 2010 (2010-01-14), pages 363-372, XP055246352, GB ISSN: 0950-1991, DOI: 10.1242/dev.035360 abstract; figures 1-3	15-17, 20,23
A	----- YANG YING ET AL: "Transcriptional Control of Lymphatic Endothelial Cell Type Specification", ADVANCES IN ANATOMY, EMBRYOLOGY AND CELL BIOLOGY, SPRINGER, HEIDELBERG, DE, vol. 214, 1 January 2014 (2014-01-01), pages 5-22, XP009188258, ISSN: 0301-5556 the whole document	1-14,18, 19,21, 22,24-28
X	----- YANG YING ET AL: "Transcriptional Control of Lymphatic Endothelial Cell Type Specification", ADVANCES IN ANATOMY, EMBRYOLOGY AND CELL BIOLOGY, SPRINGER, HEIDELBERG, DE, vol. 214, 1 January 2014 (2014-01-01), pages 5-22, XP009188258, ISSN: 0301-5556 the whole document	15-17, 20,23
A	----- EVA GÓMEZ-ORTE ET AL: "Multiple functions of the noncanonical Wnt pathway", TRENDS IN GENETICS., vol. 29, no. 9, 1 September 2013 (2013-09-01), pages 545-553, XP055246397, NL ISSN: 0168-9525, DOI: 10.1016/j.tig.2013.06.003 the whole document	1-14,18, 19,21, 22,24-28
A	----- EVA GÓMEZ-ORTE ET AL: "Multiple functions of the noncanonical Wnt pathway", TRENDS IN GENETICS., vol. 29, no. 9, 1 September 2013 (2013-09-01), pages 545-553, XP055246397, NL ISSN: 0168-9525, DOI: 10.1016/j.tig.2013.06.003 the whole document	1-28
X,P	----- J. NICENBOIM ET AL: "Lymphatic vessels arise from specialized angioblasts within a venous niche", NATURE, vol. 522, no. 7554, 20 May 2015 (2015-05-20), pages 56-61, XP055246421, United Kingdom ISSN: 0028-0836, DOI: 10.1038/nature14425 the whole document	1-28

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IL2015/051030

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2014287498	A1	NONE	