(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 September 2016 (09.09.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/141234 A1

(51) International Patent Classification:
A01K 67/00 (2006.01) *C12N 15/00* (2006.01)
A01K 67/027 (2006.01) *C12N 15/09* (2006.01)

(21) International Application Number:
PCT/US2016/020768

(22) International Filing Date:
3 March 2016 (03.03.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/127,330 3 March 2015 (03.03.2015) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title**: ETV2 AND USES THEREOF

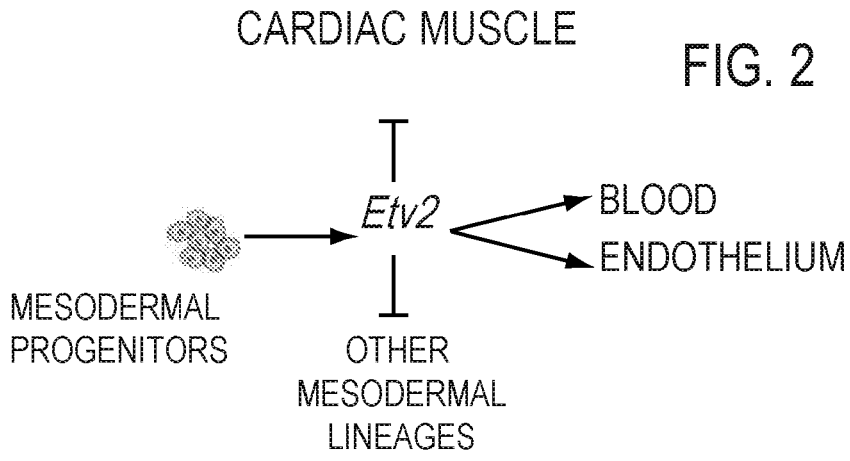

FIG. 2

(57) **Abstract**: Described herein is a method for producing a chimeric non-human animal expressing a human ETV2 gene comprising: a) generating an ETV2 null non-human animal cell, wherein both copies of the non-human ETV2 gene carry a mutation that prevents production of functional ETV2 protein in said non-human animal; b) creating an ETV2 null non-human blastocyst by somatic cell nuclear transfer comprising fusing a nucleus from said ETV2 null non-human animal cell of a) into an enucleated non-human oocyte and activating said oocyte to divide so as to form an ETV2 null non-human blastocyst; c) introducing human stem cells into the ETV2 null non-human blastocyst of b); and d) implanting said blastocyst from c) into a pseudopregnant surrogate non-human animal to generate a chimeric non-human animal expressing human ETV2.

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ETV2 AND USES THEREOF

Claim of Priority

This application claims the benefit of priority of U.S. Provisional Patent Application No. 62/127,330, filed on 3 March 2015, the benefit of priority of which is claimed hereby, and which is incorporated by reference herein in its entirety.

Sequence Listing

This document incorporates by reference herein an electronic sequence listing text file, which is filed in electronic format via EFS-Web. The text file is named "1552048.txt," is 12,288 bytes, and was created on March 1, 2016.

Background of the Invention

Blood is a major organ of the human body. It is composed of cells and plasma. The cellular part comprises red blood cells, white blood cells, and platelets. The plasma component consists of water and dissolved compounds such as proteins, sugars or electrolytes among others.

The formation of the blood begins during gastrulation. The first hematopoietic lineages appear in the blood island of the yolk sac. The differentiation of hematopoietic precursor cells is regulated by genetic programs. It was recently shown that a key factor of this genetic program is the gene *Ets variant gene 2 (Etv2)* (Stem cells. 2012 August; 30(8):1611-1623. doi:10.1002/stem.1131). Ablation of the *Etv2* gene in the mouse resulted in the loss of hematopoietic and endothelial lineages in the yolk sac. Forced overexpression of the *Etv2* gene in the *Etv2*-mutant restored the hematological and endothelial lineages. Genesis 2013 Jul:51(7):471-480. Doi: 10.1002/dvg.22396.

Blood disorders may affect the production of red blood cells (i.e. anemias), the proliferation of white blood cells (i.e. leukemias), or the clotting of platelets (i.e. coagulopathies). Donated blood is used to treat these various disorders. Red blood cells are used to treat patients with chronic anemias associated with sickle cell anemia, thalassemia, aplastic anemia, leukemia or cancer. Platelets are used to control bleeding in patients undergoing surgery. Currently, the only source of human blood is a human donor.

Summary of the Invention

The present invention is based on the novel finding that deletion of the *Etv2* gene in pigs leads to the ablation of the hematopoietic and endothelial lineages at an early step of the hematoendothelial development. The present invention generates a pig blastocyst deficient of the pig *Etv2* gene (and therefore deficient in pig vasculature and blood). By injecting human stem cells, such as iPS cells positive for the *Etv2* gene into the mutant pig (deficient in *Etv2*) blastocyst, the invention generates a pig that produces human or humanized blood and vasculature derived from the human, stem cells, such as iPS cells. Every year, more than 300,000 Americans have coronary artery bypass grafting and would benefit from the use of such engineered coronary blood vessels.

Described herein is the development of ETV2 knockout pigs or other animals, such as cow or goat, as hosts for production of personalized human/humanized blood and vasculature for clinical

applications. The progenitor cells that generate blood also generate the endothelium which represents the inner lining of blood vessels. In the absence of the endothelium, there is no vasculature and the developing embryos are non-viable. In addition to serving as a novel source of human/humanized tissues for the treatment of cardiovascular and hematopoietic disease, the humanized pigs will also serve as a large animal model to study the regeneration of human lineages and/or response(s) to pharmacological agents. Etv2 was discovered as a target of traditional cardiovascular and hematoendothelial transcription factors and signaling cascades and it was demonstrated that Etv2 regulates the specification and differentiation of hematoendothelial lineages. In addition, it was noted that the Etv2 mutant mouse embryos were nonviable and lacked endothelial/vascular and hematopoietic lineages. Using gene editing technologies, it is further established that ETV2 mutant porcine embryos lack hematopoietic and endothelial lineages. Based on the results, Etv2 is a master regulator for the hematoendothelial lineages during development. Described herein is the engineering of a humanized genetically modified animal surrogate.

One embodiment provides a non-human animal cell or blastocyst wherein the genome carries a mutation in both alleles of the ETV2 gene such that the non-human animal cell or blastocyst lacks functional ETV2 protein. In one embodiment, the mutation is a deletion of the ETV2 gene. In another embodiment, the non-human animal cell or blastocyst is a porcine, bovine, equine or goat.

One embodiment provides a chimeric non-human animal or blastocyst expressing human ETV2 and lacking expression of said non-human animal ETV2. In one embodiment, the non-human animal expresses human blood cells selected from the group consisting of white blood cells, red blood cells, platelets or a combination thereof. In another embodiment, the chimeric non-human animal expresses human endothelium. In one embodiment, the non-human animal is a porcine, bovine, equine or goat.

One embodiment provides a method for producing a chimeric non-human animal expressing a human ETV2 gene comprising: a) generating an ETV2 null non-human animal cell, wherein both copies of the non-human ETV2 gene carry a mutation that prevents production of functional ETV2 (by itself or with other genes) protein in said non-human animal; b) creating an ETV2 null non-human blastocyst by somatic cell nuclear transfer comprising fusing a nucleus from said ETV2 null non-human animal cell of a) into an enucleated non-human oocyte and activating said oocyte to divide so as to form an ETV2 null non-human blastocyst; c) introducing human stem cells into the ETV2 null non-human blastocyst of b); and d) implanting said blastocyst from c) into a pseudopregnant surrogate non-human animal to generate a chimeric non-human animal expressing human ETV2.

Another embodiment provides a method of producing human and/or humanized blood cells or vessels in a non-human animal comprising: a) generating an ETV2 null non-human animal cell, wherein both alleles of the non-human ETV2 gene carry a mutation that prevents production of functional ETV2 protein; b) creating an ETV2 null non-human blastocyst or morula by somatic cell nuclear transfer comprising fusing a nucleus from said ETV2 null non-human animal cell of a) into an enucleated non-

human oocyte and activating said oocyte to divide so as to form an ETV2 null non-human blastocyst; c) introducing human donor stem cells into the ETV2 null non-human blastocyst of b); and d) implanting said blastocyst or morula from c) into a pseudopregnant surrogate non-human animal so as to generate a non-human animal expressing human and/or humanized blood cells or vessels. In one embodiment, the blood cells are human-induced pluripotent or human umbilical cord blood stem cell-derived white blood cells, red blood cells, and/or platelets.

In one embodiment, the non-human animal is a porcine, bovine, equine or goat. In another embodiment, the human donor stem cell is a tissue specific stem cell, pluripotent stem cell, multipotent adult stem cell, induced pluripotent stem cell or umbilical cord blood stem cell (UCBSC). In one embodiment, the donor providing the stem cells is the recipient of the humanized tissue or organ produced. In another embodiment, the human induced pluripotent cell is formed from a fibroblast cell.

One embodiment provides for a non-human animal produced by the methods described herein. Another embodiment provides a progeny non-human animal produced from the mating of two non-human animals generated from the methods described herein, wherein said progeny non-human animal expresses human ETV2, wherein the genome of said progeny non-human carries a homozygous deletion of said non-human animal ETV2 gene. In one embodiment, the non-human animal is a porcine, bovine, equine or goat.

One embodiment provides a gene knockout pig cell or blastocyst wherein the genome comprises a deletion of the ETV2 gene such that the pig cell or blastocyst lacks functional ETV2 protein, wherein the pig cell or blastocyst is homozygous for the deletion. In one embodiment, the sequence of wild type pig (Sus scrofa) ETV2 (ets variant 2) is provided at ENSSSCG00000002906. In another embodiment, the predicted *sus scrofa* ets variant 2 (ETV2) mRNA and protein sequence are provided as follows:

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   1 tgagtcattg gaaacaaata cagacatcat aacacttcac tcctaaatac ctgtttcata
  61 accttataaa gatagcttcc atatcataat accattatca catctaagaa aatgactaat
 121 tatctcatat tcagttcata ctctaatttc ctcatgtgcc taaactatga cagcatggaa
 181 gggcatgatg gattctggag atgaagaaaa gtaggaggaa acgcacctca atttcccctt
 241 tcataaagtc agggtaagac tagtacccac ttcctaagag aattaaacaa aggcccatgg
 301 cacagttagt agcaagcaat gagccctcaa gaaatgttaa ccattattgt cactgttgtt
 361 attgtttata ttgttgatgt tactgtctgc tgaagcagcc ccagaacttc ctcctcaaag
 421 ccctcgaagg ggaaaacagc ctggtggaag atcccaggtc gaccaaccaa cccccaccat
 481 atcccccgca ggcccctgc ggattgtgac gtctgctgac caggggtctg gccggaaatc
 541 ccccttcctg ttgcagataa gcctggtgca gcccagctga ccccaggccc tcctccccca
 601 tcacctccct tgtcacagga tcaagtcccc aagccccctt cccctcccca ttccagtcaa
 661 cccagaaaca cccctctgca ccccaggtca tgcccatccc attgtttccc aggctcctgc
 721 tcaagtccaa gacaccccaa agctaccgtg gaggcttgag gccatcccag ggggcagagg
 781 tgggtgggga ggggggtggca cagcttggcc ccgcctcggc ccctgcaact tgacccgggc
 841 tgcgaccccc gctctgacgt cttggaaaat tccccccctgc ccaggccccc agaggagggg
 901 gtatgtggta tgaaatgggg ctgagacccc tggctggggg cacagggatc tgccagagaa
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961 cattcactac tggcatccat ggacttgtgg aactgggatg aagcatcgcc acaggaagtg
1021 cccctgggga acagactgtc agggctggaa ggagctgaat tcgacttcta tttcccctgaa
1081 ctggcactcc caggggacag gctgacagcg gagacatact ggaaaactgg ctcttcatcc
1141 ttatctgtcc cagggattcc acagccggac tgggtctccg cattaccgaa cccagaagct
1201 ccatggggcg cggaacccgt ccctcaggct cttccgtggt ccggagattg gacagacctg
1261 ccgtacagcg gctcggtccc ttggagccgg gtctcccagg ccctggggtc tggctgccta
1321 gatttccaag gtcccattca gctgtggcag ttcctcctgg agctgctcca cgacgggacg
1381 cgtagcagct gcatccgctg gacgggcaac agccgcgagt tccaactgtg cgaccccaaa
1441 gaggtggcgc ggctgtgggg cgaacgcaag aggaagcccg gcatgaatta tgagaagctg
1501 agccgaggcc tgcgttacta ctaccgccgc gacatcgtgc tcaagagcgg ggggcgcaag
1561 tacacgtacc gcttcggagg ccgagtgcca ggcctagcct atcccgaccg catgggggac
1621 ggacagggag cagcgaccca ataaaaatat ctggtcaagc c (SEQ ID NO: 20)

MDLWNWDEASPQEVPLGNRLSGLEGAEFDFYFPELALPGDRLTA
ETYWKTGSSSLSVPGIPQPDWVSALPNPEAPWGAEPVPQALPWSGDWTDLPYSGSVPW
SRVSQALGSGCLDFQGPIQLWQFLLELLHDGTRSSCIRWTGNSREFQLCDPKEVARLW
GERKRKPGMNYEKLSRGLRYYYRRDIVLKSGGRKYTYRFGGRVPGLAYPDRMGDGQGA ATQ (SEQ ID NO: 21)

One embodiment provides an ETV2 mutant pig (deficient in *Etv2*). In another embodiment, a chimeric ETV2 mutant pig expresses human or humanized blood cells selected from the group consisting of white blood cells, red blood cells, platelets or a combination thereof, as well as human or humanized endothelium.

One embodiment provides a method for producing an ETV2 mutant pig comprising: a) generating an ETV2 null pig cell; b) creating an ETV2 null pig blastocyst by somatic cell nuclear transfer (SCNT) comprising fusing a nucleus from said ETV2 null pig cell of a) into an enucleated pig oocyte and activating said oocyte to divide so as to form an ETV2 null pig blastocyst; c) introducing human stem cells into the pig ETV2 null blastocyst.

Another embodiment provides a method of producing human or humanized blood cells and human or humanized endothelium in pigs comprising: a) generating an ETV2 null pig cell; b) creating an ETV2 null pig blastocyst by somatic cell nuclear transfer (SCNT) comprising fusing a nucleus from said ETV2 null pig cell of a) into an enucleated pig oocyte and activating said oocyte to divide so as to form an ETV2 null pig blastocyst; c) introducing human stem cells into the pig ETV2 null blastocyst (blastocoel cavity) of b) and d) implanting said blastocyst from c) into a pseudopregnant/surrogate pig so as to generate a pig with human/humanized blood cells and human/humanized endothelium (human stem cells are added to the gene edited blastocyst). In one embodiment, the blood cells and endothelium are human-iPS- or human umbilical cord blood stem cell-derived white blood cells, red blood cells, platelets and/or endothelium.

It would be useful to make human or humanized tissues and organs personalized to each recipient's immune complex. As disclosed herein, it is possible to do so by using a large animal as a host

editing its genome to knock out or debilitate genes responsible for the growth and/or differentiation of a target organ and inoculating that animal at a blastocyst or zygote stage with donor stem cells to complement the missing genetic information for the growth and development of the organ. The result is a chimeric animal in which the complemented tissue (human/humanized organ) matches the genotype and phenotype of the donor. Such organs may be made in a single generation and the stem cell may be taken or generated from the patient's own body. As disclosed herein, it is possible to do so by simultaneously editing multiple genes in a cell (see, for example, WO 2015/168125, which is incorporated herein by reference). Multiple genes can be targeted for editing using targeted nucleases and homology directed repair (HDR) templates in vertebrate cells or embryos.

One embodiment provides a method to produce humanized tissues in a non-human host animal comprising: i) genetically editing one or more genes responsible for a desired tissue or organ's growth and/or development in a cell or embryo, of the host; ii) complementing the host's lost genetic information by injecting an effective amount of stem cells from a donor into the cell, embryo, zygote or blastocyst (e.g., human-porcine blastocyst) to create a chimeric animal; so as to produce humanized tissue or organ (to produce a chimeric animal through the use of cell (e.g., stem cell) complementation).

In one embodiment, the stem cell is a human induced pluripotent stem cell (iPS cell) or a human umbilical cord blood stem cell. In one embodiment, the human iPS cell was formed from a fibroblast cell or any adult or somatic cells (e.g., human cells). In one embodiment, the ETV2 null pig cell was created using gene editing with TALENS (Gene: ETV2 ENSSSCG00000002906).

Further described are methods of producing chimeric swine and methods of producing tissue from chimeric swine of the present invention. One embodiment provides a progeny swine produced from the mating of two pigs generated from methods described herein, wherein said progeny swine expresses human ETV2, wherein the genome of said progeny swine comprises a homozygous deletion of the pig ETV2 gene.

Brief Description of the Drawings

Figures 1A-D. Etv2 is a master regulator of the hematopoietic and the endothelial lineages. (A) Etv2 mutant and wild-type littermate embryos were collected at E8.5, sectioned, and analyzed by hematoxylin-eosin staining and immunohistochemistry using endomucin antibody (α-endomucin). At this stage, primary heart vein (PHV), dorsal aorta (DA), and endocardium (End) are formed in wild-type embryos, whereas Etv2 mutant mice lack these structures. Anti-endomucin staining identifies vessels (arrowheads) and endocardium (arrows), which are absent in the mutant embryo. (B) Methylcellulose colony forming assay using yolk sac cells from Etv2 wild-type (Wt), heterozygous (Het), and mutant embryos at E 8.5. Note that wild-type and heterozygous animals for Etv2 have similar colony forming activity, whereas Etv2 mutant yolk sac cells have none. (C) Stably transfected ES cells lines were engineered to express Etv2 in a doxycycline (Dox)-inducible fashion. ES cells were differentiated for 4 days and Etv2 was induced by addition of Dox. Flow cytometric analysis after 5 days of induction demonstrated significant increase of both hematopoietic ($CD45^+$) and endothelial ($PECAM^+$ or $Tie2^+$)

lineages upon Etv2 induction. (D) Colony forming activity of the EBs after induction of Etv2 from day 3 to day 6. Note the increased hematopoietic activity upon induction of Etv2. Open bars: no induction, filled bars: Dox induction.

Figure 2. Proposed role of Etv2 in specification of the mesodermal lineages.

Figures 3A-G. Etv2 but not Flk1 can rescue the endothelial and hematopoietic phenotype of Etv2 mutant ESCs/EBs. Quantification of FACS analysis demonstrating that Etv2 and Flk1 can rescue the endothelial differentiation of Flk1-/- ESCs/EBs (A) but only Etv2 can rescue the Etv2-/- ESCs/EBs (B). The same results were obtained with hematopoietic markers (data not shown). The summary of the lineages and rescue studies for Etv2 and Flk1 are presented in panel (C). Gata2 physically interacts and amplifies Etv2 activity. (D) Schematic illustration of the Etv2-Gata2 construct. Etv2 and Gata2 are linked through the 2A peptide sequence and are equally expressed. The fusion construct was translated into two proteins, Etv2 and Gata2, through the ribosome skipping mechanism. (E-F) Co-expression of Etv2 and Gata2 in EBs results in enhanced hematopoietic and endothelial lineage differentiation. The EBs were treated with doxycycline (+ Dox) or left untreated (-Dox) from EB day 3 to day 4 and harvested for FACS analysis on Day 6. The hematopoietic lineage is identified as the c-Kit$^+$/CD41$^+$ cell population (E), and the endothelial lineage is represented as Flk1$^+$/CD31$^+$ (F). Panel G highlights upstream regulators and downstream targets for Etv2 in endothelial and hematopoietic lineages.

Figures 4A-B. TALEN-mediated knockout of ETV2. (A) Three-tiered PCR assay utilized to detect gene editing. Amplification from primers a-d indicated a deletion allele was present. To distinguish between heterozygous and homozygous clones, primers a-b and c-d were used to amplify the wild type allele. Only when the a-d product is present and both a-b, c-d products are absent is the clone considered homozygous for the deletion allele. (B) Clones fitting these criteria are enclosed by a green box.

Figures 5A-H. Loss of porcine ETV2 recapitulated the mouse Etv2 mutant phenotype. Wild-type E18.0 pig embryo (A) and (B) ETV2 knockout embryo at the same developmental stage. Insets show enlarged views of the allantois. Note an abnormal overall morphology with lack of vascular plexus formation in the mutant (inset). (C-H) Sections through the allantois (C, D), the heart level (E, F) and the trunk level (G, H) of the embryos shown in A and B, respectively, were stained for Tie2, an endothelial marker; Gata4, a cardiac lineage marker; and 4',6-diamidino-2-phenylindole (DAPI), a nuclear counterstain. The wild-type allantois was highly vascularized with Tie2 positive endothelial lining and contained blood (C, arrows), whereas, the mutant lacked these populations (D). The endocardium, cardinal veins (CV), and dorsal aortae (DA) are clearly visible in the wild-type embryo (E, G). In contrast, ETV2 null embryos completely lacked these structures although the heart progenitors and gut marked by Gata4 (green) were present (F and H, respectively). Scale bars: 1000 μm (A, B), 200 μm (insets in A, B), 100 μm (C-H).

Figures 6A-H. Complementation of Etv2 mutant mouse embryo with wild-type ES cells. Blastocysts obtained from crossing Etv2 $^{-/+}$ and Etv2 $^{dEfl/+}$ mice were injected with 10-15 EYFP labeled

wild-type ES cells. Embryos were harvested at E10.5 and genotyped. A, B: Whole-mount epifluorescence image of the embryos showing the distribution of the wild-type ES cell progeny. C-H: Sections through the heart (ventricles) of the embryos shown in A and B, respectively. Panels show EYFP (C, D), Endomucin (E, F) immunohistochemistry, and overlaid images (G, H). Scale bars: 1000 μm (A, B), and100 μm (C-H).

Figures 7A-C. Co-differentiation of Etv2 mutant and wild-type ES cells. 7AC5, EYFP-labeled otherwise wild-type ES cells and Etv2 mutant ES cells ($Etv2^{-/-}$) were either differentiated separately (top two rows) or mixed and co-differentiated (bottom row) by the hanging drop method. Cells were dissociated at day 4 and analyzed for cell surface markers. Note that EYFP negative Etv2 mutant cells do not contribute to the endothelial and hematopoietic lineages, indicating that there is minimal paracrine effect from the wild-type ES cells.

Figures 8A-C. Wild-type rat ES cells rescues the endothelial potential of *Etv2* mutant mouse ES cells. Wild-type mouse ES cells **(A)**, *Etv2* mutant mouse ES cells **(B)**, or *Etv2* mutant mouse ES cells and wild-type rat ES cells at 1:1 ratio **(C)** were induced to differentiate by EB formation. Note that wild-type mouse ES cells generate $CD31^+$ cells that line the vessel-like structures (A), which are missing in the mutant EBs (B). Co-differentiation with wild-type rat ES cells restored the $CD31^+$ population (C).

Figure 9A-B. Chimeric human-porcine blastocysts. (A) Blastocyst with hUCBSCs in the ICM. Note that the blastocyst is beginning to hatch. (B) Blastocyst with DiI-labeled hiPSCs in the ICM. Note adjacent hatched blastocyst with DiI-labeled hiPSCs. ZP: zona pellucida; ICM: inner cell mass.

Detailed Description of the Invention

Described herein is the development of ETV2 (or ETV2 in combination with other gene knockouts) knockout animals, such as pigs, as hosts for production of personalized human/humanize blood and vasculature for clinical and preclinical applications. In addition to serving as a novel source of human/humanized tissues for the treatment of cardiovascular and hematopoietic disease, the humanized animals, such as pigs, will also serve as a large animal model to study the regeneration of human lineages or response(s) to pharmacological agents.

Blood and cardiovascular diseases are both common and deadly (1-3). These diseases are chronic, debilitating, and lethal and they warrant novel therapies. The blood and cardiovascular developmental programs have a number of overlapping features as both are lateral plate mesodermal derivatives and both are co-regulated by intersecting networks of transcription factors, signaling cascades and extracellular cues. Recent studies suggest that a common progenitor daughters the hematoendothelial lineages. Etv2/Etsrp71/ER71 is a target of traditional cardiovascular and hematoendothelial transcription factors and signaling cascades including: Mesp1, Flk1/Creb, and Nkx2-5. It has been demonstrated that Etv2 regulates the specification and differentiation of hematoendothelial lineages (4-17). In addition, Etv2 mutant mouse embryos were nonviable and had perturbed mesodermal (hematopoietic and endothelial) lineage development (9, 10). The data described herein support that ETV2 mutant porcine embryos are also nonviable and lack hematopoietic and endothelial lineages. Based on the results, Etv2 is

a master regulator for the hematoendothelial lineages during development. Described herein are ETV2 knockout pigs as hosts for production of personalized human blood and vasculature for clinical applications. This humanized large animal model will be an important resource for regenerative medicine and will serve as a platform for generating personalized humanized porcine organs. This strategy has the capacity to have a profound impact on the development of emerging therapies for chronic cardiovascular and hematopoietic diseases and transplantation. In addition to serving as a novel source of human tissues for the treatment of cardiovascular and hematopoietic disease, the humanized pigs will also serve as a large animal model to study the regeneration of human lineages or response(s) to pharmacological agents.

Definitions:

In describing and claiming the invention, the following terminology will be used in accordance with the definitions set forth below. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention. Specific and preferred values listed below for radicals, substituents, and ranges are for illustration only; they do not exclude other defined values or other values within defined ranges for the radicals and substituents.

As used herein, the articles "a" and "an" refer to one or to more than one, i.e., to at least one, of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

The term "about," as used herein, means approximately, in the region of, roughly, or around. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. In general, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 20%.

The term "isolated" refers to a factor(s), cell or cells which are not associated with one or more factors, cells or one or more cellular components that are associated with the factor(s), cell or cells *in vivo*.

"Cells" include cells from, or the "subject" is, a vertebrate, such as a mammal, including a human. Mammals include, but are not limited to, humans, farm animals, sport animals and companion animals. Included in the term "animal" is dog, cat, fish, gerbil, guinea pig, hamster, horse, rabbit, swine, mouse, monkey (e.g., ape, gorilla, chimpanzee, or orangutan), rat, sheep, goat, cow and bird.

The terms "pig" and "swine" and "porcine" are used interchangeably are generic terms referring to the same type of animal without regards to gender, size or breed.

Transcription Activator-Like Effector Nucleases (TALENs) are artificial restriction enzymes generated by fusing the TAL effector DNA binding domain to a DNA cleavage domain. These reagents enable efficient, programmable, and specific DNA cleavage for genome editing *in situ*. Transcription activator-like effectors (TALEs) are proteins that bind DNA in a sequence specific way. By fusing such

a TALE to a nuclease (e.g., FokI endonuclease) a highly specific DNA "scissor" is made (these molecules can be engineered to bind any DNA sequence). The term TALEN, as used herein, is broad and includes a monomeric TALEN that can cleave double stranded DNA without assistance from another TALEN. The term TALEN is also used to refer to one or both members of a pair of TALENs that are engineered to work together to cleave DNA at the same site. TALENs that work together may be referred to as a left-TALEN and a right-TALEN, which references the handedness of DNA.

Once the TALEN genes have been assembled they are inserted into plasmids; the plasmids are then used to transfect the target cell where the gene products are expressed and enter the nucleus to access the genome. TALENs can be used to edit genomes by inducing double-strand breaks (DSB) and optionally inserting a cargo/preselected gene, which cells respond to with repair mechanisms. In this manner, they can be used to correct mutations in the genome which, for example, cause disease.

Genetic engineering, including gene editing, can be carried out by any method available to an art worker, for example, by the use of tartgeted endonucleases, and homology directed repair (HDR), TALEN, CRISPR (e.g., CAS9/CRISPR), recombinase fusion molecules, synthetic porcine artificial chromosomes, meganucleases, zinc finger or rAAV based systems for gene editing (e.g., to knockout desired target genes). Further, a variety of nucleic acids can be introduced into cells, for knockout purposes, for inactivation of a gene (such as interfering RNAs (shRNA, siRNA, dsRNA, RISC, miRNA) or express a gene.

Somatic cell nuclear transfer (SCNT) is a laboratory technique for creating a viable embryo from a body cell and an egg cell. The process of somatic cell nuclear transplant involves two different cells. The first being a female gamete, known as the ovum (egg/oocyte). The second being a somatic cell, referring to the cells of the human body. Skin cells, fat cells, and liver cells are only a few examples. The nucleus of the donor egg cell is removed and discarded, leaving it 'deprogrammed.' The nucleus of the somatic cell is also removed but is kept, the enucleated somatic cell is discarded. What is left is a lone somatic nucleus and an enucleated egg cell. These are then fused by squirting the somatic nucleus into the 'empty' ovum. After being inserted into the egg, the somatic cell nucleus is reprogrammed by its host egg cell. The ovum, now containing the somatic cell's nucleus, is stimulated with a shock and will begin to divide. The egg is now viable and capable of producing an adult organism containing all the necessary genetic information from just one parent. Development will ensue normally and after many mitotic divisions, this single cell forms a blastocyst (an early stage embryo with about 100 cells) with an identical genome to the original organism (i.e. a clone). Stem cells can then be obtained by the destruction of this clone embryo for use in therapeutic cloning or in the case of reproductive cloning the clone embryo is implanted into a host mother (pseudopragnant/surrogate) for further development and brought to term.

"Chimera" refers to is a single organism composed of genetically distinct cells.

"Humanized" refers to an organ or tissue harvested from a non-human animal whose protein sequences and genetic complement are more similar to those of a human than the non-human host.

"Organ" refers to a collection of tissues joined in a structural unit to serve a common function. "Tissue" as used herein refers to a collection of similar cells from the same origin that together carry out a specific function.

A nullizygous organism carries two mutant or missing alleles for the same gene. The mutant/missing alleles are both complete loss-of-function or 'null' alleles, so homozygous null and nullizygous are synonymous.

A gene knockout (abbreviation: KO) is a genetic technique in which both of an organism's alleles are made inoperative ("knocked out" of the organism). The term knockout, inactivated, and disrupted are used interchangeably herein to mean that the targeted site is changed so that the gene expression product is eliminated or greatly reduced. Also known as knockout organisms or simply knockouts. The term also refers to the process of creating such an organism, as in "knocking out" a gene. The technique is essentially the opposite of a gene knockin.

The term gene is broad and refers to chromosomal DNA that is expressed to make a functional product. Genes have alleles. Gene editing may be mon-allelic or bi-allelic.

The terms "comprises," "comprising," and the like can have the meaning ascribed to them in U.S. Patent Law and can mean "includes," "including" and the like. As used herein, "including" or "includes" or the like means including, without limitation.

Exogenic organ production is needed to meet the demand for organ transplants.

Currently, the only definitive therapy for advanced end stage organ failure is transplantation. The limiting factor for transplantation is donor organ availability. Hundreds of thousands of patients could benefit from such therapy, but are not suitable transplant candidates due to their comorbid diseases. Therefore, there is a significant shortage of cadaveric or living-related donor organs. Furthermore, transplantation of organs requires lifelong immunosuppression which also has deleterious side effects. Herein the generation of humanized tissues in pigs is described, which will serve as an unlimited source of organs for transplantation and provide a paradigm shifting platform for the treatment of cardiovascular and hematopoietic diseases.

Intense interest has focused on xenogenic transplantation. For example, a rat pancreas was produced in a mouse by the process of blastocyst complementation (27). In these studies, blastocysts mutant for Pdx1, the master regulatory gene for pancreatic development, were injected with pluripotent stem cells from wild-type (Wt) rat (rPSCs) (27). Transfer of the rPSC-injected blastocysts into surrogate mouse dams gave rise to mouse chimeras with functional pancreata composed of rat cells. These studies emphasized the importance of generating blastocysts deficient for a key developmental regulatory factor, so that the embryo completely lacks the target organ. These mutant hosts then provide a developmental "niche", for the healthy donor stem cells to populate and generate a donor-derived organ. The blastocyst complementation strategy has also produced organs such as the kidney, thymus and liver in rodents, and recently the pancreas in pigs (28-31).

Using the gene-editing platform or any method available to an art worker, various developmental genes can be mutated to generate organ-deficient animals, such as pigs, upon which blastocyst complementation can be deployed for the generation of exogenic organs.

Etv2 (ENSSSCG00000002906) is a master regulatory gene for endothelial and hematopoietic lineages.

The *Etv2* gene locus was mutated to generate endothelial and hematopoietic deficient pig embryos for several reasons. First, the inventors have demonstrated that *Etv2* is a master regulatory gene for endothelial and hematopoietic development in mice (7-9). Using genetic lineage tracing strategies, it was demonstrated that Etv2 expressing cells give rise to endothelial and hematopoietic lineages (9, 10). Second, a global gene deletional strategy was undertaken and demonstrated that *Etv2* mutant mouse embryos were nonviable as they lacked endothelial and hematopoietic lineages (Fig. 1A, B) (8). Using transcriptome analysis, it was determined that *Tie2* was markedly dysregulated in the absence of Etv2 (7, 8). Moreover, using transgenic technologies and molecular biological techniques (transcriptional assays, EMSA, ChIP and mutagenesis), it was verified that *Spi1*, *Tie2* and *Lmo2* were direct downstream targets of Etv2 (7, 8, 11). Third, forced overexpression of Etv2 in the differentiating ES/EB system significantly increased the populations of endothelial and hematopoietic lineages, demonstrating that Etv2 is a single factor that has the capacity to govern molecular cascades that will induce both lineages (Fig. 1 C, D) (8).

Fourth, the functional role of Etv2 was defined in mesodermal lineage specification and the molecular pathways upstream of Etv2, as well as its downstream targets were defined. Microarray analysis of wild-type (Wt) and Etv2 mutant mice revealed that cardiac specific transcripts were overrepresented in Etv2 mutants, suggesting that Etv2 suppresses the differentiation of cardiac lineages (9). In support of this finding, overexpression of Etv2 suppressed cardiac differentiation in ES/EB cells while inducing the endothelial and hematopoietic programs. From these studies, a role for Etv2 was defined in the specification of the mesodermal lineages as a necessary and sufficient factor for endothelial and hematopoietic differentiation as well as an inhibitor of cardiac differentiation (Fig. 2) (9).

Several key findings regarding the molecular pathways involving Etv2 are summarized in Figure 2. First, the genetic hierarchy of Flk1 and Etv2 was determined (12). Flk1 mutant and Etv2 mutant ES cells were utilized, both unable to generate hematopoietic and endothelial lineages, and tested whether those phenotypes can be rescued by overexpression of Etv2 or Flk1. The results demonstrated that the endothelial and hematopoietic potential of Flk1 mutant ESCs/EBs were rescued by both Etv2 and Flk1, but the Etv2 mutant phenotype was rescued only by Etv2 and not by Flk1. This finding indicates that (1) Etv2 is genetically downstream of Flk1 and that (2) the down-regulation of Etv2 is responsible for the Flk1 mutant phenotype. Therefore, the hierarchy of Flk1→Etv2 in the endothelial progenitors was established. Second, Gata2 was identified as a co-factor of Etv2 function (Fig. 3D-F) (11). By overexpressing Gata2 and Etv2 in ES cells at a 1:1 stoichiometory, it was demonstrated that both hematopoietic and endothelial differentiation were enhanced compared to Etv2 alone. Gata2 by itself did not enhance differentiation, suggesting that it acts as an amplifier of Etv2 function. Third, recent studies

in mouse and zebrafish demonstrate that Etv2 regulates lineage restricted microRNAs that further govern the specification of endothelial and hematopoietic lineages (data not shown). In summary, upstream and downstream factors were defined, as well as co-factors that interact with Etv2 to promote the differentiation of hematopoietic and endothelial lineages (Fig. 3G).

These and other results support the rationale that Etv2 is a candidate for gene-editing in porcine models, as the studies predict that Etv2 mutant animals will completely lack the endothelial and hematopoietic lineages and provide a niche for human stem cells to populate. The compositions and methods described herein will yield a vascular system that will be lined or populated with human endothelial cells, making it an ideal tissue to transplant. Moreover, human blood will be produced in a porcine surrogate model.

The fifth reason that Etv2 is distinct from other genes emerges from studies of xenogenic transplantation. Although proof-of-principle studies of interspecific complementation and subsequent transplantation of exogenic organs have been successful, it was noted that vessels that serve those organs are host-derived (1, 2). These findings raise a significant concern, considering the well-known importance of vascular surfaces in organ rejection, specifically the hyperacute rejection of xenotransplanted pig tissues (13). In fact, humanization of the endothelial lineage is needed for the development of most exogenic organs. Etv2 is an ideal candidate for this purpose.

The humanized large animal model will be an important resource for regenerative medicine and will serve as a platform for making personalized organs. This strategy can transform the current clinical practice paradigms for chronic cardiovascular and hematopoietic diseases and transplantation. To date, exogenic transplantation of organs has been performed between mouse and rat (27, 29); and pig and pig (31), and no successful development of humanized organs in large animal models have been reported. Incorporated herein by reference is U.S. Provisional Application Serial No. 62/247,092.

The following examples are intended to further illustrate certain particularly preferred embodiments of the invention and are not intended to limit the scope of the invention in any way.

EXAMPLES

Materials and Methods

Sow/gilts: Domestic maternal female pigs (8-12 months old) will be used as embryo transfer recipients and cared for and maintained as regular domestic pigs in prospective gestation and farrowing under approved IACUC protocols.

Estrus Synchronization and Insemination: Sows will be given 6.8 mL of Matrix (altrenogest 2.2 mg/mL) mixed into their morning feed on days 11-22 of their estrus cycle to synchronize estrus. Lutalyse (2cc) will be administered IM on the last day of Matrix and four days later. Sows will be checked for estrus twice daily, starting day 6 after the end of Matrix administration. The sows will be inseminated with semen from selected boars up to three times after first detected in estrus. Sows will be checked for pregnancy between days 23-90 of gestation using either Doppler ultrasound or transabdominal ultrasound with a linear 5 mHz transducer. Neither form of ultrasound is invasive and does not harm the sow or

fetuses. Blood samples may be taken from the pregnant gilts/sows to determine if any diseases are present at the request of the veterinarian or for genetic analysis.

Embryo Transfer: Reconstructed cloned embryos are surgically transferred into uteri of asynchronous recipient female pigs. For surgical embryo transfer, anesthesia is induced with a combination of the following: ketamine (2 mg/kg), tiletamine/zolazepam (0.25 mg/kg), xylazine (1 mg/kg), and atropine (0.03 mg/kg; all from Iowa Veterinary Supply) General anesthesia will be maintained for the rest of the procedure with isoflurane or sevoflurane (5% induction, maintenance at 1–4% to keep at surgical plane). While in dorsal recumbence, the recipients are aseptically prepared for surgery and a caudal ventral incision is made to expose and examine the reproductive tract, including the uterus, oviducts and ovaries. Typically, 150-200 reconstructed cloned embryos are placed in the isthmus of the oviduct using a 5.5-inch TomCat® catheter (Iowa Veterinary Supply). The uterus is placed back into the peritoneal cavity, and the recipient animals are sutured and placed into postoperative recovery. During gestation, real-time ultrasound examination is used to confirm and monitor pregnancy using an Aloka 500 Ultrasound Scanner (Aloka Co. Ltd, Wallingford, CT) with an attached 3.5 MHz trans-abdominal probe. Recipient husbandry will be maintained as normal gestating sows. For piglet production, recipients will be allowed to farrow naturally or will be delivered by c-section prior to day 118 of gestation. Colostrum feeding and intensive neonatal support, including Nurtinger rearing units, are available when necessary.

***ETV2* knockout pig embryos lack hematopoietic and endothelial lineages.**

Previous studies have demonstrated that *Etv2* plays a role in vasculogenesis and hematopoiesis in the mouse, as embryos lacking *Etv2* are lethal at approximately E9.5 with an absence of vasculature and blood (7-10). To examine the role of ETV2 (ENSSSCG00000002906) in the pig, the entire *ETV2* coding sequence was removed using two TALEN pairs flanking the gene in porcine fibroblasts (Fig. 4).

1) ETV2 deletion strategy

TALENs:

ETV2 5-2 (from 5' to 3')

Left: CTGGCCGGAAATCCCC (SEQ ID NO:1)

Right: GGGCTGCACCAGGCT (SEQ ID NO:2)

ETV2 3-2 (from 5' to 3')

Left: GATCCCAAGTCACACC (SEQ ID NO:3)

Right: CCCCTAAGGGTCCTG (SEQ ID NO:4)

2) HDR Stitching template: This template fuses the 5' and 3' TALEN induced breaks in a predictable manor. It also increased the efficiency of recovering the deletion allele.

HR oligo

CGTCTGCTGACCAGGGGTCTGGCCGGAAATCCCCCTTCCTGTGGATCCaacagacacaggacccttagggg acctactgtgtgttcactg (SEQ ID NO:5)

Underline lower case = 42nt of homology from right side of ETV2 3.2 cut site

UPPER CASE BOLD = 42nt of homology from left side of ETV2 5.2 cut site

UPPER CASE UNDERLINED = Inserted BamHI site

Full sequence from ETV2 5' NJ F1 to ETV2 3' NJ R1: This is the predicted PCR product when the deletion allele is uses the above template for repair/fusion.

tgaagcagccccagaacttcctcctcaaagccctcgaaggggaaaacagcctggtggaagatcccaggtc
gaccaaccaaccccccaccatatcccccgcaggcccctgcggattgtga**CGTCTGCTGACCAGGGGTCTG**
GCCGGAAATCCCCCTTCCTGTGGATCCaacagacacaggacccttaggggacctactgtgtgttcactgt
gtggtgggccatgcagaggaatcaaattcagtagccactggcctgcctgctttgtgcctgccctgtactg
ggacttgtacatgaaacagacacaatcaataactttcgaatttacccactgtgtcccccttgagaggac
tcaagattccaaagagggcttactgtgtaccctccctgtgccggggccatcagcgaattagacctggtgc
ttgccccccagtcacctattctgttttcctacttcaagctaagggccatagaacttagatcccaaggaa
agtctaccctgttctgggaacaactgagcgctta(SEQ ID NO:6)

3) Screening Primers:

ssETV2 5' NJ F1: TGAAGCAGCCCCAGAACTTC (SEQ ID NO:7)

ssETV2 5' NJ R1: TGGCCTCCAGTGTCCTTTTC (SEQ ID NO:8)

ssETV2 3' NJ F1: TAGCCTATCCCGACCGCAT (SEQ ID NO:9)

ssETV2 3' NJ R1: TAAGCGCTCAGTTGTTCCCA (SEQ ID NO:10)

The process was 15% efficient at complete gene removal; 79/528 of the genotyped clones were homozygous for the deletion of the ETV2 gene. ETV2 homozygous knockout fibroblast clones were used for nuclear cloning (Somatic Cell Nuclear Transfer; SCNT) to generate ETV2 null embryos which were transferred to surrogate sows. The cloning efficiency was 29%.

Embryos were harvested and analyzed at E18.0 (Fig. 5). At E18.0, wild-type (Wt) embryos were vascularized with a well-developed vascular plexus in the allantois (Fig. 3A) and had evidence of blood development (Fig. 5C). In contrast, ETV2 KO embryos showed clear developmental defects. Growth was retarded in ETV2 KOs relative to the Wt embryo, though both embryos were at the 24-somite stage (Fig. 5B), and lacked both blood and vascular lineages (Fig. 7C-H). ETV2 KO embryos lacked cardinal veins, dorsal aortae, and the endocardium, that are clearly developed in the Wt embryos (Fig. 5E-H).

The results in Figures 1 and 5 reflect a similar phenotype and suggest that the function of ETV2 is conserved between mice and pigs. Further, these data demonstrate that one can direct multiple mutations into the porcine genome to support growth of chimeric organs that will be humanized in more than one cell type.

As an alternative to deleting ETV2, a mutation can be entered in to the gene, such as a frame shift mutation, wherein any protein produced would be non-functional. For example:

1) Frame-shift KO allele: In this example, a frame-shift is created in exon3 and a premature stop codon.

1) TALENs:

ETV2 3.1 (from 5' to 3')

Left: TCATCCTTATCTGTCC (SEQ ID NO:11)

Right: GCGGAGACCCAGTCC (SEQ ID NO:12)

ETV2 3.3 (from 5' to 3')

Left: TACCGAACCCAGAAG (SEQ ID NO:13)

Right: ACTGTGGGAGACACTCA (SEQ ID NO:14)

2) HDR template:

ssETV2 3.1 HR-KO

cctccctaaactcagcttcatccttatctgtcccagggat**tc***TAAGCTT*cacagccggactgggtctccg cattaccgaacccagaagct (SEQ ID NO:15)

lower case bold underlined = ssETV2 3.1 cut site

UPPER CASE ITALICS = stop codon

Capital letters = inserted bases

Underlined = inserted HindIII restriction site

ssETV2 3.3 WT sequence

cggactgggtctccgcattaccgaacccagaagctccatg**G**ggcgcgggtgagtgtctcccacagtaact ggaggtttcgatt (SEQ ID NO:16)

UPPER CASE BOLD UNDERLINED = ssETV2 3.3 cut site

ssETV2 3.3 HR-KO

cggactgggtctccgcattaccgaacccagaagctccatg**g***TAAGCTT*ggcgcgggtgagtgtctcccac agtaactggaggtttcgatt (SEQ ID NO:17)

lower case BOLD UNDERLINED = ssETV2 3.3 cut site

UPPER CASE ITALICS = stop codon

Capital letters = inserted bases

Underlined = inserted HindIII restriction site

3) Screening Primers:

ssETV2 E3 F4: CACAACTCTCGTCCCGAACA (SEQ ID NO:18)

ssETV2 E3 R4: GAACGGACCCCAAGTGAGAG (SEQ ID NO:19)

The Etv2 mutant embryos are rescued by wild-type mouse ES cells.

The underlying assumption for generating humanized tissue in pigs is that the injected cells will preferentially populate the developmental niche in the mutant host and generate the missing cell types. As a proof-of-principle study and to evaluate whether Etv2 is an ideal target gene, mouse Etv2 mutant blastocysts were complemented with EYFP-labeled wild-type mouse ES cells (Fig. 6). To positively identify mutant alleles in the presence of wild-type cells, hemizygous mice of two distinct mutant lines of Etv2 were bred (unpublished data). Embryos were collected at E10.5 (one day later than the observed lethality of the Etv2 mutant embryo), genotyped and the distribution of EYFP positive cells and an

endothelial marker, endomucin, were examined. In both Etv2 hemizygous and Etv2 mutant embryos, wild-type ES cells successfully integrated into the embryos (Fig. 6A, B). Immunohistochemical analysis revealed that EYFP-labeled wild-type cells randomly distributed to multiple cells types of all germ layers of hemizyogous animals (Fig. 6C, E, G), whereas, in the Etv2 mutants, the majority of EYFP positive cells were found in the endothelial, endocardial and hematopoietic lineages (Fig. 6D, F, H). The data supported the notion that all endomucin positive endothelial cells expressed EYFP, indicating that they were derivatives from the wild-type ES cells (Fig. 6D, F, H). Etv2 mutant embryos also had EYFP-labeled Tie2-positive cells with primitive blood-like morphology inside the vessels, suggesting that the hematopoietic lineage is also rescued (data not shown).

To further corroborate this finding, EYFP-labeled wild-type and Etv2 mutant ES cells were utilized (8), differentiated separately or together and examined the contribution of each line to the hematopoietic (CD41, CD45) and the endothelial (CD31, Tie2) lineages at day 4 of differentiation using FACS (Fig. 8). The wild-type 7AC5/EYFP cells were 99% EYFP positive, and generated 4.54% endothelial, 0.36 % hematopoietic cells (upper row). Etv2 mutant cells were EYFP negative, and generated no endothelial and no hematopoietic cells (middle row). Analysis of co-differentiated EBs revealed that contribution of EYFP negative cells to the endothelial and hematopoietic lineages were indistinguishable from that of the Etv2 mutant ES cells, and EYFP positive cells generated the endothelial and hematopoietic lineages at a similar efficiency as wild type cells cultured alone (bottom row). This result indicated that "rescued" endothelial and hematopoietic lineages in the ES/EB co-differentiation system are derived exclusively from the wild-type ES cells. These results provide the rationale to target porcine ETV2 to generate the host animal for humanized endothelial and hematopoieitic lineages.

Rat embryonic stem cells rescue the endothelial populations in Etv2 mutant mouse embryo bodies.

It was then tested whether complementation of the Etv2 null ES/EBs is possible using two different species. An *in vitro* ES/EB complementation assay was performed using Etv2 mutant mouse ES cells and wild-type rat ES cells (Fig. 7) (22). Wild-type or Etv2 mutant mouse ES cells were induced to differentiate by embryoid body (EB) formation by plating ES cells on ultra-low attachment plates in differentiation media. After 12 days of culture, EBs were fixed, sectioned and stained for an endothelial marker, CD31 (8). A strong $CD31^+$ population was observed lining the vessel-like structures in wild-type mouse EBs (Fig. 7A), but no $CD31^+$ cells were observed in the Etv2 mutant EBs (Fig. 7B). In contrast, co-differentiation cultures of Etv2 mutant ES cells and wild-type rat ES cells, revealed patches of cells with robust CD31 expression, indicating that $CD31^+$ population was rescued by this co-culture method (Fig. 7C). This success in mouse-rat complementation assay provides a proof-of-concept and the rationale to further progress to pig-pig and pig-human complementation experiments.

Human umbilical cord blood stem cells (hUCBSC) and hiPSC integrate into the inner cell mass (ICM) of porcine parthenotes (embryos electrically activated to develop without fertilization).

To evaluate the feasibility of the strategy to generate human-pig chimeras, the capacity of hUCBSC and hiPSC to integrate into the porcine blastocysts and participate in embryonic development was examined. Porcine parthenogenetic blastocysts were generated using electrical stimulation of oocytes (42). Six days following activation 9-12 DiI- or EdU (24 hr)- labeled hUCBSC or hiPSC were injected into the blastocoel cavity. Blastocysts were allowed to recover two days in culture and then imaged. Labeled hUCBSCs and hiPSCs were observed in the ICM of 90% of the porcine blastocysts (Fig. 9A, B, representative images are shown). Comparison of DiI distribution with immunohistochemistry using human nuclear antigen-specific antibody (HNA) reveals that HNA antibody detects injected human stem cells (Fig. 9A, arrows). Blastocysts injected with EdU labeled hiPSC were further pulsed with BrdU for 1 hour before harvest to detect proliferating cells. Double labeling with EdU reveals that injected human stem cells continued to proliferate after 48 hrs of injection (Fig. 9B, arrows). These results demonstrate the incorporation of human stem cells into the ICM of porcine blastocysts, and the developmental progression of the chimeric blastocysts to the hatching stage in preparation for implantation into the uterus. To examine the integration of human stem cells into porcine parthenogenetic embryos, the chimeric blastocysts were transplanted into pseudopregnant sows and analyzed embryos at E28. Normal developing embryos were obtained as previously reported (30), one of which was found to contain a cluster of human cells that stained with the HNA antibody. These results support and provide a rapid assay to examine whether human stem cell populations are compatible and/or contribute to the ICM development. Furthermore, implantation of parthenogenetic blastocysts provides a high-throughput method to examine integration and differentiation of human stem cells into developing embryos. A significant advantage of this strategy is that porcine oocytes are abundantly available as a bi-product of food production, and parthenogenetic embryos can be generated in large quantities on a regular basis. It should be noted that parthenogenetic embryos do not survive past 8 weeks, and therefore negates the concern of inadvertently giving birth to undesired human-porcine chimeras.

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The invention is described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within its scope. All referenced publications, patents and patent documents are intended to be incorporated by reference, as though individually incorporated by reference.

WHAT IS CLAIMED IS:

1. A non-human animal cell or blastocyst wherein the genome carries a mutation in both alleles of the ETV2 gene such that the non-human animal cell or blastocyst lacks functional ETV2 protein.

2. The non-human animal or blastocyst of claim 1, wherein the mutation is a deletion of the ETV2 gene.

3. The non-human animal cell or blastocyst of claim 1 or 2, wherein the non-human animal cell or blastocyst is a porcine, bovine, equine or goat.

4. A chimeric non-human animal or blastocyst expressing human ETV2 and lacking expression of said non-human animal ETV2.

5. The chimeric non-human animal of claim 4, wherein the non-human animal expresses human blood cells selected from the group consisting of white blood cells, red blood cells, platelets or a combination thereof.

6. The chimeric non-human animal of claim 4, wherein the chimeric non-human animal expresses human endothelium.

7. The chimeric non-human animal of any one of claims 4-6, wherein the non-human animal is a porcine, bovine, equine or goat.

8. A method for producing a chimeric non-human animal expressing a human ETV2 gene comprising:

a) generating an ETV2 null non-human animal cell, wherein both copies of the non-human ETV2 gene carry a mutation that prevents production of functional ETV2 protein in said non-human animal;

b) creating an ETV2 null non-human blastocyst by somatic cell nuclear transfer comprising fusing a nucleus from said ETV2 null non-human animal cell of a) into an enucleated non-human oocyte and activating said oocyte to divide so as to form an ETV2 null non-human blastocyst;

c) introducing human stem cells into the ETV2 null non-human blastocyst of b); and

d) implanting said blastocyst from c) into a pseudopregnant surrogate non-human animal to generate a chimeric non-human animal expressing human ETV2.

9. A method of producing human and/or humanized blood cells or vessels in a non-human animal comprising:

a) generating an ETV2 null non-human animal cell, wherein both alleles of the non-human ETV2 gene carry a mutation that prevents production of functional ETV2 protein;

b) creating an ETV2 null non-human blastocyst or morula by somatic cell nuclear transfer comprising fusing a nucleus from said ETV2 null non-human animal cell of a) into an enucleated non-human oocyte and activating said oocyte to divide so as to form an ETV2 null non-human blastocyst;

c) introducing human donor stem cells into the ETV2 null non-human blastocyst of b); and

d) implanting said blastocyst or morula from c) into a pseudopregnant surrogate non-human animal so as to generate a non-human animal expressing human and/or humanized blood cells or vessels.

10. The method of claim 8 or 9, wherein the non-human animal is a porcine, bovine, equine or goat.

11. The method of claim 8 or 9, wherein the human donor stem cell is a tissue specific stem cell, pluripotent stem cell, multipotent adult stem cell, induced pluripotent stem cell or umbilical cord blood stem cell (UCBSC).

12. The method of claim 9, wherein the donor providing the stem cells is the recipient of the humanized tissue or organ produced.

13. The method of claim 9, wherein the human induced pluripotent cell is formed from a fibroblast cell.

14. A non-human animal produced by the method of claim 8 or 9.

15. A progeny non-human animal produced from the mating of two non-human animals generated from method of claims 8 or 9, wherein said progeny non-human animal expresses human ETV2, wherein the genome of said progeny non-human carries a homozygous deletion of said non-human animal ETV2 gene.

16. The progeny non-human animal of claim 15, wherein the non-human animal is a porcine, bovine, equine or goat.

17. The method of claim 9, wherein the blood cells are human-induced pluripotent or human umbilical cord blood stem cell-derived white blood cells, red blood cells, and/or platelets.

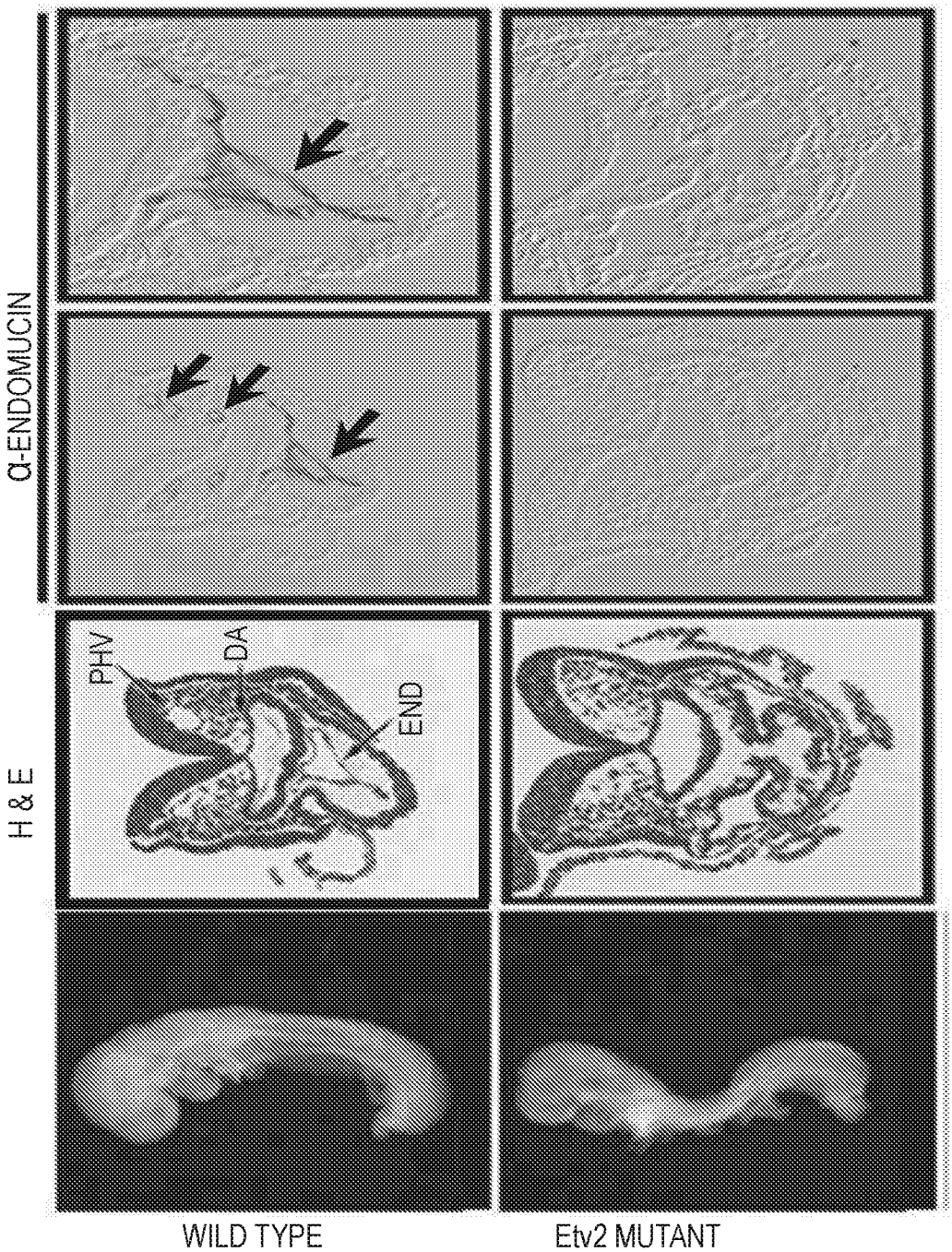


FIG. 1A

GENOTYPE	WT	HET	MUTANT
Ery-P	14 ± 5	11 ± 5	0
BFU-E	23 ± 8	27 ± 5	0
CFU-GM	9 ± 9	11 ± 6	0
CFU-G	3 ± 3	3 ± 2	0
CFU-M	18 ± 10	28 ± 19	0
CFU-GEMM	63 ± 27	55 ± 19	0
TOTAL	116 ± 39	123 ± 39	0

FIG. 1B

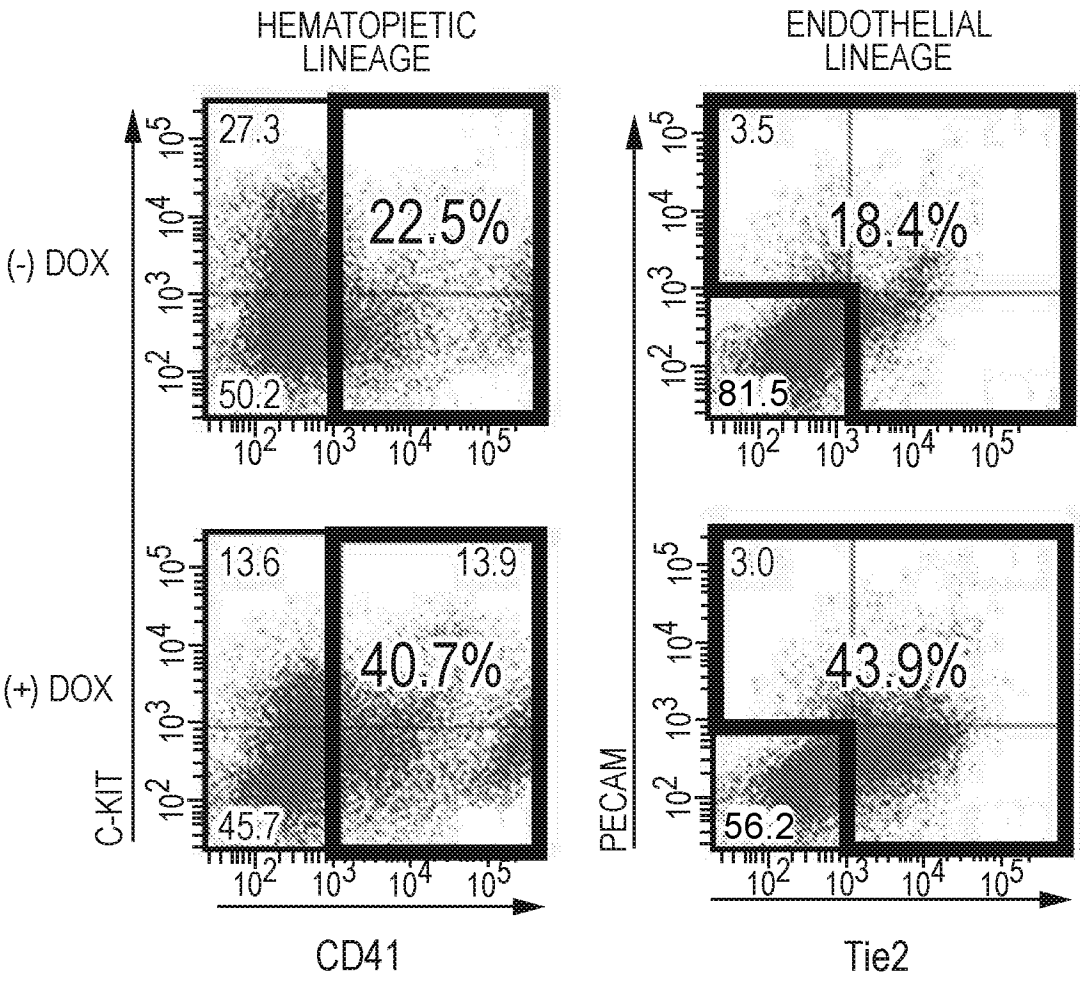
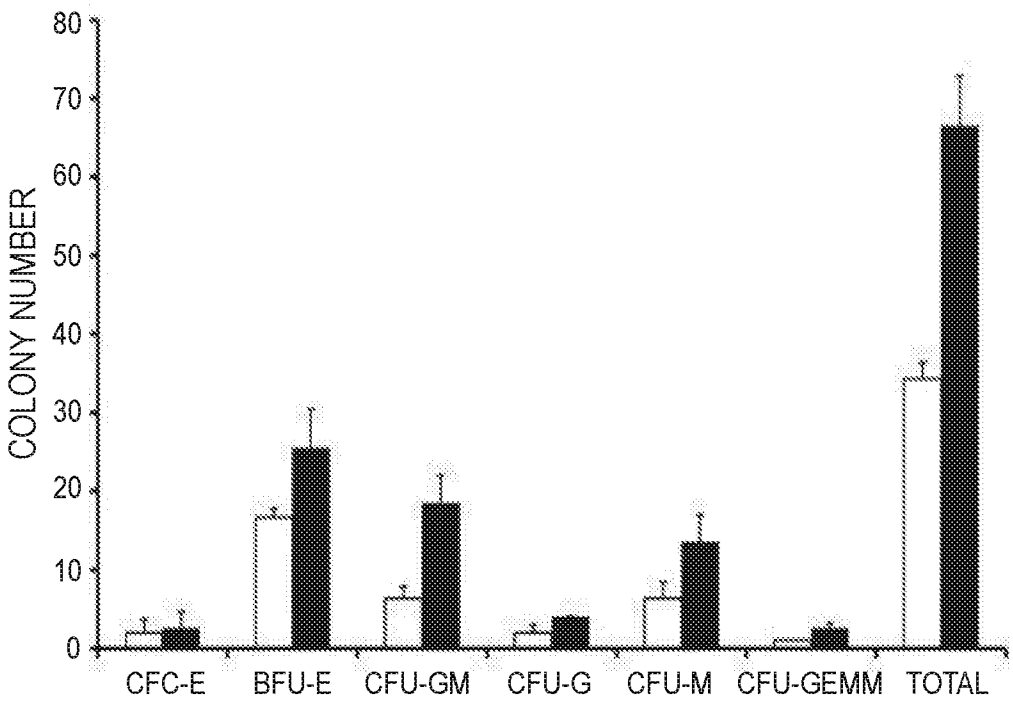

HEMATOPIETIC LINEAGE
ENDOTHELIAL LINEAGE
(-) DOX
(+) DOX
C-KIT
PECAM
CD41
Tie2
27.3
22.5%
50.2
3.5
18.4%
81.5
13.6
13.9
40.7%
45.7
3.0
43.9%
56.2

FIG. 1C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70
60
50
40
30
20
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CFC-E
BFU-E
CFU-GM
CFU-G
CFU-M
CFU-GEMM
TOTAL

FIG. 1D

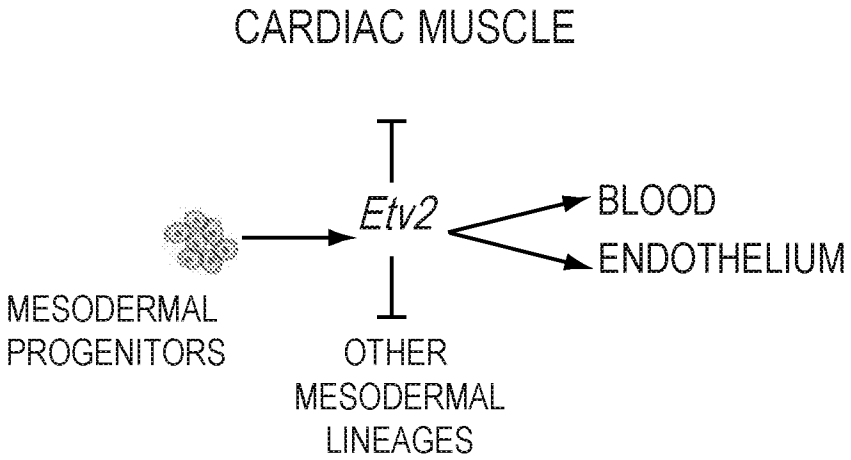
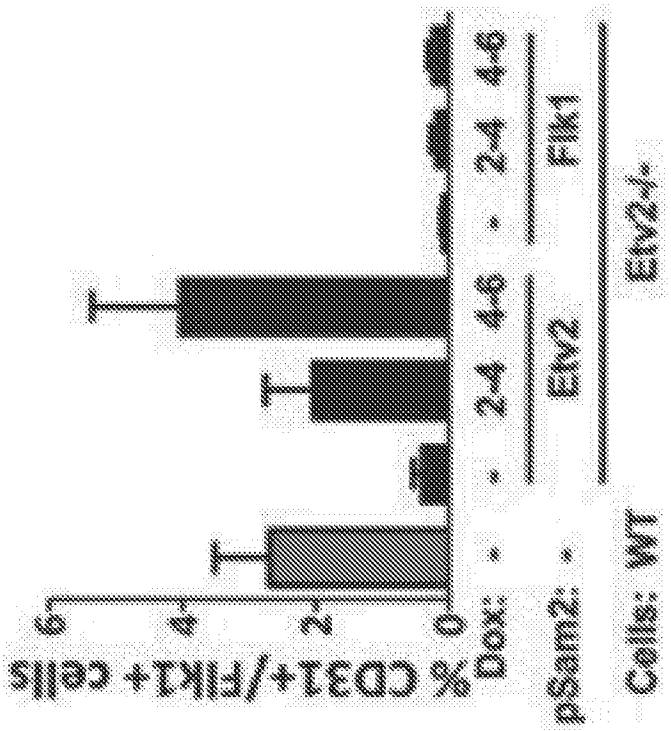

CARDIAC MUSCLE
Etv2
BLOOD
ENDOTHELIUM
MESODERMAL PROGENITORS
OTHER MESODERMAL LINEAGES

FIG. 2


% CD31+/Flk1+ cells
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2
4
6
Dox: - + - 24 48 - 24 48
pSam2: - Etv2 Flk1
Cells: WT Etv2-/-

FIG. 3B

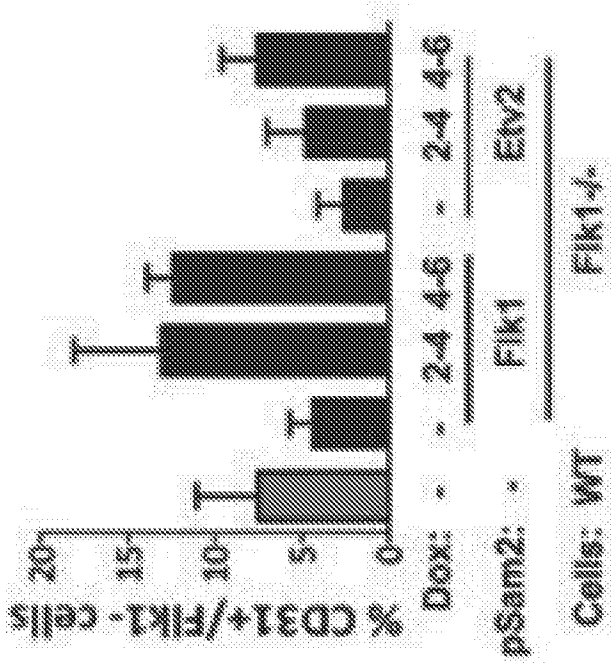

% CD31+/Flk1- cells
0
5
10
15
20
Dox: - + - 24 48 - 24 48
pSam2: - Flk1 Etv2
Cells: WT Flk1-/-

FIG. 3A

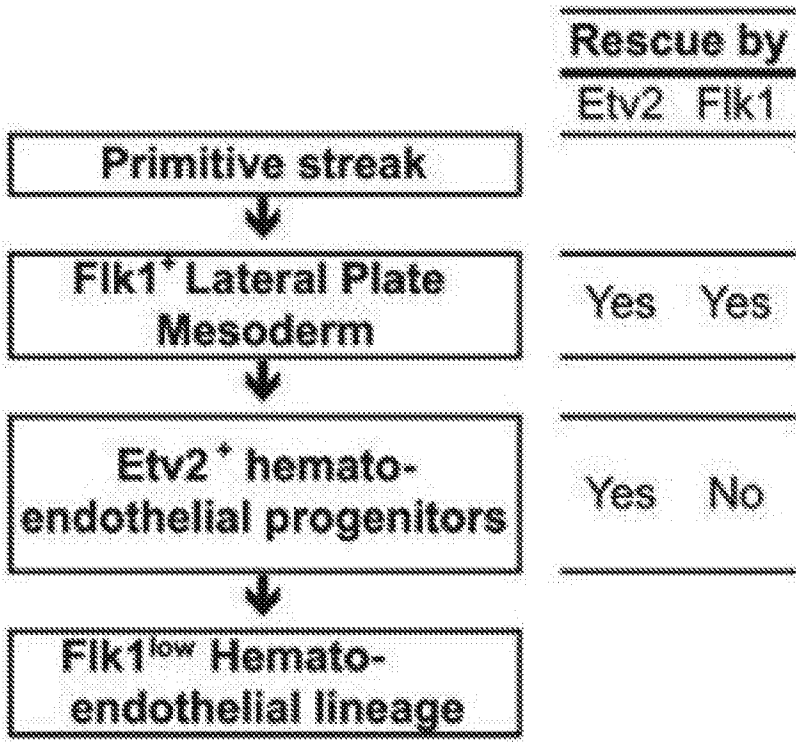

Rescue by
Etv2 Flk1
Primitive streak
Flk1+ Lateral Plate Mesoderm
Yes Yes
Etv2+ hemato-endothelial progenitors
Yes No
Flk1low Hemato-endothelial lineage

FIG. 3C

FIG. 3D

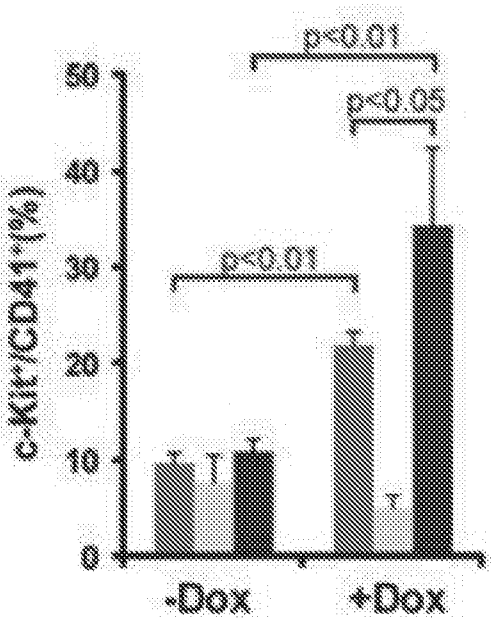


FIG. 3E

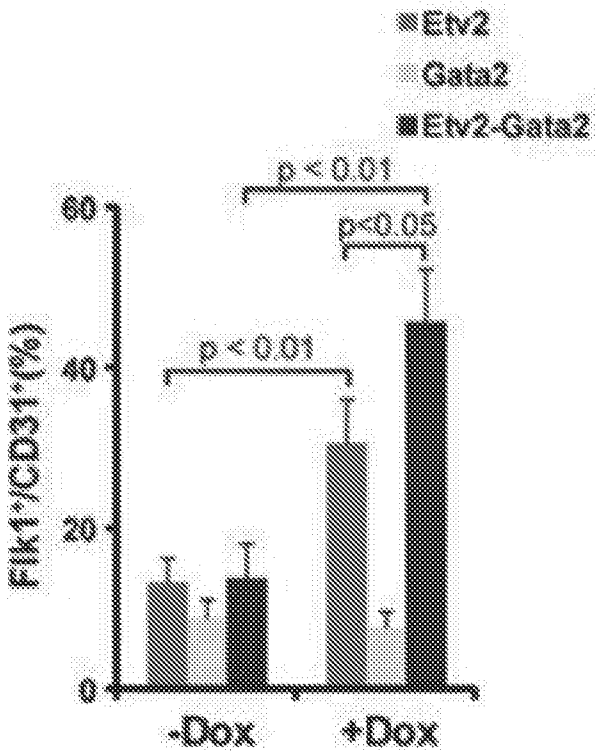


FIG. 3F

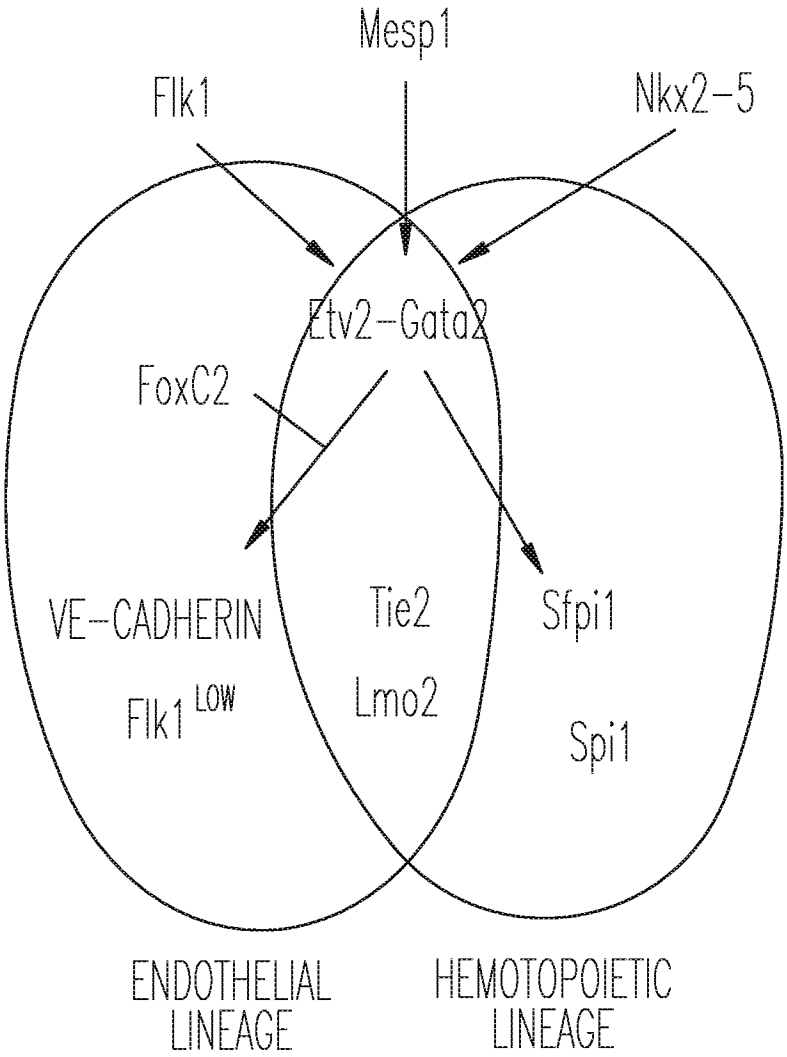
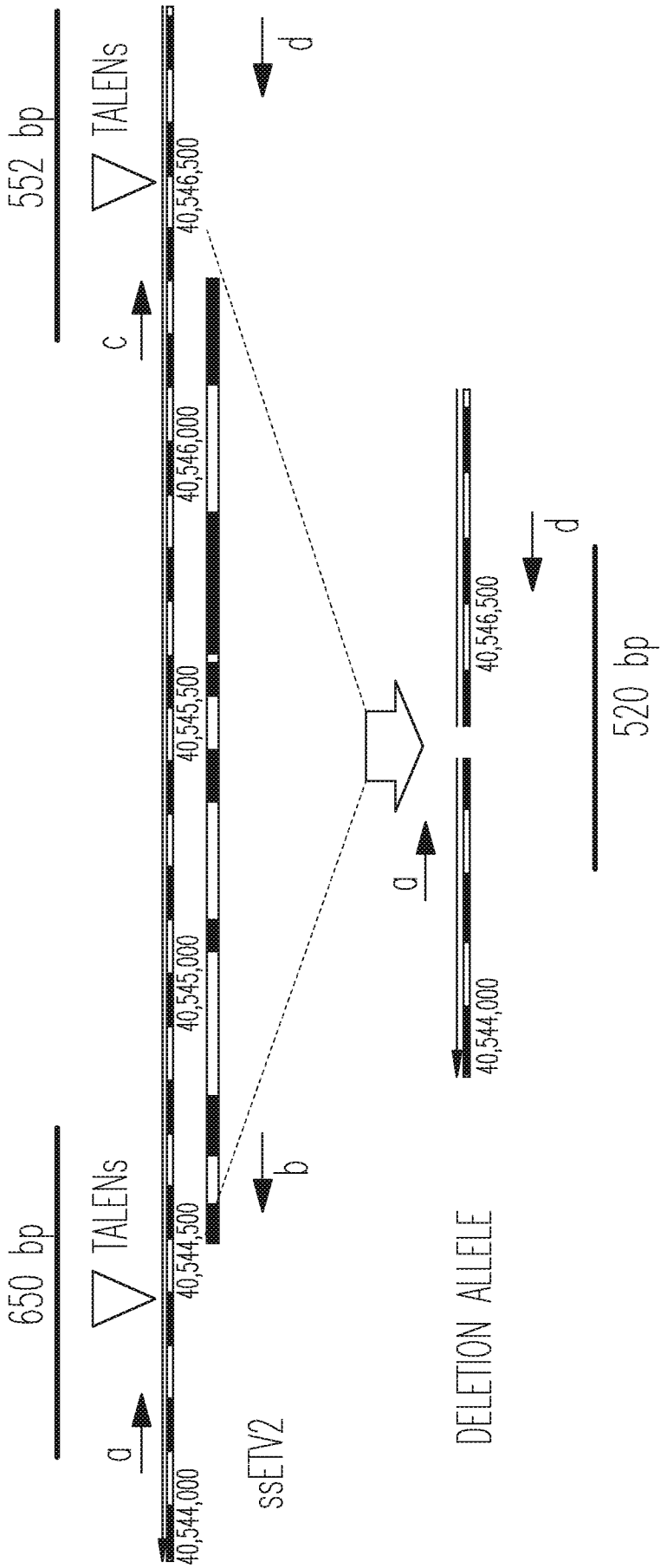

Mesp1
Flk1
Nkx2-5
Etv2-Gata2
FoxC2
VE-CADHERIN
Flk1 LOW
Tie2
Lmo2
Sfpi1
Spi1
ENDOTHELIAL LINEAGE
HEMOTOPOIETIC LINEAGE

FIG. 3G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TALENs
a
b
c
d
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ssETV2
DELETION ALLELE
520 bp

FIG. 4A

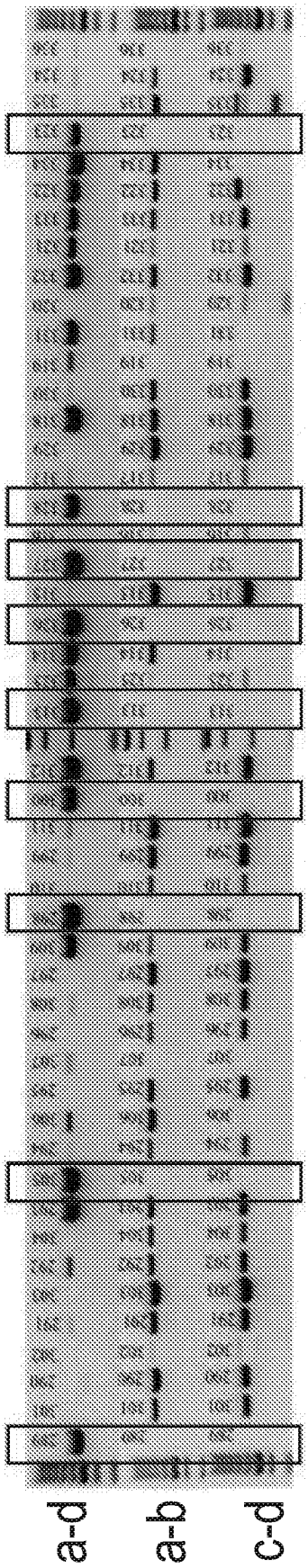

FIG. 4B

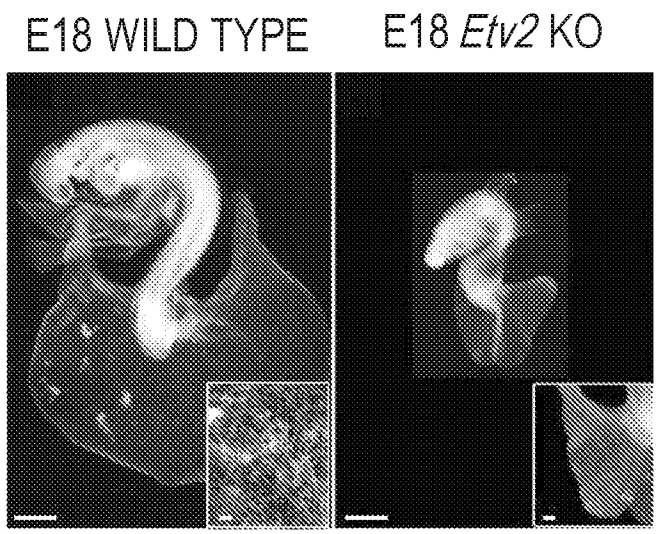

FIG. 5A FIG. 5B

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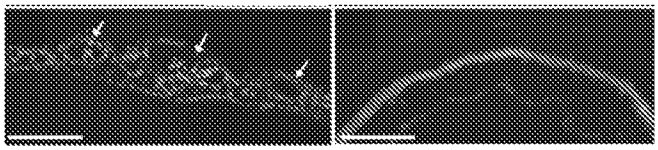


FIG. 5C FIG. 5D

Tie2/Gata4/DAPI

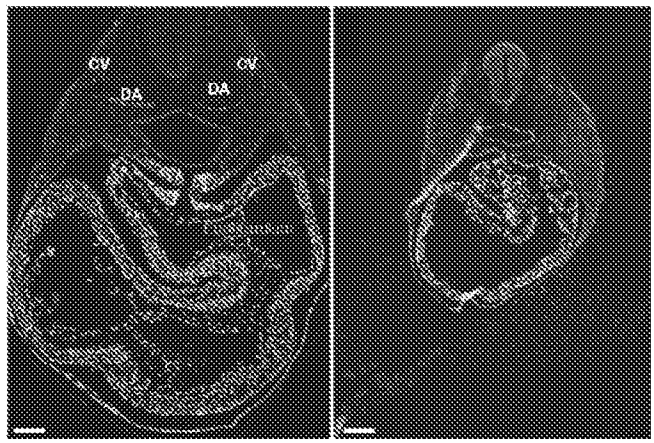


FIG. 5E FIG. 5F

Tie2/Gata4/DAPI

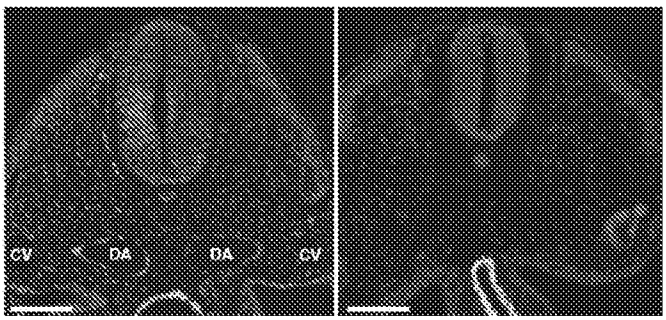


FIG. 5G FIG. 5H

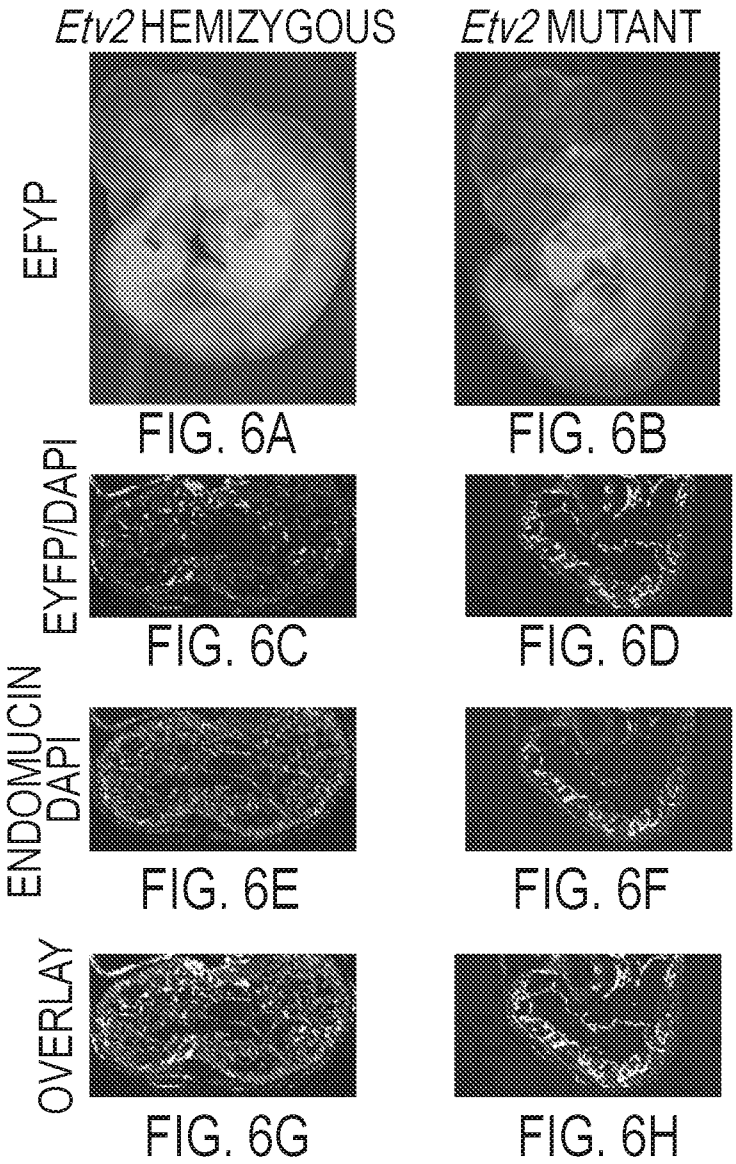


FIG. 6A FIG. 6B

FIG. 6C FIG. 6D

FIG. 6E FIG. 6F

FIG. 6G FIG. 6H

100% WT MOUSE

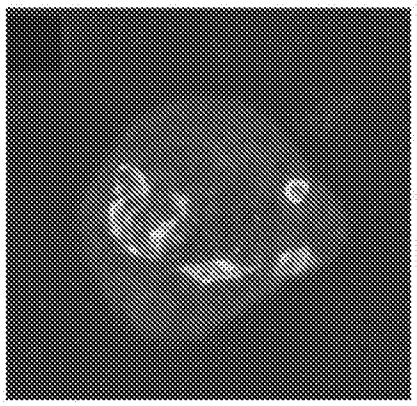


FIG. 7A

100% *Etv2*$^{-/-}$ MOUSE

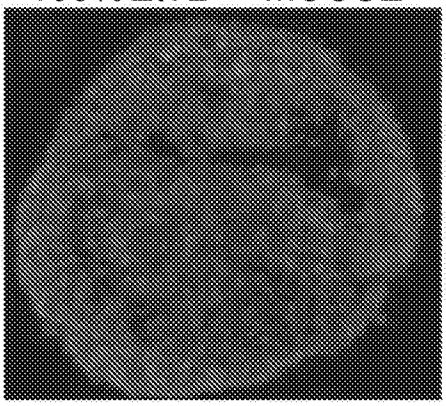


FIG. 7B

50% *Etv2*$^{-/-}$ MOUSE
50% WT RAT

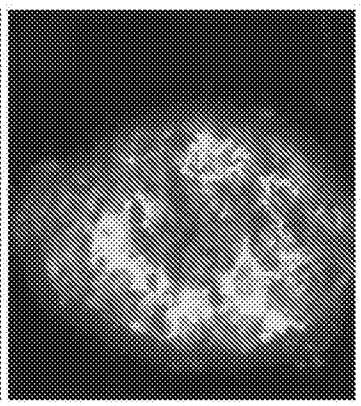
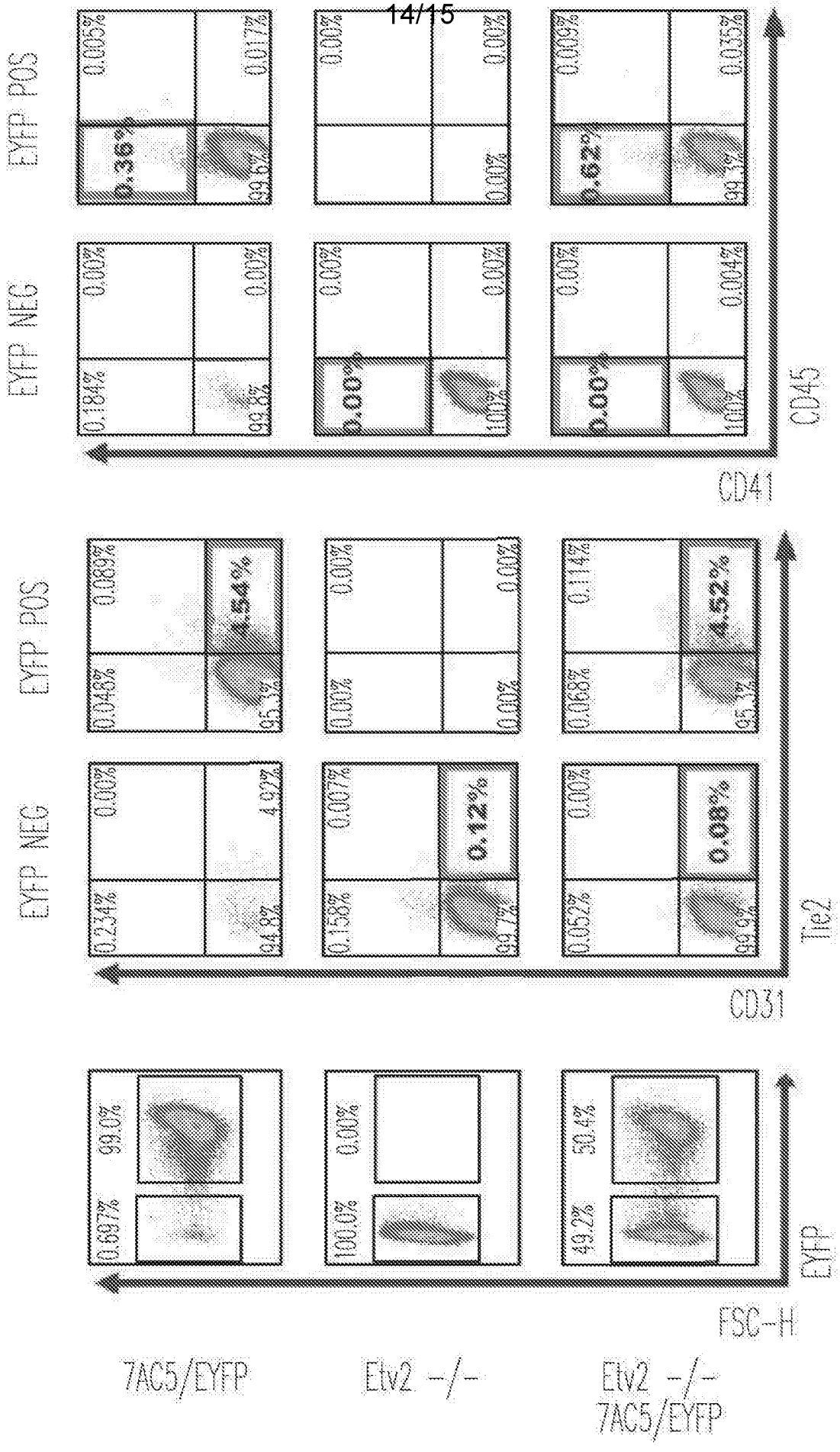


FIG. 7C


EYFP POS
EYFP NEG
0.005%
0.017%
0.36%
99.6%
0.00%
0.00%
0.00%
0.00%
0.009%
0.035%
0.62%
99.3%
0.00%
0.00%
0.184%
99.8%
0.00%
0.00%
3.00%
100%
0.00%
0.0014%
3.00%
100%
CD45
CD41
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EYFP NEG
0.089%
0.048%
4.54%
95.3%
0.00%
0.00%
0.00%
0.00%
0.114%
0.068%
4.52%
95.3%
0.00%
4.97%
0.234%
94.8%
0.007%
0.158%
0.12%
99.7%
0.00%
0.052%
0.08%
99.9%
Tie2
CD31
99.0%
0.607%
0.00%
100.0%
50.4%
49.2%
EYFP
FSC-H
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Etv2 -/-
Etv2 -/-
7AC5/EYFP

FIG. 8

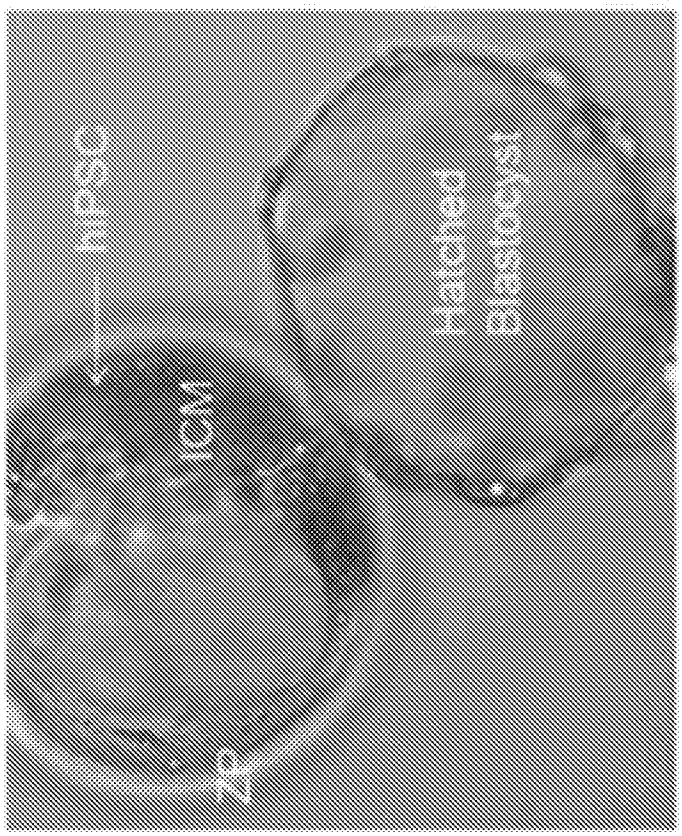

FIG. 9B

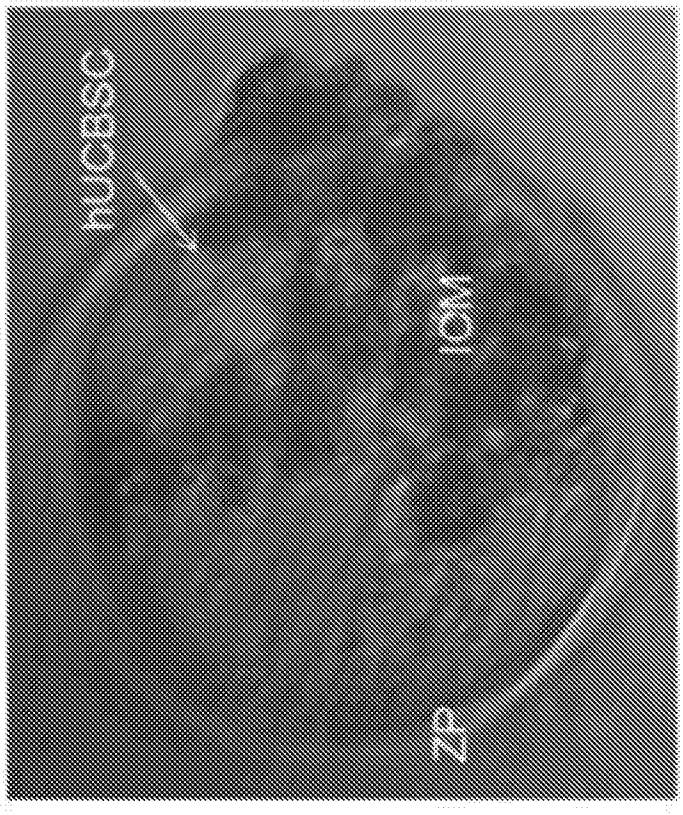

FIG. 9A

SEQUENCE LISTING

<110> Garry, Daniel J.
Garry, Mary G.
Rasmussen, Tara
Koyano-Nakagawa, Naoko
Regents of the University of Minnesota

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<151> 2015-03-03

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Lys Thr Gly Ser Ser Ser Leu Ser Val Pro Gly Ile Pro Gln Pro Asp
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Trp Val Ser Ala Leu Pro Asn Pro Glu Ala Pro Trp Gly Ala Glu Pro
65                  70                  75                  80
Val Pro Gln Ala Leu Pro Trp Ser Gly Asp Trp Thr Asp Leu Pro Tyr
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Ser Gly Ser Val Pro Trp Ser Arg Val Ser Gln Ala Leu Gly Ser Gly
            100                 105                 110
Cys Leu Asp Phe Gln Gly Pro Ile Gln Leu Trp Gln Phe Leu Leu Glu
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Leu Leu His Asp Gly Thr Arg Ser Ser Cys Ile Arg Trp Thr Gly Asn
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