# 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

In one aspect, the present invention provides a non-human blastocyst wherein the genome carries a mutation in both alleles of the ETV2 gene such that the non-human blastocyst lacks functional ETV2 protein.

In another aspect, the present invention provides a chimeric non-human animal or blastocyst expressing human ETV2 and lacking expression of said non-human animal ETV2.

In yet another aspect, the present invention provides an *in vitro* 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; and

c) introducing human stem cells into the ETV2 null non-human blastocyst of b); wherein said blastocyst from c) is to be implanted into a pseudopregnant surrogate non-human animal to generate a chimeric non-human animal expressing human ETV2.

In yet another aspect, the present invention provides an *in vitro* 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; and

c) introducing human donor stem cells into the ETV2 null non-human blastocyst of b); wherein said blastocyst or morula from c) is to be implanted 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 yet another aspect, the present invention provides a non-human animal produced by the method disclosed herein, wherein said non-human animal expresses human ETV2 and lacks expression of said non-human animal ETV2.

In yet another aspect, the present invention provides a progeny non-human animal produced from the mating of two non-human animals generated from method disclosed herein, wherein said progeny non-human animal expresses human ETV2 and does not express said non-human ETV2, wherein the genome of said progeny non-human carries a homozygous deletion of said non-human animal ETV2 gene.

In yet another aspect, the present invention provides chimeric blood made by an *in vitro* process 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; and

c) introducing human donor stem cells into the ETV2 null non-human blastocyst of b); wherein said blastocyst or morula from c) is to be implanted into a pseudopregnant surrogate non-human animal so as to generate a non-human animal expressing chimeric blood cells, wherein said blastocyst or morula from c) is to be implanted into a surrogate non-human animal so as to generate a non-human animal expressing chimeric blood, wherein said chimeric blood comprises cells descended from the human donor stem cell and proteins from said non-human animal.

In yet another aspect, the present invention provides a chimeric blood, wherein the blood comprises blood cells comprising human ETV2 and proteins from a non-human animal, wherein the blood is obtained from a non-human animal derived from an implanted non-human blastocyst or morula comprising human donor cells.

The present disclosure 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 disclosure 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 disclosure 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 example 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 example, the mutation is a deletion of the ETV2 gene. In another example, the non-human animal cell or blastocyst is a porcine, bovine, equine or goat.

One example provides a chimeric non-human animal or blastocyst expressing human ETV2 and lacking expression of said non-human animal ETV2. In one example, 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 example, the chimeric non-human animal expresses human endothelium. In one example, the non-human animal is a porcine, bovine, equine or goat.

One example 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 example 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 example, 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 example, the non-human animal is a porcine, bovine, equine or goat. In another example, 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 example, the donor providing the stem cells is the recipient of the humanized tissue or organ produced. In another example, the human induced pluripotent cell is formed from a fibroblast cell.

One example provides for a non-human animal produced by the methods described herein. Another example 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 example, the non-human animal is a porcine, bovine, equine or goat.

One example 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 example, the sequence of wild type pig (Sus scrofa) ETV2 (ets variant 2) is provided at ENSSSCG00000002906. In another example, 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 atccccccgca ggcccccctgc ggattgtgac gtctgctgac caggggtctg gccggaaatc
 541 ccccttcctg ttgcagataa gcctggtgca gcccagctga ccccaggccc tcctcccccca
 601 tcacctccct tgtcacagga tcaagtcccc aagccccctt cccctcccca ttccagtcaa
 661 cccagaaaca cccctctgca ccccaggtca tgcccatccc attgtttccc aggctcctgc
 721 tcaagtccaa gacaccccaa agctaccgtg gaggcttgag gccatcccag ggggcagagg
 781 tgggtgggga gggggtggca 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 tttccctgaa
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 example provides an ETV2 mutant pig (deficient in *Etv2*). In another example, 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 example 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 example 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 example, 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 example 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 example, the stem cell is a human induced pluripotent stem cell (iPS cell) or a human umbilical cord blood stem cell. In one example, the human iPS cell was formed from a fibroblast cell or any adult or somatic cells (e.g., human cells). In one example, 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 disclosure. One example 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 $Tie2^+$)

WHAT IS CLAIMED IS:

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

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

3. The non-human 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. An *in vitro* 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; and

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

said blastocyst from c) is to be implanted into a pseudopregnant surrogate non-human animal to generate a chimeric non-human animal expressing human ETV2.

9. An *in vitro* 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; and

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

said blastocyst or morula from c) is to be implanted 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, wherein said non-human animal expresses human ETV2 and lacks expression of said non-human animal ETV2.

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 and does not express said non-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 derived from the non-human animal are human pluripotent or human umbilical cord blood stem cell-derived white blood cells, red blood cells, and/or platelets that comprise human ETV2 and lack non-human animal ETV2.

18. Chimeric blood made by an *in vitro* process 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; and

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

said blastocyst or morula from c) is to be implanted into a pseudopregnant surrogate non-human animal so as to generate a non-human animal expressing chimeric blood cells, wherein said blastocyst or morula from c) is to be implanted into a surrogate non-human animal so as to generate a non-human animal expressing chimeric blood, wherein said chimeric blood comprises cells descended from the human donor stem cell and proteins from said non-human animal.

19. Chimeric blood, wherein the blood comprises blood cells comprising human ETV2 and proteins from a non-human animal, wherein the blood is obtained from a non-human animal derived from an implanted non-human blastocyst or morula comprising human donor cells.

20. The chimeric blood of claim 19, wherein the non-human animal is swine.

21. The method of claim 8, wherein the ETV2 mutation is carried out with the use of TALEN, wherein the TALEN pair sequences are:
Left: CTGGCCGGAAATCCCC (SEQ ID NO:1),
Right: GGGCTGCACCAGGCT (SEQ ID NO:2) and
Left: GATCCCAAGTCACACC (SEQ ID NO:3),
Right: CCCCTAAGGGTCCTG (SEQ ID NO:4).