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(54) Title: VASCULAR BED-SPECIFIC ENDOTHELIAL CELLS

(57) Abstract: This invention relates to methods for the generation and subsequent validation of vascular bed-specific endothelial cells with specific genetic and functional signatures starting from cellular precursors and use thereof. These vascular bed-specific endothelial cells with specific genetic and functional signatures from cellular precursors of the endothelial lineage of the present invention are in a particular embodiment of present invention used for the generation of in vitro bioengineered tissue culture equivalents. These in vitro bioengineered tissue culture equivalents are in an additional embodiment used for drug toxicity testing or for the generation of bioengineered vessel conduits for in vivo transplantation. In yet another embodiment these cells generated by present invention are used for treatment of vascular bed-specific diseases affecting the brain (e.g., stroke), the liver (e.g., sinusoidal obstruction syndrome), the heart (myocardial ischemia), the extremities (e.g., peripheral vascular disease), amongst others.

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VASCULAR BED-SPECIFIC ENDOTHELIAL CELLS

Background and Summary

BACKGROUND OF THE INVENTION

A. Field of the Invention

This invention relates to methods for the generation and subsequent validation of vascular bed-specific endothelial cells (ECs) with specific genetic and functional signatures starting from cellular precursors and use thereof.

These vascular bed-specific ECs with specific genetic and functional signatures from cellular precursors of the endothelial lineage of the present invention are in a particular embodiment of present invention used for the generation of *in vitro* bioengineered tissue culture equivalents. These *in vitro* bioengineered tissue culture equivalents are in an additional embodiment used for drug toxicity testing or for the generation of bioengineered vessel conduits for *in vivo* transplantation. In yet another embodiment these cells generated by present invention are used for treatment of vascular bed-specific diseases affecting the brain (*e.g.,* stroke), the liver (*e.g.,* sinusoidal obstruction syndrome), the heart (myocardial ischemia), the extremities (*e.g.,* peripheral vascular disease), amongst others.

Several documents are cited throughout the text of this specification. Each of the documents herein (including any manufacturer's specifications, instructions, etc.) are hereby incorporated by reference; however, there is no admission that any document cited is indeed prior art of the present invention.

B. Description of the Related Art

ECs line the inside ('endo') of blood and lymphatic vessels. Morphological, functional and molecular analyses revealed significant heterogeneity among ECs at different levels within the (lymph) vascular tree (Aird, 2007a, b). First, arterial, venous or lymphatic ECs clearly have different characteristics. A second level of heterogeneity is seen when comparing vascular beds of different organs (Aird, 2007b). The latter heterogeneity is mostly found in the microvasculature (*i.e.,* the capillary bed), which adapts to the unique needs of the underlying tissue (Auerbach et al., 1985). Finally, ECs have distinct characteristics in different species (Fajardo, 1989). This remarkable diversity finds its origin in a combination of factors. On the one hand, each EC has properties that are intrinsic (*i.e.,* independent of external cues) and preserved upon isolation from their *in vivo* microenvironment. On the other hand, another part of the unique EC signature is rapidly lost upon *in vitro* culture (a phenomenon called 'phenotypic drift') since it is determined by communication with surrounding tissues and by exposure to certain external biomechanical or biochemical stimuli (Amatschek et al., 2007; Wick et al., 2007). Interestingly, such heterogeneity has also been described for vascular smooth muscle cells (SMCs) and pericytes, which are the cells that surround the endothelium in a large part of the vascular bed (Majesky, 2007).

Little is currently known about the mechanisms that determine endothelial heterogeneity. Morphological diversity can be demonstrated by electron microscopy and vascular casting, thereby focusing on the presence or absence of specialised features. In general, three different EC types are distinguished morphologically: those lacking fenestrations ('continuous ECs'; *e.g.,* in cardiac muscle, brain), those featuring fenestrations sealed by a diaphragm ('fenestrated ECs'; *e.g.,* endocrine glands) and those featuring fenestrations without diaphragm ('discontinuous or sinusoidal ECs'; *e.g.,* liver) (Pries and Kuebler, 2006). Molecular heterogeneity has been studied on the DNA/RNA level by microarray, substractive hybridisation or serial analysis of gene expression (SAGE), or at the protein level using two-dimensional electrophoresis, mass spectrometry or substractive antibody expression cloning. *In vivo* approaches, most of which are not possible in humans, include specific antigen staining, injection of lectin variants, injection of antibodies, the generation of knock-in reporter mice, *in vivo* promoter activity analysis, phage

display (in which bacteriophages expressing peptides or antibody fragments are injected and specifically retained by certain vascular beds), and most recently laser capture microdissection. In the ***Examples*** of present application we show an integrated approach to study EC heterogeneity involving stem cells.

Several stem cell types have been considered as potential EC precursors, including unfractionated bone marrow cells, embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs), umbilical cord or peripheral blood mononuclear cells, adipose tissue-derived cells, endothelial progenitor cells (EPCs), blood outgrowth endothelial cells (BOECs), tissue resident progenitor cells, multipotent adult progenitor cells (MAPCs) and mesoangioblasts (MABs). There is significant heterogeneity among these progenitors in terms of their phenotypic/functional characteristics, their ability to incorporate into nascent blood vessels and their potential to be specialised into different subtypes of endothelium (Aranguren et al., 2007; Gulati et al., 2003; Yoder et al., 2007). This may in part relate to epigenetic determinants, such as differential promoter methylation or it may be speculated that organ- or vascular bed-specific endothelial precursors exist in the adult. In this context, it is interesting to mention that endothelial precursors have been found in the vascular wall of adults and embryos. Another reason for the observed heterogeneity may be that there are clonal variations within the same precursor populations.

The existence of EC heterogeneity has a tremendous clinical impact. It forms the basis of vascular bed-specific diseases (*e.g.,* atherosclerosis, varicosis or lymphedema, restricted to arteries, veins and lymphatics, respectively) and contributes to the disappointing results and side-effects obtained with 'broad spectrum' (anti-)angiogenic treatments in patients. In addition, it determines the vascular tropism of metastasising tumour cells and is the culprit for vascular bed-specific manifestations of acquired immunodeficiency syndrome (Conway and Carmeliet, 2004; Deng et al., 2006; Goerdt and Sorg, 1992; Ribatti et al., 2002). The existence of vascular bed-specific factors (*e.g.,* endocrine gland vascular endothelial growth factor or EG-VEGF and gonadotropins) and inhibitors (*e.g.,* chondromodulin-I) indeed suggests that (anti)angiogenic therapy with ubiquitous

growth factors (*e.g.,* VEGF) or inhibitors is not the ideal treatment. Moreover, EC progenitor-based revascularisation approaches have not asked whether the transplanted cells acquire the desired EC phenotype once engrafted in a diseased tissue where environmental cues are absent.

There is a need in the art for protocols to generate (stem cell-derived) pre-specialised ECs. Present invention fulfils this need by unravelling mechanisms of EC heterogeneity and offers new methods to design tailor-made therapies to cure vessel-related diseases. In addition, by revealing the 'vascular address' of each vessel, it allows targeting of systemically applied therapeutic agents to the region of interest, thereby minimising the risk for side-effects in other vascular territories.

This invention goes beyond the state-of-the-art with an unprecedented and innovative integrated *in vitro*/*in vivo* multi-disciplinary approach based on stem/progenitor cells and small animal models to: (*i*) expand our knowledge of EC diversity by obtaining EC type and vascular bed-specific gene-profiles ('signatures'); (*ii*) exploit that knowledge to design protocols to generate specialised ECs by differentiation from (stem/progenitor) cells in order to design specialised vascular therapies for (lymph)vascular disorders, to assay these signatures in pathological conditions or to use them in drug screening systems.

SUMMARY OF THE INVENTION

There is a need in the art for vascular bed-specific ECs to be used for therapeutic and diagnostic purposes, since these cells cannot be isolated in sufficient numbers from donors. According to the invention, this problem is solved by means of a method to generate these cells in sufficient numbers, which represents a significant advancement.

Such embodiment of present invention is schematically summarised in ***Figure 1*** below.

A comparative transcriptomic screen on two categories of ECs: microvascular ECs (those from capillaries or 'microvessels' in three different

murine/human organs) and macrovascular ECs (those from large vessels such as arteries or veins; we used arteries and veins from human umbilical cord and from adult mouse vena cava and thoracic aorta) delivered relevant information to develop such a method. Importantly, we used **freshly isolated** cells for the screen and not cultured cells (like the majority of existing screens), as the former are representative for vascular bed-specific ECs in their *in vivo* tissue context.

Further scope of applicability of the present invention will become apparent from the detailed description given hereinafter. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

Some embodiments of the invention are set forth in claim format directly below:

1. An engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof.
2. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec,

Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, and Zeb2; and/or with a transcription factor of the group consisting of Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16; or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof. As will be evident from the examples hereinafter, the first group is associated with differentiation into arterial endothelial cells, and the second group is associated with differentiation into microvascular endothelial cells.

3. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor of the group consisting of Prdm16, Emx2, Msx1, Tox2, Aff3 and Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof; in particular said source cell is transduced or transfected with the group of transcription factors consisting of Prdm16, Emx2, Msx1, Tox2, Aff3 and Nkx2-3, eventually including human homologues or variants, member of the same family, or orthologues thereof.
4. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.
5. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with the transcription factor Prdm16 and one or more transcription factors selected of the group consisting of Emx2, Msx1, Nkx2-3, Tox2, and Aff3; or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.
6. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell

of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Meox2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function; in particular Meox2 in combination with one ore more transcription factors selected from the group consisting of Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16: more in particular Meox2 in combination with one ore more transcription factors selected from the group consisting of Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2; even more in particular Meox2 in combination with one ore more transcription factors selected from the group consisting of Tcf15, Pparγ, Wt1, and Ebf3.

7. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell a transcription factor with Tcf15 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function; in particular TcF15 in combination with one ore more transcription factors selected from the group consisting of Meox2, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16: more in particular TcF15 in combination with one ore more transcription factors selected from the group consisting of Meox2, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2; even more in particular TcF15 in combination with one ore more transcription factors selected from the group consisting of Meox2, Pparγ, Wt1, and Ebf3.

8. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor with Pparγ or a human homologue or a variant, member of the same family, orthologue thereof,

having the same biological function; in particular Pparγ in combination with one ore more transcription factors selected from the group consisting of Meox2, TcF15, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16: more in particular Pparγ in combination with one ore more transcription factors selected from the group consisting of Meox2, TcF15, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2; even more in particular Pparγ in combination with one ore more transcription factors selected from the group consisting of Meox2, Meox2, TcF15, Wt1, and Ebf3.

9. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Wt1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function; in particular Wt1 in combination with one ore more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16: more in particular Wt1 in combination with one ore more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2; even more in particular Wt1 in combination with one ore more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, and Ebf3.

10. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Zic3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function; in particular Zic3 in combination with one or more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Wt1, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5,

Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16; more in particular Zic3 in combination with one or more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Wt1, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, and Zeb2; even more in particular Zic3 in combination with one or more transcription factors selected from the group consisting of Lef1, Foxf2, Foxf1a, and Foxc1.

11. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Lef1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function; in particular Lef1 in combination with one or more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16; more in particular Lef1 in combination with one or more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, and Zeb2; even more in particular Lef1 in combination with one or more transcription factors selected from the group consisting of Zic3, Foxf2, Foxf1a, and Foxc1.

12. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Foxf2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function; in particular Foxf2 in combination with one or more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16; more in particular Foxf2 in combination with one or more transcription factors selected from the group consisting of Meox2, Pparγ,

TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, and Zeb2; even more in particular Foxf2 in combination with one or more transcription factors selected from the group consisting of Zic3, Lef1, Foxf1a, and Foxc1.

13. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Foxf1a or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function; in particular Foxf1a in combination with one or more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16; more in particular Foxf1a in combination with one or more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, and Zeb2; even more in particular Foxf1a in combination with one or more transcription factors selected from the group consisting of Zic3, Lef1, Foxf2, and Foxc1.

14. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Foxc1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function; in particular Foxc1 in combination with one or more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxf1a, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16; more in particular Foxc1 in combination with one or more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxf1a, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, and Zeb2; even more in particular Foxc1 in

combination with one or more transcription factors selected from the group consisting of Zic3, Lef1, Foxf2, and Foxf1a.

15. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Tcfec or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function; in particular Tcfec in combination with one or more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxf1a, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16; more in particular Tcfec in combination with one or more transcription factors selected from Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxf1a, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, and Zeb2; even more in particular Tcfec in combination with one or more transcription factors selected from Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, and Zeb2.

16. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Hoxb5 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function; in particular Hoxb5 in combination with one or more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxf1a, Tcfec, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16; more in particular Hoxb5 in combination with one or more transcription factors selected from Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxf1a, Tcfec, Maf, Cux2, Gata4, Meis2, Twist1, and Zeb2; even more in particular Hoxb5 in combination with one or more transcription factors selected from Tcfec, Maf, Cux2, Gata4, Meis2, Twist1, and Zeb2.

17. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or

transfection of a source cell with a transcription factor Maf or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function; in particular Maf in combination with one or more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxf1a, Tcfec, Hoxb5, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16; more in particular Maf in combination with one or more transcription factors selected from Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxf1a, Tcfec, Hoxb5, Cux2, Gata4, Meis2, Twist1, and Zeb2; even more in particular Maf in combination with one or more transcription factors selected from Tcfec, Hoxb5, Cux2, Gata4, Meis2, Twist1, and Zeb2.

18. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Cux2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function; in particular Cux2 in combination with one or more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxf1a, Tcfec, Hoxb5, Maf, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16; more in particular Cux2 in combination with one or more transcription factors selected from Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxf1a, Tcfec, Hoxb5, Maf, Gata4, Meis2, Twist1, and Zeb2; even more in particular Cux2 in combination with one or more transcription factors selected from Tcfec, Hoxb5, Maf, Gata4, Meis2, Twist1, and Zeb2.

19. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Gata4 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function; in particular Gata4 in combination with one or more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxf1a, Tcfec, Hoxb5,

Maf, Cux2, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16; more in particular Gata4 in combination with one or more transcription factors selected from Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxf1a, Tcfec, Hoxb5, Maf, Cux2, Meis2, Twist1, and Zeb2; even more in particular Gata4 in combination with one or more transcription factors selected from Tcfec, Hoxb5, Maf, Cux2, Meis2, Twist1, and Zeb2.

20. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Meis2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function; in particular Meis2 in combination with one or more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxf1a, Tcfec, Hoxb5, Maf, Cux2, Gata4, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16; more in particular Meis2 in combination with one or more transcription factors selected from Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxf1a, Tcfec, Hoxb5, Maf, Cux2, Gata4, Twist1, and Zeb2; even more in particular Meis2 in combination with one or more transcription factors selected from Tcfec, Hoxb5, Maf, Cux2, Gata4, Twist1, and Zeb2.

21. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Twist1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function; in particular Twist1 in combination with one or more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxf1a, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16; more in particular Twist1 in combination with one or more transcription factors selected from Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxf1a, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, and Zeb2;

even more in particular Twist1 in combination with one or more transcription factors selected from Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, and Zeb2.

22. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Zeb2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function; in particular Zeb2 in combination with one or more transcription factors selected from the group consisting of Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxf1a, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16; more in particular Zeb2 in combination with one or more transcription factors selected from Meox2, Pparγ, TcF15, Ebf3, Wt1, Zic3, Lef1, Foxf2, Foxf1a, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, and Twist1; even more in particular Zeb2 in combination with one or more transcription factors selected from Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, and Twist1.

23. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Emx2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

24. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

25. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Msx1 or a human

homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

26. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Tox2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

27. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, whereby the cell is being genetically altered by transduction or transfection of a source cell with a transcription factor of the group consisting of Meox2, Tcf15, Pparγ, WT1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an organ-specific microvascular endothelial cell.

28. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, whereby the cell is being engineered by transduction or transfection of a source cell with a transcription factor of the group consisting of Prdm16, Emx2, Msx1, Tox2, Aff3 and Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an arterial endothelial cell; in particular said source cell is transduced or transfected with the group of transcription factors consisting of Prdm16, Emx2, Msx1, Tox2, Aff3 and Nkx2-3, eventually including human homologues or variants, member of the same family, or orthologues thereof.

29. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, whereby the cell is being engineered by transduction or transfection of a source cell with the transcription factor Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof,

having the same biological function, to differentiate source cell into an arterial endothelial cell.

30. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, whereby the is the cell is being engineered by transduction or transfection of a source cell with a transcription factor of the group consisting of Meox2, Tcf15, Pparγ, Wt1 and Ebf3 or a human homolog or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a cardiac microvascular endothelial cell; in particular said source cell is transduced or transfected with the group of transcription factors consisting of Meox2, Tcf15, Pparγ, Wt1 and Ebf3, eventually including human homologues or variants, member of the same family, or orthologues thereof.

31. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, whereby the is the cell being engineered by transduction or transfection of a source cell with a transcription factor of the group consisting of Zic3, Lef1, Foxf2, Foxf1a and Foxc1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a brain microvascular endothelial cell; in particular said source cell is transduced or transfected with the group of transcription factors consisting of Zic3, Lef1, Foxf2, Foxf1a and Foxc1, eventually including human homologues or variants, member of the same family, or orthologues thereof.

32. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, whereby the cell is being engineered by transduction or transfection of a source cell with a transcription factor of the group consisting of Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a liver microvascular endothelial cell; in particular said source cell is transduced or transfected with the group of transcription

factors consisting of Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2, eventually including human homologues or variants, member of the same family, or orthologues thereof.

33. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, whereby the cell is being engineered by transduction or transfection of a source cell with a transcription factor of the group consisting of Emx2, Nkx2-3, Msx1, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an arterial endothelial cell; in particular said source cell being engineered by transduction or transfection of a source cell with the transcription factor Prdm16 in combination with one or more transcription factors selected from the group consisting of Emx2, Nkx2-3, Msx1, Tox2, and Aff3; more in particular said source cell is transduced or transfected with the group of transcription factors consisting of Emx2, Nkx2-3, Msx1, Tox2, Aff3 and Prdm16, eventually including human homologues or variants, member of the same family, or orthologues thereof.

34. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to any one of the previous embodiments, whereby the transcription factors are overexpressed.

35. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to any one of the previous embodiments, whereby the source cell is a blood outgrowth endothelial cell (BOEC).

36. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to any one of the previous embodiments, whereby the source cell is a dedifferentiated cultured human umbilical vein endothelial cell (HUVEC) or a dedifferentiated HUAEC.

37. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell

according to any one of the previous embodiments, whereby the source cell is a mammalian cell of embryonic or non-embryonic origin.

38. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to any one of the previous embodiments, whereby the source cell is a source cell of the group consisting of endothelial progenitor cells (EPCs), mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), induced pluripotency stem cells (iPSCs), mesoangioblasts (MABs), multipotent adult progenitor cells (MAPCs), blood outgrowth endothelial cells (BOECs), induced endothelial cells (iECs), unfractionated bone marrow cells, umbilical cord or peripheral blood-derived mononuclear cells, adipose tissue-derived cells, tissue resident progenitor cells, human umbilical vein endothelial cells (HUVECs), human umbilical artery endothelial cells (HUAECs), human dermal microvascular endothelial cells (HDMECs), cultured endothelial cell lines from heart, cultured endothelial cell lines from liver, cultured endothelial cell lines from brain.

39. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to any one of the previous embodiments, whereby the transduction or transfection is lentiviral.

40. An engineered vascular bed-specific endothelial cell, characterized in that the cell is engineered by induced differentiation of a source cell by transferring directly to source cells the expression product of a transcription factor of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof the expression product being linked to a protein transduction domain (PTD), such as poly-arginine and HIV-derived Tat, or to small cationic peptide domains to enhance its cell membrane crossing capacity.

41. An engineered vascular bed-specific endothelial cell, characterized in that the cell is engineered by induced differentiation of a source cell by

transferring directly to source cells the expression product of a transcription factor of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, and Zeb2; and/or by induced differentiation of a source cell by transferring directly to source cells the expression product of a transcription factor of the group consisting of Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof the expression product being linked to a protein transduction domain (PTD), such as poly-arginine and HIV-derived Tat, or to small cationic peptide domains to enhance its cell membrane crossing capacity.

42. The engineered vascular bed-specific endothelial cell of embodiment 40, whereby the expression product is of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate said source cell into a vascular endothelial target cell.

43. The engineered vascular bed-specific endothelial cell of embodiment 41, whereby the expression product is of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, and Zeb2; and/or of the group consisting of Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate said source cell into a vascular endothelial target cell.

44. The engineered vascular bed-specific endothelial cell of embodiments 40 or 41, whereby the expression product is of the group consisting of Prdm16, Emx2, Msx1, Tox2, Aff3 and Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a vascular endothelial target cell.

45. The engineered vascular bed-specific endothelial cell of embodiment 40 or 41, whereby the expression product is of Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

As will be evident to the skilled artisan, in this and each of the following embodiments to the engineered vascular bed-specific endothelial cells, the particular embodiments for each of said transcription factors and the combination of each of said transcription factors with the further transcription factors as provided herein above, also apply. Said specific embodiments to each of said transcription factors and the combination of each of said transcription factors with the further transcription factors, also apply to the therapeutic and diagnostic applications (infra) as well as in the methods to obtain said cells as provided hereinafter.

46. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Meox2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.
47. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Tcf15 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.
48. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Pparγ or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.
49. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Wt1 or a

human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

50. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Zic3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

51. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Lef1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

52. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Foxf2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

53. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Foxf1a or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

54. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Foxc1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

55. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Tcfec or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

56. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Hoxb5 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

57. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Maf or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a human homolog to differentiate source cell into a vascular endothelial target cell.

58. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Cux2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

59. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Gata4 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

60. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Meis2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

61. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Twist1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

62. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Zeb2 or a human homologue or a variant, member of the same family, orthologue

thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

63. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Emx2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

64. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

65. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Msx1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

66. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Tox2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

67. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an organ-specific microvascular endothelial cell.

68. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of the group consisting of Prdm16, Emx2, Msx1, Tox2, Aff3 and Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof,

having the same biological function, or a combination thereof to differentiate source cell into an arterial endothelial cell.

69. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into an arterial endothelial cell.

70. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of the group consisting of Meox2, Tcf15, Pparγ, Wt1 and Ebf3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a cardiac microvascular endothelial cell.

71. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of the group consisting of Zic3, Lef1, Foxf2, Foxf1a and Foxc1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a brain microvascular endothelial cell.

72. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of the group consisting of Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a liver microvascular endothelial cell.

73. The engineered vascular bed-specific endothelial cell of any one of the previous embodiments, whereby the expression product is of the group consisting of Emx2, Nkx2-3, Msx1, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an arterial endothelial cell.

74. The engineered vascular bed-specific endothelial cell according to any one of the previous embodiments, whereby the transcription factors are overexpressed.

75. The engineered vascular bed-specific endothelial cell according to any one of the previous embodiments, whereby the source cell is a blood outgrowth endothelial cell (BOEC).

76. The engineered vascular bed-specific endothelial cell according to any one of the previous embodiments, whereby the source cell is a dedifferentiated cultured human umbilical vein endothelial cell (HUVEC) or a dedifferentiated HUAEC.

77. The engineered vascular bed-specific endothelial cell according to any one of the previous embodiments, whereby the source cell is a mammalian cell of embryonic or non-embryonic origin.

78. The engineered vascular bed-specific endothelial cell according to any one of the previous embodiments, whereby the source cell is a source cell of the group consisting of endothelial progenitor cells (EPCs), mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), induced pluripotency stem cells (iPSCs), mesoangioblasts (MABs), multipotent adult progenitor cells (MAPCs), blood outgrowth endothelial cells (BOECs), induced endothelial cells (iECs), unfractionated bone marrow cells, umbilical cord or peripheral blood-derived mononuclear cells, adipose tissue-derived cells, tissue resident progenitor cells, human umbilical vein endothelial cells (HUVECs), human umbilical artery endothelial cells (HUAECs), human dermal microvascular endothelial cells (HDMECs), cultured endothelial cell lines from heart, cultured endothelial cell lines from liver, cultured endothelial cell lines from brain.

79. The engineered vascular bed-specific endothelial cell according to any one of the previous embodiments, whereby the cell is terminally differentiated or in partially differentiated form.

80. The engineered cell according to any one of the previous embodiments, for use in a treatment of vascular bed-specific disorder.

81. The engineered cell according to any one of the previous embodiments, for use in a treatment of a disorder of vascular bed-specific haemostasis or for a disorder of generating and/or maintaining vascular bed-specific phenotypes.

82. The engineered cell according to any one of the previous embodiments 5, for use in a treatment of macrovascular complications evoked by a vascular bed-specific (haemostasis) disorder.

83. The engineered cell according to any one of the previous embodiments, for use in a treatment of microvascular complications evoked by a vascular bed-specific (haemostasis) disorder.

84. The engineered cell according to any one of the previous embodiments, for use in a treatment of for decreasing severity of vascular bed-specific disorder in a subject, the method comprising autologous, allogeneic or xenogeneic cell transplantation; in a subject in need of treatment with the engineered cell, thereby decreasing severity of disorder in the subject.

85. The engineered cell according to any one of the previous embodiments, for use in a treatment of for decreasing severity of vascular bed-specific disorder or diseases or circulatory or hypoxic conditions that can be treated with the compositions and the methods of the invention comprise but are not limited to: atherosclerosis, preeclampsia, erectile dysfunction, renal failure, transplant accelerated arteriosclerosis, deep vein thrombosis, sleep apnea, hypoxia during sleep, fetal hypoxia, smoking, anemia, endothelial dysfunction, sinusoidal obstruction syndrome, regional perfusion deficits (*e.g.,* limb, gut, renal ischemia), congestic heart failure, peripheral vascular disease, frost bite, decubitus ulcers, asphyxiation, poisoning (*e.g.,* carbon monoxide, heavy metal), altitude sickness, pulmonary hypertension, sudden infant death syndrome, asthma, chronic obstructive pulmonary disease, congenital circulatory anomalities (*e.g.,* Tetralogy of Fallot), erythroblastosis, myocardial infarction, aortic stenosis, or any other cardiac disease which can lead to heart failure, plaque rupture, both primary and secondary (in-stent) restenosis in coronary or peripheral arteries, coronary vascular disease, hypertension, diabetes, obesity, stroke, aneurysm, thrombosis, arrythmia, tachycardia, surgical or physical trauma in a subject, the method comprising autologous, allogeneic or xenogeneic cell transplantation; in a subject in

need of treatment the engineered cell, thereby decreasing severity of disorder in the subject.

86. The engineered cell according to any one of the previous embodiments, for use in a treatment for decreasing severity of vascular bed-specific diseases affecting the brain (*e.g.,* stroke), the liver (*e.g.,* sinusoidal obstruction syndrome), the heart (myocardial ischemia) or the extremities (*e.g.,* peripheral vascular disease) in a subject, the method comprising autologous, allogeneic or xenogeneic cell transplantation; in a subject in need of treatment the engineered cell, thereby decreasing severity of disorder in the subject.

87. The engineered cell for use in a treatment according to any one of the previous embodiments, whereby the cell is genetically altered or unaltered, by direct introduction to a site of interest, *e.g.,* on or around the surface of an acceptable matrix, or systemically, in combination with a pharmaceutically acceptable carrier so as to repair, replace or promote the growth of existing and/or new blood vessels.

88. The engineered cell to any one of the previous embodiments,, whereby said cell is administered in the treatment by any one of the following methods: localised injection, catheter administration, systemic injection, intraperitoneal injection, parenteral administration, oral administration, intracranial injection, intra-arterial injection, intra-venous injection, intra-ventricular infusion, intra-placental injection, intra-uterine injection, surgical intra-myocardial injection, transendocardial injection, transvascular injection, intra-coronary injection, intra-muscular injection, surgical injection into a tissue of interest or via direct application to tissue surfaces (*e.g.,* during surgery or on a wound).

89. A pharmaceutical composition, comprising an engineered cell according to any one of the previous embodiments.

90. An *in vitro* bioengineered tissue, comprising an engineered cell according to any one of the previous embodiments.

91. The use of an engineered cell according to any one of the previous embodiments, in a method of screening for non-toxic compounds.

92. The use of an engineered cell according to any one of the previous embodiments, in a method of *in vitro* bioengineering a tissue.

93. The use of an engineered cell according to any one of the previous embodiments, in *in vitro* drug toxicity testing, either using the cell product alone, or as a (or one of the) cellular component(s) in the context of engineered 2D or 3D tissue equivalents;

94. The use of an engineered cell according to any one of the previous embodiments as a (or one of the) cellular component(s) of tissue engineered constructs for implantation in patients (for example to coat the inside of artificial arterial conduits or as the intimal cellular component of a fully biological tissue-engineered vascular graft or to coat cardiac valves).

95. An *in vitro* method of diagnosing a vascular bed-specific disorder phenotype in a subject, said method comprising (a) analysing the level of expression or activity of expression product of at least 5 genes of the '(differential) reference signatures' of table 4 (brain ECs), of table 5 (liver ECs), of table 6 (heart ECs) and/or tables 12/16/18 (arterial or venous ECs) in a sample isolated from said subject, and (b) compare said level of expression or activity with the level of expression or activity in '(differential) reference signatures' of table 4 (brain ECs), of table 5 (liver ECs), of table 6 (heart ECs) and/or tables 12/16/18 (arterial or venous ECs); whereby a deviated level of expression or activity relative to such '(differential) reference signature' is an indication of such disorder phenotype or a propensity thereto.

96. An *in vitro* method of diagnosing a vascular bed-specific disorder phenotype in a subject, said method comprising: (a) genotyping one or more genes in the '(differential) reference signatures' of table 4 (brain ECs), of table 5 (liver ECs), of table 6 (heart ECs) and/or tables 12/16/18 (arterial or venous ECs) in a sample isolated from said subject, and (b) analyse the DNA sequence of said gene(s); whereby polymorphisms (*e.g.,* SNPs) in said genes of the 'reference signatures' is an indication of such disorder phenotype or a propensity thereto.

97. Use of the '(differential) reference signatures' of table 4 (brain ECs), of table 5 (liver ECs), of table 6 (heart ECs) and/or tables 12/16/18 (arterial or venous ECs), to design tailored therapy, *e.g.,* vascular bed-specific drug

delivery or activation of reference signature genes to increase vascularisation.

98. Use of '(differential) reference signatures' of table 4 (brain ECs), of table 5 (liver ECs), of table 6 (heart ECs) and/or tables 12/16/18 (arterial or venous ECs) to read-out the (side) effects on the ECs of the targeted organ/tissue by current or novel (anti-)angiogenic or other therapies.

99. Use of '(differential) reference signatures' of table 4 (brain ECs), of table 5 (liver ECs), of table 6 (heart ECs) and/or tables 12/16/18 (arterial or venous ECs) to read-out the efficiency of existing or to be developed alternative (combinatorial) approaches not described herein to specify source cells towards a vascular bed-specific target cell.

100. The engineered cell according to any one of the previous embodiments, for use in a treatment of for decreasing severity of vascular bed-specific disorder or diseases or circulatory or hypoxic conditions that can be treated with the compositions and the methods of the invention comprise but are not limited to: atherosclerosis, preeclampsia, erectile dysfunction, renal failure, transplant accelerated arteriosclerosis, deep vein thrombosis, sleep apnea, hypoxia during sleep, fetal hypoxia, smoking, anemia, endothelial dysfunction, sinusoidal obstruction syndrome, regional perfusion deficits (*e.g.,* limb, gut, renal ischemia), congestic heart failure, peripheral vascular disease, frost bite, decubitus ulcers, asphyxiation, poisoning (*e.g.,* carbon monoxide, heavy metal), altitude sickness, pulmonary hypertension, sudden infant death syndrome, asthma, chronic obstructive pulmonary disease, congenital circulatory anomalities (*e.g.,* Tetralogy of Fallot), erythroblastosis, myocardial infarction, aortic stenosis, or any other cardiac disease which can lead to heart failure, plaque rupture, both primary and secondary (in-stent) restenosis in coronary or peripheral arteries, coronary vascular disease, hypertension, stroke, aneurysm, thrombosis, arrythmia, tachycardia, surgical or physical trauma in a subject, the method comprising autologous, allogeneic or xenogeneic cell transplantation; in a subject in need of treatment the engineered cell, thereby decreasing severity of disorder in the subject.

101. A method to differentiate a source cell into a vascular endothelial cell (the target cell), said method comprising genetically altering the source cell by transduction or transfection of said source cell with a transcription factor of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof; in particular said method comprising genetically altering the source cell by transduction or transfection of said source cell with a transcription factor of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, and Zeb2; and/or with a transcription factor of the group consisting of Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof.

102. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with a transcription factor of the group consisting of Prdm16, Emx2, Msx1, Tox2, Aff3 and Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof; in particular said source cell is transduced or transfected with the group of transcription factors consisting of Prdm16, Emx2, Msx1, Tox2, Aff3 and Nkx2-3, eventually including human homologues or variants, member of the same family, or orthologues thereof.

103. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with a transcription factor Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

104. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Meox2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

105. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Tcf15 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

106. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Pparγ or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

107. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Wt1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

108. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Zic3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

109. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Lef1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

110. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Foxf2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

111. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Foxf1a or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

112. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Foxc1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

113. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Tcfec or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

114. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Hoxb5 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

115. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Maf or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

116. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Cux2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

117. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Gata4 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

118. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of embodiment 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Meis2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

119. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Twist1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

120. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription

factor Zeb2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

121. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Emx2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

122. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

123. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Msx1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

124. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Tox2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

125. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with a transcription factor of the group consisting of Meox2, Tcf15, Pparγ, WT1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an organ-specific microvascular endothelial cell.

126. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with a transcription factor of the group of Prdm16, Emx2, Msx1, Tox2, Aff3 and Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate said source cell into an arterial endothelial cell; in particular said source cell is transduced or transfected with the group of transcription

factors consisting of Prdm16, Emx2, Msx1, Tox2, Aff3 and Nkx2-3, eventually including human homologues, variants, members of the same family, or orthologues thereof, to differentiate said source cell into an arterial endothelial cel.

127. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with transcription factor Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into an arterial endothelial cell.

128. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with a transcription factor of the group consisting of Meox2, Tcf15, Pparγ, Wt1 and Ebf3 or a human homolog or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a cardiac microvascular endothelial cell; in particular said source cell is transduced or transfected with the group of transcription factors consisting of Meox2, Tcf15, Pparγ, Wt1 and Ebf3, eventually including human homologues, variants, members of the same family, or orthologues thereof, to differentiate said source cell into a cardiac microvascular endothelial cell

129. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with a transcription factor of the group consisting of Zic3, Lef1, Foxf2, Foxf1a and Foxc1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a brain microvascular endothelial cell; in particular said source cell is transduced or transfected with the group of transcription factors consisting of Zic3, Lef1, Foxf2, Foxf1a and Foxc1, eventually including human homologues, variants, members of the same family, or orthologues thereof, to differentiate said source cell into a brain microvascular endothelial cell.

130. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with a transcription

factor of the group consisting of Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a liver microvascular endothelial cell; in particular said source cell is transduced or transfected with the group of transcription factors consisting of Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2, eventually including human homologues, variants, members of the same family, or orthologues thereof, to differentiate said source cell into a liver microvascular endothelial cell.

131. The method of embodiment 101, the source cell being genetically altered by transduction or transfection of said source cell with a transcription factor of the group consisting of Emx2, Nkx2-3, Msx1, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an arterial endothelial cell; in particular said source cell is transduced or transfected with the group of transcription factors consisting of Emx2, Nkx2-3, Msx1, Tox2, Aff3 and Prdm16, eventually including human homologues, variants, members of the same family, or orthologues thereof, to differentiate said source cell into an arterial endothelial cell.

132. The method according to any one of the previous embodiments 97 to 127, whereby the transcription factors are overexpressed.

133. The method according to any one of the previous embodiments 97 to 128, whereby the source cell is a blood outgrowth endothelial cell (BOEC).

134. The method according to any one of the previous embodiments 97 to 128, whereby the source cell is a dedifferentiated cultured human umbilical vein endothelial cell (HUVEC) or a dedifferentiated HUAEC.

135. The method according to any one of the previous embodiments 97 to 128, whereby the source cell is a mammalian cell of embryonic or non-embryonic origin.

136. The method according to any one of the previous embodiments 97 to 128, whereby the source cell is a source cell of the group consisting of endothelial progenitor cells (EPCs), mesenchymal stem cells (MSCs),

embryonic stem cells (ESCs), induced pluripotency stem cells (iPSCs), mesoangioblasts (MABs), multipotent adult progenitor cells (MAPCs), blood outgrowth endothelial cells (BOECs), induced endothelial cells (iECs), unfractionated bone marrow cells, umbilical cord or peripheral blood-derived mononuclear cells, adipose tissue-derived cells, tissue resident progenitor cells, human umbilical vein endothelial cells (HUVECs), human umbilical artery endothelial cells (HUAECs), human dermal microvascular endothelial cells (HDMECs), cultured endothelial cell lines from heart, cultured endothelial cell lines from liver, cultured endothelial cell lines from brain.

137. The method according to any one of the previous embodiments, whereby the transduction or transfection is lentiviral.

Further embodiments of the present invention in claim format are;

1. An engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof.
2. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor of the group consisting of Prdm16, Emx2, Msx1, Tox2, Aff3 and Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof.
3. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Prdm16 or a human

homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

4. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Meox2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.
5. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell a transcription factor with Tcf15 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.
6. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor with Pparγ or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.
7. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Wt1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.
8. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Zic3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

9. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Lef1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

10. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell a transcription factor with Foxf2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

11. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Foxf1a or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

12. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Foxc1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

13. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Tcfec or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

14. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or

transfection of a source cell with a transcription factor Hoxb5 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

15. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Maf or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

16. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Cux2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

17. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Gata4 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

18. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Meis2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

19. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Twist1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

20. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Zeb2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

21. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Emx2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

22. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

23. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Msx1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

24. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor Tox2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function.

25. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, whereby the cell is being genetically altered by

transduction or transfection of a source cell with a transcription factor of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an organ-specific microvascular endothelial cell.

26. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, whereby the cell is being engineered by transduction or transfection of a source cell with a transcription factor of the group consisting of Prdm16, Emx2, Msx1, Tox2, Aff3 and Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an arterial endothelial cell.

27. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, whereby the cell is being engineered by transduction or transfection of a source cell with the transcription factor Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into an arterial endothelial cell.

28. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, whereby the is the cell is being engineered by transduction or transfection of a source cell with a transcription factor of the group consisting of Meox2, Tcf15, Pparγ, Wt1 and Ebf3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a cardiac microvascular endothelial cell.

29. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, whereby the is the cell being engineered by transduction or transfection of a source cell with a transcription factor of the

group consisting of Zic3, Lef1, Foxf2, Foxf1a and Foxc1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a brain microvascular endothelial cell.

30. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, whereby the cell is being engineered by transduction or transfection of a source cell with a transcription factor of the group consisting of Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a liver microvascular endothelial cell.

31. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, whereby the cell is being engineered by transduction or transfection of a source cell with a transcription factor of the group consisting of Emx2, Nkx2-3, Msx1, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an arterial endothelial cell.

32. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to any one of the previous claims 1 to 31, whereby the transcription factors are overexpressed.

33. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to any one of the previous claims 1 to 32, whereby the source cell is a blood outgrowth endothelial cell (BOEC).

34. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to any one of the previous claims 1 to 32, whereby the source cell is a dedifferentiated cultured human umbilical vein endothelial cell (HUVEC) or a dedifferentiated HUAEC..

35. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to any one of the previous claims 1 to 32, whereby the source cell is a mammalian cell of embryonic or non-embryonic origin.

36. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to any one of the previous claims 1 to 32, whereby the source cell is a source cell of the group consisting of endothelial progenitor cells (EPCs), mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), induced pluripotency stem cells (iPSCs), mesoangioblasts (MABs), multipotent adult progenitor cells (MAPCs), blood outgrowth endothelial cells (BOECs), induced endothelial cells (iECs), unfractionated bone marrow cells, umbilical cord or peripheral blood-derived mononuclear cells, adipose tissue-derived cells, tissue resident progenitor cells, human umbilical vein endothelial cells (HUVECs), human umbilical artery endothelial cells (HUAECs), human dermal microvascular endothelial cells (HDMECs), cultured endothelial cell lines from heart, cultured endothelial cell lines from liver, cultured endothelial cell lines from brain.

37. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to any one of the previous claims, whereby the transduction or transfection is lentiviral.

38. An engineered vascular bed-specific endothelial cell, characterized in that the cell is engineered by induced differentiation of a source cell by transferring directly to source cells the expression product of a transcription factor of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof the expression product being linked to a protein transduction domain (PTD), such as poly-arginine and HIV-derived Tat, or to small cationic peptide domains to enhance its cell membrane crossing capacity.

39. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a vascular endothelial target cell.

40. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of the group consisting of Prdm16, Emx2, Msx1, Tox2, Aff3 and Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a vascular endothelial target cell.

41. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

42. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Meox2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

43. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Tcf15 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

44. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Pparγ or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

45. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Wt1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

46. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Zic3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

47. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Lef1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

48. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Foxf2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

49. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Foxf1a or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

50. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Foxc1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

51. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Tcfec or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

52. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Hoxb5 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

53. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Maf or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a human homolog to differentiate source cell into a vascular endothelial target cell.

54. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Cux2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

55. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Gata4 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

56. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Meis2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

57. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Twist1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

58. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Zeb2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

59. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Emx2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

60. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

61. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Msx1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

62. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Tox2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a vascular endothelial target cell.

63. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an organ-specific microvascular endothelial cell.

64. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of the group consisting of Prdm16, Emx2, Msx1, Tox2, Aff3 and Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an arterial endothelial cell.

65. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into an arterial endothelial cell.

66. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of the group consisting of Meox2, Tcf15, Pparγ, Wt1 and Ebf3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a cardiac microvascular endothelial cell.

67. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of the group consisting of Zic3, Lef1, Foxf2, Foxf1a and Foxc1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a brain microvascular endothelial cell.

68. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of the group consisting of Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a liver microvascular endothelial cell.

69. The engineered vascular bed-specific endothelial cell of claim 38, whereby the expression product is of the group consisting of Emx2, Nkx2-3, Msx1, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an arterial endothelial cell.

70. The engineered vascular bed-specific endothelial cell according to any one of the previous claims 38 to 69, whereby the transcription factors are overexpressed.

71. The engineered vascular bed-specific endothelial cell according to any one of the previous claims 38 to 70, whereby the source cell is a blood outgrowth endothelial cell (BOEC).

72. The engineered vascular bed-specific endothelial cell according to any one of the previous claims 38 to 70, whereby the source cell is a dedifferentiated cultured human umbilical vein endothelial cell (HUVEC) or a dedifferentiated HUAEC.

73. The engineered vascular bed-specific endothelial cell according to any one of the previous claims 38 to 70, whereby the source cell is a mammalian cell of embryonic or non-embryonic origin.

74. The engineered vascular bed-specific endothelial cell according to any one of the previous claims 38 to 70, whereby the source cell is a source cell of the group consisting of endothelial progenitor cells (EPCs), mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), induced pluripotency stem cells (iPSCs), mesoangioblasts (MABs), multipotent adult progenitor cells (MAPCs), blood outgrowth endothelial cells (BOECs), induced endothelial cells (iECs), unfractionated bone marrow cells, umbilical cord or peripheral blood-derived mononuclear cells, adipose tissue-derived cells, tissue

resident progenitor cells, human umbilical vein endothelial cells (HUVECs), human umbilical artery endothelial cells (HUAECs), human dermal microvascular endothelial cells (HDMECs), cultured endothelial cell lines from heart, cultured endothelial cell lines from liver, cultured endothelial cell lines from brain.

75. The engineered vascular bed-specific endothelial cell according to any one of the previous claims 38 to 70, whereby the cell is terminally differentiated or in partially differentiated form.

76. The engineered cell according to any one of the previous claims 1 to 75, for use in a treatment of vascular bed-specific disorder.

77. The engineered cell according to any one of the previous claims 1 to 75, for use in a treatment of a disorder of vascular bed–specific haemostasis or for a disorder of generating and/or maintaining vascular bed–specific phenotypes.

78. The engineered cell according to any one of the previous claims 1 to 75, for use in a treatment of macrovascular complications evoked by a vascular bed-specific (haemostasis) disorder.

79. The engineered cell according to any one of the previous claims 1 to 75, for use in a treatment of microvascular complications evoked by a vascular bed-specific (haemostasis) disorder.

80. The engineered cell according to any one of the previous claims 1 to 75, for use in a treatment of for decreasing severity of vascular bed-specific disorder in a subject, the method comprising autologous, allogeneic or xenogeneic cell transplantation; in a subject in need of treatment with the engineered cell, thereby decreasing severity of disorder in the subject.

81. The engineered cell according to any one of the previous claims 1 to 75, for use in a treatment of for decreasing severity of vascular bed-specific disorder or diseases or circulatory or hypoxic conditions that can be treated with the compositions and the methods of the invention comprise but are not limited to: atherosclerosis, preeclampsia, erectile dysfunction, renal failure, transplant accelerated arteriosclerosis, deep vein thrombosis, sleep apnea, hypoxia during sleep, fetal hypoxia, smoking, anemia, endothelial dysfunction, sinusoidal obstruction syndrome, regional perfusion deficits

(*e.g.,* limb, gut, renal ischemia), congestic heart failure, peripheral vascular disease, frost bite, decubitus ulcers, asphyxiation, poisoning (*e.g.,* carbon monoxide, heavy metal), altitude sickness, pulmonary hypertension, sudden infant death syndrome, asthma, chronic obstructive pulmonary disease, congenital circulatory anomalities (*e.g.,* Tetralogy of Fallot), erythroblastosis, myocardial infarction, aortic stenosis ro any other cardiac disease which can lead to heart failure, plaque rupture, both primary and secondary (in-stent) restenosis in coronary or peripheral arteries, coronary vascular disease, hypertension, diabetes, obesity, stroke, aneurysm, thrombosis, arrythmia, tachycardia, surgical or physical trauma in a subject, the method comprising autologous, allogeneic or xenogeneic cell transplantation; in a subject in need of treatment the engineered cell, thereby decreasing severity of disorder in the subject.

82. The engineered cell according to any one of the previous claims 1 to 75, for use in a treatment for decreasing severity of vascular bed-specific diseases affecting the brain (*e.g.,* stroke), the liver (*e.g.,* sinusoidal obstruction syndrome), the heart (myocardial ischemia) or the extremities (*e.g.,* peripheral vascular disease) in a subject, the method comprising autologous, allogeneic or xenogeneic cell transplantation; in a subject in need of treatment the engineered cell, thereby decreasing severity of disorder in the subject.

83. The engineered cell for use in a treatment according to any one of the claims 76 to 82, whereby the cell is genetically altered or unaltered, by direct introduction to a site of interest, *e.g.,* on or around the surface of an acceptable matrix, or systemically, in combination with a pharmaceutically acceptable carrier so as to repair, replace or promote the growth of existing and/or new blood vessels.

84. The engineered cell according to any one ofthe previous claims 76 to 83, whereby said cell is administered in the treatment by any one of the following treatments: localised injection, catheter administration, systemic injection, intraperitoneal injection, parenteral administration, oral administration, intra-cranial injection, intra-arterial injection, intra-venous injection, intra-ventricular infusion, intra-placental injection, intra-uterine

injection, surgical intra-myocardial injection, transendocardial injection, transvascular injection, intra-coronary injection, intra-muscular injection, surgical injection into a tissue of interest or via direct application to tissue surfaces (*e.g.,* during surgery or on a wound).

85. A pharmaceutical composition, comprising an engineered cell according to any one of the previous claims 1 to 75.

86. An in vitro bioengineered tissue, comprising an engineered cell according to any one of the previous claims 1 to 75.

87. The use of an engineered cell according to any one of the previous claims 1 to 75, in a method of screening for non-toxic compounds.

88. The use of an engineered cell according to any one of the previous claims 1 to 75, in a method of *in vitro* bioengineering a tissue.

89. The use of an engineered cell according to any one of the previous claims 1 to 75, in *in vitro* drug toxicity testing, either using the cell product alone, or as a (or one of the) cellular component(s) in the context of engineered 2D or 3D tissue equivalents;

90. The use of an engineered cell according to any one of the previous claims 1 to 75 as a (or one of the) cellular component(s) of tissue engineered constructs for implantation in patients (for example to coat the inside of artificial arterial conduits or as the intimal cellular component of a fully biological tissue-engineered vascular graft or to coat cardiac valves).

91. An in vitro method of diagnosing a vascular bed-specific disorder phenotype in a subject, said method comprising (a) analysing the level of expression or activity of expression product of at least 5 genes of the '(differential) reference signatures' of table 4 (brain ECs), of table 5 (liver ECs), of table 6 (heart ECs) and/or tables 12/16/18 (arterial or venous ECs) in a sample isolated from said subject, and (b) compare said level of expression or activity with the level of expression or activity in '(differential) reference signatures' of table 4 (brain ECs), of table 5 (liver ECs), of table 6 (heart ECs) and/or tables 12/16/18 (arterial or venous ECs); whereby a deviated level of expression or activity relative to such 'reference signature' is an indication of such disorder phenotype or a propensity thereto.

92. An *in vitro* method of diagnosing a vascular bed-specific disorder phenotype in a subject, said method comprising: (a) genotyping one or more genes in the '(differential) reference signatures' of table 4 (brain ECs), of table 5 (liver ECs), of table 6 (heart ECs) and/or tables 12/16/18 (arterial or venous ECs) in a sample isolated from said subject, and (b) analyse the DNA sequence of said gene(s); whereby polymorphisms (*e.g.,* SNPs) in said genes of the 'reference signatures' is an indication of such disorder phenotype or a propensity thereto.

93. Use of the '(differential) reference signatures' of table 4 (brain ECs), of table 5 (liver ECs), of table 6 (heart ECs) and/or tables 12/16/18 (arterial or venous ECs), to design tailored therapy, *e.g.,* vascular bed-specific drug delivery or activation of reference signature genes to increase vascularisation.

94. Use of '(differential) reference signatures' of table 4 (brain ECs), of table 5 (liver ECs), of table 6 (heart ECs) and/or tables 12/16/18 (arterial or venous ECs) to read-out the (side) effects on the ECs of the targeted organ/tissue by current or novel (anti-)angiogenic or other therapies.

95. Use of '(differential) reference signatures' of table 4 (brain ECs), of table 5 (liver ECs), of table 6 (heart ECs) and/or tables 12/16/18 (arterial or venous ECs) to read-out the efficiency of existing or to be developed alternative (combinatorial) approaches not described herein to specify source cells towards a vascular bed-specific target cell.

Detailed Description

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

The following detailed description of the invention refers to the accompanying drawings. The same reference numbers in different drawings identify the same or similar elements. Also, the following detailed description does not limit the invention. Instead, the scope of the invention is defined by the appended claims and equivalents thereof.

Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein.

It is intended that the specification and examples be considered as exemplary only.

Each and every claim is incorporated into the specification as an embodiment of the present invention. Thus, the claims are part of the description and are a further description and are in addition to the preferred embodiments of the present invention.

Each of the claims set out a particular embodiment of the invention.

DEFINITIONS

As used herein, the terms below are defined by the following meanings:

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, “includes”, “including” or the like means including, without limitation.

The term “source cell” is defined as the cell that is converted, by methods described in the invention, to a vascular bed-specific endothelial cell. Any mammalian cell of embryonic or non-embryonic origin with endothelial (differentiation) potential can be used as the source cell. Examples of such cells include, but are not limited to: endothelial progenitor cells (EPCs), mesenchymal stem cells (MSCs), embryonic stem cells (ECS), induced pluripotency stem cells (iPSCs), mesoangioblasts (MABs), multipotent adult progenitor cells (MAPCs), blood outgrowth endothelial cells (BOECs), induced endothelial cells (iECs), unfractionated bone marrow cells, umbilical cord or peripheral blood-derived mononuclear cells, adipose tissue-derived cells, tissue resident progenitor cells, human umbilical vein endothelial cells (HUVECs), human umbilical artery

endothelial cells (HUAECs), human dermal microvascular endothelial cells (HDMECs) or cultured endothelial cell lines from heart, liver, brain.

The term "target cell" or the "cell product" is defined as the vascular bed-specific endothelial cell that results from the conversion imposed by the methods described in the invention, wherein the vascular-bed identifies the desired endothelial cell type; for example, the pulmonary vascular bed, refers to vascular endothelial cells of the lungs. The target cell or cell product thus refers to vascular endothelial cells in general, including subtypes based on the vascular bed, such as for example an organ-specific microvascular endothelial cell, an arterial endothelial cell, a cardiac microvascular endothelial cell, a brain microvascular endothelial cell, or a liver microvascular endothelial cell

The term "(differential) reference signature" designates a list of genes that can be used as a reference to define the endothelial subtype identity of the cell product. The latter needs to have certain expression levels of the said list of genes in order to be defined as a certain endothelial subtype, *e.g.,* an arterial endothelial cell, a capillary endothelial cell from the heart, the brain or the liver.

"Embryonic stem cells (ESCs)" are well known in the art and have been prepared from many different mammalian species for many years. ESCs are stem cells derived from the inner cell mass of an early stage embryo known as blastocyst. They are able to differentiate into all derivatives of the three germ layers (ectoderm, mesoderm and endoderm). These include each more than 220 cell types in the adult body. ESCs can become any tissue in the body, excluding placenta. Only the morula's cells are totipotent, able to become all tissues and placenta.

"Induced pluripotency stem cells (iPSCs)" are somatic cells that have been reprogrammed, for example, by introducing exogenous genes that confer on the somatic cell a less differentiated phenotype. The cells can then be induced to differentiate into less differentiated progeny. IPSCs have been derived using modifications of an approach originally discovered in 2006 (Takahashi et al., 2007). For example, in one instance, to produce iPSCs, scientists started with skin cells that were then modified by standard laboratory technique using retroviruses to insert genes into the cellular DNA. In one instance, the inserted genes were *Oct4*,

Sox2, *Klf4* and *c-myc*, known to act together as natural regulators to keep cells in an embryonic stem cell-like state. These cells have been described in the literature (Brambrink et al., 2008; Hanna et al., 2008; Jaenisch and Young, 2008; Wernig et al., 2008). These references are incorporated by reference for teaching iPSCs and methods for producing them. It is also possible that such cells can be created by specific culture conditions (exposure to specific agents).

The term "MAPC" is an acronym for "multipotent adult progenitor cell". The term "adult", with respect to MAPCs is non-restrictive. It refers to a non-embryonic somatic cell. "Multipotent", with respect to MAPCs, refers to the ability to give rise to cell types of more than one embryonic lineage. MAPCs can form cell lineages of all three primitive germ layers (*i.e.,* endoderm, mesoderm and ectoderm). Human MAPCs and methods for their isolation and growth are described in U.S. Patent 7,015,037 and U.S. Patent Application Serial No. 10/467,963 (PCT/US02/04652, published as WO 02/064748). MAPCs have also been derived from other mammals: mice (U.S. Patent 7,015,037 and U.S. Patent Application No. 10/467,963); rats (U.S. Patent Application No. 10/467,963); pigs (U.S. Patent Application PCT/US2005/038979).

"Progenitor cells" are cells produced during differentiation of a stem cell that have some, but not all, of the characteristics of their terminally-differentiated progeny. Defined progenitor cells, such as "endothelial progenitor cells", are committed to a lineage, but not to a specific or terminally-differentiated cell type. The phrase "endothelial cells" encompasses not only terminally-differentiated cell types, but also cells that are committed to a specific endothelial lineage (*e.g.,* venous and/or arterial lineage), but are not terminally-differentiated. The term "progenitor" as used in the acronym "MAPC" does not limit these cells to a particular lineage.

"Stem cell" means a cell that can undergo self-renewal (*i.e.,* progeny with the same differentiation potential) and also produce progeny cells that are more restricted in differentiation potential.

"Effective amount" generally means an amount which provides the desired local or systemic effect. For example, an effective amount is an amount sufficient to effectuate a beneficial or desired clinical result. The effective amounts can be provided all at once in a single administration or in fractional amounts that provide

the effective amount in several administrations. The precise determination of what would be considered an effective amount may be based on factors individual to each subject, including their size, age, injury, and/or disease or injury being treated, and amount of time since the injury occurred or the disease began. One skilled in the art will be able to determine the effective amount for a given subject based on these considerations which are routine in the art.

"Subject" means a vertebrate, such as a mammal, such as a human. Mammals include, but are not limited to, humans, dogs, cats, horses, cows and pigs.

"Therapeutically effective amount" refers to the amount determined to produce any therapeutic response in a mammal. For example, effective amounts of therapeutic cells or cell-associated agents may prolong the survivability of the patient, an/or inhibit overt clinical symptoms. Treatments are therapeutically effective within the meaning of the term as used herein, include treatments that improve a subject's quality of life even if they do not improve the disease outcome *per se*. Such therapeutically effective amounts are ascertained by one of ordinary skill in the art through routine application to subject populations such as in clinical and pre-clinical trials. Thus, to "treat" means to deliver such an amount.

"Treat", "treating" or "treatment" are used broadly in relation to the invention and each such term encompasses, among others, preventing, ameliorating, inhibiting, or curing a deficiency, dysfunction, disease, or other deleterious process, including those that interfere with and/or result from therapy.

METHODS AND COMPOSITION OF THE INVENTION

The invention is based on genetically altering the source cells by overexpression of transcription factor genes. It is accordingly an object of the present invention to provide methods of altering source cells into vascular endothelial cells by overexpression of transcription factor genes as provided throughout this application. As described in detail in the ***Examples*** provided herein, this has been accomplished by lentiviral transduction. Nevertheless, genetic modification by introducing DNA or RNA into the source cell can be accomplished by a variety of methods available to those skilled in the art, and are within the admit

of the present invention. These methods comprise means for either permanent or transient expression of the gene introduced and are generally grouped into four major categories: (i) viral transfer, including the use of DNA or RNA viral vectors, such as retroviruses (including lentiviruses), Simian virus 40 (SV40), adenovirus, adeno-associated viruses, alpha virus, including Sindbis virus (U.S. Patent No. 5,843,723), herpes virus and bovine papillomavirus; (ii) chemical transfer, including calcium phosphate transfection, DEAE dextran transfection methods; (iii) membrane fusion transfer, using DNA-loaded membranous vesicles such as liposomes, red blood cell ghosts and protoplasts; and (iv) physical transfer techniques, such as microinjection, microprojectile, electroporation, nucleofection or direct "naked" DNA transfer.

The genetic material can be introduced using promoters that will allow for the gene of interest to be positively or negatively induced using certain chemicals/drugs, to be eliminated following administration of a given drug/chemical/temperature, or can be tagged to allow induction by chemicals (including, but not limited to, the tamoxifen responsive mutated estrogen receptor) in specific cell compartments (including, but not limited to, the cell membrane).

Since lentiviral transduction will lead to random integration in the genome, and since random integration may cause untoward effects (*e.g.,* silencing of the transgene, or insertional activation of tumour suppressor genes), the genetic material can be introduced by site-directed homologous recombination, using Zinc finger nucleases or TALE nucleases, into gene loci that are known to have favourable chromatin configurations and are thus transcriptionally active, such as the *AASV1* locus on human chromosome 19 or the murine *ROSA26* locus.

Successful transduction or transfection of the source cells can be demonstrated using genetic markers, a technique that is known to those of skill in the art. Fluorescent proteins, *e.g.,* green fluorescent protein of *Aequorea Victoria*, Cherry protein, for example, have been routinely used. Alternative selectable markers include the *β-Gal* gene, the truncated nerve growth factor receptor, drug selectable markers (including, but not limited to: NEO, MTX, hygromycin, blasticidin).

Not only overexpression of the TFs mentioned above and below can increase their expression level or activity can also be increased in alternative ways.

Certain chemical compounds have been described to mimic or act as ligands for several of these TFs or to stabilise the protein (*e.g.,* rosiglitazone for PPARγ and PRDM16). Other chemical compounds can act by repressing the effect of specific TFs. In addition, TFs (*e.g.,* PRDM16) often act through complexing with co-factors which modulates the effect of the TFs. Thus, methods in which such chemical components or co-factors are used as a substitute for their 'target' TF represent another embodiment of the current invention.

Assessment of successful conversion of the source cell to the desired cell product can be done by quantitative real-time polymerase chain reaction (qRT-PCR) for genes from the (differential) reference signature or for genes previously described in the literature to be specific for the desired vascular bed-specific EC. In addition, whole genome gene expression of the cell product can be assayed by microarray or equivalent methods (*e.g.,* RNA-seq). Also contemplated are methods to identify successfully converted cells by their expression of proteins encoded by genes from the (differential) reference signature or genes previously published in the literature, by a variety of methods including but not limited to: fluorescence activated cell sorting (FACS), immunofluorescence staining or Western blotting. Assessment of the purity of the cell product can be performed by qRT-PCR for potentially contaminating cell types or, at the protein level, by FACS, immunofluorescence or Western blotting. In addition, successful conversion can also be monitored on life cells using reporter constructs, stably incorporated in the source cells, based on vascular bed-specific EC promoters that then become activated upon successful conversion. These promoters can drive expression of a fluorescence gene, such that successfully converted cells can be identified by FACS.

In case the cell product is not homogenous, *i.e.,* comprises a mixture of successfully converted and not successfully converted cells, then, if desirable, the successfully converted cells can be separated from the not successfully converted cells by FACS or magnetic beads, using antibodies against cell surface markers uniquely expressed on the successfully converted cells. The reporter technology based on fluorescence genes described above to monitor successful conversion of the source cells can also be used to separate them from the not successfully converted cells. Alternatively, the promoters of the reporter constructs can drive the

expression of an antibiotic resistance gene, allowing for positive selection of the successfully converted cells by exposure to antibiotics (including, but not limited to G418, hygromycin, blasticidin).

As an alternative to genetic modification by introducing DNA or RNA into the source cell, the corresponding proteins can be transferred directly to cells when they are linked to a protein transduction domain (PTD), small cationic peptide domains that can freely and rapidly cross cell membranes. Several PTDs such as poly-arginine and HIV-derived Tat have been identified that allow a fused protein to efficiently cross membranes.

In addition to the TF genes described herein, variants, members of the same family (*e.g.,* the Prdm family), homologues or orthologues of the factors/genes, which have the same biological function/activity, can be used or assayed for in methods of the invention. For example, variants, homologues or orthologues of use in the present invention may be homologous or have sequence identity (nucleotide or amino acid sequence) with the transcription factors provided herein. "Homology" refers to the percent identity between two polynucleotide or two polypeptide sequences. Examples of assays and programs to determine if a factor/gene is homologous are known in the art. Determination of the percent identity between any two sequences can be accomplished using a mathematical algorithm. Computer implementations of the mathematical algorithms can be utilised for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program, the ALIGN program and GAP, BESTFIT, BLAST, FASTA, and TFASTA.

In addition to the use of TF (combinations) described herein, to obtain vascular bed-specific ECs (*i.e.,* the cell product), the invention is also directed to use the cell product for distinct therapeutic and diagnostic purposes. Thus in a further embodiment the present invention provides the target cells obtained using the methods of the present invention, as well as the use thereof, in particular in any pharmaceutical compositions containing the cell product. Such compositions are suitable for administration to subjects in need of such cells. The cells would be administered in therapeutically effective amounts. The choice of the pharmaceutical composition for administering cells for a given application to patients will depend on a variety of factors. Prominent among these will be the species of subject, the

nature of the disorder, dysfunction, or disease being treated and its state and distribution in the subject, the nature of other therapies and agents that are being administered, the optimum route of administration, survivability via the route, the dose regimen, and other factors that will be apparent to those skilled in the art. In particular, for instance, the choice of suitable carriers and other additives will depend on the exact route of administration and the nature of the particular dosage form. A pharmaceutically acceptable preservative or stabiliser can be employed to increase the life of cell/medium compositions. If such preservatives are included, it is well within the purview of the skilled artisan to select compositions that will not affect the viability or efficacy of the cells. Those skilled in the art will recognise that the components of the compositions should be chemically inert. This will present no problem to those skilled in chemical and pharmaceutical principles.

The cell products can be administered by a variety of methods available to the art, including but not limited to: localised injection, catheter administration, systemic injection, intraperitoneal injection, parenteral administration, oral administration, intracranial injection, intra-arterial injection, intra-venous injection, intra-ventricular infusion, intra-placental injection, intra-uterine injection, surgical intra-myocardial injection, transendocardial injection, transvascular injection, intra-coronary injection, intra-muscular injection, surgical injection into a tissue of interest or via direct application to tissue surfaces (*e.g.,* during surgery or on a wound). Methods to administer the cells may be combined with methods to increase cell survival, including, but not limited to: incorporation into a biopolymer (*e.g.,* fibronectin, fibrin, fibrinogen, thrombin, collagen, proteoglycans) or synthetic polymer, encapsulation achieved by polymers (such polymeric encapsulation systems include, but are not limited to: alginate, polysaccharide hydrogels, chitosan, calcium or barium alginate, a layered matrix of alginate and polylysine, a photopolymerisable poly(ethylene glycol) (PEG) polymer, a polyanionic material termed Biodritin (U.S. Patent 6,281,341), polyacrylates, and polymers such as hydroxyethyl methacrylate methyl methacrylate), encapsulation in silicon capsules with pores applied by photolithographic techniques, encapsulation using immune-compatible polycations, including but not limited to, poly-l-lysine polycation or poly-l-ornithine or poly(methylene-co-guanidine) hydrochloride, encapsulation in biocompatible semipermeable membranes (“macroencapsulation”).

USE OF THE INVENTION

For the gene signatures or the '(differential) reference signatures', the following uses can be defined:

- since the gene or reference signature changes upon pathological conditions, it can be used as a diagnostic in patients with vascular bed-specific disorders (for instance by genotyping for the signature); furthermore, the gene or reference signature can be used to estimate the risk for acquiring certain cardiovascular disorders by looking for polymorphisms (*e.g.,* SNPs) in one or more genes of the signature;
- the gene or reference signature can be used to design tailored therapy, *e.g.,* vascular bed-specific drug delivery or activation of reference signature genes to increase vascularisation;
- the gene or reference signature can be used as a read-out for the (side) effect on the ECs of the targeted organ/tissue by current or novel (anti-)angiogenic or other therapies;
- the gene or reference signature can be used as a read-out for existing or to be developed alternative (combinatorial) approaches not described herein to specify source cells towards a vascular bed-specific EC phenotype.

For the vascular bed-specific ECs ('the target cells' or desired 'cell products'), the following applications can be defined:

- cell transplantation in patients with vascular bed-specific disorders. For the purposes described herein, either autologous, allogeneic or xenogeneic cells can be administered to a patient, either in terminally differentiated or in partially differentiated form, genetically altered or unaltered, by direct introduction to a site of interest, *e.g.,* on or around the surface of an acceptable matrix, or systemically, in combination with a pharmaceutically acceptable carrier so as to repair, replace or promote the growth of existing and/or new blood vessels;
- *in vitro* drug toxicity testing, either using the cell product alone, or as a (or one of the) cellular component(s) in the context of engineered 2D or 3D tissue equivalents;

- as a (or one of the) cellular component(s) of tissue-engineered constructs for implantation in patients (for example to coat the inside of artificial arterial conduits or as the intimal cellular component of a fully biological tissue-engineered vascular graft or to coat cardiac valves).

Examples of (vascular bed-specific) conditions or diseases or circulatory or hypoxic conditions that can be treated with the compositions and the methods of the invention comprise but are not limited to: atherosclerosis, preeclampsia, erectile dysfunction, renal failure, transplant accelerated arteriosclerosis, deep vein thrombosis, sleep apnea, hypoxia during sleep, fetal hypoxia, smoking, anemia, endothelial dysfunction, sinusoidal obstruction syndrome, regional perfusion deficits (*e.g.,* limb, gut, renal ischemia), congestic heart failure, peripheral vascular disease, frost bite, decubitus ulcers, asphyxiation, poisoning (*e.g.,* carbon monoxide, heavy metal), altitude sickness, pulmonary hypertension, sudden infant death syndrome, asthma, chronic obstructive pulmonary disease, congenital circulatory anomalities (*e.g.,* Tetralogy of Fallot), erythroblastosis, myocardial infarction, aortic stenosis, or any other cardiac disease which can lead to heart failure, plaque rupture, both primary and secondary (in-stent) restenosis in coronary or peripheral arteries, coronary vascular disease, hypertension, diabetes, obesity, stroke, aneurysm, thrombosis, arrythmia, tachycardia, surgical or physical trauma.

EXAMPLES

The following examples are provided in order to demonstrate and further illustrate aspects of the present invention and its many advantages and are not to be construed as limiting the scope thereof.

Example 1: *Identification of and induction in dedifferentiated cultured heart endothelial cells or blood outgrowth endothelial cells (BOECs) of a gene (and functional) signature specific for heart microvascular endothelial cells using individual transcription factors or combinations thereof and studies on the* in vivo *role of these transcription factors*

1.1. ABSTRACT

Endothelial cells (ECs) lining the inside of blood vessels in different organs show significant heterogeneity caused by cell-intrinsic and -extrinsic factors. They are morphologically and functionally adapted to meet the unique demands of the tissue in which they reside. In this study, by performing a comparative microarray analysis on microvascular ECs freshly isolated from liver, heart and brain, we established '*ex vivo*' gene-signatures and partly validated them at the protein level and in samples of human origin. When zooming in on heart microvascular ECs, we found that the lack of tissue microenvironment in cultured ECs wiped out the expression of the majority of heart EC-specific genes. Functional annotation analysis suggested a prominent active function of heart ECs in fatty acid transport, a process the molecular regulation of which is very elusive at present. Interestingly, when evaluating the expression of the heart EC fingerprint in a larger array of tissues, we found that ECs from metabolically highly active tissues (*i.e.,* fat and skeletal muscle) were very similar to heart ECs, unlike ECs from other tissues (*i.e.,* lung, kidney and pancreas). Furthermore, we identified a pool of 5 transcription factors (TFs; mesenchyme homeobox 2 or *MEOX2*, transcription factor 15 or *TCF15, Early B-cell factor 3 EBF3,* peroxisome proliferation-activated receptor gamma or *PPARγ*, and Wilms tumour 1 or *WT1*) which were able to induce the heart EC gene and functional signature when overexpressed alone or in combination *in vitro,* in cultured heart ECs or in blood outgrowth endothelial cells (BOECs). Accordingly, compound heterozygosity for two of these TFs (*Meox2* and *Tcf15*) in mice resulted in a significant drop in expression of heart EC signature genes – but not liver EC genes – *in vivo.* Furthermore, fatty acid transporter gene expression was decreased in ECs – but not cardiomyocytes – of *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ hearts compared to their wild-type (WT) littermates, while glucose transporter *Glut1* was upregulated. Accordingly, *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ hearts, but not livers, had a reduced uptake of fatty acids, while glucose uptake was increased. Finally, aged – but not young – *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ mice developed cardiac fibrosis and had impaired heart function.

The availability of ECs with a heart-specific molecular gene and functional signature will be useful for *in vitro* drug toxicity testing and for designing *in vivo* revascularisation strategies tailored to the heart for patients with ischemic heart

disease. In addition, this strategy based on vascular bed-specific TF overexpression to induce a heart EC-specific gene signature in BOECs, can also be tested for other EC types, *e.g.,* liver and brain ECs. Furthermore, our findings highlight a regulatory role for ECs in fatty acid transfer to the heart parenchyma and describe two of its intrinsic regulators. Our insights could be used to develop new strategies based on endothelial targeting of Meox2/Tcf15 to remedy cardiac dysfunction as a result of changes in the relative contribution of fatty acids and glucose to energy production.

1.2. BACKGROUND AND AIMS

ECs lining the inside of blood vessels in different organs show significant heterogeneity caused by cell-intrinsic and -extrinsic factors and are morphologically and functionally adapted to meet the unique demands of the tissue in which they reside. For instance, the ECs of the liver – a filter and storage organ – are organised in sieve plates and lack a basal lamina to facilitate the passive exchange of big molecules between the blood and the hepatocytes (Aird, 2007a). Brain ECs, on the other hand, rest on a basal lamina, are in close contact with astrocyte end feet and pericytes, have tight junctions between them and are equipped with specific carrier molecules to transport only the necessary substances and to avoid entrance of pathogens or toxic compounds into the central nervous system. Heart capillary ECs influence cardiac function by secreting signalling molecules (*e.g.*, nitric oxide or NO), possess a basal lamina and have a high vesicular transport activity (Brutsaert, 2003). The existence of endothelial diversity has tremendous implications for the development and treatment of diseases, because often the vasculature of the targeted organ is affected or has a direct role in the pathogenesis. From this perspective, adequate and customised EC repair is indispensable for successful restoration of organ function (Aird, 2007a). Moreover, since little is known about specific functions of heart microvascular ECs, we focussed our efforts on the study of these cells in relation to the organ where they reside. As a continuously contracting organ, the heart needs to produce ~20 times its own weight in adenosine triphosphate (ATP) each day. Mainly glucose and fatty acids (FAs) serve as substrates for ATP production. Under physiological conditions

FAs account for 60-90% of ATP generation, whereas glucose contributes for 10-40%. A broad range of cardiomyopathies that can lead to cardiac hypertrophy and failure are associated with chronic alterations in cardiac energy metabolism in general and FA utilisation in particular (Neubauer, 2007). Besides the demonstrated role of molecules involved in FA metabolism in cardiomyocytes, recent findings suggest that proteins involved in FA uptake and transport in both ECs and cardiomyocytes may also play a role in these pathologies (van der Vusse, 2000). Nevertheless, it has not been established so far whether cardiac ECs are specialised in order to fulfil the high FA delivery demanded by the heart. Recent studies identified vascular endothelial growth factor (VEGF)-B as a paracrine mediator of FA uptake through ECs in heart (and skeletal muscle) and showed that VEGF-B inhibition protected against type 2 diabetes by decreasing FA uptake and reverting muscle metabolism towards glucose consumption (Hagberg et al., 2010 and 2012). However, the EC-intrinsic (transcriptional) regulators that determine active uptake and transfer of FAs are unknown, except for peroxisome proliferator-activated receptor γ (PPARγ) (Kanda et al., 2009). We now identified two novel transcriptional regulators of the gene signature and metabolic substrate transport in heart ECs, *i.e.*, *Meox2* (also known as *Gax* or *Mox2*) and *Tcf15* (also known as *paraxis*). Meox2 and Tcf15 play a role in early specification of paraxial mesoderm to somitic dermomyotome and a defect in heart development was not reported in *Meox2*- or *Tcf15*-deficient mice (Burgess et al., 1996; Mankoo et al., 1999). Nevertheless, expression of both factors was shown in the adult mouse heart, without indication of specific cellular localisation. Meox2 participates in regulating EC homeostasis, being either pro- or anti-angiogenic depending on the expression level and EC type. *Tcf15*, on the other hand, has never been studied in ECs, except that it was initially cloned from an endothelial library. Here we demonstrate that within the adult heart, Meox2 and Tcf15 are exclusively expressed in ECs and, using gain- and loss-of-function *in vitro* and *in vivo* studies, that they are critical regulators of the balance between FA and glucose transport across heart ECs.

Our first aim was to learn more about molecular and functional differences between capillary (microvascular) ECs from three clinically relevant vascular beds (*i.e.,* those in brain, heart and liver). Certain studies have offered some insight into

specific expression of brain and liver capillary ECs (Calabria and Shusta, 2008; Geraud et al., 2010), however, for heart capillaries, almost nothing is known since most research efforts have focused on endocardial ECs or the coronary macrovasculature (Brutsaert, 2003). This transcriptional profiling effort was then complemented with functional annotation studies and a cross-species comparison in human samples. Since it is known for macrovascular (Aranguren et al., in revision; see ***Example 2***) and some (*e.g.,* brain and liver) microvascular ECs (Calabria and Shusta, 2008; Geraud et al., 2010) that vascular bed-specific genes are significantly downregulated once the cells are deprived of their microenvironment during cell culture, we here investigated the '*ex vivo*' vascular bed-specific gene expression profile of freshly isolated cells and compared it to their cultured '*in vitro*' equivalents.

A second objective was to restore or induce the '*ex vivo*' signature in cultured heart ECs or EC progenitors, respectively. From studies in macrovascular ECs, it is known that TFs play a central role in determining their specific gene signature. Hence, within our '*ex vivo*' microvascular EC gene profiles, we expected to find – and indeed found – TFs that co-determine their unique signature. To demonstrate the latter, we overexpressed heart EC-specific TFs – alone or in combination – in cultured heart ECs as well as in unspecified EC progenitors from peripheral or cord blood (blood outgrowth endothelial cells or 'BOECs') which we previously showed to be functionally integrating in newly formed vessels in wounds (Hendrickx et al., 2010).

A final aim was to explore the *in vivo* role of heart EC-specific TFs. To analyse in more detail the *in vivo* role of some TFs, corresponding knock-out mice were obtained with the specific intention to (i) explore whether lack of these TFs *in vivo* had repercussions for the specific EC signature and to (ii) study potential effects of TF deficiency on vascular and heart function.

1.3. MATERIALS AND METHODS

Procedures to achieve Aim 1

Animals and human biopsies. *Tie2-GFP* mice (Motoike et al., 2000) were used

as EC donors for expression profiling. After obtaining informed consent, human ECs were isolated from heart biopsies (right atrial appendage from patients undergoing left-sided valve surgery without pulmonary hypertension or right heart failure), brain biopsies (cortical tissue from epileptic patients undergoing amygdalo-hippocampectomy) or liver biopsies (patients undergoing elective cholecystectomy). Experimental procedures with animals and human-derived samples were approved by the Ethics Committee on Animal Use of KU Leuven and of University Hospitals Leuven, respectively. Human samples were handled according to the Declaration of Helsinki.

EC isolation and culture. For murine microvascular EC isolation, tissues from 8-12 weeks-old mice were dissected out, surrounding connective tissue and visible large vessels removed and tissues enzymatically digested using optimised procedures for each organ (*i.e.*, 1.2 U/ml dispase, followed by Percoll gradient centrifugation for liver; 0.7 mg/ml crude collagenase + 39 U/ml DNAse I followed by BSA density gradient centrifugation for brain; 0.7 mg/ml crude collagenase for kidney, lung and pancreas; 1.5 mg/ml collagenase I for heart, skeletal muscle, BAT and WAT). After a final wash in PBS, cells were resuspended in FACS buffer (PBS/EDTA 1mM/HEPES 25 mM /1 % BSA, pH7), filtered with a 40 μm mesh and sorted directly in RLT (Qiagen) or TRIzol® (+ 1% β-mercapto-ethanol [BME]) for RNA extraction or in Gey's buffer for protein extraction. For profiling, sorting was done based on the GFP^+ fraction from *Tie2-GFP* donors and for each organ, samples were a mix of male and female in a 1:1 ratio. For assaying gene expression in $Meox2^{+/-}$:$Tcf15^{+/-}$ donors (Aim 3) and their single-heterozygous or Wild-type littermates, sorting was based on the $CD31^+CD34^+CD45^-$ fraction and either males or females were used.For human heart ECs, epicardial tissue was removed and biopsies were digested with 1.5 mg/ml collagenase I; for human liver ECs, biopsies were digested with 0.08 Wunsch U/ml liberase and 39 U/ml DNAse I; for human brain ECs, meninges and the most external layer of white matter were removed and biopsies were digested with 0.7 mg/ml crude collagenase and 39 U/ml DNAse I followed by BSA density gradient centrifugation. After a final wash in PBS, cells were resuspended in FACS buffer, filtered with a 40 μm mesh and sorted directly in RLT or TRIzol® (+ 1% BME) for RNA extraction. Sorting was done

either based on the $Tie2^+podoplanin^-CD45^-$ or the $CD31^+CD34^+CD45^-$ fraction. An antibody list for FACS, immunofluorescence and Western blot is provided in ***Table 1.*** To obtain cultured human heart ECs, cells obtained from freshly digested biopsies were grown on plates coated with 0.1% gelatine in EBM2 medium supplemented with EGM2-MV bullet kit (Lonza) in standard conditions (95% O_2/5% CO_2/37° C). When confluent, the $Tie2^+podoplanin^-CD45^-$ or $CD31^+Tie2^+CD45^-$ EC fraction was sorted and replated, or RNA from ~10^5 sorted ECs was collected for qRT-PCR analysis. Before every experiment cell purity was assessed by CD31 FACS staining.

Cardiomyocyte isolation and culture. Single ventricular myocytes were enzymatically dissociated from 3- to 4-months-old mice. Mice were injected i.p. with heparin, anaesthetised with pentobarbital, and the heart was quickly excised. After cannulation of the aorta, hearts were mounted on a Langendorff perfusion set. The heart was briefly rinsed with normal Tyrode solution, containing 137 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 11.8 mM Hepes, 10 mM glucose and 10 mM 2,3-butanedione monoxime (BDM; Sigma Aldrich), pH adjusted to 7.4 with NaOH. Subsequently it was perfused with a Ca^{2+}-free Tyrode solution for 10 min. The Ca^{2+}-free Tyrode solution contained 130 mM NaCl, 5.4 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 6 mM Hepes, 20 mM glucose and 10 mM BDM, pH adjusted to 7.2 with NaOH. Collagenase type II 672 U/ml (Worthington), and 30 μM $CaCl_2$ added to the Ca^{2+}-free Tyrode solution, were recirculated for 8-10 min. The enzymes were washed out with low Ca^{2+}Tyrode solution, *i.e.*, the Ca^{2+}-free solution to which 0.18 mM $CaCl_2$ was added, supplemented with 0.5% Bovine Serum Albumin (BSA; Sigma) for 3 min. and then again with low Ca^{2+}Tyrode solution without BSA for 3 min. All solutions used were continuously gassed with 95% O_2/5%CO_2. The heart was then removed from the perfusion apparatus, the ventricles dissociated into single cells by pipetting and afterwards with 5 min. gentle shaking. Cells were sieved through a 250 μm mesh opening, washed twice with Ca^{2+}-free Tyrode solution supplemented with increasing doses of Ca^{2+} (0.5 and 1 mM) and pelleted by centrifugation at 50g for 1 min. after each wash. Finally, cardiomyocytes were collected in TRIzol® or RIPA buffer for *ex-vivo* qPCR or Western blot analysis, or resuspended in culture medium (M-199 medium, Gibco-Life Technologies),

supplemented with 2 mM carnitine, 5 mM taurine, 5 mM creatine (all Sigma Aldrich), and 10 mM BDM, plated onto dishes previously coated with laminin (Sigma Aldrich), and allowed to attach for four hours under standard conditions (95% O_2/5% CO_2, 37° C) before using them for the *in vitro* fatty acid (FA) uptake experiments.

Microarray analysis and data filtering. Microarrays on pooled EC samples were performed by the VIB Nucleomics Core. Briefly, RNA quality control was done using a Bioanalyser 2100 (Agilent Technologies) and 500 pg of RNA from the EC fraction of 5 selected samples per tissue was amplified, biotin-labelled and run on a mouse genome-wide microarray (Affymetrics Mo Gene1-0ST). Microarray data were filtered to obtain a validated tissue-specific EC signature: first, only genes that were statistically significantly and differentially overexpressed at least 4-fold ($Log_2 \geq 2$) *versus* the other two tissues-ECs and that had a mean Log_2 probe intensity greater than 6 were retained. Next, for the heart, only genes that were significantly enriched in the EC fraction (by comparing *versus* the non-EC fraction and/or freshly isolated cardiomyocytes) were retained. For brain and liver, the validaton against the non-EC fraction was performed for only 30 genes (representing all TFs and the most differentially expressed genes). Functional annotation analysis was performed with DAVID (http://david.abcc.ncifcrf.gov/) software on the filtered list of the heart and on the full lists of brain and liver.

RNA/protein isolation, cDNA preparation, qRT-PCR and Western blot. RNA isolation, qRT-PCR and Western blot were performed as described extensively in ***Example 3***. Briefly, total RNA from cell lysates was extracted using TRIzol® reagent or RLT lysis buffer. mRNA was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen) and cDNA underwent 40 rounds of amplification on an ABI PRISM 7700 cycler (Perkin Elmer/Applied Biosystems) for standard SYBER GREEN qRT-PCR. Primer sequences are listed in ***Table 2***. mRNA levels were normalised using *GAPDH, ACTB or TUBB* as housekeeping gene. Proteins were extracted with RIPA buffer (Sigma) supplemented with protease inhibitors. For Western blotting, 10 to 40 μg of proteins was used. Blot pictures were recorded with a Bio-Rad Chemidoc XRS+ molecular imager, equipped with Image Lab

software (Bio-Rad laboratories). An antibody list for Western blotting is provided in ***Table 1***.

Procedures to achieve Aim 2

Lentivirus production and overexpression. The lentiviral construct for overexpressing human *MEOX2* (EX-Z3242-Lv114) was purchased from Genecopoeia; *PPARG1* was cloned from human heart EC cDNA and other TFs were amplified starting from cDNA plasmids (Thermo Scientific Molecular Biology; (maps for the used lentiviruses can be found in ***Figure 2.A-D*** and cloning information in ***Table 3***). Lentiviruses were produced in HEK293 cells using two helper plasmids (psPax2 and PMD2G) and Fugene® transfection reagent (Roche Applied Science) and virus productions were titered to use the minimum amount of virus giving 100% transduction. For transduction, cultured human heart ECs and BOECs were plated on 0.1% gelatin-coated and collagen type I-coated 24-well plates, respectively, and transduced the next day. The medium was changed 12 hours post-transduction. When multiple transductions were performed, the different viruses were added with an interval of 24 hours. Cells were collected 3 days after the last overexpression for RNA extraction, cDNA synthesis and qRT-PCR analysis or used for *in vitro* FA uptake or Western blot experiments.

BOEC isolation and culture. BOECs were isolated and grown from peripheral and umbilical cord blood (after obtaining informed consent), as previously published (Hendrickx et al., 2010). Briefly, two times 25 ml peripheral (venous) blood was taken using a 20 ml syringe filled with 2.5 ml heparin (5,000 U/ml). Cord blood (50 ml) was collected in dedicated plastic collector bags (Fenwal) containing sodium citrate as anticoagulant. After dividing the blood equally in two 50 ml falcon tubes, the blood was diluted with an equal volume of PBS containing 1% PSA (penicillin/streptomycin/amfotericinB; Invitrogen). Ten falcon tubes (10 ml) were prepared by filling them with 2.5 ml histopaque (Ficoll Paque™ Plus, GE Healthcare). The layer of 10 ml diluted blood was carefully put onto each histopaque layer taking care not to mix the two layers and the 10 ml falcon tubes were centrifuged for 30 min. at 750 g in a centrifuge with a swing-out rotor of which the brake was switched off. Buffy coats were collected into a new 50 ml falcon tube. 45 ml of PBS/1% PSA/2% FBS (FBS: fetal bovine serum; Hyclone) was added to

the falcon tube and the pellet was washed thoroughly by resuspension. After centrifugation for 5 min. at 750 g (with the brakes on), the supernatant was taken off and the cell pellet was resuspended in 50 ml PBS/1% PSA/2% FBS. After repeating the last two steps one more time, the supernatant was taken off and the remaining cell pellet was resuspended in EBM-2 (EGM-2 basal medium + EGM-2 singlequots; Lonza). The cells were plated on one or more collagen type-I coated six-well plate(s) (3 ml/well; Greiner Bio-One) and incubated at 37°C and 5% CO_2. The media of the cells was changed 100% every day during the first week and thereafter every other day until colonies appeared (usually between day 11-28 days after plating). Once colonies had reached a sufficient size (usually 2-4 weeks after plating), the colonies were harvested together by trypsinisation. Cells were resuspended in EBM-2 growth medium and 3 ml/well was plated in collagen-coated six-wells. Once the wells were confluent, cells were split (1:3). From then on cells were plated in 10 cm collagen type I-coated plates (Greiner Bio-One).

In vitro *FA uptake.* FA uptake assays were performed in human heart ECs 72 hours post-transduction or in freshly isolated adult murine cardiomyocytes, after 4 hours of culture. Briefly, cells were medium-deprived and washed with Gey's buffer. For FFA uptake measurement, cells were incubated with 500 nM BODIPY-PA (Molecular Probes) in Gey's buffer for 30 min. (for cardiomyocytes 1 mM 2,3-butanedione monoxime was also added), or 5 μM BODIPY-PA in PBS containing 0.1% FA free-BSA. For VLDL-associated FA uptake, cells were incubated for 2 hours with 10 μg/ml DiI-Human VLDL (Kalen Biomedical) in Gey's buffer. Cells were washed twice and maintained in Gey's buffer for 1 hour, then fixed with 4% paraformaldehyde and stained with DAPI. Pictures were taken with a Zeiss Axiovert 200M microscope at 40X magnification. The number of fluorescent vesicles in the cytoplasm was determined by a blinded investigator in 20-40 cells per condition, in 3-5 independent experiments.

Procedures to achieve Aim 3

Animals. *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ mice were obtained by intercrossing *Meox2*$^{Cre/+}$ (C57Bl/6 background; Jackson Laboratories; stockN°003755) and *Tcf15*$^{+/-}$ mice (129S7/SvEvBrd*C57Bl/6 background; provided by E.N. Olson and J.A. Rawls,

Dallas, TX and Tempe, AZ, USA; Burgess et al., 1996). Mice were analysed at 3-4 months of age or at 11 months of age. Heart ECs or liver ECs ($CD31^+CD34^+CD45^-$ cells) were isolated as described above from 7 to 17 weeks-old littermates, either males or females, and gene expression was evaluated by qRT-PCR.

Histology. Oil Red-O, Hematoxylin-Eosin, Sirius red and immunofluorescence stainings were performed as described (Hendrickx et al., 2010). Briefly, for Oil Red-O staining mice were anesthetised with pentobarbital, hearts were rapidly excised, washed in KCl 1M and PBS, directly snap-frozen in liquid N_2 and conserved at -80°C until used. Frozen hearts were embedded in Tissue-tek freezing medium (Leica Biosystems), cryo-sectioned at 10 μm, air-dried for 10 min. and immediately stored at -20°C to be used the same day. Tissue slides were rinced with milliQ water and fixed 1h with 3.7% formaldehyde in milliQ water. After one rinse in milliQ water, slides were immersed in a freshly prepared, Wattman-filtered Oil Red-O solution (0.3% Oil Red-O in 60% Triethyl-phosphate-H_2O) for 30 min. Then rinsed with milliQ water and deionised water and mounted with Aquadrop (Merck). Pictures were taken with a Zeiss Axiovert 200M microscope at 40X magnification and quantification was carried out with the use of ImageJ software evaluating the percentage of stained tissue surface. The procedure for immunofluorescence/immunohistochemical staining was performed on 3-6 μm thick sections of paraffin embedded tissues. An antibody list for IF is provided in ***Table 1***. Images were recorded on a Zeiss Axiovert 200M microscope equipped with a Zeiss MRc5 camera and Axiovision 4.8 software. Oil red-O staining, capillary/cardiomyocyte ratio, cardiomyocyte cross-sectional area and Sirius red staining were quantified using Image J software by a blinded investigator.

Echocardiography. Mice (*n*=8-9) were sedated with 1.5% isoflurane and standard views were obtained in 2 dimensions by transthoracic echocardiography. Echocardiography was performed using an MS400 transducer (18-38 mHz probe frequency) on a Vevo 2100 scanner (VisualSonics). Body temperature was monitored and maintained at 37°C. Image analysis was performed by a blinded investigator using the manufacturer's software package. Mice were analysed at 3-4 or 11 months of age.

In vivo *FA uptake.* For the assessment of FA uptake *in vivo*, 7 Wild-type and 8 *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ mice were injected intravenously with a 2 µCi dose of [1-^{14}C]-Oleic Acid (^{14}C-OA; Perkin Elmer) dissolved in saline (1:4). For the analysis of ^{14}C-OA in tissues, and after 30 min. of intravenous injection, mice were anaesthetised with an i.p. injection of ketamine (75 mg/kg) and xylazine (10 mg/kg) mixture and perfused with 0.9% saline solution. Organs (heart and liver) were dissected, weighed and solubilised by the addition of Solvable (Perkin Elmer, 1 ml/100 mg tissue) in a glass scintillation vial and incubated at 60°C overnight. After cooling to room temperature, 30% (w/w) hydrogen peroxide (100 µl per 1 ml of sample) was added followed by heating at 60°C for another hour to minimise colour quenching of samples. Finally, scintillation fluid (4 ml per 200 µl; Normascint, Scharlab) was added to each sample followed by vigorous shaking. The vials were then allowed to equilibrate in the dark for at least 60 min. before scintillation counting using an LKB Wallac Rackbeta 1214 Counter (Perkin Elmer). Final data are expressed as % injected dose per gram of tissue.

[^{18}F]FDG PET imaging. Glucose metabolism in the heart was measured by positron emission tomography (PET) with the radiotracer 2-deoxy-2-[^{18}F]fluoro-D-glucose ([^{18}F]FDG), synthetised by standard nucleophilic substitution methods at the Clínica Universidad de Navarra PET-GMP laboratory. PET imaging was performed in a dedicated small animal scanner (Philips Mosaic, Cleveland, OH), with 2 mm resolution at full width half maximum (FWHM), 11.9 cm axial field of view (FOV) and 12.8 cm transaxial FOV. Mice (*n*=12 Wild-type; *n*=11 *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$) were anaesthetised with 2% isoflurane in 100% O_2 gas and placed horizontally on the PET scanner bed. The [^{18}F]FDG (7.9 ± 1.5 MBq in 100 µl saline) was injected through the tail vein simultaneously at the beginning of a list mode study of 60 min. For each study, a summed sinogram of the whole emission study and an 18 frame dynamic sinogram (2×15″; 7×30″; 1×60″; 1x120″; 1x180″; 2x300″; 4x300″) were created. From these sinograms, a summed and a dynamic image were generated containing information about the corresponding time intervals. All images were reconstructed using the 3D Ramla algorithm with 2 iterations and a relaxation parameter of 0.024 into a 128×128 matrix with a 1 mm voxel size applying dead time, decay, random and scattering corrections. For the assessment of [^{18}F]FDG

uptake in the heart, all studies were exported and analysed using the PMOD software (PMOD Technologies Ltd., Adliswil, Switzerland). For each mouse, a volume of interest (VOI) of the heart was drawn on coronal 1-mm-thick slices of summed PET images. Then, VOI was applied to the dynamic image to obtain the [^{18}F]FDG uptake values for each time point. The obtained data were normalised by the specific dose at the time of injection.

Statistics. All data are expressed as mean ± s.e.m. from pooled data of 3-12 independent experiments. For single comparisons *P*-values were calculated with two-tailed unpaired Student's *t*-test, for multiple comparisons *P*-values were calculated with one-way ANOVA, followed by contrast analysis. For a time course of a single variable comparison, repeated measures ANOVA was applied, followed by Student's *t*-test for each time point, $P<0.05$ was considered significant. Statistical analysis of the microarray data was performed by the VIB Nucleomics Core. Briefly, differential expression was assessed via the moderated *t*-statistic and to control the false discovery rate, multiple testing correction was performed according to Benjamini and Hochberg. Differences ≥ 4-fold with $P<0.05$ and Log_2 probe intensities ≥ 6 were considered significant.

1.4. RESULTS

Results for Aim 1: To gain insight into the molecular heterogeneity of microvascular ECs

1° Generate and validate tissue-specific microvascular EC '*ex vivo*' gene signatures

Step 1: Defining a differential gene signature.

To obtain a specific signature of heart microvascular ECs, we performed a whole genome microarray comparison of GFP$^+$ ECs freshly sorted from hearts, livers and brains of adult *Tie2-GFP* reporter mice (in which the *Tie2* promoter-enhancer is active in blood vascular but not lymphatic ECs; Motoike et al., 2000). FACS analysis confirmed that the sorted GFP$^+$ fraction constituted a pure population of cells positive for the general EC marker CD31 and for the microvascular EC marker CD36 (see Supplementary Note 1 at the end of ***Example 1***) and negative for the hematopoietic marker CD45 (***Figure 3A***). Cluster and

principal component analysis of microarray data revealed that the 5 samples for each tissue nicely clustered together and away from the two other tissues, suggesting that there was a high degree of intra-organ homogeneity and inter-organ heterogeneity (***Figure 3B***). Despite the large number of genes commonly expressed by all three microvascular EC types studied, a high degree of heterogeneity was present: we identified a pool of 49, 168 and 117 genes (corresponding to 92, 191 and 159 probes) specifically enriched in heart, liver or brain ECs, respectively (***Figure 3C*** and ***Table 4***).

The microarray output was representative for the three microvascular EC types, as evidenced by the high expression of general (*i.e.*, *Pecam1* and *Tek*) EC markers, the low expression of genes corresponding to contaminating cells and from the expected enrichment of previously known tissue-specific microvascular EC markers (***Figure 3D-E***).

Step 2: Filtering against non-EC genes.

Since the design of the microarray experiment did not allow excluding detection of transcripts shared between ECs and the surrounding stromal cells, we performed a qRT-PCR comparison between the sorted EC and non-EC fractions. For liver and brain we performed this comparison for only 30 genes (*i.e.,* the most differentially expressed genes including all TFs. For the liver ~77%, and for brain ~93% of the tested genes were enriched in the EC fraction (***Figure 4A-B***; and in ***Table 5*** a list of the TFs belonging to the fingerprints of the three vascular beds studied). For the heart, we conducted this analysis for all 49 genes, and we determined the enrichment in the endothelial fraction in comparison to both the non-EC fraction and a population enriched in cardiomyocytes (***Figure 4C***). 31 genes (~63%) were retained after this analysis, corresponding to the final list of validated murine heart ECs (***Figure 5*** and ***Table 6***). Next, we performed a functional annotation analysis on the gene lists. Since only a limited number of genes was not enriched in the EC fraction, for brain and liver, we considered the whole list, since the amount of genes that was not enriched in the EC fraction was more substantial for the heart, and a functional role specific for heart ECs has not been determined so far, we limited the functional analysis for this organ to the validated gene list. The main functions represented by this gene set were 'lipid

binding' and 'lipid/FA transport' (***Table 7A***). For brain ECs '(selective) transport' and 'metabolism' were highly represented functional terms, whereas for liver 'coagulation' and 'scavenger receptor activity' were listed as highly represented functions (***Table 7B,C***), in accordance with previously reported data sets.

Step 3: Test the heart EC signature in additional tissue ECs.

To consolidate the heart EC-specific gene signature, we measured its expression by quantitative RT-PCR in ECs freshly isolated from additional tissues, *i.e.*, pancreas, lung and kidney. We found that most genes (~81% compared to at least 2 of these tissues and ~48% compared to all three tissues) were at least 4-fold enriched in heart ECs (***Figure 6A***). On the other hand, ECs isolated from tissues with continuous endothelium that, like the heart, are highly active in FA uptake for energy production or storage (*i.e.*, skeletal muscle or SkM, brown and white adipose tissue or BAT and WAT, respectively) shared the expression of most genes with heart ECs (***Figure 6B***). This observation strengthened our hypothesis that the molecular signature of heart ECs reflects their specialisation in active FA transport.

Step 4: Cross-over analysis with human ECs.

A cross-species validation in human ECs confirmed a partially retained enrichment of the signature in human heart ECs when compared with brain and liver ECs (***Figure 7***). Similar results were obtained for brain and liver ECs (data not shown).

2° Compare the '*ex vivo*' with the *in vitro* heart EC signature

To determine the effect of the lack of microenvironment on the gene expression profile, we cultured the bulk cells from the digested human heart biopsies, sorted the blood EC fraction ($Tie2^+$:$podoplanin^-$:$CD45^-$; or $Tie2^+$:$CD31^{+-}$:$CD45^-$; ***Figure 8A***) and analysed their gene expression profile by qRT-PCR directly or either after further passages *in vitro*. As previously shown for other EC types (Geraud et al., 2010), when human heart ECs were cultured *in vitro*, they lost their specific gene expression profile (***Figure 8B***). Similar results were obtained for brain and liver ECs (data not shown).

Results for Aim 2*: *To induce the heart EC gene expression and specific function by overexpressing heart EC-specific TFs

1° Restoring the '*ex vivo*' heart EC gene and functional signature in cultured heart ECs

Step 1: Transciptional regulation of the heart EC fingerprint

In addition to genes encoding FA transport-related proteins, the heart EC gene signature also contained five genes encoding a TF, *i.e.*, *Meox2*, *Tcf15*, *early B-cell factor 3* (*Ebf3*), *Pparg* and *Wilms tumour 1* (*Wt1*; ***Table 5C*** and ***Table 6***). Meox2, Tcf15 and Ebf3 have never been associated with the heart vasculature, whereas Pparγ has been described for its involvement in FA uptake in ECs in the heart (among other tissues; Kanda et al., 2009) and Wt1 for its detection in heart vessels under ischemic conditions (Wagner et al., 2002). Moreover, Wt1 is involved in cardiac vascular development (Wagner et al., 2005). We confirmed at the protein level that Meox2 and Tcf15 were exclusively expressed in the EC compartment of the murine heart and not detectable in liver or brain tissue under identical staining conditions (***Figure 9A,B***). We next tested the hypothesis that these TFs intrinsically regulate the heart EC signature by assessing in a stepwise manner whether their lentiviral overexpression would restore this signature in cultured human heart ECs. When overexpressing *MEOX2* and *Tcf15* alone or in combination in cultured heart ECs, we observed a synergistic effect between them, together significantly inducing ~31% of the genes in the signature (***Figure 10A***). Interestingly, *MEOX2/Tcf15* did neither affect each other's expression nor that of *WT1*, but had a small yet significant inductive effect on *PPARG*. Expression of *EBF3*, known to be involved in differentiation into multiple non-endothelial cell types, was strongly induced by *MEOX2/Tcf15*. Yet, overexpression of *EBF3* alone only induced a limited number of signature genes and thus did not recapitulate the combined effect of *MEOX2* and *Tcf15*. Furthermore, combining *EBF3* overexpression with *MEOX2/Tcf15* only additionally induced aquaporin 7 (*AQP7*) and *FABP9,* and only boosted the upregulation of *EEPD1* (***Figure 10B***). When we overexpressed *PPARG* alone, we measured a strong upregulation of three genes, two of which were known targets – *RBP7* and *AQP7* – and one novel target (*FABP9*). When *PPARG* was combined with *MEOX2/Tcf15*, for multiple genes, a superior inductive effect was obtained

compared to *MEOX2/Tcf15*, resulting in the significant induction of ~48% of the gene signature (***Figure 10C***). When we finally overexpressed the most abundant isoform (Kamarzova et al., 2012 and Supplementary Note 2 at the end of ***Example 1***) of *WT1*, we detected a significant upregulation of multiple genes (~38%) of the signature, however combining overexpression of *WT1* with *MEOX2/Tcf15/PPARG* did not have an additive effect (***Figure 10D***). Induction of mRNA expression for a random gene subset was confirmed at the protein level (***Figure 10E***).

Step 2: Transcriptional regulation of FA uptake in heart ECs

Since lipid binding and FA transport are major functions represented by the heart EC gene signature, we next wondered whether the uptake of FAs in cultured human heart ECs can be restored by overexpressing TF (combination)s with a significant inductive effect on the heart EC signature. *In vivo*, FAs reach the heart EC barrier in mainly two forms, either bound to albumin (free-fatty acids or FFA) or contained in circulating lipoproteins (*e.g.*, chylomicrons or very low density lipoproteins [VLDL]) from which they can be released by lipoprotein lipase (Lpl), present at the luminal EC surface. While it was previously assumed that, within the heart, cardiomyocytes are the prime – if not the only – source of Lpl (Davies et al., 2012), we document for the first time *Lpl* mRNA expression in heart ECs (***Table 5***). Furthermore, *LPL* was the gene most upregulated by *MEOX2/Tcf15*, *MEOX2/Tcf15/PPARG* or *MEOX2/Tcf15/PPARG/WT1* overexpression (***Figure 10A-D***), thereby restoring its expression to levels approximating those detected in freshly isolated heart ECs (data not shown). As a test model for FFA uptake we incubated cultured human heart ECs overexpressing the TFs or a reporter gene with fluorescently-labelled palmitic acid (BODIPY-PA) and counted the number of lipid-containing vesicles (***Figure 11A***). Furthermore, as a model for Lpl-dependent FA uptake we incubated ECs overexpressing the TFs or a reporter gene with VLDL containing DiI-labelled FA (DiI-VLDL; ***Figure 11B***). In both models, we measured that all TF conditions improved FA uptake compared to control and that combined overexpression of *MEOX2/Tcf15* outperformed single overexpression of *PPARG* or *WT1* in VLDL-derived FA uptake. Moreover, in the FFA model, the uptake induced by *MEOX2/Tcf15* was further enhanced when *PPARG* was added, while this synergistic effect was not observed for Lpl-dependent FA uptake. Finally, in

accordance with the observed effect on the heart EC signature (***Figure 10D***), the four-TF combination did not significantly increase the effect produced by *MEOX2/Tcf15/PPARG*. In both tests, the uptake was saturable in a competition assay with non-labelled FA. Thus, we here demonstrate for the first time that FA uptake from VLDL can be heart EC-autonomous, *i.e.*, independent of Lpl produced by cardiomyocytes and that in addition to *PPARG* (Kanda et al., 2009), also *MEOX2/Tcf15* and *WT1* regulate FA uptake in heart ECs.

2° Inducing the '*ex vivo*' heart EC signature in endothelial progenitors

We next attempted to induce a specific heart EC signature by TF overexpression in totally unrelated and unspecialised endothelial progenitors (BOECs). The latter TF-induced differentiation approach is in fact opposite to the TF-induced reprogramming of differentiated cells to stem cells ('induced pluripotent stem cells'), first described by Yamanaka *et al.* (Takahashi et al., 2007). Like we observed with cultured heart ECs, our TF cocktail (MEOX2 + TCF15 + PPARγ + WT1 + rosiglitazone 10 μM) induced strong upregulation of the heart EC fingerprint in BOECs (***Figure 12***).

Results for Aim 3: To explore the* in vivo *role of heart EC-specific TFs

1° Meox2/Tcf15 determine heart EC identity *in vivo*

To address whether Meox2/Tcf15 synergised to affect the adult heart EC expression profile *in vivo,* transgenic mice lacking one allele of each TF were generated by intercrossing $Tcf15^{+/-}$ with $Meox2^{Cre/+}$ (further referred to as $Meox2^{+/-}$) mice. Mice homozygously deficient for *Tcf15* (Burgess et al., 1996) or *Meox2* (Supplementary Note 3 at the end of ***Example 1***) die perinatally, while heterozygously deficient mice are viable. ECs were isolated from the heart and, as a control, from the liver of adult $Meox2^{+/-}$:$Tcf15^{+/-}$ mice and their single-heterozygous or Wild-type littermates. While haplodeficiency for *Meox2* or *Tcf15* alone only slightly affected the heart EC signature, we observed a significant downregulation of ~45% of the signature genes in $Meox2^{+/-}$:$Tcf15^{+/-}$ hearts, supporting the synergistic genetic interaction of both TFs also *in vivo* (***Figure 13***).

Complementary to our *in vitro* findings, *Pparg* and *Ebf3* were significantly downregulated, whereas *Wt1* was not affected, confirming the transcription factor hierarchy we unravelled *in vitro* (***Figure 10A-D***) General EC markers were not affected in heart ECs, and specific liver sinusoidal EC genes were not altered in liver ECs (***Figure 14A,B***), suggesting that Meox2/Tcf15 specialise ECs and do not affect EC identity *per se* in the heart, and that these factors do not affect EC specialisation in unrelated tissues.

2° Meox2/Tcf15 regulate energy substrate uptake in heart ECs *in vivo*

To further investigate whether Meox2 and Tcf15 regulate energy substrate uptake by heart ECs *in vivo*, we tested additional genes encoding glucose or FA transport-related molecules, previously described in heart ECs but not included in our signature, either because they were not enriched in heart ECs, or because they were less than 4-fold enriched in comparison to both liver and brain ECs (***Figure 15***). *CD36*, *Fabp4*, *Fabp5*, and *Gpihbp1*, the latter encoding the anchoring protein for Lpl on the luminal EC surface (Davies et al., 2012), were significantly downregulated in *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ heart ECs (but not in liver sinusoidal ECs), while *Fatp3*, a FA transporter regulated by VEGF-B interaction with heart ECs (Hagberg et al., 2010) as well as the glucose transporter *Glut1* were upregulated in *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ heart ECs, possibly in a compensatory fashion (***Figure 16A,B***). Notably, expression of *Fatp4*, another FA transporter gene induced by VEGF-B in heart ECs, VEGF-B receptors (*Flt1* and *Nrp1*) or *Glut4,* were not affected by *Meox2/Tcf15* (combined) haplodeficiency (***Figure 16B*** and data not shown). Reduced *CD36* expression in *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$heart ECs was confirmed at the protein level (***Figure 16C***). Upon *Meox2/Tcf15* combined heterozygous deficiency the expression of none of the lipid or glucose transport-related genes tested was affected in cardiomyocytes (***Figure 17A***). Accordingly, isolated adult *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ cardiomyocytes did not have an impaired FA uptake *in vitro* (***Figure17B***). Together, these observations demonstrate an EC-autonomous role within the heart, as also supported by the unique expression of Meox2 and Tcf15 in the endothelial fraction (***Figure 9A***).

To monitor FA uptake by the heart *in vivo* we administered ^{14}C-labelled oleic acid (^{14}C-OA) via tail vein injection to 12-16 weeks-old *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ and Wild-

type males, and measured the radioactive signal in the heart after 30 min. We detected a ~30% reduction in FA uptake in *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ compared to Wild-type hearts (***Figure 18A***), while FA uptake in the liver was not compromised (injected dose/g tissue: 12 ± 1 in Wild-type *versus* 10 ± 3 in *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ livers; *n*=7-8; *P*=NS). To investigate whether reduced FA handling is compensated by increased glucose uptake, we measured 2-deoxy-2-[^{18}F]fluoro-D-glucose ([^{18}F]FDG) uptake in the heart, and detected a ~38% increase in *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ compared to Wild-type mice at ~60 min. post-injection (***Figure 18B,C***).

3° Aged *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ mice develop cardiac dysfunction

Since a shift from FA to glucose uptake due to a defect in FA transport-related proteins can be associated with cardiac dysfunction in animal models upon aging, we performed histology and functional testing on young and aged *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ and Wild-type male mice. In accordance with the *in vivo* ^{14}C-OA uptake assay, in young (8-16 weeks-old) mice, Oil red-O staining revealed that lipid accumulation decreased by ~61% in the heart muscle of *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ compared to Wild-type hearts. However, this did not cause fibrosis or cardiac contractile dysfunction, nor was it accompanied by changes in cardiomyocyte size or vessel number (n° of capillaries/n° of cardiomyocytes: 0.99 ± 0.07 in Wild-type *versus* 0.97 ± 0.08 in *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ hearts. *n*=4; *P*=NS; ***Figure 19***). However, aged (32-44 weeks-old) *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ mice, in which we measured a more pronounced (~79%) decrease in heart lipid accumulation, displayed fibrosis and heart systolic dysfunction (as evidenced by a reduced ejection fraction and fractional shortening), in the absence of significant differences in cardiomyocyte size or vessel counts (***Figure 20*** and data not shown).

1.5. CONCLUSIONS AND PERSPECTIVES

We determined for the first time a unique '*ex vivo*' molecular fingerprint for heart capillary ECs (as well as brain and liver microvascular ECs) and demonstrated that this signature can be restored or induced in cultured heart ECs or BOECs, respectively, using a combination of heart EC-specific TFs. For two of these TFs, Meox2 and Tcf15, we showed an equivalent important role in determining the heart EC signature *in vivo*. These findings will be a platform for

studies in which we will exploit the use of the generated heart ECs, including their use for *in vitro* drug toxicity tests and as *in vivo,* for treatment in animal models of ischemic heart disease. We hypothesise that BOECs pre-specified to heart ECs by overexpression of our heart-specific TF cocktail will have a more profound and durable effect on revascularisation (and refunctionalisation) upon transplantation in the ischemic heart. We will test this hypothesis first in mice and then later in a clinically more relevant porcine model.

In addition, this strategy based on TF overexpression to induce a heart EC-specific gene signature in BOECs, can also be tested for other EC types, *e.g.*, liver and brain ECs. Indeed, in the gene list of murine liver ECs we found 8 genes functionally annotated as having TF activity (*i.e., Tcfec*, *Hoxb5*, *Maf*, *Cux2*, *Gata4*, *Meis2*, *Twist1* and *Zeb2*; ***Table 5A***) and in the murine brain ECs, we indentified 5 TFs (*i.e., Zic3*, *Lef1*, *Foxf2*, *Foxf1a* and *Foxc1*; ***Table 5B***) Resulting pre-specified ECs can than be tested for their therapeutic efficacy in models of liver (*e.g.*, sinusoidal obstruction syndrome) or brain (*e.g.,* stroke) vascular disease.

Moreover, here we unravel for the first time that heart, skeletal muscle, BAT and WAT, the major tissues involved in whole body metabolism, share specialised endothelial cells, active in the transport of fatty acids to the parenchyma, and we provide a fingerprint of genes specifically expressed by these ECs.

We show that *Meox2* and *Tcf15* EC-autonomously determine the expression of these fingerprint genes both *in vitro* and *in vivo*, and that they can modulate energy substrate delivery to contracting cardiomyocytes. Meox2/Tcf15 acted upstream of another endothelial regulator of FA uptake, *i.e.*, PPARγ (Kanda et al., 2009), as suggested by the increased *PPARG* expression upon *MEOX2/Tcf15* overexpression and by the significant downregulation of *Pparg* and some of its known target genes (*CD36*, *Fabp4* and *Aqp7*) in *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ heart ECs. On the other hand, *MEOX2/Tcf15* and *PPARG* also had a synergistic effect *in vitro* on the regulation of most genes of the signature, which were not induced by the overexpression of *PPARG* alone. These findings thus establish a previously unrecognised TF network in charge of endothelial FA uptake regulation

Understanding the intrinsic regulation of FA uptake by heart ECs offers new

therapeutic perspectives for the use of ECs as a target to modify the metabolic status of the heart, which has been extensively described to be affected in a large list of pathologies leading to heart failure. For example, pathological situations where there is an increase in circulating FAs, like obesity or type 2 diabetes, lead to an excess of fat uptake in tissues, resulting in lipotoxicity and the so-called "diabetic heart". The latter results from metabolic reprogramming of the cardiomyocytes which occurs in parallel with the overload of FA, leading to the development of insulin resistance, oxidative stress, mitochondrial damage and ultimately heart failure (Abel et al., 2012). It is also known that a variety of pathological insults in the heart, which initially are not related to cardiac metabolism, including hypertension, myocardial infarction, and aortic stenosis, if protracted or severe, can lead to the reprogramming of the cardiac energy production machinery and lead to activation of lipid anabolic pathways, inefficient ATP production, and therefore impaired contractility, cytotoxicity and heart failure (Neubauer, 2007). Further studies will be necessary to assess whether our insights could be used to develop new strategies based on endothelial targeting of Meox2/Tcf15 to remedy cardiac dysfunction as a result of changes in the relative contribution of FAs and glucose to energy production. Such therapeutic strategies could be complementary to other approaches aimed at shifting the energy substrate source of the heart (Jaswal et al., 2011; Stanley et al., 2012).

Another EC-targeted approach, *i.e.*, inhibition of VEGF-B as a paracrine factor affecting the ECs' FA transporter expression, was shown to decrease FA uptake and increase glucose uptake in the heart (Hagberg et al., 2010). *Meox2/Tcf15 in vivo* regulated a set of membrane FA and glucose transporters (*Cd36* and *Glut1*) that was not overlapping with those regulated by VEGF-B. Furthermore, *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ heart ECs had lower expression the glycerol transporter *Aqp7* and of intracellular FA transporters (*Fabp4* and *Fabp5*). Finally, we revealed for the first time that besides cardiomyocytes, cardiac ECs – in large part driven by Meox2 and Tcf15 – produced their own *Lpl* and that combined deficiency of the two transcription factors reduced expression of both *Lpl* and its prime endothelial transporter, *Gpihbp1* (Davies et al., 2012) *in vivo*. Collectively, this suggests that *Meox2* and *Tcf15* co-regulate several molecules involved at different levels of

endothelial FA uptake, complementary to VEGF-B. Hence, combined targeting of these intrinsic and paracrine pathways could synergistically reinforce the endothelial barrier of the heart against pathological fat uptake.

Of note, within the heart EC fingerprint, there were also genes encoding secreted molecules that could be involved in local EC-to-cardiomyocyte communication and influence the cardiomyocyte's FA usage. For instance, *C1qtnf9* – the expression of which was decreased in *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ heart ECs and increased *in vitro* upon *MEOX2-Tcf15* combined overexpression – is a paralogue of the adipokine *Adipoq*. Although adipose tissue is the prime organ for C1qtnf9 secretion, significant local production has also been detected in the heart. In mice, type 2 diabetes and obesity, as well as cardiac ischaemic damage, induced a significant decrease in cardiac C1qtnf9, and its exogenous supplementation reduced cardiac damage after ischaemia-reperfusion (Kambara et al., 2012). Mechanistic studies revealed that C1qtnf9 exerts its effect in cardiomyocytes through activation of the AMPK pathway, which is known to increase FA oxidation in myocytes. Finally, the heart EC fingerprint also harboured genes encoding proteins not linked to FA handling but relevant for cardiac remodelling, such as metalloproteinase *Adamts9* and metalloproteinase inhibitor *Timp4*. Given their known involvement in collagen homeostasis, their lowered expression in *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ heart ECs may have contributed to the cardiac fibrosis we observed, which is a phenotype common to other animal models of impaired cardiac FA uptake (Augustus et al., 2006).

In summary, here we provide for the first time a fingerprint of genes enriched in ECs lining the capillaries of tissues with high metabolic capacity for FA, suggesting a common specialisation of these vascular beds. We identified Meox2 and Tcf15 as transcriptional determinants of the heart EC fingerprint and demonstrated that they are critical regulators of the balance between FA and glucose transport across heart ECs (***Figure 21***). These findings have broadened our understanding of the genetic identity and physiological role of heart microvascular endothelium and provide a platform for further mechanistic studies for therapeutic exploitation of specific EC targeting aimed at modulation of the energy substrate uptake in the heart.

SUPPLEMENTARY NOTES TO EXAMPLE 1

Supplementary note 1. Microvascular EC purity.

As previously described, CD36 is highly expressed in microvascular ECs and absent in ECs from large vessels (Chi et al., 2003). Nevertheless, between capillary ECs from different organs there is a different degree of expression of CD36, being very low in brain ECs and high in liver and heart ECs (as evident from our microarray analysis; ***Figure 15***). Indeed, for heart and liver, microvascular EC purity could be determined by FACS for CD36 (as shown for the heart in ***Figure 3A***), whereas under identical FACS staining conditions we could not detect CD36 positive cells in brain ECs. Therefore, to confirm microvascular EC purity of brain EC preparations, we relied upon the significant enrichment for known brain-specific microvascular EC markers such as *Glut1*, *Lat1*, *Ocln* and *Tfrc* (***Figure 3E***).

Supplementary note 2. Relative abundance of WT1 isoforms in cardiac ECs.

Several WT1 isoforms have been described with different tissue specificities and functions (Kamarzova et al., 2012). We quantified the relative abundance of the long *versus* short isoforms (the latter generated from an alternative start codon in intron 1) and of the isoforms carrying or not the KTS domain in freshly isolated human and mouse heart ECs and found that the most abundant WT1 isoform in heart ECs is the isoform expressing exon1 and the KTS domain (ratio isoform - exon1/isoform + exon1 = 1:1,500, and 71±8% carrying the KTS element). We therefore overexpressed the isoform D (expressing exon1, exon5 and carrying KTS), the most prevalent isoform of WT1 in normal tissues, to test its effect on the heart EC gene signature.

Supplementary note 3. Mendelian ratios of Meox2$^{+/-}$ x Meox2$^{+/-}$ intercrosses.

Meox2$^{Cre/+}$ mice were obtained from the Jackson Laboratories (stockN°003755) and crossed for one additional generation in C57Bl/6 to establish the colony. When setting up *Meox2$^{Cre/+}$* x *Meox2$^{Cre/+}$* crosses to obtain *Meox2$^{cre/cre}$* (or *Meox2$^{-/-}$*) mice, we observed that *Meox2$^{-/-}$* pups died within a few hours after birth, while the ratios just before birth (at embryonic day 19.5) were Mendelian. This observation is at variance with what is reported on the Jackson Laboratory website

where it is mentioned that *Meox2*$^{-/-}$ mice are viable at birth and die just before weaning (http://jaxmice.jax.org/strain/003755.html).

Example 2: *Identification of and induction in dedifferentiated cultured human umbilical vein endothelial cells of a gene signature specific for human arterial endothelial cells using individual transcription factors or combinations thereof*

2.1. ABSTRACT

Endothelial cells (ECs) lining arteries and veins have distinct molecular and functional features. The underlying regulatory mechanisms in human ECs are incompletely understood. Here, we established a specific fingerprint of freshly isolated arterial (HUAECs) and venous EC (HUVECs) from human umbilical cord comprising 64 arterial and 12 venous genes, representing distinct functions and pathways. Among the arterial genes were 8 transcription factors (TFs), including Notch target *HEY2*, the current 'golden standard' denominator for arterial (A)EC specification. Culture of HUAECs or HUVECs abrogated differential gene expression at least in part due to loss of canonical Notch activity and *HEY2* expression. Notably, restoring *HEY2* expression or Delta-like4-induced Notch signalling in cultured ECs only partially reinstated the aEC gene signature while combined overexpression of the 8 TFs restored this fingerprint more robustly. While some TFs only stimulated few genes, others boosted a large proportion of arterial genes. Interestingly, although there was some overlap and cross-regulation, the TFs largely complemented each other in regulating the aEC gene profile. Additionally, overexpression of the 8 arterial TFs improved the arteriogenic capacity of HUVECs in a Matrigel plug assay *in vivo*. Thus, our study showed that Notch signalling determines only part of the AEC signature and identified additional novel and complementary transcriptional players in the complex regulation of human arteriovenous EC identity.

2.2. BACKGROUND AND AIMS

Endothelial cells (ECs) of different vessels across the body differ in morphology, function and gene expression profile, a phenomenon known as 'endothelial heterogeneity' (Aird, 2007a, b). Endothelial diversity is due to exposure to different microenvironments (extracellular matrix, surrounding cells, blood flow) and different intrinsic genetic programs which are present at very early stages of development even before the vascular system is functional (Aird, 2007a). Genetic programs determining arterial or venous EC identity have been mainly studied in zebrafish and mice and much less attention has been given to human cells, mainly because of the difficulty to obtain ECs of human origin. It is well documented that canonical Notch signalling including the ligand delta-like 4 (Dll4), the receptors Notch1 and Notch4 and the downstream TFs Hey1 and Hey2 (*gridlock* in zebrafish) determine the arterial phenotype across species (Swift and Weinstein, 2009), in part by blocking the TF chicken ovalbumin upstream promoter-TF (COUP-TF)II which is expressed in venous ECs (VECs) (You et al., 2005). The Notch-Hey pathway induces ephrinB2 expression and blocks the expression of the corresponding receptor EphB4 in arterial ECs (AECs) whereas in vECs COUP-TFII mediates the opposite effect (Swift and Weinstein, 2009; You et al., 2005). This ephrinB2-EphB4 differential expression establishes a polarity that assists in segregating arteries from veins down to the capillary level (Wang et al., 1998).

Many other molecules and pathways like Shh, VEGF, FoxC1/C2, adrenomedullin or cAMP, spreds, Wnt, Sry-related HMG box (Sox), TGF-β and phosphatidylinositol-3 kinase play a role in arteriovenous specification (Corada et al., 2010; Swift and Weinstein, 2009). Nevertheless, most studies have been focused on one or only a few regulatory elements in this specification process. An exception to this is the study by Chi *et al.* (Chi et al., 2003) who elegantly showed endothelial heterogeneity between arterial and venous ECs as well as microvascular ECs on a genome-wide scale. Importantly, for this analysis they used human ECs in culture. However, studies in microvascular ECs (Calabria and Shusta, 2008; Geraud et al., 2010) and lymphatic ECs (Amatschek et al., 2007; Wick et al., 2007) have shown that the culture process, even short-term, can erase a significant part of the specific gene expression profile. This loss of identity upon culture has not been systematically studied for arterial and venous ECs.

Therefore, our aim was to perform a stringent genome-wide profiling study on ECs isolated from arteries and veins from different human vascular beds, including cultured and freshly isolated cells, and to analyse in more detail intrinsic regulators of arteriovenous endothelial heterogeneity.

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2.3. ***MATERIALS AND METHODS***

EC isolation and culture. Commercial EC lines used: HAECs (Lonza, Barcelona, Spain; CatN°CC-2535), HCAECs (Lonza, CatN°CC-2585), HIAECs (ATCC, Barcelona, Spain; CatN° CRL-2475), HPAECs (ATCC, CatN° CRL-2598), HIVECs 10 (ATCC, CatN° CRL-2606), and HPVECs (ATCC, CatN° CRL-2607). EC lines were cultured according to the provider's instructions. HHAECs, HHVECs, HUVECs and HUAECs were isolated at the Clinica Universidad de Navarra (after obtaining informed consent) by perfusing the corresponding vessel with collagenase type I (Invitrogen, Carlsbad, CA). Harvested cells were cultured for 24 hours, washed to 15 discard non-attached cells, grown until 100% confluence and split 1:3 every 3-4 days. For the analysis of fresh cells or short time culture for time course analysis, ECs from umbilical arteries or veins were magnetically selected using anti-human CD34 magnetic beads (Miltenyi-Biotec, Madrid, Spain) and an AutoMACS magnetic selector (Miltenyi-Biotec) according to the manufacturer's instructions. Purity of 20 magnetically sorted HUVEC-F or HUAEC-F was assayed by FACS. Only samples with more than 95% purity for $CD31^+CD34^+CD45^-$ cells were eligible for microarray hybridisation or short time culture (***Figure 22***). All TFs and a random subset of the genes from the fingerprint were validated using FACS sorting for the $Tie2^+CD31^+CD45^-$ population (data not shown).

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RNA isolation, quality control and qRT-PCR. Total RNA from cell lysates was extracted using TRIzol® reagent or RLT lysis buffer (Qiagen). The RNA integrity/quality of the samples used for microarray hybridisation were determined with a Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA). When necessary, 30 mRNA was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and cDNA underwent 40 rounds of amplification on an ABI PRISM 7700 cycler (Perkin Elmer/Applied Biosystems, Foster City, CA) for

standard quantitative Real-Time PCR as described in ***Example 3***. Primer sequences are listed in ***Table 8***. mRNA levels were normalised using *GAPDH* as housekeeping gene. Data, expressed as mean ± s.e.m. comparing two groups were analysed by Student's *t*-test. SPSS software was used for statistical analyses and differences were considered significant when $P<0.05$.

Microarray hybridisation and statistical analysis. RNA hybridisation of the 38 human EC samples was done in collaboration with the Department of Hematology, Hospital Universitario de Salamanca, using the Affymetrix HG-U133 Plus 2.0 GeneChip Oligonucleotide Microarray (Affymetrix, Santa Clara, CA, USA). All steps were carried out according to the manufacturer's protocol. 100 ng of RNA was amplified and 15 μg of amplified and labeled cRNA was hybridised on the array. Arrays were scanned using a GeneChip Scanner 7G. Background correction and normalisation were done using the RMA (Robust Multichip Average) algorithm. The method for differential gene expression analysis was the one contained in the LIMMA Bioconductor package. To control the false discovery rate, multiple testing correction was performed and probes with a corrected *P*-value below 0.05 were selected. For the classification analysis, a filtering process was applied first to eliminate probe sets with low expression values. Applying the criterion of an expression value greater than 32 in 5 samples for each experimental condition, 32,939 probe sets were selected for statistical analysis using the LIMMA Bioconductor package. Prediction analysis for microarrays was applied to classify arterial and venous samples and identify genes that were associated with each specific class. This algorithm ranked genes using a penalised *t*-test and identified a gene set for classification with soft thresholding. Gene number was controlled by a thresholding parameter, which was determined with a 10-fold cross-validation. This parameter was manually selected to minimise the overall error rate (t=7). The obtained classifier required 78 probes. Functional and pathway enrichment analysis was done using Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA, http://www.ingenuity.com).

Time course analysis with TLDA. Taqman® Low Density Array (TLDA) plates with Taqman® primers for our described $HUAEC_{fresh}$/$HUVEC_{fresh}$ fingerprint, previously described arteriovenous markers and some general endothelial markers

were obtained from Applied Biosystems. $HUAEC_{fresh}$/$HUVEC_{fresh}$ samples and samples from HUAECs and HUVECs cultured for 24 hours, 48 hours or 6 days were run on a 7900 HT fast real time PCR system (Applied Biosystems) and analysed according to the manufacturer's instructions. *GAPDH* was used as housekeeping gene. Each condition was run in quadruplicate.

nCounter analysis. DLL4-Fc activated or BSA-treated HUAECs (*N*=3) and HUVECs transduced with cherry, each of the 8 TFs or a combination of them (*N*=4-6) were used for nCounter analysis. Briefly, RNA, extracted using TRIzol® reagent was quantified and quality controlled with a Bioanalyser 2100 (Agilent Technologies) and samples were processed in collaboration with the VIB Nucleomics Core Facility. 100-500 ng of total RNA was hybridised according to the manufacturer's instructions. Some of the results were confirmed by qRT-PCR and genes for which the probe intensity value was low were analysed by qRT-PCR. Data were normalised by scaling to the *GAPDH* gene. The scaled counts were base log 2-transformed. Testing whether a contrast was significantly different from 0 was done by using a moderated *t*-test, as implemented in LIMMA. The resulting *P*-values were corrected for multiple testing with Benjamini-Hochberg to control the false discovery rate. $P<0.05$ and at least 50% change differences in comparison with control (BSA-treated HUAECs or cherry-transduced HUVECs) was considered as significant. The Nanostring probe list is provided in ***Table 9***. The global expression profiles of the samples were represented in the form of a heat map, using the gplots package of R. Hierarchical clustering was used to cluster the individual gene expression profiles based on Pearson correlation and complete linkage.

Immunofluorescence staining and Western blot. The procedure for IF staining was done on cord blood samples as described previously (Aranguren et al., 2007). Antibodies used were: Rabbit anti-human Rasgrf2 (Sigma, CatNº HPA018679), Alexa488-labelled mouse anti-human smooth muscle α-actin (Sigma, CatN° F3777), Rabbit anti-human Nr3c2 (Santa-Cruz, CatN° SC-11412) and goat anti-human Msx1 (R&D Systems, CatN° AF5045). Western blot was performed as described (Hendrickx et al., 2010). Samples were collected in RIPA buffer (Sigma, Bornem, Belgium) and protein concentration was measured by the BCA assay. 40

μg of protein was used for blotting. Antibodies used were: Rabbit anti-human Rasgrf2 (Sigma, CatNº HPA018679), rabbit anti-human A2M (Sigma, CatN° HPA002265), Rabbit anti-human MAP9 (Sigma, CatN° HPA037864) and mouse anti-human α-tubulin (Sigma, CatN°T6199; used as housekeeping reference).

siRNA treatment and Notch activity assays. siRNA knockdown was performed using *Silencer*® Select pre-designed siRNA from Applied Biosystems for *RBPJ* (siRNA ID#: s7251 and s7253) or Negative Control 1 (siRNA ID#: am4636). Briefly, 2,500 HUAECs/cm^2 were cultured overnight. The next day, cells were transfected with 5 pmol siRNA mixed with 0.5 μl of lipofectamine 2000 (Invitrogen) in 100 μl of OPTI-MEM (Invitrogen). The day after transfection, media was replaced and cells were maintained for 6 days, with an additional siRNA transfection at day 3. The canonical Notch pathway was induced by immobilised DLL4 ligand activation. Briefly, DLL4-Fc (R&D Systems, CatN° 1389-D4) was incubated overnight at 4ºC at 1 μg/ml in 0.1% gelatin 1% BSA in PBS with gently shaking to allow its adsorption to the cell culture dish. The next day, DLL4-Fc coated plates were incubated at 37ºC for 1 hour. Non-attached DLL4-Fc was removed by washing and 2,500 HUAECs/cm^2 were seeded and cultured for 72 hours. The canonical Notch pathway was blocked by γ-secretase inhibitor DAPT (Calbiochem, San Diego, CA, USA; CatN° 565784; alone or in combination with immobilised DLL4-Fc) at 3 μM concentration. The corresponding DMSO volume was used as a control.

Lentivirus production and overexpression. The lentiviral construct for overexpression of *HEY2* was obtained from Genecopoeia (Rockville, USA; ***Table 10***). Open reading frames (ORF) for MSX1, EMX2, Prdm16, NKX2-3, Aff3 and TOX2 were cloned from cDNA (Open Biosystems) or total cDNA (BD) after the cytomegalovirus (CMV) promoter in pRRL2-CMV-PGK-Cherry (***Figure 23*** and ***Table 10***). The lentiviral construct for overexpression of *SOX17* was kindly provided by C. Verfaillie (Stem Cell Institute, KU Leuven; ***Figure 23***). For lentiviral particle production HEK293 cells were plated (5x10^6 cells/10 cm dish) and the next day transfected with the plasmid of interest together with two helper plasmids (psPax2 and PMD2G) using Fugene transfection reagent (Roche). In brief, 400 μl of OPTIMEM (Gibco) was mixed with 1 μg PMD2G, 3 μg psPax2 and 4 μg lentiviral

construction plasmid (***Table 10***). 24 μl of Fugene was added and the mixture was incubated for 20 min. at RT and gently applied to the cells. The next day, medium was replaced and lentiviral particle-containing supernatant was collected 36 hours later. Viruses were concentrated by centrifugation using 50.000 MWCO Vivaspin(R) 20ml centrifugal concentrators (Sartorius Stedim). Transduced cells were kept for 6 days and collected into TRIzol® buffer. For long-term overexpression of *HEY2* and *Prdm16,* transduced cells were cultured up to 28 days.

In vivo *Matrigel implantation assay.* $0.5x10^6$ HUVECs transduced with control Cherry lentivirus or with the combination of the 8 TFs were mixed with pre-cooled 0.5 ml of Growth Factor Reduce Matrigel containing 300 ng/ml $VEGF_{165}$ (R&D Systems) and 700 ng/ml bFGF (R&D Systems) and subcutaneously injected in the back of 8-weeks-old athymic nu/nu mice (*N*=5 per group). 14 days later, mice were sacrificed by cervical dislocation and the Matrigel plug was dissected out. The Matrigel plug was divided in two equal pieces, one piece was processed for cryosectioning and the other for paraffin sectioning. Human cells were detected by the Cherry fluorescent signal or by human-specific CD31 staining (Dako). Smooth muscle coverage was analysed on smooth muscle α-actin (Sigma) stained sections and collagen deposition was quantified on Sirius red stained sections.

2.4. ***RESULTS***

Freshly isolated but not cultured AECs and VECs differ in gene expression profile

To identify an arteriovenous fingerprint in human ECs across different vascular beds, we used microarrays on RNA from 38 human (H) EC samples (***Figure 24***) corresponding to 6 cultured AEC types (hepatic artery ECs or HHAECs, *N*=3; aorta ECs or HAECs, *N*=2; coronary artery ECs or HCAECs, *N*=2; iliac artery ECs or HIAECs, *N*=2; pulmonary artery ECs or HPAECs, *N*=3; and umbilical artery ECs or HUAEC-C, *N*=5), 4 cultured VEC types (hepatic vein ECs or HHVECs, *N*=3; iliac vein ECs or HIVECs, *N*=3; pulmonary vein ECs or HPVECs, *N*=2; and umbilical vein ECs or HUVEC-C, *N*=5), freshly isolated HUAECs (HUAEC-F, *N*=4) and freshly isolated HUVECs (HUVEC-F, *N*=4). Due to the

difficulty to obtain biopsies from healthy donors, we did not have access to freshly isolated AECs or VECs matched for all cultured EC types. Despite the homogeneity within EC subtypes, when considering all 30 cultured EC samples together, we did not find statistically significant differences in gene expression of AECs *versus* vECs.

We next performed profiling analysis on the freshly isolated HUVEC/HUAEC subset using two statistical approximations: differential gene expression or classification analysis (***Figure 25A***). Both analyses detected significant differences between HUAEC-F and HUVEC-F yielding a combined panel of 76 genes (~102 probes; ***Table 12***) with 64 HUAEC-F genes and 12 HUVEC-F genes, ~55% of which were commonly picked up by both statistical methods (***Figure 25A***). In the remainder of the text, we refer to this 76 genes set as the 'arteriovenous fresh profile'. This profile included 16 genes with documented (differential) expression in AECs/VECs and 13 genes associated with a vascular phenotype in mice, 4 of which were described to be involved in AEC specification/development (*NOTCH4*, *HEY2*, *KDR* and *SOX17*), revealing that our extracted gene list was reliable (***Figure 25A***; ***Table 11***). Functional and pathway enrichment analysis produced several relevant terms, including (cardio)vascular disease, cardiovascular system development and Notch signalling (***Figure 25B-C***). Thorough study of the literature revealed that 10 genes (or a related family member or orthologue) had a previously documented interaction with the Notch pathway (***Table 11***). Finally, the arteriovenous fresh profile harboured 9 genes encoding a TF (8 arterial and 1 venous; ***Figure 25D***). We validated some of the new AEC (*i.e.,* RASGRF2; ***Figure 25E-F***) and VEC (*i.e.,* NR3C2; ***Figure 25G-H***) markers at the protein level. All TFs and a random subset of the genes from the fingerprint were validated using FACS sorting for $Tie2^{+}CD31^{+}CD45^{-}$ population (data not shown).

The culturing process rapidly erases differential arteriovenous gene expression

Hierarchical clustering analysis for the arteriovenous fresh profile showed a perfect separation of the 4 HUAEC-F and HUVEC-F samples, confirming a high degree of difference between the two EC subtypes (***Figure 26A***). The influence of

the culture process on EC gene expression has been previously described for brain microvascular ECs, lymphatic ECs and venular ECs but not for AECs or large VECs. Hence, we next compared freshly isolated HUVECs/HUAECs with their cultured counterparts. Global differential expression analysis showed dramatic changes in gene expression upon culturing, with 410 probe sets (~327 annotated genes) upregulated and 442 probe sets (~332 annotated genes) downregulated in HUAEC-C *versus* HUAEC-F and 66 probe sets (~56 annotated genes) upregulated and 105 probe sets (~69 annotated genes) downregulated in HUVEC-C *versus* HUVEC-F. Strikingly, when considering the arteriovenous fresh profile genes alone, there were no obvious differences between HUVEC-C and HUAEC-C (***Figure 26B***; ***Table 12***). Expression of the 8 arterial TFs was completely lost upon culturing HUAECs (***Figure 26C***). This culture-induced assimilation was also underscored by incorrect clusters generated to distinguish HUVEC-C and HUAEC-C, unlike their fresh equivalents, which clustered according to their arterial or venous origin (***Figure 26A***). These incorrect clusters were also apparent when considering all cultured AEC and VEC types from our screen, suggesting that AECs and VECs from these different vascular beds had lost their specific expression profiles upon culturing (***Figure 24***).

Since during the observed assimilation process arterial and venous-specific genes of the arteriovenous fresh profile as well as the two cell types may behave differently, we next analysed the arterial and venous part of the signature separately in HUAECs and HUVECs. When HUAECs were cultured for several passages, there was strong silencing of most of the 64 arterial genes of the signature, while there was no obvious change in the venous arm (***Figure 26D-F***; ***Table 12***). Conversely, when culturing HUVECs, venous genes were significantly – albeit less dramatically than arterial genes in HUAECs – downregulated, while arterial genes did not change (***Figure 26E-F***; ***Table 12***). Thus, HUAECs were more susceptible than HUVECs to assimilation. To determine the kinetics of the downregulation, we cultured freshly isolated cells for different periods of time before determining the arteriovenous fresh profile. Twenty-four hours of culture was sufficient to induce changes for a majority of the genes (62% of arterial genes in HUAECs and 73% of venous genes in HUVECs; ***Figure 27A-B***). The changes

became more obvious after 48 hours and 6 days (***Figure 27C-F***), revealing that the majority of gene expression changes occurred very rapidly independent of passaging.

Reactivation of Notch signalling only partially restores arterial gene expression in HUAEC-C

It is well established that canonical Notch signalling induces arterial specification during vascular development, by boosting the expression of AEC markers like ephrinB2, and blunting the expression of VEC markers like EphB4. Due to the silencing of the Notch target *HEY2* in HUAEC-C compared to HUAEC-F (***Figure 26***; ***Table 12***), we hypothesised that the canonical Notch pathway might be inactive *in vitro*, which could explain the loss of the arterial phenotype upon culture. During canonical Notch signalling, ligand binding to the Notch receptor induces γ-secretase-mediated cleavage of the receptor, thereby releasing the Notch intracellular domain (NICD). The latter subsequently migrates to the nucleus and binds to recombination signal binding protein for immunoglobulin kappa J region (RBPJ). This association releases RBPJ-associated co-inhibitors and makes RBPJ accessible to co-activators that induce expression of Notch-responsive genes like *HEY1/2* (***Figure 28A***). If the canonical Notch pathway were active in HUAEC-C, then blocking it by using DAPT (a γ-secretase inhibitor) should induce silencing of Notch targets *HEY1/2* and downstream gene *EFNB2*, while compensatorily upregulating VEC markers (*i.e., NR2F2*, *NRP2* and *EPHB4*; ***Figure 28Bleft***). None of this happened, suggesting that the canonical Notch pathway is inactive in HUAEC-C (***Figure 28C***). In this inactive state, there is no NICD that binds to RBPJ, so the latter would be associated to co-inhibitors and repress *HEY1/2* expression (***Figure 28Bleft***). Accordingly, upon siRNA-mediated knockdown of *RBPJ* expression this repressive mark was lost and *HEY1/2* expression was induced, suggesting that RBPJ in HUAEC-C is in a repressive mode (***Figure 28Bmiddle***; ***Figure 28D***). In accordance, *HEY1, HEY2* and *EFNB2* expression could be reactivated in HUAEC-C by stimulation with the Notch-ligand DLL4, and this could

be blocked again by DAPT treatment (***Figure 28Bright***; ***Figure 28E***). To evaluate the overall importance of canonical Notch signalling in the arteriovenous fresh profile regulation in HUAEC-C, we exposed them to anchored DLL4-Fc or bovine serum albumin (BSA). Only a subset (20/64: ~31%) of arterial markers were significantly upregulated upon DLL4-mediated Notch activation and 4 were even downregulated, suggesting additional pathways are involved to fully specify AECs (***Figure 28F***; ***Table 13***).

Combined overexpression of 8 TFs robustly induces the arteriovenous fresh profile in HUVEC-C

The HUAEC-F-specific fingerprint contained 8 TFs (***Figure 25D***), only 2 of which were previously associated with arterial specification, *i.e.,* HEY2 and SOX17 (***Table 11***). Since canonical Notch pathway induction in HUAEC-C was not able to completely restore the arteriovenous fresh profile, some of the newly identified TFs might be important for arterial specification. To determine the role of the 8 TFs we attempted to convert HUVEC-C into cells with a HUAEC-Fexpression profile by overexpression of these factors. Six days after transduction, we analysed expression of the genes within the arteriovenous fresh profile (***Figure 29A-B***), of markers previously reported to be enriched in AEC or VEC (***Figure 29C***) and of general EC markers (***Figure 29D***). ***Figure 29A*** shows the heat map analysis of the arteriovenous fresh profile for individual TFs or the combination of all 8 of them. More distant localisation from the cherry control sample indicates a stronger capacity of the TF (combination) to induce arterial specification. As can be observed from the red gene blocks in the hierarchical clustering analysis, the TF largely complemented each other for arteriovenous fingerprint regulation, although some of these blocks also overlapped (***Figure 29B***). Overall, both analyses revealed that the 8 TF combination delivered the most complete restoration of the arteriovenous fresh profile, while the 'golden standard' for arterialisation *HEY2*, did not induce strong changes in the arteriovenous fresh profile (***Figure 29A-B***). Many established AEC markers were also significantly upregulated, while classical venous markers were downregulated by overexpression of the TF combination (***Figure 29C***). Importantly, except for *TEK*, we did not observe significant changes in general EC marker expression, suggesting that the TF combination specifically

drives arterial specification (***Figure 29D***). When comparing the converted HUVEC-C to HUAEC-F, for the majority (73%) of arterial genes, the TF combination induced expression levels that were above 2%, 60% of which were in the mid-range (2-25% of HUAEC-F levels) and 40% in the high-range (> 25% of HUAEC-F levels) of expression (***Figure 29E***). Some of the gene expression changes were validated at protein level by Western blot (***Figure 29F***). Additionally, analysis of gene expression profile at 28 days post-transduction showed that long-term induction of the arterial phenotype is possible (***Figure 30***)

Individual TFs interact in a complex network to regulate the arteriovenous fresh profile

We next zoomed in on the relative contribution of individual TFs to the regulation of the arteriovenous fresh profile by evaluating their effect on a gene-by-gene basis. When looking at the arterial arm of the profile, overall, a large proportion of genes (~73%) were regulated by at least 1 TF, with ~51% co-regulated by at least 2 TFs (***Figure 31A***). In contrast, only 1 third of the genes was regulated at the venous side (***Figure 31B***). While some TFs, like Aff3, almost did not exhibit any regulatory effect, others regulated a high percentage of arterial genes from the arteriovenous fresh profile (~41% for Prdm16, ~36% for NKX2-3 and ~30% for EMX2; ***Table 14***). Consistent with the global heat map and hierarchical clustering analyses, HEY2 contributed less robustly to arterial gene regulation (~22%; ***Table 14***). Although the hierarchical cluster analysis revealed a significant degree of complementarity between TFs, we also observed overlap, suggesting complex interactions. Therefore, we mapped these interactions in a network (***Figure 31C***). While some genes (*e.g., APO*, *GRB14*, *ARL15*) were exclusively regulated by one TF, most genes were co-regulated by more than one TF (e.g. *FAT1* was co-regulated by 6 TFs). Furthermore, we also observed cross-regulation between the different TFs, for instance *MSX1* being regulated by EMX2, NKX2-3, Prdm16 and TOX2 and *HEY2* being regulated by Prdm16 and MSX1. Finally, some TFs (*i.e.,* TOX2 and SOX17) modulated their own expression. Thus, induction of the arterial gene signature requires a complex interaction of multiple TFs.

***Arterialised HUVECs form more arterial-like vascular structures in a Matrigel plug implantation assay* in vivo.**

To determine whether HUVECs transduced with the combination of the 8 arterial TFs were able to form vascular structures which possess more arterial characteristics *in vivo*, we performed a subcutaneous Matrigel plug implantation assay (***Figure 32***). In comparison to Cherry-infected HUVECs, cells infected with the 8 TFs formed more elaborate vascular networks (***Figure 32A,B***), which were significantly more covered by smooth muscle cells (***Figure 32C,D***). Moreover, the smooth muscle-covered fractional area (***Figure 32E***) as well as organised collagen deposition was greater in the Matrigel containing the 8 TF-treated HUVECs (***Figure 32F***).

2.5. ***CONCLUSIONS AND PERSPECTIVES***

Several studies have addressed the question how the identity of arterial endothelium is established. While each of these studies has highlighted a specific pathway active during the arterial specification process, none of them has taken a genome-wide approach. Only one study reported a differential gene screen between several arterial and venous EC types (Chi et al., 2003). However, this study only considered cultured cells. Here, we have shown that the culturing process rapidly 'erases' the specific gene expression profile of arterial ECs. Therefore, to get insight into the *in vivo* expression pattern of arterial endothelium, we performed a genome-wide differential screen on freshly isolated arterial and venous ECs from human umbilical cord. In this way we established a specific arteriovenous 'fresh' signature of 76 genes. This signature was not only determined by canonical Notch signalling – currently considered as the 'golden standard' for arterial specification – but was co-regulated by additional TFs many of which have not been previously associated with arterial endothelial specification. Overexpression of these TFs in HUVECs revealed that they regulated arterial identity in a complementary but also interactive fashion. Some of the newly identified TFs, such as Prdm16, Emx2 and Nkx2-3, performed better than the current golden standard. Simultaneous overexpression of all eight TFs resulted in the induction of the majority of arterial genes within the newly established arteriovenous 'fresh' signature.

To have insight in arterial specification can lead to important applications. Many diseases with a vascular origin are restricted to a certain vascular bed. For instance, atherosclerosis, leading to ischemia, only affects arteries. Restoration of the perfusional defect mostly requires the expansion of arterial blood (*i.e.,* oxygen) supply to the tissues affected by ischemia. Current 'general' revascularisation strategies based on growth factors or stem cells have not taken into account this specific need for arterial supply and this may in part explain their limited clinical success (Conway and Carmeliet, 2004; Deng et al., 2006; Pearson, 2009). Although the Matrigel plug implantation assay we used here is not a pathological model, the fact that we found a more elaborate and arterial-like vascular network (characterised by smooth muscle coating and collagen deposition) in the presence of cells overexpressing the 8 arterial TFs suggests that this overexpression strategy could improve the outcome of cell transplantation in clinically relevant animal models of arterial growth, such as limb ischemia. In addition, increased insight in the arterial specification may provide new targets for specific growth factor therapy. The arterialising TF combination that we identified, along with the arteriovenous signature, can be used in different approaches. One approach would be to pre-specify endothelial progenitors (*e.g.,* blood outgrowth endothelial cells or BOECs) by TF overexpression to an arterial fate before transplantation into ischemic tissues. Another approach would be to use these arterialised BOECs to coat the inside of artificial vessel conduits to reduce their thrombogenicity when implanted in ischemic patients. These cells could also be used as the endothelial component of a 'biological' arterial conduit consisting of an endothelial and smooth muscle cell layer.

Example 3: *Identification and validation of Prdm16 as an important arterialising transcription factor acting in part upstream of Notch which induces an arterial endothelial-specific gene (and functional) signature in dedifferentiated cultured human umbilical vein endothelial cells (HUVECs) or blood outgrowth endothelial cells (BOECs)*

3.1. ABSTRACT

By using a genome-wide transcriptomic screen on freshly isolated murine arterial and venous endothelial cells (ECs), we identified a murine arteriovenous fingerprint that was strikingly different from our human arteriovenous signature. However, meta-analysis on our human and murine arterial and venous endothelial samples revealed a conserved set of genes, defining the arterial and venous identity of ECs, independent of species, developmental stage, anatomical location and haemodynamic parameters. Intriguingly, we identified an arterial-exclusive expression pattern for the transcription factor Prdm16 in both humans and mice. In addition, Prdm16 retained this arterial expression pattern throughout evolution, as demonstrated by whole-mount *in situ* hybridisation in zebrafish. *Prdm16* deficiency in both zebrafish and mice resulted in arterial vascular defects. Furthermore, the lack of a single *Prdm16* allele in adult mice impaired their recovery from an ischemic insult, a process that requires expansion of pre-existing arterial collateral vessels. Micro-array analysis of Prdm16-, Hey2-, or control (Cherry) lentivirus-treated endothelial progenitors (blood outgrowth endothelial cells or BOECs) revealed that Prdm16 also instructs ECs to adopt the murine arterial gene signature in a superior fashion than Hey2. These *in vitro* studies further demonstrated that Prdm16 induces many conserved arterial signature genes in BOECs. Intriguingly, using our conserved arteriovenous fingerprint as a read-out, this arterialising capacity again exceeded that of the Notch target Hey2, the current 'golden standard'. Moreover, the arterialising effect of Prdm16 on the conserved arteriovenous fingerprint was more profound than its effect on our species-restricted fingerprints. Hence, Prdm16 orchestrates the arterial programme of ECs via regulating a set of conserved arterial- and venous-specific genes, in addition to its effect on the species-restricted arteriovenous signatures. Notably, the arterialising effect of Prdm16 on all three arteriovenous fingerprints described outcompeted the arterialising effect of Hey2. Of interest, ectopic Prdm16 overexpression resulted in a robust induction of many Notch components in both HUVECs and BOECs, raising the hypothesis that Prdm16 lies directly upstream of Notch during the arterial specification of ECs. Indeed, concomitant blocking of the canonical Notch pathway upon Prdm16 overexpression severely hampered or completely abolished the inductive effect of Prdm16 on key Notch pathway genes. Likewise, Prdm16 was able to induce several conserved arterial-specific genes,

including *Gja5* and *Dkk2* in the presence of DMSO, but not DAPT. Intriguingly, treatment with DAPT only partially blunted the arterialising effect of Prdm16. Hence, Prdm16 arterialises ECs via canonical Notch-independent and –dependent manners, thereby outperforming the current 'golden standard' Hey2. Indeed, *in vivo* studies clearly indicated that prdm16 and notch genetically interact during arterial development in zebrafish. In addition, using mutational studies, we found that both the DNA binding- and the CtBP binding-domain of Prdm16 are required for full-blown Prdm16-mediated arterialisation. Finally, Prdm16 strongly induced the expression of semaphorin 3C and – to a lesser extent - semaphorin 3G, known to be attractants for smooth muscle cells (SMCs). Accordingly, SMC coating of arteries of *Prdm16*-deficient embryos was strongly reduced. Thus, Prdm16 induced a specific function of arterial ECs, *i.e.* SMC attraction. Therefore, our data indicate for the first time a role for Prdm16 in the establishment of an arterial gene and functional signature in ECs, in part by acting directly upstream of Notch.

3.2. ***BACKGROUND AND AIMS***

Multiple studies have been devoted to understanding the molecular pathways that determine the development of arteries and veins. Several factors and pathways have been identified, the canonical Notch pathway being considered as the master regulator of arterial fate (Chi et al., 2003). Nevertheless, due to the lack of genome-wide approaches in studying arteriovenous specification, many of the regulating factors remain unknown. Our genome-wide transcriptional profiling studies on freshly isolated human arterial and venous ECs revealed a specific arteriovenous signature (see ***Example 2***). Within this signature we identified eight transcription factors (TFs) that co-regulate arterial endothelial identity of which Prdm (PRD1-BF1-RIZ1 homologous domain containing)16 emerged as one of the most powerful TFs.

Prdm16 is a member of a large family of factors characterised by a PR domain at their N-terminal side. In humans, 17 members of the Prdm family have been identified, and 15 members in mice. Given the fact that members of the Prdm family control processes such as cell commitment, differentiation, growth and

apoptosis, it is not surprising that Prdm proteins play important roles during bidirectional cell fate decisions. Perhaps this is reflected by their highly specific expression profiles. Indeed, Prdm members are generally expressed in a cell type- and tissue-specific manner. Prdm16, for instance, is highly enriched in brown adipose tissue (BAT), while its expression is absent in white adipose tissue (WAT) and induces browning of pre-adipocytes (Seale et al., 2007). Although Prdm family members have been extensively described as key molecules in cellular differentiation processes, the underlying molecular mechanism by which they do so remains to be further elucidated.

Prdm family members can regulate gene expression according to different mechanisms. First, they can cause epigenetic changes in gene expression profiles, depending or not on their intrinsic histone methyltransferase capacity. Secondly, Prdm molecules also have DNA-binding capacities, through their Zn-finger domains. Thirdly, Prdm molecules may regulate gene expression independent of their DNA binding capacity by complexing with other DNA-binding molecules. Prdm16 for instance can form transcriptional complexes with C-terminal binding proteins (CtBPs) and peroxisome proliferator-activated receptor gamma co-activator 1 (PGC1) in adipose tissue. Both are in direct competition with each other to bind Prdm16 and define whether Prdm16 acts as a transcriptional repressor or activator (Kajimura et al., 2008; Seale et al., 2007).

Interestingly, while several Prdm family members have been described in bidirectional cell fate decisions, none, with the exception of Prdm6, has been described to play a role in the vasculature in general, nor in the establishment of endothelial specification in particular. Interestingly, interaction with CtBP molecules is a common feature of multiple Prdm family members and CtBPs have been implicated in vascular development since *CtBP2*$^{-/-}$ mice display extraembryonic vascularisation defects. In addition, *CtBP1*$^{+/-}$*:CtBP2*$^{-/-}$ mice suffered from severe haemorrhaging, a hallmark of vascular defects (Hildebrand and Soriano, 2002). Moreover, a complex formed by Tel1 (Etv6) and CtBPs regulates the endothelial response to angiogenic stimuli by linking VEGF receptor signalling with intracellular Notch signalling (Roukens et al., 2010). Together with the fact that Prdm16 binds CtBPs in multiple tissues (Endo et al., 2012; Kajimura et al., 2008), this raises the

intriguing question whether a Prdm16-CtBP complex could act at the level of arteriovenous cell fate decisions.

As mentioned above, (canonical) Notch signalling has been hailed as the holy grail for arterialising ECs. Indeed, *Dll4*$^{+/-}$ mice, *Hey1:Hey2* combined knockout and *Su(H)*-deficient mice all display vascular defects characterised by the reduced expression of arterial markers (most notably *EfnB2*) and ectopic expression of venous markers (mainly identified by *EphB4*) on the arterial branch of the vasculature (Swift and Weinstein, 2009). Zebrafish studies confirmed the absolute requirement for Notch signalling for proper arterial development (Swift and Weinstein, 2009). However, although its role in arterial differentiation seems to be evolutionary conserved, Notch-deficient animals retain expression of some arterial-specific genes indicating that other factors, potentially upstream of or in parallel to Notch, must act during the arteriovenous cell fate decisions. Intriguingly, Hamlet (the Drosophila orthologue of Prdm16/Prdm3) modifies the accessibility of Su(H) to Notch target gene promoters (Endo et al., 2012). Moreover, mice deficient for Notch downstream Hes effectors display reduced levels of Prdm16 in the telencephalon during murine neurogenesis (Kinameri et al., 2008). Given the arteriovenous expression pattern of Prdm16 in humans, an interesting question one therefore might raise is whether Prdm16 is tightly linked to Notch in the context of endothelial differentiation.

This study was aimed at further elucidating the potential involvement of Prdm16 in (arterial) vascular development in both physiological and pathological conditions, investigating its potential interaction with Notch signalling and testing its potency to arterialise endothelial (progenitor) cells.

3.3. Materials and Methods

Isolation of macrovascular ECs from different species

Mice. The thoracic aorta and vena cava from *Tie2-GFP* mice (expressing GFP in the blood-vascular ECs or but not in lymphatic ECs (Motoike et al., 2000)) were dissected out and placed in ice-cold MCDB131 medium (Gibco). Excess fat and contaminating tissue around the vessels were removed and vessels were cut

into smaller pieces before incubation in 1X PBS (Gibco, pH 7.4) containing 2 mg/ml crude extract collagenase (Roche) at 37°C until fully dissociated. To obtain a single cell solution, tubes were repeatedly shaken, filtered through a 100 µm nylon mesh (BD Biosciences), spun down (600 g, 7 min.), resuspended in PBS supplemented with 1% BSA and run through a FACS Aria™ device (Beckton Dickinson) to separate the endothelial (GFP^+) from the non-endothelial (GFP^-) fraction.

Humans. After dissecting the umbilical cord after birth, the arterial and venous vessels were flushed with PBS to remove remaining blood cells prior to incubation with collagenase type I (Gibco) at 37°C. EC-containing collagenase solution was collected and vessels were flushed with PBS once more to obtain maximal cell yield. Arterial or venous ECs (HUAECs and HUVECs, respectively) were washed with PBS and resuspended in 300 µl MACS buffer (Miltenyi-biotec), with addition of 100 µl FcγR blocking reagent and 100 µl anti-CD34 magnetic beads (Miltenyi-biotec). After incubation at 4°C, cells were washed with PBS to remove non-attached anti-CD34 magnetic beads prior to resuspension in 500 µl MACS buffer and magnetic isolation by the AutoMACS system (Miltenyi-biotec). Afterwards, purity was assessed by FACS using EC markers (CD31 and CD34) and a hematopoietic marker (CD45). Purity typically exceeded 95%.

Zebrafish. FACS sorting of zebrafish ECs was performed on single cell suspensions of 24 hours post-fertilisation (hpf), 48 hpf, 72 hpf and 6 days post-fertilisation (dpf) *Tg(Kdr:eGFP)*^{s843} embryos (Jin et al., 2005) obtained by trypsinisation. Vascular ($eGFP^+$) and non-vascular ($eGFP^-$) cell fractions were sorted with high purity in TRIzol® using a FACS Aria™ device.

RNA extraction and cDNA preparation

RNA extraction. To extract total RNA, 1 ml TRIzol® (Invitrogen) was added to the cells and stored at -80°C. After thawing, samples were vortexed and 200 µl chloroform was added (Merck) prior to incubation for 10 min. at RT. The samples were centrifuged at full speed for 15 min., after which the upper transparent layer was taken off and transferred into a new Eppendorf tube. 500 µl isopropanol (Merck) was added, 200 µl 4 M LiCl (Merck) and 1 µl glycogen (Invitrogen) was added and the samples were stored for at least 10 min. at RT. After centrifugation at full speed for 10 min., the isopropanol layer was carefully discarded and 1 ml of

cold 70% EtOH was added. Afterwards, the samples were centrifuged at top speed for 5 min. and EtOH was carefully discarded. Again, 1 ml of cold 70% EtOH was added and samples were centrifuged at full speed for 10 min.. After discarding the entire EtOH layer, samples were air-dried for at least 10 min. and the remaining pellet was resuspended in 33 µl RNase-free-water prior to storage at -80°C.

cDNA preparation. For cDNA preparation from RNA samples, 32 µl of an RNA sample was mixed with 4 µl random hexamer primers and 4 µl 10 mM dNTP mix (Superscript III First Strand Kit). The sample was denatured for 5 min. at 65°C and cooled down for 2 min. at 4°C. Simultaneously, a solution of 8 µl 10X RT buffer, 16 µl $MgCl_2$ (25 mM), 8 µl DTT (0.1 M), 6 µl H_2O, 1 µl RNase OUT and 1 µl SSIII reverse transcriptase (Superscript III First Strand Kit) was prepared. Subsequently, 40 µl of the solution was added to each sample and the first cDNA synthesis cycle was started: 10 min. at 25°C, 50' min. at 50°C and 5 min. at 85°C. The samples were cooled down to 4°C and 1 µl RNaseH was added prior to incubation for 20 min. at 37°C.

Quantitative reverse-transcription PCR

cDNA was subjected to PCR-based amplification and detected with a non-specific fluorescent dye (SYBR green) which intercalates in the de novo formed double stranded DNA. Each PCR reaction was performed by adding 1 µl cDNA to 6 µl SYBR green (Applied Biosystems), 4 µl MQ water and 1 µl primer mix (2.5 µM forward and reverse primer; a primer list is provided in ***Table 15***). The polymerase reaction was performed on a Real-Time PCR system (Step One Plus, Applied Biosystems): 2 min. at 50°C, 10 min. at 95°C and subsequently 40 rounds of amplification at 95°C for 50 sec., each time followed by 45 sec. at 60°C. *GAPDH* or *β-actin* were used as housekeeping genes to standardise for the total amount of cDNA in the samples.

Microarray on HUVECs and HUAECs

Microarray analysis was also performed on RNA from freshly isolated HUAECs and HUVECs (*N*=5 per group), and hybridised on a human genome-wide microarray (Affymetrix Human U133 array), as more elaborately described in ***Example 2***. Statistical analysis and data interpretation was performed in close

collaboration with the University of Navarra, (Pamplona, Spain) and Integromics (Madrid, Spain).

Microarray on mAECs and mVECs

Pooled RNA of ECs from 8-12 week-old mice was extracted (RNeasy minikit, Qiagen). The quality and purity of the RNA samples was analysed by the VIB Nucleomics Core facility with a Bioanalyser 2100 and validated by PCR analysis prior to further proceedings. 500 pg of RNA from 5 selected isolations per tissue was amplified, labeled with biotin and hybridised on a mouse genome-wide microarray (Affymetrics Mo Gene1-0 ST array). The qualitative and statistical analysis of the microarray output was performed by the Nucleomics Core. The analysis was based on the RMA expression levels of the probe sets that had at least once a present MAS 5.0 detection call. Differential expression was assessed via the moderated *t*-statistic. To control the false discovery rate, multiple testing correction was performed according to Benjamini and Hochberg. We considered genes to be differentially expressed between mAECs and mVECs if they displayed at least 2-fold enrichment in either of these two fractions ([log-value]≥ 1) with a corrected *P*-value < 0.001. Such stringent approach was chosen to obtain 'workable' amounts of genes.

To further define a conserved arteriovenous signature, we conducted a meta-analysis on all microarray samples discussed above, using a Fischer *t*-test on the combined data sets. To map the probe sets of both microarray platforms (*i.e.,* Human Genome U133 Plus 2.0 Array and the GeneChip Mouse Gene 1.0 ST Array), we mapped the probe sets of the Human U133 Plus 2.0 array to the Mouse Gene 1.0 ST array. This was done by mapping of homologous/orthologous probe sets with the package annotation Tools of BioConductor (annotation files *HG-U133_Plus_2.na32.annot.csv* and *Mouse Gene_1.na32.annot.csv* as downloaded from the Affymetrix website and HomoloGene database homologene.data build 66 as downloaded from NCBI). In case a probe set of one array maps to several probe sets of the other array, they are mentioned multiple times in the data table.

Nanostring on HUVECs overexpressing arterialising TFs

RNA from Prdm16-overexpressing, Hey2-overexpressing or Cherry control-overexpressing HUVECs (*N*=4 per group) was used for a comparative Nanostring experiment (which also included 6 additional TFs, as decribed in detail in ***Example 2***). RNA quality/concentration were determined with a Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA) and 100-500 ng of total RNA was hybridised, according to the manufacturers' instructions, in collaboration with the VIB Nucleomics Core Facility at KU Leuven. Some of the results were confirmed by qRT-PCR and for those genes for which the Nanostring probe intensity value was low, results were analysed by qRT-PCR.

Whole-mount* in situ *hybrydisation (WISH) on zebrafish embryos

Probe synthesis. Primers were designed using Primer 3.0 software (http://frodo.wi.mit.edu/primer3/) to amplify a 601 bp long fragment of zebrafish *prdm16*. The following primers were used: 5'GTGACCAGTGCCCCAAAG3' and 5'TTCTTTCCCTCGCAAAAGC3'. A cDNA sample containing a mix of cDNA derived from whole zebrafish (uniZF) was used as a scaffold for the PCR based amplification of *prdm16*. Half of the PCR product was run on a gel to confirm proper fragment length and 2 µl of the remaining product was ligated according to the manufacturers' protocol into a pCRII-TOPO® TA vector (Invitrogen). Linearised DNA (1 µg) underwent transcription to produce either labelled sense or antisense probe by incubation for 2 h at 37°C with labelled NTPs, 10X buffer (Roche), DEPC-treated water, RNA polymerase (either for Sp6 or for T7; Roche) and Protector RNAse inhibitor (Roche). At last, the RNA probe was purified added to hybridisation buffer (Hyb+) and stored at -20°C. From this stock concentration, a 1:100 working concentration was used for performing WISH.

WISH. AB zebrafish embryos were collected at 24 and 48 hpf and fixed using Memfa fixative (1 M MOPS, 10 mM $MgSO_4$ and 20 mM EGTA) for 1h on a shaker. Afterwards, the fixative was replaced by 100% EtOH. After a few minutes, fresh EtOH was added to the embryos for storage at -20°C. Alternatively, embryos were directly submitted to WISH. First, embryos were rehydrated and rinsed three times with PBS-T (PBS containing 0.1% Tween20). Afterwards, embryos were permeabilised with 1X proteinase K in PBS-T for 15 min. and subsequently transferred into 0.1 M triethanolamine, into 0.1 M triethanolamine with 1X acetic

anhydride solution, into 0.1 M triethanolamine with 2X acetic anhydride solution and washed two times with PBS-T before fixation with formaldehyde-PBS-T for 20 min.. After four washes with PBS-T, the embryos were shortly incubated in the settling buffer (50:50 mix of hybridisation buffer and PBS-T). After a brief pre-incubation in hybridisation buffer (10 min. at 60°C), embryos were incubated overnight at 60°C in the hybridisation solution containing the digoxygenin (DIG)-labelled *prdm16* probe. The next day, embryos were washed several times at room temperature (RT) with 2X saline sodium citrate (SSC), 2 times with 0.2X SSC and ultimately two times with MAB (maleic acid buffer). Subsequently, 1 ml of MAB-BR (blocking reagent) was added for 30 min., prior to incubation for 4 hours with 1 ml of MAB-BR-serum with anti-DIG-AP antibody (1:200, Roche). Finally, embryos were four times incubated with MAB for 20 min. and one time overnight at 4°C. The following day, the embryos were rinsed two times with alkaline phosphatase (AP) buffer before putting them overnight on 1 ml BM purple AP substrate in the dark (i.e. covered with aluminum foil) at RT. The next day (or after several days depending on the staining intensity), the chromogenic reaction was stopped by adding PBS-T. Subsequently embryos were fixed with multiple aliquots of Memfa fixative and finally, embryos were bleached, rehydrated and stored in glycerol until further processing. Whole mount pictures were taken using a Zeiss Axio Imager Z1 microscope. Alternatively, embryos were paraffin-embedded, cross-sectioned (7 µm) on a microtome (Leica) and counterstained with nuclear fast red (NFR). Briefly, slides were deparaffinised and placed into the NFR solution for 5-10 min.. Afterwards, slides were rinsed with MQ water, dehydrated and mounted with DPX. Cross-sections were examined on a Zeiss Axio Imager Z1 microscope.

Production of wild-type and mutant Prdm16 expressing lentivirus

Vector cloning. A pYX-Asc vector containing the full cDNA for mPrdm16 was purchased from Open Biosystems (clone ID 6409778). *mPrdm16* was subsequently amplified by means of PCR (primer list: see ***Table 10***) using Phusion® Hot Start II DNA polymerase according to the manufacturers' protocol and cloned into a pRRL2-PGK-Cherry vector using XbaI and XhoI restriction enzymes. Plasmids were transformed and purified by miniprep. Correct insertion

was confirmed by sequencing and maxipreps were made prior to lentiviral production.

Mutagenesis. To insert mutations in the sequence coding for the DNA-binding region of Prdm16 (Prdm16*ΔDNA*), we used the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturers' protocol. In brief, the wild-type pRRL2-Prdm16 PGK-Cherry plasmid served as a template for PCR amplification, using primers carrying the desired mutation (primers used for mutation were: 5'AACCTCCAGCGGCACGTGCAG3' and 5'TTCTCTTTGTTGTGGATGTTC3'). After amplification of the plasmid DNA with the designed mutagenic primers, the non-mutated template string was eliminated by restriction digest with DpnI for 1 hour at 37°C, specifically breaking down parental adenine-methylated GATC sites. Mutated plasmid was then transformed into TOP10 bacteria and plated on ampicillin containing agar plates. Single bacterial colonies were picked from which plasmid DNA was isolated as miniprep. The vector samples were sequenced to verify if the desired mutation was present. The Prdm16 mutant inable to interact with CtBP (*Prdm16ΔCtBP*) was kindly provided by S. Kajimura (UCSF, CA, USA).

Virus production. HEK293 cells were plated ($5x10^6$ cells/10 cm dish) and the next day transfected with our plasmid of interest together with two helper plasmids (psPax2 and PMD2G) using Fugene transfection reagent (Roche). In brief, 400 μl of OPTIMEM (Gibco) was mixed with 1 μg PMD2G, 3 μg psPax2 and 4 μg pRRL2 plasmid. 24 μl of Fugene was added and the mixture was incubated for 20 min. at RT and gently applied to the cells. The next day, medium was replaced and lentiviral particle-containing supernatant was collected 36 h later. Viruses were concentrated by centrifugation and used directly to transduce HUVECs/BOECs (or stored at -80°C). The lentiviral construct for overexpression of *HEY2* was obtained from Genecopoeia (Rockville, USA; ***Table 10***).

αSMA/Prdm16 double staining on frozen sections

E14.5 and E17.5 FvB embryos were dissected out, rinsed in RNA later and put in a 15 ml falcon tube to snap-freeze in liquid nitrogen. Unfixed cryo-preserved embryos were sectioned (7 μm) on a cryostat (Leica, CM3000) and sections were

stained for Prdm16. Therefore, air-dried sections were incubated with 4% paraformaldehyde (PFA) for 10 min. at 4°C, sections were washed with MQ water and three times with Tris-HCl-NaCl-Tween (TNT) before they were immersed in PBS-Tr (PBS with 0.1% Triton-X) for 30 min.. The sections were blocked for 1 h with tris-NaCl-BMP buffer (TNB) containing 20% pre-immune donkey serum (PID; Sigma-Aldrich) and incubated overnight on a shaker with Sheep-anti-Prdm16 primary antibody (1:20 in 20% PID/TNB; R&D Systems) at 4°C. After washing three times with TNT buffer, the sections were incubated with secondary Donkey-anti-Sheep-Texas Red antibody (Jackson Laboratories; 1:100) in TNB and FITC-conjugated anti-αSMA (Sigma; 1:500) for 2 h at RT and once again washed three times with TNT before mounting with a glue (prolong gold) containing DAPI.

Haematoxylin and eosin staining on paraffin sections

Slides were briefly submerged in MQ water prior to deparaffinisation. Afterwards, they were incubated in hemaluin dye for 3 min., washed with flowing water and put in eosin dye for 10 sec.. Finally, slides were washed with MQ water until its red colour disappeared and subsequently dehydrated again before being mounted with DPX.

αSMA/Coup-TFII double staining on paraffin sections

Sections were deparaffinised and subjected to antigen retrieval (based on a pH 6-citrate buffer) and microwave heating. Slides were washed three consecutive times with tris-buffered saline (TBS) prior to blocking with 10% pre-immune goat serum (PIG) in TNB for 1 h. In a next step, slides were incubated overnight with primary Ab against Coup-TFII (Perseus Proteomics; 1:200) at 4°C. The next day, slides were washed multiple times with TNT, incubated with Goat-anti-Mouse Alexa 568 secondary Ab to visualise Coup-TFII and with FITC-conjugated anti-αSMA antibody (Sigma; 1:500) to visualise the SMC layer. Finally, slides were washed several times with TNT and mounted with prolong gold containing DAPI. SMC density of the jugular aorta was measured as the difference between the outer and inner (luminal) surface area of the artery using NIH Image J software. 5 to 10 images were analysed for each mouse.

Whole-mount β-galactosidase staining on murine tissues

Adult vessels (aorta, vena cava) from *Prdm16*$^{+/-}$ embryos, containing the *β-galactosidase* gene (http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=alleleDetail&key=37049), or E10.5 embryos derived from *Prdm16*$^{+/-}$ intercrosses, were processed as follows: tissues were briefly washed with PBS prior to fixation at 4°C with fixing solution for 20 min.. Tissues were subsequently washed repeatedly with PBS and incubated with staining solution at 30°C overnight. Next day, tissues were washed three times with PBS and post-fixed with 4% PFA for 2 h at RT. Tissues were washed multiple times and further processed for paraffin sectioning and nuclear fast red (NFR) staining. Adult tissues from *Prdm16*$^{+/+}$ mice were used as an internal negative control.

Morpholino knockdown and live screening in zebrafish embryos

Knockdown of *zPrdm16* was achieved by injecting *Tg(kdr:eGFP)*^{s843} zebrafish with a morpholino (Mo) at the one cell stage. The *prdm16* Mo is a 25 bp oligomer (purchased from GeneTools LLC; sequence 5'-3': ATATGCTGCCCAAGACTAGAAATAC) which complementary binds to the region containing the ATG start codon and thereby blocks translation. The Mo was diluted in phenol red (resulting in a better contrast during and after injection) to obtain the correct Mo concentration. First, the injection needle was calibrated at 5X magnification of a Stema 2000-C microscope (Zeiss). After injection at 0.8X of the same microscope, at 200-300 hPa for 0.2 sec., the embryos were incubated at 28°C (in a Binder incubator). We used up to 14.4 ng *prdm16* Mo per embryo. Our findings were confirmed with a second splice-site morpholino (5'-ACTCACACTATCACCCACCTTATCA-3'). To address the genetic interaction between prdm16 and notch *in vivo*, we injected *Tg(kdr-eGFP)*^{s843} zebrafish with a suboptimal dose (10.8 ng) of *prdm16* Mo at the single cell stage and manually dechorionated the embryos 6 to 7 h later. Dechorionated embryos were incubated in 0.3% Danieau water to which we added a final concentration of 12.5 μM DAPT or an equal volume of DMSO. Zebrafish embryos were screened for vascular defects at 48 hpf. Alternatively, we injected *Tg(kdrl-eGFP)*^{s843} zebrafish with morpholinos targeting either *prdm16* or *grl* or the combination of the latter two. Briefly, 10.8 ng *prdm16* Mo or 3.6 ng *grl* Mo were injected alone or together. Ns Mo was used to compensate for the differential amount of Mo between conditions. A

bolus injection of 14.4 ng ns Mo was used as a control.

Live screening or confocal imaging of *Tg(kdr:eGFP)*^{s843} zebrafish embryos was performed using a Zeiss Lumar V.12 fluorescence stereomicroscope equipped with a Zeiss AxioCam MrC5 digital camera or a laser-scanning microscope LSM510, respectively. The development of the intersomitic vessels (ISVs) was analysed in 10 consecutive somite segments. Scores ranging from 0 to 10 were given to each embryo, depending on the number of normally developed ISV in those 10 segments. Typically, blood vascular defects were analysed at 48 hpf. Only embryos that were alive and had a heart beat were included in the analyses.

Hind limb ischemia in mice

Prior to surgery, mice were anaesthetised with 200 µl of a (26:8:66) mixture of ketamin: xylazin: NaCl. During the surgery, mice were placed on a heating pad and the temperature was monitored to remain at 37°C. The fur of both hind limbs was removed by Veet® treatment. After making an incision to open the skin, the nerve was carefully dissociated from the femoral artery in the right upper limb. Subsequently, to induce femoral artery occlusion, two surgical clamps were placed onto the right femoral artery, one above and one below the branching point with the arteria caudalis femoralis. Additionally, to ensure a complete lack of blood flow, an incision was made in the femoral artery between the two clamps. The skin was stitched and the closed wound was disinfected with iodine. While the mouse was still under anesthesia, successful interruption of blood flow was monitored by Laser Doppler scanning (Lisca; see below). Mice were kept on the heating pad until they awoke.

The follow-up of the ischemic mice implied non-invasive monitoring of blood flow during recovery of the ischemic insult by Laser Doppler scanning. This system scans a preselected area of the hind limb with a laser beam. When the laser beam hits red blood cells in motion, a sound wave is sent back to a detector in the scanning head. The intensity of this feedback signal is proportional to the amount of moving red blood cells (*i.e.,* the blood flow) and is transformed into a colour code by the Lisca software, red representing high blood flow, blue/black representing low/no blood flow. The left non-ligated limb was also scanned and used as an internal reference to calculate the relative perfusion in the ligated right limb. In

addition to the scanning session immediately following surgery, Laser Doppler measurements were performed at day 3, 7, 10, 14 and 21 after surgery, under isoflurane anaesthesia and temperature monitored (37°C) conditions.

At day 21, mice were sacrificed. Therefore, mice were sedated with a (26:8:66) mixture of ketamine: xylazine: NaCl. The vessels were perfused (by inserting a needle into the apex (left ventricle) of the heart and making an incision in the right atrium) with adenosine to induce vasodilatation. Next, vessels were perfused with Zinc Fix (zinc formalin fixative) for 10 min. The right adductor and gastrocnemius muscles were dissected out and placed in Zinc Fix overnight. The next day, after washing the tissues three times with MQ water, they were stored in 70% EtOH at 4°C until further histological processing.

Sirius red staining on paraffin sections and analysis

To measure fibrosis, cross-sections of the gastrocnemius muscle were stained with the sulphonated azo-dye Sirius red. Briefly, after deparaffinisation, sections were incubated for 90 min. in freshly prepared Sirius red dye solution, followed by differentiation in HCl. Slides were dehydrated and mounted with DPX. Fibrosis was determined by measuring the area taken up by Sirius red staining using ImageJ software on 4 images randomly chosen from three different sections per mouse.

Lentiviral transduction of HUVECs or BOECs

HUVECs were obtained as described in ***Example 2***. BOECs from peripheral blood or umbilical cord blood were obtained as described under ***Example 1***. HUVECs or hBOECs were plated one day prior to transduction into a 24-well plate (25,000 cells/well). The next day, 0.5 to 10 μl of virus (depending on the virus and the virus production) was added to the medium. The morning following transduction, medium was changed and cells were directly harvested 5 days later (6 days after transduction) on TRIzol® lysis buffer. RNA/cDNA was made and analysed by qRT-PCR. Alternatively, we added 50 μM DAPT or its solvent DMSO to the medium.

For luciferase assays, cells were transduced as described above. However, cells were co-transduced with a RBPJκ-luciferase reporter virus (Cignal Lenti RBP-

Jk Reporter (luc) Kit: CLS-014L; SABiosciences; Qiagen) and a lentiviral Renilla control to normalise for transduction efficiency (Cignal™ Lenti Renilla Control (luc): CLS-RCL; SABiosciences; Qiagen). Medium was changed the next day and again at day 3 and cells were collected in 50 μl 1x luciferase lysis buffer 6 days after transduction. Firefly and Renilla luciferase activity were measured from 20 μl of lysate according to the manufacturers' protocol using the Dual-Luciferase® Reporter Assay System (E1910) from Promega on a Microplate Luminometer LB 96V (EG&G Berthold).

Statistics

Data, expressed as mean ± s.e.m comparing two groups were analysed by Student's *t*-test: for single comparisons *P*-values were calculated with two-tailed unpaired Student's *t*-test; for multiple comparisons *P*-values were calculated with one-way ANOVA, followed by contrast analysis. For the limb ischemia study, repeated measures ANOVA was applied, followed by Student's *t*-test for each time point. For phenotypic scoring of zebrafish, the penetrance of the phenotype, the number of embryos exhibiting the different severities of morphant phenotype was counted and Chi-square analysis was used to determine whether this fraction differed between control or dose groups. SPSS software was used for statistical analysis and differences were considered significant when $P<0.05$.

3.4. ***RESULTS***

Prdm16 has an arterial-exclusive expression pattern throughout evolution

Our microarray screen on human ECs from human umbilical cord arteries and veins revealed that *PRDM16* was highly enriched in arterial ECs (***Example 2***). In a next step, we were eager to establish an arteriovenous fingerprint and identify their transcriptional regulators in another species, since evolutionary conserved mechanisms most likely play the most pivotal role in the establishment of the arterial/venous identity of ECs. Since it was technically not feasible to derive sufficient ECs from murine umbilical arteries and veins, we sorted GFP^+ ECs from the thoracic aorta and vena cava of adult *Tie2-GFP* mice and performed gene profiling on these samples. As for the human profiling studies, we used a stringent statistical comparison to define our murine arteriovenous fingerprint. Remarkably,

there was only a very limited overlap of our human arteriovenous fingerprint with the murine arteriovenous gene signature. More specifically, almost none of the genes from the human fingerprint were also significantly differentially expressed between murine (m)AECs and mVECs. Thus, the murine arteriovenous signature represented a completely novel set of 68 and 85 probe sets differentially expressed between mAECs and mVECs, corresponding to 65 arterial- and 62 venous-specific genes, respectively (***Table 16***).

COUP-TFII, a TF known as the prime VEC denominator (You *et al.*, 2005) was significantly enriched in mVECs compared to mAECs. However, due to our stringent statistical standards, COUP-TFII was not part of our human arteriovenous fingerprint, despite its differential expression pattern. This prompted us to evaluate more broadly the expression profile of all TFs differentially expressed between AECs and VECs of either human or murine origin, to avoid missing out on potentially strong candidates which could play pivotal roles in arteriovenous specification. A summary of the average probe intensities of these TFs in both AECs and VECs from human and murine origin is listed in ***Table 17***. Intriguingly, this analysis revealed that only few TFs retained their differential expression pattern across species. Indeed, many of the arterial TFs of the human (including *AFF3*, *EMX2* and *TOX2*) and the murine (*Dlx5* and *Isl1*) fingerprint only displayed a differential expression in the original species in which they were identified. Intriguingly however, Coup-TFII remained a valid VEC determinant in both species, confirming the validity of our data. Additionally, some arterial TFs were significantly enriched in both human and murine AECs, compared to their venous counterparts. Indeed, both Hey2 and Sox17, as well as Prdm16 were significantly differentially expressed between both HUAECs *versus* HUVECs and mAECs versus mVECs, although only Hey2 made the stringent cut-off values set by us in both species. As Hey2 and Sox17 have been previously associated with the arterial vasculature and given the strong arterialising capacity of Prdm16 in HUVECs (see ***Example 2***), we decided to further elaborate on the role of Prdm16 during arterial development.

Our profiling studies suggested that, even though the arteriovenous gene signature may be different depending on species (or other variables), these signatures may be determined by common TFs. Hence, we next wondered whether

Prdm16 also regulated the murine arteriovenous signature, and whether it outperformed Hey2, parallel to our findings concerning the human signature in HUVECs. Therefore, we overexpressed Prdm16, HEY2 or a negative control (Cherry) in human endothelial progenitor cells (i.e. blood outgrowth endothelial cells or BOECs) and performed microarray analysis on these samples (*N*=3 for each condition). Prdm16 overexpression resulted in the significantly increased expression of a subset of genes (14 out of 48 detectable genes; ~29%) preferentially expressed on mAECs. Conversely, unlike Prdm16 overexpression in HUVECs, Prdm16 significantly suppressed the expression of ~31% (15 out of 48 detectable genes) of the mVEC-specific genes in BOECs, while only enhancing the levels of ~10% of these genes. Thus, Prdm16 clearly induced an arterial shift in BOECs when considering the murine arteriovenous fingerprint. Surprisingly, unlike previously shown in HUVECs (Chi *et al.*, 2003), under our experimental conditions in BOECs, HEY2 significantly upregulated or downregulated only a limited number of genes upon overexpression and none of these were part of the murine arteriovenous fingerprint. This further suggests that also for the murine arteriovenous fingerprint, Prdm16 was a stronger 'arterialiser' than the golden standard Hey2.

Determination of the arteriovenous fingerprint across species: towards a conserved arteriovenous signature

When performing stringent statistical analysis on the human and murine data sets separately, we obtained two distinct, almost non-overlapping arteriovenous gene signatures. Yet, some of the transcriptional regulators were common to both species. We next wondered whether a meta-analysis considering both data sets together and thereby using an alternative biostatistics approach would deliver a common – thus conserved – gene signature, the regulation of which would potentially be better than for the two separate signatures by the common TFs, *i.e.,* Prdm16, Hey2 and Coup-TFII. When only considering genes that were at least two-fold enriched in AECs or VECs in both species, the meta-analysis on our human and murine microarray data gave us a third list of genes differentially expressed between arteries and veins (21 arterial and 30 venous genes; ***Table 18***). The

arterial versus venous log_2 ratios of these genes are plotted in ***Figure 33*** for both human and murine samples.

Notably, Prdm16, Hey2 and Coup-TFII were retained in this list. In addition to these master regulators of arteriovenous identity, among the arterial-specific genes, several were previously associated with arteriovenous differentiation or arterial defects in mice or zebrafish (e.g. *HEY2*, *GJA5* and *PTPRJ*) upon knockdown/knockout (***Table 18***). Thus, this third fingerprint exhibits hallmarks of a conserved signature that defines the arterial or venous identity of ECs across species. Therefore, we took this third list as our read-out for mechanistic studies discussed below.

Like our 2 species-restricted fingerprints, this conserved arteriovenous signature responded in the expected fashion to lentiviral Prdm16 overexpression, as evidenced by our microarray results in BOECs. More importantly, the arterialising effect of Prdm16 on this final list exceeded that on our species-restricted fingerprints, as Prdm16 induced ~63% of the arterial genes, while suppressing ~64% of the venous markers, thereby clearly shifting the balance towards a more arterialised phenotype (*N*=3-3; ***Figure 34A***). Furthermore, like for the species-specific arteriovenous gene signatures, comparative microarray profiling of HEY2 overexpressing or Prdm16 overexpressing BOECs, revealed that the arterialising effect of HEY2 was inferior to that of Prdm16. In fact, when considering the conserved arteriovenous gene list, HEY2 overexpression only induced the expression of ~47% of the arterial genes, while suppressing ~43% of the venous markers (***Figure 34B***).

Finally, to further validate this conserved arteriovenous signature, we conducted microarray analysis on HUVECs treated with an siRNA against NR2F2 (siNR2F2) or a negative control (siNS). We hypothesised that silencing of NR2F2 – a prime determinant of venous specification by inhibiting Notch (You *et al.*, 2005) – would augment the expression of our newly identified arterial-specific genes, while suppressing the venous-specific genes. As expected, knockdown of *COUP-TFII* enhanced the levels of several arterial-specific genes, including *HEY2*. However, the arterialising effect of siCOUP-TFII was less dramatic than that observed in our Prdm16 overexpression studies, mainly because of a modest inhibitory effect on

the venous genes (***Figure 34C***). In conclusion, the evolutionary conserved arterial-specific TF Prdm16 is a firm driver of EC arterialisation by regulating a set of well-established arterial and venous enriched genes, thereby outperforming the Notch pathway.

To confirm our microarray results, we performed PCR analysis for *PRDM16* and two endogenous controls, *HEY2* (for arterial ECs) and *NR2F2* (also known as COUP-TFII; for venous ECs), on independently derived human umbilical cord samples and ECs from murine aorta and vena cava. PCR data confirmed the arterial enrichment of *PRDM16* and *HEY2*, while *NR2F2* was clearly enriched in venous ECs ([fold upregulation HUAECs versus HUVECs]: 15 ± 2, $P<0.001$ for *HEY2*; 45 ± 6, $P<0.001$ for *PRDM16*; 0.11 ± 0.01, $P<0.01$ for *NR2F2*; $N=4$; ***Figure 35A***). Similar results were obtained in ECs isolated from murine aorta (mAECs) and vena cava (mVECs; [fold upregulation mAECs versus mVECs]: 4.7 ± 0.2, $P<0.05$ for *Hey2*; 47 ± 18, $P<0.05$ for *Prdm16*; 0.16 ± 0. 12, $P<0.05$ for *Nr2f2*; $N=4$; ***Figure 35B***).

PRDM16 staining confirmed the presence of human PRDM16 protein on HUAECs, while absent on HUVECs (***Figure 35C,D***). To further validate the strictly arterial expression pattern of Prdm16 in mice, we studied Prdm16 protein expression taking advantage of *Prdm16*$^{+/-}$ mice carrying one allele in which the *β-galactosidase* gene was knocked-in into the *Prdm16* locus. 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) stainings revealed clear Prdm16 expression in the arterial endothelium of E10.5 embryos, including in the DA and the vitelline artery (***Figure 35E,F***). Conversely, we could not detect Prdm16 on ECs from the (common) cardinal or vitelline vein (***Figure 35E,F***). Prdm16 immunofluorescence staining using a commercially available antibody confirmed our findings on later developmental stages: at E14.5, Prdm16 was readily detected in the endothelium of intercostal arteries and the jugular artery, while it was absent in their venous counterparts (***Figure 35G,H***). Similarly, at E17.5, ECs from coronary arteries, but not coronary veins stained for Prdm16 (***Figure 35I,J***). Likewise, X-gal staining on adult thoracic aorta and vena cana of *Prdm16*$^{+/-}$ mice confirmed the persistent exclusive arterial expression profile of Prdm16 (***Figure 35K-M***). Thus, the expression of Prdm16 is restricted to the arterial branch of the vasculature in both

humans and mice from early development through adulthood. Notably, Prdm16 was not only detected on AECs, but was also frequently found on arterial SMCs (***Figure 35G,H***).

Early vascular expression of prdm16 in zebrafish

Next, we investigated whether Prdm16 is also expressed in the vasculature of the zebrafish, a vertebrate organism that is taxonomically quite distant from humans and mice, but has been well documented for vascular research. Therefore, we separated endothelial from non-endothelial fractions using *Tg(kdr:eGFP)*^{s843} zebrafish embryos, at different developmental stages after which we analysed zebrafish *prdm16* expression by means of qRT-PCR. With this approach, a temporal expression profile during embryogenesis was obtained revealing an endothelial expression peak at 24 hours post-fertilisation (hpf; ***Figure 36A***). This is a very relevant timeframe since the two major trunk vessels (the dorsal aorta or DA and posterior cardinal vein or PCV) are properly formed and obtain their arteriovenous identity around this time in zebrafish. After 24 hpf, *prdm16* expression – although still detectable - gradually declined on the vasculature.

These timeline experiments did however not allow us to make a subdivision between arterial and venous ECs. Therefore, to gain insight into the spatial distribution of *prdm16* expression, we performed whole-mount *in situ* hybridisation (WISH; ***Figure 36B,C***). Whole mount pictures of 24 hpf AB zebrafish, demonstrated *prdm16* expression in the DA but not the PCV. Additionally, some embryos were cross-sectioned and counterstained with nuclear fast red (NFR). A representative picture (***Figure 36C***) shows signal in the DA, while *prdm16* expression remained absent in the PCV. Since zebrafish do not have SMCs or pericytes in the trunk vessels at this developmental stage, vascular expression of *prdm16* at this stage can be regarded as arterial endothelial-exclusive. At 48 hpf the expression profile of *prdm16* remained exclusively arterial, however less prominent as its expression in the notochord became predominant (data not shown). Therefore, we conclude that Prdm16 displays an arterial exclusive expression pattern in the zebrafish, murine and human vasculature.

Prdm16 *deficiency causes severe (arterial) vascular defects in zebrafish and mice*

Prdm16 deficiency in zebrafish results in (arterial) vascular defects

To investigate its functional importance in AECs, we knocked down *prdm16* in *Tg(kdr-eGFP)*^{s843} zebrafish embryos by injecting a morpholino specifically targeting its ATG region (*prdm16* Mo). *Prdm16* morphants displayed a clear vascular phenotype (***Figure 36D-F***) at 48 hpf, characterised by defective development of the (arterial) ISVs and an improperly formed DLAV. Indeed, 48% of zebrafish injected with *prdm16* Mo suffered impaired ISV formation, while nearly all (92%) control (ns Mo) embryos had a normal ISV phenotype (P<0.0001 *versus* ns Mo; N=99-99). Moreover, in more than half (52%) of the *prdm16* morphants, the DLAV did not or only partially form, while 97% ns Mo-treated embryos showed normal DLAV development (P<0.0001 *versus* ns Mo, N=99-99). In addition, *prdm16* morphants often displayed aortic hypoplasia, making it hard to distinguish between the DA and PCV, although there was no evident fusion between the two axial vessels. All these defects could be recapitulated via injection of a splice-site morpholino (data not shown).

As Prdm16 is a likely candidate to act during arteriovenous cell fate decisions, we analysed the expression of several arterial and venous markers on sorted eGFP$^+$ ECs from *prdm16* and ns Mo-treated embryos at both 24 and 48 hpf. However, we failed to detect any sigificant downregulation of either *dll4*, *efnb2a* or *grl*. *Vice versa*, eGFP$^+$ ECs from *prdm16* Mo-treated embryos did not have elevated levels of the venous marker *coup-tfII* (data not shown). As these results were unexpected, we turned to WISH to see whether *dll4*, *efnb2a* and *grl* displayed ectopic expression on the PCV or other vascular structures. WISH for *dll4*, *efnb2a* and *grl* did however not reveal a mispatterned expression profile as none of these genes showed ectopic expression on the PCV or a reduced arterial expression. WISH for the venous marker *coup-tfII* also did not reveal an aberrant expression pattern (data not shown).

Prdm16 deficiency in mice results in (arterial) vascular defects

In order to address the role of Prdm16 in murine vascular development, we performed timed-matings between *Prdm16*$^{+/-}$ x *Prdm16*$^{+/-}$ intercrosses and initially collected embryos at E14.5 as we had done for previous studies. Collecting embryos at this specific stage allowed us to examine all three macrovascular vessels within one region (***Figure 37A***). Indeed, at this developmental stage, embryos possess a major artery, vein and lymphatic within their jugular region, and are therefore particularly useful to study macrovascular endothelial heterogeneity. Close examination of these embryos revealed subtle, but consistent vascular defects. Indeed, H&E stainings demonstrated that *Prdm16*$^{-/-}$, but not *Prdm16*$^{+/+}$ or *Prdm16*$^{+/-}$ E14.5 embryos displayed haemorrhages throughout their body (***Figure 37B,C***). Moreover, in some of these *Prdm16*$^{-/-}$ embryos, red blood cells could be seen leaking out of the artery (***Figure 37D-G***). This vascular leakage is most likely secondary to the fragile structure of arteries of *Prdm16*-deficient embryos. Indeed, αSMA staining on sections of the jugular region of these embryos revealed a reduced coverage by vascular SMCs in the artery compared to their *Prdm16*$^{+/-}$ or *Prdm16*$^{+/+}$ littermates ([αSMA$^+$area in μm^2]: 3777 ± 102 *versus* 2662 ± 88 for *Prdm16*$^{+/+}$ versus *Prdm16*$^{-/-}$ mice; *P*<0.0001; *N*=7-7 and 3625 ± 213 *versus* 2662 ± 88 for *Prdm16*$^{+/-}$ *versus Prdm16*$^{-/-}$ mice; *P*<0.01; *N*=11-7; ***Figure 37D-H***). Moreover, the residual SMCs that were present failed to organise properly and revealed altered morphology. Analogous to our experiments in zebrafish, we failed to detect ectopic expression of the venous TF Coup-TFII on AECs (not shown).

Heterozygous deficiency of *Prdm16* impairs collateral arterial reperfusion

The subtle but consistent vascular defects seen in our knockout embryos, prompted us to hypothesise that *Prdm16*-deficient mice might have impaired vascular recovery upon an ischemic insult. As *Prdm16*$^{-/-}$ mice do not survive beyond birth, we therefore submitted *Prdm16*$^{+/+}$ and *Prdm16*$^{+/-}$ mice to a model of moderate limb ischemia – also known as intermittent claudication, as the recovery of such an ischemic insult is highly dependent on the expansion of native arterial collaterals, in a process termed adaptive arteriogenesis. The recovery of *Prdm16*$^{+/-}$ mice was indeed significantly impaired compared to their *Prdm16*$^{+/+}$ littermates as determined by laser Doppler perfusion measurements (*P*<0.001; *N*=12-12; ***Figure 38A-C***). One week after surgery, for example, *Prdm16*$^{+/+}$ mice had already 37 ± 3%

of normal blood perfusion, while *Prdm16*$^{+/-}$ mice only returned to 25 ± 2% of their normal perfusion profile (*P*<0.01; *N*=11-12), suggesting impaired collateral growth specifically in adductor muscles of *Prdm16*$^{+/-}$ mice, as Pecam1 staining revealed no difference in vascular density of the gastrocnemius muscle between the two genotypes (***Figure 38D,E***).

In accordance with their reduced blood flow during the recovery phase, a higher percentage of *Prdm16*$^{+/-}$ mice displayed macroscopic signs of necrosis, compared to their control littermates throughout the experiment ([% of mice with macroscopic signs]: 92% of *Prdm16*$^{+/-}$ mice *versus* 50% of *Prdm16*$^{+/+}$ mice at day 3; 83% of *Prdm16*$^{+/-}$ mice *versus* 17% *Prdm16*$^{+/+}$ mice at day 21; *P*<0.05; *N*=12-12; ***Figure 39A***). Representative images of the lack of macroscopically observable necrosis in *Prdm16*$^{+/+}$ or its presence in *Prdm16*$^{+/-}$ mice are given in ***Figure 39B*** and ***C,*** respectively.

Finally, histological examination of the gastrocnemius muscle of these mice revealed a two-fold higher degree of fibrosis in *Prdm16*$^{+/-}$ mice, compared to their *Prdm16*$^{+/+}$ littermates ([% fibrotic area]: 4 ± 1 in *Prdm16*$^{+/+}$ mice *versus* 8 ± 1 in *Prdm16*$^{+/-}$ mice (*P*<0.05; *N*=11-12; ***Figure 40***).

Prdm16 induces an arterial phenotype in ECs in part through canonical Notch

Prdm16 and Hey2 co-regulate multiple arterial-specific genes

Since the ultimate goal of this project was to generate new tailor-made vascular therapies, using pre-differentiated stem/progenitor cells, we were keen on finding out whether Prdm16 could direct ECs towards an arterial phenotype. Therefore, we first overexpressed Prdm16 in established venous ECs (HUVECs) using a lentiviral construct. Nanostring analysis revealed that Prdm16 significantly induced many (23/64; ~36%) of the arterial genes we found in our human microarrays (see also ***Example 2***). Moreover, plotting the arterialising effect of Prdm16 against that of the 'golden standard' Hey2 in these experiments, clearly indicated that Prdm16 induced a more robust arterial phenotype in HUVECs than HEY2. Indeed, not only was the overall number of genes significantly induced by Prdm16 higher than for HEY2 (see ***Example 2***), the increase in expression of many genes was far more enhanced upon Prdm16 treatment. Of note, while several

arterial specific genes were indeed only induced by either Prdm16 or HEY2, we detected several arterial genes regulated by both TFs. Thus, Prdm16 and Hey2 not only act in a parallel fashion to direct ECs towards an arterial phenotype, they also co-regulate part of the arterial signature. A striking overlap between the genes regulated by HEY2 and Prdm16 was indeed observed: 9 out of the 13 (69%) arterial genes (*MFAP5*, *NPR3*, *RASGRF2*, *SEMA3C*, *SYTL2*, *FAT1*, *MAP9*, *ODAM*, *CUBN*) significantly induced by HEY2 also revealed significantly elevated expression levels upon Prdm16 overexpression.

Prdm16 activates Hey1/2 and their downstream target ephrinB2

The arterial-specific TFs Prdm16 and Hey2 were significantly differentially expressed in our different comparisons of arterial *versus* venous ECs and therefore are most likely key determinants of arterial identity. Secondly, the capacity of Prdm16 exceeded Hey2's capability of arterialising both HUVECs (see ***Example 2***) and BOECs. Thirdly, several members of the Prdm family – including Prdm16 – genetically interact with the Notch pathway. Fourthly, Prdm16 and HEY2 co-regulate many human arterial-specific genes upon overexpression in HUVECs (see above). Finally, even though these genes were not part of the conserved arterial fingerprint, half of the – few – genes significantly regulated by HEY2 in BOECs are driven in the same direction by Prdm16. Indeed, microarray analysis of control (Cherry), Prdm16 or HEY2-overexpressing BOECs (N=3 for all) indicated that 13 out of the 27 genes significantly regulated by HEY2, were driven in the same direction by Prdm16 (data not shown). Therefore, we wondered whether Prdm16 and Notch acted through a single cascade to drive endothelial arterialisation. To address this question, we analysed in more depth the effect of Prdm16 overexpression on the Notch pathway via qRT-PCR. Intriguingly, overexpressing Prdm16 in HUVECs resulted in a robust induction of key canonical Notch pathway related members, including *DLL4*, *HEY1* and *HEY2* ([fold upregulation Prdm16 *versus* Cherry]: 10.6 ± 3.4; P<0.05 for *DLL4*, 6.8 ± 1.3; P<0.01 for *HEY1;* 4.5 ±1.1; P<0.05 for *HEY2*). Conversely, the established venous markers EPHB4, NRP2 and COUP-TFII were not or only slighty suppressed by Prdm16 ([fold upregulation Prdm16 versus Cherry]: 1.0 ± 0.4; P=*NS* for *EPHB4*, 0.4 ± 0.1; P<0.05 for *NRP2;* 0.7 ±0.1; P=*NS* for *COUP-TFII*). Moreover, the expression of the downstream

Notch target gene *EFNB2* was also elevated by ectopic Prdm16 expression ([fold upregulation Prdm16 *versus* Cherry]: 4.0 ±0.6; *P*<0.01 for *EFNB2*; ***Figure 41***). Similar results were obtained in BOECs ([fold upregulation Prdm16 *versus* Cherry]: 5.5 ± 3.6*; P=NS* for *DLL4*, 5.6 ± 1.2; *P*<0.01 for *HEY1;* 8.5 ±4.0; *P*<0.05for *HEY2,* 3.7 ± 1.0*; P*<0.05 for *EFNB2,* 1.1 ± 0.2*; P=NS* for *EPHB4,* 0.5 ± 0.1*; P*<0.05 for *NRP2* and1.1 ± 0.2*; P=NS* for *COUP-TFII*; ***Figure 41***). Hence, Prdm16 directly activates Notch signalling *in vitro*. Since no clear upregulation of *PRDM16* was observed upon ectopic Hey2 expression, we hypothesised that Prdm16 lays directly upstream Hey2 during arterial endothelial differentiation.

Prdm16 lies directly upstream of canonical Notch during arterial differentiation

Prdm16 enhances RBPJκ's transcriptional activity

To confirm these findings, we conducted co-transduction experiments with Prdm16 lentiviral particles and a luciferase-based canonical Notch (RBPJκ) reporter virus. Co-transduction with Prdm16 lentivirus increased the luciferase signal compared to co-transduction with a control virus (Cherry). Data were normalised to Renilla luciferase for transduction efficiency ([ratio luciferase/Renilla signal x 10^{-3}]: 3.0 ± 0.5 and 1.2 ± 0.2 for Prdm16 *versus* Cherry, respectively; **P*<0.05; *N*=3 for all; ***Figure 42***).

The arterialising effect of Prdm16 *in vitro* is partially abolished by DAPT

If Prdm16 would act at least in part through canonical Notch signalling, its ability to induce the Notch ligand *DLL4*, the Notch downstream effectors *HEY1/2* and the Notch downstream target *EFNB2* should be attenuated by addition of a γ-secretase inhibitor, such as DAPT. Indeed, qRT-PCR demonstrated that the induction of all four of these genes was severely compromised in the presence of DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester), compared to its control DMSO ([fold upregulation Prdm16 DMSO/Cherry DMSO]: 3.4 ± 0.7 for *DLL4; P*<0.05, 13.5 ± 5.8; *P*<0.05 for *HEY1*, 27.1 ± 5.7; *P*<0.01 for

HEY2 and 2.9 ± 0.4; *P=NS* for *EFNB2*; [fold upregulation Prdm16 DAPT/Cherry DAPT]: 1.4 ± 0.3; *P*=NS for *DLL4*, 2.7 ± 0.8; *P*=NS for *HEY1,* 6.3 ± 2.3; *P*<0.05 for *HEY2*, 1.7 ± 0.5; *P*=NS for *EFNB2*). Data are depicted in ***Figure 43*** as expression levels *versus* Cherry-treated BOECs that were not treated with a compound. Furthermore, we found that the Prdm16-mediated induction of several arterial genes of our conserved arteriovenous signature, including *GJA5* and *DKK2,* was also severely hampered or completely abrogated in the presence of DAPT (***Figure 44***).

Prdm16 knockdown in zebrafish does not lead to aberrant canonical Notch activity

To assess whether Prdm16 also instructs Notch signalling *in vivo*, we purchased rbpja reporter zebrafish, expressing eGFP under the control of an element containing 12 RBPJκ-binding sites (*Tg(Tp1-Mmu.Hbb:eGFP)*). However, we failed to detect differences between ns Mo and *prdm16* Mo-treated *Tg(Tp1-Mmu.Hbb:eGFP)* embryos, suggesting that canonical notch signalling was not abolished by *prdm16* silencing (***Figure 45***). Thus, similar to what we observed in mice and consistent with our WISH results in zebrafish, *prdm16* deficiency does not lead to reduced Notch signalling or diminished expression of key Notch effectors, such as grl, despite the strong induction of Notch pathway genes upon ectopic Prdm16 expression *in vitro*. Most likely, other Prdm family members compensate for the loss of Prdm16.

***Prdm16 and Notch genetically interact* in vivo**

DAPT induces aortic abnormalities in prdm16 Mo, but not ns Mo-treated zebrafish

Collectively, the results mentioned in the previous section indicate that ectopic Prdm16 expression drives arterialisation, at least in part by directly acting through canonical Notch signalling. However, despite the obvious (arterial) vascular defects, *Prdm16* deficiency in mice nor in zebrafish resulted in impaired Notch signalling or dramatic shifts in the arteriovenous identity of ECs. In order to confirm our *in vitro* data, we incubated *Tg(kdr-eGFP)*^{s843} zebrafish treated with suboptimal

doses of ns or *prdm16* Mo with the canonical Notch inhibitor DAPT or its control DMSO and scored these embryos for aortic hypoplasia or other morphological anomalies of the DA at 48 hpf (***Figure 46***). Intriguingly, while DAPT treatment in ns Mo-injected zebrafish did not cause increased levels of aortic defects, compared to DMSO-treated zebrafish (P=0.64; N=99-100), it had a clear deleterious effect on the appearance of the DA in *prdm16* morphants (P=0.01; N=99-109). Consequently, while injection of a suboptimal dose of *prdm16* Mo in zebrafish incubated with DMSO only resulted in marginal arterial defects (ns Mo/DMSO *versus* *prdm16* Mo/DMSO; P<0.05; N=99-99), the phenotype of zebrafish injected with the same dose of morpholino was severely aggravated in the presence of DAPT (ns Mo/DAPT *versus* *prdm16* Mo/DAPT; P<0.0001; N=100-109). Representative images of the zebrafish embryos subjected to the different treatments are shown in ***Figure 46B-E***.

Grl and prdm16 co-operate during arterial development in zebrafish

As grl – the zebrafish orthologue of Hey2 - is the main determinant of the arterial identity in zebrafish, we next performed co-injection experiments in which we simultaneously knocked-down *grl* and *prdm16*. Indeed, co-injecting *grl* and *prdm16* morpholino's significantly worsened the vascular defects seen with suboptimal dose injections of either of these morpholino's alone (P<0.0001 for *grl* Mo versus *grl* Mo + *prdm16* Mo; N=100-100; P<0.0001 for *prdm16* Mo *versus* *grl* Mo + *prdm16* Mo; N=100-100; ***Figure 47A***). Hence, prdm16 and notch/grl signalling genetically interact during the establishment of the arterial vasculature in zebrafish. Representative images of the zebrafish embryos subjected to the different treatments are shown in ***Figure 47B-E***.

Prdm16-mediated arterialisation is partially dependent on its interaction with CtBPs and DNA

The Prdm16-mediated arterial shift requires CtBP and DNA binding

To unravel the precise mechanisms behind the driving forces of Prdm16-mediated arterialization, we conducted parallel overexpression experiments in BOECs with wild-type (WT) Prdm16 and two well-described Prdm16 mutants, one that fails to bind to DNA (Prdm16*ΔDNA*) and another that is incapable of forming a

complex with CtBP proteins (Prdm16*ΔCtBP*). Intriguingly, both mutant Prdm16 isoforms exhibited only a partial inductive effect on *HEY1*, *HEY2* and *EFNB2* expression levels compared to WT Prdm16, underscoring the importance of both regions for Prdm16-mediated Notch signaling. Likewise, Prdm16*ΔCtBP*, but not Prdm16*ΔDNA*, displayed lower inductive activity on Dll4 expression levels compared to WT Prdm16 ([fold upregulation versus Cherry]: 5.1 ± 2.0 *for* WT Prdm16, 1.7 ± 0.4*;* for Prdm16*ΔCtBP*, 4.4 ± 2.0 for Prdm16*ΔDNA*). Moreover, like we observed with our compound experiments (see 5.3.2.2), both *GJA5* ([fold upregulation versus Cherry]: 93 ± 39 for WT Prdm16, 3.6 ± 1.8 for Prdm16*ΔCtBP*, 45 ± 20 for Prdm16*ΔDNA*) and *DKK2* ([fold upregulation versus Cherry]: 268 ± 91 for WT Prdm16, 85 ± 29 for Prdm16*ΔCtBP*, 34 ± 19 for Prdm16*ΔDNA*) elicited only a limited response upon ectopic overexpression of the mutated Prdm16 variants (***Figure 48***), despite the fact that all Prdm16 isoforms were equally overexpressed (data not shown).

Prdm16-mediated Notch signalling requires CtBP and DNA binding

From the above, it was clear that Prdm16 must interact with CtBP and DNA to establish part of its arterialising effect. We next wondered whether these interactions were also involved in Prdm16-mediated canonical Notch signalling. Analogous to the previous section, we assayed the Prdm16 mutants and their WT equivalent for their capacity to induce canonical Notch signalling, using the RBPJκ-luc reporter virus as a read-out. We found that, while Prdm16 was able to strongly activate canonical Notch signalling, the mutants did so to a far lesser extent (***Figure 49***). Thus, Prdm16-mediated canonical Notch signalling is dependent on its interaction with CtBP and DNA.

Prdm16 leads the way to proper arterial SMC coating

Our initial microarray analysis on freshly isolated HUAECs and HUVECs revealed arterial enrichment of two semaphorins, *SEMA3C* and *SEMA3G*. Intriguingly, these genes exhibited highly enhanced expression levels, upon Prdm16 overexpression in HUVECs, especially *SEMA3C* (fold-upregulation *versus* control virus: 155 ± 25.3; $P<0.0001$ and 1.8 ± 0.24; $P<0.05$ for *SEMA3C* and *SEMA3G*, respectively; ***Figure 50A***). Since semaphorins act as guidance

molecules for migrating SMCs, as was already described for Sema3G, Prdm16 might specifically instruct arterial ECs to secrete these molecules to attract multiple layers of SMCs, explaining our *in vivo* phenotype of deficient arterial SMC coating in *Prdm16* knockout embryos (***Figure 50B***). Hence, Prdm16 defines both the molecular and functional identity of arterial endothelial cells.

3.5. SUMMARY AND CONCLUSIONS

Together, our data indicate for the first time a role for Prdm16 during arterial endothelial differentiation. Indeed, via whole-genome transcriptomic profiling, we identified an arterial-exclusive expression pattern of Prdm16 in both human and murine ECs. Moreover, via meta-analysis of our human and murine arrays, we report here a conserved arteriovenous fingerprint, defining the arterial and venous state of ECs independent of species, anatomical location and haemodynamic parameters. Interestingly, this list contained Hey2 and Coup-TFII – two well-described key determinants of arterial and venous specification – and Prdm16. Ectopic Prdm16 induced many of the arterial-specific genes of both our human and murine aretriovenous fingerprint, clearly indicating that Prdm16 orchestrates the arterial program of both human and murine ECs. More importantly, the arterialising capacity of Prdm16 on the conserved arteriovenous signature exceeded that observed on the species-restricted fingerprints. Hence, Prdm16 is a key determinant of the arterial identity of ECs, by regulating a conserved set of genes in addition to the regulation of a species-specific set of genes. One intriguing finding was that the arterialising capacity of Prdm16 was superior to that of the current 'golden standard' Hey2. Using a variety of assays, we were able to place Prdm16 directly upstream of canonical Notch signalling and thus Hey2, both *in vitro* and *in vivo*. Indeed, Prdm16 and Hey2 co-regulate several genes and concommittant inhibition of canonical Notch attenuated in great part the arterialising power of Prdm16 *in vitro*. *Vice versa*, reduced Notch signalling *in vivo* aggravated the vascular defects observed *prdm16* Mo-treated zebrafish. Finally, we identified 2 domains of Prdm16 that are indispensable for its full arterialising capacity. Several, non-mutually exclusive mechanisms might underlie the observed effects (***Figure 50B***). First, Prdm16 might directly activate the expression of the Notch ligand Dll4, thereby triggering the release of NICD, which will ultimately lead to the induction of

Notch target genes HEY1/2. Notably however, the DNA binding deficient mutant of Prdm16 fails to induce the expression levels of HEY1/2 to the same extent as its WT variant, despite comparable DLL4 expression levels between these two variants. Secondly, Prdm16 might bind CtBP proteins, thereby converting the RBPJκ-complex from an inhibitory to an activating state, similar to the function of NICD. Moreover, both Prdm16-CtBP and NICD could be necessary for full activation of the RBPJκ complex. Finally, Prdm16 could merely sequester CtBP, thereby releasing it from the RBPJκ-complex and hence augmenting endogenous Hey1/2 levels. However, if this would be the case, inhibiting *CtBP1/2* via small interfering RNAs would result in elevated levels of Hey1/2 in BOECs, a finding we did not observe. Moreover, adding DAPT to Prdm16-treated BOECs severely attenuated or nearly abolished the induction of Hey1/2, indicating that Prdm16 does not just act as a permissive factor by sequestering CtBPs, but actively drives Hey1/2 expression. Finally, Prdm16 is one of the few Prdm family members with intrinsic methyltransferase activity. Hence, once directed to the promoter of its target genes by transcriptional co-regulators, Prdm16 might induce methylation and thus alter the transcriptional activity of the loci poised with binding sites for the Prdm16 complex. Moreover, Endo *et al.* reported that the binary switches instructed by *hamlet* occur in a methylation-dependent fashion: *hamlet* binds to RBPJκ binding sites and trimethylates H3K27, while preventing trimethylation of H3K4. Hamlet also increases histone H3 levels, all hallmarks of a dense chromatin structure, not compatible with active transcription. Alternatively, as described for multiple Prdm proteins, histone acetyltransferases (*e.g.,* HDACs) might be at play in the Prdm16-mediated arterialisation of ECs. On a more speculative basis, Prdm16 might have non-histone post-transcriptional targets. Nevertheless, further studies are warranted to pinpoint the exact mechanism via which Prdm16 induces canonical Notch activity. Altogether, Prdm16 is a main orchestrator of the arterial molecular and functional fingerprint. Thus, Prdm16 could be used as a novel therapeutic target in vascular therapies. Furthermore, the generation of an endothelial-specific Prdm16 knockout mouse could lead to a better understanding of its role and importance during early arteriovenous specification. Finally, it would be of great interest to assess the role of Prdm16 during the development of atherosclerotic lesions or potentially other arterial defects.

Drawing Description

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will become more fully understood from the detailed description given herein below and the accompanying drawings which are given by way of illustration only, and thus are not limitative of the present invention, and wherein:

FIGURE 1 *provides a chart which presents a schematic summary* of an embodiment of the invention' and of the examples. Example 1 relates to studies on the establishment of microvascular EC gene and reference signatures and on the use of TF (combinations) to induce a heart EC-specific target cell. Example 2 relates to studies on the establishment of macrovascular EC gene and reference signatures and on the use of TF (combination)s to induce an arterial EC-specific target cell. Example 3 relates to studies validating one of the 8 arterial TFs, *i.e.,* Prdm16, discovered in the studies related to Example 2.

FIGURE 2 *displays the lentiviral maps for overexpression of Tcf15, EBF3, PPARγ, and WT1.* **A-E**. Schematic circular plasmid maps of the Cherry control vector (panel A; without insertion of a gene in the multiple cloning site or MCS) or constructs containing the open reading frame of murine *Tcf15* (*mTcf15*; panel B), human *EBF3* (*hEBF3*; panel C), human *PPARγ* (h*PPARγ* panel D) and human *Wilms tumour 1* (*hWT1;* panel E) inserted in the MCS. Expression of the Cherry reporter gene is driven by the phosphoglycerate kinase (PGK) promoter while expression of the inserted transcription factor gene is driven by the cytomegalovirus (CMV) promoter.

FIGURE 3 *displays the microarray results and validation.* **A.** Representative FACS plots of heart homogenates from *Tie2-GFP* mice showing gate (G1) setting for sorting of GFP^+ endothelial cells (ECs) and G1 analysis revealing ~99% purity for EC marker CD31 and ~96% for microvascular EC marker CD36, and negligible contamination (<1%) with hematopoietic cells (expressing CD45). **B.** Microarray heat map and principal component analysis of heart EC, liver EC and brain EC samples. For the heat map dark grey represents a high degree of similarity in gene expression profile between the matching samples on the two axes of the plot; light

grey represents a low degree of similarity. **C.** Venn diagramme representing the genes differentially expressed in the three vascular beds studied. **D.** Representative plot of microarray probe intensities for EC markers and markers of potential contaminant cell types for a single EC preparation from brain, heart and liver. **E.** The diagram shows the proportions of microarray probe intensities for known vascular bed-specific markers for each of the three vascular beds. Data represent mean ± s.e.m.; *P<0.05 *versus* corresponding specific organ and a minimum 4-fold difference and Log_2 probe intensity ≥6.

FIGURE 4. *provides a graphic display on the signature cross-validation versus the non-EC fraction of murine organs.* **A.** Validation of a selected subset of the murine liver EC signature: transcription factors and genes most differentially expressed according to the microarray vs. heart ECs and liver ECs for a total number of 30 genes. qRT-PCR data represent fold enrichment in the EC fraction *versus* the non-EC fraction sorted from mouse liver and expressed as mean ± s.e.m.; N=3-5; *P<0.05. **B.** Validation of a selected subset of the murine liver EC signature: transcription factors and genes most differentially expressed according to the microarray *versus* heart ECs and brain ECs for a total number of 30 genes. qRT-PCR data represent fold enrichment in the EC fraction *versus* the non-EC fraction sorted from mouse brain and expressed as mean ± s.e.m.; N=3-5; *P<0.05; #P<0.1. **C.** Validation of the murine heart EC signature. qRT-PCR data represent fold enrichment in the EC fraction *versus* the non-EC fraction (depicted with white bars) and in the EC fraction *versus* the freshly isolated cardiomyocytes (depicted as black bars). Data are expressed as mean ± s.e.m.; N=3-5; *P<0.05.

FIGURE 5. *provides a graphic display of Log_2 probe-set intensity of validated genes from the heart EC fingerprint in murine heart, liver and brain ECs.* N=5; *P<0.05 *versus* heart ECs.

FIGURE 6 *provides graphics related to the expanded expression analysis of the heart EC signature in additional tissues* revealing that the signature was very similar to that of other metabically active tissues (*i.e.,* brown adipose tissue, white adipose tisse and skeletal muscle; panel A), but very different from other tissues (*i.e.,* lung, pancreas and kidney; panel B). Data are presented relative to

expression in heart ECs (=1). (*N*=3-4). **P*<0.05 *versus* heart ECs and a minimum 4-fold difference.

FIGURE 7 *provides a graphic display of the mRNA expression determined by qRT-PCR of genes of the heart EC fingerprint in human (h) heart, brain or liver ECs relative to human heart ECs*, revealing that part of the signature is also enriched in human heart ECs *versus* brain and liver ECs. ND: not detectable. Data represent mean ± s.e.m. (*n*=3-10; **P*<0.05; $^{\#}$*P*<0.1 *versus* heart ECs)

FIGURE 8 *displays the heart EC signature analysis in cultured ECs from human biopsies.* **A.** Schematic representation of isolation and culture of ECs from human biopsies with subsequent sorting set-up to have a pure $Tie2^{+}$:$Podoplanin^{-}$ (non-lymphatic) population. Right: heart ECs in culture uniformly stained for the EC marker VE-Cadherin, DAPI was used as nuclear counterstain. **B.** qRT-PCR analysis of the heart EC signature in cultured ECs relative to freshly isolated heart ECs. Data represent mean ± s.e.m.; *N*=3-10; **P*<0.05; #*P*<0.1.

FIGURE 9 *provides pictures that demonstrate the validation of the heart EC-specific TFs at protein level.* **A.** Western blots show Meox2 and Tcf15 in sorted cardiac EC and their absence in non-EC from the heart and freshly isolated cardiomyocytes. **B,** Murine heart, brain and liver tissue cross-sections stained for Meox2, and Tcf15 and co-stained for an EC marker (BS-I lectin for brain and heart, CD105 for liver; DAPI was used as nuclear counterstain). Arrowheads indicate nuclei positively stained for the corresponding TF. Scale bars correspond to 20 μm.

FIGURE 10 *provides graphics on the effect of TF overexpression on the heart EC signature in cultured human heart ECs*. All diagrams in (**A**-**D**) represent mRNA expression determined by qRT-PCR for 29 genes of the heart EC fingerprint in cultured human heart ECs overexpressing certain transcription factor (combination)s relative to a 'Cherry' reporter control. (*n*=3-9 independent experiments; in each experiment all the conditions were tested and analyzed in parallel. **P*<0.05 or $^{\#}$*P*<0.1 *versus* Cherry shown under the bars; or *versus* another condition as indicated above the bars). Primers in the 3'UTR region were used to distinguish endogenous expression from that caused by lentiviral overexpression. Data represent means ± s.e.m. Two of the 31 signature genes were not included, *i.e.*, *Klra9* and *Klra10*, as there is no human equivalent. **A,** Overexpression of

MEOX2, *Tcf15* or *MEOX2/Tcf15versus* Cherry. **B,** Overexpression of *MEOX2/Tcf15*, *EBF3* or *MEOX2/Tcf15/EBF3 versus* Cherry. **C,** Overexpression of *MEOX2/Tcf15,PPARG* or *MEOX2/Tcf15/PPARGversus* Cherry (in all experiments where *PPARG* was overexpressed, an agonist, rosiglitazone, was added at 10 μM). **D,** Overexpression of *MEOX2/Tcf15/PPARG*, *WT1* or *MEOX2/Tcf15/PPARG/WT1 versus* Cherry. **E,** Western blots for ZDHHC2, RBP7 and TIMP4 in cultured human heart ECs transduced with Cherry or *MEOX2/Tcf15/PPARG*. α-TUBULIN was used as loading control.

FIGURE 11 *provides photographs on the fatty acid uptake in cultured heart ECs overexpressing TFs*. **A,** Fluorescence micrographs of cultured human heart ECs transduced with Cherry, *WT1* (W), *PPARG* (P), *MEOX2/Tcf15* (MT), *MEOX2/Tcf15/PPARG* (MTP) or *MEOX2/Tcf15/PPARG/WT1* (MTPW) exposed to BODIPY-palmitic acid (PA; green), counterstained with DAPI for nuclei (blue). An excess (10X) of non-labeled PA was added in a competition assay. Corresponding quantification of the number of lipid-laden vesicles per cell is shown as mean ± s.e.m. (n=4-5; $^{\#}P$=0.059, $^{*}P$<0.05, $^{**}P$<0.01 *versus* Cherry shown inside the bars; or *versus* another condition as indicated above the bars). **B,** Fluorescence micrographs of cultured human heart ECs transduced with GFP, W, P, MT,MTP or MTPW exposed to DiI-VLDL (red), counterstained with DAPI for nuclei (blue). An excess (10X) of non-labeled VLDL was added in a competition assay. Corresponding quantification of the number of lipid-laden vesicles per cell is shown as mean ± s.e.m. (n=3; $^{*}P$<0.05, $^{**}P$<0.01 *versus* GFP shown inside the bars; or *versus* another condition as indicated above the bars). Scale bars: 20μm. NS: not significant.

FIGURE 12 *provides a graphic display on results of the programming of BOECs into heart ECs by TF overexpression.* Bar diagram reporting expression levels of heart EC-specific genes following overexpression of MEOX2+TCF15+PPARγ+WT1 with addition of rosiglitazone (10 μM; Ros.) in blood outgrowth endothelial cells (BOECs). Data represent fold change ± s.e.m. relative to BOECs transduced with a negative control (Cherry virus); N=4; $^{*}P$<0.05; #P<0.1.

FIGURE 13 *provides a graphic display on the cardiac EC expression pattern of Meox2/Tcf15 deficient mice*. Bar diagrams reporting mRNA expression determined

by qRT-PCR of genes of the heart EC fingerprint in heart ECs from of adult *Meox2*$^{+/-}$, (white bars),*Tcf15*$^{+/-}$ (grey bars) or *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ (black bars) mice, relative to Wild-type (=1, indicated by dashed line) littermates. Data represent mean ± s.e.m.; *n*=3-8; **P*<0.05 *versus* WT.

FIGURE 14 *provides a graphic display on the analysis of genes outside the heart EC fingerprint in Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ *mice*. **A,** mRNA expression determined by qRT-PCR of general EC markersin heart ECs sorted from *Meox2*$^{+/-}$, *Tcf15*$^{+/-}$, or *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ littermates relative to Wild-type heart ECs (*n*=6-8) revealing no significant differences in expression. **B,** mRNA expression determined by qRT-PCR of liver sinusoidal (LS)EC markers in liver ECs sorted from *Meox2*$^{+/-}$, *Tcf15*$^{+/-}$, or *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ littermates relative to Wild-type heart ECs (*n*=4-8) revealing no significant differences in expression. Data represent mean ± s.e.m.

FIGURE 15 *provides a graphic display of the expression levels of fatty acid and glucose transporter genes in heart, brain and liver ECs.* The graph shows Log_2 probe set intensity in heart, brain and liver ECs. *Fabp4* and *Cd36*, two well-known fatty acid transporter molecules, are significantly higher in heart ECs compared to brain and liver. (*n*=5). **P*<0.05.

FIGURE 16 *provides a graphic display of the expression levels of fatty acid and glucose transporters in Meox2*$^{+/-}$, *Tcf15*$^{+/-}$ *mice.* **A.** qRT-PCR analysis of genes involved in lipid uptake commonly expressed between heart and liver ECs, show heart-specific downregulation in *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ ECs. (*N*=5-8). **P*<0.05. **B.** qRT-PCR analysis of glucose transporters on freshly isolated ECs from *Meox2*$^{+/+}$*:Tcf15*$^{+/+}$and *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ mice show increased expression of Glut1 and the FA transporter Fatp3, a downstream target of VEGFB in *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ mice. (*N*=3) **P*<0.05. **C.** Representative FACS plot showing downregulation of Cd36 protein expression in heart ECs from Meox2$^{+/-}$Tcf15$^{+/-}$ mice (darg grey line) compared to *Meox2*$^{+/+}$*:Tcf15*$^{+/+}$ (light grey line). The experiment was repeated three times.

FIGURE 17 *provides graphic displays showing the lack of effect of Meox2/Tcf15 heterozygous deficiency on FA uptake in cardiomyocytes.* **A,** mRNA expression determined by qRT-PCR of genes encoding FA or glucose transporters in cardiomyocytes isolated from *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ littermates relative to Wild-type

cardiomyocytes (*n*=4) revealing no significant differences in expression. **B,** Representative fluorescence micrographs of cultured cardiomyocytes from Wild-type or *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ littermates exposed to BODIPY-palmitic acid (PA; in green), counterstained with DAPI for nuclei (in blue), revealing no differences in PA uptake between genotypes. An excess (10X) of non-labelled PA was used as competition assay. Quantitative data represent mean ± s.e.m.

FIGURE 18 *provides a graphic display of the in vivo uptake of fatty acids and glucose in Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ *mice.* **A.** Graph showing the uptake of radio-labelled ^{14}C-oleic acid in *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ and *Meox2*$^{+/+}$*:Tcf15*$^{+/+}$ littermates showing a reduction in uptake upon Meox2/Tcf15 heterozygous deficiency. (*N*=7-8) **P*<0.05. **B.** Time-lapse quantification of of [^{18}F]FDG uptake into *Meox2*$^{+/+}$*:Tcf15*$^{+/+}$ and *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$hearts showing a significant increase in glucose uptake upon Meox2/Tcf15 heterozygous deficiency. **C.** Representative µPET image of *Meox2*$^{+/+}$*:Tcf15*$^{+/+}$ and *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ hearts (arrow) is shown. (*N*=11-12) **P*<0.05

FIGURE 19 *provides graphic displays related to the heart phenotype and function of young adult Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ *mice*. All histological data are relative to *Meox2*$^{+/+}$*:Tcf15*$^{+/+}$ or *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ hearts from 8-16 weeks-old male mice. **A.** Representative Oil Red-O staining and relative quantification (*N*=4; **P*<0.05), scale bar 20 µm. **B.** Representative Sirius red staining showing the absence of fibrosis. (*N*=4), scale bar 200 µm. **C.** Representative merged pictures of laminin staining of extracellular matrix (in green) and lectin staining of blood vessels (in red) and relative quantification of the cardiomyocyte cross-sectional area revealing no significant differences (*N*=4), scale bar 20µm. **D.** Echocardiographic phenotype of *Meox2*$^{+/+}$*:Tcf15*$^{+/+}$ or *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ male mice of 12-16 weeks old expressed as % ejection fraction (%EF) and % fractional shortening (%FS) (*N*=9) revealing no functional impairment.

FIGURE 20 *provides graphic displays related to the heart phenotype and funtion of aged adult Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ *mice* .All histological data are relative to *Meox2*$^{+/+}$*:Tcf15*$^{+/+}$ or *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ hearts from 11 months-old male mice. **A.** Oil Red-O staining and relative quantification revealing a significant reduction in fat accumulation in cardiomyocytes upon Meox2/Tcf15 heterozygous deficiency (*N*=3; **P*<0.05), scale bar 20 µm. **B.** Sirius red staining and relative quantification

revealing a significant increase in fibrosis upon Meox2/Tcf15 heterozygous deficiency (*N*=3; **P*<0.05), scale bar 200 µm. **C.** Merged pictures of laminin staining of extracellular matrix (in green) and lectin staining of blood vessels (in red) and relative quantification the cardiomyocyte cross-sectional area revealing no significant differences (*N*=3;), scale bar 20 µm. **D.** Echocardiographic phenotype of *Meox2*$^{+/+}$*:Tcf15*$^{+/+}$ or *Meox2*$^{+/-}$*:Tcf15*$^{+/-}$ male mice (11 months old) expressed as % ejection fraction (%EF) and % fractional shortening (%FS) revealing functional impairment upon Meox2/Tcf15 heterozygous deficiency. (*N*=8-9; **P*<0.05).

FIGURE 21 *displays a schematic summary of the findings in Example 1.* In Wild-type heart endothelial cells (ECs), Meox2 and Tcf15 together regulate the balance between fatty acid (FA) and glucose uptake by orchestrating the expression of multiple signature genes, encoding membranous or intracellular regulators of FA transport, supporting the preferential use of FAs as a source of energy production in cardiomyocytes. Combined *Meox2/Tcf15* heterozygous deficiency results in downregulation of these genes and an increase in *Glut1*, together causing a shift to higher glucose and lower FA delivery to cardiomyocytes. This combined deficiency in the long run causes fibrosis and systolic dysfunction. Lpl: lipoprotein lipase; FAPB: fatty acid binding protein; HSPG: heparan sulphate proteoglycans; Glut1: glucose transporter 1; Alb: albumin; VLDL: very low density lipoprotein; CM: chylomicron.

FIGURE 22 *displays a graphical display of the purity of EC preparations from human umbilical cord.*FACS analysis of freshly MACS column-sorted HUAEC (A) or HUVEC (B) revealing only minimal (< 1% in both cell populations) contamination with CD45^{+} blood or inflammatory cells and high purity (> 97% in both cell populations) for CD31^{+} and CD34^{+} endothelial cells.

FIGURE 23 *displays the lentiviral maps for overexpression of the arterial TFs.* **A-G.** Schematic circular plasmid maps of the Cherry control vector (panel A; without insertion of a gene in the multiple cloning site or MCS) or constructs containing the open reading frame of murine *Aff3* (*mAff3*; panel B), human *MSX1* (*hMSX1*; panel C), human *EMX2* (*hEMX2*; panel D), human *NKX2-3* (*hNKX2-3*; panel E), human *TOX2* (*hTOX2*; panel F), or murine *Prdm16* (*mPrdm16*; panel G) inserted in the MCS of the pRRL2 backbone. Expression of the Cherry reporter gene is driven by

the phosphoglycerate kinase (PGK) promoter while expression of the inserted transcription factor gene is driven by the cytomegalovirus (CMV) promoter. **H.** Schematic circular plasmid map of the construct containing the open reading frame of human *SOX17* (*hSOX17*) in the pLVX backbone. Expression of the transcription factor is driven by the CMV promoter.

FIGURE 24 *provides a graphic display of how the cell culture process assimilates arterial and venous endothelial cells.* Hierarchical clustering analysis of all 38 endothelial cell (EC) samples for all 102 probes (corresponding to 76 annotated genes of the arteriovenous fresh profile) reveals that freshly isolated cells (on the left) cluster according to their venous or arterial origin, while for cultured cell types (on the right) the clustering does not classify the sample groups correctly, suggesting that the differences in expression profile have been largely erased. Arterial cell types are represented by a red colour while venous cell types are represented by a blue colour in the colour bar below. The colour code for expression levels is displayed on top. HUAEC: human umbilical artery EC; HUVEC: human umbilical vein EC; HPVEC: human pulmonary vein EC; HHAEC: human hepatic artery EC; HHVEC: human hepatic vein EC; HIVEC: human iliac vein EC; HCAEC: human coronary artery EC; HAEC: human aortic EC; HPAEC: human pulmonary artery EC; HIAEC: human iliac artery EC; NA: not assigned.

FIGURE 25 *provides photographs and graphics on the genome-wide analysis of freshly isolated HUVECs and HUAECs.* **A.** *Upper*: Pie diagrams showing the number of annotated arterial (red) and venous (blue) genes emerging as differentially expressed between freshly isolated HUAEC and HUVEC from the differential gene statistical analysis (left) or classification analysis (right). *Lower*: Diagram showing the total amount of annotated genes and corresponding percentages (black lettering; arterial-enriched genes in red, venous-enriched genes in blue lettering) uniquely identified by the differential gene analysis (pink), from the classification analysis (yellow) or emerging from both analyses (orange; which included 4 genes, *i.e., HEY2*, *NOTCH4*, *SOX17* and *KDR*, previously associated with arterial specification). Other displayed names correspond to the most differentially expressed genes in each part of the diagram. **B,C.** Bar diagrams showing functional terms (B) and pathways (C) significantly enriched in the 76

differential gene set. Numbers of associated genes per function/pathway are mentioned on top of the bars. Yellow bars represent subcategories of the main functional term 'cardiovascular diseases'. **D.** Bar diagram showing microarray probe set intensities (± s.e.m.; *N*=4) for the 8 arterial (A) and 1 venous (V) transcription factors identified among the arteriovenous fresh profile, revealing their differential expression in freshly isolated HUAEC (red) and HUVEC (blue). *: *P*<0.05 *versus* arterial probe set intensity. **E-H.** Immunofluorescence stainings on paraffin cross-sections of human umbilical cord (umbilical vein is shown in E,G, umbilical artery in F-H) for α-smooth muscle cell actin (αSMA; in green) and RASGRF2 (E-F; in red) or NR3C2 (G-H; in red). DAPI (blue) was used as nuclear counterstain. White arrowheads indicate positive staining in the endothelial lining. Scale bars: 20 μm.

FIGURE 26 *displays how the culturing process rapidly erases differential arteriovenous gene expression.* **A.** Hierarchical clustering analysis for freshly isolated or cultured HUAEC (red) or HUVEC (blue) revealing that for fresh samples replicates of each cell type tightly cluster together and both clusters are nicely separated. For cultured samples, incorrect clustering occurred. **B.** Diagram representing the probe set intensity difference between HUVEC and HUAEC for each gene of the arteriovenous fresh profile (showing arterial genes (A) on the left and venous genes (V) on the right) for cultured (filled diamond) or freshly isolated (open triangles) cells, revealing only minor differences in cultured cells. **C.** Diagram representing the expression (± s.e.m.; *N*=4) determined by qRT-PCR for arterial transcription factors in freshly isolated cells (open triangles) or cultured cells (filled diamonds) relative to their expression in freshly isolated cells. *: *P*<0.05 *versus* freshly isolated cells. **D-E.** Diagrams showing probe set intensity (± s.e.m.; *N*=4) for arterial (A) and venous (V) genes of the arteriovenous fresh profile for cultured (filled diamonds) or freshly isolated (open triangles) HUAEC (D; symbols in red) or HUVEC (E; symbols in blue). **F.** Diagram showing that the average probe set intensity for all arterial genes (dashed lines) or venous genes (full lines) in freshly isolated HUAEC (left; red) or HUVEC (right; blue) 'bleach' to a default average expression level (grey dashed line) upon culturing (middle; pink). *: *P*<0.05 *versus* fresh; NS: not significant *versus* fresh (*N*=4-5).

FIGURE 27 *is a graphic display of the kinetics of the culture-induced assimilation process.* Diagrams on the left represent expression levels for all arterial genes (*N*=64) of the arteriovenous fresh profile in HUAEC. Panel A shows expression levels after 24 hours of culture (filled red diamonds) relative to those in freshly isolated HUAEC (open black triangles). Panel C represents expression levels after 48 hours of culture (open red circles) relative to those after 24 hours of culture (filled black diamonds). Panel E represents expression levels after 6 days of culture (filled red squares) relative to those after 48 hours of culture (open black circles). Diagrams on the right show expression levels for all venous (*N*=12) genes of the arteriovenous fresh profile in HUVEC. Panel B represents expression levels after 24 hours of culture (filled blue diamonds) relative to those in freshly isolated HUVEC (open black triangles). Panel D represents expression levels after 48 hours of culture (open blue circles) relative to those after 24 hours of culture (filled black diamonds). Panel F represents expression levels after 6 days of culture (filled blue squares) relative to those after 48 hours of culture (open black circles). All diagrams together reveal that loss of expression occurs within 24 hours after culturing for the majority of arterial and venous genes in HUAEC and HUVEC, respectively, an effect that further increases rapidly independent of cell passaging. The percentage of genes with expression lower than the reference (corresponding to the expression levels in freshly isolated cells indicated by a dashed line) are mentioned on the right side of each diagram.

FIGURE 28 *provides a display of how reactivation of Notch signalling only partially restores arterial gene expression in HUAEC-C.* **A-B.** Schematic diagrams showing 4 numbered consecutive steps during normal canonical Notch signalling*in vivo* (A) or under several experimental conditions *in vitro* (B), i.e. in the presence of DAPT (left), siRNA against RBPJ (middle) or Delta-like (DLL)4-Fc anchored to the cell culture dish (right). **C.** Diagram representing expression (± s.e.m.; *N*=4) of several Notch pathway members and downstream genes in cultured HUAEC treated with DAPT (black) or DMSO (white) relative to DMSO. *: *P*<0.05 *versus* DMSO. **D.** Diagram representing expression (± s.e.m.; *N*=4) of the same gene panel in cultured HUAEC treated with siRBPJ (black) or non-silencing siRNA (siNS; white) relative to siNS. *: *P*<0.05 *versus* siNS. **E.** Diagram representing expression (± s.e.m.; *N*=4) of the same gene panel in cultured HUAEC treated with bovine serum

albumin (BSA)/DAPT (light grey), BSA/DMSO (white), DLL4-Fc/DMSO (black) or DLL4-Fc/DAPT (dark grey) relative to BSA/DMSO. *: *P*<0.05 *versus* BSA/DMSO or DLL4-Fc/DMSO. **F.** Diagram representing expression of all arterial (left) and venous (right) genes of the arteriovenous fresh profile in cultured HUAEC exposed to DLL4-Fc relative to BSA (red dotted line indicates expression in BSA-treated cells). Genes with no change in expression are in white, those that are upregulated in black and those that are downregulated in grey. *: *P*<0.05 *versus* BSA.

FIGURE 29 *provides a graphic display of how combined overexpression of eight transcription factors robustly induces the arteriovenous fresh profile in HUVEC-C.* **A.** Heat map analysis of the arteriovenous fresh profile upon transduction of cultured HUVEC with cherry control virus, lentivirus expressing one transcription factor, or a combination of all 8 transcription factor-expressing lentiviruses ('ALL'). The farther the condition is removed from the cherry control condition (or the greener the colour in the cherry column for a certain condition), the better the induction of the arteriovenous fresh profile. Note that the 'golden standard' HEY2 certainly is not the best performing individual transcription factor. **B.** Hierarchical clustering analysis of the arteriovenous fresh profile upon transduction of cultured HUVEC with cherry control virus, lentivirus expressing one transcription factor, or a combination of all 8 transcription factor-expressing lentiviruses ('ALL'). Red colour in the bar on the left represents an arterial gene, while the blue colour represents a venous gene. While almost all the genes are expressed at low levels (in green) in the cherry control condition, each of the individual transcription factors, except for Aff3, upregulated a subset of (mostly arterial) genes in a largely complementary but in some cases overlapping fashion. As expected from the complementarity, the 8 transcription factors together induce the majority of the (arterial) genes. **C,D.** Diagrams representing expression for classical arterial (C, left) or venous (C, right) or general endothelial (D) markers in cultured HUVEC transduced with cherry control virus (white) or all 8 transcription factor-expressing lentiviruses ('ALL TF') relative to cherry control. *: *P*<0.05 *versus* cherry control. **E.** *Upper*: Pie diagram representing the proportion of arterial genes with expression levels < 2% (light pink) or ≥ 2% (light red) in cultured HUVEC transduced with all 8 transcription factor-expressing lentiviruses ('ALL TF') relative to those in freshly isolated HUAEC. *Lower*: Pie diagram representing, within the subset of arterial genes with ≥ 2 %

values, the proportion of genes with mid-range (< 25%; dark pink) or high-range (≥ 25%; dark red) expression. **F.** Western blot showing the validation of some genes being upregulated by trancription factor overexpression. α-TUBULIN was used as loading control. 'D' represents proportional values for the density of the protein band, kDA represents expected protein size.

FIGURE 30 *provides a graphic representation of how TFs can induce long-term expression of the arterial gene profile.* Diagram representing the expression of selected genes of the fingerprint upon long-term overexpression of transcription factors Prdm16 and Hey2, respectively (data are presented as mean ± s.e.m.; *N*=3). Although some variation across time is present, transduced cells acquire and maintain TF-dependent gene expression for up to 1 month.

FIGURE 31 *provides a graphic representation of how individual transcription factors interact in a complex network to regulate the arteriovenous fresh profile.* **A,B.** Pie diagrams representing the proportion of arterial (A) or venous (B) genes of the arteriovenous fresh profile regulated by 0 (blue), 1 (purple), 2 (orange), 3 (green) or more than 3 (beige) transcription factors (TF). Corresponding absolute numbers are listed in the table on the right of the panel. **C.** To summarise all information on the effect of transcription factor overexpression on the arteriovenous fresh profile, we composed an interaction network of the 8 transcription factors and the arteriovenous fresh profile. Since Aff3 did not regulate any gene of the signature, nor was it regulated by the other transcription factors, it is not included in the network. Each transcription factor hub (represented by rounded boxes) is drawn in a different colour and interactions originating from each hub are shown by arrows (induction) or vertical lines (inhibition) in the corresponding colour. Genes are assigned to the hubs according to the transcription factor by which they were most strongly regulated. For those genes exclusively regulated by 1 transcription factor the box of that gene is drawn in dark colour, whereas for genes that are regulated by more than one transcription factor the box is drawn in light colour. Transcription factors are in oval boxes, arterial genes are in rectangular boxes with full line and venous genes in rectangular boxes with dashed lines.

FIGURE 32 *displays the effect of the 8 TFs on HUVECs in an* in vivo *Matrigel plug implantation assay system.* **A,B.** Fluorescent micrographs of Matrigel implants

containing HUVECs transduced with Cherry reporter gene or HUVECs transduced with the combination of the 8 arterial TFs and Cherry. (*A*) *Ex vivo* explant and (*B*) cryosections showing more elaborate Cherry$^+$ vascular structures (white arrows) upon overexpression of the 8 arterial TFs. **C.** Cryosections stained with smooth muscle α-actin, and the corresponding quantification (right panel), showing a higher % of smooth muscle-coated Cherry$^+$ vessels in Matrigels with the 8 TF-transduced HUVECs. **D.** Human-specific CD31 (red) and smooth muscle α-actin (green) staining, demonstrating the presence of vessels of human origin in the implant containing the 8 TF-transduced HUVECs. The white arrow indicates an autofluorescent erythrocyte inside a functional human vessel. **E.** Smooth muscle α-actin (green) staining and the corresponding quantification (right panel) showing a larger smooth muscle-covered area in the Matrigels containing HUVECs transduced with the 8 arterial TFs. **F.** Sirius red staining, analysed under polarised light, and its corresponding quantification, show more deposition of organised collagen in Matrigels with the 8 TFs transduced HUVECs (right panel). Dashed lines delineate the borders of the Matrigel. All quantitative data are shown as mean ± s.e.m. (*N*=5 per group); **P*<0.05. Scale bars: 100 μm in *A,B*,10 μm in *C,D*, and 200 μm in *E,F*.

FIGURE 33 *displays the conserved arteriovenous fingerprint between human and murine EC samples.* AEC/VEC log-ratio's of probe set intensities for the conserved arteriovenous signature genes. Black bars represent log-ratio's of our human microarray, white bars indicate log-ratio's of the murine array.

FIGURE 34 *displays microarray results for the conserved arteriovenous signature in Prdm16 overexpressing BOECs, HEY2 overexpressing BOECs or siNR2F2-treated HUVECs.* **A**. Prdm16 overexpression in BOECs resulted in augmented levels of several arterial-specific genes (dark grey), while the expression of the bulk of venous genes (light grey) was suppressed. A Cherry containing vector was used as a control. **B**. Overexpression of HEY2 in BOECs only resulted in a modest modulation of the conserved arteriovenous fingerprint in BOECs. **C**. siNR2F2 treatment of HUVECs resulted in a strong induction of multiple arterial genes (dark grey) compared to a non-silencing siRNA (siNS). However, only half of the venous-specific genes (light grey) were inhibited by siNR2F2 treatment.

Figure 35 *displays the validation of the arterial-exclusive expression pattern of Prdm16 across species at different developmental stages.* **A**. qRT-PCR demonstrating the arterial enrichment for *HEY2* and *PRDM16* in HUAECs *versus* HUVECs. *Vice versa*, *COUP-TFII* was predominantly expressed on HUVECs (*N*=4-4). **B**. qRT-PCR indicating the arterial enrichment for *Hey2* and *Prdm16* in mAECs *versus* mVECs. *Vice versa*, *Coup-TFII* was predominantly expressed on mVECs (*N*=4-4). **C,D.** Immunofluorescence staining for PRDM16 (in grey) on cross-sections of human umbilical cords confirming its presence on HUAECs (white asterisks in *C*), while absent on HUVECs (white arrows in *D*). Smooth muscle cells (SMCs) are stained with α-SMC-actin in white. **E, E', E"**. X-gal staining (dark grey) in E10.5 *Prdm16*$^{+/-}$ 'knock-in' embryos demonstrating its expression on the dorsal aorta (DA; *E'*), while the common cardinal vein (CCV) is devoid of Prdm16 expression (*E"*). **F, F', F"**. X-gal staining in E10.5 *Prdm16*$^{+/-}$ 'knock-in' mice demonstrating its expression in the DA, but not the cardinal vein (*F'*). Likewise, Prdm16 is expressed on the vitelline artery, but not its venous counterpart (*F"*). **G,H**. Prdm16 staining (grey) on E14.5 embryos indicating its presence on intercostal arteries and the carotid artery (white asterisks in *G,H*). Conversely, Prdm16 was not detected on ECs from intercostal veins or the carotid vein (white arrows in *G,H*). Slides were counterstained with α-SMC-actin in white. **I,J**. Prdm16 immunofluorescence staining on an E17.5 embryo visualising its presence on coronary AECs (white asterisks in *J*), but not on coronary VECs (white arrows in *I*). **K,L**. X-gal staining (dark grey) on adult tissues: Prdm16 is expressed on ECs from the aorta (black asterisks in *K*), but not the vena cava (black arrows in *L*). **M**. X-gal staining on *Prdm16*$^{+/+}$ aorta demonstrating the specificity of the staining as X-gal could not be detected on aortic ECs of a *Prdm16*$^{+/+}$ mouse (black asterisks). Scale bars: 50 μM in *E, E', E", F, F', F",G, I* and *J* and 100 μM in *C, D, H, K, L* and *M*. **P*<0.05 *versus* VECs.

FIGURE 36 *displays the Prdm16 expression profile and knockdown phenotype in* Tg(kdr-eGFP)^{s843} *zebrafish.* **A**. Expression levels of *prdm16* in *Tg(kdr-eGFP)*^{s843} zebrafish at different developmental stages for vascular (black bars) and non-vascular (white bars) fractions. Vascular expression of *prdm16* peaks at 24 hpf. Data are expressed as fold-expression versus universal zebrafish RNA (uniZ). **B**.

Prdm16 whole-mount *in situ* hybridisation (WISH) shows *prdm16* expression (dark grey) in the dorsal aorta (DA; arrowheads), but not in the posterior cardinal vein (PCV). **C**. Panel *C* shows a cross-section of an embryo stained for *prdm16* RNA by WISH. Inset in *C* zooms in on the region of the DA and PCV, revealing *prmd16* expression in the DA, but not in the PCV. **D,E**. Confocal images of *Tg(kdr-eGFP)*^{s843} zebrafish at 48 hpf, injected either with non-silencing (*D*) morpholino (ns Mo) or *prdm16* Mo (E). *Prdm16* morphants show a clear vascular phenotype, characterised by impaired formation of the dorsal longitudinal anastomotic vessel (DLAV) and improperly formed or absent intersomitic vessels (ISVs, indicated by arrowheads). **D',E'**. Higher magnification of the images in *D,E*. **F**. Quantification of the ISV developmental defects in ns Mo and *prdm16* Mo-treated embryos, expressed as the percentage of embryos showing either normal (white), abnormal (grey) or abolished (black) ISV formation. Numbers of embryos analysed are mentioned in the bar graphs. Scale bars represent: 80 μm in *B*, 40 μm in *C*, 100 μm in *D,E* and 200 μm in *D',E'*.****P<0.0001 *versus* ns Mo.

FIGURE 37 *displays* Prdm16$^{-/-}$ *mouse embryos with haemorrhages and signs of reduced SMC coverage*. **A**. Schematic drawing of an E14.5 embryo, highlighting the region of interest in these embryos. Inset corresponds to region shown in (*B,C*). **B,C**. Haematoxylin&Eosin (H&E) staining of *Prdm16*$^{+/+}$ (*A*) or *Prdm16*$^{-/-}$ (*B*) embryonic day (E)14.5 embryos showing severe bleedings (dotted area in (*C*) in the *Prdm16*$^{-/-}$ mice. **D,E**. α-smooth muscle cell-actin (αSMA) staining (white) for *Prdm16*$^{+/+}$ (*D*) and *Prdm16*$^{-/-}$ (*E*) E14.5 embryos showing reduced SMC coverage, with abnormal morphology. **F,G**. Areas corresponding to the insets in panels D,E revealing reduced/discontinuous SMC coating and blood cells leaking out (arrows) of the artery in *Prdm16*$^{-/-}$ mice (*G*) but not in WT littermates (*F*). (**H**) Quantification of SMC coating in *Prdm16*$^{+/+}$ (black bar), *Prdm16*$^{+/-}$ (grey bar), and *Prdm16*$^{-/-}$ (white bar) E14.5 embryos (expressed as αSMA^{+} area in μm ± s.e.m. clearly revealing reduced SMC investment in *Prdm16*$^{-/-}$ mice *versus* *Prdm16*$^{+/+}$and *Prdm16*$^{+/-}$ mice. Scale bars correspond to 40 μm.**P<0.01 *versus* *Prdm16*$^{+/-}$, ****P<0.0001 *versus* *Prdm16*$^{+/+}$.

FIGURE 38 *displays* Prdm16$^{+/-}$ *mice with impaired perfusional recovery upon limb ischemia.* **A**. Graph displaying the time-dependent gradual recovery of blood

perfusion in the paw of the operated limb, relative to its perfusion in the control limb for *Prdm16*$^{+/+}$ (full line) and *Prdm16*$^{+/-}$ (dotted line) mice. *Prdm16*$^{+/-}$ mice display hampered recovery as evidenced by their reduced perfusion levels (*N*=12-12; *P*<0.001). **B,C**. Representative laser Doppler images of a *Prdm16*$^{+/+}$ (*B*) and *Prdm16*$^{+/-}$ (*C*) mouse 1 week after surgery, demonstrating reduced blood flow in *Prdm16*$^{+/-}$ mice compared to their *Prdm16*$^{+/+}$ siblings in the paw of the ischemic limb (compare left squares in *B* and *C*), while perfusion in the non-ischemic control limb is similar in both genotypes (compare right squares in *B* and *C*). **D,E**. Pecam1 immunofluorescence staining (in grey) on gastrocnemius muscle of *Prdm16*$^{+/+}$ (*D*) and *Prdm16*$^{+/-}$ (*E*) mice indicating equal capillary density between both genotypes (*N*=12-12). Scale bars represent 100 μm.

FIGURE 39 *displays* Prdm16$^{+/-}$ *mice with macroscopic signs of necrosis upon limb ischemia.* **A**. Graph representing the fraction of mice (%) displaying macroscopic signs of necrosis during the recovery of an ischemic insult. *Prdm16*$^{+/-}$ mice (black bars) clearly exhibit more necrosis compared to their WT littermates (white bars) throughout the experiment (*N*=12-12). **B,C**. Representative images of the ischemic paw of a *Prdm16*$^{+/+}$ (B) or *Prdm16*$^{+/-}$ (*C*) mouse 7 days after ligation, demonstrating necrotic digits (arrows in *C*) and signs of inflammation in *Prdm16*$^{+/-}$, but not *Prdm16*$^{+/+}$ mice (compare brackets in *B* and *C*). **P*<0.05 *versus Prdm16*$^{+/+}$.

FIGURE 40 *displays* Prdm16$^{+/-}$ *mice with increased fibrosis in ischemic hind limbs compared to their* Prdm16$^{+/+}$*littermates.* **A,B**. Sirius red images of the gastrocnemius muscle of *Prdm16*$^{+/+}$ (*A*) and *Prdm16*$^{+/-}$ (*B*) mice 21 days after induction of hind limb ischemia, demonstrating elevated levels of fibrosis (grey) in *Prdm16*$^{+/-}$ mice compared to their *Prdm16*$^{+/+}$ littermates. Also note the increased replacement of muscle by adipose tissue in *Prdm16*$^{+/-}$ mice. **C**. Quantification of the amount of fibrosis between *Prdm16*$^{+/+}$ (white bar) and *Prdm16*$^{+/-}$ (black bar) mice showing a 2-fold increase in the amount of fibrotic tissue in *Prdm16*$^{+/-}$ *versus Prdm16*$^{+/+}$ mice. Scale bars represent 100 μM. **P*<0.05 *versus Prdm16*$^{+/+}$.

FIGURE 41 *shows how ectopic Prdm16 drives the onset of multiple Notch pathway genes.* **A**. qRT-PCR showing the relative expression levels of several Notch-related genes in HUVECs treated with Prdm16 lentivirus (black bars) or a control (Cherry; white bars) virus, demonstrating clear upregulation of *DLL4, HEY1, HEY2* and

EFNB2 upon ectopic Prdm16 expression. Prdm16 had no effect on the expression of the venous markers *EPHB4* and *COUP-TFII*, but was able to suppress *NRP2*. **B**. qRT-PCR showing the relative expression levels of several Notch-related genes in BOECs treated with Prdm16 lentivirus (black bars) or a control (Cherry; white bars) virus, revealing enhanced expression levels of Notch pathway genes *DLL4*, *HEY1*, *HEY2* and *EFNB2* upon excess Prdm16. *Vice versa*, Prdm16 was able to suppress *NRP2*, nut not *EPHB4* or *COUP-TFII*. **P*<0.05 *versus* Cherry; ***P*<0.01 *versus* Cherry.

FIGURE 42 *shows how Prdm16 induces canonical Notch signalling* in vitro**.** BOECs were transduced with a Renilla control virus and an RBPJκ-luciferase (RBPJκ-luc) reporter virus alone (white bar) or in combination with either a control (Cherry; grey bar) or *Prdm16*-encoding lentivirus (black bar). Co-transduction with Prdm16 resulted in a 2.4 fold induction of canonical Notch signalling, as measured by the ratio of luciferase *versus* Renilla signal. **P*<0.05 versus Cherry; *N*=3.

FIGURE 43 *shows how Prdm16 activates the canonical Notch pathway.* BOECs were treated with a control (Cherry, white bars) or a Prdm16-containing (black bars) lentivirus in the presence of the canonical Notch inhibitor DAPT, or its control DMSO. qRT-PCR analysis revealed that Prdm16-mediated induction of the Notch ligand *DLL4* (**A**) and the Notch downstream target *EFNB2* (**D**) was completely abolished in the presence of DAPT, but not DMSO. Likewise, Prdm16 induces *HEY1* (**B**) and *HEY2* (**C**) in the presence of DMSO, while its effect on the latter two Notch effector genes was severely hampered in the presence of DAPT. **P*<0.05 *versus* Cherry; ***P*<0.01 *versus* Cherry; *N*=3.

FIGURE 44 *shows how Prdm16 arterialises BOECs through activation of canonical Notch.*qRT-PCR analysis of BOECs treated with a control (Cherry, white bars) or a *Prdm16*-containing (black bars) virus demonstrating that Prdm16 augments the expression levels of both the arterial-specific genes *GJA5* (**A**) and *DKK2* (**B**) in the presence of DMSO, but not upon incubation with the canonical Notch inhibitor DAPT. **P*<0.01 *versus* Cherry; *N*=3.

FIGURE 45 *shows how Prdm16 deficiency in zebrafish does not result in reduced notch signalling.* **A**. Representative image of ns Mo-treated canonical notch reporter zebrafish (*Tg*(*Tp1-Mmu.Hbb:eGFP*)) embryo at 72 hpf. Note the clear

signal in the dorsal aorta (DA), while the posterior cardinal vein (PCV) is devoid of canonical notch activity. Canonical Notch activity is also high in the neural tube (NT). **B**. Representative image of a *prdm16* Mo-treated *Tg*(*Tp1-Mmu.Hbb:eGFP*) embryo at 72 hpf, indicating no observable difference between notch activity in the DA of *prdm16* Mo *versus* ns Mo (*A*)-treated zebrafish. Scale bars represent 100 μm.

FIGURE 46 *shows how DAPT induces aortic abnormalities in* prdm16 *Mo, but not ns Mo-treated zebrafish.* **A**. Quantification of the aortic defects seen in ns Mo or *prdm16* Mo-treated *Tg(kdr-eGFP)*^{s843} zebrafish simultaneously treated with DMSO or DAPT, demonstrating that, while DAPT had no effect on ns Mo-treated, it clearly resulted in increased aortic defects in *prdm16* Mo-treated embryos. Numbers of embryos analysed per condition are mentioned in the graph bars. **B-E**. Representative images of ns Mo DSMO (*B*), ns Mo DAPT (*C*), *prdm16* Mo DMSO (*D*) and *prdm16* Mo DAPT (*E*) treated embryos, illustrating the aortic hypoplasia readily observed in *prdm16* Mo DAPT treated zebrafish embryos (see also inset in (**E**)). Dimensions of the DA are demarcated by dotted lines. Scale bars represent 200 μm. **P*<0.05; ***P*<0.01; *****P*<0.0001.

FIGURE 47 *displays how co-injections of* grl *Mo with* prdm16 *Mo severely aggravates the vascular defects observed in zebrafish treated with only grl Mo or* prdm16 *Mo.* **A**. Quantification of the aortic defects seen in ns Mo, *grl* Mo, *prdm16* Mo or *grl* + *prdm16* Mo-treated *Tg(kdr-eGFP)*^{s843} zebrafish, demonstrating increased percentage of embryos presenting aortic defects (hypoplasia) in *grl* Mo + *prdm16* Mo-treated zebrafish compared to single Mo-treated zebrafish. Numbers of embryos analysed per condition are mentioned in the graph bars. **B-E**. Representative images of ns Mo (*B*), *grl* Mo (*C*), *prdm16* Mo (*D*) and *grl* Mo + *prdm16* Mo DAPT (*E*) treated embryos, illustrating the aggravated aortic hypoplasia observed in *grl* Mo + *prdm16* Mo-treated zebrafish embryos. Dimensions of the DA are demarcated by dotted lines. Scale bars represent 200 μm. **P*<0.05; ***P*<0.01; *****P*<0.0001.

FIGURE 48 *shows that Prdm16, but not Prdm16ΔCtBP or Prdm16ΔDNA exerts full arterialising capacity in BOECs.* qRT-PCR analysis revealing only partial or completely abolished upregulation of Notch pathway genes (*DLL4, HEY1, HEY2*

and *EFNB2*) in BOECs upon overexpression of Prdm16*ΔCtBP* (dark grey) or Prdm16*ΔDNA* (light grey) mutants, compared to overexpression of WT Prdm16 (black). Expression values are relative to control (Cherry; indicated by dotted line) treated BOECs. Similarly, WT Prdm16, but not Prdm16*ΔCtBP* or Prdm16*ΔDNA* resulted in enhanced levels of the arterial-specific genes *GJA5* and *DKK2*. **P*<0.05 *versus* WT Prdm16; *N*=8-11.

FIGURE 49 *shows that Prdm16, but not Prdm16ΔCtBP or Prdm16ΔDNA induces full canonical Notch activation.* BOECs were transduced with a Renilla control virus and an RBPJκ-luciferase (RBPJκ-luc) reporter virus in combination with a control (Cherry; white), WT Prdm16 (black), Prdm16*ΔCtBP* (dark grey) or Prdm16*ΔDNA* (light grey) mutants. While WT Prdm16 clearly activated canonical Notch activity as measured by luciferase/Renilla signal, this effect was drastically reduced when either of the mutant Prdm16 variants was applied. Results are given as fold-induction *versus* Cherry treated BOECs, the latter indicated by a dotted line. **P*<0.05 *versus* WT Prdm16; *N*=7.

FIGURE 50 *shows how Prdm16 leads the way to proper arterial differentiation and development* .**A.** Diagram representing qRT-PCR results demonstrating that both class 3 semaphorins, SEMA3C (left diagram) and SEMA3G (right diagram) are upregulated by ectopic mPrdm16 expression compared to transduction with a Cherry-control virus. **P*<0.05; ****P*<0.001 *versus* Cherry-control virus; *N*=4. **B. (Left panel)** Since Prdm16 is able to induce *SEMA3G* and more notably *SEMA3C*, Prdm16 might instruct AECs, but not VECs to secrete a gradient of Sema3c/Sema3g to attract multiple layers of SMCs during arterial development, thereby functionally defining arterial identity. (**Right panel**) Alternatively, but not mutually exclusive, Prdm16 interacts with the Notch pathway to induce arterial differentiation via multiple potential mechanisms. Indeed, Prdm16 might directly bind to CtBP to convert the RBPJκ-containing repressor complex to an activating state. Alternatively, Prdm16 exerts epigenetic functions via its PR domain or via its interaction with histone modifying enzymes such as HMTs and HDACs. Finally, Prdm16 might activate both Hey1 and Hey2 in a Dll4-independent or Dll4-dependent manner by directly triggering the release of NICD. **DLL4**: delta-like ligand 4; **SEMA**: semaphorin; **SMC**: smooth muscle cell: **(A/V)EC**: arterial/venous

endothelial cell; **NICD**: Notch intracellular domain; **NECD**: Notch extracellular domain; **HDAC**: histone deacetylase; **CtBP**: C-terminal binding protein; **PRDM16**: PR domain containing protein 16; RBPJκ: recombination signal binding protein for immunoglobulin kappa J region.

TABLE 1 provides an Antibodies list for work described in Example 1

TABLE 2 provides a Primer list for work described in Example 1

TABLE 3 provides heart TF cloning information

TABLE 4 on on General differential signatures of brain, liver and heart murine endothelial cells (ECs)

TABLE 5 provides the transcription factors in the tissue EC-related fingerprints

TABLE 6 provides the Reference signature of (murine) heart ECs

TABLE 7 provides afunctional annotation list for the (validated) signatures

TABLE 8 provides a Primer list for work described in Example 2

TABLE 9 provides a Nanostring probe list

TABLE 10 provides the arterial TF cloning information

TABLE 11 provides characteristics of the arteriovenous human differential reference signature

TABLE 12 provides the probe set intensities in HUAEC/HUVEC of genes contained within the arteriovenous fresh profile. The table represents the average microarray probe set intensities for all 102 differentially expressed probe sets (corresponding to 64 annotated arterial genes and 12 annotated venous genes) in freshly isolated HUAEC (column A; *N*=4) and HUVEC (column B; *N*=4) or cultured HUAEC (column C; *N*=5) or HUVEC (column D; *N*=5). Rows for both arterial and venous markers are sorted according to the degree of differential expression between freshly isolated HUVEC and HUAEC (column E). Calculated differences between freshly isolated HUAEC or HUVEC and their cultured counterparts reveal dramatic loss of expression of arterial markers in cultured HUAEC (column F) and venous markers in cultured HUVEC (column G). As a result, expression differences between

cultured HUAEC and HUVEC for the majority (~73%) of the probe sets in the arteriovenous fresh profile were small (≥ -1 or ≤ 1; column H). NA: not annotated.

TABLE 13 demonstrates the expression of genes from the arteriovenous fresh profile in DLL4-Fc treated HUAEC. The table represents relative expression levels (± s.e.m.; *N*=4) for the 76 genes in the arteriovenous fresh profile upon exposure of cultured HUAEC to DLL4-Fc compared to bovine serum albumin (BSA)-treated HUAEC. Genes are sorted according to the same order as in ***Table 12***. *: *P*<0.05 *versus* BSA.

is TABLE 14 provides expression of genes from the arteriovenous fresh profile in cultured HUVEC overexpressing transcription factors. The table represents relative expression levels (± s.e.m.; *N*=4-6) for the 76 genes in the arteriovenous fresh profile upon lentiviral transduction of HUVEC with a single transcription factor or a combination of all 8 transcription factors, compared to those in HUVEC transduced with a cherry control lentivirus. Genes are sorted according to the same order as in ***Table 12***. *: *P*<0.05 *versus* cherry control. #: $x10^3$; aEC: arterial endothelial cell; vEC: venous endothelial cell; ND: not detectable.

TABLE 15 provides the primer list for work described in Example 3

TABLE 16 provides the gene list of arterial- and venous-specific genes differentially expressed ([logvalue]≥1 and *P*<0.001) between mAECs and mVECs

TABLE 17 provides the average arterial and venous probe intensities and corresponding log2 ratios for all TFs identified in our human and murine microarrays

TABLE 18 provides the list of conserved arterial/venous-specific genes

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TABLE 1: Antibodies list for work described in Example 1

Antigen	Species	Supplier, catalog N°	Label
CD31	Mouse	BD 558738	FITC
CD31	Mouse	BD 551262	APC
CD34	Mouse	BD 55138	PE
CD45	Mouse	BD 559864	APC
CD36	Mouse	Biolegend 102612	APC
CD31	Human	BD 555446	PE
CD31	Human	BD 555445	FITC
CD34	Human	BD 345804	APC
CD45	Human	BD 345808	FITC
PODOPLANIN	Human	Angiobio 11-009PE	PE
TIE2	Human	Reliatech 101-NBi54	biotin
Endoglin	Mouse	R&D AF1320	none
Laminin	Mouse	Sigma L9393	none
Meox2	Mouse	Eurogentec	none
Tcf15	Mouse	Santa Cruz sc-46438	none
Gapdh	Human/Mouse	Cell Signaling 2118	none
α-Tubulin	Human/Mouse	Sigma T6199	none
TIMP4	Human	Abcam ab58425	none
ZDHCC2	Human	Santa Cruz sc-98219	none
RBP7	Human	Sigma HPA034749	none
BSI Lectin	Mouse	Sigma L3759	biotin

TABLE 2: Primerlist for work described in Example 1

Gene	Species	Forward primer (5'3')	Reverse primer (5'3')
GAPDH	H	TGGTATCGTGGAAGGACTCATGAC	ATGCCAGTGAGCTTCCCGTTCAGC
ACTB	H	TGGCACCACACCTTCTACAATG	TAGCAACGTACATGGCTGGG
ABLIM3	H	CTTCATCACAGGCGAAGTCA	TTGGTCCACGAATCTTGATG
ADAMTS9	H	TACACCGCAAACGACTGTGT	TCACGATCGGGAGGTTTATC
ADH1	H	ATGGAGGTGTGGATTTTTCG	GTGCGTCCAGTCAGTAGCAG
AKAP5	H	GACGCCCTACGTTGATCTTT	CCAGGCATATTCATACCTTTCA
AQP7	H	GACTGGGGACACAGGGATAG	GGATGCTTGAACCATGTTTTG
C1QTNF9	H	AATGGTCTGCCTGGAAGAGA	CCTTTGATGCCTTTTGCTTC
EBF3	H	TGCCACCGTCATCATAATTG	TGCAGAACTGCTTGGATTTG
EEPD1	H	GTCACGACCTGACCCTTGTT	GGGTTCTTGGTGCTGATGTT
FAPB9	H	TGATGGGAAAATGATGACCA	CTCTTTGCCAAGCCATTTTT
H19	H	GAGCTCTCAGGAGGGAGGAT	CCAGCCTAAGGTGTTCAGGA
HN1I	H	TCCTTCCAGCAGGCCTAATA	CCAAAAATGTCGCTGGTCTT
KCNA5	H	CAGTTCCCCAACACACTCCT	CAGGGGCTTCTCCTCTTCTT
LPL	H	GTCCGTGGCTACCTGTCATT	TGTCCCACCAGTTTGGTGTA
MAP3K5	H	GCCCCTGCATGAAGAAATAG	TTAATGGACCCCATTTGGAA
MCTP1	H	TAATGCAGAAGTCCCCTTGG	CCACACAGGGTTGAGGTTCT
MEOX2	H	CCAGGAAAGAAAGGACAGCA	TCCACCCTTTACCCTCTTCC
MEOX2 UTR	H	TCTGGGACCACCTTCTTTTG	CCACCACCCTCTGTCACTTT
NAV3	H	GGACCGAGTGGTACTGGAAA	CAGAGAGCCCACATGATGAA
NTS	H	AAGCACATGTTCCCTCTTGG	AAGCCCTGCTGTGACAGATT
PAPSS2	H	GAGGTGGCTAAGCTGTTTGC	AAGCACACGCTCAGGAGTTT
PDPN	H	TGC TCT TCG TTT TGG GAA GC	TCGCTGGTTCCTGGAGTCAC
PMP2	H	GGGGTTAGCCACCAGAAAAC	TCTCTTTGCCATCCCATCTC
PPARG	H	GACCACTCCCACTCCTTTGA	CGACATTCAATTGCCATGAG
PPARG UTR	H	CTCGAGGACACCGGAGAG	GGCTGACTCTCGTTTGAGAAA
PRND	H	ACCAAGGAGGCATTTGTCAC	GCTGCTGCACTCTGTACTGC
RBP7	H	TGCTGAAGCCACAGAAAGTG	AGGTGAGCCTGTCATTGTCC
RTN1	H	GAGTTTCCTGCTGCTGCTCT	AGGGTGATCTCAAGCTCCAA
SLC28A2	H	CCTTGGGGACATGACACTCT	GCTTTACCCCCTCCTCACTC
TCF15	H	GCAGCTGCTTGAAGGTGAG	CGGTCCCTACACAAAGAAGG
TCF15 UTR	H	TCCTGGAGAGCTGTGAGGAT	CCCAGAACATCTGTGCCTCT
TIMP4	H	CAGACCCTGCTGACACTGAA	AGACTTTCCCTCTGCACCAA
WT1	H	CAAATGACATCCCAGCTTGA	GACACCGTGCGTGTGTATTC
WT1 UTR	H	CAGGCTGCTAACCTGGAAAG	CTCCATTTGTGCAAGGAGGT
ZDHHC2	H	TTGCGGCAACAGATTTACAG	TGCTGACTAGCCAACAATGA
ACTA2	H	TGGTGTGTGACAATGGCTCT	CTTTTCCATGTCGTCCCAGT
PROX1	H	CAGTACTGAAGAGCTGTCTATAACCAGAG	TCTGAGCAACTTCCAGGAATCTC
PTPRC	H	GACAACAGTGGAGAAAGGACG	GGGAAGGTGTTGGGCTTTG
TIE2	H	ACACCTGCCTCATGCTCAGC	AGCAGTACAGAGATGGTTGCATTC
PECAM1	H	TCTGCACTGCAGGTATTGACAA	CTGATCGATTCGCAACGGA
Tubb	M	GGGAGGTGATAAGCGATGAA	CCCAGGTTCTAGATCCACCA

Gapdh	M	CCGCATCTTCTTGTGCAGT	GAATTTGCCGTGAGTGGAGT
Actb	M	GACGGCCAGGTCATCACTAT	CTTCTGCATCCTGTCAGCAA
Ablim3	M	CAGTCACTTCTGGCACTGGA	CCCCTCAGTGAACATCTGGT
Actc1	M	CAGCTGCATCTTCCTCCTCT	ATGGTGGTGCCTCCAGATAG
Adamst9	M	ACCCGGATGAGATACGTCAG	GGTCAGTCTCGGGGATGTAA
Adh1	M	ACAAACCCTTCACCATCGAG	CCTTCTCCAACGCTCTCAAC
Airn	M	CGGCAGTTTGGTTTACAGGT	CTGGTGTCCCTGTTGGTTCT
Akap5	M	AGCAGAGCTGGCCAGAGAC	TGTGACTTCTTCAGGACACGA
Akr1c19	M	AGCTCTAGAGGCTGGGTTCC	CAGCTCTGGCCTATGGAAAG
Aqp7	M	CTCGGTGTCAACTTGGGTTT	CCTTGGAACCTGTCACCAAC
C1qtnf9	M	AGGTCACAATGGCCTACCTG	AGCCTGGATCACCTTTGATG
Cox8b	M	TGCGAAGTTCACAGTGGTTC	TGCTGCGGAGCTCTTTTTAT
Dmtp	M	CAATGGAACTACGCCTGCAT	AATATGGGCACCTCTTGCTG
Ebf3	M	CTCGACACATCCCTGGAGTT	GTAACCTTTCGGGGTCACCT
Eepd1	M	TTTGGAGGTATGAGGGATGG	GCCTCTTTGTCAAGCAGCTC
Enpp3	M	CAGCTACGGGAACAATGGAT	CCTCTGTGCGATGAGTCAAA
Fabp3	M	GACGAGGTGACAGCAGATGA	TGCCATGAGTGAGAGTCAGG
Fabp9	M	ATGGACATCCAAGCAGGAAG	CTGTTTGCCAAGCCATTTTT
Gda	M	GTGGTGCTTCAAACCATGTG	TTCTTCAGCCACATCAGTGC
H19	M	AATGGTGCTACCCAGCTCAT	GCAGAGTTGGCCATGAAGAT
Hn1l	M	AGGCAAAGGAAGTGGGATCT	TGGAGGATTTGTCCTCTTGG
Kcna5	M	CGCTACTTCGATCCCTTGAG	CAGCTGGTAAAAGCGGATCT
Klra10	M	GGCCAAGCAATGAACTTCTG	ATGCTGGCAGTTCGCTTTAC
Klra9	M	AGATTCCTCACGGGACACAG	ATTGGCCATTGTCAATCCAT
Lamb1	M	GGAACCGGAGTTCAGCTATG	GGGTTGAGGGTCTCGTGATA
Lpl	M	TTTGGCTCCAGAGTTTGACC	GTCTTGCTGCTGTGGTTGAA
Map3k5	M	GGCCAACAACATCATCCTCT	GCCACCTTCAAAAGCTGAAC
Mb	M	CACCCTGAGACCCTGGATAA	GAGCATCTGCTCCAAAGTCC
Mctp1	M	CAGTAATGCAGATGTCCCTTTG	CAAGAAAGGCTGAGCCCATA
Meox2	M	GGCAGAATTTGCCCATCATA	TGCTCAGAGCTGTGGTCACT
Myh6	M	CCACCCAAGTTCGACAAGAT	AGGCGTTGTCAGAGATGGAG
Myl2	M	GACCCAGATCCAGGAGTTCA	TCAGCCTTCAGTGACCCTTT
Myl3	M	AAGGGCGAGATGAAGATCAC	GCAGGAACGTCTCAAAATCC
Myoz2	M	CCATGCAGAATGGGAGAGTT	TTTGGGTACAAAGCCTCCAG
Nav3	M	AAGCAAGCAGTGACCTGGAT	AGGGGACAGTGATGTTGGAG
Nts	M	TTCACAATGAGTTGCCAGGA	GTGTGGACCTGCTTGTCAGA
Papss2	M	GTCTACCAGGCCCATCATGT	GACATTGTCCCCATCCAGAG
Pmp2	M	GTGGGGTTAGCCAACAGAAA	TCTCCAGTGTCACGATGCTC
Pparg	M	CAGGCCTCATGAAGAACCTT	GGATCCGGCAGTTAAGATCA
Prg4	M	GCCACCTGCAACTGTGATTA	CTGCACAGCACTTGCCATAC
Prnd	M	CTGGGTTTCGTTTGGTTCAT	CCCTGGCAGATACTCCGTTT
Rbp7	M	TCAGCGGTACCTGGAATCTT	AGGCTCGTGCATTTTCTGTT
Rtn1	M	CATCAGCTTCCGCATCTACA	AAAGCCTCCGTAGCTCCTTC
Slc28a2	M	GAGCAGCTGATCTCCTTTGC	ATCTGATCTCCCAGCCATTG
Tcf15	M	GCTCCATCTGCACCTTCTGT	TTGTCCTCCGGTCCTTACAC

Timp4	M	ACACGCCATTTGACTCTTCC	CAGCCACAGTTCTGGTGGTA
Tnnc1	M	CAGCAAAGGGAAGTCTGAGG	CGTGCAAGACCAGCATCTAC
Tnni3	M	GAAGCAGGAGATGGAACGAG	TGACTTTTGCTTCCACGTCA
Tnnt2	M	CTGAGACAGAGGAGGCCAAC	TTCTCGAAGTGAGCCTCGAT
Wt1	M	AGGTTTTCTCGCTCAGACCA	GCTGAAGGGCTTTTCACTTG
Zdhhc2	M	GCTGTACTGCCTTTTCATTGC	CCAAACAGAGAGGACAAGCTG
Acta2	M	CGCTGTCAGGAACCCTGAGA	CGAAGCCGGCCTTACAGA
Ptprc	M	GCCCCGGGATGAGACAGT	TTTGAAAGCCCGAGTGCCT
Cdh5	M	ATTGAGACAGACCCCAAACG	TTCTGGTTTTCTGGCAGCTT
Ng2	M	CCTTCCTACAAGTGACCATTGC	CCTGAATTCACTGCCTCCTG
Pdpn	M	GCCAGTGTTGTTCTGGGTTT	AGAGGTGCCTTGCCAGTAGA
Pecam1	M	GTCATGGCCATGGTCGAGTA	CTCCTCGGCGATCTTGCTGAA
Prox1	M	CGCGTGGGTTTCTTCTCTGC	GGGCTGTGCTGTCATGGTCA
Tek	M	GAAACATCCCTCACCTGCAT	TGGCCTTTTCTCTCTTCCAA
Fabp5	M	CAAAACCGAGAGCACAGTGA	CCCTCATTGCACCTTCTCAT
Slc2a4	M	ATTCTGGTTGCC CAGGTGCT	ATTGGACGCTCTCTCTCCAA
Slc2a1	M	CCGCCTCATGTTGGCTGT	TGTGGTGGATGGGATGGG
Gpihbp1	M	GGGCACAAGAAGATGGTGAT	CTGGAGCAGCTCTGTGTCTG
Slc27a1	M	ACCACTCTGCAGGGAACATC	AGCGGCAGATTTCACCTATG
Slc27a4	M	CTACCCAAAGCTGCCATTGT	GAACTTCTTCCGGATCACCA
CD36	M	TGCCAGTCGGAGACATGCT	GCCACGTCATCTGGGTTTTG
Fabp4	M	TGGAAGCTTGTCTCCAGTGA	TCGACTTTCCATCCCACTTC
Flt1	M	TGGCCAGAGGCATGGAGT	TCGCAAATCTTCACCACATGG
Nrp1	M	ACACCTGAGCTTCGGACGTT	CCACTGTGTGTGGCTCTCTCA

H: human; M: mouse

TABLE3. ***Heart TF cloning information***

Gene Symbol	Forward Primer (5'->3')	Reverse Primer (5'->3')	Cloned mRNA sequence (bases)	Restriction enzymes used	Backbone
control GFP					Genecopoeia (ref. EX-EGFP-Lv114)
hMeox2					Genecopoeia (ref. EX-Z3242-Lv114)
hWT1	CCCTCTAGACGATCCTGGACTTCCTCTTG	TTTGTCGACTGGAGAGTCAGACTTGAAAGCA	171-1817	XbaI-SalI	pRRL2-GPK-Cherry
mTCF15	CCCTCTAGAATGGCGTTCGCGCTGCTGC	TTTGCTAGCTTGTCCTCCGGTCCTTACAC	18-733	XbaI-NheI	pRRL2-GPK-Cherry
hPPRGamma	CCCTCTAGAATGACCATGGTTGACACAGA	TTTCTCGAGGTACAAGTCCTTGTAGATCT	248-1678	XbaI-XhoI	pRRL2-GPK-Cherry
hEBF3	CCCTCTAGAGCAGTTTTCATGTTTGGGAT	TTTGTCGACTCCCTCACATTGGCGGGACT	51-1719	XbaI-SalI	pRRL2-GPK-Cherry

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TABLE4. ***General differential signature of brain, liver and heart murine endothelial cells (ECs)***

A. ENRICHED IN BRAIN 159 PROBES, 117 DESCRIBED GENES, SORT ORDER: HIGHEST DIFFERENCE BRAIN-HEART

		Brain ECs	Heart ECs	Liver ECs	Heart - Brain	Brain - Liver	Heart - Liver
ANNOTATION 1	SYMBOL	Probe intensity (Log2)			Probe intensity (Log2)		
NM_021471	Slco1c1	12.1	5.2	4.7	-6.9	7.4	0.5
NM_030687	Slco1a4	10.9	4.0	3.7	-6.9	7.2	0.3
NM_030556	Slc19a3	11.2	4.5	4.5	-6.6	6.7	0.0
NM_013657	Sema3c	11.1	5.0	4.7	-6.0	6.4	0.3
ENSMUST00000049544	2610301F02Rik	10.8	4.9	4.1	-6.0	6.7	0.7
NM_029662	Mfsd2	10.9	5.1	5.0	-5.8	5.9	0.1
NM_008973	Ptn	11.6	5.9	5.0	-5.7	6.7	1.0
NM_001025576	2610301F02Rik	10.8	5.3	4.5	-5.5	6.3	0.7
NM_011400	Slc2a1	12.7	7.2	6.4	-5.5	6.2	0.8
ENSMUST00000049544	2610301F02Rik	10.9	5.4	4.9	-5.5	6.0	0.5
NM_031194	Slc22a8	10.9	5.6	5.3	-5.4	5.7	0.3
ENSMUST00000049544	2610301F02Rik	10.0	4.7	3.4	-5.3	6.6	1.3
NM_009738	Bche	9.4	4.1	4.5	-5.3	4.9	-0.4
BC116955	1190003M12Rik	10.3	5.1	4.6	-5.3	5.7	0.4
NM_008756	Ocln	10.0	4.9	4.7	-5.1	5.3	0.2
NM_008409	Itm2a	12.0	6.9	4.5	-5.1	7.6	2.5
NM_021436	Tmeff1	9.8	4.8	4.9	-5.0	4.9	-0.1
NM_009402	Pglyrp1	11.7	6.7	6.0	-5.0	5.6	0.7
NM_016672	Ddc	10.2	5.2	5.2	-5.0	5.0	0.0
NM_010703	Lef1	10.9	6.0	5.9	-5.0	5.0	0.1
NM_023805	Slc38a3	10.6	5.7	5.7	-4.9	4.9	0.0
NM_028060	Slc35f2	9.7	4.9	4.9	-4.9	4.9	0.0
NM_133237	Apcdd1	10.6	5.9	5.7	-4.7	5.0	0.2
NM_052994	Spock2	11.1	6.4	6.0	-4.7	5.1	0.4
BC047154	A930038C07Rik	9.0	4.3	4.1	-4.7	4.9	0.2
NM_172471	Itih5	10.3	5.6	5.0	-4.7	5.3	0.6
NM_172479	Slc38a5	10.1	5.4	5.3	-4.6	4.8	0.2
NM_009728	Atp10a	10.1	5.5	5.1	-4.6	5.1	0.5
NM_011638	Tfrc	11.2	6.7	5.3	-4.4	5.9	1.5
NM_009575	Zic3	9.0	4.6	4.7	-4.4	4.4	0.0
NM_027299	Degs2	10.9	6.6	5.5	-4.3	5.4	1.0
NM_009196	Slc16a1	9.8	5.5	5.4	-4.3	4.4	0.1
ENSMUST00000098351	Foxf1a	10.7	6.4	5.9	-4.3	4.8	0.5
NM_011198	Ptgs2	10.5	6.3	5.5	-4.3	5.1	0.8
NM_178797	Far2	9.7	5.5	5.4	-4.2	4.3	0.1
NM_172476	Tmc7	9.2	5.1	5.0	-4.1	4.2	0.2
ENSMUST00000049544	2610301F02Rik	7.4	3.4	3.3	-4.0	4.1	0.1
NM_013825	Ly75	8.9	4.9	4.8	-4.0	4.1	0.2
NM_028943	Sgms2	9.4	5.5	5.1	-3.9	4.3	0.4
NM_008935	Prom1	11.3	7.4	5.5	-3.9	5.9	2.0
NM_175105	Aqp11	9.7	6.0	5.8	-3.7	3.9	0.2
NM_029001	Elovl7	10.7	7.1	7.0	-3.6	3.7	0.2
NM_028802	Prei4	10.6	7.0	7.4	-3.6	3.2	-0.4
NM_007515	Slc7a3	8.6	5.0	4.8	-3.5	3.8	0.2
NM_001038602	Marveld2	8.8	5.2	5.3	-3.5	3.4	-0.1
NM_010225	Foxf2	8.2	4.7	4.5	-3.5	3.7	0.2

		Brain ECs	Heart ECs	Liver ECs	Heart - Brain	Brain - Liver	Heart - Liver
ANNOTATION 1	SYMBOL	Probe intensity (Log2)			Probe intensity (Log2)		
NM_025807	Slc16a9	9.8	6.3	6.6	-3.5	3.1	-0.3
NM_016719	Grb14	9.3	5.8	5.6	-3.4	3.7	0.2
NM_013467	Aldh1a1	9.5	6.1	6.2	-3.4	3.4	-0.1
NM_016678	Reck	10.2	6.8	5.9	-3.4	4.3	0.9
NM_029116	Kbtbd11	8.8	5.4	5.7	-3.4	3.2	-0.2
NM_026385	Pllp	9.1	5.7	5.3	-3.4	3.8	0.5
NM_009255	Serpine2	9.3	6.0	5.2	-3.4	4.2	0.8
NM_146136	Slc16a4	10.8	7.5	6.3	-3.4	4.6	1.2
NM_008256	Hmgcs2	8.6	5.3	5.7	-3.4	2.9	-0.4
NM_001080813	Rab11fip1	8.6	5.2	5.1	-3.3	3.5	0.2
NM_015744	Enpp2	10.7	7.4	5.6	-3.3	5.1	1.8
NM_008982	Ptprj	9.3	6.1	6.2	-3.2	3.2	-0.1
NM_001099634	Myof	9.0	5.9	6.0	-3.1	3.0	-0.2
NM_008982	Ptprj	9.1	6.0	6.0	-3.1	3.0	-0.1
NM_009199	Slc1a1	10.2	7.1	5.1	-3.1	5.0	2.0
NM_028916	Efhc2	7.1	4.0	3.9	-3.1	3.1	0.1
NM_001004173	Sgpp2	8.7	5.7	5.4	-3.0	3.3	0.2
NM_145447	Mfsd7c	8.7	5.6	5.2	-3.0	3.5	0.4
NM_133857	Usp53	10.6	7.6	6.9	-3.0	3.8	0.7
NM_026778	Cthrc1	9.6	6.6	6.2	-3.0	3.4	0.4
NM_172778	Maob	7.9	4.9	4.7	-3.0	3.3	0.3
NM_053073	Lrp8	8.6	5.6	5.4	-3.0	3.2	0.2
NM_173740	Maoa	10.9	8.0	5.6	-3.0	5.3	2.4
NM_018811	Abhd2	11.4	8.5	7.8	-2.9	3.6	0.6
NM_009199	Slc1a1	9.6	6.7	5.7	-2.9	3.9	1.0
NM_008869	Pla2g4a	8.2	5.3	5.4	-2.9	2.8	-0.1
NR_000004	Snord35b	10.5	7.6	5.3	-2.9	5.2	2.3
NM_153574	Fam13a	7.7	4.8	5.1	-2.9	2.6	-0.3
NM_009367	Tgfb2	9.2	6.3	5.6	-2.9	3.6	0.7
NM_021451	Pmaip1	8.9	6.1	6.3	-2.8	2.6	-0.2
NM_028757	Nebl	8.9	6.1	5.0	-2.8	3.9	1.1
NM_007470	Apod	8.7	5.9	5.5	-2.8	3.3	0.5
NM_013869	Tnfrsf19	8.3	5.5	5.4	-2.8	2.9	0.1
NM_007513	Slc7a1	10.3	7.5	6.5	-2.8	3.7	1.0
NM_027153	Pir	8.8	6.1	5.7	-2.7	3.1	0.3
NM_198702	Lphn3	7.7	4.9	4.6	-2.7	3.1	0.3
NM_010513	Igf1r	10.5	7.8	7.5	-2.7	3.0	0.3
NM_001126490	Ism1	8.4	5.6	5.2	-2.7	3.2	0.5
NM_009890	Ch25h	8.2	5.5	5.3	-2.7	2.9	0.2
NM_028748	Paqr5	9.4	6.7	6.4	-2.7	2.9	0.2
NM_010104	Edn1	10.0	7.3	6.1	-2.7	3.9	1.2
NM_001042611	Cp	10.0	7.3	5.8	-2.7	4.2	1.5
NM_029614	Prss23	10.8	8.2	6.9	-2.7	3.9	1.3
NM_153546	Mboat1	7.5	4.9	4.5	-2.6	3.0	0.4
NM_001033336	Abcc4	10.1	7.4	7.5	-2.6	2.5	-0.1
NM_015732	Axin2	9.0	6.4	6.6	-2.6	2.5	-0.1
NM_146102	Afap1l2	8.8	6.2	5.5	-2.6	3.3	0.7
NM_001039556	Rad54b	7.4	4.8	4.7	-2.6	2.7	0.1
NM_001024720	Hmcn1	8.3	5.8	6.1	-2.5	2.2	-0.3
NM_019671	Net1	9.7	7.2	6.3	-2.5	3.4	0.9

		Brain ECs	Heart ECs	Liver ECs	Heart - Brain	Brain - Liver	Heart - Liver
ANNOTATION 1	SYMBOL	Probe intensity (Log2)			Probe intensity (Log2)		
NM_026672	Gstm7	8.2	5.8	5.5	-2.5	2.7	0.3
NM_009768	Bsg	11.4	9.0	9.1	-2.5	2.4	-0.1
NM_023395	Wfdc1	8.3	5.9	5.9	-2.4	2.4	0.0
NM_001024720	Hmcn1	8.1	5.7	5.7	-2.4	2.4	0.0
NM_009759	Bmx	8.8	6.4	5.3	-2.4	3.5	1.1
NM_009846	Cd24a	10.3	7.9	7.3	-2.4	3.0	0.6
NM_172752	Sorbs2	10.0	7.6	5.1	-2.4	4.9	2.5
NM_008846	Pip5k1b	7.8	5.4	4.7	-2.4	3.1	0.7
NM_017405	Lsr	8.6	6.2	5.8	-2.3	2.7	0.4
NM_001024720	Hmcn1	10.1	7.8	7.5	-2.3	2.6	0.2
NM_010357	Gsta4	9.5	7.2	6.1	-2.3	3.4	1.1
NM_001024720	Hmcn1	10.4	8.1	8.1	-2.3	2.3	-0.1
NM_054041	Antxr1	8.7	6.3	5.7	-2.3	3.0	0.7
NM_001024720	Hmcn1	9.9	7.6	7.9	-2.3	2.0	-0.3
NM_172653	Slc39a10	11.9	9.6	8.3	-2.3	3.6	1.3
NM_001024720	Hmcn1	8.5	6.2	6.0	-2.3	2.5	0.3
NM_172872	Kank4	7.4	5.1	4.9	-2.3	2.5	0.2
NM_020559	Alas1	9.2	6.9	7.1	-2.3	2.1	-0.2
NM_001033289	Slc9a2	7.2	5.0	5.0	-2.3	2.3	0.0
---	NA	9.4	7.2	4.7	-2.3	4.8	2.5
NM_175193	Golim4	11.3	9.1	8.8	-2.2	2.5	0.2
NM_001024720	Hmcn1	9.5	7.3	7.0	-2.2	2.6	0.3
NM_177025	Cobll1	10.9	8.6	6.1	-2.2	4.8	2.5
NM_001024720	Hmcn1	10.4	8.1	8.3	-2.2	2.1	-0.1
BC048939	A130022J15Rik	11.3	9.1	7.9	-2.2	3.4	1.2
NM_001024720	Hmcn1	10.0	7.8	7.5	-2.2	2.5	0.3
NM_001024720	Hmcn1	9.8	7.6	7.5	-2.2	2.4	0.2
NM_001024720	Hmcn1	9.5	7.3	7.1	-2.2	2.3	0.1
NM_001024720	Hmcn1	10.4	8.2	8.2	-2.2	2.2	0.0
NM_001024720	Hmcn1	9.2	7.0	6.8	-2.2	2.4	0.2
NM_001024720	Hmcn1	9.6	7.4	7.3	-2.2	2.3	0.1
NM_001024720	Hmcn1	11.2	9.0	9.4	-2.2	1.8	-0.3
NM_008592	Foxc1	7.9	5.7	5.0	-2.2	2.9	0.7
NM_001024720	Hmcn1	8.9	6.7	6.7	-2.2	2.2	0.0
NM_175263	Notum	7.7	5.6	5.6	-2.2	2.2	0.0
NM_001024720	Hmcn1	10.4	8.2	8.4	-2.1	2.0	-0.1
NM_001024720	Hmcn1	11.3	9.1	9.1	-2.1	2.2	0.1
NM_001024720	Hmcn1	11.1	9.0	8.6	-2.1	2.5	0.3
NM_001024720	Hmcn1	8.7	6.6	6.6	-2.1	2.1	0.0
NM_001024720	Hmcn1	10.4	8.3	8.0	-2.1	2.4	0.3
NM_178728	Napepld	7.4	5.3	4.8	-2.1	2.6	0.5
NM_001082974	Neurl2	9.1	7.0	6.5	-2.1	2.6	0.5
NM_011158	Prkar2b	6.9	4.8	4.9	-2.1	2.0	-0.2
NM_010274	Gpd2	10.3	8.2	8.1	-2.1	2.2	0.1
NM_198161	Bhlhb9	7.7	5.6	4.5	-2.1	3.1	1.0
NM_010216	Figf	8.6	6.5	5.7	-2.1	2.9	0.8
NM_023627	Isyna1	8.8	6.7	5.9	-2.1	2.9	0.8
NM_010233	Fn1	11.6	9.5	6.3	-2.1	5.3	3.2
NM_028803	Gbe1	10.3	8.2	8.3	-2.1	1.9	-0.1
NM_001024720	Hmcn1	10.3	8.2	7.7	-2.1	2.6	0.5

		Brain ECs	Heart ECs	Liver ECs	Heart - Brain	Brain - Liver	Heart - Liver
ANNOTATION 1	SYMBOL	Probe intensity (Log2)			Probe intensity (Log2)		
NM_001024720	Hmcn1	9.5	7.4	7.0	-2.1	2.4	0.4
ENSMUST00000095134	Nebl	7.6	5.5	4.8	-2.1	2.8	0.8
NM_001024720	Hmcn1	10.5	8.5	8.5	-2.1	2.0	0.0
NM_011125	Pltp	11.8	9.8	7.6	-2.0	4.2	2.2
NM_001024720	Hmcn1	10.2	8.2	8.3	-2.0	2.0	-0.1
NM_147776	Vwa1	9.0	7.0	5.5	-2.0	3.5	1.5
NM_001024720	Hmcn1	10.2	8.1	8.2	-2.0	2.0	-0.1
NM_029614	Prss23	8.9	6.8	6.0	-2.0	2.9	0.8
NM_001024720	Hmcn1	10.0	7.9	8.0	-2.0	2.0	0.0
NM_001024720	Hmcn1	10.8	8.8	8.4	-2.0	2.3	0.3
NM_001024720	Hmcn1	8.3	6.3	6.3	-2.0	2.0	0.0
NM_001024720	Hmcn1	11.1	9.1	8.8	-2.0	2.2	0.2
NM_001024720	Hmcn1	11.7	9.7	9.8	-2.0	1.9	-0.1

B. ENRICHED IN LIVER 191 PROBES, 168 DESCRIBED GENES, SORT ORDER HIGEST DIFFERENCE LIVER-HEART

		Liver ECs	Heart ECs	Brain ECs	Heart - Liver	Brain - Liver	Heart - Brain
ANNOTATION 1	SYMBOL	Probe intensity (Log2)			Probe intensity (Log2)		
NM_001134675	Mup7	10.5	3.0	2.9	-7.6	-7.6	0.0
NM_029465	Clec4g	12.5	5.1	5.1	-7.4	-7.4	0.0
NM_008647	Mup2	10.3	2.9	2.9	-7.4	-7.4	0.0
NM_007870	Dnase1l3	12.0	4.7	4.8	-7.3	-7.2	-0.1
NM_008647	Mup2	10.1	2.9	2.9	-7.2	-7.2	0.0
NM_010554	Il1a	11.2	4.1	4.2	-7.1	-7.1	-0.1
NM_031195	Msr1	11.2	4.0	3.6	-7.1	-7.6	0.5
NM_008625	Mrc1	12.2	5.1	4.8	-7.1	-7.4	0.3
NM_001045550	Mup2	9.9	2.9	3.0	-6.9	-6.9	0.0
NM_008647	Mup2	9.8	3.0	3.0	-6.8	-6.8	0.0
NM_001045550	Mup2	9.9	3.1	3.1	-6.8	-6.8	0.0
NM_008278	Hpgd	11.9	5.3	4.9	-6.6	-7.0	0.4
NM_001045550	Mup2	9.5	2.9	2.9	-6.6	-6.6	0.0
NM_001045550	Mup2	9.7	3.0	3.1	-6.6	-6.6	-0.1
NM_007553	Bmp2	11.7	5.2	6.1	-6.5	-5.6	-0.9
NM_018797	Plxnc1	11.7	5.2	4.7	-6.4	-7.0	0.5
NM_138673	Stab2	12.2	5.9	5.9	-6.3	-6.3	0.0
NM_031198	Tcfec	10.2	4.2	4.1	-6.0	-6.1	0.1
NM_001077189	Fcgr2b	11.2	5.2	5.0	-5.9	-6.2	0.2
NM_008012	Akr1b8	10.6	4.7	3.8	-5.8	-6.8	0.9
NM_001163011	Mup1	8.5	2.7	2.8	-5.8	-5.8	0.0
NM_007977	F8	11.9	6.3	5.7	-5.7	-6.2	0.6
NM_009654	Alb	9.9	4.3	4.2	-5.6	-5.7	0.1
NM_010959	Oit3	11.0	5.4	5.2	-5.6	-5.8	0.2
NM_182783	Fam167b	11.7	6.2	6.5	-5.5	-5.2	-0.3
NM_008356	Il13ra2	9.0	3.6	3.4	-5.5	-5.6	0.2
NM_020578	Ehd3	11.3	5.9	6.3	-5.4	-5.0	-0.4
AF285583	Tex18	8.4	3.0	3.2	-5.4	-5.2	-0.2
NM_177909	Slc9a9	9.6	4.3	3.9	-5.3	-5.7	0.3
NM_017399	Fabp1	8.8	3.5	3.4	-5.3	-5.4	0.1
NM_019985	Clec1b	10.2	5.3	5.0	-4.9	-5.2	0.3
NM_001136072	Meis2	10.4	5.6	5.1	-4.9	-5.3	0.5

		Liver ECs	Heart ECs	Brain ECs	Heart - Liver	Brain - Liver	Heart - Brain
ANNOTATION 1	SYMBOL	Probe intensity (Log2)			Probe intensity (Log2)		
NM_007934	Enpep	11.2	6.3	5.5	-4.8	-5.7	0.8
NM_007412	Gpr182	11.7	7.1	6.6	-4.6	-5.1	0.5
NM_010016	Cd55	11.1	7.0	5.9	-4.2	-5.2	1.0
NM_010074	Dpp4	11.9	6.3	8.0	-5.6	-3.8	-1.8
NM_177789	Vsig4	8.9	4.1	4.1	-4.8	-4.8	0.0
NM_138304	Calml4	10.7	6.1	7.0	-4.5	-3.7	-0.8
NM_008555	Masp1	9.8	5.3	5.0	-4.5	-4.9	0.4
NM_009924	Cnr2	9.3	5.0	4.7	-4.4	-4.7	0.3
NM_033314	Slco2a1	10.8	6.5	6.5	-4.3	-4.4	0.1
NM_010427	Hgf	8.6	4.3	4.3	-4.3	-4.3	0.0
NM_027711	Iqgap2	9.7	5.5	5.6	-4.3	-4.2	-0.1
NM_009245	Serpina1c	8.1	3.8	4.0	-4.3	-4.1	-0.1
NM_011175	Lgmn	11.5	7.3	8.0	-4.2	-3.6	-0.7
NM_013489	Cd84	8.4	4.3	4.2	-4.2	-4.2	0.0
NM_172508	Dse	9.1	5.1	5.0	-4.0	-4.2	0.1
NM_028749	Npl	10.0	6.0	5.9	-4.0	-4.0	0.0
NM_152803	Hpse	8.5	4.5	4.3	-4.0	-4.2	0.2
NM_173036	Gpr97	10.2	6.3	6.1	-3.9	-4.1	0.2
NM_019413	Robo1	9.5	5.6	5.6	-3.9	-3.9	0.0
BC027185	2210023G05Rik	9.5	5.7	6.0	-3.8	-3.5	-0.3
NM_133720	Cysltr2	7.1	3.3	3.8	-3.8	-3.3	-0.5
---	NA	9.8	6.1	6.0	-3.7	-3.8	0.1
NM_013474	Apoa2	9.9	6.2	6.0	-3.7	-3.9	0.2
NM_001001309	Itga8	9.3	5.7	5.4	-3.6	-3.9	0.3
NM_001159965	Ralgps2	9.4	5.8	6.4	-3.6	-2.9	-0.7
NM_011026	P2rx4	9.5	5.9	6.6	-3.6	-2.9	-0.7
NM_001033767	Gm4951	9.9	6.3	6.2	-3.6	-3.7	0.1
NM_139198	Plac8	7.5	3.9	3.7	-3.6	-3.7	0.2
NM_144830	Tmem106a	10.1	6.6	6.2	-3.5	-4.0	0.4
NM_013737	Pla2g7	10.9	7.4	6.5	-3.5	-4.4	0.9
---	NA	9.9	6.4	6.6	-3.5	-3.3	-0.2
NM_007469	Apoc1	8.5	5.1	5.0	-3.5	-3.5	0.0
NM_001081957	Gm11428	7.9	4.4	4.3	-3.5	-3.6	0.1
NM_145429	Arrb2	10.4	7.0	6.9	-3.4	-3.5	0.1
NM_008772	P2ry1	9.2	5.8	7.0	-3.4	-2.2	-1.2
NM_028351	Rspo3	7.6	4.3	4.4	-3.4	-3.3	-0.1
NM_030750	Sgpp1	10.2	6.9	7.2	-3.3	-3.0	-0.3
NM_028810	Rnd3	11.1	7.8	8.4	-3.3	-2.6	-0.6
ENSMUST00000055842	Armcx4	9.4	6.2	7.3	-3.3	-2.1	-1.2
NM_007547	Sirpa	9.5	6.2	6.3	-3.3	-3.2	0.0
NM_008495	Lgals1	11.9	8.8	7.5	-3.2	-4.4	1.3
BC080730	3200002M19Rik	10.5	7.4	7.1	-3.2	-3.4	0.3
NM_008867	Pla2r1	8.8	5.7	5.1	-3.1	-3.7	0.5
BC006736	3200002M19Rik	10.5	7.4	7.2	-3.1	-3.3	0.2
NM_009696	Apoe	12.4	9.3	10.2	-3.1	-2.1	-0.9
NM_138672	Stab1	10.7	7.6	7.5	-3.0	-3.1	0.1
NM_022024	Gmfg	12.0	9.0	8.2	-3.0	-3.8	0.8
NM_016923	Ly96	9.5	6.4	6.2	-3.0	-3.2	0.2
NM_133721	Itga9	10.7	7.7	6.9	-3.0	-3.8	0.8
NM_011594	Timp2	10.4	7.4	7.7	-3.0	-2.6	-0.4

		Liver Ecs	Heart ECs	Brain Ecs	Heart - Liver	Brain - Liver	Heart - Brain
ANNOTATION 1	SYMBOL	Probe intensity (Log2)			Probe intensity (Log2)		
NM_008096	Gc	7.7	4.7	4.8	-3.0	-2.9	-0.1
NM_009899	Clca1	8.3	5.3	4.7	-3.0	-3.6	0.6
NM_010696	Lcp2	8.8	5.8	6.0	-3.0	-2.8	-0.2
NM_008608	Mmp14	9.5	6.6	6.0	-2.9	-3.5	0.6
NM_001012323	Mup20	5.8	2.9	2.8	-2.9	-3.0	0.0
NM_008029	Flt4	10.0	7.1	7.6	-2.9	-2.4	-0.6
NM_053247	Lyve1	9.9	7.0	6.2	-2.9	-3.7	0.8
NM_019517	Bace2	10.3	7.4	6.5	-2.9	-3.8	0.9
BC116748	9030625A04Rik	7.7	4.8	4.8	-2.9	-3.0	0.1
NM_001039544	Mup3	6.7	3.8	3.9	-2.9	-2.8	-0.1
NM_010774	Mbd4	9.8	7.0	7.1	-2.9	-2.7	-0.1
NM_177373	Ppfia2	7.8	5.0	5.0	-2.8	-2.8	0.0
NM_020282	Nqo2	9.7	6.9	7.0	-2.8	-2.7	-0.1
NM_025408	Acer3	8.7	5.9	5.8	-2.8	-2.9	0.1
NM_011662	Tyrobp	9.9	7.1	7.0	-2.8	-2.9	0.1
NM_021272	Fabp7	9.5	6.7	5.8	-2.8	-3.6	0.8
NM_026439	Ccdc80	9.8	7.0	5.4	-2.8	-4.5	1.7
NM_008599	Cxcl9	9.1	6.3	5.2	-2.8	-4.0	1.2
---	NA	8.9	6.1	6.2	-2.8	-2.8	0.0
NM_133862	Fgg	6.1	3.3	3.3	-2.8	-2.8	0.1
NM_023635	Rab27a	9.9	7.2	7.5	-2.8	-2.4	-0.4
NM_031997	Tmem2	10.8	8.0	7.3	-2.8	-3.5	0.7
NM_011882	Rnasel	7.6	4.8	4.2	-2.8	-3.4	0.6
NM_001101479	Pabpc4l	7.7	5.0	4.7	-2.7	-3.0	0.3
NM_026731	Ppp1r14a	9.7	7.0	6.9	-2.7	-2.9	0.2
NM_007398	Ada	9.6	6.9	6.5	-2.7	-3.1	0.4
NM_010158	Khdrbs3	9.1	6.4	6.5	-2.7	-2.6	-0.1
---	NA	5.3	2.6	2.6	-2.7	-2.7	0.1
NM_139232	Fgd4	7.4	4.7	4.6	-2.7	-2.8	0.1
NM_016667	Sntb1	9.4	6.8	5.9	-2.6	-3.5	0.8
NM_032541	Hamp	7.3	4.7	4.5	-2.6	-2.8	0.2
NM_007454	Ap1b1	9.3	6.7	7.3	-2.6	-2.0	-0.6
NM_010517	Igfbp4	9.7	7.1	7.2	-2.6	-2.5	-0.1
BC150711	AI607873	7.5	4.9	4.2	-2.6	-3.3	0.7
NM_010045	Darc	7.5	5.0	5.4	-2.6	-2.1	-0.5
NM_013584	Lifr	11.8	9.2	9.6	-2.6	-2.2	-0.4
NM_009178	St3gal4	9.8	7.2	6.7	-2.6	-3.1	0.5
NM_023132	Renbp	8.5	5.9	6.0	-2.6	-2.5	-0.1
NM_024495	Car13	7.2	4.6	4.4	-2.6	-2.8	0.2
XR_033405	Gm5970	6.9	4.3	4.1	-2.5	-2.7	0.2
NM_023537	Rab3b	7.1	4.6	4.6	-2.5	-2.5	0.0
NM_010230	Fmn1	8.0	5.5	5.4	-2.5	-2.7	0.1
NM_001014423	Abi3bp	8.4	5.9	5.5	-2.5	-2.9	0.3
NM_016751	Clec4f	8.2	5.7	5.4	-2.5	-2.8	0.3
ENSMUST00000111752	Cux2	8.7	6.2	5.9	-2.5	-2.8	0.3
BC053070	Rnf157	8.3	5.8	5.5	-2.5	-2.8	0.4
NM_177157	Gchfr	9.0	6.5	6.1	-2.5	-2.9	0.4
NM_007853	Degs1	11.1	8.7	8.4	-2.5	-2.7	0.3
NM_018761	Ctnnal1	8.5	6.0	6.3	-2.5	-2.2	-0.3
NM_172961	Abat	8.8	6.3	6.3	-2.5	-2.4	0.0

		Liver Ecs	Heart ECs	Brain Ecs	Heart - Liver	Brain - Liver	Heart - Brain
ANNOTATION 1	SYMBOL	Probe intensity (Log2)			Probe intensity (Log2)		
NM_173388	Slc43a2	7.6	5.2	5.5	-2.5	-2.1	-0.4
NM_011815	Fyb	7.0	4.6	4.3	-2.4	-2.8	0.3
NM_008969	Ptgs1	9.7	7.2	6.6	-2.4	-3.1	0.7
NM_054077	Prelp	9.8	7.3	6.9	-2.4	-2.8	0.4
NM_011243	Rarb	8.9	6.4	5.7	-2.4	-3.1	0.7
ENSMUST00000099728	Gm10808	7.5	5.1	4.5	-2.4	-3.0	0.6
ENSMUST00000090166	Adh6b	7.7	5.3	4.4	-2.4	-3.3	0.9
NM_028275	1700112E06Rik	7.2	4.8	4.6	-2.4	-2.6	0.3
NM_146126	Sord	9.1	6.8	6.7	-2.4	-2.4	0.0
NM_008092	Gata4	9.0	6.7	6.3	-2.4	-2.7	0.4
NM_026792	Agpat5	9.8	7.4	7.2	-2.4	-2.6	0.3
NM_011405	Slc7a7	9.6	7.3	6.9	-2.4	-2.8	0.4
NM_009690	Cd5l	6.6	4.3	4.1	-2.4	-2.5	0.2
NM_146126	Sord	9.2	6.8	6.7	-2.3	-2.4	0.1
NM_008268	Hoxb5	8.8	6.4	5.5	-2.3	-3.2	0.9
NM_007574	C1qc	7.6	5.3	5.5	-2.3	-2.2	-0.2
NM_001025577	Maf	8.3	6.0	6.2	-2.3	-2.2	-0.2
NM_023653	Wnt2	7.8	5.4	5.4	-2.3	-2.4	0.1
NM_145839	Rasgef1b	7.9	5.6	4.9	-2.3	-3.0	0.6
NM_019467	Aif1	8.0	5.7	5.4	-2.3	-2.6	0.3
NM_181988	Rerg	8.0	5.7	5.5	-2.3	-2.5	0.2
NM_020283	B3galt1	7.0	4.7	4.6	-2.3	-2.4	0.1
NM_153528	Gramd1c	7.2	4.9	5.0	-2.3	-2.2	-0.1
NM_010807	Marcksl1	9.9	7.7	6.3	-2.3	-3.6	1.4
NM_010353	Gsg2	7.5	5.3	5.1	-2.3	-2.4	0.2
NM_008327	Ifi202b	7.2	5.0	4.0	-2.2	-3.2	0.9
NM_172523	Slc18a2	7.6	5.4	5.3	-2.2	-2.3	0.1
NM_009692	Apoa1	8.5	6.3	6.2	-2.2	-2.3	0.1
NM_011576	Tfpi	10.7	8.5	8.7	-2.2	-2.1	-0.2
NM_024413	Plekhf1	7.7	5.5	5.4	-2.2	-2.2	0.0
NM_024225	Snx5	11.1	8.9	9.0	-2.2	-2.1	-0.2
NM_017372	Lyz2	7.9	5.7	4.7	-2.2	-3.3	1.1
NM_022880	Slc29a1	9.2	7.0	6.6	-2.2	-2.6	0.4
NM_010378	H2-Aa	8.2	6.0	5.2	-2.2	-3.0	0.8
NM_026820	Ifitm1	8.4	6.3	6.4	-2.2	-2.0	-0.1
AK148800	2810055G20Rik	5.5	3.3	3.3	-2.2	-2.2	0.0
NM_029023	Scpep1	9.2	7.0	7.1	-2.2	-2.1	-0.1
NM_145594	Fgl1	6.8	4.7	4.7	-2.2	-2.1	0.0
NM_009658	Akr1b3	10.1	8.0	7.9	-2.2	-2.2	0.0
NM_008663	Myo7a	8.2	6.1	5.7	-2.2	-2.5	0.4
NM_023158	Cxcl16	8.5	6.3	5.5	-2.2	-3.0	0.8
NM_001142701	Hmha1	7.7	5.5	5.6	-2.1	-2.1	0.0
NM_008511	Lrmp	7.9	5.8	5.4	-2.1	-2.5	0.3
NM_008492	Ldhb	9.9	7.8	7.2	-2.1	-2.7	0.5
NM_011658	Twist1	7.7	5.6	5.5	-2.1	-2.2	0.1
NM_001162532	Fam174b	10.2	8.1	6.9	-2.1	-3.3	1.2
NM_007490	Art2a	7.1	5.0	5.0	-2.1	-2.1	0.0
NM_016673	Cntfr	8.7	6.6	6.4	-2.1	-2.3	0.2
NM_026820	Ifitm1	9.7	7.6	7.6	-2.1	-2.1	0.0
NM_001037717	Slc38a6	8.6	6.6	6.6	-2.1	-2.0	-0.1

		Liver Ecs	Heart ECs	Brain Ecs	Heart - Liver	Brain - Liver	Heart - Brain
ANNOTATION 1	SYMBOL	Probe intensity (Log2)			Probe intensity (Log2)		
NM_001033335	Serpina3f	6.5	4.5	4.5	-2.1	-2.1	0.0
NM_175034	Slc24a5	9.0	6.9	6.7	-2.1	-2.3	0.2
NM_015753	Zeb2	9.1	7.0	6.6	-2.0	-2.4	0.4
NM_007651	Cd53	7.7	5.7	5.5	-2.0	-2.2	0.2
NM_026972	Cd209b	6.6	4.6	4.6	-2.0	-2.0	0.0
NM_008859	Prkcq	8.9	6.8	5.2	-2.0	-3.7	1.6
NM_172546	Cnksr3	9.4	7.4	7.3	-2.0	-2.1	0.1
NM_008152	Gpr65	6.0	4.0	3.9	-2.0	-2.1	0.1
NM_007646	Cd38	11.2	9.2	7.5	-2.0	-3.8	1.8

C. ENRICHED IN HEART 88 PROBES, 48 DESCRIBED GENES, SORT ORDER: HIGEST DIFFERENCE HEART-LIVER

		Liver ECs	Heart ECs	Brain ECs	Heart - Brain	Heart - Liver	Brain - Liver
ANNOTATION 1	SYMBOL	Probe intensity (Log2)			Probe intensity (Log2)		
NM_010861	Myl2	10.5	4.4	4.4	6.0	6.1	0.0
NM_022020	Rbp7	12.0	7.7	6.6	4.2	5.4	1.2
NM_007473	Aqp7	10.5	5.0	5.2	5.5	5.4	-0.1
NM_001130174	Tnnt2	11.4	6.3	6.3	5.1	5.1	0.0
NM_183175	C1qtnf9	10.2	6.3	5.1	3.9	5.1	1.1
ENSMUST00000090647	Gm10021	10.7	6.1	5.7	4.6	5.0	0.4
NM_008509	Lpl	11.0	6.5	6.1	4.6	5.0	0.4
NM_008584	Meox2	9.2	4.1	4.3	5.1	4.9	-0.2
NM_001024712	544988	9.7	5.1	5.0	4.5	4.7	0.1
ENSMUST00000090652	544988	9.7	5.3	5.1	4.5	4.6	0.2
NM_080639	Timp4	9.6	5.2	5.1	4.4	4.6	0.1
EF651836	ENSMUSG00000(	9.6	5.1	5.0	4.5	4.5	0.0
NM_009393	Tnnc1	9.9	5.9	5.4	4.0	4.5	0.6
NM_009608	Actc1	10.3	5.7	5.9	4.6	4.5	-0.2
EF651808	Hn1l	10.0	5.7	5.5	4.3	4.5	0.1
NM_145983	Kcna5	9.8	6.5	5.4	3.3	4.4	1.1
NM_001024712	544988	10.0	5.7	5.6	4.3	4.4	0.1
EF651808	Hn1l	9.7	5.4	5.3	4.3	4.4	0.1
EF651810	B930046C15Rik	9.7	5.4	5.4	4.2	4.3	0.1
BC172015	LOC100038847	9.6	5.4	5.3	4.1	4.3	0.1
EF651820	Gm3264	9.2	4.9	4.9	4.3	4.3	-0.1
EF651820	Gm3264	9.2	4.9	4.9	4.3	4.3	-0.1
NM_001024706	Gm5458	9.2	5.0	5.0	4.2	4.2	0.0
NM_177135	D830030K20Rik	9.8	5.6	5.6	4.1	4.2	0.1
NM_198649	Ablim3	8.6	4.5	4.4	4.1	4.2	0.1
AK049619	ENSMUSG00000(	7.3	3.4	3.1	4.0	4.2	0.2
NM_177135	D830030K20Rik	9.7	5.6	5.6	4.1	4.1	0.0
---	NA	8.8	4.9	4.7	3.9	4.1	0.3
EF651835	ENSMUSG00000(	9.7	5.6	5.5	4.0	4.1	0.1
NR_002853	Airn	10.1	6.9	6.1	3.3	4.0	0.7
NM_001024706	Gm5458	8.2	4.2	4.2	4.1	4.0	-0.1
NM_134005	Enpp3	8.8	5.5	4.8	3.3	4.0	0.6
EF651825	D830030K20Rik	9.5	5.6	5.6	3.9	3.9	0.0
NM_177135	D830030K20Rik	9.5	5.6	5.6	3.9	3.9	0.0
NM_177135	D830030K20Rik	7.4	3.5	3.5	4.0	3.9	0.0
NM_008459	Klra10	7.1	3.3	3.3	3.8	3.8	0.0

		Liver ECs	Heart ECs	Brain ECs	Heart - Brain	Heart - Liver	Brain - Liver
ANNOTATION 1	SYMBOL	Probe intensity (Log2)			Probe intensity (Log2)		
NM_175314	Adamts9	9.7	7.0	6.0	2.7	3.8	1.1
BC151018	A430107O13Rik	10.0	7.0	6.3	3.0	3.7	0.7
NM_001024706	Gm5458	9.6	5.9	5.9	3.7	3.7	0.0
NM_175314	Adamts9	10.5	7.7	6.8	2.8	3.7	0.9
NM_011864	Papss2	8.9	5.5	5.3	3.4	3.6	0.3
NM_010651	Klra9	7.0	3.3	3.4	3.7	3.6	-0.1
NM_011598	Fabp9	7.7	3.8	4.1	3.9	3.6	-0.3
NM_177135	D830030K20Rik	6.6	2.9	3.0	3.6	3.6	-0.1
NM_001081035	Nav3	8.7	5.3	5.1	3.3	3.5	0.2
BC100412	1700001E04Rik	8.8	5.3	5.3	3.5	3.5	-0.1
NM_008482	Lamb1-1	9.5	7.0	6.0	2.5	3.5	1.0
NM_177135	D830030K20Rik	7.0	3.5	3.6	3.6	3.5	-0.1
NM_001127330	Pparg	10.1	5.5	6.7	4.6	3.4	-1.2
NM_008580	Map3k5	9.4	7.1	6.1	2.4	3.4	1.0
ENSMUST00000067972	Gm9956	8.8	6.0	5.5	2.9	3.4	0.5
NM_019759	Dpt	7.8	4.5	4.5	3.3	3.3	0.0
NM_175314	Adamts9	9.8	7.5	6.6	2.4	3.2	0.9
NM_010651	Klra9	7.0	3.6	3.8	3.4	3.2	-0.2
NM_021503	Myoz2	7.2	4.0	4.0	3.2	3.2	0.0
NM_172980	Slc28a2	6.9	3.9	3.8	3.0	3.1	0.1
NM_010859	Myl3	8.8	5.7	5.7	3.1	3.1	0.0
NM_023043	Prnd	8.2	6.0	5.1	2.1	3.0	0.9
NM_175314	Adamts9	10.2	8.0	7.3	2.2	2.9	0.7
---	NA	6.9	3.9	4.0	3.0	2.9	-0.1
NM_024435	Nts	6.9	3.8	4.0	3.0	2.8	-0.2
NM_010174	Fabp3	7.5	5.3	4.6	2.2	2.8	0.6
NM_026189	Eepd1	8.7	6.2	5.9	2.5	2.8	0.3
NM_178395	Zdhhc2	7.2	4.9	4.4	2.3	2.8	0.5
NM_001113415	Ebf3	8.1	5.5	5.3	2.6	2.8	0.2
NM_001101471	Akap5	8.9	6.0	6.1	2.9	2.8	-0.1
BC100412	1700001E04Rik	7.7	4.9	4.9	2.8	2.8	0.0
NM_001024706	Gm5458	7.7	4.9	4.9	2.8	2.8	0.0
ENSMUST00000104964	Gm16503	7.3	5.0	4.5	2.3	2.8	0.5
NM_007751	Cox8b	8.0	5.9	5.3	2.1	2.7	0.6
NM_009328	Tcf15	9.1	6.9	6.4	2.1	2.7	0.6
NM_010266	Gda	8.0	5.7	5.3	2.3	2.7	0.4
ENSMUST00000049189	Adamts9	7.8	5.7	5.2	2.1	2.6	0.5
NM_013593	Mb	8.8	6.2	6.2	2.6	2.6	0.0
NM_153457	Rtn1	8.1	5.8	5.6	2.3	2.5	0.3
NM_010856	Myh6	7.7	5.2	5.2	2.5	2.5	0.0
NM_010174	Fabp3	7.5	5.5	5.0	2.0	2.5	0.5
NM_009406	Tnni3	10.2	7.5	7.7	2.8	2.5	-0.3
NR_001592	H19	7.5	5.4	5.0	2.2	2.5	0.3
NM_144783	Wt1	8.8	6.2	6.3	2.5	2.5	-0.1
NM_001024706	Gm5458	7.2	4.8	4.8	2.4	2.4	0.0
NM_001024712	544988	8.3	6.1	6.0	2.2	2.3	0.1
NM_001013785	Akr1c19	6.9	3.4	4.6	3.5	2.3	-1.3
NM_007409	Adh1	9.8	7.8	7.6	2.0	2.2	0.2
NM_030174	Mctp1	8.3	5.8	6.1	2.5	2.2	-0.3
NM_001030305	Pmp2	6.2	3.8	4.0	2.4	2.2	-0.2

		Liver ECs	Heart ECs	Brain ECs	Heart - Brain	Heart - Liver	Brain - Liver
ANNOTATION 1	SYMBOL	Probe intensity (Log2)			Probe intensity (Log2)		
NM_133187	1110032E23Rik	9.3	7.3	7.2	2.1	2.1	0.0
NM_021400	Prg4	7.0	4.9	4.9	2.1	2.0	0.0

TABLE 5. ***Transcription factors in the tissue-related ECs fingerprints***

A. *TRANSCRIPTION FACTORS IN THE LIVER ECs FINGERPRINT*

		Liver EC	Heart EC	Brain EC	Heart -Liver	Brain -Liver	Heart - Brain
ANNOTATION 1	SYMBOL	Probe intesity (Log2)			Probe intesity (Log2)		
NM_031198	Tcfec	10.2	4.2	4.1	-6.0	-6.1	0.1
NM_001136072	Meis2	10.4	5.6	5.1	-4.9	-5.3	0.5
ENSMUST000001117	Cux2	8.7	6.2	5.9	-2.5	-2.8	0.3
NM_008092	Gata4	9.0	6.7	6.3	-2.4	-2.7	0.4
NM_008268	Hoxb5	8.8	6.4	5.5	-2.3	-3.2	0.9
NM_001025577	Maf	8.3	6.0	6.2	-2.3	-2.2	-0.2
NM_011658	Twist1	7.7	5.6	5.5	-2.1	-2.2	0.1
NM_015753	Zeb2	9.1	7.0	6.6	-2.0	-2.4	0.4

B. *TRANSCRIPTION FACTORS IN THE BRAIN ECs FINGERPRINT*

		Brain EC	Heart EC	Liver EC	Heart - Brain	Brain -Liver	Heart -Liver
ANNOTATION 1	SYMBOL	Probe intesity (Log2)			Probe intesity (Log2)		
NM_010703	Lef1	10.9	6.0	5.9	-5.0	5.0	0.1
NM_009575	Zic3	9.0	4.6	4.7	-4.4	4.4	0.0
ENSMUST00000098351	Foxf1a	10.7	6.4	5.9	-4.3	4.8	0.5
NM_010225	Foxf2	8.2	4.7	4.5	-3.5	3.7	0.2
NM_008592	Foxc1	7.9	5.7	5.0	-2.2	2.9	0.7

C. *TRANSCRIPTION FACTORS IN THE HEART ECs FINGERPRINT*

		Heart ECs	Brain ECs	Liver ECs	Heart - Brain	Heart -Liver	Brain -Liver
ANNOTATION 1	SYMBOL	Probe intesity (Log2)			Probe intesity (Log2)		
NM_008584	Meox2	9.2	4.1	4.3	5.1	4.9	-0.2
NM_001127330	Pparg	10.1	5.5	6.7	4.6	3.4	-1.2
NM_001113415	Ebf3	8.1	5.5	5.3	2.6	2.8	0.2
NM_144783	Wt1	8.8	6.2	6.3	2.5	2.5	-0.1
NM_009328	Tcf15	9.1	6.9	6.4	2.1	2.7	0.6

TABLE6. ***Validated signature of (murine) heart endothelial cells (ECs)***

		Heart ECs	Brain ECs	Liver ECs	Heart - Brain	Heart -Liver	Brain -Liver
ANNOTATION 1	SYMBOL	Probe intesity (Log2)			Probe intesity (Log2)		
NM_007473	Aqp7	10.5	5.0	5.2	5.5	5.4	-0.1
NM_008584	**Meox2**	**9.2**	**4.1**	**4.3**	**5.1**	**4.9**	**-0.2**
NM_001127330	**Pparg**	**10.1**	**5.5**	**6.7**	**4.6**	**3.4**	**-1.2**
NM_008509	Lpl	11.0	6.5	6.1	4.6	5.0	0.4
NM_080639	Timp4	9.6	5.2	5.1	4.4	4.6	0.1
EF651808	Hn1l	10.0	5.7	5.5	4.3	4.5	0.1
NM_022020	Rbp7	12.0	7.7	6.6	4.2	5.4	1.2
NM_198649	Ablim3	8.6	4.5	4.4	4.1	4.2	0.1
NM_183175	C1qtnf9	10.2	6.3	5.1	3.9	5.1	1.1
NM_011598	Fabp9	7.7	3.8	4.1	3.9	3.6	-0.3
NM_008459	Klra10	7.1	3.3	3.3	3.8	3.8	0.0
NM_010651	Klra9	7.0	3.3	3.4	3.7	3.6	-0.1
NM_011864	Papss2	8.9	5.5	5.3	3.4	3.6	0.3
NM_001081035	Nav3	8.7	5.3	5.1	3.3	3.5	0.2
NM_145983	Kcna5	9.8	6.5	5.4	3.3	4.4	1.1
NM_172980	Slc28a2	6.9	3.9	3.8	3.0	3.1	0.1
NM_024435	Nts	6.9	3.8	4.0	3.0	2.8	-0.2
NM_001101471	Akap5	8.9	6.0	6.1	2.9	2.8	-0.1
NM_175314	Adamts9	9.7	7.0	6.0	2.7	3.8	1.1
NM_001113415	**Ebf3**	**8.1**	**5.5**	**5.3**	**2.6**	**2.8**	**0.2**
NM_144783	**Wt1**	**8.8**	**6.2**	**6.3**	**2.5**	**2.5**	**-0.1**
NM_026189	Eepd1	8.7	6.2	5.9	2.5	2.8	0.3
NM_030174	Mctp1	8.3	5.8	6.1	2.5	2.2	-0.3
NM_001030305	Pmp2	6.2	3.8	4.0	2.4	2.2	-0.2
NM_008580	Map3k5	9.4	7.1	6.1	2.4	3.4	1.0
NM_178395	Zdhhc2	7.2	4.9	4.4	2.3	2.8	0.5
NM_153457	Rtn1	8.1	5.8	5.6	2.3	2.5	0.3
NR_001592	H19	7.5	5.4	5.0	2.2	2.5	0.3
NM_009328	**Tcf15**	**9.1**	**6.9**	**6.4**	**2.1**	**2.7**	**0.6**
NM_023043	Prnd	8.2	6.0	5.1	2.1	3.0	0.9
NM_007409	Adh1	9.8	7.8	7.6	2.0	2.2	0.2

TABLE7. ***Functional annotation list for the validated heart EC signature and the brain and liver EC signatures***

A. HEART EC VALIDATED SIGNATURE FUNCTIONAL ANNOTATIONS

Term	Genes	PValue
GO:0008289~lipid binding	LPL, RBP7, FABP9, PPARG, PMP2, MCTP1	0,00
BP00028:Lipid and fatty acid transport	LPL, RBP7, FABP9, PMP2	0,00
mmu03320:PPAR signaling pathway	LPL, PPARG, AQP7	0,00
GO:0005215~transporter activity	RBP7, FABP9, AQP7, KCNA5, PMP2, SLC28A2	0,03
BP00274:Cell communication	RBP7, FABP9, NTS, PPARG, PMP2	0,05

B. BRAIN EC SIGNATURE FUNCTIONAL ANNOTATIONS

Term	Genes	PValue
GO:0044255~cellular lipid metabolic process	PTGS2, SGMS2, PIP5K1B, ALDH1A1, FAR2, PRKAR2B, PLA2G4A, ISYNA1, NAPEPLD, HMGCS2, CH25H, MBOAT1, ELOVL7, DEGS2	0,00
GO:0030246~carbohydrate binding	LY75, LPHN3, A930038C07RIK, BSG, SERPINE2, ENPP2, SPOCK2, PGLYRP1, PTN, CD24A, FN1	0,00
signal peptide	CTHRC1, PTGS2, SLC39A10, ENPP2, SPOCK2, EDN1, PGLYRP1, NOTUM, APCDD1, LSR, CD24A, ISM1, TGFB2, LPHN3, A930038C07RIK, IGF1R, APOD, SERPINE2, BCHE, TNFRSF19, PTN, ITIH5, SEMA3C, VWA1, FIGF, PLTP, FN1, PTPRJ, RECK, LY75, BSG, TMEFF1, PROM1, A130022J15RIK, WFDC1, LRP8, ANTXR1, CP, PRSS23	0,00
GO:0008610~lipid biosynthetic process	ALDH1A1, FAR2, ISYNA1, SGMS2, PTGS2, HMGCS2, CH25H, MBOAT1, ELOVL7, DEGS2	0,00
GO:0022857~transmembrane transporter activity	SLC38A3, SLC39A10, SLC9A2, SLC22A8, ATP10A, SLC19A3, SLCO1A4, SLC16A1, TFRC, SLC7A3, SLC7A1, SLC16A9, SLC2A1, ABCC4, PLLP, SLC1A1	0,00
lipid synthesis	FAR2, PTGS2, HMGCS2, CH25H, ELOVL7, DEGS2	0,00
GO:0001568~blood vessel development	PTPRJ, RECK, FOXF1A, EDN1, SEMA3C, FOXC1, FIGF, TGFB2	0,00
IPR011497:Protease inhibitor, Kazal-type	RECK, SLCO1A4, SPOCK2, SLCO1C1	0,00
BP00016:Amino acid transport	SLC7A3, SLC7A1, SLC1A1, NET1	0,00

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GO:0009308~amine metabolic process	A930038C07RIK, DDC, SPOCK2, MAOA, PGLYRP1, ITIH5, FOXC1, TGFB2	0,01
GO:0006066~alcohol metabolic process	GPD2, DDC, ISYNA1, GBE1, HMGCS2, CH25H, MAOA, TGFB2	0,01
GO:0016021~integral to membrane	OCLN, SGMS2, SGPP2, SLC9A2, ATP10A, APCDD1, LSR, LPHN3, FAR2, SLC16A1, AQP11, CH25H, SLC2A1, SLCO1C1, ELOVL7, SLC1A1, PTPRJ, TMEFF1, BSG, SLC22A8, PLLP, DEGS2, SLC38A5, SLC38A3, ENPP2, SLC39A10, SLC19A3, ABHD2, PAQR5, ITM2A, SLCO1A4, IGF1R, MFSD7C, MARVELD2, SLC35F2, TNFRSF19, MYOF, LY75, TMC7, MAOA, MAOB, GOLIM4, PROM1, SLC7A3, TFRC, SLC7A1, SLC16A9, MBOAT1, ABCC4, LRP8, ANTXR1	0,01
GO:0006082~organic acid metabolic process	ALDH1A1, GPD2, FAR2, PRKAR2B, DDC, PTGS2, CH25H, ELOVL7, DEGS2	0,01
BP00081:Coenzyme and prosthetic group metabolism	GPD2, ALAS1, APOD, HMGCS2, SLC19A3	0,01
GO:0016053~organic acid biosynthetic process	FAR2, PTGS2, CH25H, ELOVL7, DEGS2	0,01
GO:0042180~cellular ketone metabolic process	ALDH1A1, GPD2, FAR2, PRKAR2B, DDC, PTGS2, CH25H, ELOVL7, DEGS2	0,01
GO:0006584~catecholamine metabolic process	DDC, MAOA, TGFB2	0,02
GO:0006885~regulation of pH	AQP11, EDN1, SLC9A2	0,02
GO:0031012~extracellular matrix	CTHRC1, A930038C07RIK, HMCN1, SPOCK2, PTN, VWA1, FN1	0,02
mmu00260:Glycine, serine and threonine metabolism	ALAS1, MAOA, MAOB	0,02
MF00102:Protease inhibitor	RECK, SERPINE2, SPOCK2, ITIH5, WFDC1	0,02
GO:0005975~carbohydrate metabolic process	GPD2, A930038C07RIK, ISYNA1, GBE1, SPOCK2, PGLYRP1, ITIH5, FOXC1	0,02
oxidoreductase	ALDH1A1, GPD2, FAR2, PTGS2, CH25H, MAOA, MAOB, CP, DEGS2	0,03
IPR003961:Fibronectin, type III	PTPRJ, A930038C07RIK, IGF1R, VWA1, FN1	0,03
GO:0055080~cation homeostasis	TFRC, AQP11, EDN1, SLC9A2, CD24A	0,03
BP00110:Other receptor mediated signaling pathway	FOXF1A, FOXF2, SEMA3C, FOXC1, TGFB2	0,03
BP00013:Amino acid metabolism	DDC, SLC7A3, SLC7A1, SLC1A1, NET1	0,04
BP00001:Carbohydrate metabolism	GPD2, IGF1R, FOXF1A, GBE1, FOXF2, SLC2A1, PGLYRP1, FOXC1	0,04

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C. LIVER EC SIGNATURE FUNCTIONAL ANNOTATIONS

Term	Genes	PValue
signal peptide	SCPEP1, ART2A, MASP1, LGMN, PTGS1, DNASE1L3, DSE, C1QC, WNT2, APOA2, LYZ2, BMP2, MUP1, MUP3, FLT4, APOA1, HPSE, APOE, HAMP, ROBO1, RSPO3, OIT3, IL13RA2, GPR97, LY96, LIFR, F8, SLC24A5, MMP14, SIRPA, PRELP, CD84, BACE2, PLA2G7, TFPI, H2-AA, GC, PLXNC1, CXCL9, APOC1, CNTFR, TIMP2, FGG, ALB, SERPINA1C, FGL1, PLA2R1, TYROBP, MRC1, FAM174B, MUP7, MUP2, CCDC80, CD5L, STAB2, HGF, LYVE1, CD55, FCGR2B, STAB1, ITGA8, CXCL16, IGFBP4	0,00
GO:0008034~lipoprotein binding	APOA2, APOA1, MSR1, STAB1, APOE, CXCL16	0,00
GO:0006954~inflammatory response	BMP2, MASP1, LY96, F8, CXCL9, C1QC, CD55, STAB1, DARC, CNR2, PLA2G7, IGFBP4, IL1A	0,00
GO:0030246~carbohydrate binding	MRC1, LGALS1, CCDC80, STAB2, CD209B, ABI3BP, LYVE1, CLEC4F, APOE, STAB1, RSPO3, CLEC4G, PLA2R1, CLEC1B	0,00
IPR016186:C-type lectin-like	MRC1, LYVE1, CLEC4F, STAB1, CLEC4G, STAB2, PLA2R1, CD209B, CLEC1B	0,00
GO:0030247~polysaccharide binding	LYVE1, STAB1, APOE, RSPO3, CCDC80, STAB2, CD209B, ABI3BP	0,00
GO:0016044~membrane organization	PLEKHF1, MRC1, MSR1, AP1B1, FCGR2B, CLEC4F, MYO7A, STAB2, PLA2R1, CD209B, SIRPA	0,00
IPR000566:Lipocalin-related protein and Bos/Can/Equ allergen	MUP7, MUP20, MUP1, MUP3, MUP2, FABP1, FABP7	0,00
mmu04610:Complement and coagulation cascades	FGG, CD55, MASP1, F8, TFPI, C1QC	0,00
GO:0016616~oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	AKR1B8, LDHB, BMP2, SORD, AKR1B3, HPGD	0,01
GO:0006869~lipid transport	APOA2, APOA1, MSR1, APOE, APOC1, SLCO2A1	0,01
GO:0005044~scavenger receptor activity	MSR1, STAB1, CXCL16, CD5L	0,01
GO:0030169~low-density lipoprotein binding	MSR1, STAB1, CXCL16	0,01
GO:0050670~regulation of lymphocyte proliferation	CD38, PRKCQ, FCGR2B, VSIG4, ADA	0,01
GO:0031012~extracellular matrix	WNT2, ALB, HPSE, LGALS1, CCDC80, TIMP2, MMP14, ABI3BP, PRELP	0,01
GO:0045576~mast cell activation	FYB, FCGR2B, LCP2	0,01
GO:0005887~integral to plasma membrane	SLC29A1, ITGA9, P2RX4, GPR97, CLCA1, FCGR2B, STAB1, FLT4, ITGA8, SIRPA, SLCO2A1, CLEC1B	0,01
GO:0002682~regulation of immune system process	CD38, PRKCQ, CD55, MASP1, FCGR2B, H2-AA, VSIG4, C1QC, ADA	0,01

GO:0016192~vesicle-mediated transport	MRC1, MSR1, AP1B1, FCGR2B, CLEC4F, MYO7A, STAB2, PLA2R1, CD209B, SIRPA, RAB27A	0,02
GO:0030301~cholesterol transport	APOA1, MSR1, APOE	0,02
GO:0005764~lysosome	PLEKHF1, HPSE, MYO7A, LGMN, H2-AA, RAB27A	0,03
GO:0042311~vasodilation	WNT2, P2RX4, APOE	0,03
GO:0042157~lipoprotein metabolic process	APOA2, APOA1, APOE, APOC1	0,04
GO:0002274~myeloid leukocyte activation	FYB, FCGR2B, LCP2	0,05

Table 8. ***Primer list for qRT-PCR.***

Gene symbol	Forward primer	Reverse primer	Gene symbol	Forward primer	Reverse primer
A2M	CGCCTCAGTCTCTGGAAAAC	GCCTCCAGGTCAGTGAAGAG	*MFAP5*	ATACCCCTGGGGGCTAAATAG	CGTCGTAAACTGGTGAAGCA
ACE2	ATACTGTGACCCCGCATCTC	ATGCTAGGGTCCAGGGTTCT	*MGST1*	GACCTCACCCAGGTAATGGA	TACAGGAGGCCAATTCCAAG
ADRB1	CGAGACCCTGTGTGTCATTG	AGCACTTGGGGTCGTTGTAG	*MPP7*	AAAAAGCCTGCATTCATTGG	AGGCATAGGAGGCAACACTG
AFF3	TTTCAGTCATCAGCCAGCAG	AAGTGTTCTGGATCCGGTTG	*MPPED2*	GGTTTAATGGATGGGGCTTT	CATGGATTCCACCAAACACA
AFF3-UTR	GCCGCCTGTGTATGTGTGTA	ACGGTTTAGCACTGGAATGG	*MSX1*	ACTCCTCAAGCTGCCAGAAGA	AGCTCTGCCTCTTGTAGTCTCTTT
APM2	TGCTCTTGACGACTCCACAG	TCAGAGGCCTGGTTAGCAGT	*NAV1*	CCCAAAGAACTTCGGATCAA	GGAGGGTGAAGCAGTCTCTG
ARL15	AAGAACTTGGAGGGGCTGAT	CTGCTGGCTTGTCTTGATGA	*NKX2-3*	GAGCCCAAGGAACATGGAGA	CTGGGCTTGCGAGAAGAG
ACTB	TGGCACCACACCTTCTACAATG	TAGCAACGTACATGGCTGGG	*NKX2-3-UTR*	ATTTCGTTTCGCAGCTGTCT	AAAGGAAACCCGGTAACACC
CCDC3	TTGCCTCACGGAGTCAATTT	CGAGGAGCACATGAGCCTA	*NOS1*	CCCTTCAGTGGCTGGTACAT	CGGAGTGATGGTCAACAATG
CDH5	GTTCACGCATCGGTTGTTC	TCTGCATCCACTGCTGTCA	*NOTCH1*	CCACGGGCGACGTCACCC	TCCACTCTGGCGGGCACG
CMBL	GCAGTTGCCCAATACCAGAT	TCTGGGCATGACACTGTTGT	*NOTCH4*	ATGTCTCAATGGCGGCTCC	GGAGAAGGTGCCAGGCCT
CNTN3	CCAGCAGCTAAAGGTTCGAC	GTAAGTGAGACGCCCTCTGG	*NPR3*	GGAGACCGATATGGGGATTT	CACTGCCGATTCTTCTAGGC
COL4A1	CTGGCCAGAAAGGAGAGATG	TCATTGCCTTGCACGTAGAG	*NR2F2*	CGCCTCAAAAAGTGCCTCA	GCATCCTGCCCCTCTGC
COL4A2	CACCTTCCACCCAGATCAGT	CTCTGGCACCTTTTGCTAGG	*NR3C2*	AACAGGTAGACGGCGAGAGA	TTTGGAATAGCACCGGAAAC
CUBN	ACTGTGAAGGGGGTTCTGTG	GACAGGCCCCACAGTAGAAA	*NRCAM*	TCACCATTGTGGACCAAAGA	CTCTGGGAGGACATTGGAAA
CYSLTR1	TGAGAACAAACGCAAAAGGA	CTTGATTGCGGAAGTCATCA	*NRP2*	GCGAGTGGATTGTTTACGCC	CAGTCTTTGCCCAGGAGGTC
DLL4	ATGACCACTTCGGCCACTATG	GCCCGAAAGACAGATAGGCTG	*ODAM*	CAGGCCAAGTTGATCCCTTA	TGAGGTTGTTCCCAGGGTAG
EFNB1	GTTCTCGACCCCAACGTGTT	CAGGCTTCCATTGGATGTTGA	*OLFML3*	AACGCCGACTAGCTGCTTTA	GCTCCAGACGATCCACTCTC
EFNB2	CTGCTGGATCAACCAGGAAT	TCCGGTACTTCAGCAAGAGG	*PAPLN*	GGCAAGAGGGATGTCTGTGT	AGCTCGGCTGAGGTCATTAG
EMX2	ACCTTCTACCCCTGGCTCAT	AGCCTTCTTCCTCCAGCTTC	*PDE4D*	CCAGTCTGCGAACTGTACGA	TGCCTGGTCTGTAGGGTCTC
ENG	TGCCACTGGACACAGGATAA	CCTTCGAGACCTGGCTAGTG	*PECAM1*	TCTGCACTGCAGGTATTGACAA	CTGATCGATTCGCAACGGA
EPHB4	GAGCTGTGTGGCAATCAAGA	GAACTGTCCGTCGTTTAGCC	*PRDM16*	CAAACGCTTCGAATGTGAAA	CGTGTAGGACTTGTGGCAGA
FAM19A5	CGCAGTTCCTCAAAGAAGGT	GACACGGAAGCATGTCACAC	*PRDM16-UTR*	CTGCAAAACACTTGCCTGAA	GACCAGGAGCAGCTATGTCC
FAP	CCAGGAGATCCACCTTTTCA	ACGCAGGGTAAGTGGTATCG	*PSMAL*	TTGGAATCTTCCTGGAGGTG	CTGCTATCTGGTGGTGCTGA
FAT1	GTGGAAAAGGGGACAGTGAA	CCTGATCGGTTGCAAAGACT	*PTPRR*	TTGTCTGCCAGCTTCGTATG	GGGCTTCAGAGCTTCTCCTT
FGL2	TTGGATGGCAAATGTTCAAA	CCATGGTCTCCATGTCACAG	*RASGRF2*	ACCTTGCCATCGAAAGAATG	ACAGAACCCCACCTGTCTTG
FLRT3	ACAGTGTATCCTGCCCAAGG	CTGCGTTCCCCTGTTACAAT	*RBPJ*	GGCAGTGGATGGAAGAAAA	CTTTTATCCGCTTGCTGAGG
FOLH1	TTGGAATCTTCCTGGAGGTG	CTGCTATCTGGTGGTGCTGA	*RUNDC3B*	GTATCTGCAGCATTGAAAATATGG	CAATAGCATTGAGTCCTAGAAGCA
FREM1	ATGGATGTAGTGGGGCAGAC	GTCAGCTGAAGGAGGTCAGG	*RYR3*	AAATTGCTGGCTGCTCTCAT	TTAAGGCTTCTGGGCTTTCA
GALNTL4	CGACAAGAAGCTGGAGGAAG	GGTAGGCGTTGTAGCCGTAG	*SCARA3*	CGCTGCCAGAAGAACCTATC	CAGCTCCTCCTGCAGTTTTC

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GAPHD	TGGTATCGTGGAAGGACTCATGAC	ATGCCAGTGAGCTTCCCGTTCAGC	*SEMA3C*	TCATTCCATGATTGCTCGAA	CCTCGGGTTATCAGTTTCCA
GLIPR2	GCTCTGCAAGAACCTCAACC	ATACCATGGCCGTGAAGTGT	*SEMA3G*	ACGGAGCACAATAGCACCTT	GACCACAGTCTGGGAGAAGC
GRB14	AAATCCCACTGAAGCCCTTT	CCCGTACCAAGAAAACTCCA	*SLC2A1*	CCTGCAGGAGATGAAGGAAG	ACAGCGACACGACAGTGAAG
H19	GAGCTCTCAGGAGGGAGGAT	CCAGCCTAAGGTGTTCAGGA	*SLC2A3*	TGGTTATTGGCCTCTTCTGC	AAGGGCTGCACTTTGTAGGA
HECW2	TCCAGCATTCCCTATGAAGG	GCTTCTGAAACTGCCTGTCC	*SNTB2*	GCAAAGATACAGCCACAGCA	TGTCCACGGCATACAGTCAT
HEY1	GAGAAGGCTGGTACCCAGTG	GCTCAGATAACGCGCAACTT	*SOX17*	CAGCAGAATCCAGACCTGCA	CAGCGCCTTCCACGACTT
HEY2	CCCCTGCGAGGAGACGA	ATCTAATCACAGAGCTAGTACTTTGCCC	*SOX17-UTR*	TGACTCCGGTGTGAATCTCC	GCAACAACAAAAACCCAGGA
HEY2-UTR	TTCAAGGCAGCTCGGTAACT	CAGGCACTTACGAAACACGA	*SOX18*	AGAACCCGGACCTGCACA	CAGCTCCTTCCACGCTTTG
HSF2BP	TCGTTAGACGGTGATGTCCA	CCCAGAAGCTGCAATATGGT	*SOX7*	GGCGGCCCATGAACG	TCCACGTACGGCCTCTTCTG
JAG1	CCAATGACTGCAGCCCTCAT	GCTCCAAAGGCACAAGGTGA	*SYTL2*	TGTTTTTGTGGCCCAGTGTA	AGTTCCACCTCCCCTAGGAA
JAG2	TCATCCCCTTCCAGTTCGC	AGGCTCTTCCAGCGGTCCT	*TFPI2*	GTCGATTCTGCTGCTTTTCC	CACTGGTCGTCCACACTCAC
KDR	TGGCATCGCGAAAGTGTATC	AAAGGGAGGCGAGCATCTC	*TEK*	ACACCTGCCTCATGCTCAGC	AGCAGTACAGAGATGGTTGCATTC
KLHL6	TTTGAGACCGTGATGAGCTG	CTCATTGCCAGAAAGGTGGT	*TMEM200A*	GAGCAGCATTTGCATTCTGA	ACCAGTGTACATGCCGTTCA
LAMA2	AGGTGAATGTGGAAGGCATC	GGGGTAGAATGGTCTGCTCA	*TMEM200C*	CAGTAGCAGTGGCAGCAAAA	CCGAAGACCTTGAGCTTGTC
LMCD1	CCAAGAGGACCACTGCCTAA	GTAAAAGGCACCCTCTGTGC	*TOX2*	TCAGGAAGAGGAGTCGGAAG	CACGATTTTGGACACGTCAC
LOC401022	TCAGGTCCCTGGATAAGGTG	GTGAGAGCTTTCCCTGCAAC	*TOX2-UTR*	ATCTCTGAGTTCCCCAGCAG	TTTTCTGCTGTCAGCCCTTC
LRCH1	GAGCATACAAGCATGCCAGA	TGGCACATTGAGGCTAACAG	*VWF*	TGCTGGTATGGAGTATAGGCAGTG	CCGGAATGCACGCAGG
MAP9	ACATGGAGGAGAAGGATGGA	CAGATGCGTTTCCCTCAGAT	*XG*	AGCTGGGAGACCAGAAGTCA	GGCATCTGCCAAATCAAAGT

Table 9. ***Nanostring probelist.***

Probe set ID	Gene	Targeted region	Target sequence
Arterial genes from the arteriovenous fresh profile			
NM_003480.2	*MFAP5*	55-155	CTCATCTCATTGTTTCAGCGGAGGCCAAATCTGAAGTCCTTTCCAGGGAGTGGCTCTGTTCATCTTATTCGCCAGCCAAAGTAGGAACAGCGTAAGAGGA
NM_015381.3	*FAM19A5*	320-420	GCCGGCACCACGAGAGCCCGGCCCGCCTGTGTGGACGCAAGAATCATCAAGACCAAGCAGTGGTGTGACATGCTTCCGTGTCTGGAGGGGGAAGGCTGCG
NM_130846.1	*PTPRR*	670-770	GTCCAACGTATCTCTTACATTGGACATGAGTAGCTTGGGGAACATTGAACCCTTTGTGTCTATACCAACACCACGGGAGAAGGTAGCAATGGAGTATCTG
NR_002196.1	*H19*	1592-1692	GGCCTTTGAATCCGGACACAAAACCCTCTAGCTTGGAAATGAATATGCTGCACTTTACAACCACTGCACTACCTGACTCAGGAATCGGCTCTGGAAGGTG
NM_006682.2	*FGL2*	250-350	CAATTCAGCAGGATCGAGGAGGTGTTCAAAGAAGTCCAAAACCTCAAGGAAATCGTAAATAGTCTAAAGAAATCTTGCCAAGACTGCAAGCTGCAGGCTG
NM_000908.2	*NPR3*	35-135	TTGCAGAGAAGGACGCTTCCTCTCTATCTTTTGGCGCATTAGTGAAGGGGGTATTCTATTTTGTTAAAGCGCCCAAGGGGGCGCAGGGACCTTGGAGAGA
NM_020872.1	*CNTN3*	3030-3130	CCAGTAGTGAACAGATCAGGATTCCACGAATAACCAGTATGGATGCAAGAGGATCCACTTCAGCCATCTCGAATGTCCACCCTATGTCAAGTTATATGCC
NM_006909.1	*RASGRF2*	2675-2775	GAGTCCACCAGGCTTTAACAACACCGAGAGAACATGTGATAAAGAGTTTATTATACGGAGAACGGCTACCAATCGAGTTCTGAACGTCCTCCGTCACTGG
NM_020163.1	*SEMA3G*	2591-2691	CCCAAGGGATCTGCCATTTGTTCTCAGAGATGGCCTGGCTTCCGCAACACATTTCCGGGTGTGCCCAGAGGCAAGAGGGTTGGGTGGTTCTTTCCCAGCC
NM_199454.2	*PRDM16*	4455-4555	AACCCGCACTGTGCCTGGGTGTATTCTTTATACTGTAGATAATGGAGAAATTTTCTATCTCTGTCCCTATTTGTATAAGCCAAGGTGATGCTGGGTGCCC
NM_001584.2	*MPPED2*	550-650	TGAAACCAGAGGACTTTGACAATGTTCAGTCCCTCCTGACAAACAGTATTTACTTACAAGATTCGGAGGTAACAGTGAAGGGATTCAGGATATACGGTGC
NM_000014.4	*A2M*	1685-1785	TCCATCTCAATCCCTGTGAAGTCAGACATTGCTCCTGTCGCTCGGTTGCTCATCTATGCTGTTTTACCTACCGGGGACGTGATTGGGGATTCTGCAAAAT
NM_014059.2	*RGC-32*	765-865	TCCGAGGACCTGCTAAAATCAGCTACTAGAATCTGCTGCCAGAGGGGACAAAGACGTGCACTCAACCTTCTACCAGGCCACTCTCAGGCTCACCTTAAAA
NM_000426.3	*LAMA2*	3855-3955	GGCAAACTCAAGTATGCAATCTATTTCGAGGCTCGGGAAGAAACAGGTTTCTCTACATATAATCCTCAAGTGATCATTCGAGGTGGGACACCTACTCATG
NM_052913.2	*TMEM200A*	3355-3455	CCTTGTGGTTCCTTTGCCCAACACCAGTGAATCCTTCCAGCCCGTCAGCACAGTGCTACCAAGGAATAATTCCATTGGGGAGTCGTTGTCGAGTCAGTAC
NM_000684.1	*ADRB1*	795-895	TGTGCATCATGGCCTTCGTGTACCTGCGGGTGTTCCGCGAGGCCCAGAAGCAGGTGAAGAAGATCGACAGCTGCGAGCGCCGTTTCCTCGGCGGCCCAGC
NM_006528.2	*TFPI2*	600-700	TTTAATCCAAGATACAGAACCTGTGATGCTTTCACCTATACTGGCTGTGGAGGGAATGACAATAACTTTGTTAGCAGGGAGGATTGCAAACGTGCATGTG
NM_022454.3	*SOX17*	1374-1474	AGCCACGGGGCCATTTCCTCGGTGGTGTCCGACGCCAGCTCCGCGGTATATTACTGCAACTATCCTGACGTGTGACAGGTCCCTGATCCGCCCCAGCCTG
NM_006931.2	*SLC2A3*	35-135	GGCTGGGGGCTTGTCGCCCTTTCAGGCTCCACCCTTTGCGGAGATTATAAATAGTCATGATCCCAGCGAGACCCAGAGATGCTGTAATGGTAAGACTTTG
NM_032379.3	*SYTL2*	1760-1860	CATCATGTGTTTCACACCACAATTCTGTGCCACAGTTAAGAGGGTCTGGTACCCTTGCAGGACCTTTGTAGGTTGTGGGAAAAAGTCGCAGAAAGATACT
NM_130446.2	*KLHL6*	3365-3465	CCGGAACTGTCATTGCCAGACAGAATTGTACCTTTAGCACATTCTGGTGCCCTGGCCTCTTAGTGTTCACCAGTTGTACTGCAATATAGACAAGATGTCT
NM_145285.2	*NKX2-3*	1646-1746	CTCCGCGAAGCGTTGGCGGGGAGCCCAAGGACATAACAAATTAAAAGCATGAAGGAGAGAAAAATGGGGGTCGTGGCTTGAGAAATTCCAGGCCCTACCG
NM_020760.1	*HECW2*	3895-3995	GAAAAGACCTGCAGAGAAATAAGCTATATGTCACCTTCGTTGGGGAGGAAGGGCTGGATTACAGTGGGCCTTCTAGAGAGTTTTTCTTCCTGGTATCCAG
NR_023391.1	*CYP4F30P*	1740-1840	GAGGTCAGACCTTGGACAGTAGTCTTGACTTCCTGCTATAGAACACATTGTTAACACTGAAAAAGATGATCTGTTCTAGGGGAATGGTGAAAGCTGACTC
NM_144966.5	*FREM1*	1942-2042	GCCATTCTGAGAGGAGACATGATGAGGTAGAATTGGAGGTATACGACTTCTTCTTTGAAAGGAGTGCACCTATGACAGTCCACATCTCCATCAGAACAGC
NM_001846.2	*COL4A2*	1150-1250	GGCATTTCCTTGAAGGGAGAAGAAGGAATCATGGGCTTTCCTGGACTGAGGGGTTACCCTGGCTTGAGTGGTGAAAAAGGATCACCAGGACAGAAGGGAA
NM_012259.2	*HEY2*	1575-1675	GAGAGAGAGTGAGAGAGTCGTGTTTCGTAAGTGCCTGAGCTTAGGAAGTTTTCTTCTGGATATATAACATTGCACAAGGGAAGACGAGTGTGGAGGATAG
NM_005010.4	*NRCAM*	875-975	CATTGTTGTCCGCCCATCCAGATCACCATTGTGGACCAAAGAAAAACTTGAACCAATCACACTTCAAAGTGGTCAGTCTTTAGTACTTCCCTGCAGACCC
NM_001845.4	*COL4A1*	780-880	TGGGCTTAAGTTTTCAAGGACCAAAAGGTGACAAGGGTGACCAAGGGGTCAGTGGGCCTCCAGGAGTACCAGGACAAGCTCAAGTTCAAGAAAAAGGAGA
NM_006379.2	*SEMA3C*	945-1045	TGTCTGTGGGAGTGGCGCTTTCAGTCCTGTCTGTACTTACTTGAACAGAGGGAGGAGATCAGAGGACCAAGTTTTCATGATTGACTCCAAGTGTGAATCT
NM_004460.2	*FAP*	1490-1590	GCATTGGAAGCTATCCTCCAAGCAAGAAGTGTGTTACTTGCCATCTAAGGAAAGAAAGGTGCCAATATTACACAGCAAGTTTCAGCGACTACGCCAAGTA
NM_001098798.1	*TOX2*	784-884	GAAGCCTGTGTCGGCCTACGCACTCTTCTTCAGAGACACTCAGGCCGCCATCAAGGGTCAGAACCCCAGTGCCACTTTCGGTGACGTGTCCAAAATCGTG
NM_002253.2	*KDR*	1420-1520	CAATCACACAATTAAAGCGGGGCATGTACTGACGATTATGGAAGTGAGTGAAAGAGACACAGGAAATTACACTGTCATCCTTACCAATCCCATTTCAAAG
NM_175569.2	*XG*	27-127	AGCTGTGGAGTTTGGGATCTGAGCTTGGAGCCCATTTGTTTCTGGCAGTTCCGCTCATATTTTCCACTTGAAGACATCGCCTCCCTTCCTTCCAAGCTGG
NM_005245.3	*FAT1*	780-880	ACCCAACCAGTGGTGTGATAGTGTTAACTGGTAGACTTGATTACCTAGAGACCAAGCTCTATGAGATGGAAATCCTCGCTGCGGACCGTGGCATGAAGTT
NM_001014986.1	*FOLH1*	1530-1630	GAAGAATTTGGTCTTCTTGGTTCTACTGAGTGGGCAGAGGAGAATTCAAGACTCCTTCAAGAGCGTGGCGTGGCTTATATTAATGCTGACTCATCTATAG
NM_198516.1	*GALNTL4*	995-1095	ACAGCAGTAACGAGGAACTGAAGGAGAAGCTGACCGAATATGTGGACAAGGTGAACAGCCAGAAGCCAGGCTTCATCAAAGTCGTGCGTCACAGCAAGCA
NM_001036.3	*RYR3*	3655-3755	TCTCAGATCGGCCGCATGAATCTCGGGACAGATGCCAGTACCTTCAAGTTTTATACCATGTGCGGTCTCCAAGAGGGCTTTGAGCCTTTTGCTGTCAACA
NM_001025108.1	*AFF3*	1174-1274	AAGCACCTGGCAAAGTGGAACCAACCAAATTTCCATTTCCAAATAAGGACTCTCAGCTTGTATCCTCTGGACACAATAATCCAAAGAAAGGTGATGCAGA

NM_004557.3	*NOTCH4*	6420-6520	ACCCCTAAGTTGGAACCAAGAATTGCAGGCATATGGGATGTAAGATGTTCTTTCCTATATATGGTTTCCAAAGGGTGCCCCTATGATCCATTGTCCCCAC
NM_004098.3	*EMX2*	2505-2605	ACATTCCCTTTCCTAACATCCTGAGGCTTAAAACCCTGATGCAAACTTCTCCTTTCAGTGGTTGGAGAAATTGGCCGAGTTCAACCATTCACTGCAATGC
NM_001167738.1	*NAV1*	294-394	CTCAGTTCAGAAGAATTCAATGCCAGCTCCTCACTCAACTCCCTCCCAAGTACTCCCACTGCTTCTCGCAGGAACTCAACAATAGTGCTACGCACAGACT
NM_019087.2	*ARL15*	75-175	GGATGCTTTAGGCTGCCGGATGTCTGATCTCCGAATAACTGAGGCGTTTCTGTACATGGATTATCTGTGTTTTAGAGCACTTTGCTGCAAGGGACCACCA
NM_001039580.1	*MAP9*	547-647	ATAAATCAAACGGTAACATAACCAAAGATGAGCCAGTGTGTGCCATCAAAAATGAAGAGGAAATGGCACCTGATGGGTGTGAAGACATTGTTGTAAAATC
NM_022343.2	*GLIPR2*	216-316	CCAGTGTGGGGAGAACCTTGCATGGGCATCCTATGATCAGACAGGAAAGGAGGTGGCTGATAGATGGTACAGTGAAATCAAGAACTATAACTTCCAGCAG
NM_001193329.1	*APO*	1375-1475	AGACCCTTCTGCGGCTGATCCCTCCTTGCCTCTCAGCAGCACATTCTGTTCTGGGAGCACACCCGTTCTCTCGGCTGGATGTTCTCATCGTCCCTGCCAA
NM_015116.2	*LRCH1*	715-815	CTGCCTGTGTGGTCTGCCTCTCAAAGTCTTAATCGCAAGTAACAACAAACTTGGATCATTACCAGAAGAGATAGGTCAGCTCAAACAGTTAATGGAGCTG
NM_002448.3	*MSX1*	1225-1325	GGCACCGCCAGCCGCCTTCCCTTTAACCCTCACACTGCTCCAGTTTCACCTCTTTGCTCCCTGAGTTCACTCTCCGAAGTCTGATCCCTGCCAAAAAGTG
NM_006516.2	*SLC2A1*	2500-2600	AGGCTCCATTAGGATTTGCCCCTTCCCATCTCTTCCTACCCAACCACTCAAATTAATCTTTCTTTACCTGAGACCAGTTGGGAGCACTGGAGTGCAGGGA
NM_004490.2	*GRB14*	980-1080	ACAGCTGGACCCTTTTTGAGCACCTGCCTCACATAGGTGTAGAAAGAACAATAGAAGACCACGAACTGGTGATTGAAGTGCTATCCAACTGGGGGATAGA
NM_017855.3	*ODAM*	445-545	TCAATACTATCCAGTTTACATGGTCCTACCCTGGGAACAACCTCAGCAAACAGTTCCAAGGTCACCTCAACAAACAAGACAGCAACAGTATGAGGAGCAG
NM_031455.3	*CCDC3*	470-570	ACCGTGGTCCAGGACTACTCCTATTTCTTCTTCCTCAGGATGGATGAAAATTATAACCTCTTGCCTCACGGAGTCAATTTCCAAGATGCCATCTTCCCAG
NM_001081.3	*CUBN*	8930-9030	TGACTTTTGTGTCCTTCCACTTAGAAGCTCGTTCCGCTGTGACGGGAAGCTGTGTCAACGATGGCGTGCACATTATCAGAGGTTACAGCGTCATGTCCAC
NM_001197221.1	*PDE4D*	2120-2220	ATTGTTTCCAAAGTGCATGTCACATGCCACAACCACGGTCACACCTCACTGTCATCTGCCAGGACGTTTGTTGAACAAAACTGACCTTGACTACTCAGTC
NM_001134406.1	*RUNDC3B*	929-1029	GCTTCTAGGACTCAATGCTATTGATTTCAGTTTCTGCCTAAAGGGAGAGGGGCTGGATGGCAGTTTTCCTGCTGTAATAGACTATACACCATATTTGAAG
NM_006639.2	*CYSLTR1*	90-190	AAAGAAGCTTGCCTATAGAGCAGGCACTCTGTGAATGGACTGTGCTTTTACGACCCTACAGGGTATCAAGATACTGTGCAGCTCGCCAACAAGGATTAAT
NM_021804.2	*ACE2*	1825-1925	GGCCCTCTGCACAAATGTGACATCTCAAACTCTACAGAAGCTGGACAGAAACTGTTCAATATGCTGAGGCTTGGAAAATCAGAACCCTGGACCCTAGCAT
NM_001080392.1	*LCHN*	4440-4540	CCAGGTGGGAGTGGAATTCAGGTTTGGGGCTCGTTGGTATCCATGCAAAATATGACAAAGGCCTGTTCAAGAGGGCATTTTCAATTCTGTAGGCTCAGCA
NM_014583.2	*LMCD1*	1470-1570	TTGTTTCCCAGCTACAACCAACTAAAGACACAAATGGCGTTCTGCAAGGGGACTCTGGGAGGAGTTTTCCAGAATGCAATTCCGAGTGAGCAAATCGCAT
NM_007031.1	*HSF2BP*	800-900	ACTGGTCAAACAATGGAGAGTTTTGTGAAGTCGTTAGACGGTGATGTCCAGGAGCTGGATTCGGATGAAAGTCAGTTTGTTTTCGCTCTGGCTGGAATTG
NM_001135032.1	*FAM176A*	510-610	CGTGGAGATGGCTTTGCTCAGCAACATCCTAGCGGCCTATTCCTTTGTCTCAGAAAATCCTGAGCGAGCAGCTCTGTACTTTGTTTCTGGCGTGTGCATC

Venous genes from the arteriovenous fresh profile

NM_000901.3	*NR3C2*	430-530	CCTACAGAGAGGACCGATGAGAATAACTACATGGAGATTGTCAACGTAAGCTGTGTTTCCGGTGCTATTCCAAACAACAGTACTCAAGGAAGCAGCAAAG
NM_016240.2	*SCARA3*	280-380	AGAACCTATCTTTGCACACATCGGTGCGGATTCTTTACCTCTTCCTGGCCCTGCTCCTGGTGGCCGTGGCTGTGTTGGCCTCTCTGGTTTTCAGAAAAGT
NM_173462.3	*PAPLN*	424-524	CCTACTACAGCGCCCCAAACAAGTGTGAACTGAACTGCATTCCCAAGGGGGAGAACTTCTACTACAAGCACAGGGAGGCTGTGGTTGATGGGACGCCCTG
NM_006750.3	*SNTB2*	2645-2745	GTTTGAACAAGATAAATCCTAAAGAGCCAGCCTGCTTGAGGAGTAGACTTGGTGGGTGAAGCCAGCAATTCCGCACAAACGTCATGTTGAATTGTTTTGG
NM_001042521.1	*MGC13057*	550-650	ATCTTGGAATATGCACACCGCCTGTCTCAGGATATCTTGTGTGATGCCTTGCAGCAATGGGCATGCAATAACATCAAGTACCATGACATTCCATACATTG
NM_013281.2	*FLRT3*	1145-1245	GACAATATAACACAACTGATTCTTCGCAACAATCCCTGGTATTGCGGGTGCAAGATGAAATGGGTACGTGACTGGTTACAATCACTACCTGTGAAGGTCA
NM_006829.2	*APM2*	8-108	CCCTGGGGCGCCTTAAAAACCGGAGCTGGCGCTTGGCATCGCCACTCTGGGCAGGATCCAACGTCGCTCCAGCTGCTCTTGACGACTCCACAGATACCCC
NM_138809.3	*CMBL*	2425-2525	TTGTCTCCTAAGCAGTGGATTTCCAAGCACTGGCATTTTTGGGTCCCAGGGCCTGAGGCTACCTCAGAAATATCTCCTGTGTTTCTGGTGGTGGCGTAAA
NM_020190.2	*OLFML3*	1525-1625	GCCCGAAGAGTCAAAACCCTCAATGTTCCCTCCTGCTCTCCTGCCCCATGTCAACAAATTTCAGGCTAAGGATGCCCCAGACCCAGGGCTCTAACCTTGT
NM_145764.1	*MGST1*	330-430	TCTTGGAATTGGCCTCCTGTATTCCTTGAGTGGTCCCGACCCCTCTACAGCCATCCTGCACTTCAGACTATTTGTCGGAGCACGGATCTACCACACCATT
NM_173496.3	*MPP7*	573-673	TACACTACTATGAGAAGCAGAGTCCGGTGCCCATTCTCCATGGTGCGGCGGCCTTGGCCGATGATCTGGCCGAAGAGCTTCAGAACAAGCCATTAAACAG
NM_001204213.1	*NOS1*	1201-1301	GGCGCTGGTGGAGATCAATATCGCGGTTCTCTATAGCTTCCAGAGTGACAAAGTGACCATTGTTGACCATCACTCCGCCACCGAGTCCTTCATTAAGCAC

Established arterial EC genes

NM_018419.2	*SOX18*	1475-1575	CCTGCGAGGGTGCCTGGAGTTCCCACGTGTCCCGGGGCTTTTCCAGGAAGCCCGAGCCCAGGACCTGTTGGCAGAGTTGCCAGGGTTACATTTTTGAAGC
NM_004093.2	*EFNB2*	620-720	TTGTAAAACCAAATCCAGGTTCTAGCACAGACGGCAACAGCGCCGGACATTCGGGGAACAACATCCTCGGTTCCGAAGTGGCCTTATTTGCAGGGATTGC
NM_017617.3	*NOTCH1*	735-835	CTGCCAGGCTTCACCGGCCAGAACTGTGAGGAAAATATCGACGATTGTCCAGGAAACAACTGCAAGAACGGGGGTGCCTGTGTGGACGGCGTGAACACCT

NM_000214.2	*JAG1*	915-1015	TTGCTTGTGGAGGCGTGGGATTCCAGTAATGACACCGTTCAACCTGACAGTATTATTGAAAAGGCTTCTCACTCGGGCATGATCAACCCCAGCCGGCAGT
NM_004429.4	*EFNB1*	137-237	CGAGGCTTCGGGGGCGCAAACTAATGGGACTGGCTCGCTCGGCAGCATCTCCCCGCTCTTCTAAGTACACTGAGCAGGGCCCGCGCTGAAGTAGAAGCTG
NM_019074.2	*DLL4*	893-993	AATGACCACTTCGGCCACTATGTGTGCCAGCCAGATGGCAACTTGTCCTGCCTGCCCGGTTGGACTGGGGAATATTGCCAACAGCCTATCTGTCTTTCGG

Probe set ID	Gene	Targeted region	Target sequence
Established arterial EC genes, continued			
NM_145159.1	*JAG2*	4225-4325	ATTTTTGTAAAGTTTCCGTGCGTGGCACTCGCTGTATGAAAGGAGAGAGCAAAGGGTGTCTGCGTCGTCACCAAATCGTAGCGTTTGTTACCAGAGGTTG
NM_001001392.1	*CD44*	429-529	ACACCATGGACAAGTTTTGGTGGCACGCAGCCTGGGGACTCTGCCTCGTGCCGCTGAGCCTGGCGCAGATCGATTTGAATATAACCTGCCGCTTTGCAGG
NM_031439.2	*SOX7*	2635-2735	CTGTGAGAATTTGTCTTCCTCACCAGCCAGGTCCTCAGGCAAAGTCCTCAGCCAGTGCTTTAGAGCAACTTCCCGCAAATCAGAAACTCACTGTGATTCC
NM_012258.3	*HEY1*	398-498	AACAGTTTGTCTGAGCTGAGAAGGCTGGTACCCAGTGCTTTTGAGAAGCAGGGATCTGCTAAGCTAGAAAAAGCCGAGATCCTGCAGATGACCGTGGATC
Established venous EC genes			
NM_201264.1	*NRP2*	805-905	TCTCACCTGGGTTTTCTTAGCCCTCTACTTTTCAAGACACCAAGTGAGAGGCCAACCAGACCCACCGTGCGGAGGTCGTTTGAATTCCAAAGATGCTGGC
NM_021005.2	*NR2F2*	1530-1630	CCATAGTCCTGTTCACCTCAGATGCCTGTGGTCTCTCTGATGTAGCCCATGTGGAAAGCTTGCAGGAAAAGTCTCAGTGTGCTTTGGAAGAATACGTTAG
NM_004444.4	*EPHB4*	1680-1780	GTCCTGACTTCACCTATACCTTTGAGGTCACTGCATTGAACGGGGTATCCTCCTTAGCCACGGGGCCCGTCCCATTTGAGCCTGTCAATGTCACCACTGA
General EC genes			
NM_000442.3	*PECAM1*	1365-1465	ATCTGCACTGCAGGTATTGACAAAGTGGTCAAGAAAAGCAACACAGTCCAGATAGTCGTATGTGAAATGCTCTCCCAGCCCAGGATTTCTTATGATGCCC
NM_000552.3	*VWF*	8115-8215	CACCTGCAACCCCTGCCCCCTGGGTTACAAGGAAGAAAATAACACAGGTGAATGTTGTGGGAGATGTTTGCCTACGGCTTGCACCATTCAGCTAAGAGGA
NM_000459.2	*TEK*	615-715	CGAGTTCGAGGAGAGGCAATCAGGATACGAACCATGAAGATGCGTCAACAAGCTTCCTTCCTACCAGCTACTTTAACTATGACTGTGGACAAGGGAGATA
NM_001795.3	*CDH5*	3405-3505	TCTCCCCTTCTCTGCCTCACCTGGTCGCCAATCCATGCTCTCTTTCTTTTCTCTGTCTACTCCTTATCCCTTGGTTTAGAGGAACCCAAGATGTGGCCTT
NM_001114753.1	*ENG*	1480-1580	GTCCTTGATCCAGACAAAGTGTGCCGACGACGCCATGACCCTGGTACTAAAGAAAGAGCTTGTTGCGCATTTGAAGTGCACCATCACGGGCCTGACCTTC
Housekeeping genes			
NM_000194.1	*HPRT1*	240-340	TGTGATGAAGGAGATGGGAGGCCATCACATTGTAGCCCTCTGTGTGCTCAAGGGGGGCTATAAATTCTTTGCTGACCTGCTGGATTACATCAAAGCACTG
NM_001101.2	*ACTB*	1010-1110	TGCAGAAGGAGATCACTGCCCTGGCACCCAGCACAATGAAGATCAAGATCATTGCTCCTCCTGAGCGCAAGTACTCCGTGTGGATCGGCGGCTCCATCCT
NM_022551.2	*RPS18*	256-356	TGCAGAATCCACGCCAGTACAAGATCCCAGACTGGTTCTTGAACAGACAGAAGGATGTAAAGGATGGAAAATACAGCCAGGTCCTAGCCAATGGTCTGGA
NM_002046.3	*GAPDH*	35-135	TCCTCCTGTTCGACAGTCAGCCGCATCTTCTTTTGCGTCGCCAGCCGAGCCACATCGCTCAGACACCATGGGGAAGGTGAAGGTCGGAGTCAACGGATTT

TABLE10. ***Arterial TF cloning information***

Gene Symbol	Forward Primer (5'->3')	Reverse Primer (5'->3')	Cloned mRNA sequence (bases)	Restriction enzymes used	Backbone
hHey2					Genecopoeia (ref. EX-U0515-Lv114)
mPRDM16	CCATCTAGAATGCGATCCAAGGCGAGGG	TCACTCGAGTCAGAGGTGGTTGATGGGG	115-3770	XbaI-XhoI	pRRL2-GPK-Cherry
mAFF3	AGGGCTAGCATGGACAGCTTCGACTTAGC	TGAGCTAGCCTAGGACAGGTGTACGCTGC	202-3888	NheI	pRRL2-GPK-Cherry
hTox2	AAGTCTAGACCTACGTGGGGATGAGTGAC	GTCCTCGAGCTCTGAGCAGTGCCTTCCA	209-1663	XbaI-XhoI	pRRL2-GPK-Cherry
hMsx1	TTTTGCTAGCATGGCCCCGGCTGCTGACA	GGGCTCGAGCTATGTCAGGTGGTACATGC	236-1147	NheI-XhoI	pRRL2-GPK-Cherry
hEmx2	CCCCGCTAGCATGTTCCAGCCGGCGCCCAA	AAACTCGAGTTAATCATCTGAGGTCACGT	824-1582	NheI-XhoI	pRRL2-GPK-Cherry
hNkx2-3	CCCCAGCTAGCATGATGTTACCAAGCCCGG	TAGCTCGAGCTACCAGGCCCGGATGCCCT	200-1294	NheI-XhoI	pRRL2-GPK-Cherry
hSox17	ATCGTCCTCGAGCACCATGAGCAGCCCGGATGCGGG	ATGACTCTAGATCACACGTCAGGATAGTTGCAGT	203-1449	XhoI-XbaI	pLVX-IRES-Hyg (Clontech ref. 632185)

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Table 11. Characteristics of the arteriovenous fresh profile gene list

Characteristic	Number of genes	Gene names
Knock-out mouse has vascular phenotype*	13	*COL4A1, COL4A2, CUBN, CYSLTR1, EMX2, FGL2, HEY2, KDR, MSX1, NKX2-3, NOTCH4, SEMA3C, SOX17*
Previously associated with cardiovascular disease/risk factors in humans†	7	*ARL15, CNTN3, COL4A1, GRB14, MFAP5, NPR3, TOX2*
Coding for cell surface protein	22	*A2M, ACE2, ADRB1, CNTN3, CYSLTR1, FAP, FAT1, FOLH1, KDR, NOTCH4, NPR3, NRCAM, PTPRR, SLC2A1, SLC2A3, SYTL2, XG, FLRT3, MPP7, NOS1, TMEM200A, TMEM200C*
Coding for secreted protein	14	*CCDC3, COL4A1, COL4A2, FAM19A5, FGL2, FREM1, LAMA2, MFAP5, ODAM, SEMA3C, SEMA3G, TFPI2, OLFML3, PAPLN*
Coding for intracellular protein	27	*AFF3, APO, CUBN, EMX2, FAM176A, GALNTL4, GLIPR2, GRB14, HECW2, HEY2, KLHL6, LMCD1, MAP9, MSX1, NAV1, NKX2-3, PDE4D, PRDM16, PSMAL, RGC-32, RYR3, SOX17, TOX2, CMBL, MGST1, SCARA3, SNTB2*
Coding for transcription factor	9	*AFF3, EMX2, HEY2, MSX1, NKX2-3, PRDM16, SOX17, TOX2, NR3C2*
Documented expression in aEC and/or vEC	16	*APO, CCDC3, COL4A1, COL4A2, CYSLTR1, GLIPR2, HEY2, KDR, MFAP5, MSX1, NKX2-3, NOTCH4, RGC-32, SEMA3G, SOX17, NR3C2*
Previously associated with arteriovenous specification	4	*HEY2, KDR, NOTCH4, S0X17*
Previously linked with the Notch pathway†	10	*CNTN3, EMX2, HECW2, HEY2, KDR, MFAP5, MSX1, NKX2-3, NOTCH4, PRDM16*

*gene from the arteriovenous profile list alone and/or in combination with another gene; † gene from the arteriovenous profile list or an orthologue/family member

Table 12. ***Probe set intensities in HUAEC/HUVEC of genes contained within the arteriovenous fresh profile.***

		A	B	C	D	E	F	G	H
Probe set ID	Gene	HUAEC-F	HUVEC-F	HUAEC-C	HUVEC-C	B-A	C-A	D-B	D-C
		Arterial probe sets							
213764_s_at	*MFAP5*	12,0	3,9	6,0	4,1	-8,1	-6,0	0,2	-1,9
229459_at	*FAM19A5*	11,6	3,7	4,1	3,6	-7,9	-7,5	-0,1	-0,5
213765_at	*MFAP5*	11,4	3,9	5,7	4,2	-7,6	-5,7	0,4	-1,5
206084_at	*PTPRR*	11,4	4,0	6,7	4,6	-7,4	-4,6	0,6	-2,2
224646_x_at	*H19*	13,5	6,3	6,6	6,8	-7,1	-6,9	0,4	0,2
209758_s_at	*MFAP5*	11,3	4,6	6,9	5,6	-6,7	-4,4	0,9	-1,3
237094_at	*FAM19A5*	10,1	3,5	3,8	3,7	-6,6	-6,4	0,1	-0,1
224997_x_at	*H19*	11,6	5,1	5,3	5,1	-6,6	-6,4	0,0	-0,2
227265_at	*FGL2*	10,4	4,1	4,0	3,8	-6,3	-6,5	-0,4	-0,2
219789_at	*NPR3*	10,0	3,9	4,8	4,1	-6,1	-5,3	0,1	-0,7
229831_at	*CNTN3*	9,6	3,5	7,3	5,0	-6,0	-2,3	1,4	-2,3
210675_s_at	*PTPRR*	10,2	4,2	5,4	4,2	-6,0	-4,7	0,1	-1,2
228109_at	*RASGRF2*	11,0	5,0	7,9	7,4	-6,0	-3,1	2,4	-0,5
219689_at	*SEMA3G*	10,5	4,6	5,1	6,0	-6,0	-5,4	1,4	0,8
232424_at	*PRDM16*	9,3	3,4	4,8	3,6	-5,9	-4,6	0,2	-1,2
204834_at	*FGL2*	10,0	4,1	4,3	4,0	-5,9	-5,7	0,0	-0,2
205413_at	*MPPED2*	9,1	3,2	3,3	3,4	-5,8	-5,7	0,2	0,1
230943_at	*NA*	10,3	4,5	7,8	8,9	-5,8	-2,6	4,4	1,1
217757_at	*A2M*	10,7	4,9	4,6	4,7	-5,8	-6,1	-0,2	0,1
237727_at	*FREM1*	10,1	4,4	4,0	4,3	-5,7	-6,1	-0,2	0,3
211966_at	*COL4A2*	10,8	5,3	10,0	8,6	-5,5	-0,9	3,3	-1,4
219743_at	*HEY2*	9,8	4,3	6,2	5,8	-5,5	-3,6	1,5	-0,5
204105_s_at	*NRCAM*	9,3	3,9	10,3	8,6	-5,5	1,0	4,7	-1,7
211981_at	*COL4A1*	12,2	6,8	10,8	9,9	-5,4	-1,4	3,1	-0,9
203789_s_at	*SEMA3C*	9,6	4,3	10,1	7,3	-5,3	0,5	3,1	-2,8
209955_s_at	*FAP*	11,2	5,9	9,9	6,8	-5,3	-1,3	0,9	-3,1
222921_s_at	*HEY2*	10,9	5,6	8,5	8,0	-5,3	-2,3	2,5	-0,5
228737_at	*TOX2*	9,8	4,5	6,2	7,6	-5,2	-3,6	3,1	1,5
219054_at	*NPR3*	12,0	6,8	6,9	6,3	-5,2	-5,1	-0,5	-0,6
203934_at	*KDR*	10,6	5,5	7,4	8,7	-5,1	-3,2	3,2	1,3
1554062_at	*XG*	8,8	3,7	7,2	4,5	-5,1	-1,5	0,8	-2,7
229523_at	*TMEM200C*	10,3	5,4	7,4	7,1	-5,0	-2,9	1,7	-0,3
218723_s_at	*RGC_32*	9,1	4,2	6,0	7,9	-4,9	-3,1	3,6	1,9
216840_s_at	*LAMA2*	8,7	4,0	4,5	4,5	-4,8	-4,2	0,5	0,0
234994_at	*TMEM200A*	8,6	3,9	9,1	8,8	-4,7	0,5	4,9	-0,4
		A	B	C	D	E	F	G	H
Probe set ID	Gene	HUAEC-F	HUVEC-F	HUAEC-C	HUVEC-C	B-A	C-A	D-B	D-C

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236031_x_at	*FREM1*	8,4	3,7	3,6	3,7	-4,7	-4,8	0,0	0,1		
229309_at	*ADRB1*	8,3	3,6	4,3	4,1	-4,7	-4,0	0,5	-0,2		
209278_s_at	*TFPI2*	12,0	7,4	8,8	11,3	-4,6	-3,2	3,9	2,5		
219993_at	*SOX17*	8,8	4,3	6,3	7,8	-4,6	-2,5	3,6	1,5		
202498_s_at	*SLC2A3*	10,6	6,2	5,4	5,8	-4,4	-5,1	-0,5	0,3		
209277_at	*TFPI2*	10,8	6,4	7,7	10,0	-4,3	-3,0	3,5	2,2		
232914_s_at	*SYTL2*	9,4	5,1	5,5	4,7	-4,3	-3,9	-0,5	-0,9		
228233_at	*FREM1*	8,8	4,5	3,9	4,1	-4,2	-4,8	-0,4	0,2		
228167_at	*KLHL6*	8,1	3,9	5,2	5,3	-4,2	-2,9	1,5	0,1		
1553808_a_at	*NKX2-3*	8,1	4,0	4,4	4,1	-4,1	-3,7	0,1	-0,3		
232080_at	*HECW2*	8,6	4,4	9,7	9,0	-4,1	1,1	4,5	-0,7		
226809_at	*CYP4F30P*	8,5	4,5	4,4	4,4	-4,0	-4,1	-0,1	0,0		
201579_at	*FAT1*	9,7	5,8	11,8	9,0	-3,9	2,0	3,1	-2,8		
215363_x_at	*FOLH1*	9,9	6,0	4,6	4,6	-3,9	-5,4	-1,4	0,0		
213519_s_at	*LAMA2*	8,8	4,9	5,2	5,3	-3,9	-3,6	0,3	0,1		
1554079_at	*GALNTL4*	9,7	5,8	5,4	6,1	-3,9	-4,3	0,2	0,7		
206306_at	*RYR3*	7,2	3,3	3,4	3,3	-3,9	-3,8	0,0	0,0		
227198_at	*AFF3*	7,3	3,6	3,8	3,8	-3,7	-3,5	0,2	-0,1		
222088_s_at	*SLC2A3*	11,6	8,0	6,2	6,8	-3,7	-5,5	-1,2	0,6		
205247_at	*NOTCH4*	9,6	5,9	5,3	6,5	-3,7	-4,3	0,5	1,1		
205116_at	*LAMA2*	8,6	4,9	5,3	5,2	-3,7	-3,3	0,3	-0,1		
221950_at	*EMX2*	7,0	3,4	4,8	4,0	-3,6	-2,2	0,6	-0,8		
224772_at	*NAV1*	10,4	6,8	10,4	9,6	-3,5	0,0	2,8	-0,8		
219842_at	*ARL15*	9,2	5,6	6,6	5,6	-3,5	-2,5	0,0	-1,0		
225496_s_at	*SYTL2*	8,8	5,4	5,3	5,3	-3,4	-3,5	-0,1	0,0		
211303_x_at	*FOLH1*	7,6	4,3	3,8	3,7	-3,3	-3,8	-0,5	0,0		
228423_at	*MAP9*	6,9	3,6	8,1	5,7	-3,3	1,2	2,1	-2,4		
1556128_a_at	*RASGRF2*	9,3	6,1	7,2	7,9	-3,2	-2,0	1,8	0,6		
225602_at	*GLIPR2*	8,1	4,9	9,7	9,4	-3,2	1,6	4,5	-0,2		
237716_at	*APO*	8,0	4,8	4,4	4,2	-3,2	-3,6	-0,6	-0,2		
226795_at	*LRCH1*	8,5	5,3	8,1	7,7	-3,2	-0,3	2,4	-0,4		
205932_s_at	*MSX1*	8,4	5,3	7,3	7,9	-3,1	-1,1	2,6	0,6		
201250_s_at	*SLC2A1*	11,3	8,2	9,5	8,6	-3,1	-1,9	0,4	-0,8		
229655_at	*FAM19A5*	8,0	4,9	5,0	5,2	-3,0	-2,9	0,3	0,2		
206204_at	*GRB14*	10,5	7,5	6,6	6,8	-3,0	-4,0	-0,7	0,2		
220133_at	*ODAM*	6,8	3,8	5,0	4,2	-3,0	-1,9	0,3	-0,8		
223316_at	*CCDC3*	13,8	10,9	8,0	7,9	-2,9	-5,7	-3,0	-0,1		
201249_at	*SLC2A1*	7,1	4,3	5,5	4,7	-2,8	-1,7	0,4	-0,8		
		A	B	C	D	E	F	G	H		
Probe set ID	Gene	HUAEC-F	HUVEC-F	HUAEC-C	HUVEC-C	B-A	C-A	D-B	D-C		
238169_at	*NA*	6,4	3,7	4,3	5,4	-2,7	-2,1	1,6	1,0		
202497_x_at	*SLC2A3*	10,3	7,7	6,2	7,0	-2,7	-4,1	-0,6	0,8		

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206775_at	*CUBN*	8,6	5,9	6,3	6,0	-2,7	-2,3	0,1	-0,2	
228151_at	*NA*	12,7	10,0	9,9	8,8	-2,6	-2,8	-1,3	-1,1	
228962_at	*PDE4D*	7,6	5,0	6,1	5,6	-2,6	-1,5	0,6	-0,4	
241703_at	*RUNDC3B*	6,2	3,6	6,7	4,5	-2,6	0,5	0,9	-2,2	
225604_s_at	*GLIPR2*	6,3	3,9	8,3	8,3	-2,4	2,0	4,4	0,0	
213484_at	*NA*	7,2	4,9	4,8	5,1	-2,3	-2,4	0,2	0,3	
231747_at	*CYSLTR1*	6,5	4,2	3,9	4,1	-2,3	-2,6	-0,1	0,2	
227828_s_at	*FAM176A*	7,0	4,7	8,8	8,8	-2,3	1,7	4,1	0,0	
209227_at	*NA*	6,0	3,8	6,4	5,6	-2,2	0,4	1,8	-0,8	
222257_s_at	*ACE2*	6,8	4,6	4,5	4,6	-2,2	-2,3	0,0	0,1	
228601_at	*LOC401022*	8,8	6,7	7,4	7,8	-2,2	-1,4	1,1	0,4	
227502_at	*LCHN*	9,0	6,9	6,3	6,3	-2,1	-2,7	-0,6	0,0	
218574_s_at	*LMCD1*	11,7	10,1	8,3	7,7	-1,6	-3,4	-2,4	-0,7	
207020_at	*HSF2BP*	6,5	4,9	6,5	6,0	-1,5	0,0	1,1	-0,4	
Venous probe sets										
205259_at	*NR3C2*	5,6	7,3	5,6	5,3	1,7	0,0	-2,0	-0,3	
219416_at	*SCARA3*	7,6	9,4	6,9	8,1	1,8	-0,7	-1,3	1,2	
219250_s_at	*FLRT3*	4,8	6,7	7,4	5,9	1,9	2,6	-0,8	-1,5	
226435_at	*PAPLN*	4,2	6,2	4,5	4,4	1,9	0,3	-1,8	-0,1	
229004_at	*NA*	5,3	7,3	6,7	5,5	2,0	1,4	-1,9	-1,3	
227312_at	*SNTB2*	6,2	8,4	6,1	5,5	2,2	-0,2	-3,0	-0,6	
228195_at	*MGC13057*	5,0	7,2	3,8	3,9	2,2	-1,2	-3,3	0,1	
203571_s_at	*APM2*	5,8	8,1	5,8	6,2	2,3	-0,1	-1,9	0,4	
227522_at	*CMBL*	6,6	9,0	8,8	9,6	2,4	2,2	0,6	0,8	
218162_at	*OLFML3*	5,1	7,6	5,2	7,0	2,4	0,1	-0,6	1,8	
224918_x_at	*MGST1*	4,0	6,6	8,5	9,6	2,6	4,5	2,9	1,0	
238778_at	*MPP7*	5,0	8,4	5,1	3,3	3,3	0,0	-5,1	-1,8	
239132_at	*NOS1*	6,6	10,0	5,0	4,9	3,4	-1,6	-5,0	-0,1	

Table 13. ***Expression of genes from the arteriovenous fresh profile in DLL4-Fc treated HUAEC.***

Arterial genes	BSA			DLL4-Fc		
MFAP5	100	±	8	169	±	8
FAM19A5	100	±	34	107	±	4
PTPRR	100	±	14	185	±	22
H19	100	±	18	1298	±	258*
FGL2	100	±	27	3	±	8
NPR3	100	±	1	233	±	64
CNTN3	100	±	2	241	±	46
RASGRF2	100	±	18	115	±	14
SEMA3G	100	±	33	757	±	141*
PRDM16	100	±	22	76	±	13
MPPED2	100	±	55	152	±	109
A2M	100	±	28	194	±	21
FREM1	100	±	33	145	±	81
COL4A2	100	±	15	315	±	33*
HEY2	100	±	17	992	±	43*
NRCAM	100	±	3	64	±	3
COL4A1	100	±	15	428	±	47*
SEMA3C	100	±	26	159	±	48
FAP	100	±	6	133	±	4
TOX2	100	±	9	116	±	6
KDR	100	±	15	46	±	2*
TMEM200C	100	±	25	111	±	35
XG	100	±	18	209	±	13*
RGC-32	100	±	4	43	±	0.2*
LAMA2	100	±	2	117	±	8
TMEM200A	100	±	9	52	±	6*
ADRB1	100	±	24	88	±	26
TFPI2	100	±	4	180	±	21*
SOX17	100	±	3	91	±	8
SLC2A3	100	±	4	109	±	12
SYTL2	100	±	7	89	±	6
KLHL6	100	±	7	102	±	7
NKX2-3	100	±	15	412	±	290
HECW2	100	±	9	178	±	10*
CYP4F30P	100	±	11	222	±	22
FAT1	100	±	7	311	±	36*
FOLH1	100	±	10	242	±	35*
GALNTL4	100	±	24	133	±	5
RYR3	100	±	10	164	±	25*

Arterial genes	BSA			DLL4-Fc		
AFF3	100	±	85	71	±	15
NOTCH4	100	±	12	129	±	7
EMX2	100	±	17	137	±	31
NAV1	100	±	3	95	±	7
ARL15	100	±	8	125	±	5
PSMAL	100	±	11	246	±	14*
MAP9	100	±	19	179	±	134
GLIPR2	100	±	8	132	±	8
APO	100	±	4	103	±	11
LRCH1	100	±	4	105	±	2
MSX1	100	±	1	256	±	19*
SLC2A1	100	±	6	162	±	2*
GRB14	100	±	4	46	±	1*
ODAM	100	±	97	776	±	1807
CCDC3	100	±	18	2735	±	407*
CUBN	100	±	16	330	±	7*
PDE4D	100	±	2	113	±	5
RUNDC3B	100	±	11	94	±	9
CYSLTR1	100	±	8	584	±	40*
FAM176A	100	±	4	210	±	4*
ACE2	100	±	9	100	±	86
LOC401022	100	±	12	110	±	4
LCHN	100	±	16	351	±	30*
LMCD1	100	±	9	193	±	33*
HSF2BP	100	±	5	121	±	21

Venous genes	BSA			DLL4-Fc		
NR3C2	100	±	6	91	±	2
SCARA3	100	±	14	83	±	2
FLRT3	100	±	14	169	±	11*
PAPLN	100	±	17	161	±	15*
SNTB2	100	±	7	96	±	6
MGC13057	100	±	2	107	±	19
APM2	100	±	21	87	±	15
CMBL	100	±	2	115	±	3
OLFML3	100	±	30	21	±	95
MGST1	100	±	2	70	±	2
MPP7	100	±	1	112	±	13
NOS1	100	±	26	132	±	21

Table 14. ***Expression of genes from the arteriovenous fresh profile in cultured HUVEC overexpressing transcription factors.***

aEC genes	cherry			HEY2			MSX1			EMX2			Prdm16		
MFAP5	100	±	69	919	±	229*	91	±	71	696	±	209	919	±	0*
FAM19A5	100	±	15	158	±	11	91	±	22	151	±	29	148	±	15
PTPRR	100	±	57	207	±	133	165	±	21	643	±	92	441	±	107
H19	100	±	4	129	±	44	196	±	68	310	±	85*	39540	±	10556*
FGL2	100	±	71	706	±	539*	212	±	130	121	±	60	339	±	160
NPR3	100	±	77	798	±	403*	182	±	168	490	±	193*	419	±	64*
CNTN3	100	±	96	180	±	111	51	±	28	27	±	22	546	±	63*
RASGRF2	100	±	37	919	±	378*	31	±	16	319	±	101	393	±	78*
SEMA3G	100	±	52	81	±	31	288	±	212	174	±	45	179	±	25*
PRDM16	100	±	20	208	±	38	184	±	44	177	±	42	125	±	27
MPPED2	100	±	79	371	±	605	197	±	237	222	±	275	217	±	370
A2M	100	±	41	70	±	22	50	±	7	141	±	31	2061	±	394*
FREM1	100	±	12	125	±	35	106	±	6	167	±	22	96	±	56
COL4A2	100	±	21	179	±	49	146	±	42	384	±	54*	77	±	8
HEY2	100	±	116	152	±	144	746	±	272*	255	±	208	767	±	263*
NRCAM	100	±	39	221	±	62	55	±	42	43	±	7*	110	±	10
COL4A1	100	±	22	193	±	56	141	±	55	340	±	49*	86	±	10
SEMA3C	100	±	41	985	±	820*	356	±	75	43	±	19	15578	±	2529*
FAP	100	±	9	125	±	4	117	±	12	115	±	10	129	±	8
TOX2	100	±	16	91	±	11	116	±	12	104	±	9	77	±	5
KDR	100	±	28	61	±	16	120	±	33	65	±	10	29	±	3*
TMEM200C	100	±	91	56	±	22	83	±	108	85	±	19	14	±	9*
XG	100	±	25	201	±	54	297	±	37*	222	±	39	373	±	71*
RGC-32	100	±	21	41	±	14*	82	±	22	113	±	20	169	±	8
LAMA2	100	±	12	107	±	10	122	±	12	159	±	8*	120	±	7
TMEM200A	100	±	31	130	±	15	64	±	20	52	±	4	53	±	2
ADRB1	100	±	10	102	±	24	113	±	14	120	±	7	60	±	12
TFPI2	100	±	27	342	±	268*	154	±	45	1706	±	197*	94	±	9
SOX17	100	±	12	105	±	2	112	±	7	110	±	11	86	±	3

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SLC2A3	100	±	5	102	±	14	103	±	10	139	±	14	77	±	6
SYTL2	100	±	43	479	±	143*	85	±	55	138	±	31	738	±	254*
KLHL6	100	±	13	96	±	8	137	±	16	148	±	2	68	±	2
NKX2-3	100	±	6	124	±	38	117	±	21	119	±	27	48	±	18
HECW2	100	±	19	118	±	21	141	±	13	95	±	9	38	±	2*
CYP4F30P	100	±	15	165	±	42	118	±	29	194	±	13*	123	±	37
FAT1	100	±	35	220	±	66*	349	±	49*	314	±	31*	177	±	14*
FOLH1	100	±	61	252	±	92	256	±	102	172	±	83	43	±	42
GALNTL4	100	±	10	108	±	16	106	±	15	157	±	7*	112	±	11
RYR3	100	±	24	106	±	7	99	±	9	115	±	13	103	±	27
AFF3	100	±	22	89	±	38	246	±	64	162	±	145	47	±	41
NOTCH4	100	±	24	133	±	12	112	±	13	144	±	15	118	±	10
EMX2	100	±	17	113	±	11	85	±	8	99	±	4	116	±	16
NAV1	100	±	14	113	±	12	100	±	14	109	±	4	111	±	8

aEC genes	**NKX2-3**			**Aff3**			**SOX17**			**TOX2**			**All**		
MFAP5	178	±	125	382	±	236	5#	±	4*#	620	±	403	219#	±	25*#
FAM19A5	105	±	40	119	±	11	126	±	30	172	±	10	133	±	18
PTPRR	95	±	34	110	±	52	228	±	60*	94	±	41	595	±	127*
H19	97	±	7	122	±	22	247	±	118	99	±	16	11953	±	3906*
FGL2	510	±	196*	146	±	58	238	±	146	459	±	215	2851	±	1436*
NPR3	315	±	207	128	±	105	154	±	61	355	±	86	723	±	310*
CNTN3	134	±	60	43	±	29	197	±	122	98	±	65	176	±	39
RASGRF2	1074	±	261*	139	±	50	331	±	26	105	±	22	323	±	189
SEMA3G	373	±	160*	117	±	60	1037	±	333*	69	±	12	531	±	312*
PRDM16	119	±	24	135	±	21	139	±	51	114	±	5	186	±	28
MPPED2	153	±	119	213	±	186	141	±	303	1619	±	1481*	553	±	784
A2M	470	±	71*	55	±	13	156	±	6	259	±	52*	338	±	103*
FREM1	141	±	15	91	±	29	117	±	64	66	±	10	144	±	26
COL4A2	190	±	61*	89	±	20	167	±	39	58	±	8	124	±	15
HEY2	307	±	54	56	±	32	179	±	97	73	±	24	275	±	99
NRCAM	147	±	29	154	±	21	180	±	14	185	±	11	140	±	11
COL4A1	165	±	67	95	±	25	198	±	40	59	±	8	148	±	11
SEMA3C	1255	±	392*	101	±	21	439	±	160	86	±	48	1238	±	1125*

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Transcription factor code for arterial EC identity

FAP	120	±	8	96	±	4	100	±	4	81	±	3	108	±	7			
TOX2	88	±	10	89	±	16	94	±	10	6848	±	1314*	2542	±	111*			
KDR	253	±	33*	89	±	14	95	±	20	182	±	4	86	±	13			
TMEM200C	29	±	43	24	±	13	17	±	5	18	±	5	22	±	9			
XG	296	±	71*	107	±	24	81	±	11	157	±	33	553	±	120*			
RGC-32	163	±	20	73	±	16	69	±	20	54	±	11	161	±	8			
LAMA2	105	±	25	94	±	5	113	±	6	80	±	1	135	±	9			
TMEM200A	93	±	15	124	±	6	88	±	8	102	±	7	40	±	5*			
ADRB1	86	±	9	67	±	10	86	±	20	41	±	2*	64	±	9			
TFPI2	201	±	36	105	±	28	51	±	20	28	±	4	368	±	71*			
SOX17	81	±	9	103	±	11	166	±	24*	78	±	2	160	±	34*			
SLC2A3	145	±	11	89	±	7	118	±	8	95	±	7	103	±	7			
SYTL2	499	±	94*	97	±	22	64	±	40	257	±	135	307	±	196			
KLHL6	122	±	6	102	±	9	95	±	4	93	±	13	93	±	9			
NKX2-3	93	±	13	83	±	14	107	±	25	72	±	5	69	±	16			
HECW2	48	±	4*	90	±	8	110	±	7	86	±	7	96	±	10			
CYP4F30P	93	±	20	66	±	8	107	±	36	68	±	6	108	±	13			
FAT1	186	±	48*	115	±	18	138	±	25	285	±	31*	358	±	26*			
FOLH1	278	±	100	106	±	53	125	±	72	148	±	97	153	±	95			
GALNTL4	165	±	6*	90	±	11	159	±	12*	145	±	23	136	±	15			
RYR3	105	±	30	93	±	7	117	±	16	92	±	7	87	±	22			
AFF3	85	±	37	132	±	23	68	±	9	45	±	15	76	±	17			
NOTCH4	254	±	47	92	±	18	438	±	79	79	±	15	164	±	15			
EMX2	66	±	46	89	±	14	118	±	37	140	±	5	120	±	23			
NAV1	94	±	7	100	±	13	112	±	10	90	±	7	103	±	6			

aEC genes	cherry			Hey2			Msx1			Emx2			Prdm16		
ARL15	100	±	6	115	±	6	107	±	9	118	±	4	194	±	5*
PSMAL	100	±	25	363	±	27*	523	±	65*	313	±	54	44	±	8
MAP9	100	±	44	329	±	110*	104	±	27	125	±	40	857	±	345*
GLIPR2-	100	±	25	94	±	14	189	±	45*	178	±	17*	98	±	5
APO	100	±	15	129	±	10	108	±	14	141	±	3	132	±	10
LRCH1	100	±	24	100	±	11	123	±	13	162	±	14*	111	±	5
MSX1	100	±	10	185	±	31*	142	±	21	227	±	29*	128	±	6
SLC2A1	100	±	13	149	±	21	162	±	26	275	±	41*	162	±	11*
GRB14	100	±	13	63	±	11	112	±	32	156	±	9*	56	±	2
ODAM	100	±	54	151	±	717*	133	±	77	162	±	117	2891	±	1008*
CCDC3	100	±	31	115	±	56	101	±	28	32	±	26	78	±	26
CUBN	100	±	16	224	±	40*	136	±	50	88	±	13	205	±	33*
PDE4D	100	±	13	133	±	17	117	±	17	133	±	10	73	±	6
RUNDC3B	100	±	20	149	±	35	60	±	28	74	±	8	209	±	12*
CYSLTR1	100	±	20	100	±	21	90	±	19	148	±	14	98	±	10
FAM176A	100	±	22	163	±	21	71	±	27	236	±	12*	116	±	4
ACE2	100	±	68	241	±	175	184	±	98	75	±	165	556	±	155
LOC401022	100	±	33	90	±	21	42	±	11	102	±	21	231	±	27*
LCHN	100	±	22	121	±	15	102	±	9	114	±	5	179	±	7*
LMCD1	100	±	24	163	±	47	181	±	34	767	±	204*	211	±	28*
HSF2BP	100	±	17	112	±	13	105	±	28	171	±	12*	111	±	7
vEC genes	**cherry**			**HEY2**			**MSX1**			**EMX2**			**Prdm16**		
NR3C2	100	±	35	151	±	8	64	±	23	117	±	7	143	±	12
SCARA3	100	±	15	101	±	24	92	±	2	108	±	9	69	±	3
FLRT3	100	±	6	138	±	7	111	±	8	104	±	9	75	±	10
PAPLN	100	±	34	126	±	17	86	±	18	113	±	9	111	±	19
SNTB2	100	±	15	120	±	10	83	±	17	107	±	5	101	±	7
MGC13057	100	±	13	135	±	12	117	±	22	119	±	9	548	±	40*
APM2	100	±	34	114	±	11	165	±	18	134	±	20	119	±	6
CMBL	100	±	14	91	±	7	120	±	18	129	±	8	90	±	5
OLFML3	100	±	66	444	±	63*	201	±	254	14703	±	5481*	222	±	65

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MGST1	100	±	6	105	±	6	86	±	10	63	±	6	99	±	3
MPP7	100	±	36	111	±	45	47	±	18	90	±	6	546	±	18*
NOS1	ND			ND			ND			ND			ND		

aEC genes	**Nkx2-3**			**Aff3**			**Sox17**			**Tox2**			**All**		
ARL15	148	±	8	99	±	7	136	±	10	107	±	2	143	±	13*
PSMAL	550	±	61*	209	±	75	133	±	13	151	±	31	259	±	78
MAP9	226	±	87	191	±	77	172	±	78	252	±	79	775	±	259*
GLIPR2-	53	±	4	92	±	7	99	±	12	53	±	6	116	±	11
APO	261	±	25*	87	±	5	114	±	10	108	±	2	123	±	6
LRCH1	413	±	62*	69	±	13	129	±	14	151	±	6	147	±	16
MSX1	217	±	38*	116	±	11	165	±	32	183	±	42*	244	±	12*
SLC2A1	228	±	72*	97	±	11	102	±	17	77	±	4	221	±	19*
GRB14	108	±	8	81	±	8	85	±	9	102	±	15	110	±	4
ODAM	99	±	52	178	±	186	376 2	±	664*	71	±	34	2569	±	3349*
CCDC3	189	±	29	116	±	42	174	±	44	242	±	83	200	±	101*
CUBN	246	±	33*	113	±	12	171	±	17	156	±	6	114	±	15
PDE4D	119	±	16	110	±	21	101	±	11	102	±	5	74	±	12
RUNDC3B	196	±	46*	107	±	13	109	±	9	105	±	12	117	±	12
CYSLTR1	154	±	22	84	±	11	125	±	9	122	±	15	97	±	17
FAM176A	261	±	12*	108	±	10	121	±	6	91	±	3	129	±	7
ACE2	962	±	128 *	148	±	106	241	±	195	602	±	366	888	±	466*
LOC4010 22	346	±	48*	67	±	15	115	±	19	79	±	20	122	±	53
LCHN	167	±	21*	84	±	16	162	±	22*	102	±	7	126	±	16
LMCD1	155	±	30	84	±	7	170	±	16	73	±	3	325	±	33*
HSF2BP	279	±	30*	104	±	13	117	±	30	98	±	4	125	±	15

vEC genes	**NKX2-3**			**Aff3**			**SOX17**			**TOX2**			**All**		
NR3C2	158	±	12	130	±	19,3	161	±	8	100	±	8	133	±	13
SCARA3	121	±	16	62	±	8,6	98	±	9	79	±	12	70	±	8
FLRT3	85	±	8	102	±	5,6	131	±	16	102	±	15	99	±	6
PAPLN	178	±	36	67	±	12,6	108	±	24	413	±	130*	162	±	36*
SNTB2	131	±	13	109	±	12	126	±	17	86	±	5	84	±	8
MGC1305 7	152	±	23	84	±	17	88	±	14	139	±	9	369	±	22*

Transcription factor code for arterial EC identity

APM2	151	±	37	84	±	26	100	±	15	66	±	4	177	±	6
CMBL	96	±	16	100	±	4	89	±	7	103	±	4	100	±	10
OLFML3	190	±	63	58	±	17	60	±	14	119	±	43	2075	±	1394*
MGST1	134	±	15	96	±	6	94	±	6	67	±	5	77	±	5
MPP7	139	±	43	92	±	27	95	±	31	230	±	94	301	±	152*
NOS1	ND			ND			ND			ND			ND		

TABLE15. ***Primerlist for work described in Example 3***

Gene Symbol	Species	Forward Primer (5'->3')	Reverse Primer (5'->3')	working concentration (nM)
PRDM16	H	CAAACGCTTCGAATGTGAAA	CGTGTAGGACTTGTGGCAGA	200
DLL4	H	ATGACCACTTCGGCCACTATG	GCCCGAAAGACAGATAGGCTG	200
HEY1	H	GAGAAGGCTGGTACCCAGTG	GCTCAGATAACGCGCAACTT	200
HEY2	H	CCCCTGCGAGGAGACGA	ATCTAATCACAGAGCTAGTACTTTGCCC	200
NOTCH1	H	CCACGGGCGACGTCACCC	TCCACTCTGGCGGGCACG	200
NOTCH4	H	ATGTCTCAATGGCGGCTCC	GGAGAAGGTGCCAGGCCT	200
EFNB2	H	CTGCTGGATCAACCAGGAAT	TCCGGTACTTCAGCAAGAGG	200
GJA5	H	TACAACACAAGGCAGCAAGC	TCGTATCACACCGGAAATCA	200
NR2F2	H	CGCCTCAAAAAGTGCCTCA	GCATCCTGCCCCTCTGC	200
NRP2	H	GCGAGTGGATTGTTTACGCC	CAGTCTTTGCCCAGGAGGTC	200
EPHB4	H	GAGCTGTGTGGCAATCAAGA	GAACTGTCCGTCGTTTAGCC	200
B-ACTIN	H	TGGCACCACACCTTCTACAATG	TAGCAACGTACATGGCTGGG	200
ODAM	H	CAGGCCAAGTTGATCCCTTA	TGAGGTTGTTCCCAGGGTAG	200
SEMA3C	H	TCATTCCATGATTGCTCGAA	CCTCGGGTTATCAGTTTCCA	200
SEMA3G	H	ACGGAGCACAATAGCACCTT	GACCACAGTCTGGGAGAAGC	200
FGL2	H	TTGGATGGCAAATGTTCAAA	CCATGGTCTCCATGTCACAG	200
MFAP5	H	ATACCCCTGGGGGCTAAATAG	CGTCGTAAACTGGTGAAGCA	200
RASGRF2	H	ACCTTGCCATCGAAAGAATG	ACAGAACCCCACCTGTCTTG	200
NPR3	H	GGAGACCGATATGGGGATTT	CACTGCCGATTCTTCTAGGC	200
ACE2	H	ATACTGTGACCCCGCATCTC	ATGCTAGGGTCCAGGGTTCT	200
FAT	H	GTGGAAAAGGGGACAGTGAA	CCTGATCGGTTGCAAAGACT	200
SYTL2	H	TGTTTTTGTGGCCCAGTGTA	AGTTCCACCTCCCCTAGGAA	200
MAP9	H	ACATGGAGGAGAAGGATGGA	CAGATGCGTTTCCCTCAGAT	200
PTPRR	H	TTGTCTGCCAGCTTCGTATG	GGGCTTCAGAGCTTCTCCTT	200
CUBN	H	ACTGTGAAGGGGGTTCTGTG	GACAGGCCCCACAGTAGAAA	200
XG	H	AGCTGGGAGACCAGAAGTCA	GGCATCTGCCAAATCAAAGT	200
Prdm16	M	CACGGAAGAGCGTGAGTACA	GGCAGACCTCGCATATGAAT	200

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Nr2f2	M	GACTCCGCCGAGTATAGCTG	CCTACCAAACGGACGAAAAA	200
Hey2	M	GCGTCGGGATCGAATAAATA	AGCATGGGCATCAAAGTAGC	100
B-actin	M	GACGGCCAGGTCATCACTAT	CTTCTGGATCCTGTCAGCAA	200
prdm16	ZF	CATTCGCCATCAGATGTCAC	TGTGACGGCACAGGTTAGAG	200
ef1a	ZF	TCACTGGTACTTCTCAGGCTGACT	TTCTTGGAGATACCAGCCTCAAA	200

Table 16. Gene list of arterial- and venous-specific genes differentially expressed ([log-value]≥1 and *P*<0.001) between mAECs and mVECs

ARTERIAL GENES					
Ace	*Edn1*	*Gkn3*	*Krt80*	*Pcolce2*	*Sod3*
Atp2b2	*Ehd4*	*Glce*	*Lox*	*Pde3b*	*Spon1*
Bgn	*Enpp6*	*Glt8d4*	*Mall*	*Plcg2*	*Ssfa2*
Bmp6	*Ephx2*	*Gria3*	*Mettl7a1*	*Plxdc2*	*Sulf1*
Cbr2	*Fam3c*	***Hey2***	*Mkl2*	*Prss23*	*Tagln*
Ccdc3	*Fblim1*	*Htra1*	*Naaladl2*	*Ptprj*	*Thbs1*
Clu	*Fbln2*	***Isl1***	*Nmt2*	*Serping1*	*Timp2*
Cmklr1	*Fbln5*	*Kazald1*	*Nox4*	*Serpini1*	*Tnfrsf26*
Cp	*Fgfr1*	*Klk10*	*Nupr1*	*Sfrp1*	*Wwp1*
Cytl1	*Frzb*	*Klk11*	*Pam*	*Slc4a4*	
Dlx5	*Gja5*	*Krt18*	*Pcdh9*	*Smoc1*	
VENOUS GENES					
Abcb1a	*Emcn*	*Hamp*	*Ltbp1*	*Nts*	*Serpina3n*
Aqp1	*Eng*	*Hmcn1*	*Lyve1*	*Pitpnc1*	*Stra6*
Arhgap18	*Enpp2*	*Ifi203*	*Mctp1*	*Pla2g7*	*Tmem100*
Bmp2k	*Epha7*	*Igfbp5*	*Mest*	*Plxnc1*	*Tppp*
Casp6	*Flt1*	*Igfbp6*	*Myo10*	*Postn*	*Vav3*
Ccdc141	*Fmnl2*	*Inhba*	*Myo1b*	***Prox1***	***Zfp462***
Cdh11	*Fmo1*	*Itga6*	*Nckap5*	*Rasgef1b*	***Zfp521***
Ctla2a	*Gimap6*	*Kdr*	*Npr3*	*Rasgrp3*	
Ctla2b	*Gja1*	*Lama4*	***Nr2f2***	*Rgs16*	
Ebf1	*Gpm6a*	*Lbh*	*Nrp2*	*Rgs5*	
Eltd1	*Gpr116*	*Lsamp*	*Nsg1*	*Rspo3*	

Transcription factors are indicated in boldface.

Table 17. *Average arterial and venous probe intensities and corresponding log_2 ratios for all TFs identified in our human and murine microarrays.*

GENE	HUAECs	HUVECs	Log_2 ratio (HUAECs / HUVECs)	mAECs	mVECs	Log_2 ratio (mAECs/ mVECs)
			Arterial			
Aff3	7.23	3.59	3.64	5.91	6.12	-0.21
Dlx5	4.03	4.06	-0.03	8.49	6.19	2.30
Emx2	6.91	3.42	3.49	5.02	4.83	0.19
Hey2	10.9	5.59	5.26	9.96	8.03	1.93
Isl1	4.49	4.89	-0.40	7.80	5.86	1.94
Msx1	8.28	5.30	2.98	7.54	7.16	0.38
Nkx2-3	7.83	3.95	3.88	6.05	5.89	0.16
Prdm16*	9.40	3.38	6.02	8.27	7.12	1.15
Sox17	8.84	4.30	4.54	8.03	7.22	0.81
Tox2	9.66	4.53	5.13	7.01	7.04	-0.03
			Venous			
Ebf1	5.01	5.06	-0.05	7.59	9.41	-1.82
Coup-TFII*	6.67	10.0	-3.33	6.69	8.07	-1.38
Nr3c2	5.62	7.30	-1.68	6.53	6.32	0.21
Prox1	4.63	5.97	-1.34	5.63	8.32	-2.65
Znf462	6.97	9.15	-2.18	7.09	8.39	-1.30
Znf521	9.26	10.3	-1.04	7.03	8.78	-1.75

*While Prdm16 was not present in our murine arteriovenous fingerprint due to the stringent tresholds we utilized, this TF was significantly differentially expressed between mAECs and mVECs (P<0.01) and was more than 2-fold enriched in AECs in both human and mouse ECs.
**Likewise, Nr2f2 did not make our cut-off in the human signature although it was significantly enriched in HUVECs versus HUAECs (P<0.01).

Table 18. ***List of conserved arterial/venous-specific genes***

ARTERIAL GENES		VENOUS GENES		
Antxr1$^{\$}$	*Hey2**	*Abcg2**	*Gpm6a*	***Prox1***
*Atp2a3**	*Igf2*	***Bex1***	*Lrg1**	*Pthlh*
Bcap29	*Lox**	*Cdh11*	*Ltbp1**	*Rgs5*$^{\$}$
*Camk4**	*Nox4**	*Chn2*	*Lyve1*	*Samd5*
Ccdc3	***Prdm16***	*Ctsk**	***Meox2****	*Sntb2*
Cp	*Ptprj**	*Dcn*$^{\$}$	*Mest**	***Tbx5****
Dkk2$^{\$}$	*Rps6ka5*	*Emcn*	*Nostrin**	***Zfp462***
*Edn1**	*Slc4a4*	*Enpp2**	*Nr2f2**	***Zfp521***
*Efna5**	*Thbs1**	*Entpd1**	*Nrp2**	
*Ehd4***	***Tox***	*Flrt2**	*Piptnc1*	
*Gja5**		*Fmnl2*	*Pkhd1l1*	

*knockouts mice has cardiovascular phenotype; ***Ehd4-/-Ehd3-/-* *has vascular phenotype;* $^{\$}$*described to have a role during pathological angiogenesis; genes already described to be expressed in an arterial/venous-specific manner are in red, TFs are in bold.*

VASCULAR BED-SPECIFIC ENDOTHELIAL CELLS

Claims

What is claimed is:

96. An engineered vascular endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof.

97. A method to differentiate a source cell into a vascular endothelial cell (the 'target cell'), said method comprising genetically altering the source cell by transduction or transfection of said source cell with a transcription factor of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof.

98. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell of claim 1, the cell being genetically altered by transduction or transfection of a source cell with a transcription factor of the group consisting of Prdm16, Emx2, Msx1, Tox2, Aff3 and Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof.

99. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, whereby the cell is being genetically altered by transduction or transfection of a source cell with a transcription factor of the

group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an organ-specific microvascular endothelial cell.

100. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, whereby the cell is being engineered by transduction or transfection of a source cell with a transcription factor of the group consisting of Prdm16, Emx2, Msx1, Tox2, Aff3 and Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an arterial endothelial cell.

101. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, whereby the cell is being engineered by transduction or transfection of a source cell with the transcription factor Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into an arterial endothelial cell.

102. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, whereby the is the cell is being engineered by transduction or transfection of a source cell with a transcription factor of the group consisting of Meox2, Tcf15, Pparγ, Wt1 and Ebf3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into a cardiac microvascular endothelial cell.

103. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, whereby the is the cell being engineered by transduction or transfection of a source cell with a transcription factor of the group consisting of Zic3, Lef1, Foxf2, Foxf1a and Foxc1 or a human

homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a brain microvascular endothelial cell.

104. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, whereby the cell is being engineered by transduction or transfection of a source cell with a transcription factor of the group consisting of Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a liver microvascular endothelial cell.

105. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to claim 1, whereby the cell is being engineered by transduction or transfection of a source cell with a transcription factor of the group consisting of Emx2, Nkx2-3, Msx1, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an arterial endothelial cell.

106. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to any one of the previous claims 1 to 10, whereby the transcription factors are overexpressed.

107. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to any one of the previous claims 1 to 11, whereby the source cell is a source cell of the group consisting of endothelial progenitor cells (EPCs), mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), induced pluripotency stem cells (iPSCs), mesoangioblasts (MABs), multipotent adult progenitor cells (MAPCs), blood outgrowth endothelial cells (BOECs), induced endothelial cells (iECs), unfractionated bone marrow cells, umbilical cord or peripheral blood-derived mononuclear cells, adipose tissue-derived cells, tissue resident progenitor cells, human umbilical vein

endothelial cells (HUVECs), human umbilical artery endothelial cells (HUAECs), human dermal microvascular endothelial cells (HDMECs), cultured endothelial cell lines from heart, cultured endothelial cell lines from liver, cultured endothelial cell lines from brain.

108. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to any one of the previous claims 1 to 11, whereby the source cell is a dedifferentiated cultured human umbilical vein endothelial cell (HUVEC) or a dedifferentiated HUAEC..

109. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to any one of the previous claims 1 to 11, whereby the source cell is a mammalian cell of embryonic or non-embryonic origin.

110. The engineered vascular bed-specific endothelial cell (the 'target cell') or the engineered source cell to differentiate into a vascular endothelial target cell according to any one of the previous claims, whereby the transduction or transfection is lentiviral.

111. An engineered vascular bed-specific endothelial cell, characterized in that the cell is engineered by induced differentiation of a source cell by transferring directly to source cells the expression product of a transcription factor of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof the expression product being linked to a protein transduction domain (PTD), such as poly-arginine and HIV-derived Tat, or to small cationic peptide domains to enhance its cell membrane crossing capacity.

112. The engineered vascular bed-specific endothelial cell of claim 16, whereby the expression product is of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1, Zeb2, Emx2, Msx1, Nkx2-3, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family,

orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a vascular endothelial target cell.

113. The engineered vascular bed-specific endothelial cell of claim 16, whereby the expression product is of the group consisting of Prdm16, Emx2, Msx1, Tox2, Aff3 and Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a vascular endothelial target cell.

114. The engineered vascular bed-specific endothelial cell of claim 16, whereby the expression product is of the group consisting of Meox2, Tcf15, Pparγ, Wt1, Ebf3, Zic3, Lef1, Foxf2, Foxf1a, Foxc1, Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an organ-specific microvascular endothelial cell.

115. The engineered vascular bed-specific endothelial cell of claim 16, whereby the expression product is of the group consisting of Prdm16, Emx2, Msx1, Tox2, Aff3 and Nkx2-3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an arterial endothelial cell.

116. The engineered vascular bed-specific endothelial cell of claim 16, whereby the expression product is of Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, to differentiate source cell into an arterial endothelial cell.

117. The engineered vascular bed-specific endothelial cell of claim 16, whereby the expression product is of the group consisting of Meox2, Tcf15, Pparγ, Wt1 and Ebf3 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a cardiac microvascular endothelial cell.

118. The engineered vascular bed-specific endothelial cell of claim 16, whereby the expression product is of the group consisting of Zic3, Lef1,

Foxf2, Foxf1a and Foxc1 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a brain microvascular endothelial cell.

119. The engineered vascular bed-specific endothelial cell of claim 16, whereby the expression product is of the group consisting of Tcfec, Hoxb5, Maf, Cux2, Gata4, Meis2, Twist1 and Zeb2 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into a liver microvascular endothelial cell.

120. The engineered vascular bed-specific endothelial cell of claim 16, whereby the expression product is of the group consisting of Emx2, Nkx2-3, Msx1, Tox2, Aff3 and Prdm16 or a human homologue or a variant, member of the same family, orthologue thereof, having the same biological function, or a combination thereof to differentiate source cell into an arterial endothelial cell.

121. The engineered vascular bed-specific endothelial cell according to any one of the previous claims 16 to 25, whereby the transcription factors are overexpressed.

122. The engineered vascular bed-specific endothelial cell according to any one of the previous claims 16 to 26, whereby the source cell is a source cell of the group consisting of endothelial progenitor cells (EPCs), mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), induced pluripotency stem cells (iPSCs), mesoangioblasts (MABs), multipotent adult progenitor cells (MAPCs), blood outgrowth endothelial cells (BOECs), induced endothelial cells (iECs), unfractionated bone marrow cells, umbilical cord or peripheral blood-derived mononuclear cells, adipose tissue-derived cells, tissue resident progenitor cells, human umbilical vein endothelial cells (HUVECs), human umbilical artery endothelial cells (HUAECs), human dermal microvascular endothelial cells (HDMECs), cultured endothelial cell lines from heart, cultured endothelial cell lines from liver, cultured endothelial cell lines from brain.

123. The engineered vascular bed-specific endothelial cell according to any one of the previous claims 16 to 26, whereby the source cell is a dedifferentiated cultured human umbilical vein endothelial cell (HUVEC) or a dedifferentiated HUAEC.

124. The engineered vascular bed-specific endothelial cell according to any one of the previous claims 16 to 26, whereby the source cell is a mammalian cell of embryonic or non-embryonic origin.

125. The engineered cell according to any one of the previous claims 16 to 29, for use in a treatment of vascular bed-specific disorder.

126. The engineered cell according to any one of the previous claims 1 to 29, for use in a treatment of a disorder of vascular bed–specific haemostasis or for a disorder of generating and/or maintaining vascular bed–specific phenotypes.

127. The engineered cell according to any one of the previous claims 1 to 29, for use in a treatment of for decreasing severity of vascular bed-specific disorder or diseases or circulatory or hypoxic conditions that can be treated with the compositions and the methods of the invention comprise but are not limited to: atherosclerosis, preeclampsia, erectile dysfunction, renal failure, transplant accelerated arteriosclerosis, deep vein thrombosis, sleep apnea, hypoxia during sleep, fetal hypoxia, smoking, anemia, endothelial dysfunction, sinusoidal obstruction syndrome, regional perfusion deficits (*e.g.,* limb, gut, renal ischemia), congestic heart failure, peripheral vascular disease, frost bite, decubitus ulcers, asphyxiation, poisoning (*e.g.,* carbon monoxide, heavy metal), altitude sickness, pulmonary hypertension, sudden infant death syndrome, asthma, chronic obstructive pulmonary disease, congenital circulatory anomalities (*e.g.,* Tetralogy of Fallot), erythroblastosis, myocardial infarction, aortic stenosis ro any other cardiac disease which can lead to heart failure, plaque rupture, both primary and secondary (in-stent) restenosis in coronary or peripheral arteries, coronary vascular disease, hypertension, diabetes, obesity, stroke, aneurysm, thrombosis, arrythmia, tachycardia, surgical or physical trauma in a subject, the method comprising autologous, allogeneic or xenogeneic cell transplantation; in a subject in

need of treatment the engineered cell, thereby decreasing severity of disorder in the subject.

128. The engineered cell according to any one of the previous claims 1 to 29, for use in a treatment for decreasing severity of vascular bed-specific diseases affecting the brain (*e.g.,* stroke), the liver (*e.g.,* sinusoidal obstruction syndrome), the heart (myocardial ischemia) or the extremities (*e.g.,* peripheral vascular disease) in a subject, the method comprising autologous, allogeneic or xenogeneic cell transplantation; in a subject in need of treatment the engineered cell, thereby decreasing severity of disorder in the subject.

129. A pharmaceutical composition, comprising an engineered cell according to any one of the previous claims 1 to 29.

130. An in vitro bioengineered tissue, comprising an engineered cell according to any one of the previous claims 1 to 29.

131. The use of an engineered cell according to any one of the previous claims 1 to 29, in a method of screening for non-toxic compounds.

132. The use of an engineered cell according to any one of the previous claims 1 to 29, in a method of *in vitro* bioengineering a tissue.

133. The use of an engineered cell according to any one of the previous claims 1 to 29 as a (or one of the) cellular component(s) of tissue engineered constructs for implantation in patients (for example to coat the inside of artificial arterial conduits or as the intimal cellular component of a fully biological tissue-engineered vascular graft or to coat cardiac valves).

134. An in vitro method of diagnosing a vascular bed-specific disorder phenotype in a subject, said method comprising (a) analysing the level of expression or activity of expression product of at least 5 genes of the '(differential) reference signatures' of table 4 (brain ECs), of table 5 (liver ECs), of table 6 (heart ECs) and/or tables 12/16/18 (arterial or venous ECs) in a sample isolated from said subject, and (b) compare said level of expression or activity with the level of expression or activity in '(differential) reference signatures' of table 4 (brain ECs), of table 5 (liver ECs), of table 6 (heart ECs) and/or tables 12/16/18 (arterial or venous ECs); whereby a

deviated level of expression or activity relative to such 'reference signature' is an indication of such disorder phenotype or a propensity thereto.

FIGURES

FIGURE 1: *Schematic summary of the invention.*

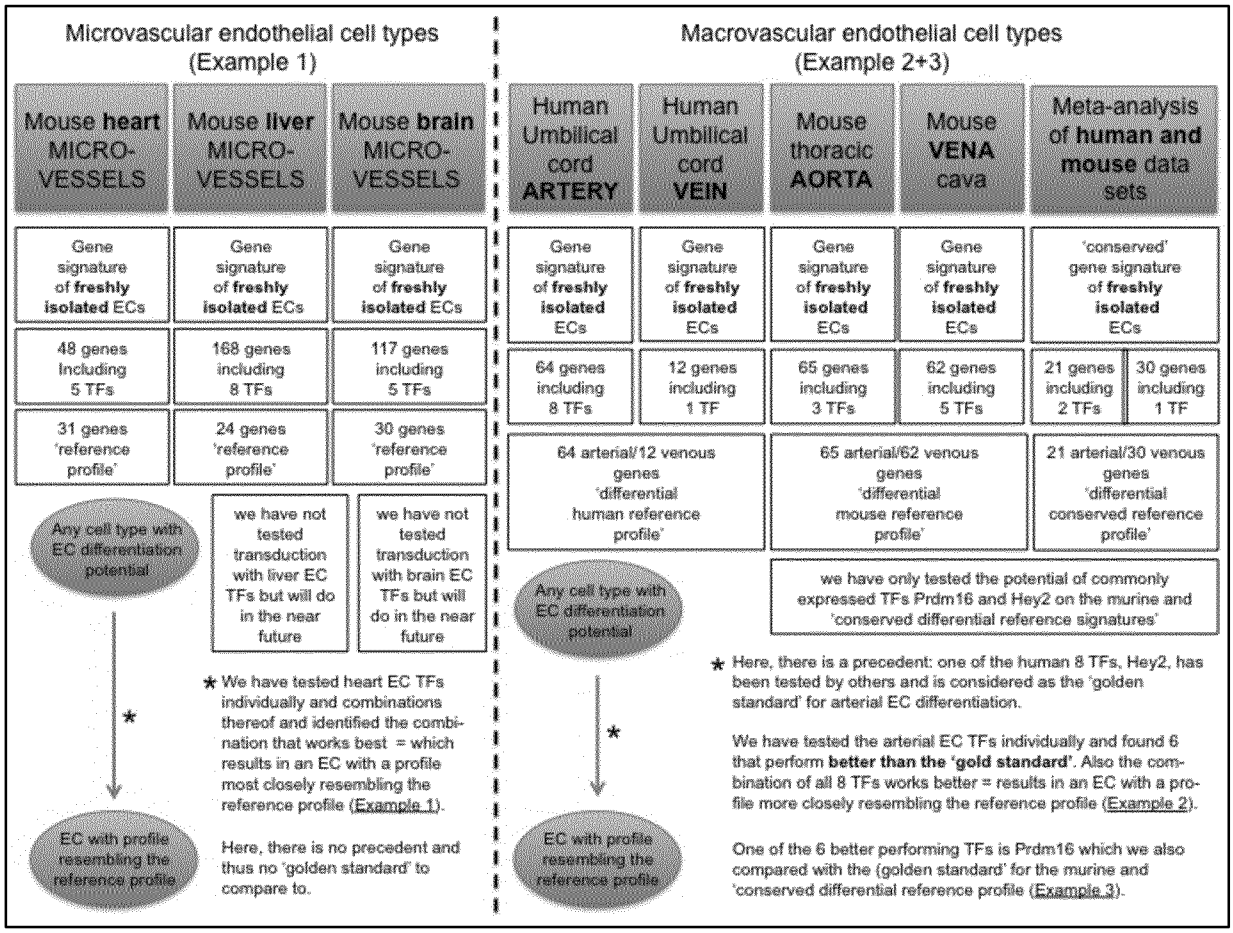


FIGURE 2A: *Lentiviral plasmid map presenting a multiple cloning site (MCS) and a Cherry reporter gene used as a backbone for cloning and overexpression of all the transcription factors, as indicated.*

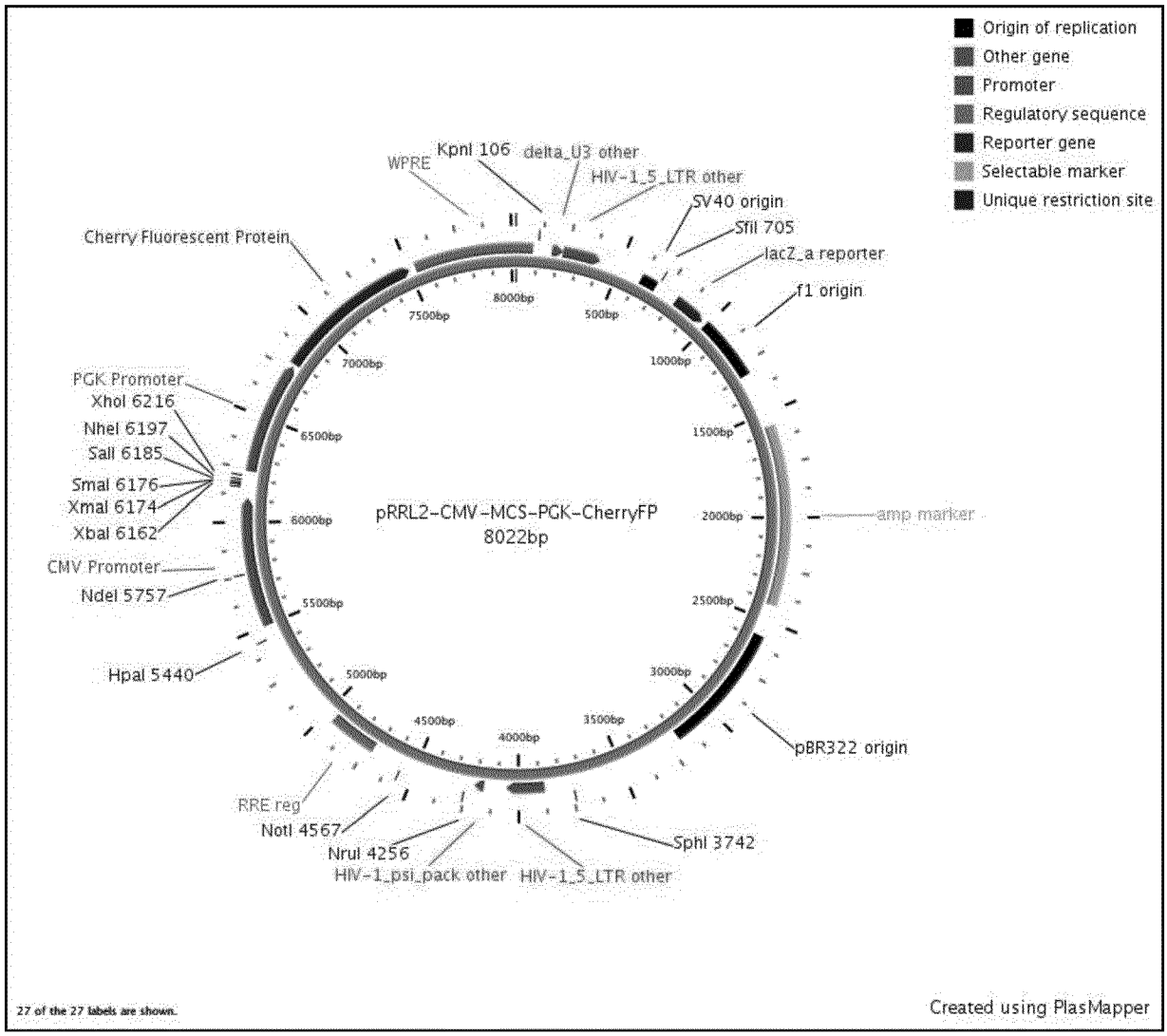


FIGURE 2B: *Lentiviral plasmid map for overexpression of Tcf15.*

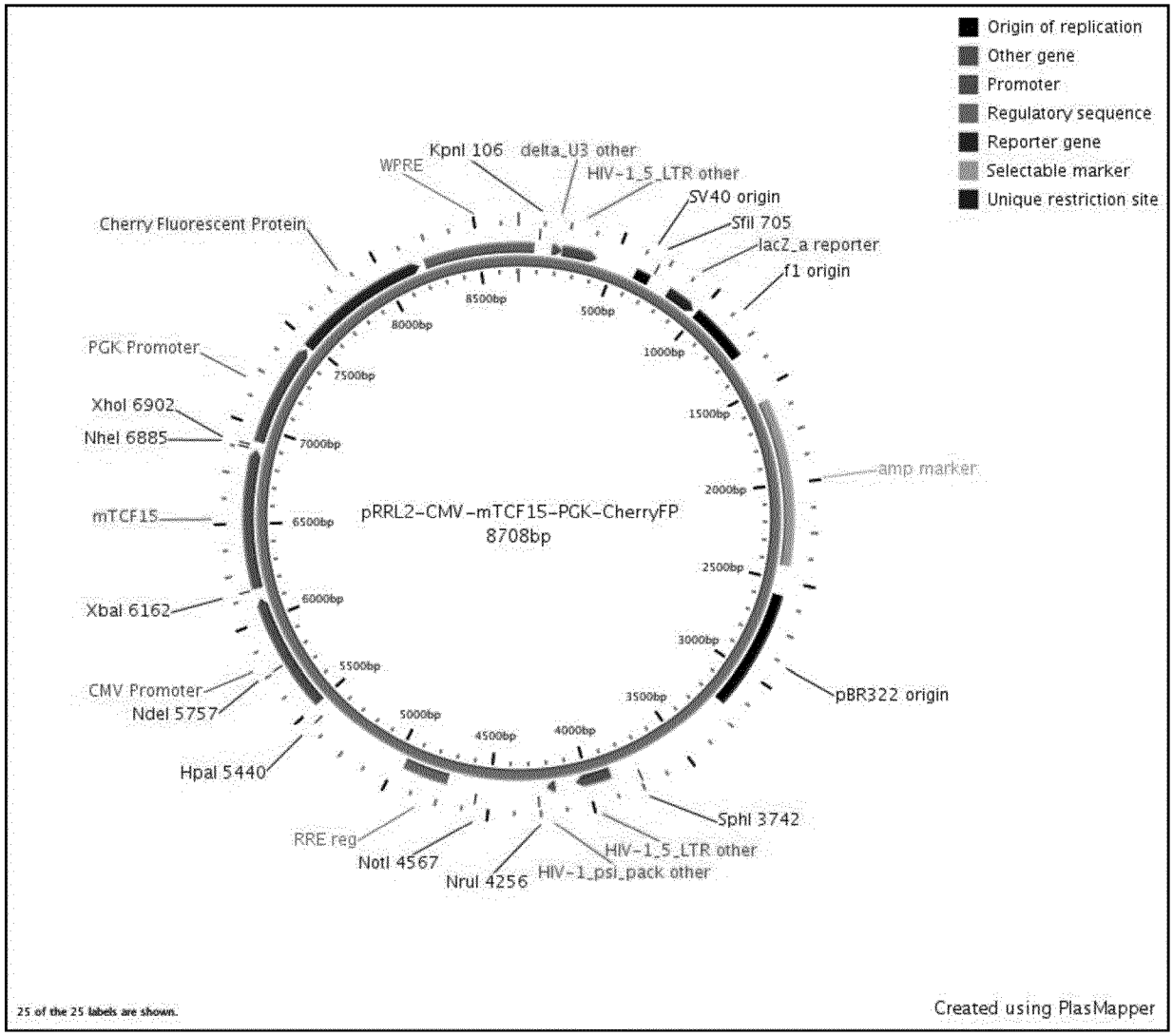


FIGURE 2C: *Lentiviral plasmid map for overexpression of EBF3.*

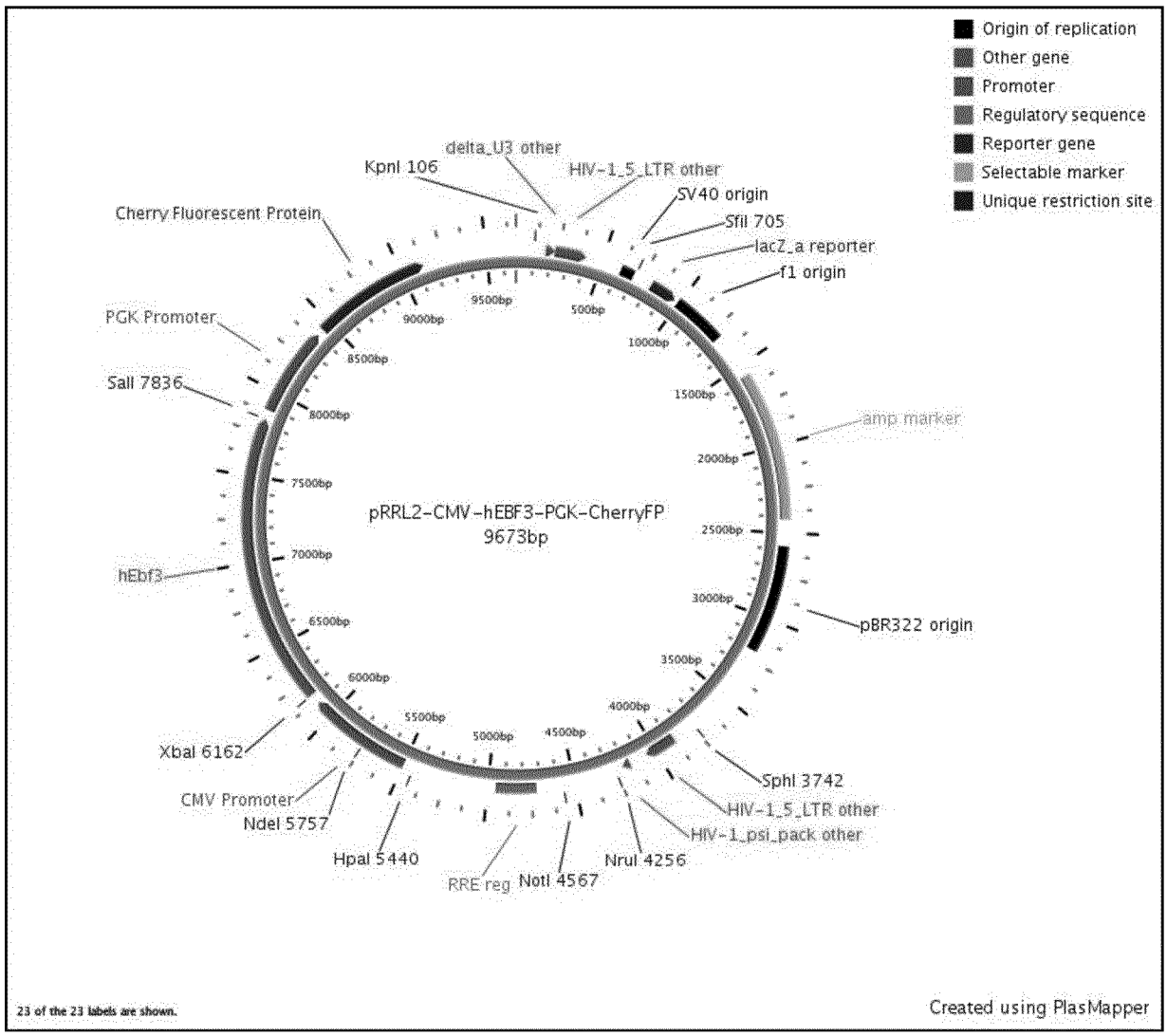


FIGURE 2D: *Lentiviral plasmid map for overexpression of PPARγ.*

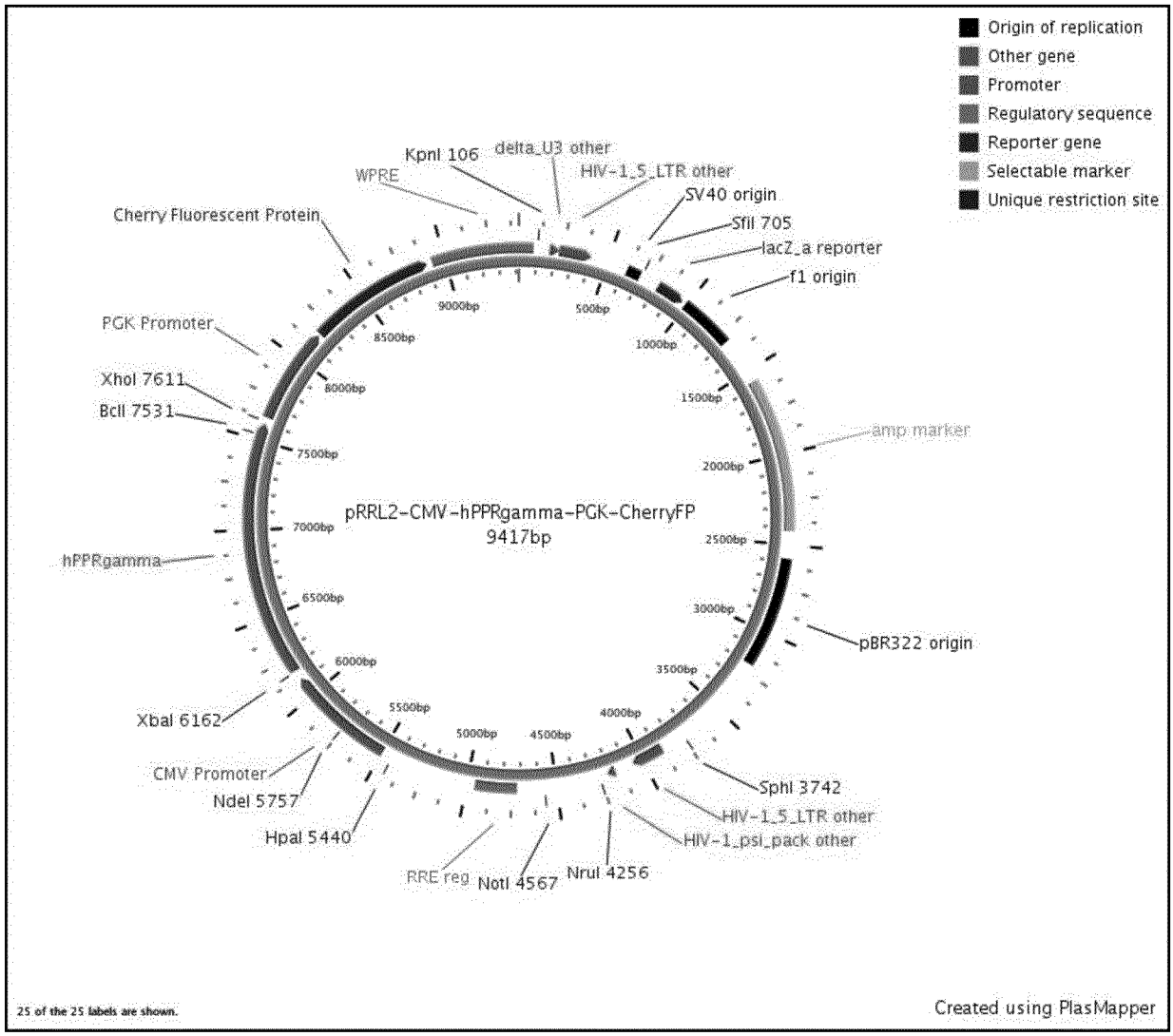


FIGURE 2E: *Lentiviral plasmid map for overexpression of WT1.*

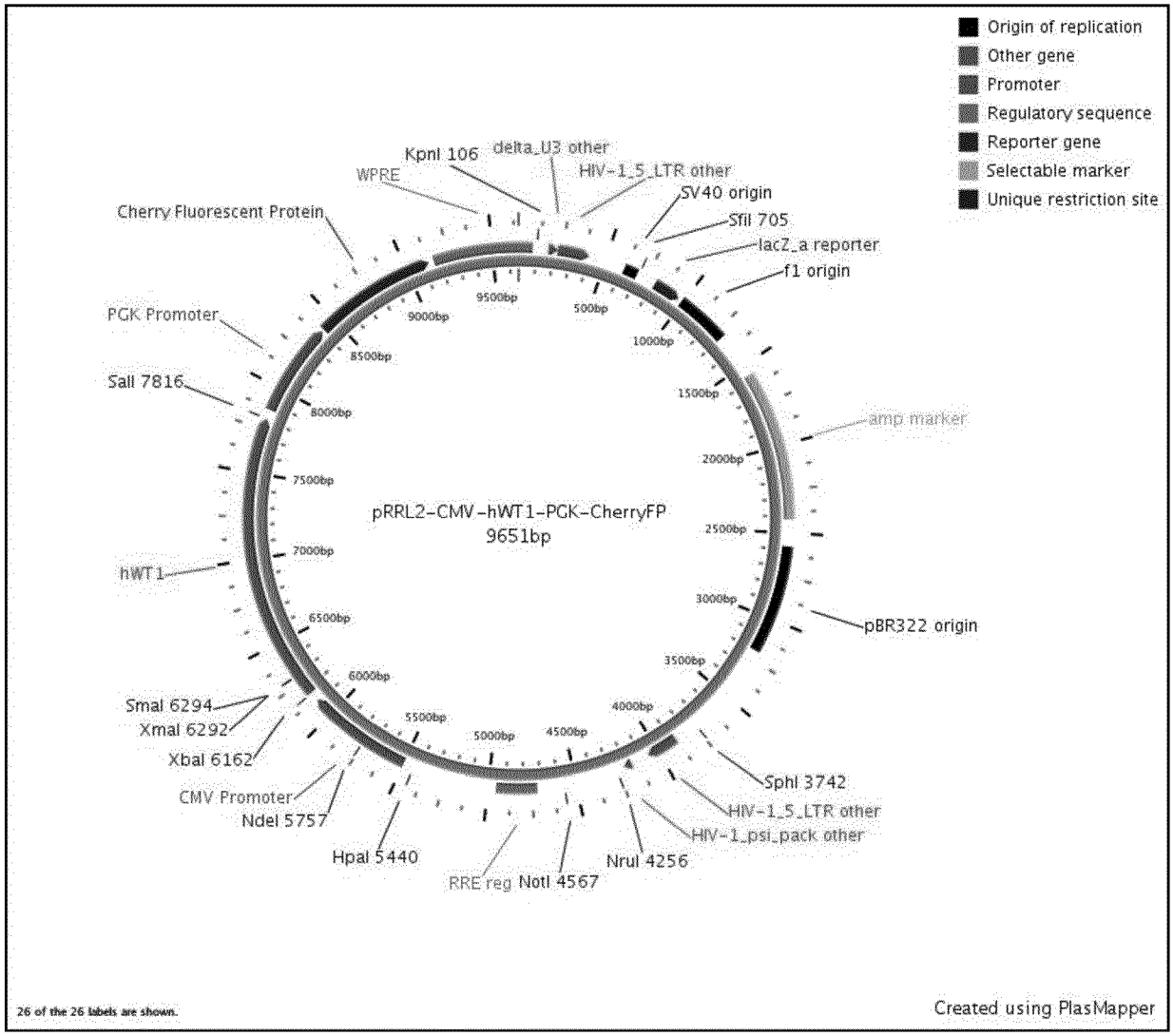


FIGURE 3A-E: *Microarray sample purity validation by FACS and microarray results.*

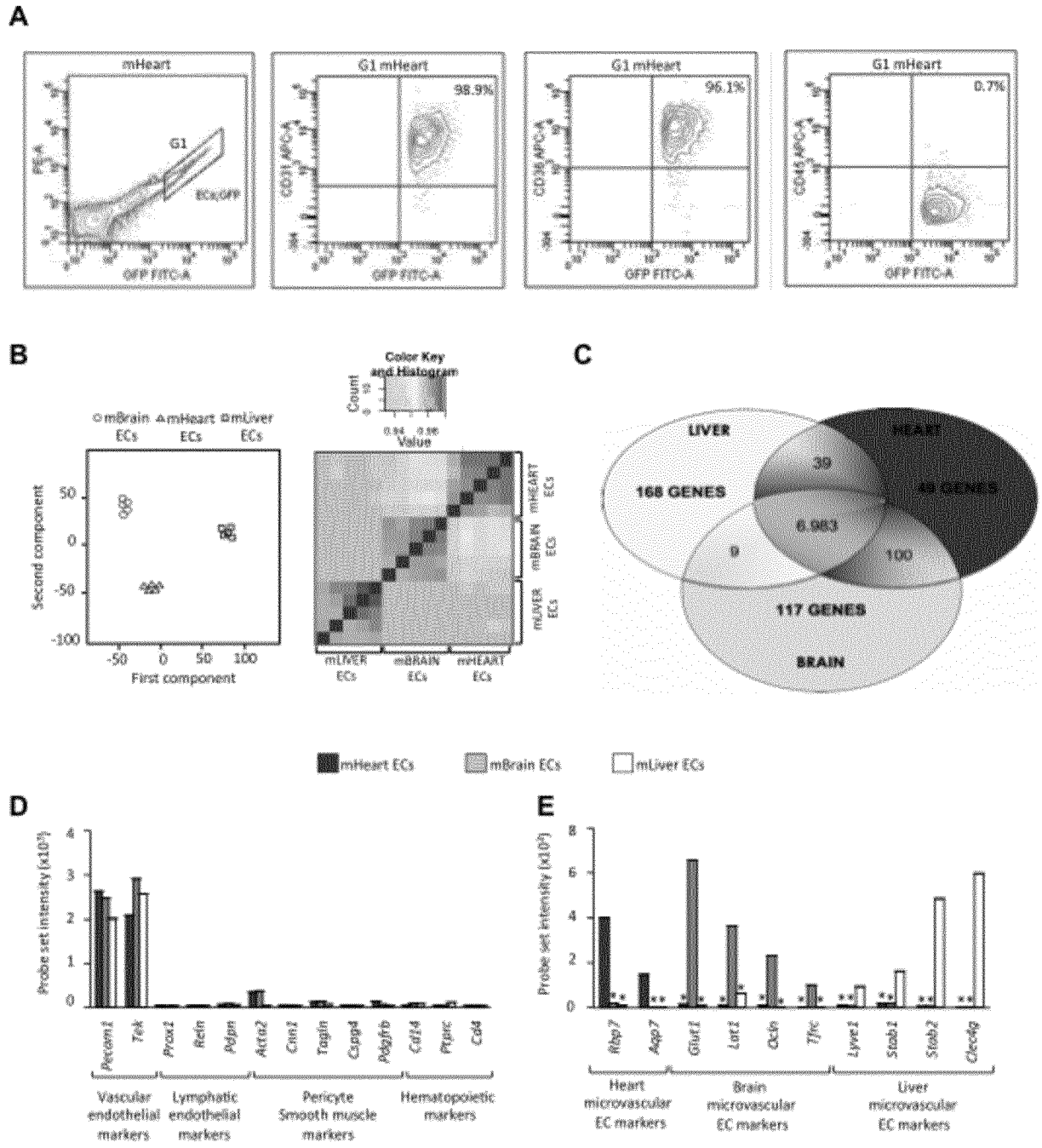


FIGURE 4A-C: *Validation of the liver (A), brain (B) and heart (C) EC fingerprints.*

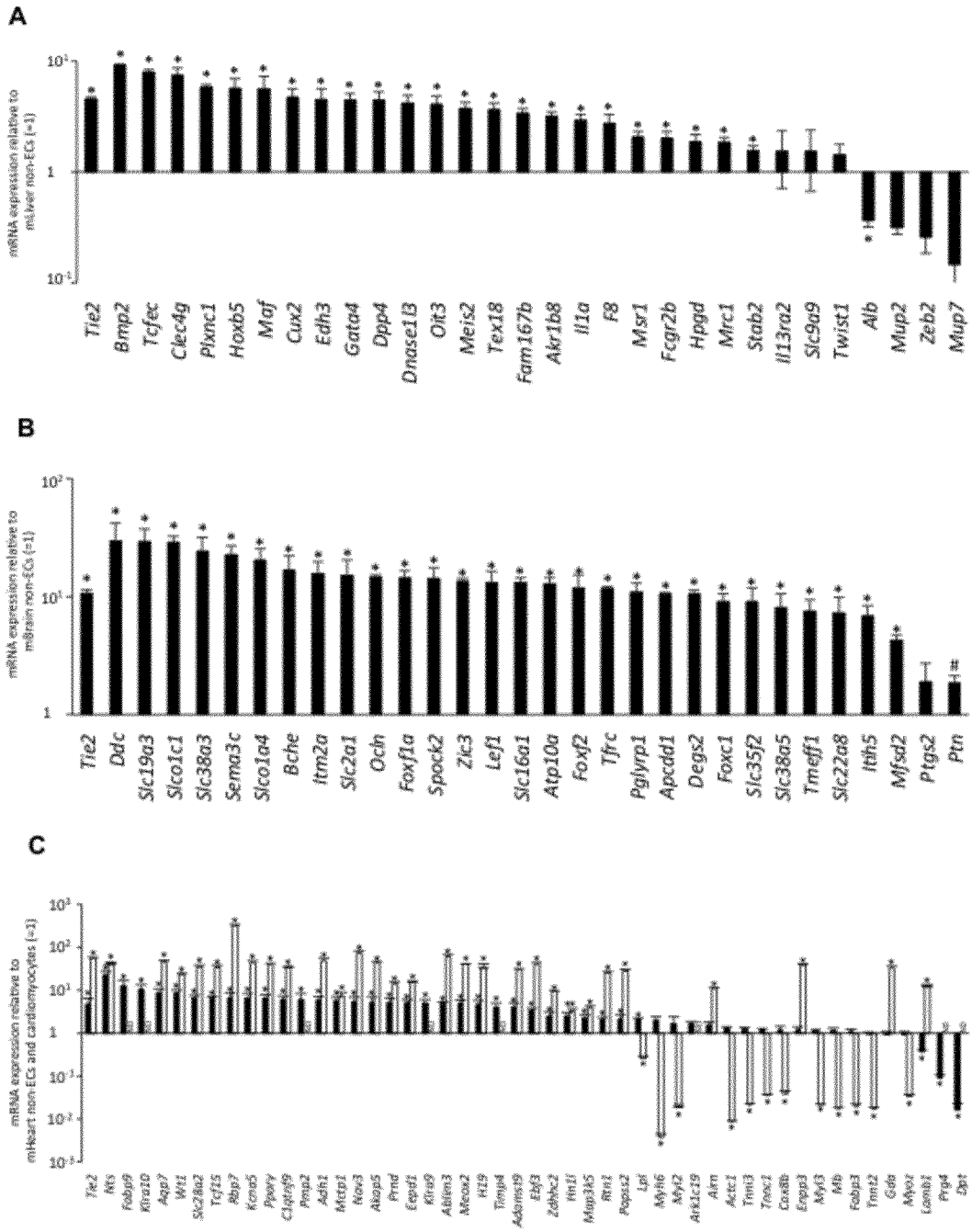


FIGURE 5: *Microarray probe intensities for the genes of the validated heart EC fingerprint in heart, brain and liver ECs.*

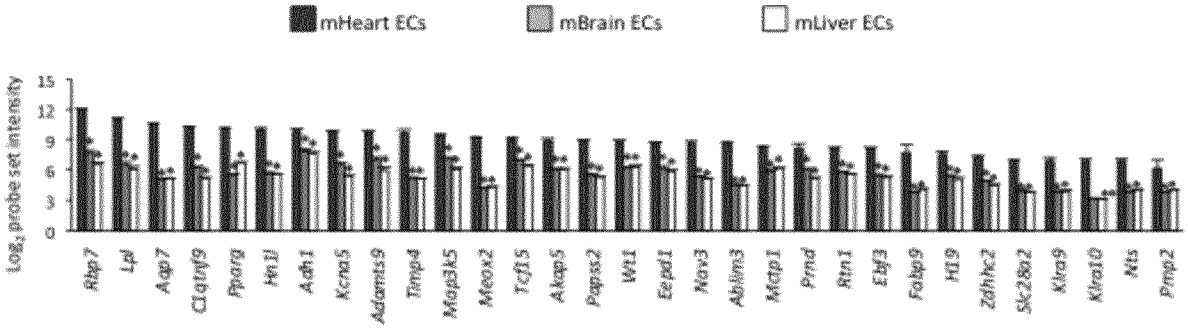


FIGURE 6A,B: *The heart microvascular EC fingerprint in ECs from additional tissues.*

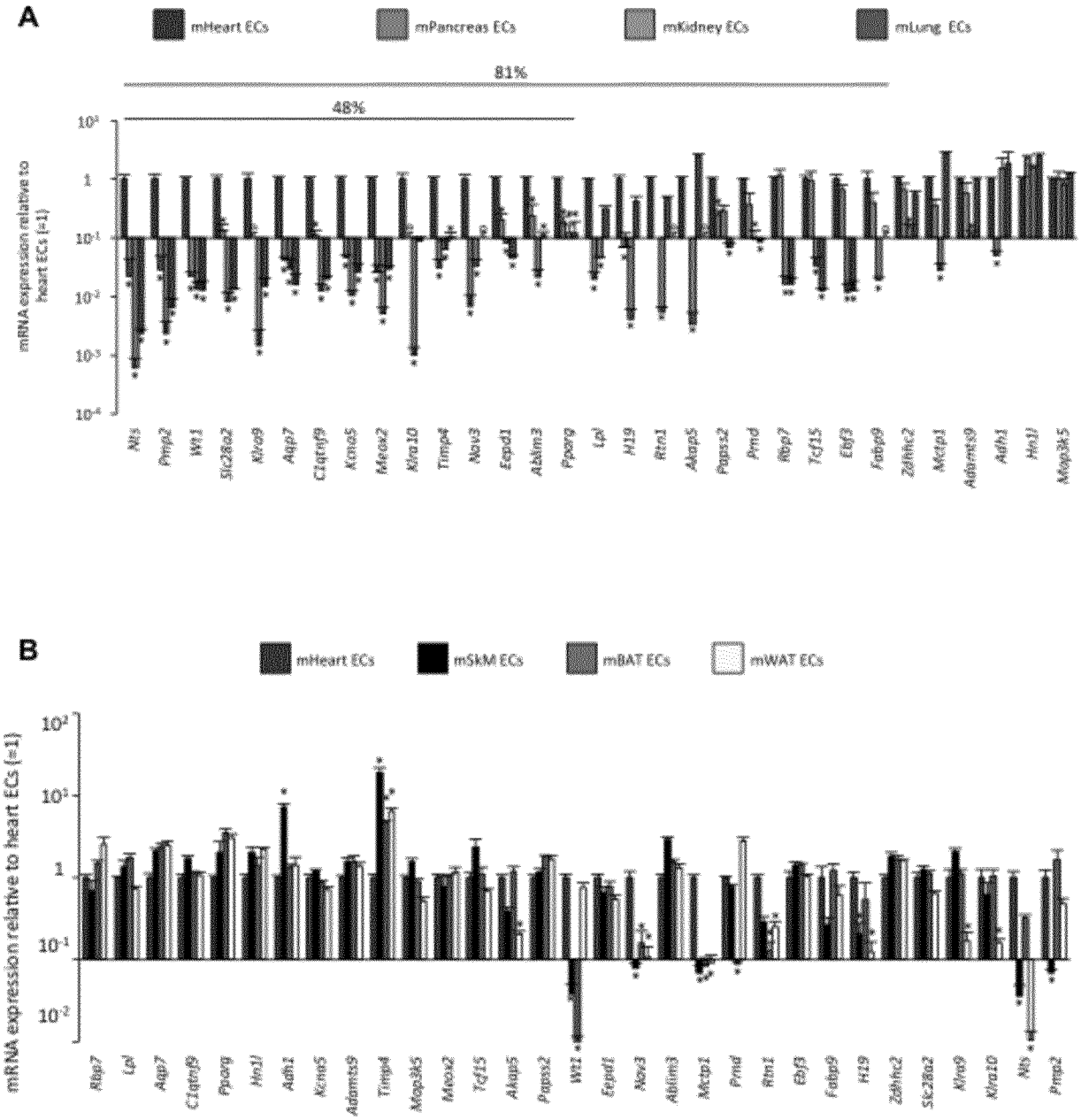


FIGURE 7: *The heart microvascular EC fingerprint in human samples.*

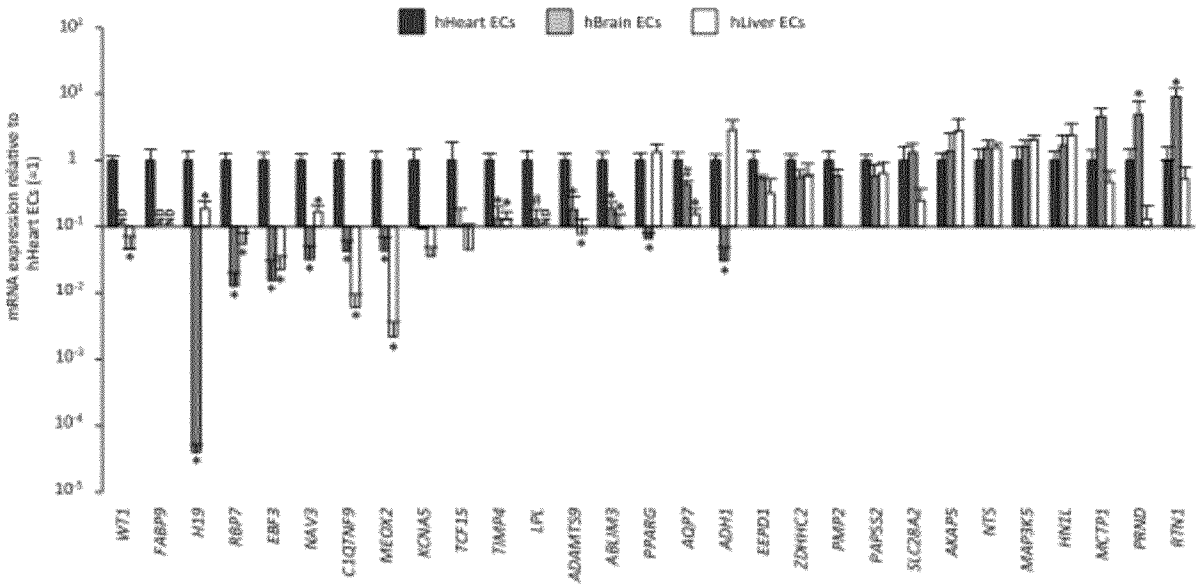


FIGURE 8A,B: *The heart microvascular EC fingerprint in human heart ECs in culture.*

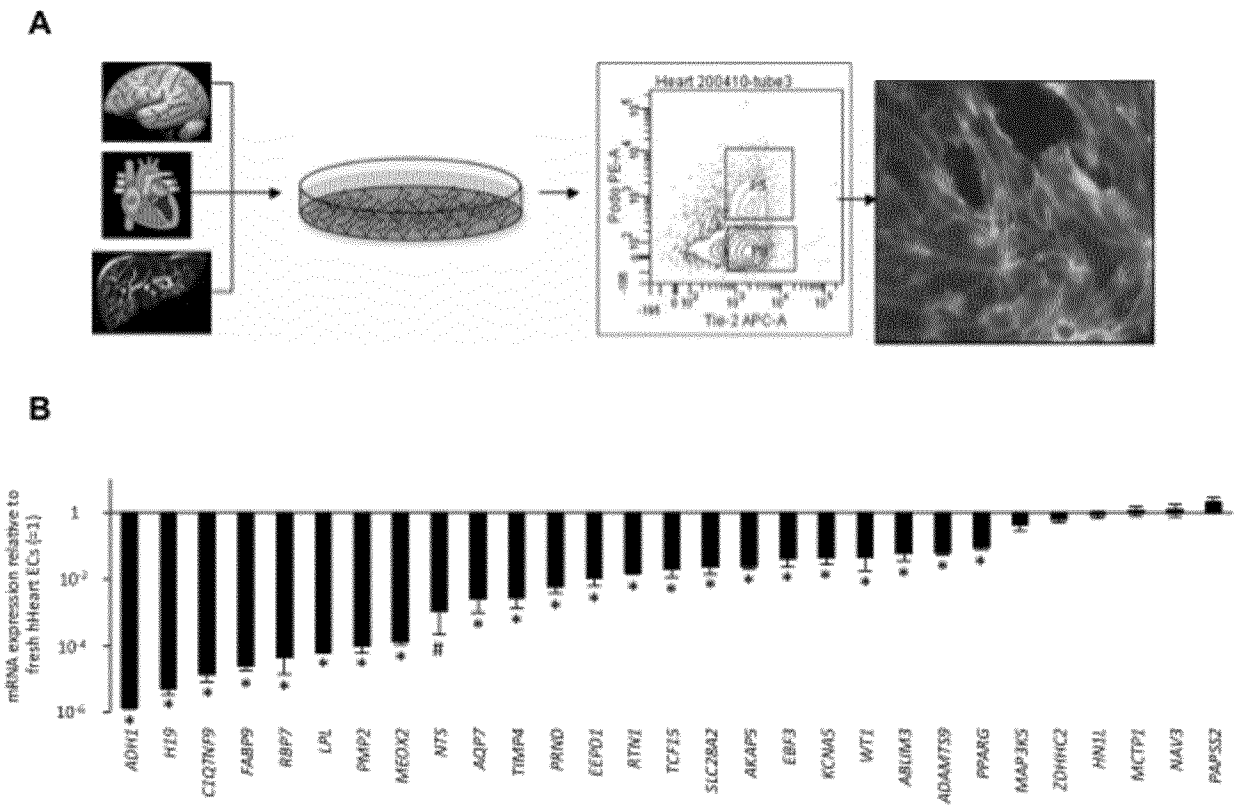


FIGURE 9A,B: *Validation of Meox2 and Tcf15 expression in heart ECs at protein level.*

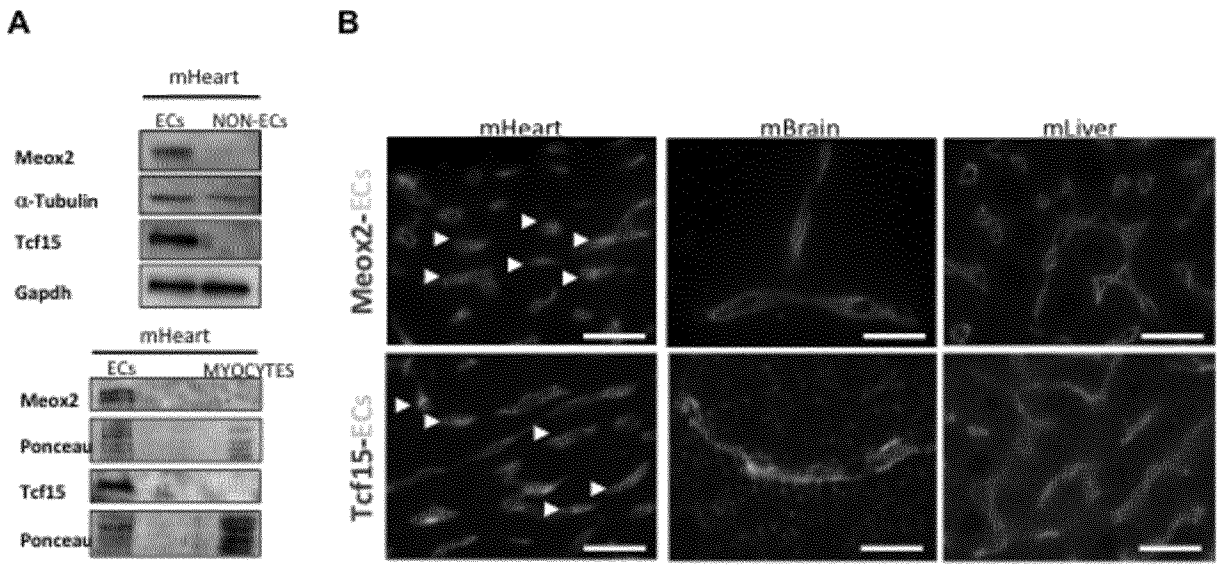


FIGURE 10A,B: *Effect of TF overexpression on the heart EC signature in cultured human heart ECs.*

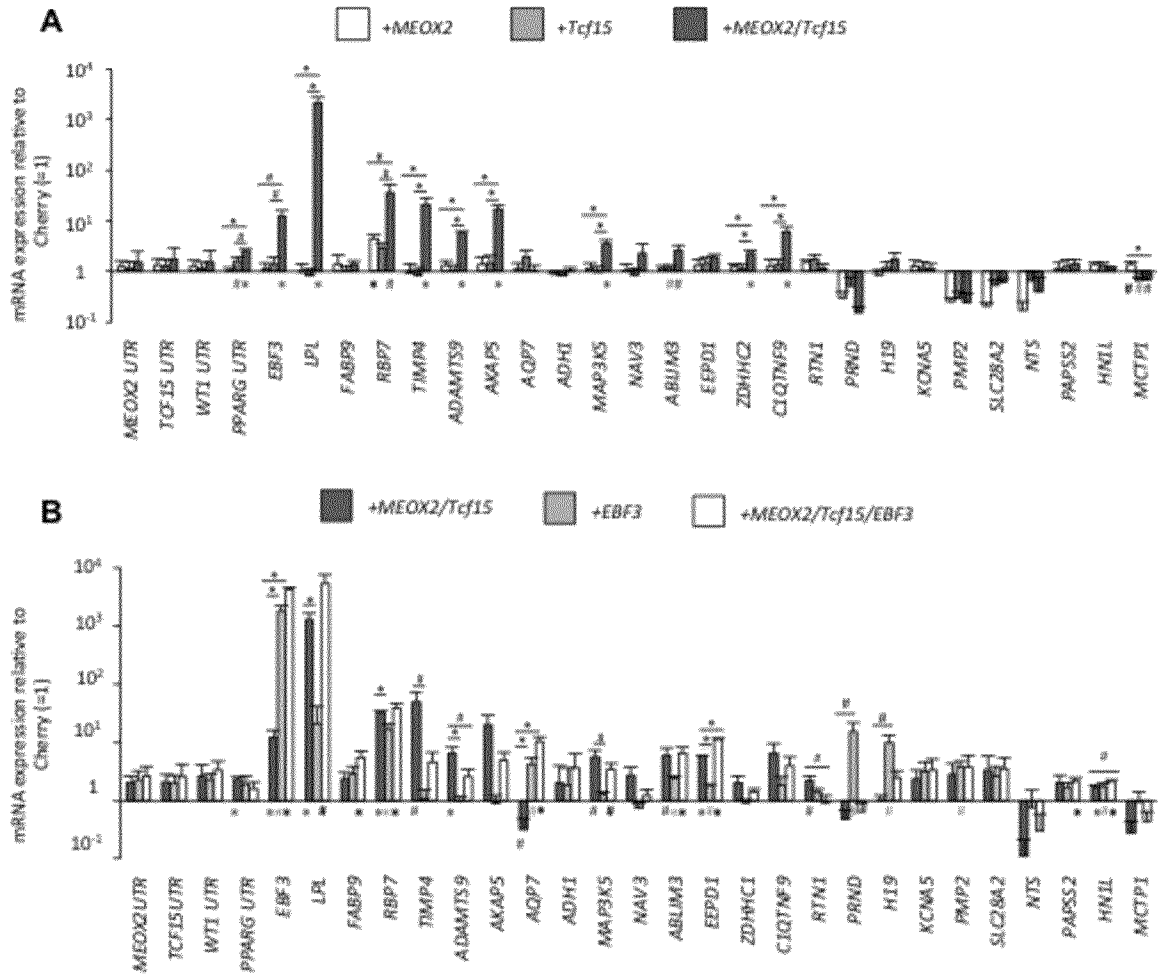


FIGURE 10C-E: *Effect of TF overexpression on the heart EC signature in cultured human heart ECs, continued.*

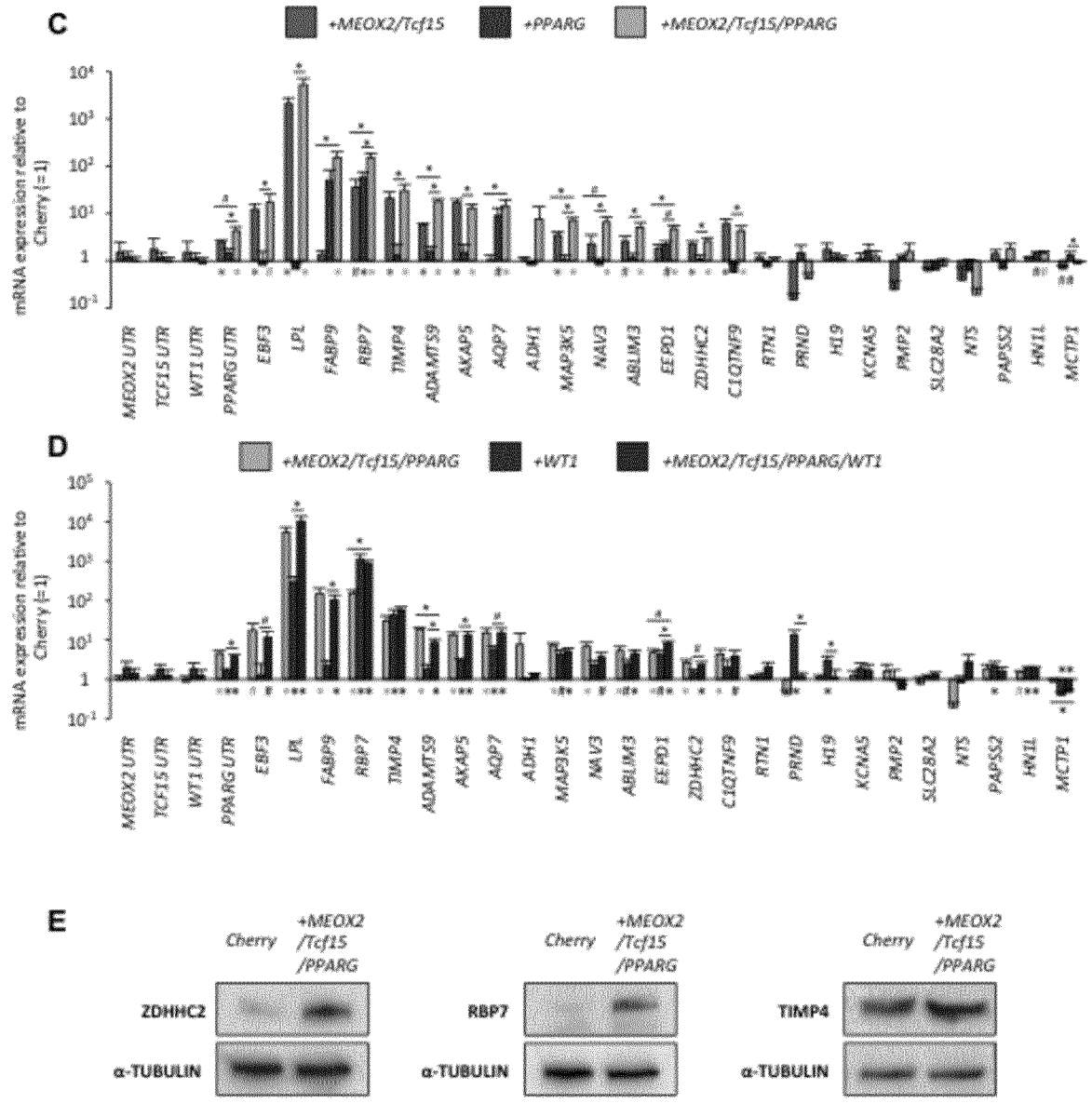


FIGURE 11A,B: *Effect of TF overexpression on fatty acid uptake in cultured human heart ECs.*

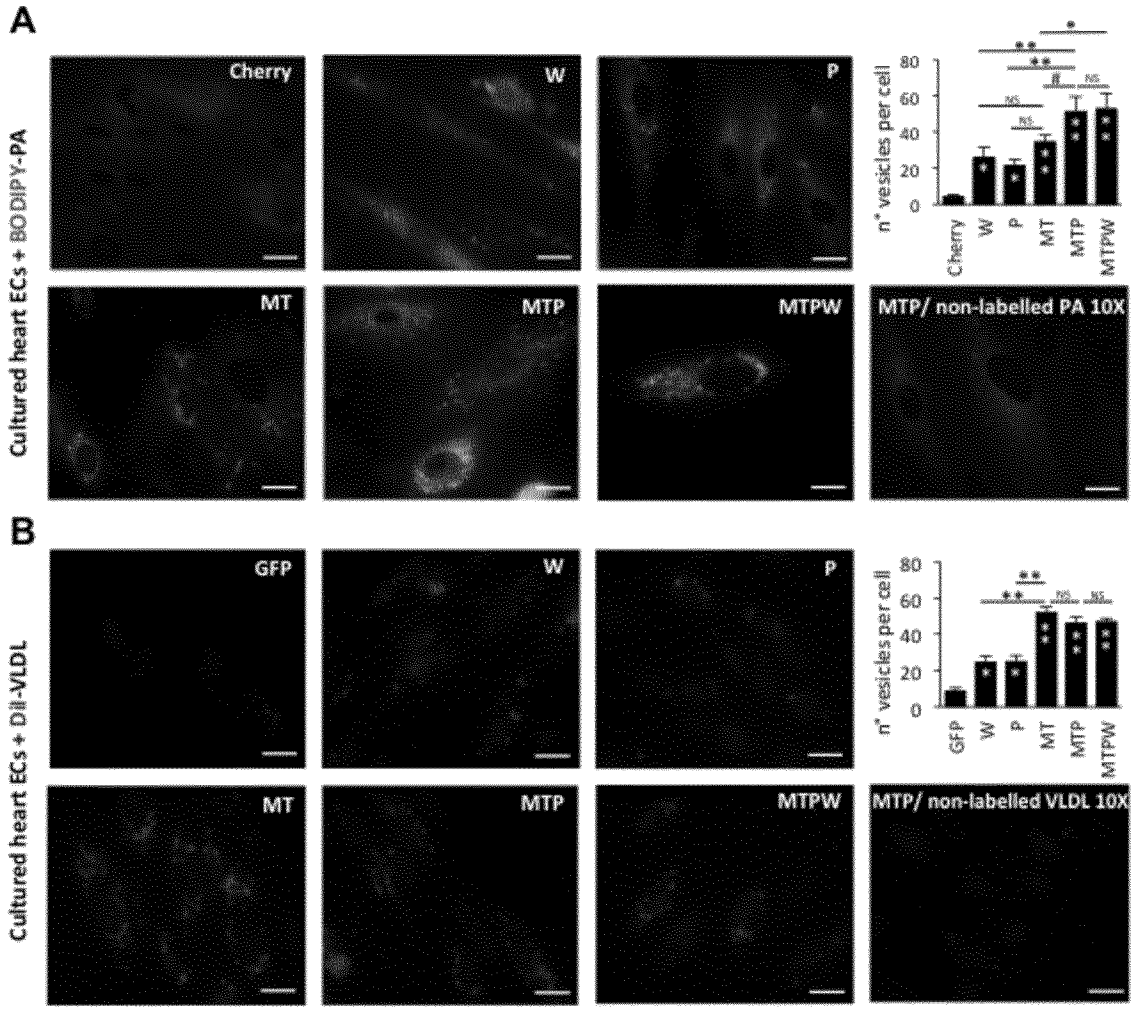


FIGURE 12: *Effect of TF overexpression on the heart EC signature in human BOECs.*

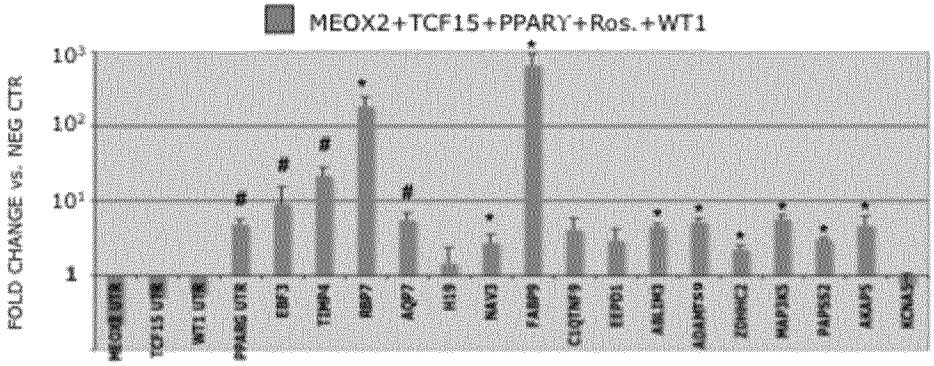


FIGURE 13: *Expression pattern of the heart EC fingerprint in Meox2$^{+/-}$:Tcf15$^{+/-}$ mice.*

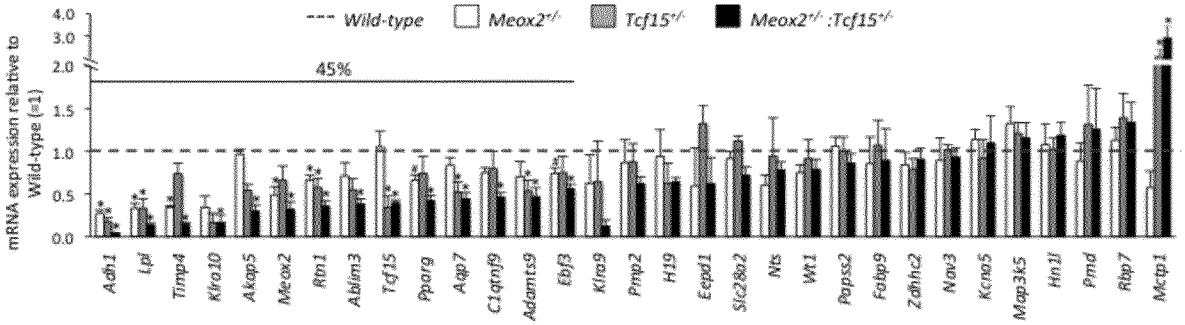


FIGURE 14A,B: *Analysis of genes outside the heart EC fingerprint in Meox2$^{+/-}$:Tcf15$^{+/-}$ mice.*

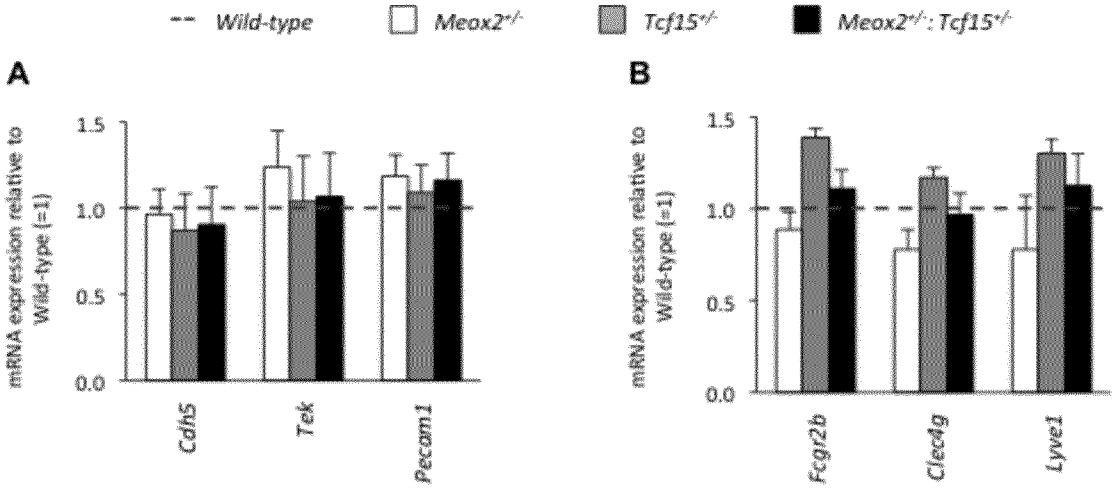


FIGURE 15: *Microarray probe-set intensity for glucose and fatty acid transporter-related genes.*

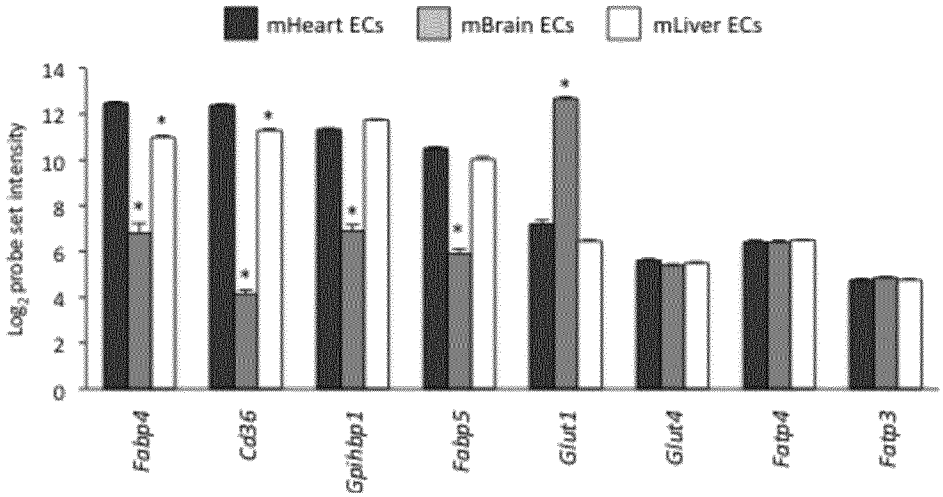


FIGURE 16A-C: *Glucose and fatty acid transporter-related gene expression profile in Meox2$^{+/-}$:Tcf15$^{+/-}$ mice.*

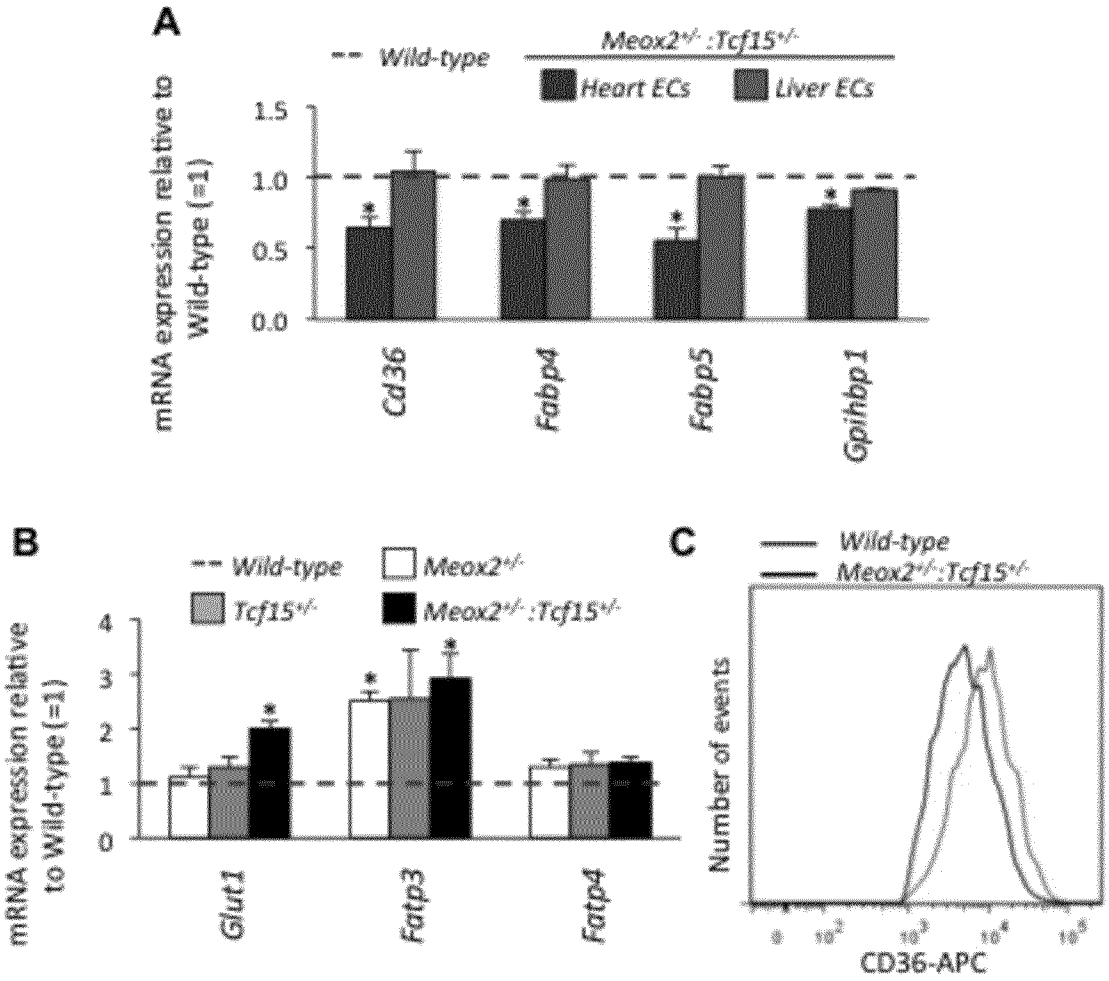


FIGURE 17: *Effect of Meox2 and Tcf15 combined heterozygous deficiency on fatty acid uptake in cardiomyocytes.*

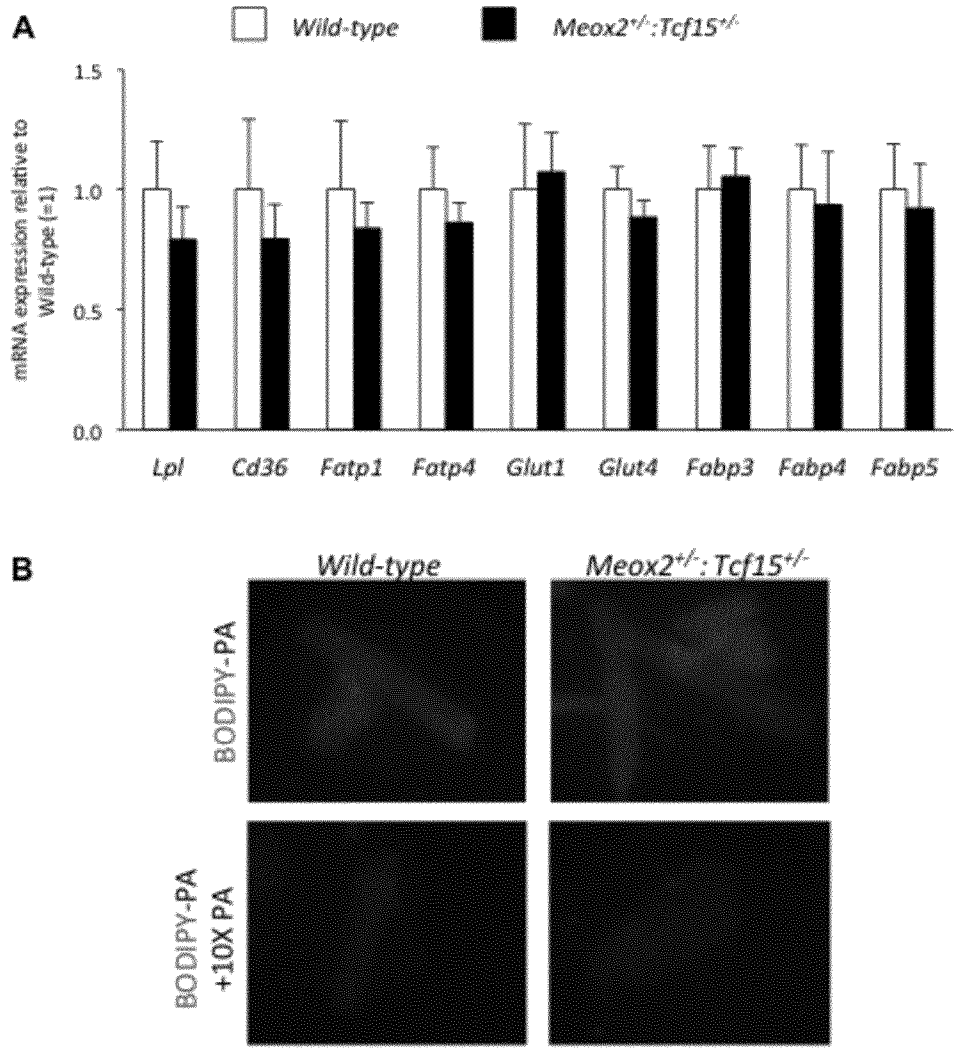


FIGURE 18: *Meox2$^{+/-}$:Tcf15$^{+/-}$ mice have lower fatty acid uptake and higher glucose uptake in the heart.*

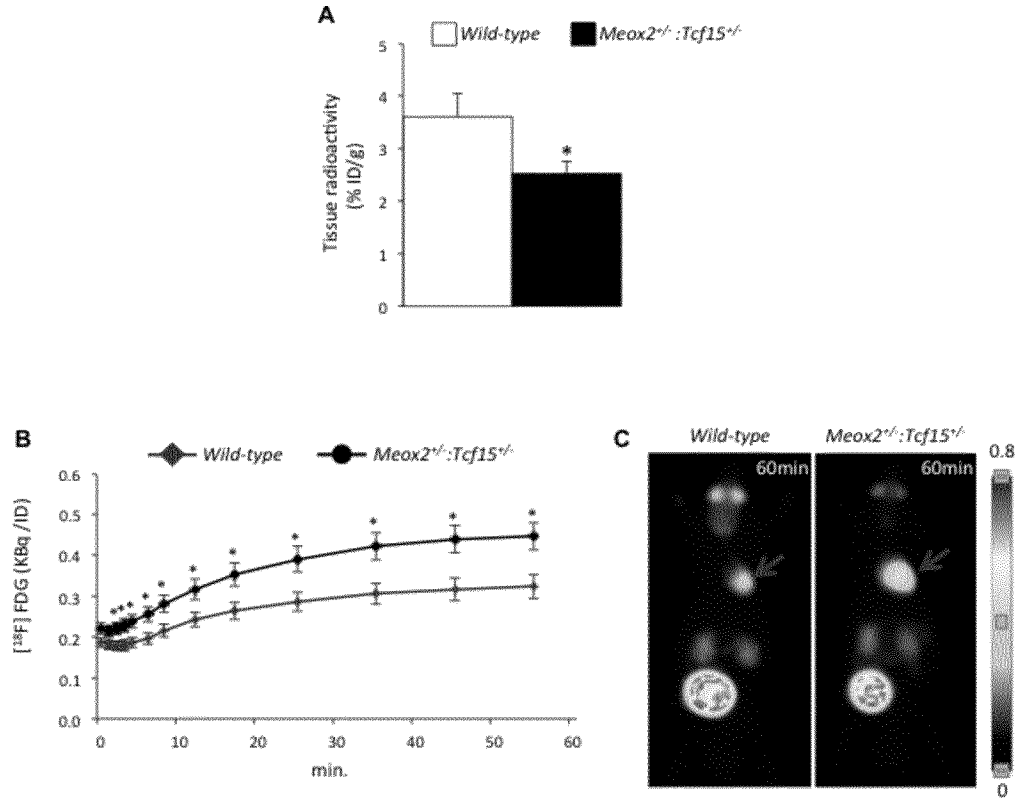


FIGURE 19: *Young Meox2$^{+/-}$:Tcf15$^{+/-}$ mice have lower neutral lipid storage in the heart but do not develop cardiac fibrosis or systolic dysfunction.*

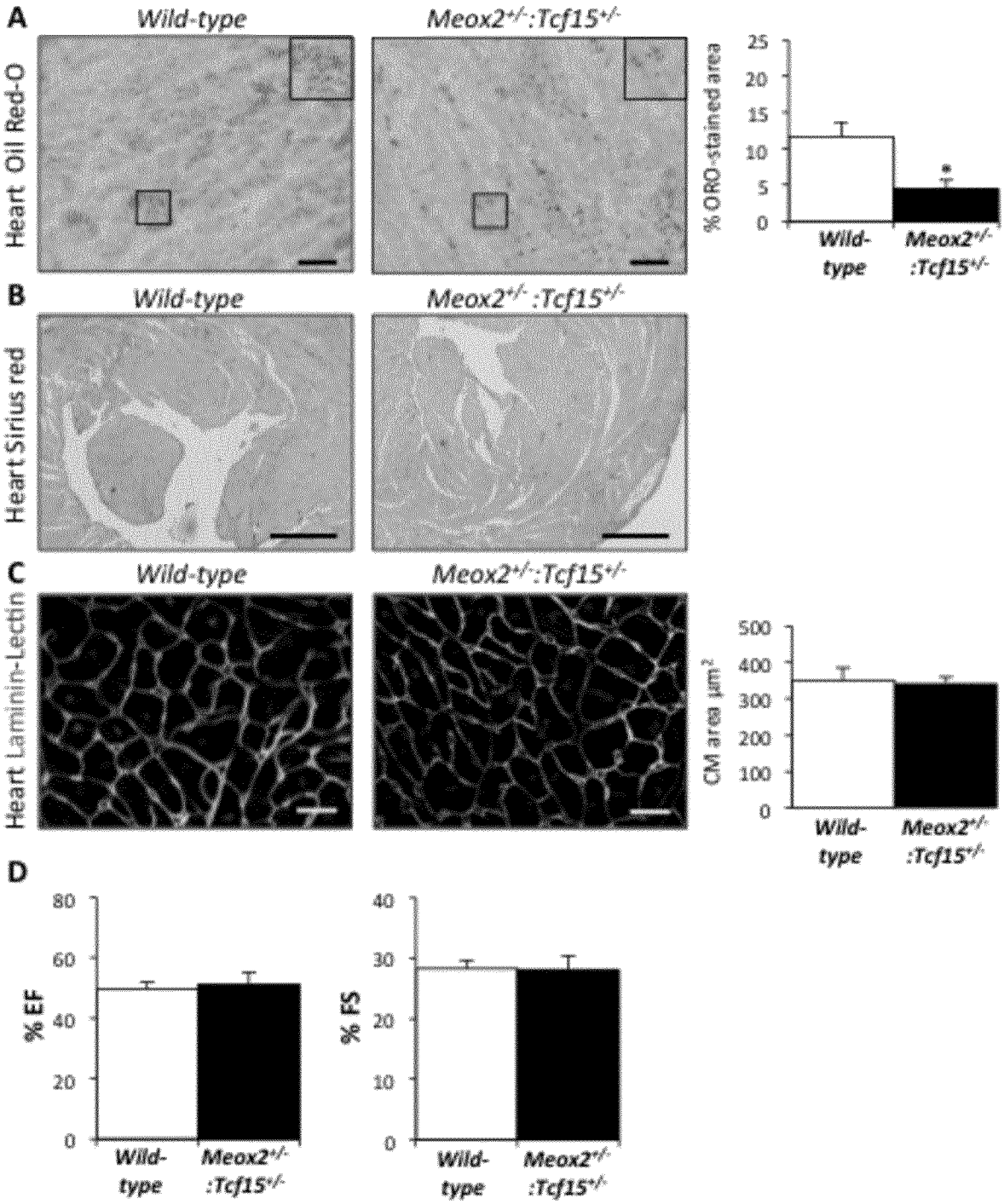


FIGURE 20: *Aged Meox2$^{+/-}$:Tcf15$^{+/-}$ mice develop cardiac fibrosis and systolic dysfunction.*

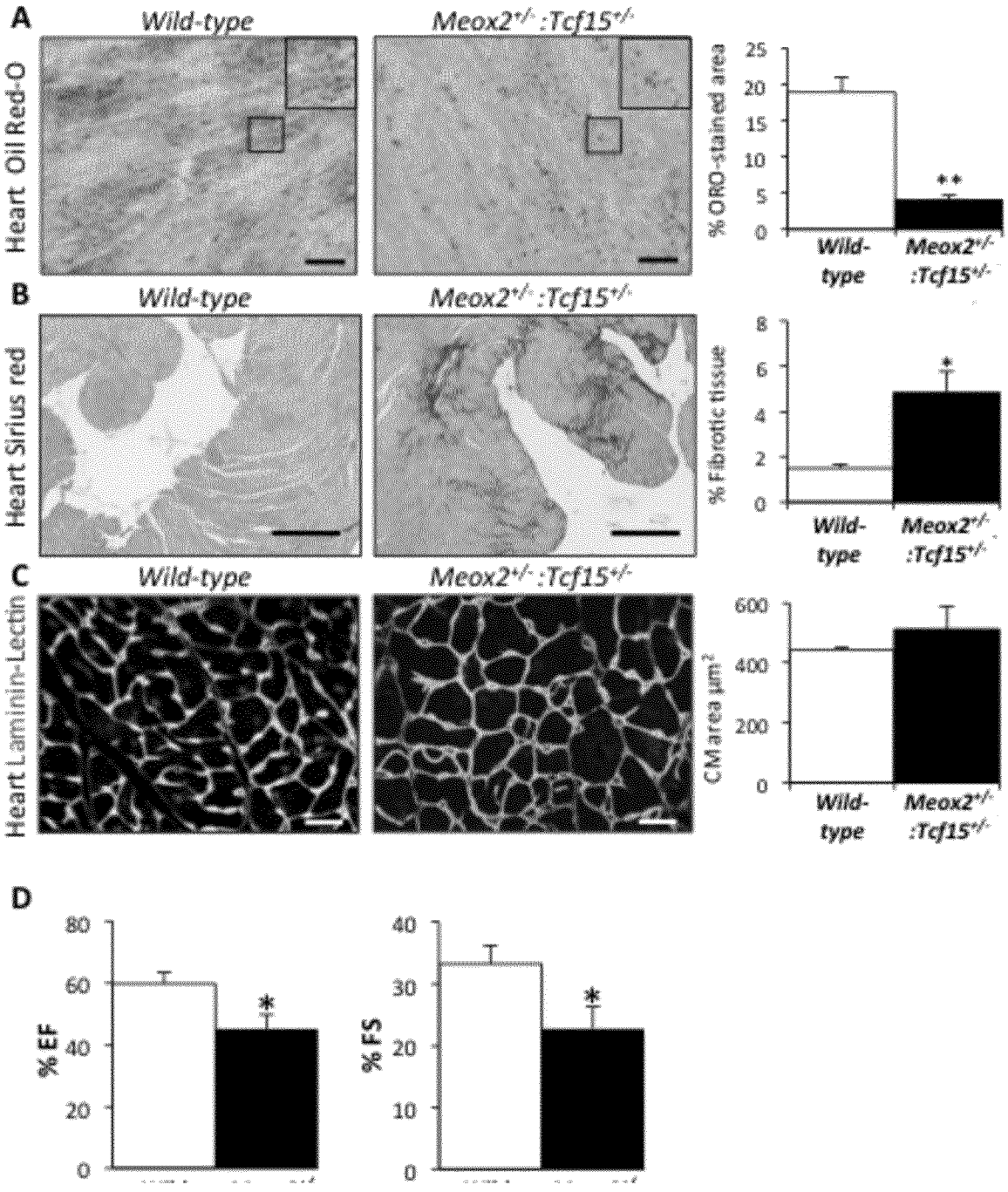


FIGURE 21: *Graphic summary of the findings in Example 1.*

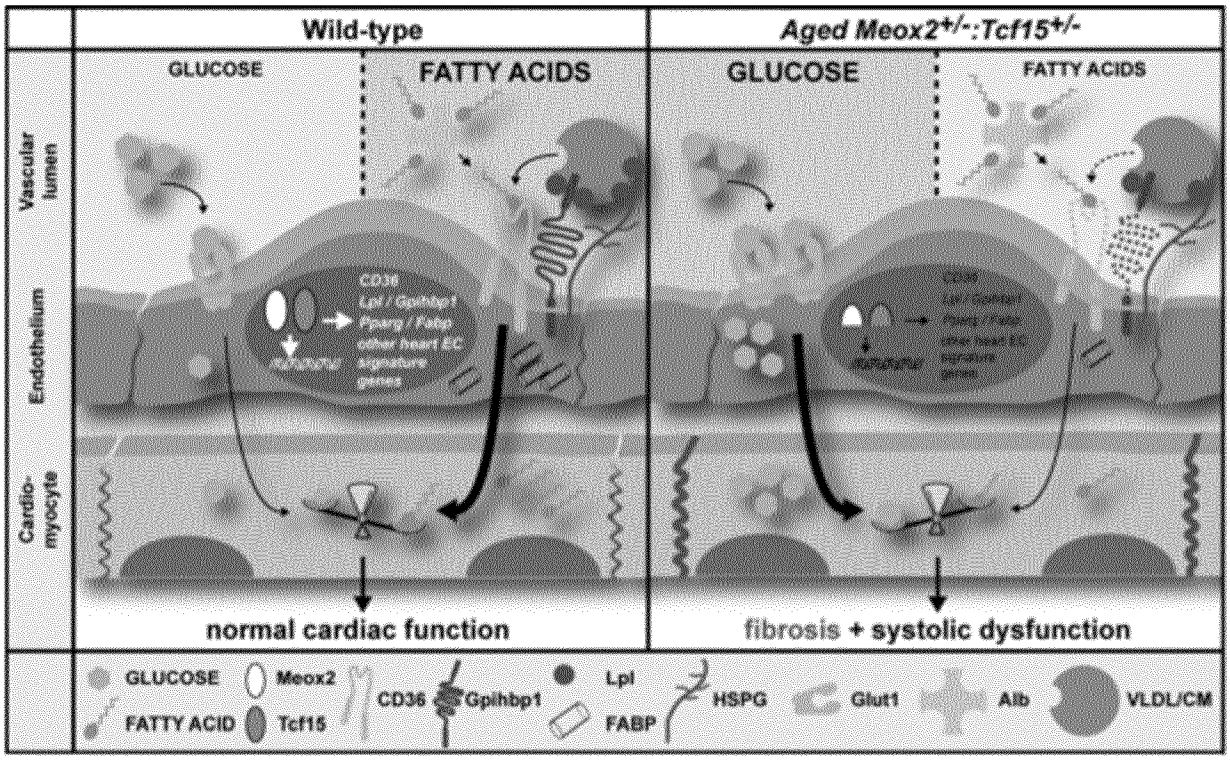


FIGURE 22A,B: *is a graphical display of the purity of EC preparations from human umbilical cord.*

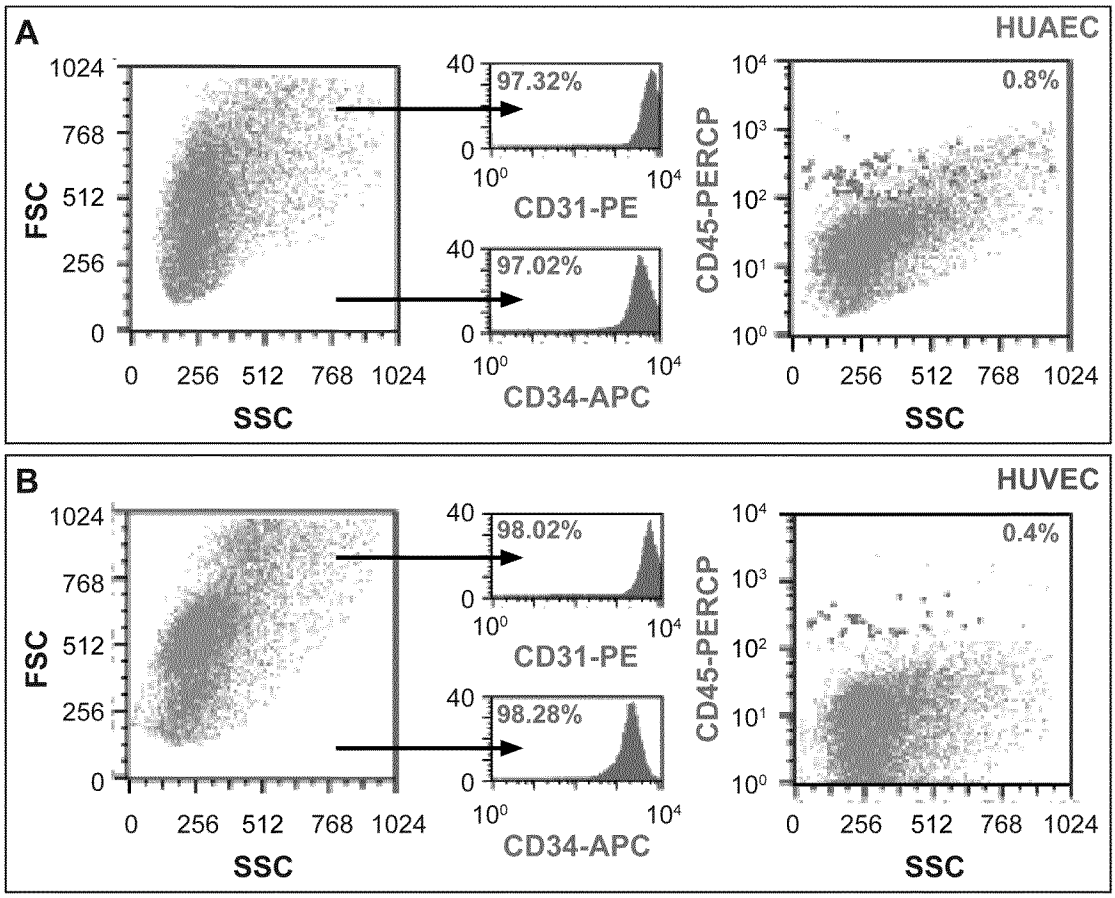


FIGURE 23A: *displays the lentiviral maps for overexpression of the arterial TFs.*

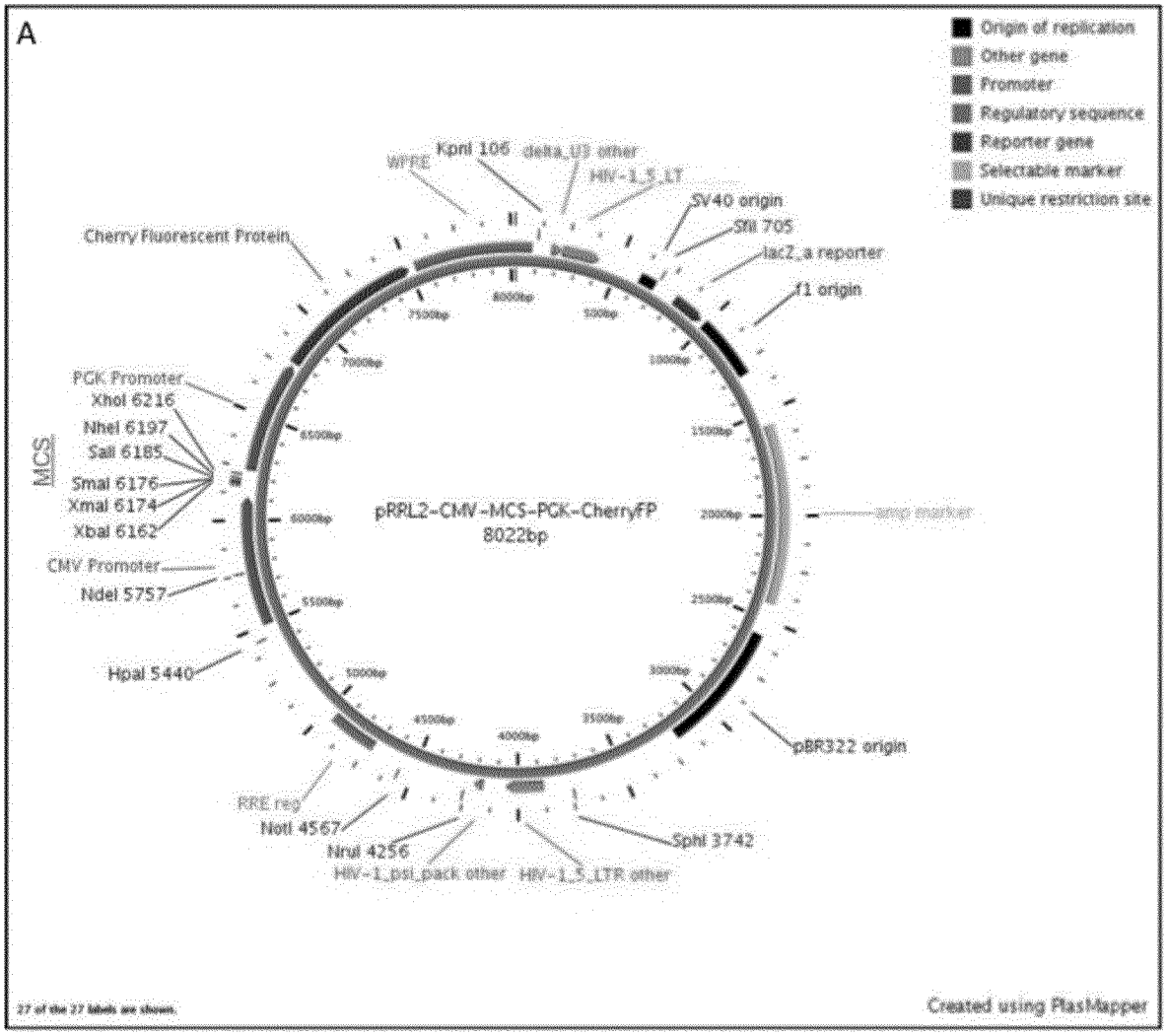


FIGURE 23B: *displays the lentiviral maps for overexpression of the arterial TFs.*

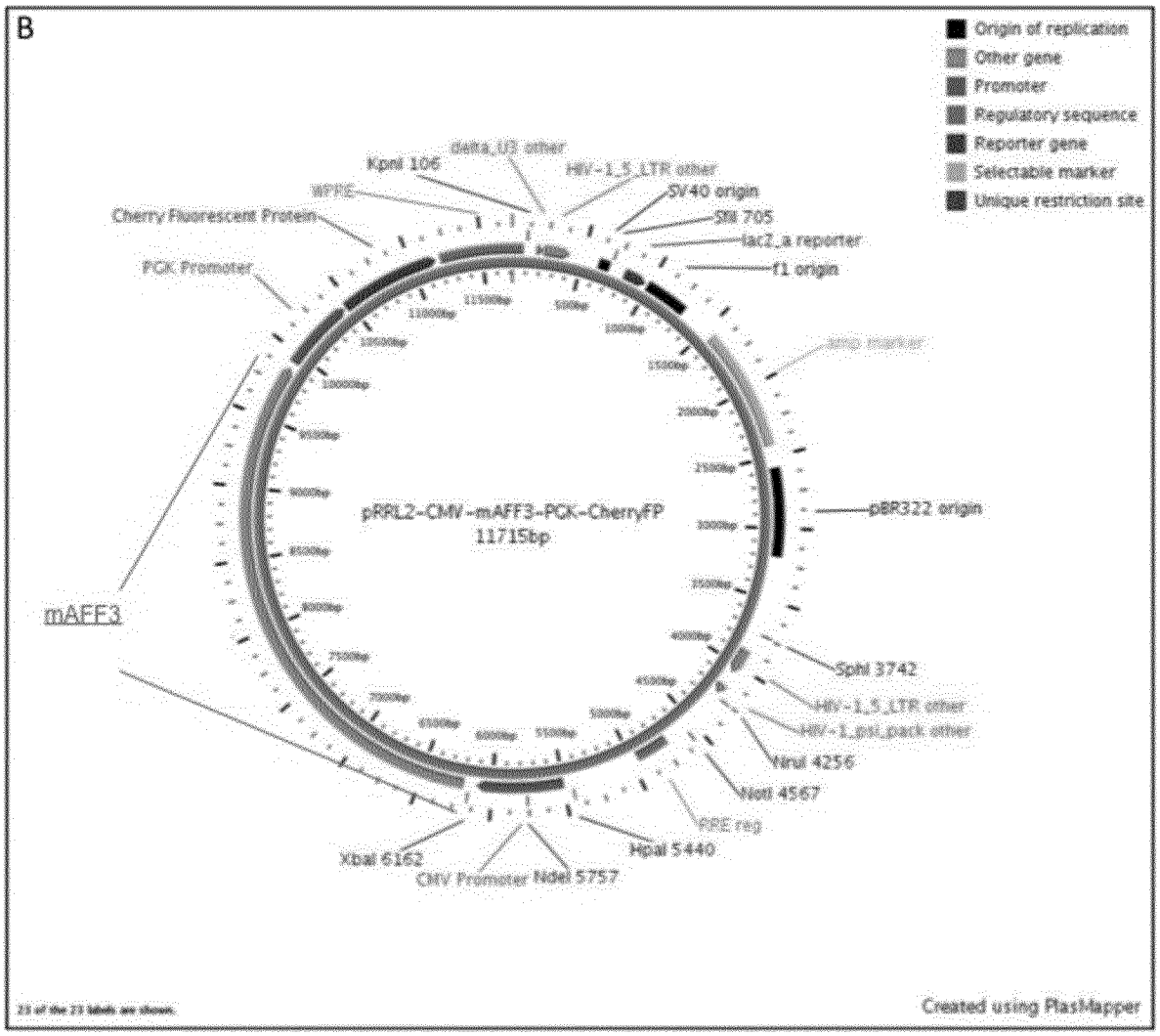


FIGURE 23C: *displays the lentiviral maps for overexpression of the arterial TFs.*

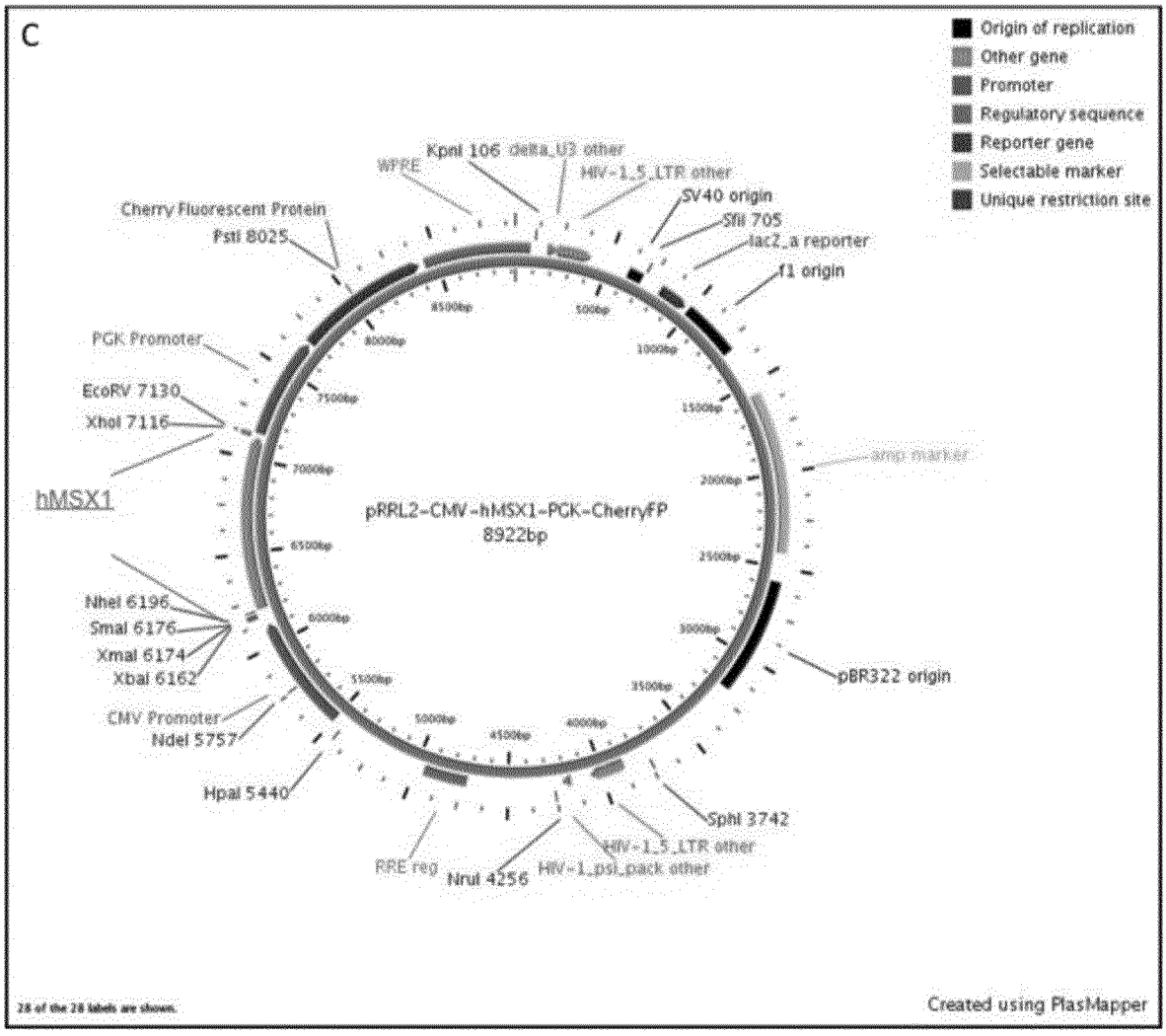


FIGURE 23D: *displays the lentiviral maps for overexpression of the arterial TFs.*

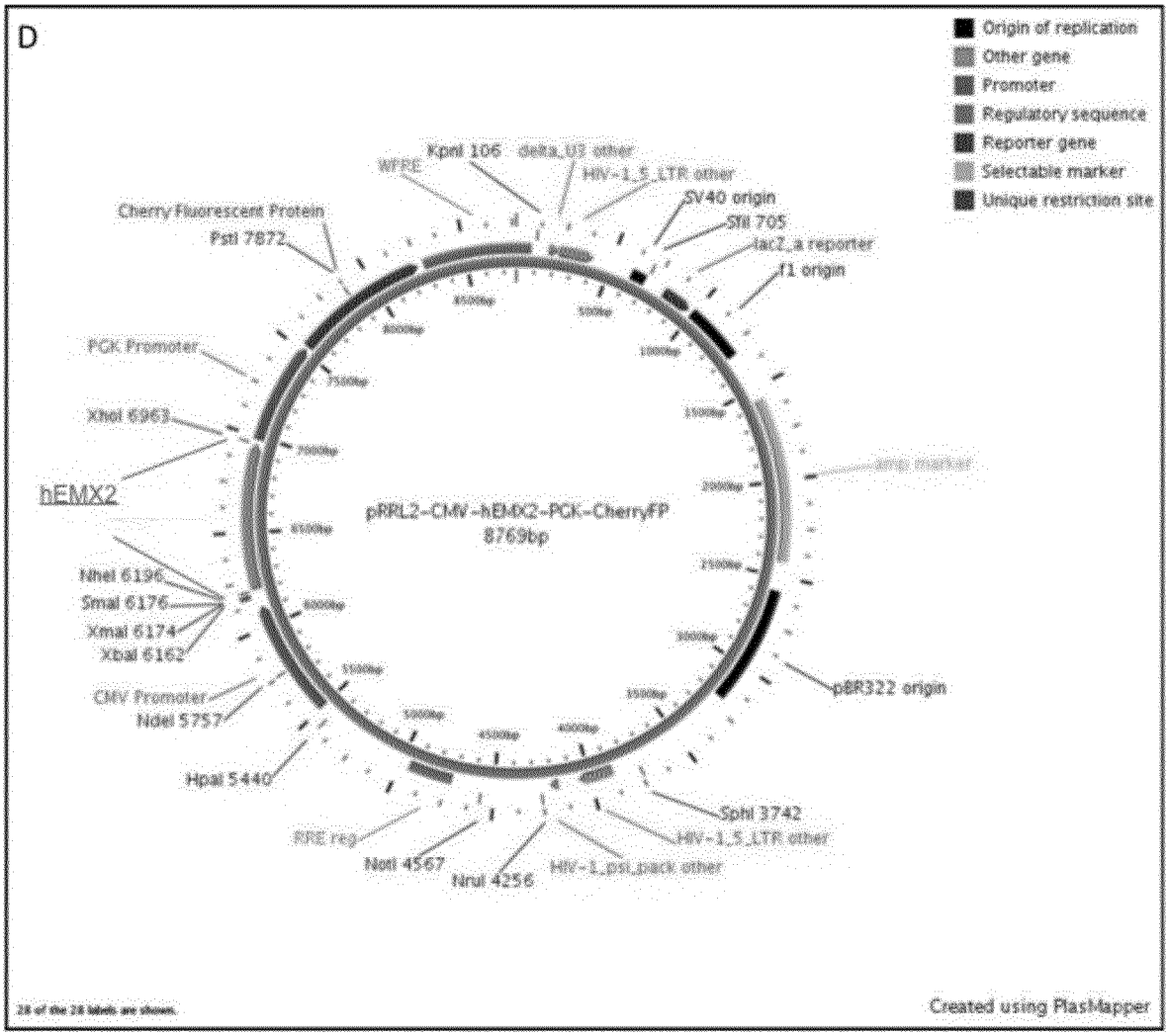


FIGURE 23E: *displays the lentiviral maps for overexpression of the arterial TFs.*

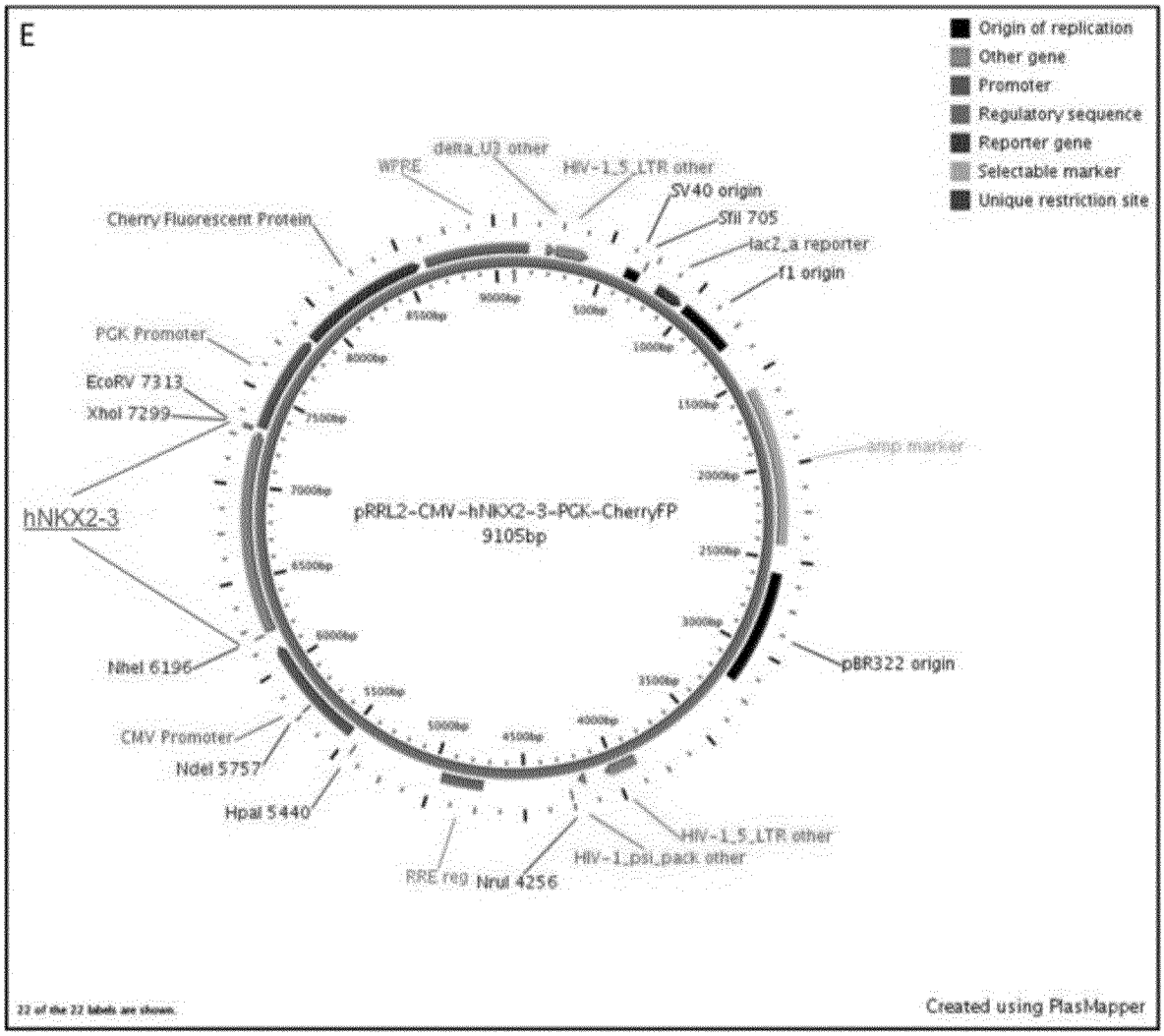


FIGURE 23F: *displays the lentiviral maps for overexpression of the arterial TFs.*

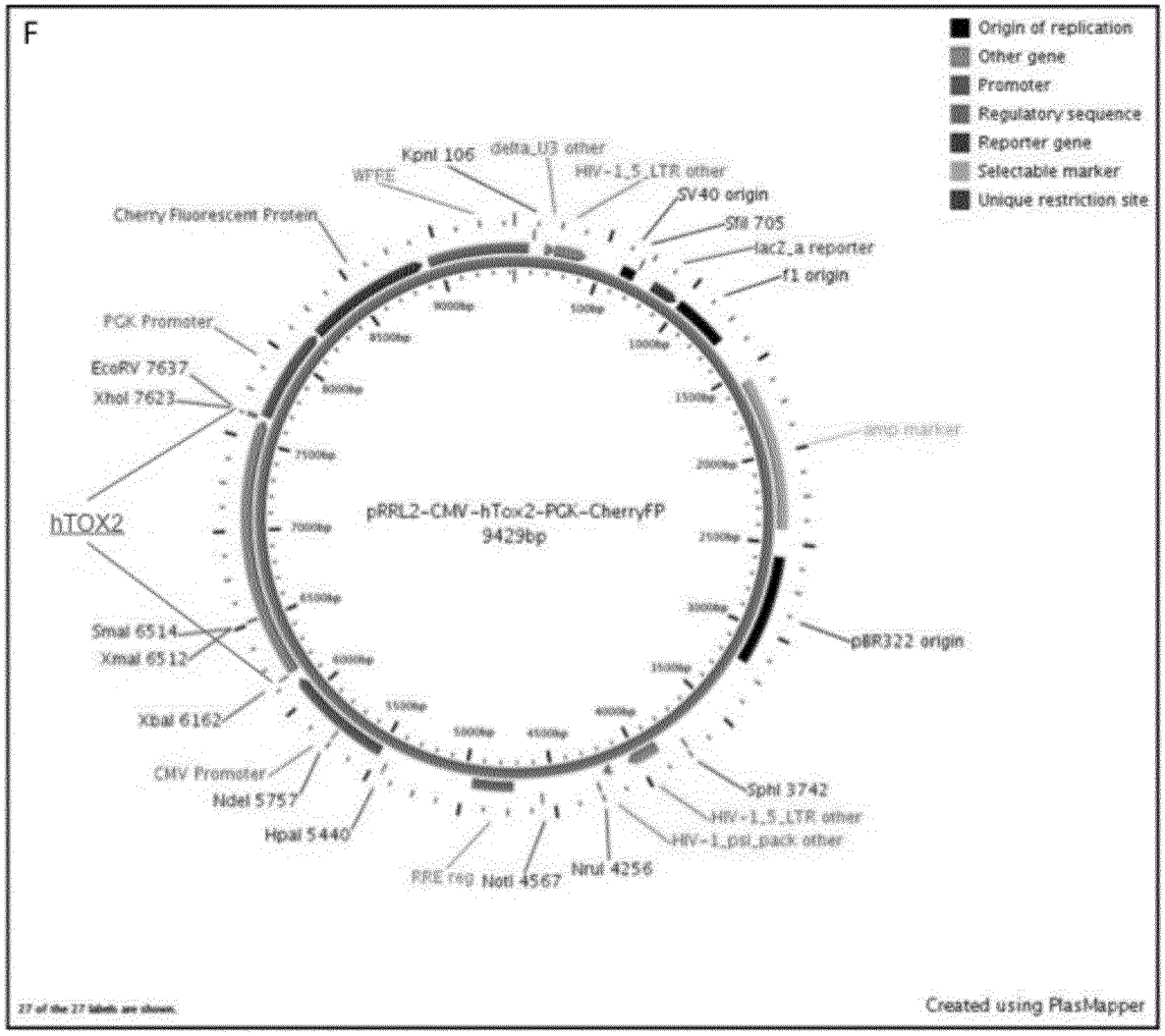


FIGURE 23G: *displays the lentiviral maps for overexpression of the arterial TFs.*

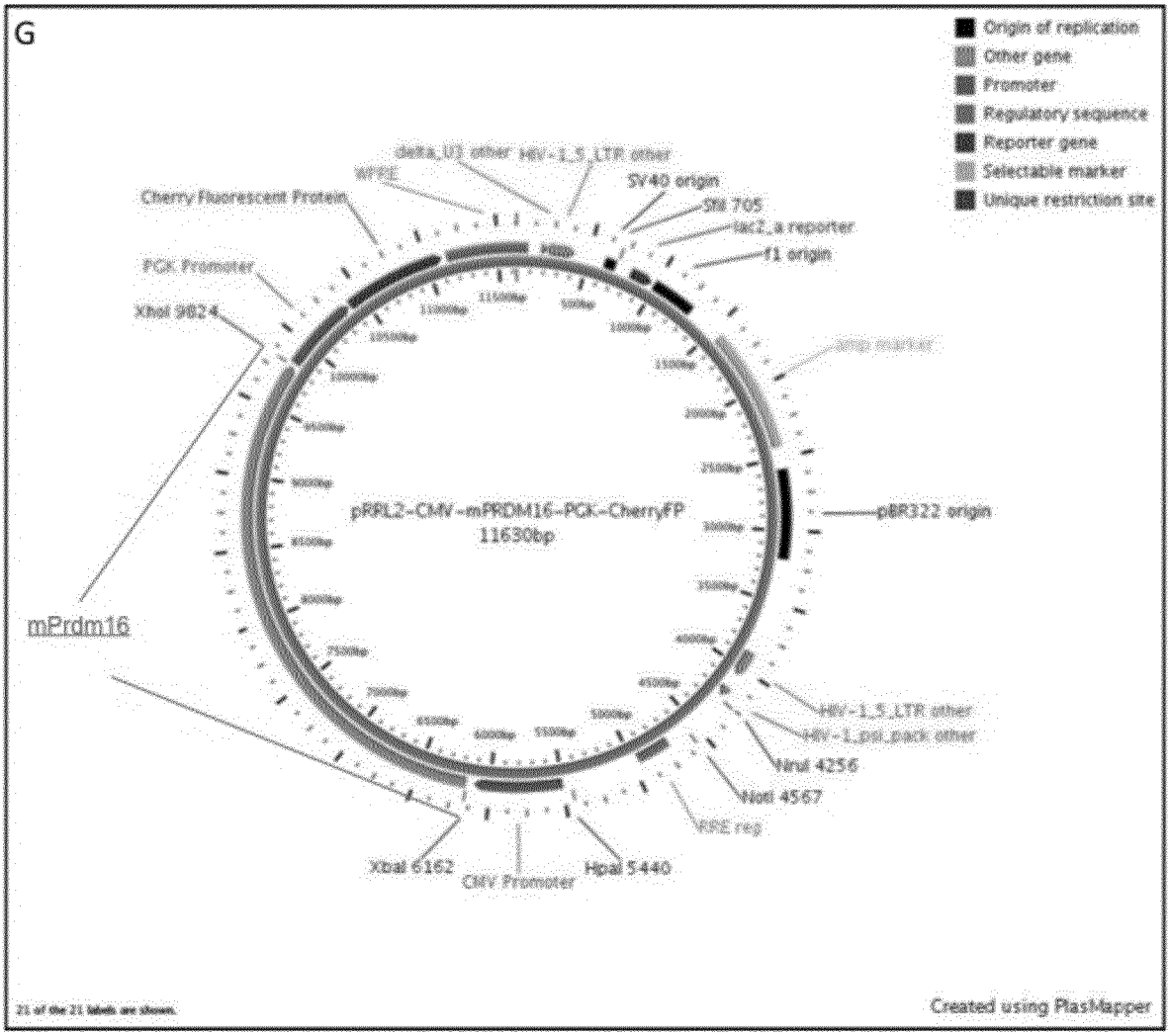


FIGURE 23H: *displays the lentiviral maps for overexpression of the arterial TFs.*

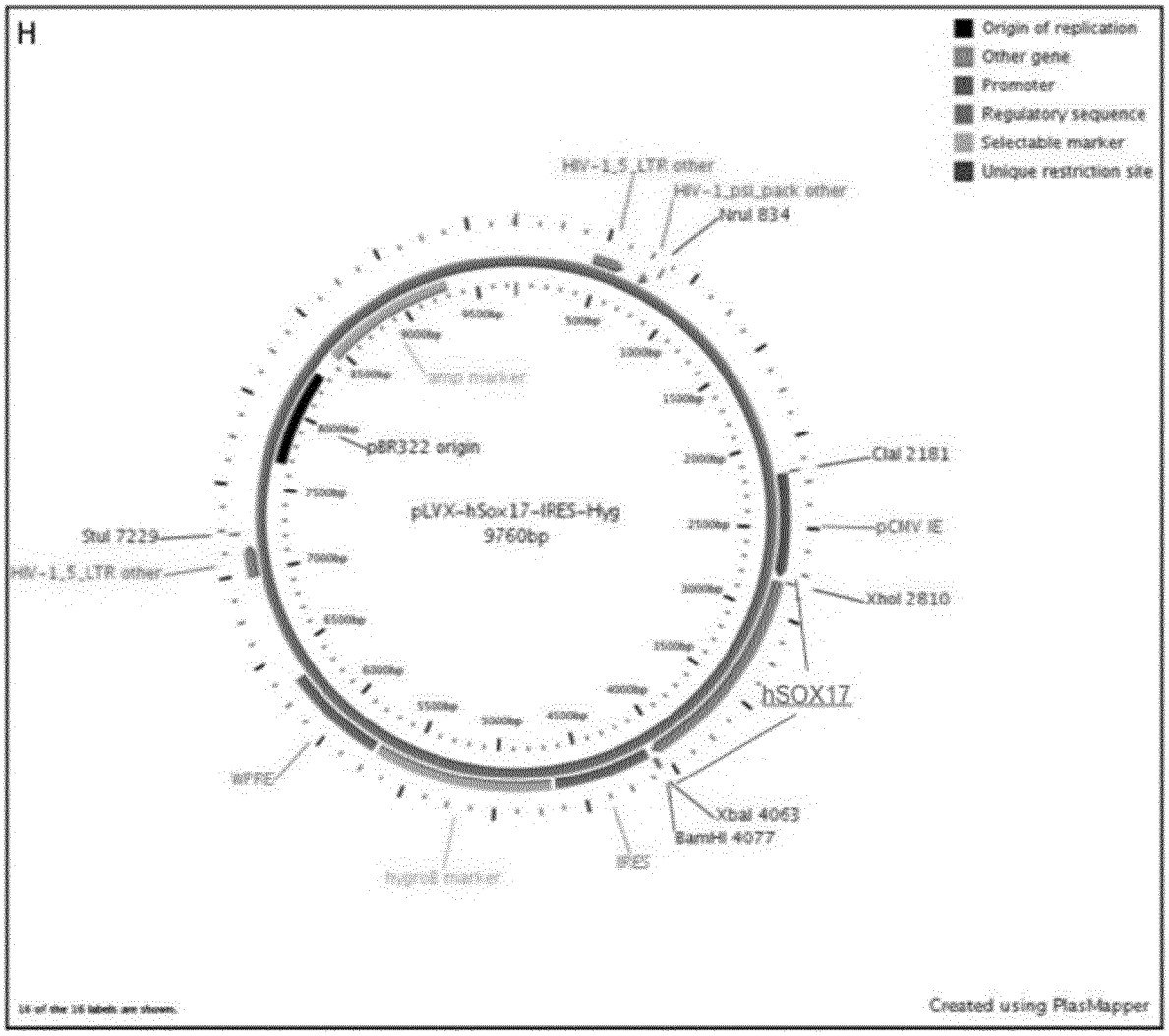


FIGURE 24: *provides a graphic display of how the cell culture process assimilates arterial and venous endothelial cells.*

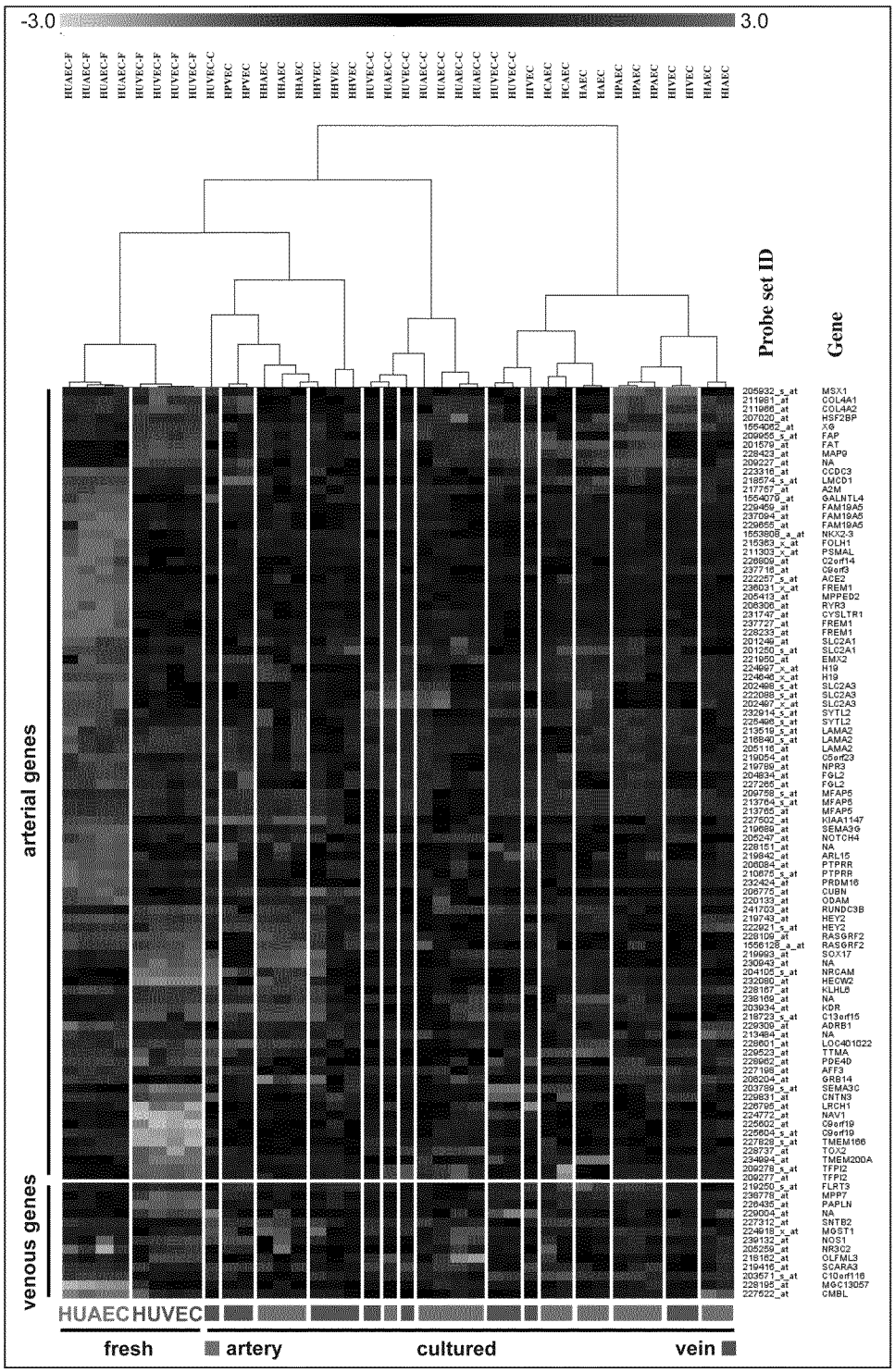


FIGURE 25A: *provides photographs and graphics on the genome-wide analysis of freshly isolated HUVECs and HUAECs.*

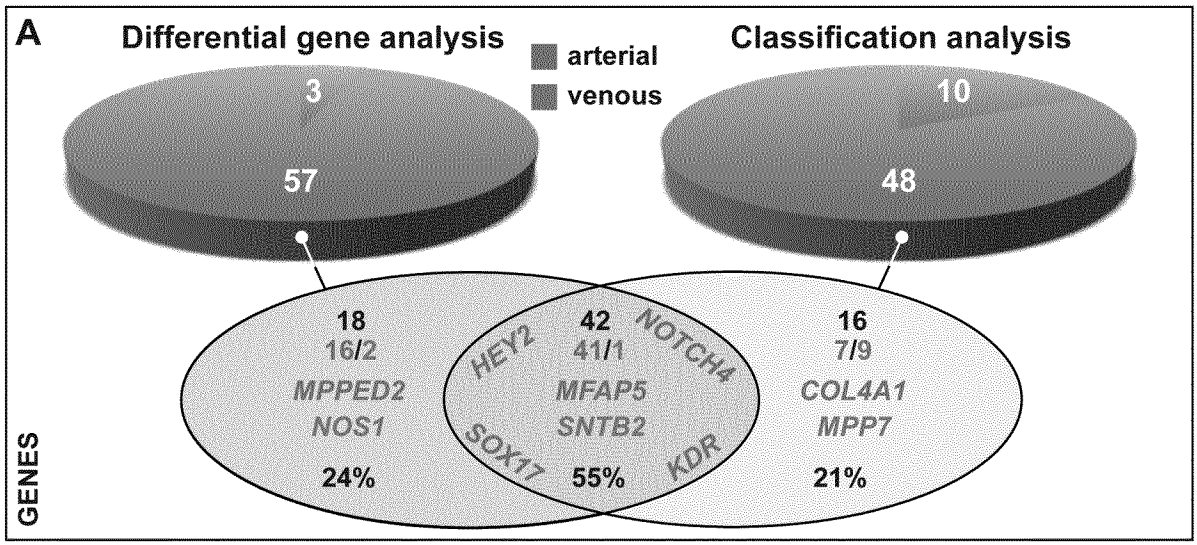


FIGURE 25B: *provides photographs and graphics on the genome-wide analysis of freshly isolated HUVECs and HUAECs.*

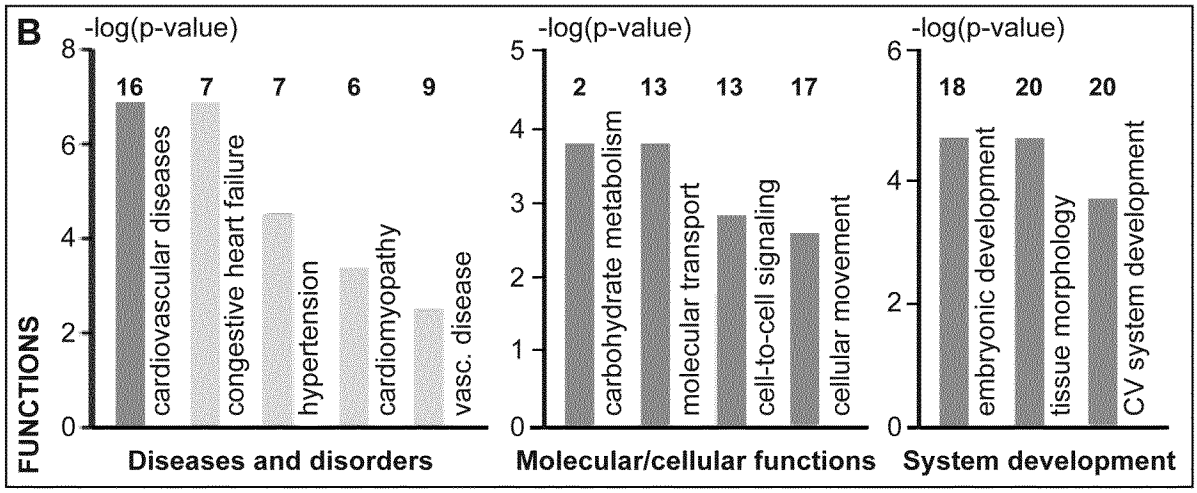


FIGURE 25C,D: *provides photographs and graphics on the genome-wide analysis of freshly isolated HUVECs and HUAECs.*

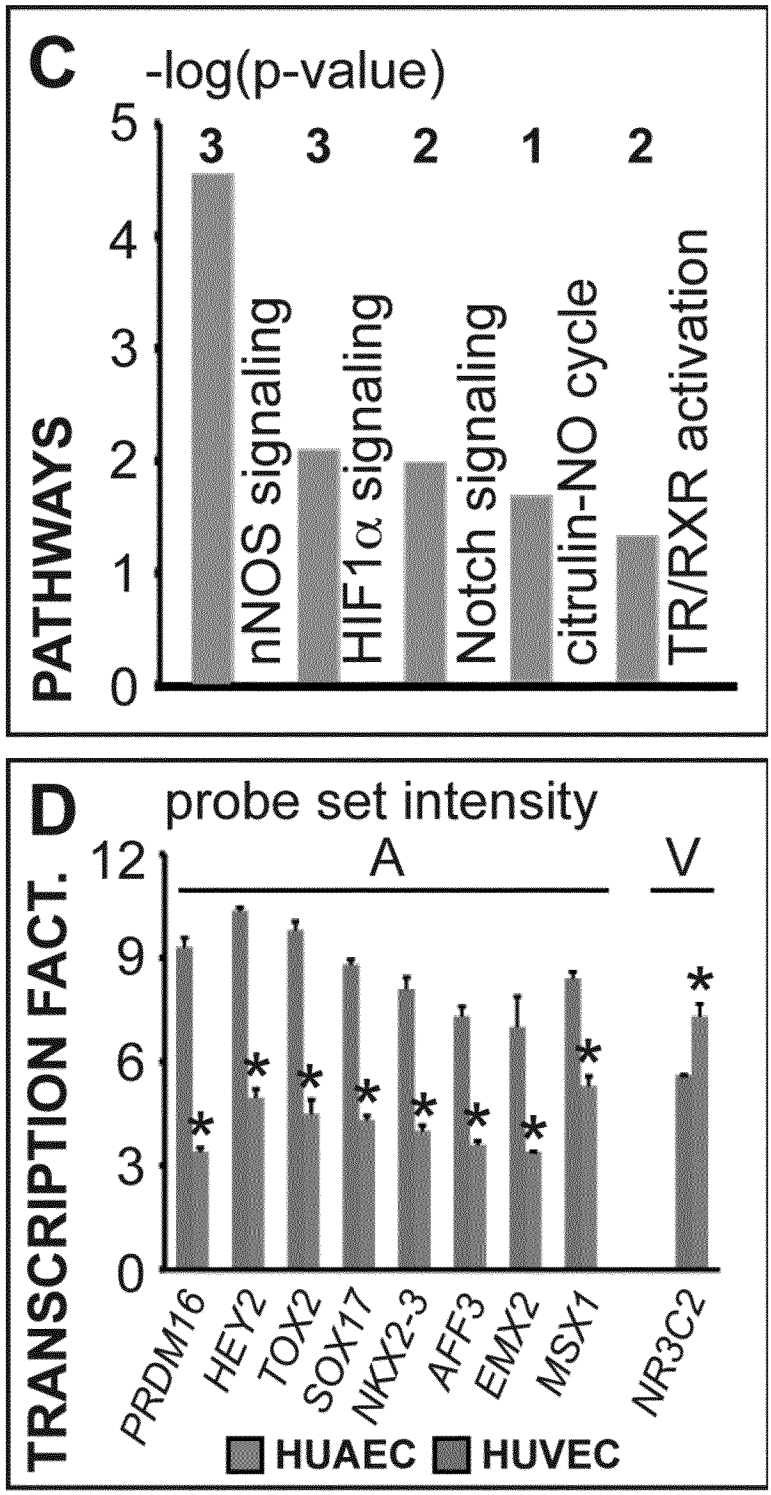


FIGURE 25E-H: *provides photographs and graphics on the genome-wide analysis of freshly isolated HUVECs and HUAECs.*

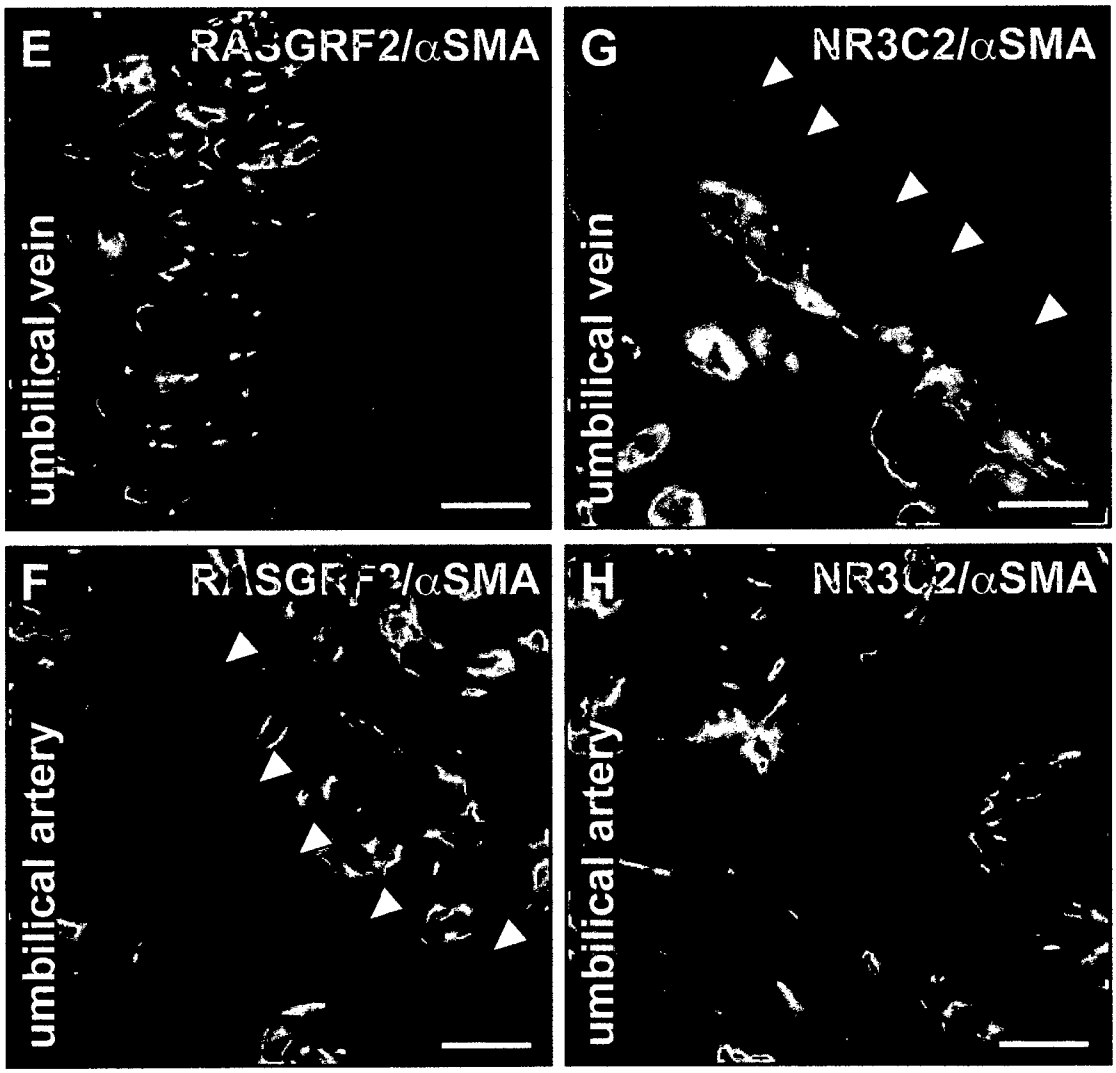
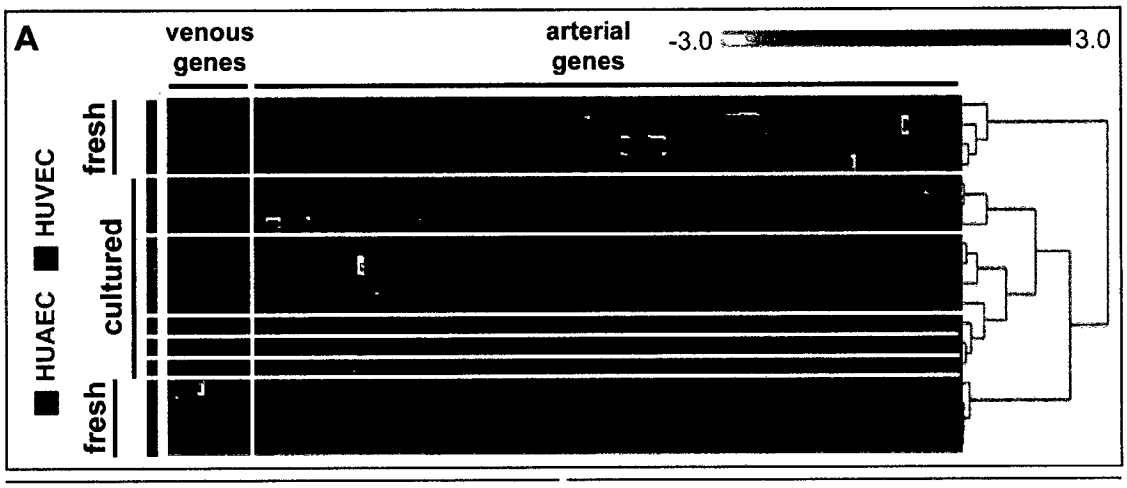
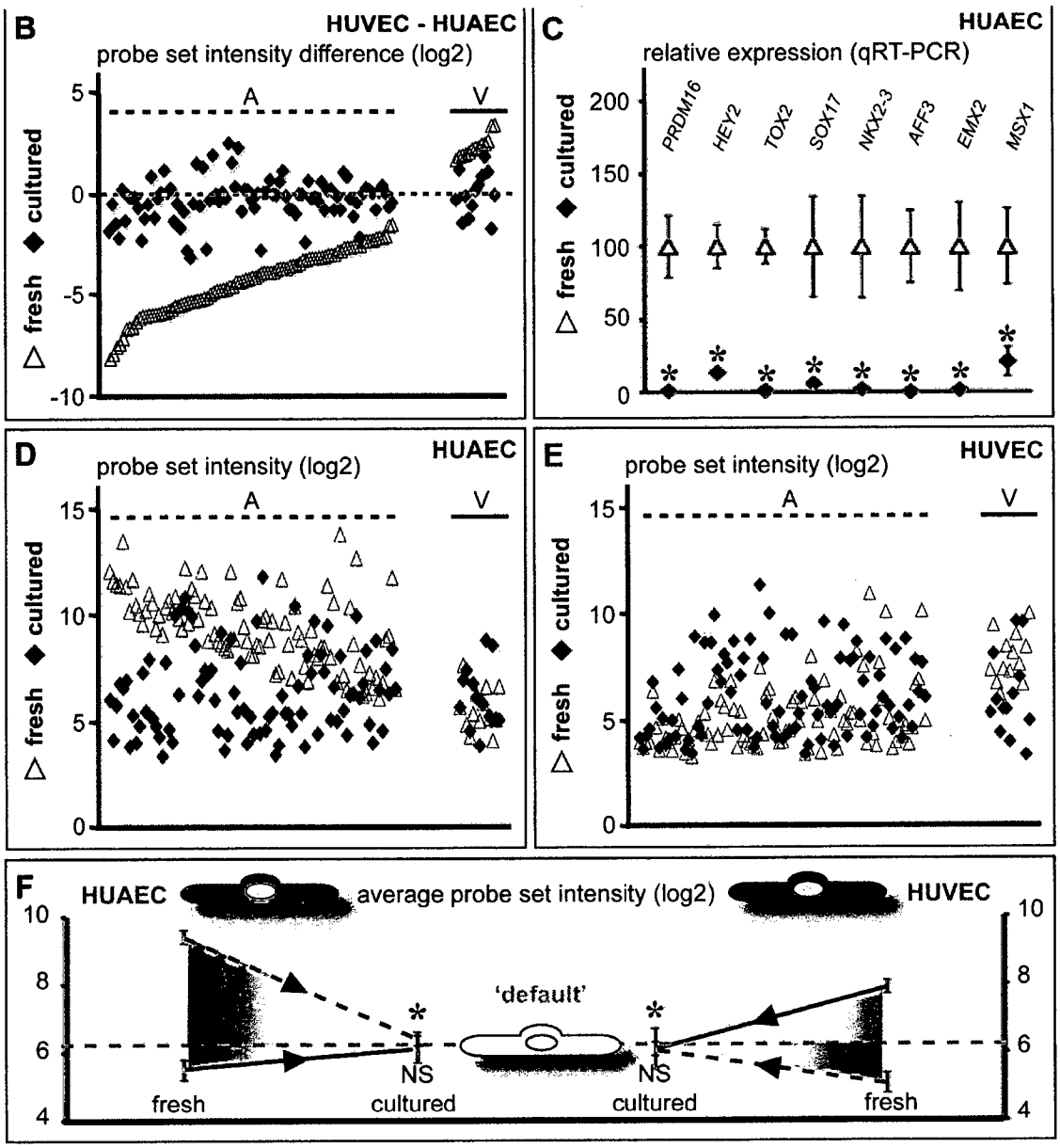


FIGURE 26A: *displays how the culturing process rapidly erases differential arteriovenous gene expression.*



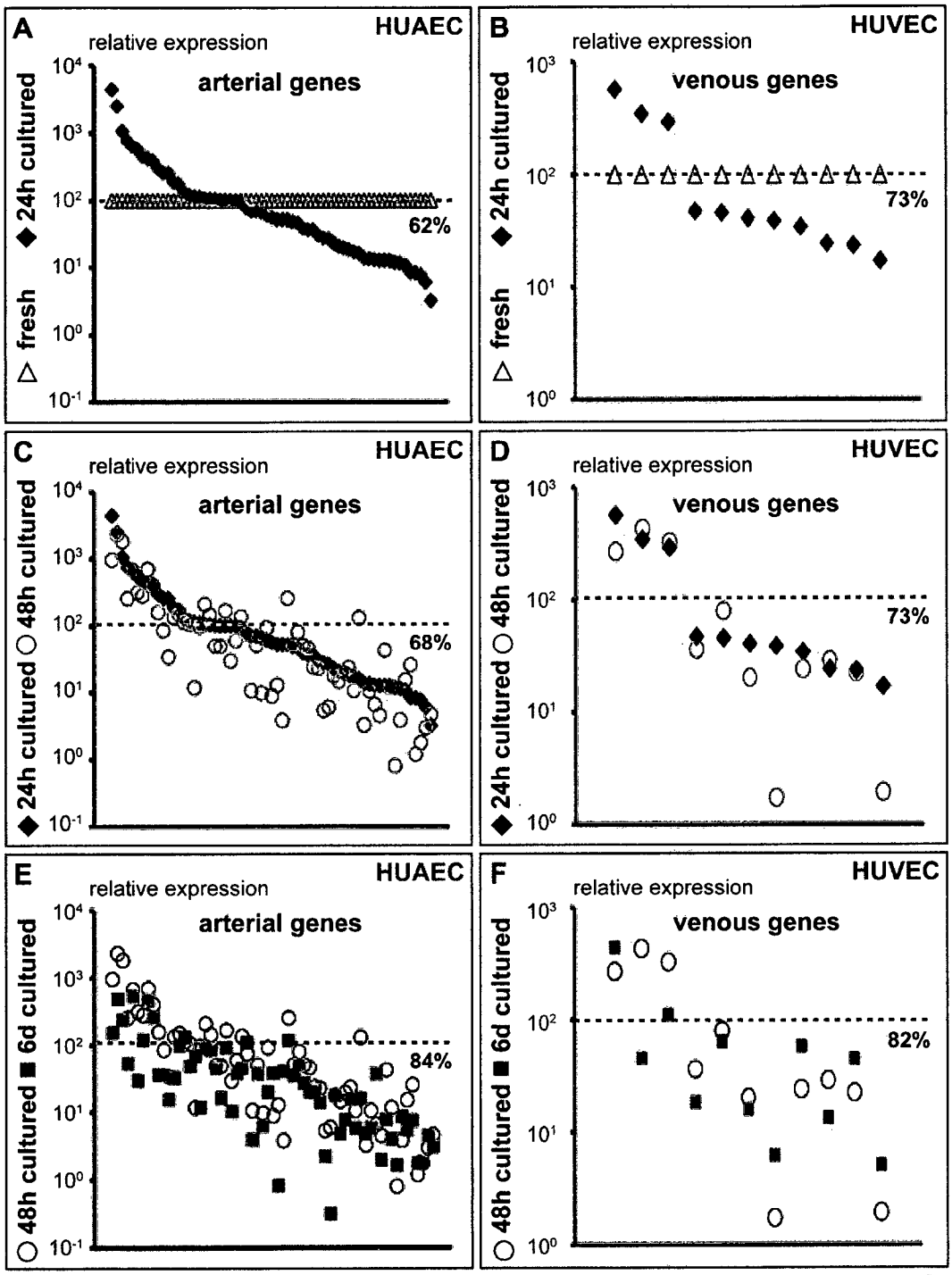
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 26B-F: *displays how the culturing process rapidly erases differential arteriovenous gene expression.*



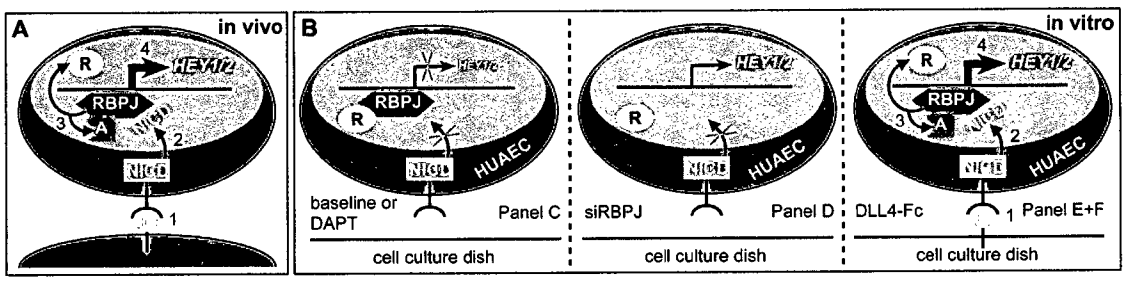
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 27A-F: *is a graphic display of the kinetics of the culture-induced assimilation process.*



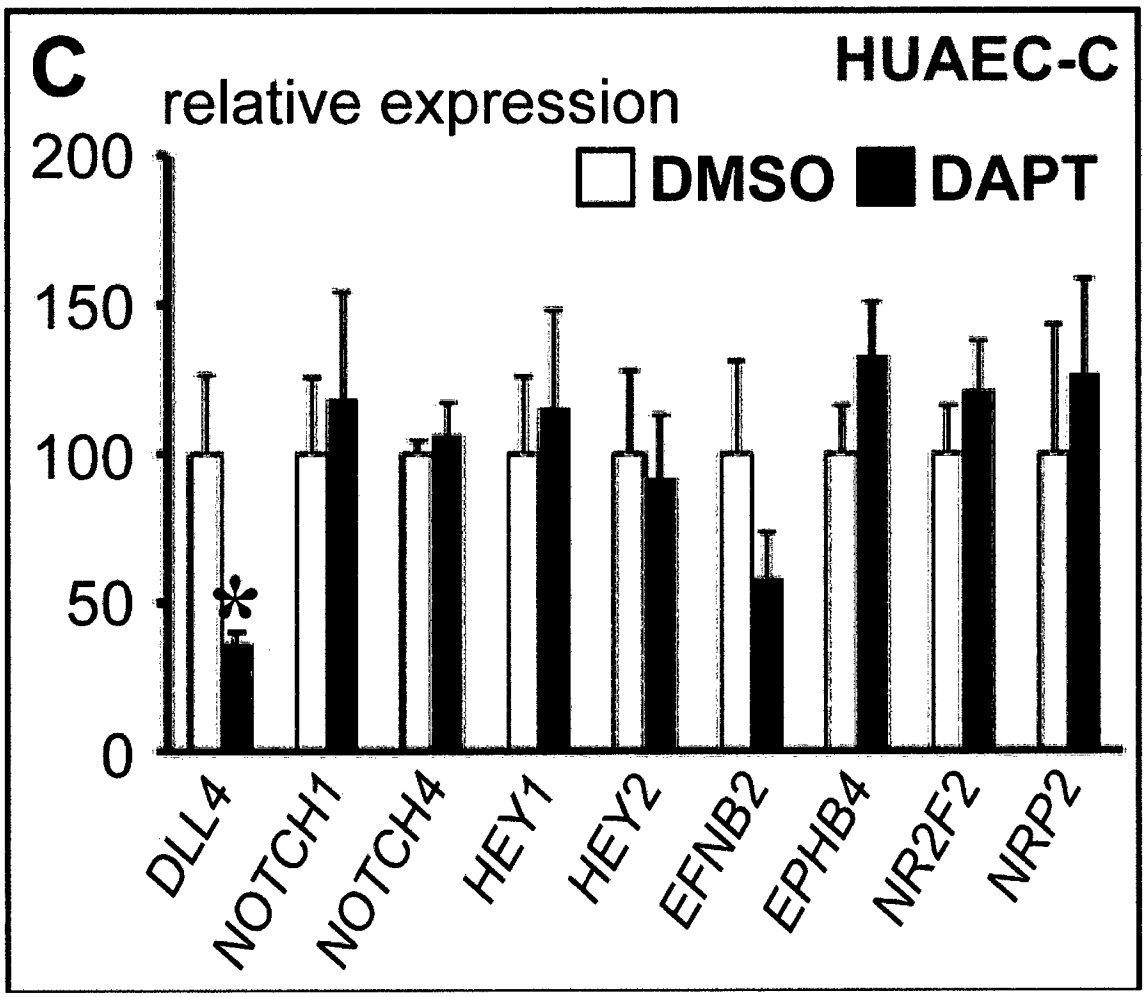
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 28A,B: *provides a display of how reactivation of Notch signalling only partially restores arterial gene expression in HUAEC-C.*



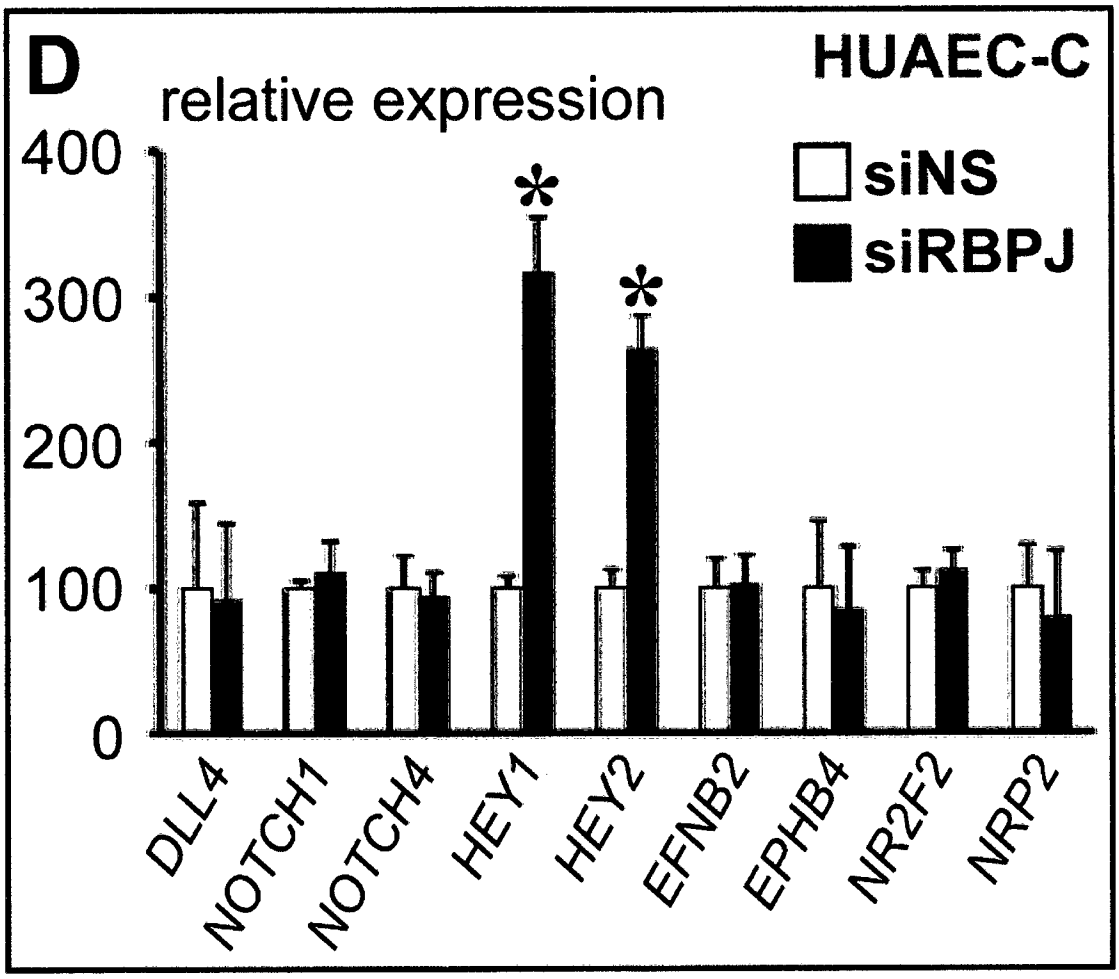
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 28C: *provides a display of how reactivation of Notch signalling only partially restores arterial gene expression in HUAEC-C.*



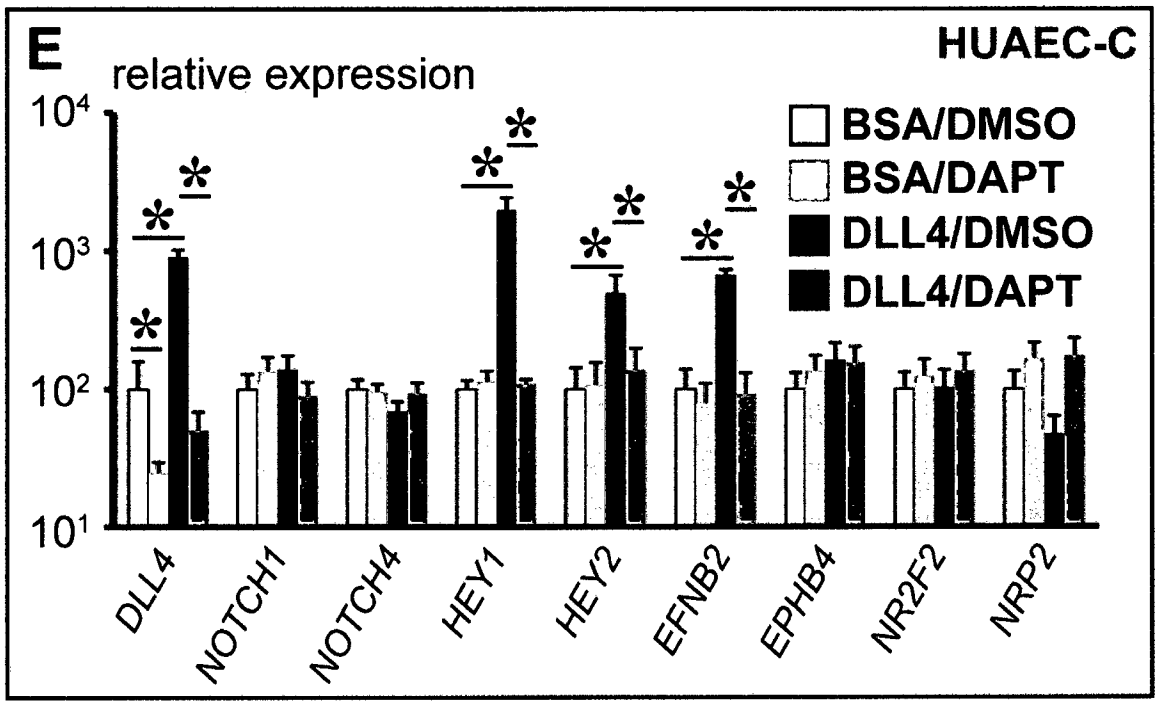
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 28D: *provides a display of how reactivation of Notch signalling only partially restores arterial gene expression in HUAEC-C.*



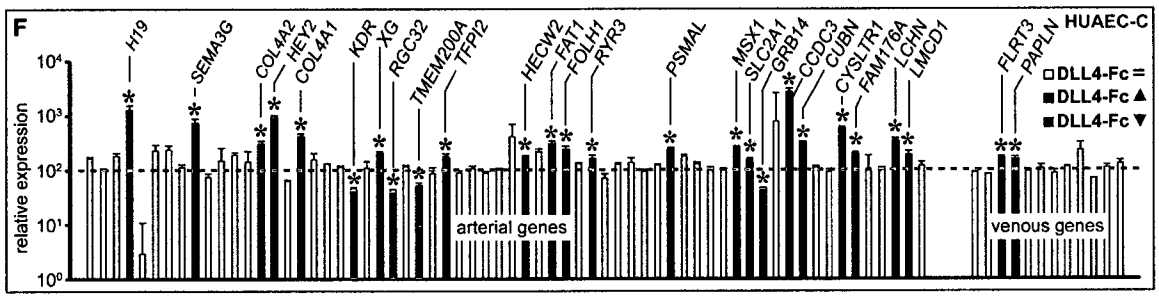
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 28E: *provides a display of how reactivation of Notch signalling only partially restores arterial gene expression in HUAEC-C.*



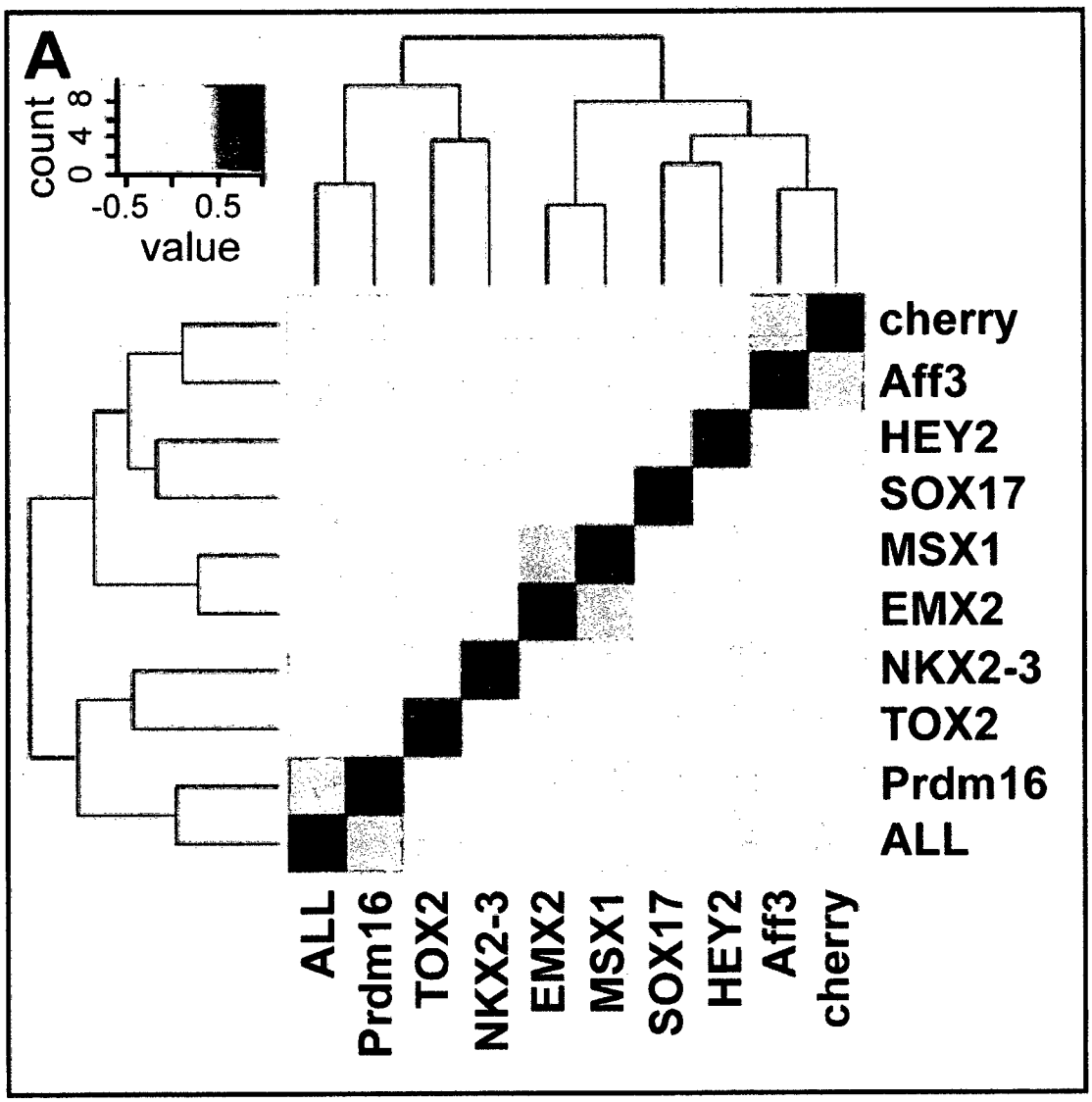
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 28F: *provides a display of how reactivation of Notch signalling only partially restores arterial gene expression in HUAEC-C.*



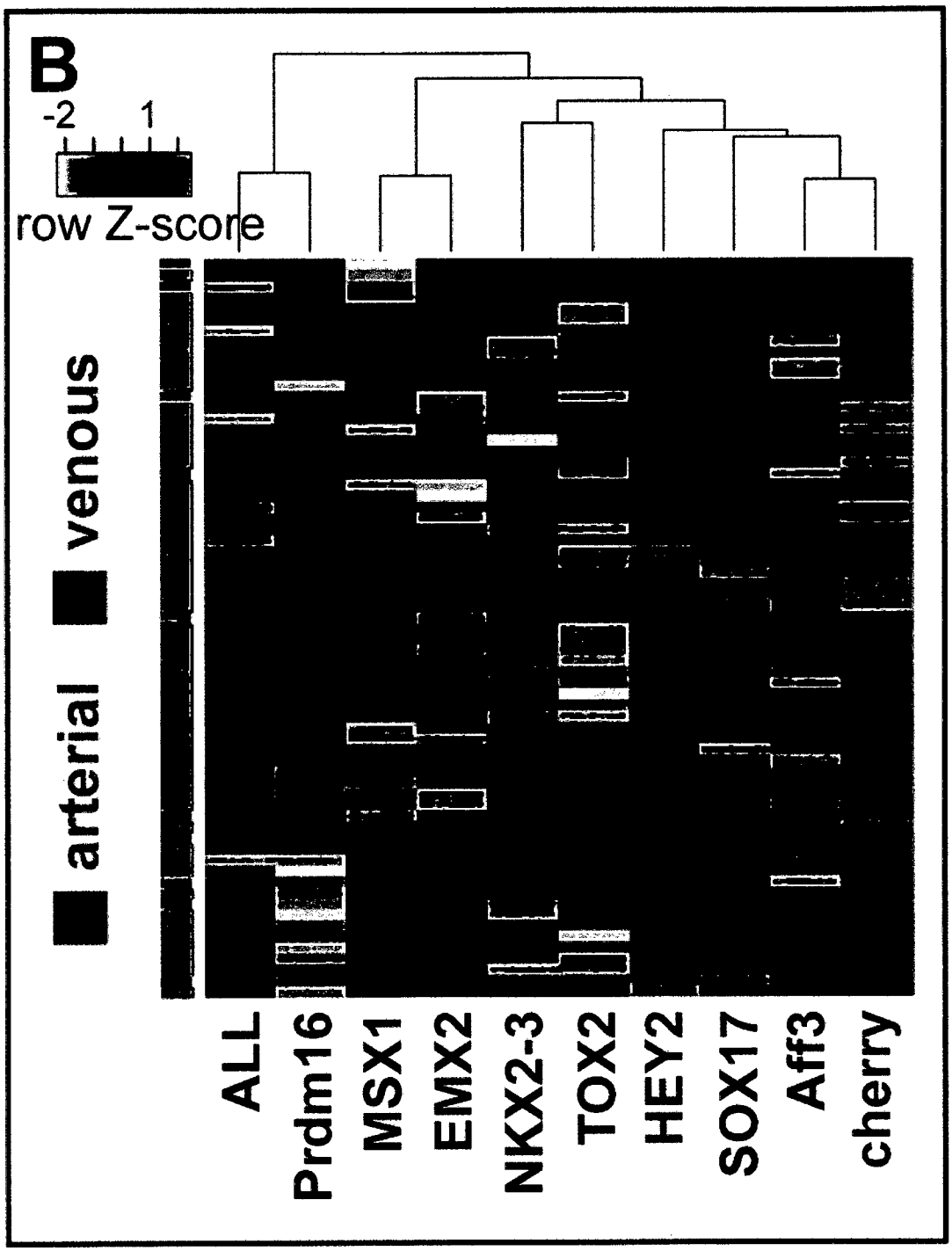
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 29A: *provides a graphic display of how combined overexpression of eight transcription factors robustly induces the arteriovenous fresh profile in HUVEC-C.*



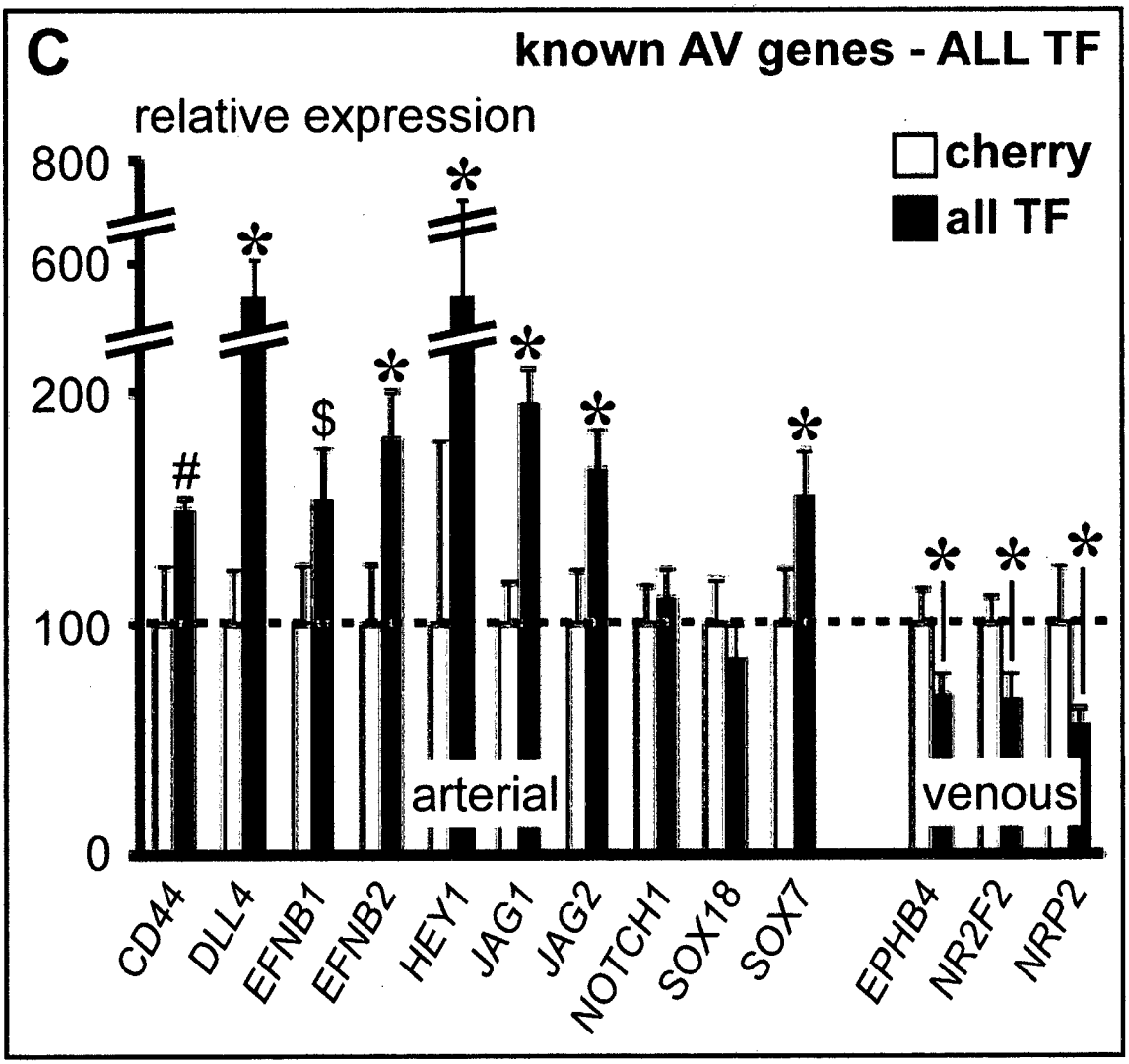
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 29B: *provides a graphic display of how combined overexpression of eight transcription factors robustly induces the arteriovenous fresh profile in HUVEC-C.*



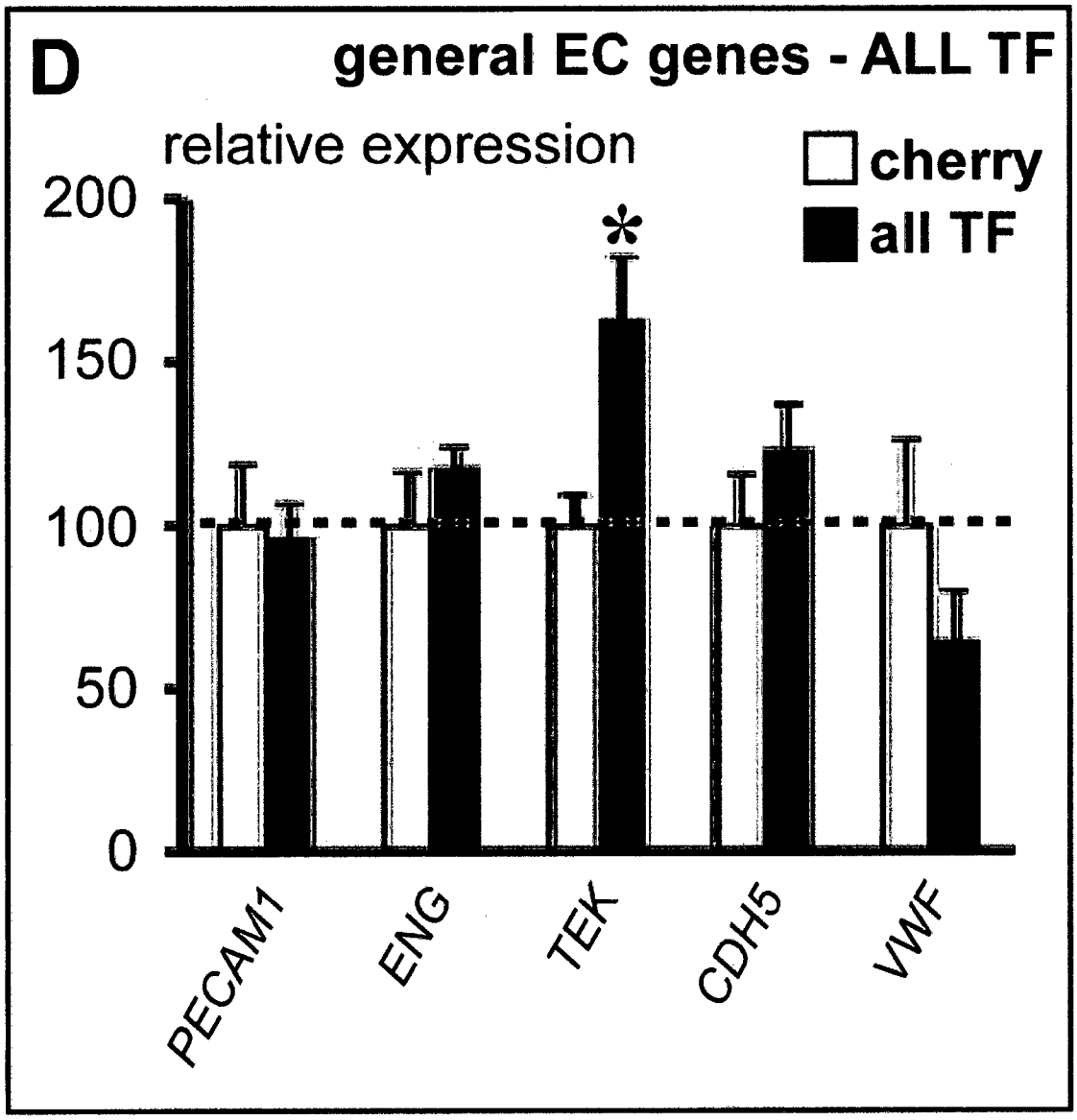
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 29C: *provides a graphic display of how combined overexpression of eight transcription factors robustly induces the arteriovenous fresh profile in HUVEC-C.*



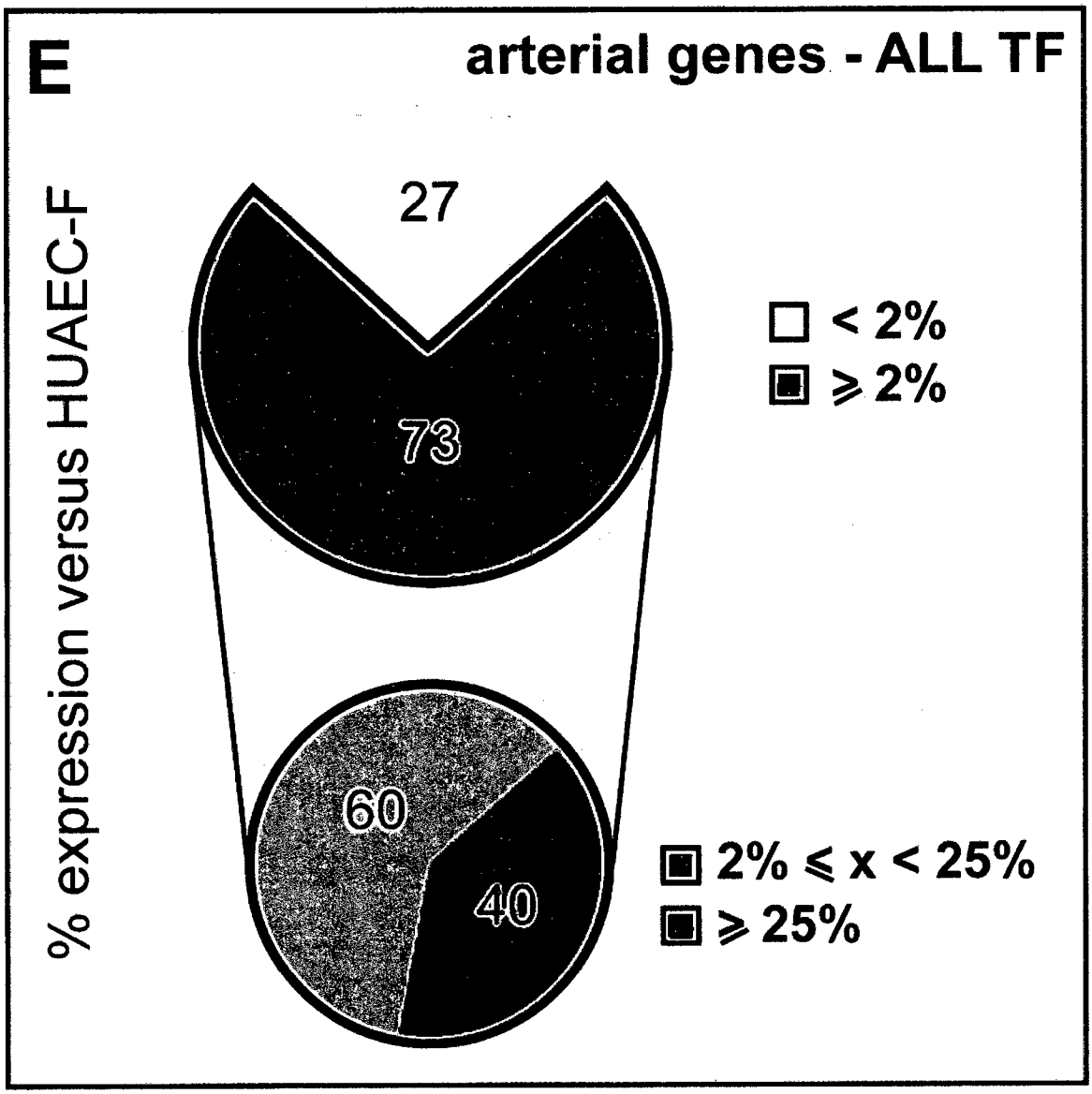
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 29D: *provides a graphic display of how combined overexpression of eight transcription factors robustly induces the arteriovenous fresh profile in HUVEC-C.*



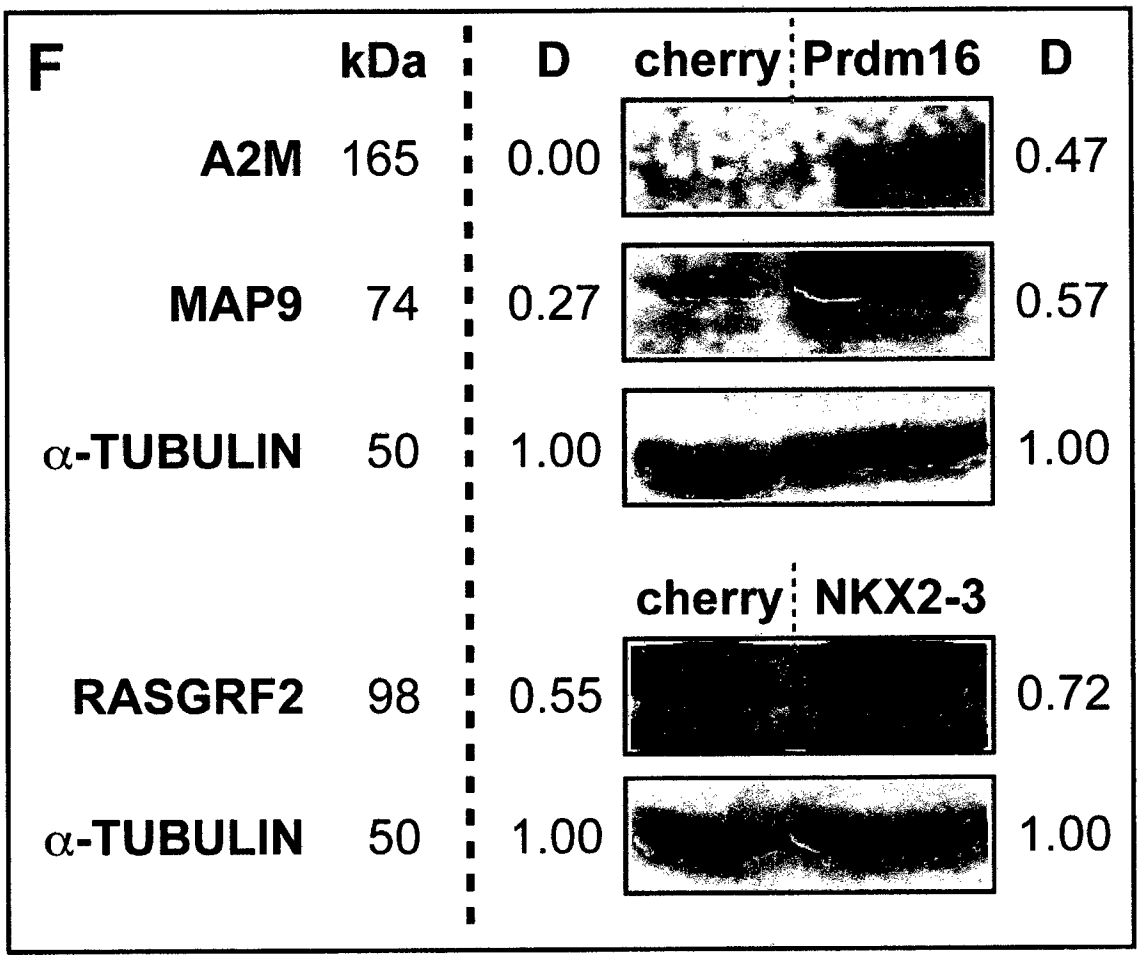
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 29E: *provides a graphic display of how combined overexpression of eight transcription factors robustly induces the arteriovenous fresh profile in HUVEC-C.*



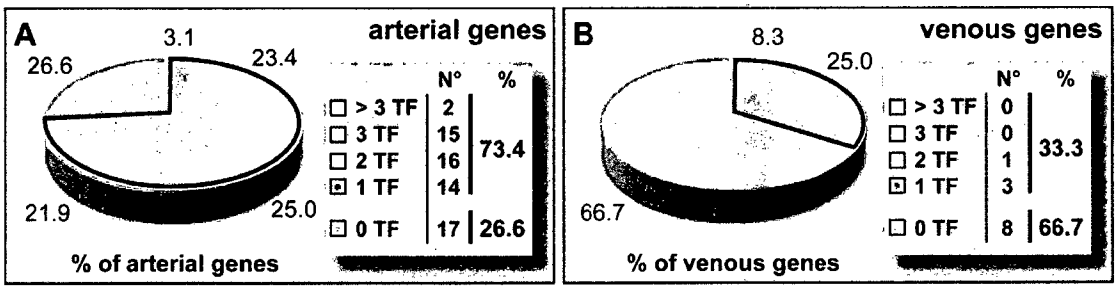
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 29F: *provides a graphic display of how combined overexpression of eight transcription factors robustly induces the arteriovenous fresh profile in HUVEC-C.*



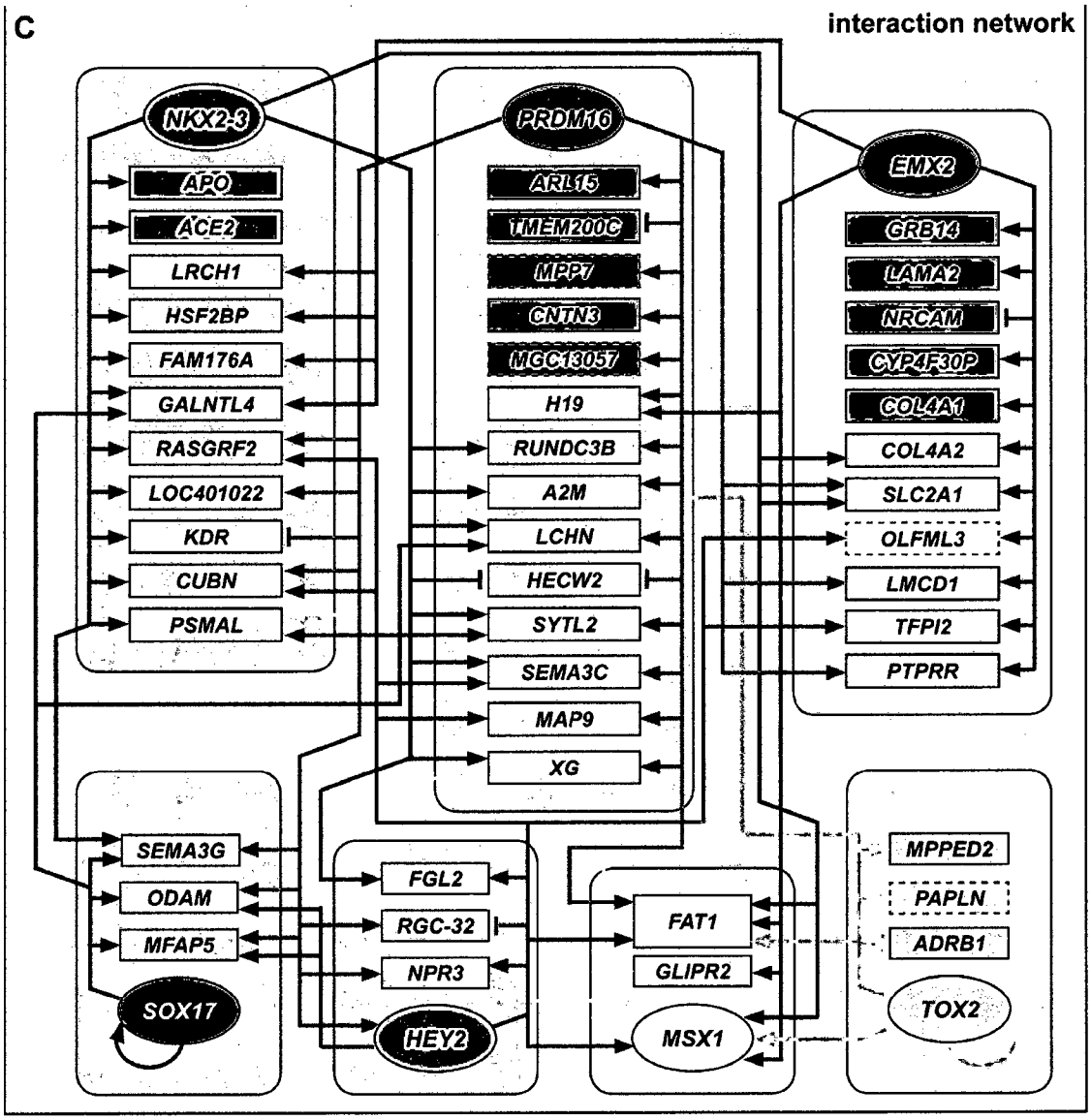
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 30 A,B: *provides a graphic representation of how individual transcription factors interact in a complex network to regulate the arteriovenous fresh profile.*



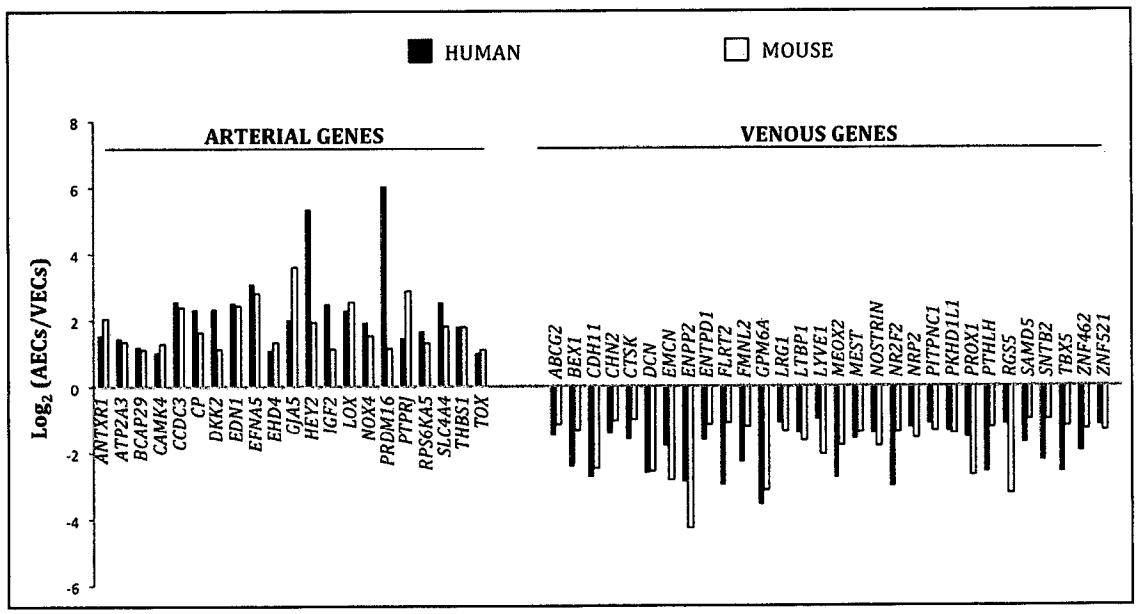
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 30C: *provides a graphic representation of how individual transcription factors interact in a complex network to regulate the arteriovenous fresh profile.*



INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 31 : *displays the conserved arteriovenous fingerprint between human and murine EC samples.*



INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 32 A-C: *displays microarray results for the conserved arteriovenous signature in Prdm16 overexpressing BOECs, HEY2 overexpressing BOECs or siNR2F2-treated HUVECs.*

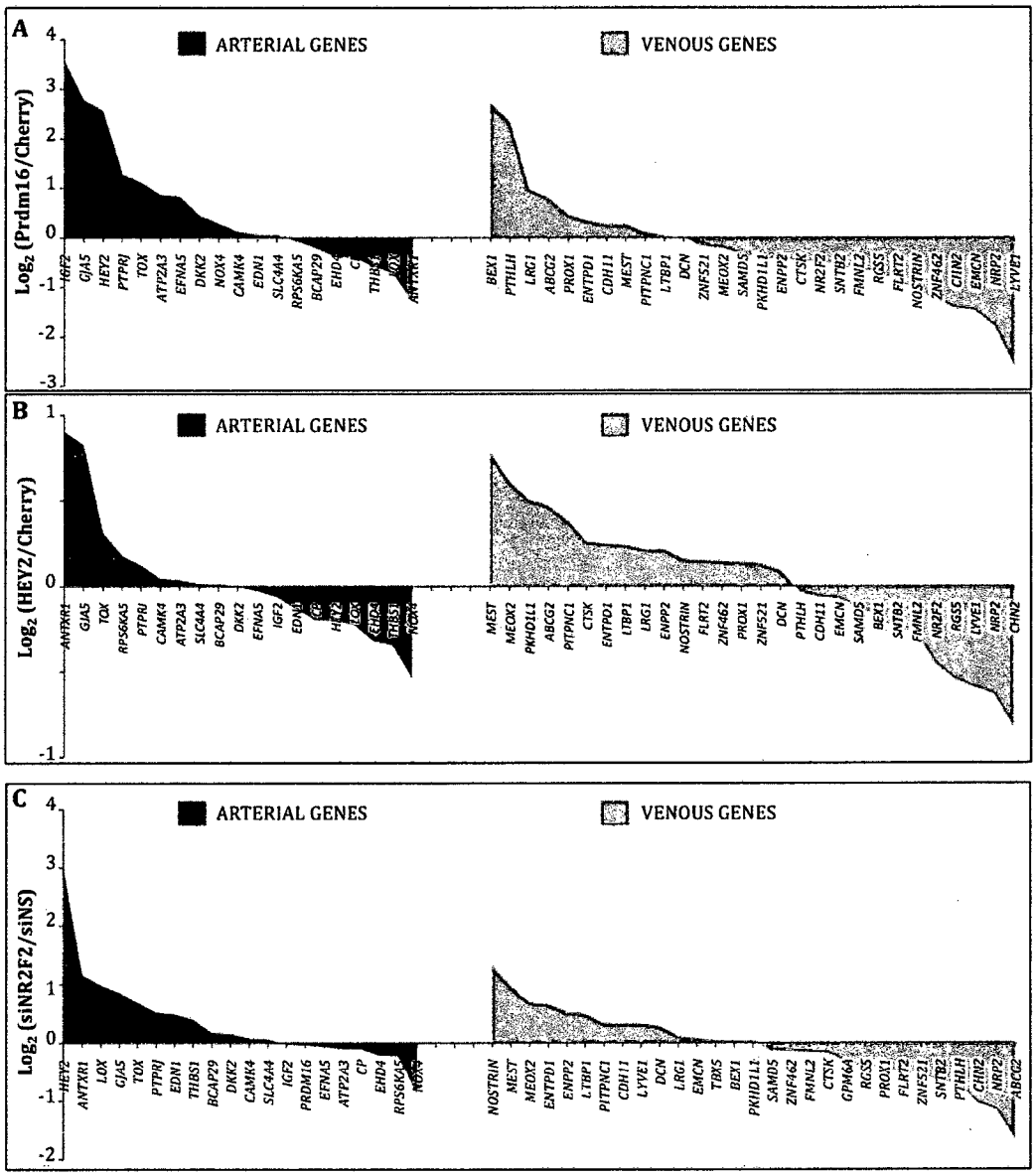


FIGURE 33A-M: *displays the validation of the arterial-exclusive expression pattern of Prdm16 across species at different developmental stages.*

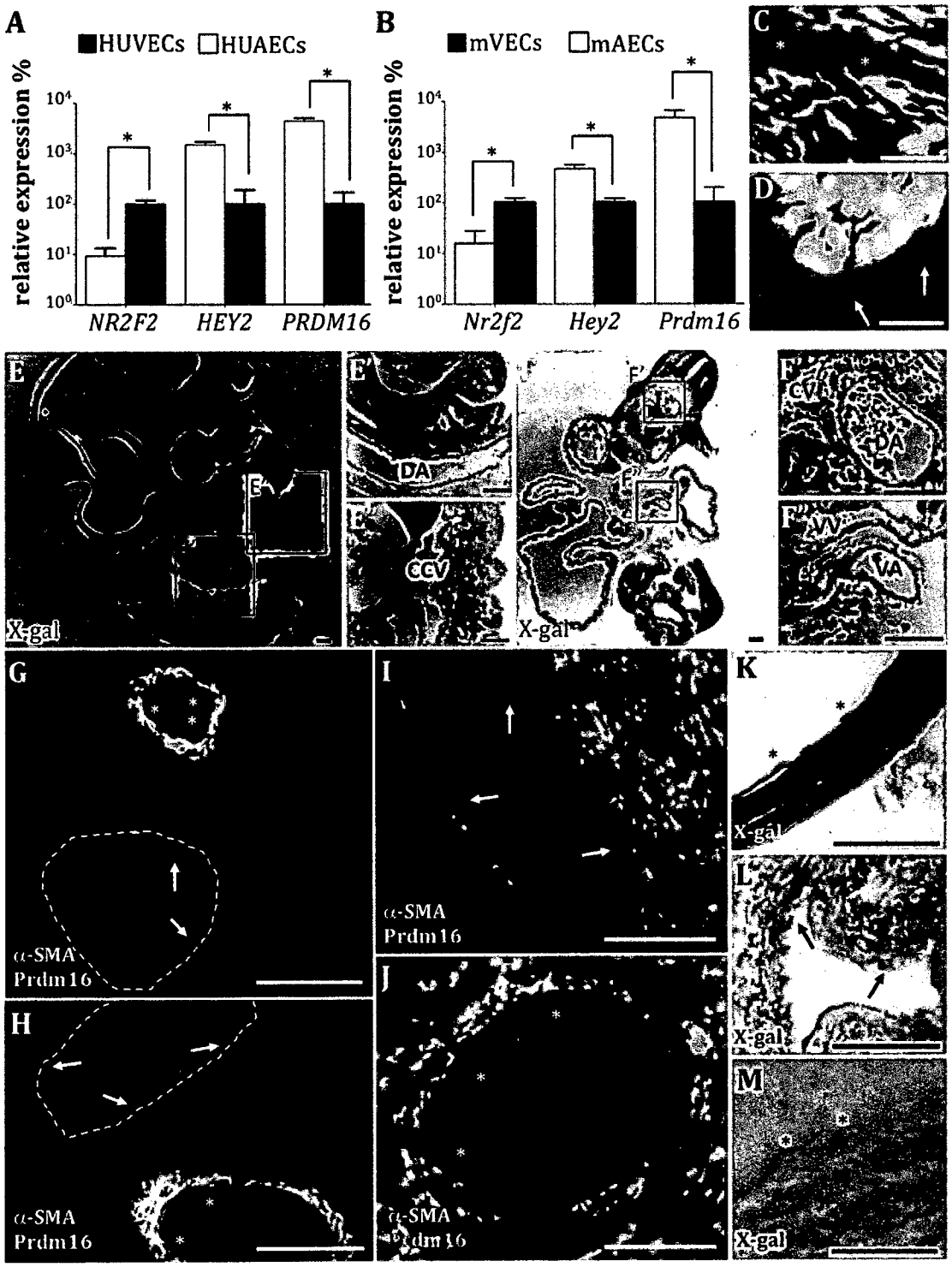
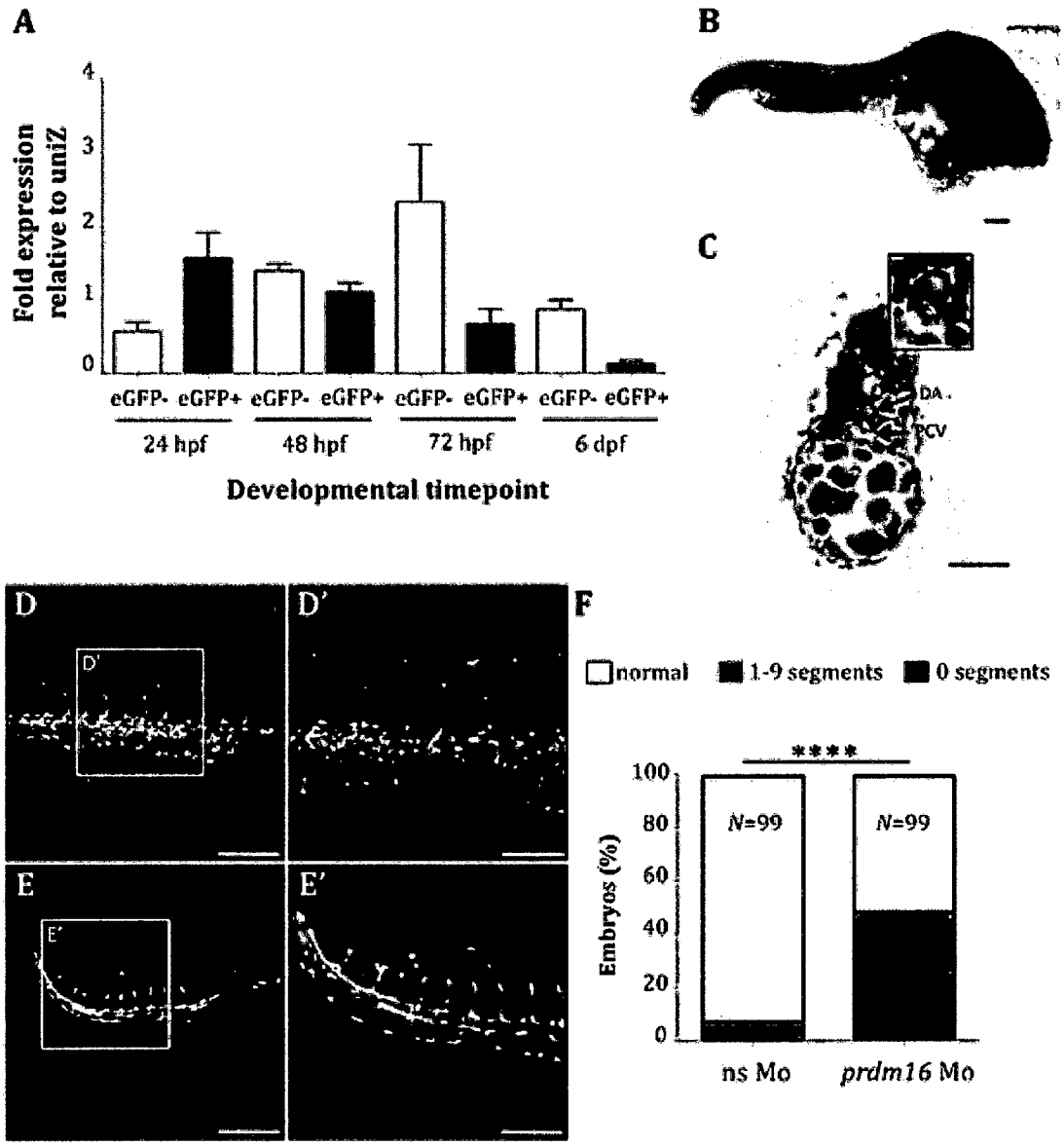


FIGURE 34A-F: *displays the Prdm16 expression profile and knockdown phenotype in* Tg(kdr-eGFP)^{s843} *zebrafish.*



INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 35 A-H: *displays* Prdm16$^{-/-}$ *mouse embryos with haemorrhages and signs of reduced SMC coverage.*

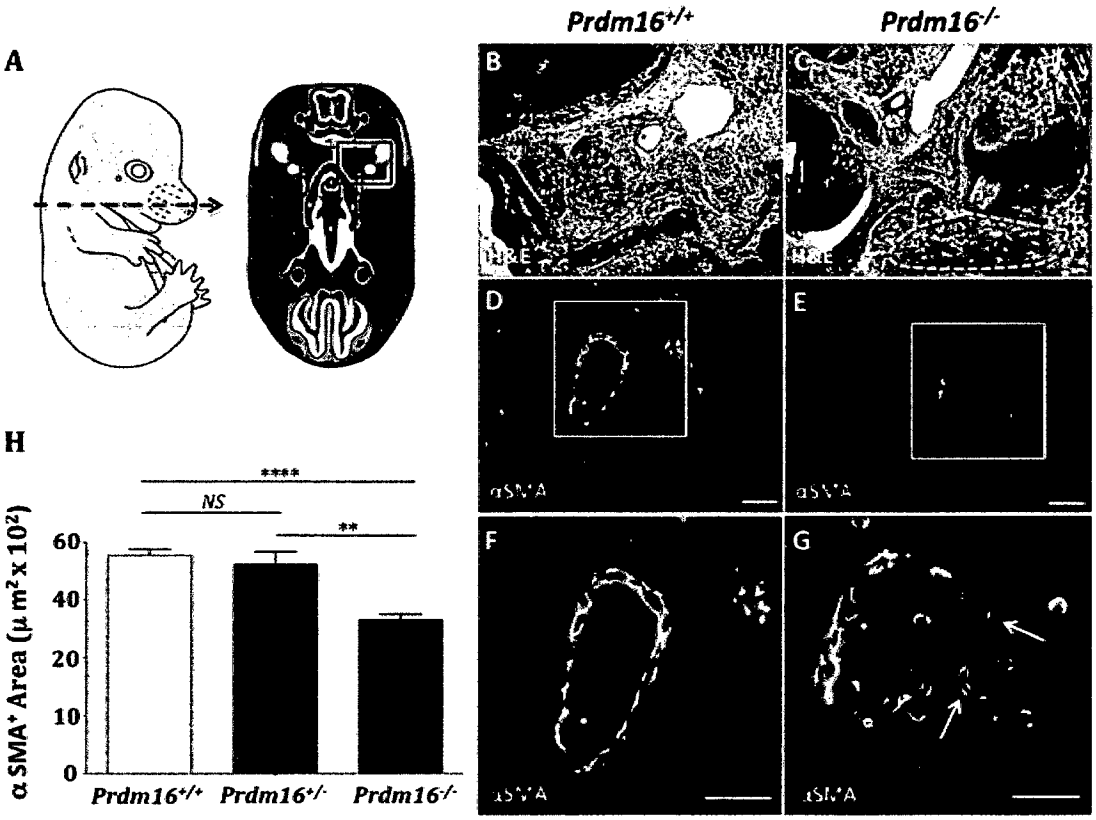


FIGURE 36 A-E: *displays* Prdm16$^{+/-}$ *mice with impaired perfusional recovery upon limb ischemia.*

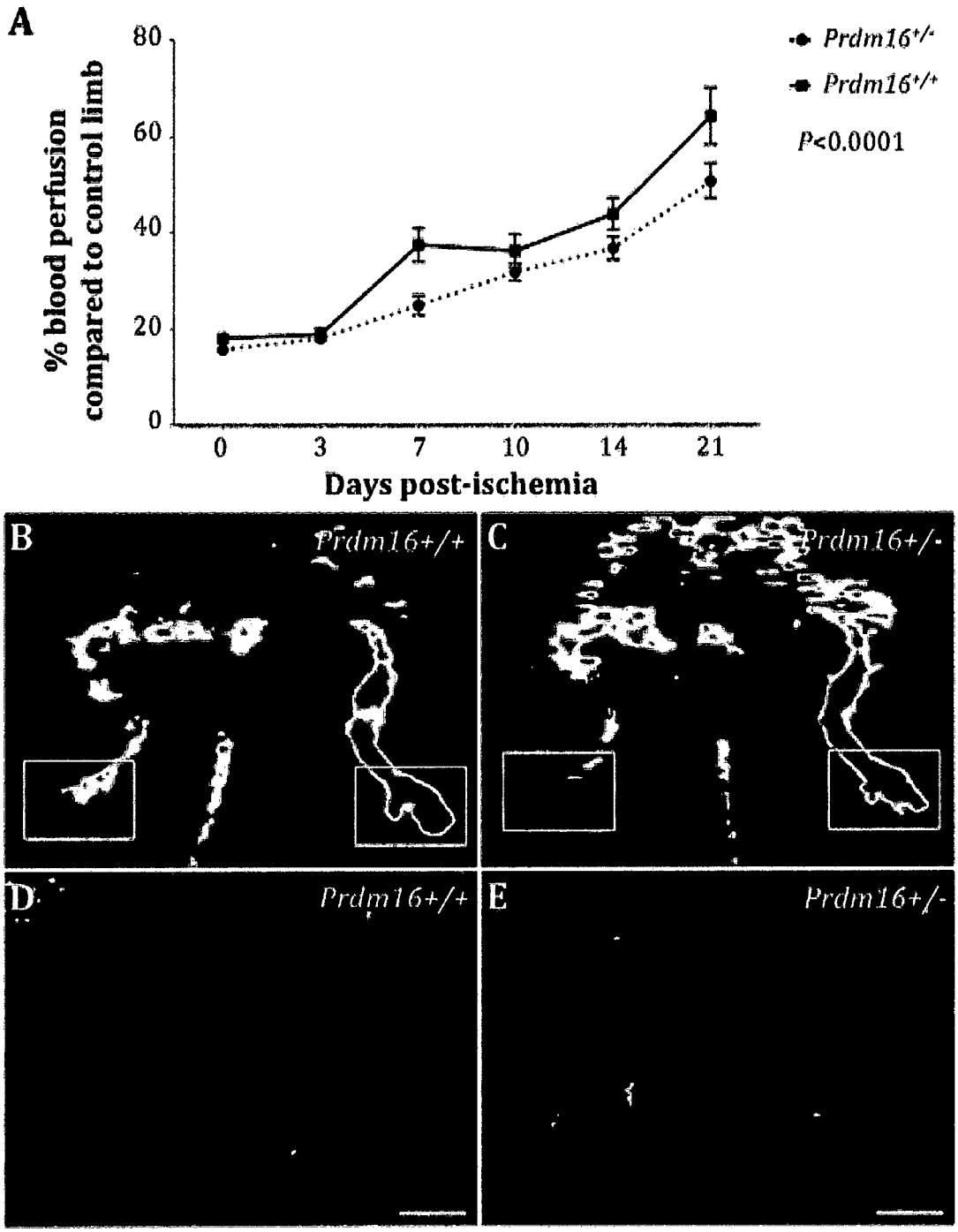
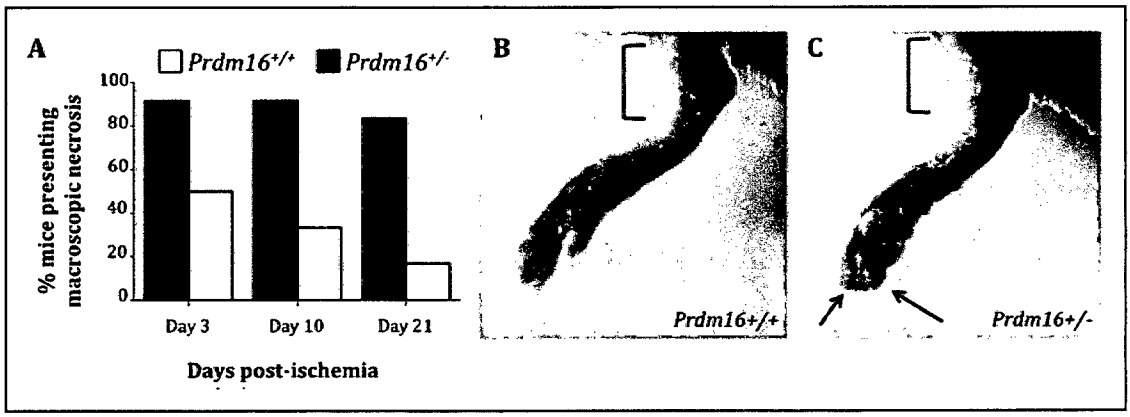
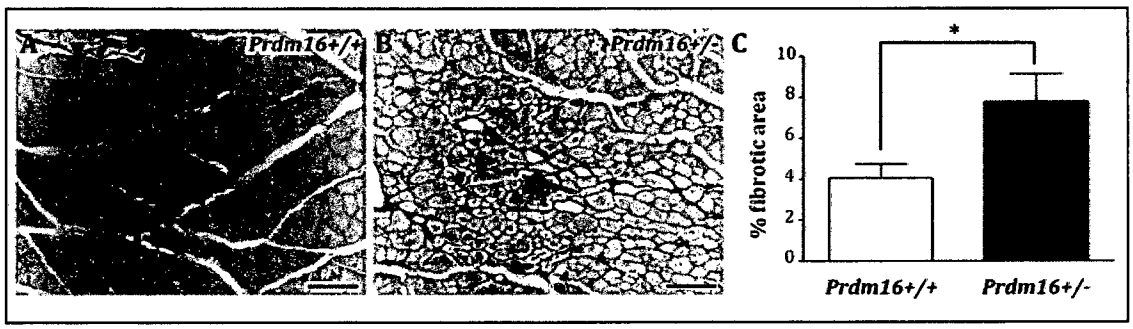


FIGURE 37 A-C: *displays* Prdm16$^{+/-}$ *mice with macroscopic signs of necrosis upon limb ischemia.*



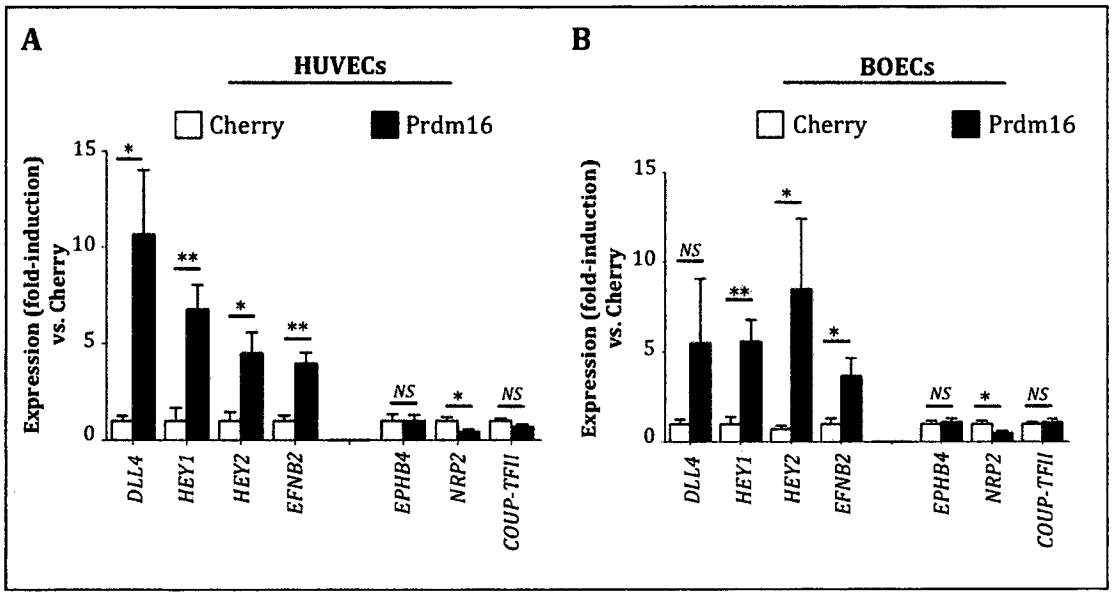
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 38 A-C: *displays* Prdm16$^{+/-}$ *mice with increased fibrosis in ischemic hind limbs compared to their* Prdm16$^{+/+}$ *littermates.*



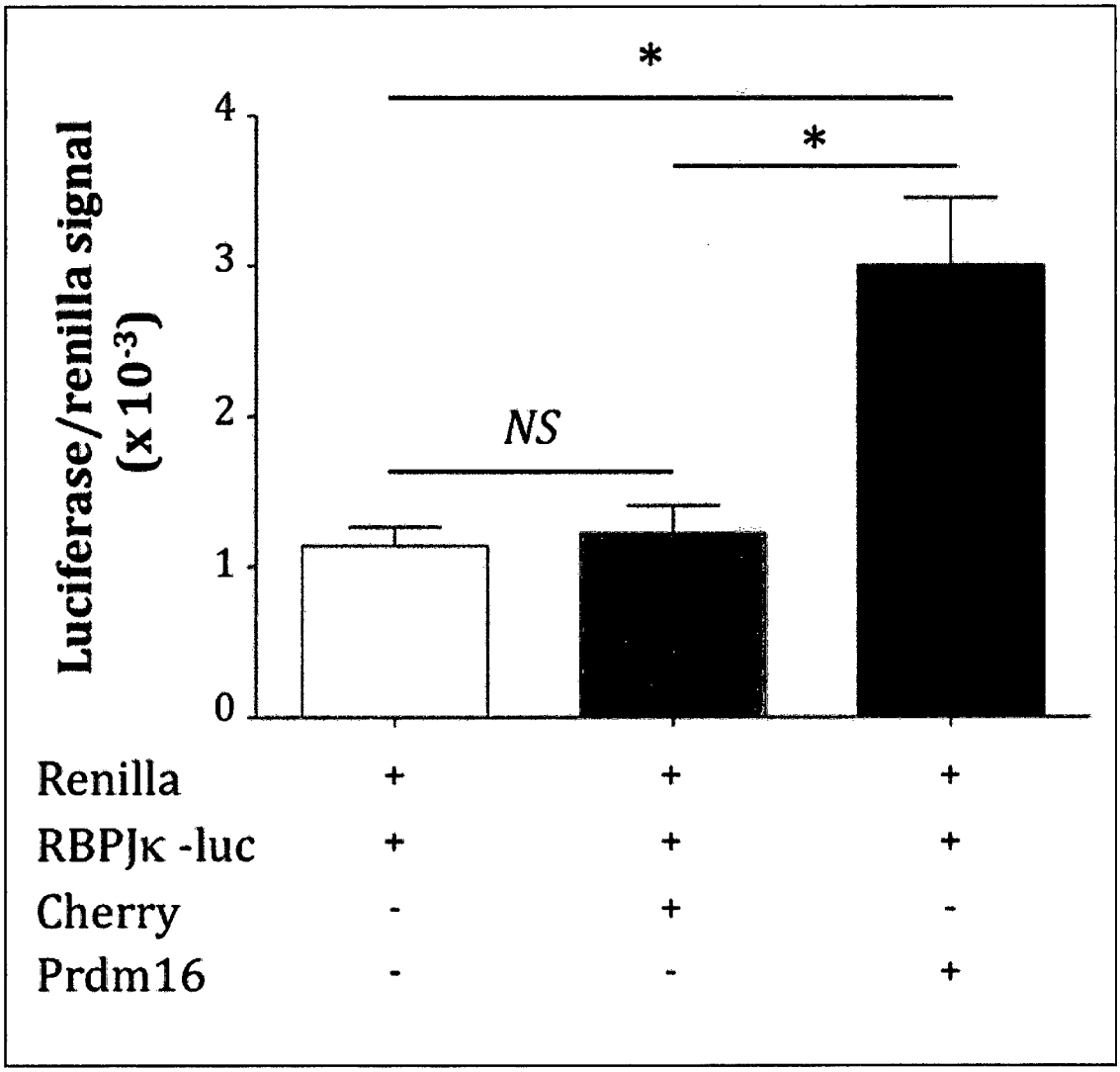
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 39A,B: *shows how ectopic Prdm16 drives the onset of multiple Notch pathway genes.*



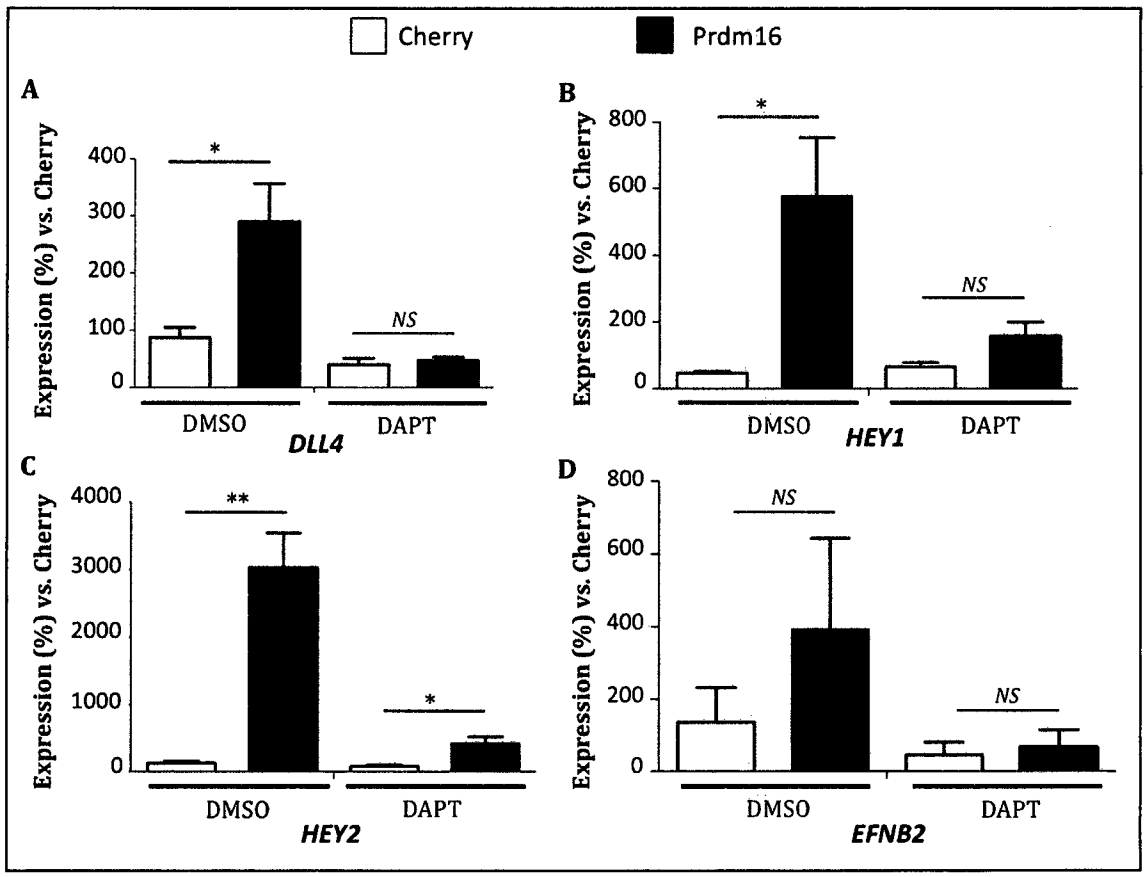
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 40: *shows how Prdm16 induces canonical Notch signalling* in vitro.



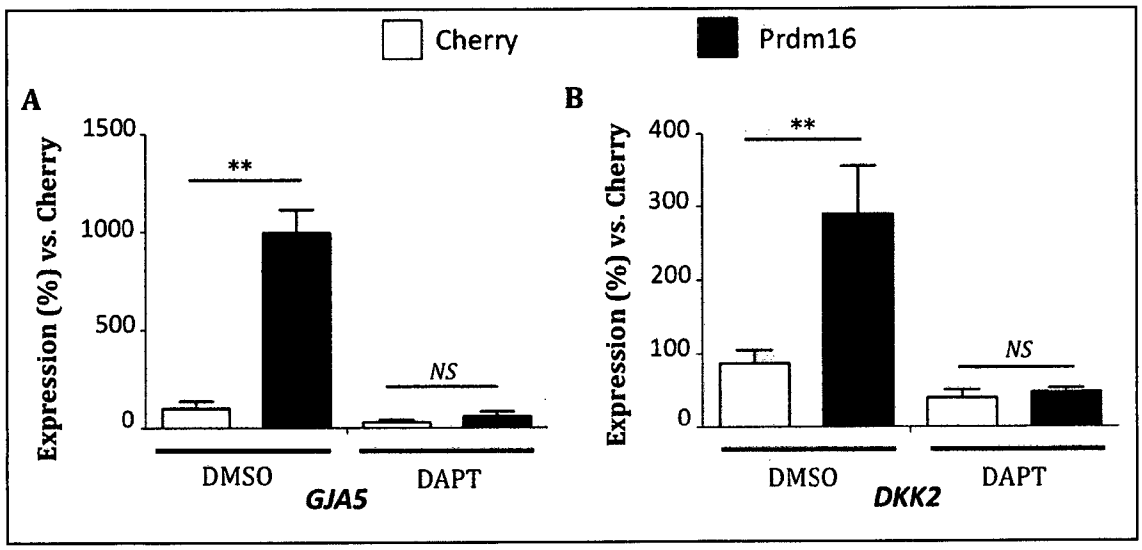
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 41 A-D: *shows how Prdm16 activates the canonical Notch pathway.*



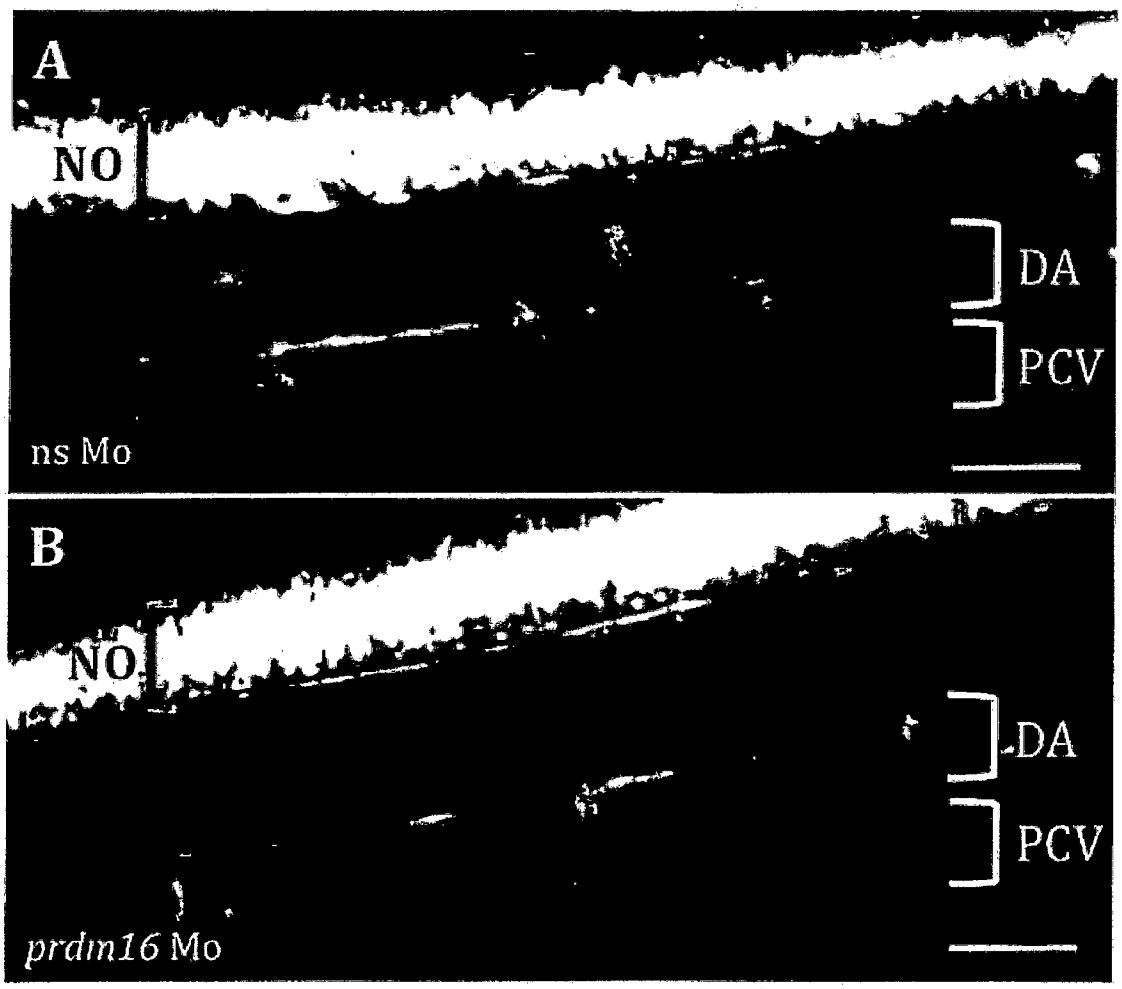
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 42A,B: *shows how Prdm16 arterialises BOECs through activation of canonical Notch.*



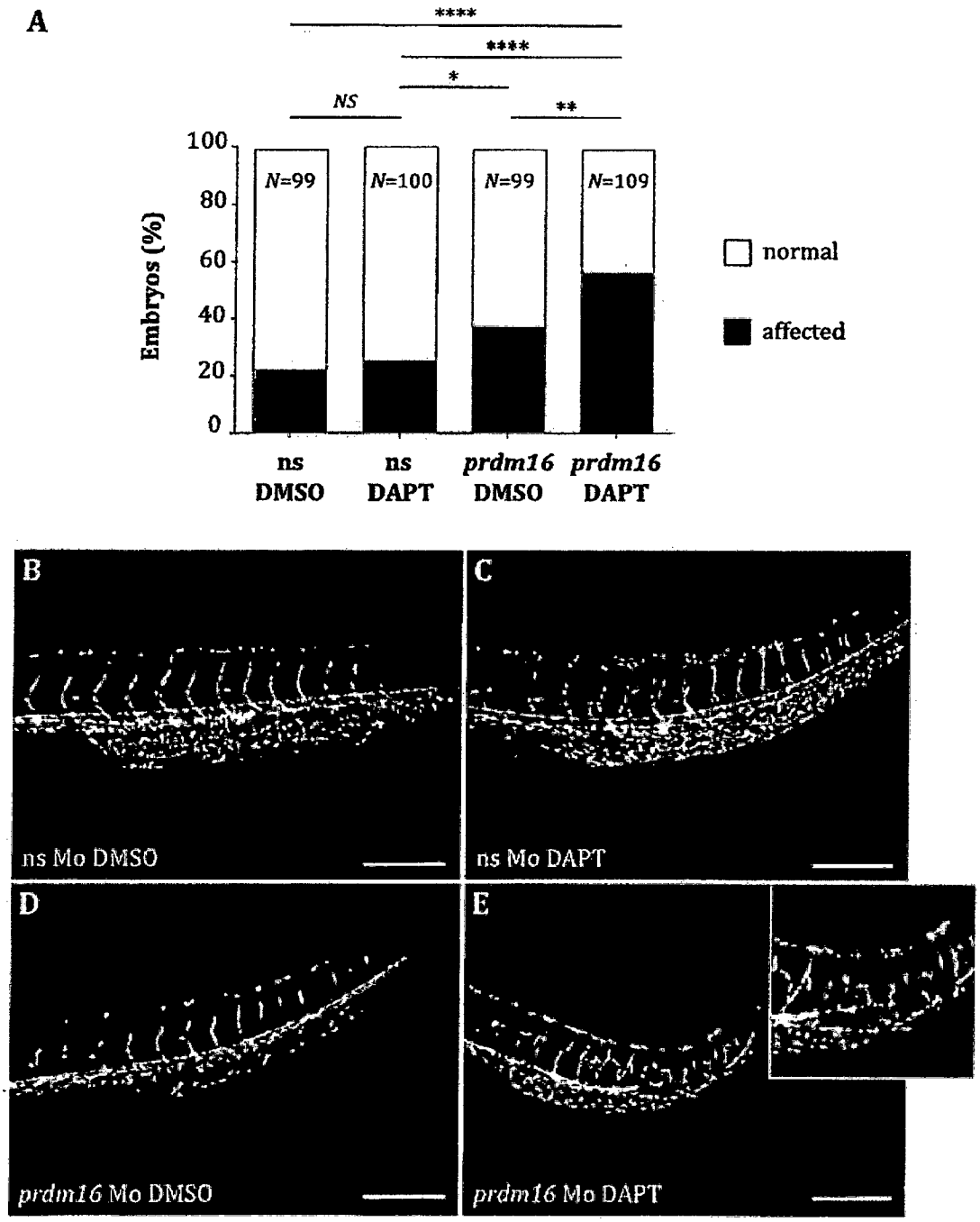
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 43A,B *shows how Prdm16 deficiency in zebrafish does not result in reduced notch signalling.*



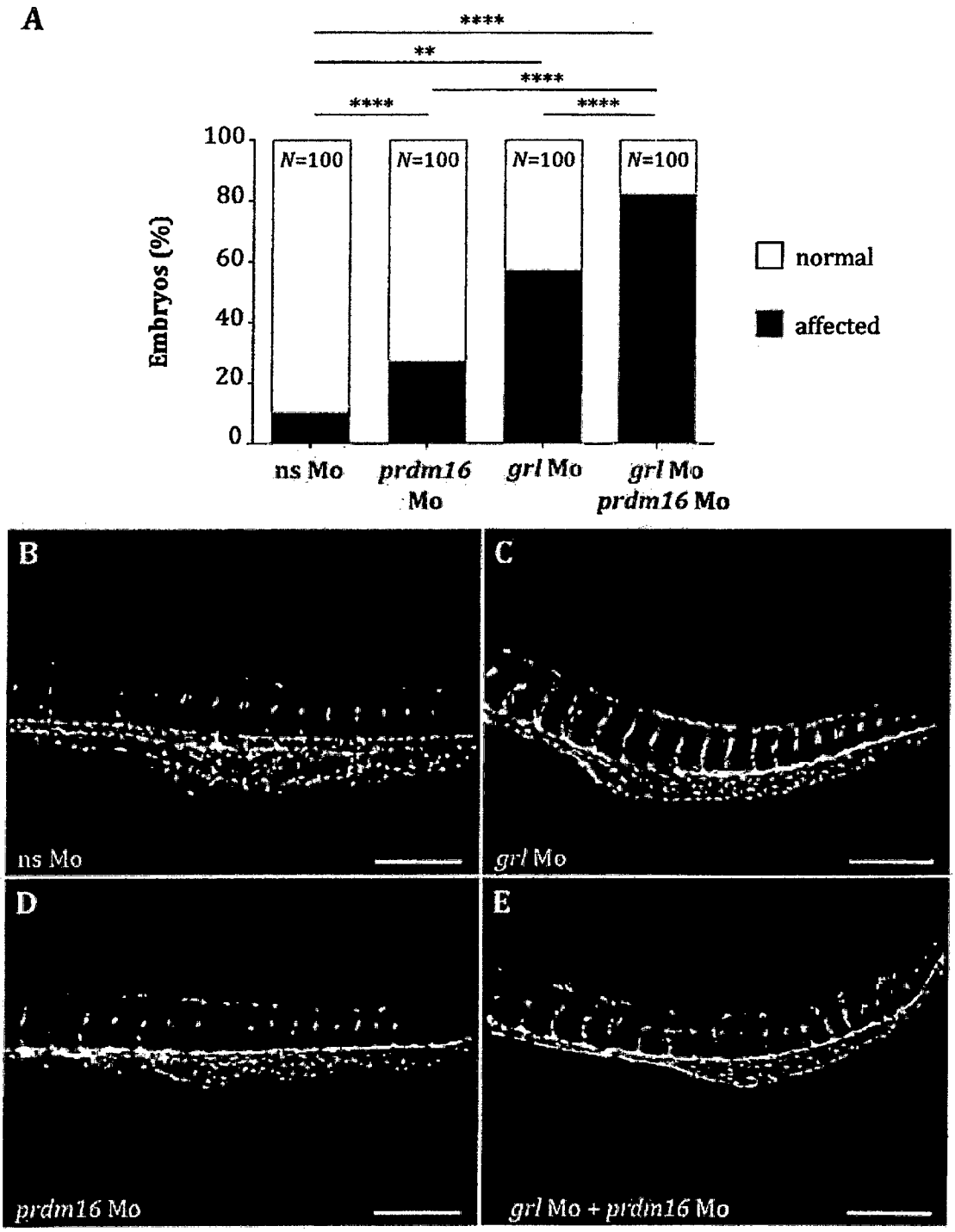
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 44A-E *shows how DAPT induces aortic abnormalities in* prdm16 *Mo, but not ns Mo-treated zebrafish*.



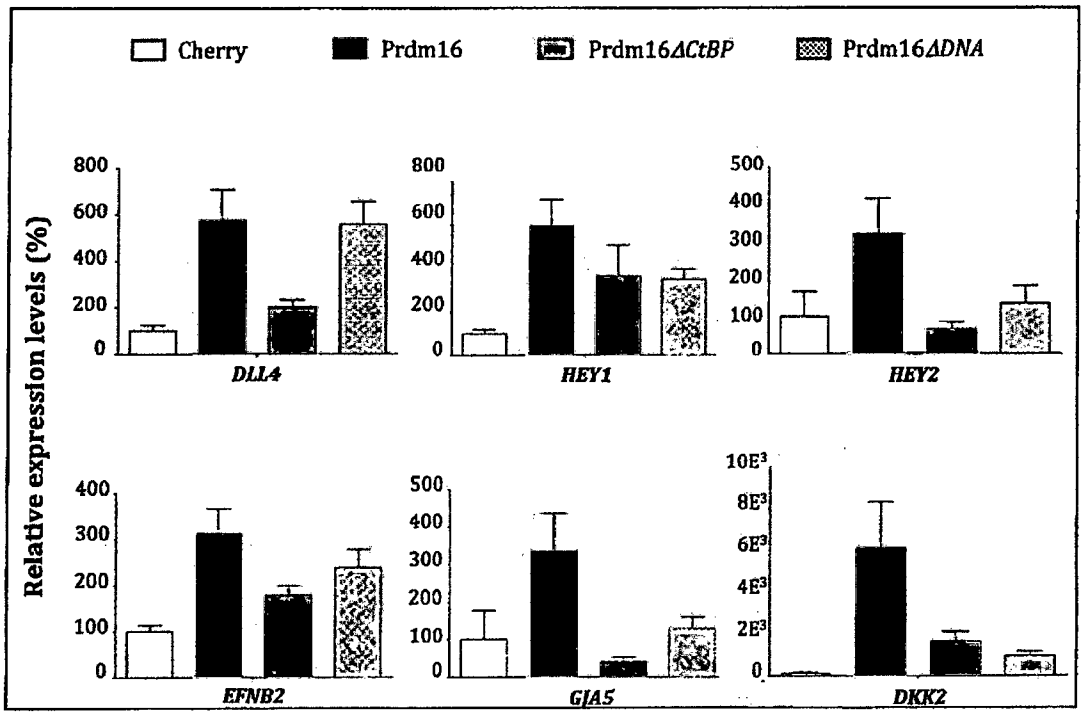
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 45A-E *displays how co-injections of* grl *Mo with* prdm16 *Mo severely aggravates the vascular defects observed in zebrafish treated with only grl Mo or* prdm16 *Mo.*



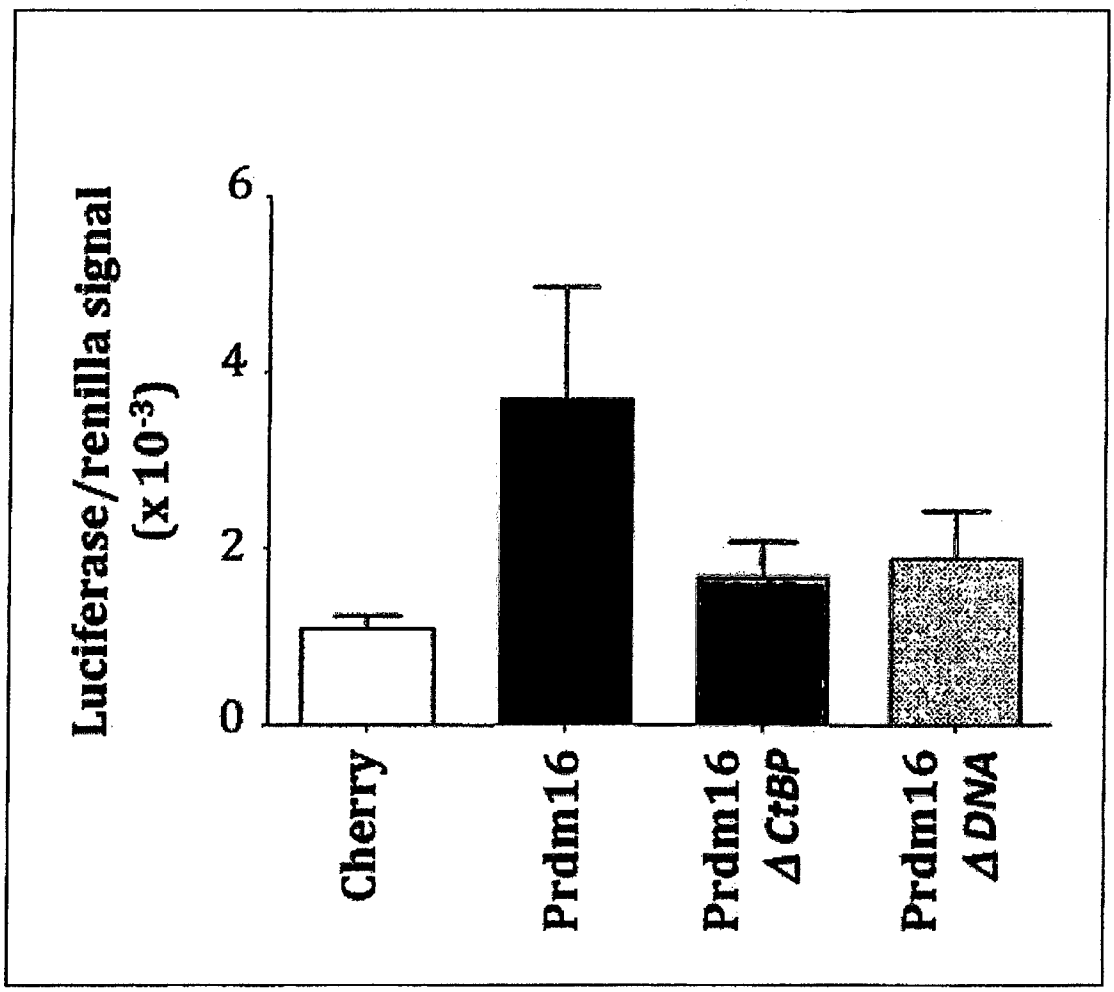
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 46 *shows that Prdm16, but not Prdm16ΔCtBP or Prdm16ΔDNA exerts full arterialising capacity in BOECs.*



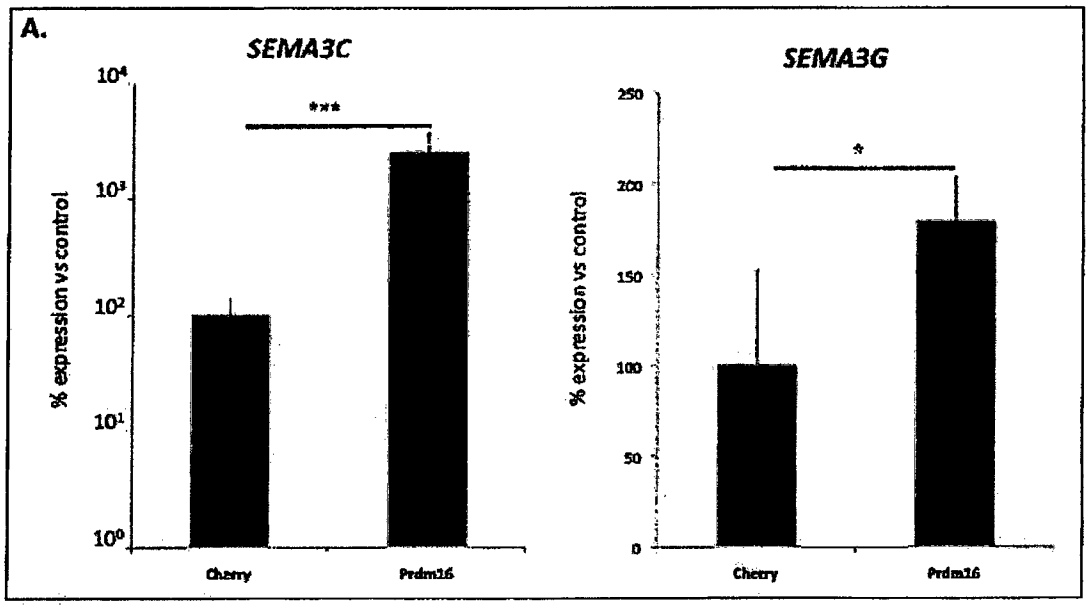
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 47 *shows that Prdm16, but not Prdm16ΔCtBP or Prdm16ΔDNA induces full Notch activation.*



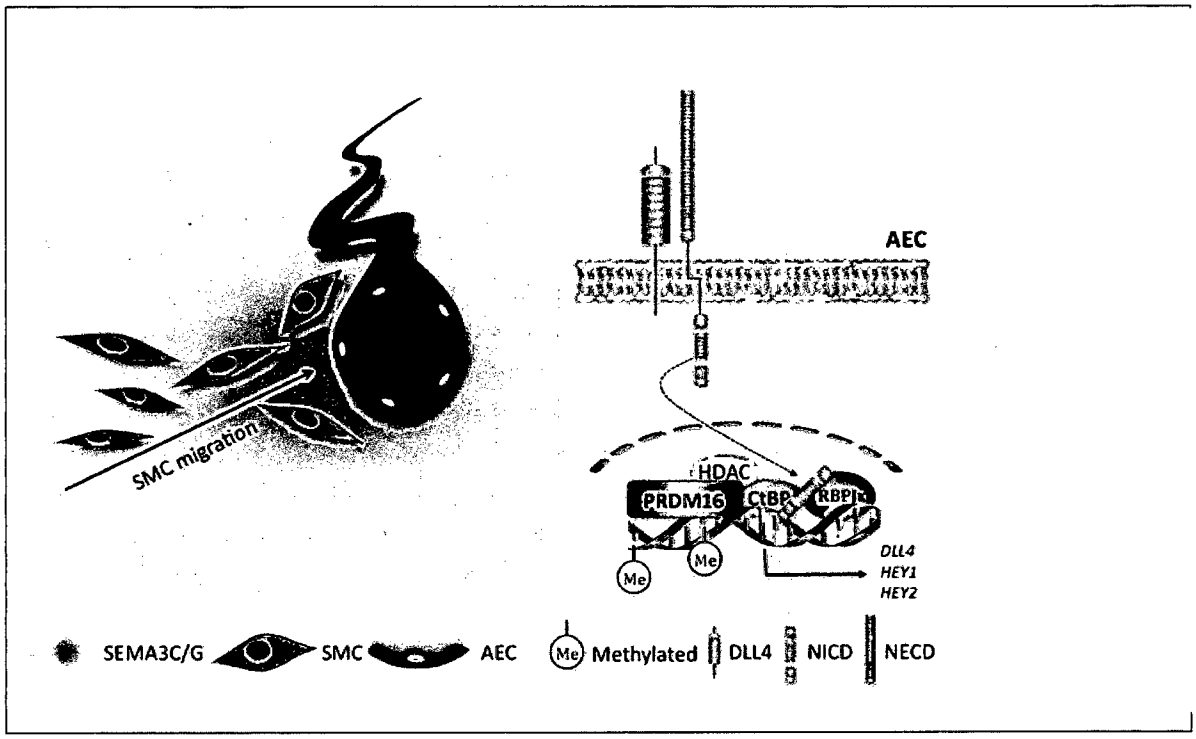
INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 48A *shows how Prdm16 leads the way to proper arterial SMC coating.*



INCORPORATED BY REFERENCE (RULE 20.6)

FIGURE 48B: *shows how Prdm16 leads the way to proper arterial SMC coating.*



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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/064410

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/071
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LUTTUN AERNOUT ET AL: "Differentiation of multipotent adult progenitor cells into functional endothelial and smooth muscle cells.", CURRENT PROTOCOLS IN IMMUNOLOGY / EDITED BY JOHN E. COLIGAN ... [ET AL.] DEC 2006, vol. Chapter 22, December 2006 (2006-12), XP9174035, ISSN: 1934-368X the whole document	1-39

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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Date of the actual completion of the international search	Date of mailing of the international search report
8 November 2013	15/11/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Novak-Giese, Sabine

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Information on patent family members

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