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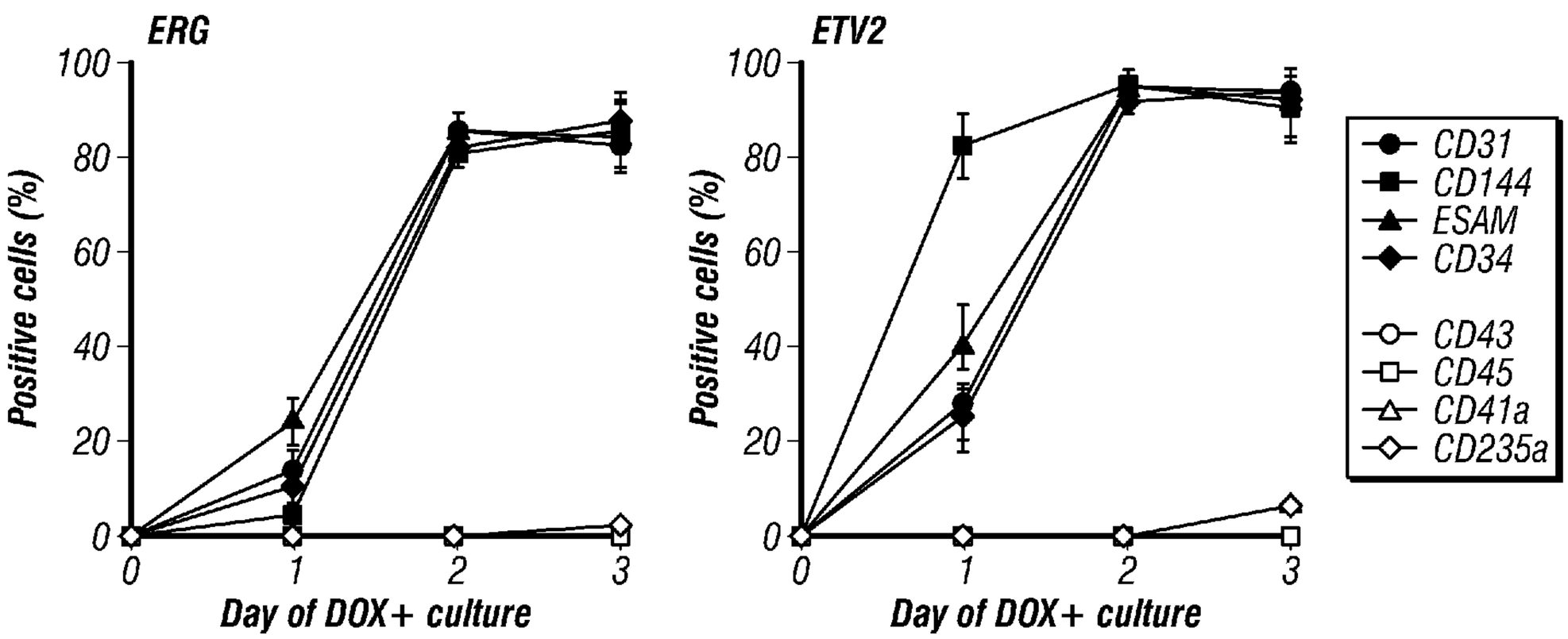
(54) **Titre : PRODUCTION DE CELLULES ENDOTHELIALES PAR PROGRAMMATION**
(54) **Title: ENDOTHELIAL CELL PRODUCTION BY PROGRAMMING**



(57) **Abrégé/Abstract:**
The invention generally regards methods for providing endothelial cells and precursors of endothelial cells from a variety of cell sources, such as pluripotent stem cells. Also provided are therapeutic compositions including the provided endothelial cells, and methods of using them for the treatment of subjects.

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(54) Title: ENDOTHELIAL CELL PRODUCTION BY PROGRAMMING

(57) Abstract: The invention generally regards methods for providing endothelial cells and precursors of endothelial cells from a variety of cell sources, such as pluripotent stem cells. Also provided are therapeutic compositions including the provided endothelial cells, and methods of using them for the treatment of subjects.

DESCRIPTION

ENDOTHELIAL CELL PRODUCTION BY PROGRAMMING

BACKGROUND OF THE INVENTION

[0001] This application claims priority to U.S. Provisional Application No. 61/362,085 filed on July 7, 2010.

1. Field of the Invention

[0002] The present invention relates generally to the field of molecular biology, stem cells, and differentiated cells. More particularly, it concerns programming of somatic cells and undifferentiated cells toward specific cell lineages, particularly endothelial cells and precursors of endothelial cells, such as endothelial progenitor cells.

2. Description of Related Art

[0003] Endothelial cells and precursors of endothelial cells have many potential therapeutic uses, including treatment of tissue ischemia—*e.g.*, as occurs in atherosclerosis, myocardial infarction, and limb ischemia—repair of injured blood vessels, and bioengineering of grafts. Preliminary studies have shown that transplantation of endothelial progenitor cells (EPCs) may be useful in treating ischemia in patients with myocardial infarction or limb ischemia (Dzau *et al.*, 2005). However, the clinical usefulness of EPCs obtained from patients is limited because patients in need of endothelial cell therapies often produce too few EPCs or EPCs that are functionally deficient.

[0004] In addition to such clinical applications, endothelial cells are in high demand for use in screening compounds and drugs for vascular toxicity, vascular permeability, and anti-cancer activity. However, primary endothelial cells have a finite proliferative potential due to their age, donor, and organ-type specific variations, all of which limit the ability to standardize endothelial cell culture protocols and to expand these cells in sufficient numbers for drug-screening purposes.

[0005] Endothelial cells may also be obtained from human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), both of which are capable of unlimited proliferation *in vivo* and retain their potential to differentiate into all somatic cell types.

Differentiation of human ESCs or iPSCs into cells of endothelial lineage *in vitro* recapitulates normal *in vivo* development and includes stages of mesoderm induction and specification of angiogenic mesodermal precursors. The process requires the addition of specific inductive factors. Endothelial cells derived from human ESCs or iPSCs are functional in *in vitro* assays and capable of transplantation *in vivo* (Li *et al.*, 2009). However, differentiation of endothelial cells from human ESCs or iPSCs is an inefficient process.

[0006] Therefore, there is a need for efficient production of endothelial cells and endothelial cell precursors for therapeutic and research uses.

SUMMARY OF THE INVENTION

[0007] The present invention overcomes a major deficiency in the art in providing endothelial cells and precursors of endothelial cells by forward programming or transdifferentiation to provide an unlimited supply of endothelial cells or precursors of endothelial cells. The methods may be particularly useful in providing an unlimited supply of patient-specific endothelial cells.

[0008] Methods disclosed herein provide endothelial cells or endothelial precursor cells by programming a variety of cell types. In certain aspects, programming methods include culturing pluripotent stem cells or somatic cells under conditions that increase the expression level of one or more genes that, when expressed alone or in combination with other programming factor genes, are capable of promoting programming to the endothelial lineage. Such genes are termed "endothelial programming factor genes." Endothelial programming factor genes useful in the invention may include any genes that, alone or in combination, directly impose endothelial fate upon non-endothelial cells and may include transcription factor genes or other genes that are important in endothelial cell differentiation or function. The process of programming alters the type of progeny a cell can produce and includes the distinct processes of forward programming and transdifferentiation. In some embodiments, forward programming of multipotent cells or pluripotent cells provides endothelial cells or endothelial precursor cells. In other embodiments, transdifferentiation of non-endothelial somatic cells provides endothelial cells or endothelial precursor cells. In certain aspects, programming may comprise increasing the expression level of a sufficient number of endothelial programming factor genes to cause forward programming or transdifferentiation of non-endothelial cells to endothelial precursor cells or endothelial cells. Sources of cells suitable for endothelial programming may include any stem cells or non-

endothelial cell somatic cells. For example, the stem cells may be pluripotent stem cells or any non-pluripotent stem cells. As used herein, a "pluripotent cell" or "pluripotent stem cell" is a cell that has the capacity to differentiate into essentially any fetal or adult cell type. Exemplary types of pluripotent stem cells may include, but are not limited to, embryonic stem cells and induced pluripotent stem cells (or iPS cells). Such a pluripotent stem cell may be a mammalian pluripotent stem cell. In certain embodiments, the pluripotent stem cell is a human pluripotent stem cell. Sources of cells suitable for programming of endothelial precursors or endothelial cells by transdifferentiation may include any non-endothelial somatic cells. Such somatic cells may be any cells forming the body of an organism. In a particular aspect, the somatic cells may be immortalized to provide an unlimited supply of cells, for example, by increasing the level of telomerase reverse transcriptase (TERT). For example, the level of TERT can be increased by increasing the transcription of TERT from the endogenous gene, or by introducing a transgene through any gene delivery method or system.

[0009] Pluripotent stem cells useful in the invention may be induced pluripotent stem cells, embryonic stem cells, or pluripotent stem cells derived by nuclear transfer or cell fusion. The stem cells may also include multipotent stem cells, oligopotent stem cells, or unipotent stem cells. The stem cells may also include fetal stem cells or adult stem cells, such as hematopoietic stem cells, mesenchymal stem cells, neural stem cells, epithelial stem cells, or skin stem cells. In certain aspects, the stem cells may be isolated from umbilical tissue, placenta, amniotic fluid, chorion villi, blastocysts, bone marrow, adipose tissue, brain, peripheral blood, cord blood, menstrual blood, blood vessels, skeletal muscle, skin or liver.

[0010] A "progenitor cell" or "precursor cell" refers to a lineage-committed cell derived from a pluripotent stem cell. Thus, progenitor cells or precursor cells are more differentiated than pluripotent stem cells, but still have the capacity to differentiate into more than one type of cell. Endothelial cells provided by methods disclosed here may be mature endothelial cells. In other embodiments, the disclosed methods provide endothelial progenitor cells or endothelial precursor cells. Such cells are more differentiated than pluripotent stem cells but are capable of differentiating into endothelial cells or into other types of cells. In some aspects, the disclosed methods provide hematoendothelial (or hemangioblast) progenitor cells, which are capable of differentiating into hematopoietic cells or endothelial cells. In yet other embodiments, methods are provided for differentiating

endothelial progenitor cells or endothelial precursor cells into endothelial cells by forward programming.

[0011] In certain embodiments, endothelial cells or endothelial precursor cells are provided by forward programming of pluripotent stem cells or transdifferentiation of somatic cells. Such a method may comprise providing the endothelial cells or endothelial precursor cells by culturing the pluripotent stem cells or somatic cells under conditions to increase the expression level of one or more endothelial programming factor genes capable of causing forward programming of the pluripotent stem cells or transdifferentiation of the somatic cells into endothelial cells or endothelial precursor cells, thereby forward programming the pluripotent stem cells or transdifferentiating the somatic cells into endothelial cells or endothelial precursor cells.

[0012] As a skilled artisan would understand, methods for increasing the expression of the endothelial programming factor genes in the cells to be programmed may include any method known in the art, for example, by induction of expression of one or more expression cassettes previously introduced into the cells, or by introduction of nucleic acids such as DNA or RNA, polypeptides, or small molecules to the cells. Increasing the expression of certain endogenous but transcriptionally repressed programming factor genes may also comprise reversing the silencing or inhibitory effect on the expression of these programming factor genes by regulating the upstream transcription factor expression or epigenetic modulation.

[0013] In certain aspects, endothelial cells or endothelial precursor cells are provided by forward programming of pluripotent stem cells. Such pluripotent stem cells may be induced pluripotent stem cells. In other aspects, endothelial cells or endothelial precursor cells are provided by transdifferentiation of somatic cells. In some embodiments, the somatic cells are human somatic cells such as skin fibroblasts, adipose tissue-derived cells, keratinocytes, or blood cells. Somatic cells useful for transdifferentiation may be immortalized somatic cells. In a particular aspect, the somatic cells may be immortalized to provide an unlimited supply of cells, for example, by increasing the level of telomerase reverse transcriptase (TERT). For example, the level of TERT can be increased by increasing the transcription of TERT from the endogenous gene, or by introducing a transgene through any gene delivery method or system.

[0014] Endothelial cells or endothelial precursor cells may be provided by forward programming of pluripotent stem cells or transdifferentiation of somatic cells that comprise at least one exogenous expression cassette. The expression cassette may comprise one or more endothelial programming factor genes. In some aspects, pluripotent stem cells or somatic cells are contacted with one or more such endothelial programming factors comprising gene products of the one or more endothelial programming factor genes in an amount sufficient to cause forward programming of the pluripotent cells or transdifferentiation of the somatic cells into endothelial cells or endothelial precursor cells. In some embodiments, the one or more gene products are polypeptide products of one or more endothelial programming factor genes. In certain aspects, the one or more endothelial programming factors include a protein transduction domain to facilitate intracellular entry of polypeptides of the endothelial programming factor genes. Such protein transduction domains are well known in the art, such as an HIV TAT protein transduction domain, HSV VP22 protein transduction domain, Drosophila Antennapedia homeodomain ,or variants thereof. In other embodiments, the one or more gene products are RNA transcripts of one or more endothelial programming factor genes.

[0015] Endothelial programming factor genes useful in the invention may include any genes that, alone or in combination, directly impose endothelial fate upon non-endothelial cells, especially transcription factor genes or genes that are important in endothelial cell differentiation or endothelial cell function when expressed in cells. Endothelial cell programming factor genes include, but are not limited to v-ets erythroblastosis virus E26 oncogene homolog (avian) (ERG), v-ets erythroblastosis virus E26 oncogene homolog 1 (avian) (ETS1), v-ets erythroblastosis virus E26 oncogene homolog 2 (avian) (ETS2), ELF-1, ELF-4, FLI-1, TEL, ETV2 (ets variant 2, ER71, or Etsrp71), TAL1 (SCL), GATA2, or the Forkhead (FOX) transcription factors (*e.g.*, FoxC, FoxF, FoxH, and FoxO families). For example, one, two, three, four, five, six, seven, eight, nine, ten, or more of these exemplary genes, isoforms of such genes, or variants thereof may be used in certain aspects of the invention. Many of these genes have different isoforms, which may have similar functions and thus are contemplated for use in certain aspects of the invention.

[0016] In particular aspects, the endothelial programming factor gene is ERG. In certain embodiments, the endothelial programming factor gene is ERG isoform 3 (ERG-3); however, the programming factor gene may be any isoform of ERG, including ERG isoform

1 (ERG-1), ERG isoform 2 (ERG-2), and ERG isoform 4 (ERG-4). In yet other particular embodiments, the endothelial programming factor gene is ETV2.

[0017] "Forward programming," as used herein, refers to a process having essentially no requirement to culture cells through intermediate cellular stages using culture conditions that are adapted for each such stage and/or, optionally, having no need to add different growth factors during different time points between the starting cell source and the desired end cell product, *e.g.*, endothelial cells or endothelial cell precursors, as exemplified in the upper part of **FIG. 1**. On the other hand, the bottom part of **FIG. 1** demonstrates various developmental stages present in a step-wise differentiation process and the need to add different growth factors at different times during the process, which involves more labor, time, and expense than methods described in certain aspects of the current invention. Therefore, the methods of forward programming in certain aspects of the present invention are advantageous by avoiding the need to add different growth factors at different stages of programming or differentiation to improve efficiency.

[0018] In certain aspects, the cells for endothelial cell or endothelial precursor programming, such as, for example, pluripotent stem cells or somatic cells, comprise at least one exogenous expression cassette, wherein the expression cassette comprises one or more endothelial programming factor genes. One or more expression cassettes may drive expression of one or more endothelial programming factor genes in an amount sufficient to cause forward programming of pluripotent cells into endothelial cells or transdifferentiation of somatic cells into endothelial cells. In certain embodiments, one or more expression cassettes drive expression of v-ets erythroblastosis virus E26 oncogene homolog (avian) (ERG). In other certain aspects, one or more expression cassettes may drive expression of ETV2. Alternatively, the expression of one or more endothelial programming factor genes may be increased without the use of an expression cassette.

[0019] In methods utilizing one or more exogenous expression cassettes, such an expression cassette may include an externally inducible transcriptional regulatory element for inducible expression of one or more endothelial programming factor genes. For example, an exogenous expression cassette useful in the invention may contain an inducible promoter, such as a promoter that includes a tetracycline response element. In some embodiments, the exogenous expression cassette is comprised in a gene delivery system. For example, such a gene delivery system may be a transposon system, a viral gene delivery system, or an

episomal gene delivery system. A viral gene delivery system useful in the invention may be an RNA-based or DNA-based viral vector. An episomal gene delivery system useful in the invention may be a plasmid, an Epstein-Barr virus (EBV)-based episomal vector, a yeast-based vector, an adenovirus-based vector, a simian virus 40 (SV40)-based episomal vector, a bovine papilloma virus (BPV)-based vector, or the like. In certain aspects, an expression cassette for use in forward programming or transdifferentiation may include an endothelial-specific transcriptional regulatory element operably linked to a reporter gene.

[0020] In certain methods, cells for endothelial cell programming, such as pluripotent stem cells, are contacted with one or more endothelial programming factors in an amount sufficient to cause forward programming of the cells into endothelial cells. Endothelial programming factors include endothelial programming factor genes, products of such genes, or fragments of products of such genes. Endothelial programming factors may be gene products of one or more endothelial programming factor genes. For example, the one or more gene products may be polypeptides of one or more endothelial programming factor genes or fragments of polypeptides of one or more endothelial programming factor genes. In particular embodiments, an endothelial programming factor is a product of the ERG gene (including any isoform thereof), ETS1 gene, ETS2 gene, ELF-1 gene, ELF-4 gene, FLI-1 gene, TEL gene, ETV2 (ER71 or Etsrp71) gene, TAL1 (SCL) gene, GATA2 gene, or a Forkhead (FOX) transcription factor gene (*e.g.*, a member of the FoxC, FoxF, FoxH, or FoxO family).

[0021] In some embodiments, methods of providing endothelial cells or endothelial precursor cells by forward programming of pluripotent stem cells or transdifferentiation of somatic cells are provided wherein the pluripotent stem cells, somatic cells, or progeny cells of pluripotent stem cells or somatic cells contain a reporter expression cassette. Such an expression cassette may comprise an endothelial programming factor gene. In certain embodiments, such an expression cassette may comprise an endothelial cell-specific transcriptional regulatory element operably linked to a reporter gene. In particular embodiments, an endothelial cell-specific promoter may be operably linked to a reporter. For example, the promoter of FLT-1, von Willebrand factor (vWF), or TIE1 may be operably linked to a reporter in an expression cassette in some embodiments.

[0022] Endothelial cells or endothelial precursor cells generated by any of the methods provided here may have one or more characteristics of endothelial cells. For

example, such endothelial cells may express one or more endothelial cell markers. Endothelial cell markers include, but are not limited to, VE-cadherin (CD144), ACE (angiotensin-converting enzyme) (CD143), BNH9/BNF13, CD31, CD34, CD54 (ICAM-1), CD62E (E-Selectin), CD105 (Endoglin), CD146, Endocan (also called ESM-1), Endoglyx-1, Endomucin, Eotaxin-3, EPAS1 (Endothelial PAS domain protein 1), Factor VIII related antigen, FLI-1, Flk-1 (KDR, VEGFR-2), FLT-1 (VEGFR-1), GATA2, GBP-1 (guanylate-binding protein-1), GRO-alpha, HEX, ICAM-2 (intercellular adhesion molecule 2), LMO2, LYVE-1, MRB (magic roundabout), Nucleolin, PAL-E (pathologische anatomie Leiden-endothelium), RTKs, sVCAM-1, TAL1, TEM1 (Tumor endothelial marker 1), TEM5 (Tumor endothelial marker 5), TEM7 (Tumor endothelial marker 7), Thrombomodulin (TM, CD141), VCAM-1 (vascular cell adhesion molecule-1) (CD106), VEGF (Vascular endothelial growth factor), vWF (von Willebrand factor, also called Factor VIII), ZO-1, endothelial cell-selective adhesion molecule (ESAM), CD102, CD93, CD184, CD304, and DLL4. In particular embodiments, an endothelial cell marker useful in the invention is one or more of CD144, CD31, CD34, ESAM, CD102, CD143, CD93, CD184, CD105, CD146, von Willebrand factor, ZO-1, CD304, and DLL4. In some embodiments, the endothelial cells produced by forward programming or transdifferentiation do not express certain markers or exhibit decreased expression of certain markers, such as markers of mesenchymal cells (*e.g.*, CD140a, CD140b), markers of hematopoietic cells (*e.g.*, CD43, CD45, CD235a, or CD41a) or markers of human pluripotent stem cells (*e.g.*, TRA1-60).

[0023] Other characteristics of endothelial cells useful in the invention are functional characteristics of endothelial cells. For example, one such functional characteristic is the ability to take up acetylated low density lipoprotein (ac-LDL). Yet another functional characteristic of endothelial cells is the ability to form tube-like structures in a three dimensional matrix, such as Matrigel™. An additional functional characteristic of endothelial cells is barrier function. Another characteristic of endothelial cells useful in the invention is the ability to respond to one or more pro-inflammatory stimuli (*e.g.*, TNF, and IL-1) by upregulating the expression of cell-adhesion molecules (*e.g.*, CD54 (ICAM-1), CD106, and CD62E). Yet another characteristic of endothelial cells useful in the invention is the expression of tight junction proteins (*e.g.*, Claudin 5 and ZO-1). Other additional characteristics of endothelial cells useful in the invention are morphological features, such as a flattened (or squamous) appearance and a large, central nucleus.

[0024] In certain embodiments, methods may further include one or more steps that select or enrich for endothelial cells. For example, the selected or enriched endothelial cells may express a reporter gene that is operably linked to an endothelial cell-specific transcriptional regulatory element. In other embodiments, the selected or enriched endothelial cells may exhibit one or more endothelial cell characteristics. For example, the selected or enriched endothelial cells may express one or more endothelial cell markers, exhibit one or more functional characteristics of endothelial cells, or exhibit one or more morphological characteristics of endothelial cells.

[0025] In certain embodiments, pluripotent stem cells used in methods disclosed here are cultured in a medium that contains one or more growth factors. For example, the medium may contain basic FGF, VEGF, or both. Such culturing may be prior to, during, or after the increased expression of endothelial programming factors.

[0026] Endothelial cells provided by methods disclosed herein may be provided at least, about or up to 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 days (or any range derivable therein) after the increased expression or culturing in the presence or absence of growth factors. In some particular methods, the provided endothelial cells are obtained after up to 10 days of the increased expression of one or more endothelial programming factor genes. In other embodiments, the provided endothelial cells are obtained after up to 4 days of the increased expression.

[0027] In certain aspects, the methods include one or more additional steps wherein cell groupings are dispersed into essentially individual cells. The dispersing may be performed, for example, at least about 24 hours after the increased expression. In some embodiments, the dispersing is performed at least 1, 2, 3, 4, or more days after the increased expression. The methods may also include one or more steps wherein the essentially individual cells are dispersed onto a surface coated with a matrix component. For example, the surface may be coated with fibronectin, gelatin, collagen, poly-d-lysine, matrigel, or an RGD peptide. Cells plated onto a surface coated with a matrix component may be cultured. In some embodiments, cells plated onto a surface coated with a matrix component are cultured for at least about 12 hours. After the culturing, unattached cells may be removed, and the attached cells may be further cultured. For example, the attached cells may be further cultured for at least two days.

[0028] Dispersing of cell groupings may be performed by mechanical or enzymatic means. For example, the cells may be dispersed by treatment with an effective amount of one or more enzymes, such as trypsin or trypLE, or a mixture of enzymes such as Accutase®. Dispersed cells may be cultured in a medium comprising one or more growth factors. For example, the dispersed cells may be cultured in a medium that contains basic FGF, VEGF, or both.

[0029] Also provided are methods of providing endothelial progenitor cells by forward programming of pluripotent stem cells or transdifferentiation of somatic cells. In such methods, the endothelial progenitor cells may be provided by culturing pluripotent stem cells or somatic cells under conditions to increase the expression level of one or more endothelial programming factor genes capable of causing forward programming of the pluripotent cells or transdifferentiation of the somatic cells into endothelial progenitor cells, thereby forward programming the pluripotent stem cells into endothelial progenitor cells or transdifferentiating the somatic cells into endothelial progenitor cells.

[0030] Methods of providing arterial endothelial cells are also provided. In some aspects, the method includes increasing the expression of one or more endothelial programming factors such as, for example ERG or ETV2. In certain embodiments, the arterial endothelial cells express one or more arterial endothelial cells markers such as, for example, CD304, CD184, or DLL4.

[0031] In certain aspects, methods of providing hemogenic endothelial cells are provided. In some aspects, the hemogenic endothelial cells are provided by increasing expression of an endothelial programming factor, such as, for example, ETV2. Such hemogenic endothelial cells may be used to generate hematopoietic cells when cultured in a hematopoietic culture medium. The hematopoietic culture medium may be any medium suitable for generating hematopoietic cells. For example, the hematopoietic culture medium may include one or more components selected from the group consisting of ESFM, StemLine HSC medium (Sigma), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), stem cell factor (SCF), thrombopoietin (TPO), interleukin-3 (IL-3), and interleukin-6 (IL-6). In particular embodiments, the hematopoietic culture medium includes ESFM, StemLine HSC medium (Sigma), FGF, VEGF, SCF, TPO, IL-3, and IL-6. The generated hematopoietic cells may comprise one or more hematopoietic cell markers selected from the

group consisting of CD43, CD45, CD235a, and CD41a. In certain aspects, the hematopoietic cells are CD43+, CD45+, CD235a+ and/or CD41a+.

[0032] In other aspects, methods of providing mesenchymogenic endothelial cells are provided. In some aspects, the mesenchymogenic endothelial cells are provided by increasing expression of an endothelial programming factor, such as ERG or ETV2. In particular embodiments, the mesenchymogenic endothelial cells are provided by increasing expression of ERG. Such mesenchymogenic endothelial cells may be used to generate mesenchymal cells when cultured in a mesenchymal culture medium. The mesenchymal culture medium may be any medium suitable for generating mesenchymal cells. For example, the mesenchymal culture medium may include one or more components selected from the group consisting of FGF and a TGF-beta inhibitor such as, for example, A83-01. In particular embodiments, the mesenchymal culture medium includes FGF and A83-01. The generated mesenchymal cells may comprise one or more mesenchymal cell markers selected from the group consisting of CD73 and CD105. In some aspects, the generated mesenchymal cells are CD31-CD73+CD105+.

[0033] The endothelial cells, endothelial progenitor cells, or precursors of endothelial cells provided herein may be used in any methods and applications currently known in the art for endothelial cells, such as clinical or screening applications. For example, the invention provides methods of assessing a compound for an effect on an endothelial cell. In such methods, an endothelial cell, which may be provided by any method disclosed here, may be contacted with a compound, and the effect of the compound on the endothelial cell may be assayed. For example, a pharmacological or toxicological effect on the endothelial cell may be assayed. In certain embodiments, endothelial cells of the invention are used to assess drug vascular toxicity or vascular permeability. In other embodiments, endothelial cells of the invention are used for development of anti-cancer drugs. Arterial endothelial cells may be used to study diseases such as thrombosis, atherosclerosis, and hypertension.

[0034] In some aspects, methods of treating a subject are provided. For example, the subject may have, or is at risk for, a cardiovascular disease or a cardiovascular injury. In some embodiments, the subject has, or is at risk for, ischemia. In yet other embodiments, the subject has a tissue injury or is in need of a tissue graft. In certain aspects, any such subject is treated by administering to the subject a therapeutically effective amount of endothelial cells or endothelial progenitor cells that are provided by any method disclosed herein. In addition,

in some embodiments, endothelial cells provided by methods of the invention may be used to bioengineer a tissue graft that is administered to a patient in need of such therapy. In some embodiments, arterial endothelial cells are used in methods of treatment, such as in methods of treating arterial insults, injuries, or diseases.

[0035] In certain embodiments, the invention is directed to an endothelial cell or endothelial precursor cell. Such an endothelial cell or endothelial precursor may be provided by a process in accordance with any of the methods disclosed herein. In other certain embodiments, the invention is directed to an endothelial progenitor cell or endothelial precursor cell. Such endothelial progenitor cells or precursor cells may be provided by a process in accordance with any of the methods disclosed herein.

[0036] In yet other embodiments, a cell population is provided. Such a cell population may comprise pluripotent stem cells, somatic cells, endothelial cells, endothelial progenitor cells, other precursors of endothelial cells, stem cells, or progeny of any of these. For example, the cell population may consist of endothelial cells, wherein at least 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 99% or more of the endothelial cells, or any range derivable therein, carry an exogenous expression cassette that includes one or more endothelial programming factor genes. In particular embodiments, 80% of the endothelial cells carry an exogenous expression cassette that includes one or more endothelial programming factor genes. In other aspects, the cell population may consist of endothelial progenitor cells, wherein at least 80% of the endothelial progenitor cells carry an exogenous expression cassette that includes one or more endothelial programming factor genes. In yet other aspects, a cell population is provided that contains pluripotent stem cells or somatic cells where 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 99% or more of the cells, or any range derivable therein, carry an exogenous expression cassette that includes one or more endothelial programming factor genes. For example, the endothelial programming factor gene may be ERG or ETV2.

[0037] Also provided is a composition comprising a cell population comprising two cell types, *i.e.*, the cells to be programmed to endothelial cells and endothelial cells, and essentially free of other intermediate cell types. For example, such a cell population may have two cell types including stem cells and endothelial cells, but essentially free of other cell types in the intermediate developmental stages along the endothelial cell differentiation process. In particular, a composition comprising a cell population consisting of stem cells and

endothelial cells may be provided. The stem cells may be particularly pluripotent stem cells, *e.g.*, induced pluripotent stem cells. Endothelial cells may be at least, about, or up to 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 99% of the cell population, or any range derivable therein.

[0038] In certain embodiments, endothelial cells are provided by forward programming of endothelial progenitor cells. For example, the endothelial progenitor cells may be cultured under conditions to increase the expression level of one or more endothelial programming factor genes, such as those described herein, capable of causing forward programming of the endothelial progenitor cells into endothelial cells, thereby forward programming the progenitor cells into endothelial cells. In other embodiments, endothelial cells are provided by transdifferentiation of non-endothelial immortalized somatic cells. For example, the non-endothelial immortalized somatic cells may be cultured under conditions to increase the expression level of one or more endothelial programming factor genes, such as those described herein, capable of causing transdifferentiation of the somatic cells to endothelial cells, thereby transdifferentiating the somatic cells into endothelial cells.

[0039] Embodiments discussed in the context of methods and/or compositions of the invention may be employed with respect to any other method or composition described herein. Thus, an embodiment pertaining to one method or composition may be applied to other methods and compositions of the invention as well.

[0040] As used herein the terms "encode" or "encoding" with reference to a nucleic acid are used to make the invention readily understandable by the skilled artisan however these terms may be used interchangeably with "comprise" or "comprising" respectively.

[0041] As used herein the specification, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising," the words "a" or "an" may mean one or more than one.

[0042] The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or." As used herein "another" may mean at least a second or more.

[0043] Throughout this application, the term "about" is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

[0044] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the 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.

BRIEF DESCRIPTION OF THE DRAWINGS

[0045] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0046] FIG. 1. Alternative approaches for endothelial cell differentiation from human ESCs/iPSCs. ECs can be efficiently induced from human ESCs/iPSCs via expression of appropriate transgene(s) (top box), bypassing most, if not all, developmental stages observed during normal differentiation (bottom box).

[0047] FIG. 2. The strategy employed for identifying transgenes that directly convert human ESC/iPSCs to endothelial cells. Human ESCs/iPSCs were engineered to constitutively express rtTET protein for inducible gene expression. Transgenes under the control of the inducible promoter Ptight are introduced into the engineered hESCs/iPSCs by electroporation. Upon doxycycline (Dox) addition, transgene expression is induced, and EC differentiation is monitored by the characteristic EC morphology, along with expression of EC markers (CD31, CD144 (VE-cadherin)) by flow cytometry.

[0048] FIG. 3. The establishment of human ESC/iPSC inducible lines for endothelial cell differentiation. The human Rosa26 locus on chromosome 3 was selected to allow the expression of rtTET, while minimizing the chromosome location-dependent silencing effect. First, the LoxP recombination sites (LOX71 and LOX2272) were introduced into a site between exon 1 and exon 2 of the human ROSA 26 gene via homologous recombination.

The targeting construct (KI construct) used the phosphoglycerate kinase promoter (PGK)-driven expression of diphtheria toxin A fragment gene (DTA) for negative selection, and contains a ~ 2.0 kb 5' arm and a 4.5 kb 3' arm. A splicing acceptor signal from human BCL2 gene (SA) was placed in front of LOX71 site to allow the expression of selection markers from the endogenous human ROSA26 promoter. The coding region for thymidine kinase (TK) was included to enable negative selection against incorrect Cre/LoxP recombination events at step 2 using ganciclovir. The neomycin phosphotransferase (Neo) was used for positive selection during homologous recombination (step 1). The foot-and-mouth disease virus peptide (2A) was used to co-express the TK and Neo genes from the endogenous human ROSA26 promoter. BGHpA: polyadenylation signal derived from bovine growth hormone gene. The homologous recombination yielded parental human ESC/iPSC lines for efficient cassette exchange via Cre/LoxP recombination. To establish inducible cell lines for EC differentiation, rtTET driven by the constitutively active eukaryotic elongation factor 1α promoter (pEF) was introduced into the Rosa 26 locus by lipid-mediated cotransfection of the recombination mediated cassette exchange (RMCE) vector and a Cre-expressing plasmid. The puromycin N-acetyl-transferase (Puro) was used to select for recombination events. The correctly recombined inducible cells are resistant to puromycin (Puro+) and ganciclovir (TK-), and sensitive to geneticin selection (Neo-).

[0049] FIGS. 4A, 4B. Confirmation of Tet-On inducible gene expression in human H1 ESC inducible lines. **FIG. 4A.** A two-vector *PiggyBac* stable gene expression system; Ptight is an rtTET-responsive inducible promoter; pEF is the eukaryotic elongation factor 1α promoter; hPBase is the coding region for the *PiggyBac* transposase with codons optimized for expression in human cells. **FIG. 4B.** Flow cytometric analysis of EGFP expression in human ESC inducible lines after 4 days induction with or without Doxycycline (1 μg/mL). Gray lines: Human ESC inducible lines with transfection of the EGFP vector; Black lines: Human ESC R/I lines with stable PiggyBac transposon integration after 4 days induction with or without Doxycycline.

[0050] FIG. 5. Bright-field images of direct endothelial cell (EC) induction from human ESC inducible lines via ERG expression. ERG-3 was cloned into the PiggyBac vector (Fig. 4A) under the control of the Ptight promoter and introduced into the human ESC inducible line by electroporation, along with an hPBase-expressing vector. Transfected cells were cultured in TeSR medium on matrigel in the presence of geneticin (100 μg/ml) for

selection of transformants having stable genomic transgene integration. Doxycycline (0.2 μg/ml) was added to induce ERG-3 expression, and the TeSR was replaced with endothelial serum-free medium (ESFM; Invitrogen) supplemented with 10 ng/ml basic FGF and 20 ng/ml VEGF (both from Peprotech). Differentiated cells acquire the EC morphology on day 2-3 of ERG induction. Although ERG-3 expression was used in these experiments to provide the results shown here, similar results were obtained with the other ERG isoforms including ERG isoform 1, ERG isoform 2, and ERG isoform 4 (data not shown).

[0051] FIG. 6. Bright-field images of forward programming of ECs from human ESC inducible lines via ETV2 expression. ETV2 was cloned into the PiggyBac vector (Fig. 4A) under the control of the Ptight promoter and then introduced into the human ESC inducible line by electroporation along with the hPBase-expressing vector. Transfected cells were cultured in TeSR medium on matrigel in the presence of geneticin (100 μg/ml) for selection of transformants having stable genomic transgene integration. Doxycycline (0.2 μg/ml) was added to induce ETV2 expression, and the TeSR was replaced with endothelial serum-free medium (ESFM; Invitrogen) supplemented with 10 ng/ml basic FGF and 20 ng/ml VEGF (both from Peprotech). Differentiated cells acquire EC morphology on day 2-3 of ETV2 induction.

[0052] FIG. 7. Flow cytometric expression analysis of the human pluripotent stem cell-specific marker TRA-1-60 and the EC markers (CD144/VE-cadherin and CD31) during ERG-induced EC differentiation from human ESCs. The ERG-induced differentiated cells up-regulated the expression of the EC markers (CD144 and CD31), while down-regulating the expression of the human pluripotent stem cell marker TRA-1-60.

[0053] FIG. 8. Flow cytometric expression analysis of the human pluripotent stem cell-specific marker TRA-1-60 and the EC markers (CD144/VE-cadherin and CD31) during ETV2-induced EC differentiation from human ESCs. The ETV2-induced differentiated cells up-regulated the expression of the EC markers (CD144 and CD31), while down-regulated the expression of the human pluripotent stem cell marker TRA-1-60.

[0054] FIG. 9. Kinetic analysis of the expression of EC markers (CD31, CD144, ESAM, CD34) and hematopoietic markers (CD43, CD45, CD41a, CD235a) in ERG- and ETV2-induced hESC cultures.

[0055] FIG. 10. Bright-field images of established ECs obtained from human ESCs either through normal differentiation (EEC) or via the expression of ERG (ERG-EC) or ETV2 (ETV2-EC). Cell cultures on day 3 of induction were dissociated into single-cell suspension by Accutase treatment (Invitrogen) and plated on gelatin-coated plastic in ESFM supplemented with 10 ng/ml basic FGF. After 2 hours of plating, medium containing non-adherent cells was removed and attached cells were cultured in ESFM supplemented with 10 ng/ml basic FGF and 5 µg/ml human fibronectin (Invitrogen). The morphology of ERG-ECs and ETV2-ECs was highly similar to that of HUVEC and EECs.

[0056] FIG 11. Flow cytometric expression analysis of EC and hematopoietic markers. The expression of all three markers for arterial ECs (CD304/NRP1, CD184/CXCR4 and DLL4) in ERG-ECs and ETV2-ECs might suggest an arterial fate of these induced ECs, different from HUVEC and EECs.

[0057] FIG. 12. Immunofluorescence analysis of ERG-ECs and ETV2-ECs: DAPI was used to counterstain the nuclei. Although the staining was generally weaker than in HUVEC, vWF was clearly expressed in both ERG-ECs and ETV2-ECs.

[0058] FIG. 13. Flow cytometric and Immunofluorescence analysis of tight junction proteins (Claudin 5 and ZO-1) expression in ERG-ECs and ETV2-ECs.

[0059] FIG. 14. Ac-LDL incorporation by ERG-ECs and ETV2-ECs. ECs were incubated with AcLDL-Dil conjugate (Invitrogen, 2 µg/ml) for 4 hours at 37°C, followed by incubation with 0.5 µg/ml Hoechst 33258 for 5 minutes for counterstaining. For flow cytometric analysis, the Ac-LDL-treated cultures were cultured in fresh medium overnight prior to Accutase dissociation. Non-treated ECs were used as control.

[0060] FIG. 15. Barrier function analysis of ERG-ECs and ETV2-ECs by measuring their transendothelial resistance (TER) using the ECIS ZΘinstrument (Applied Biophysics). The upper line shows the baseline TER, while the lower line shows the disruption and recovery of barrier function in response to thrombin (0.5 U/ml). The ERG-ECs showed similar kinetics in barrier function recovery as compared to HUVEC, while the ETV2-ECs were slower, suggesting that the ERG-ECs and ETV2-ECs are different.

[0061] FIG. 16. Tube formation by ERG-induced ECs. ERG-ECs were plated on the solidified matrigel at 25000 cells/cm2 in ESFM supplemented with 40 ng/ml VEGF and incubated 12 hours.

[0062] FIG. 17. Inflammatory responses of ERG-ECs and ETV2-ECs by increased expression of CD54, CD62E and CD106 activation markers in response to TNF treatment. EC cultures were treated with 25 ng/ml TNF for 24 hours and analyzed by flow cytometry.

[0063] FIG. 18. Hemogenic function of ETV2-ECs. ETV2 and ERG induction was performed in medium containing 50% ESFM, 50% StemLine HSC medium (Sigma), 10 ng/ml FGF, 5 ng/ml VEGF, 50 ng/ml SCF, 20 ng/ml SCF, 10 ng/nl TPO, 10 ng/ml IL3 and 20 ng/ml IL6. Hematopoietic cells defined by CD31+CD43+ phenotype were detected in ETV2, but not in ERG-induced cultures. The majority of the hematopoietic cells in the day 9 ETV2 culture were also CD235a/CD41a-CD45+, suggesting definitive hematopoiesis.

[0064] FIG. 19. Mesenchymogenic potential of ERG-ECs. ERG-ECs were cultured in ESFM containing 10 ng/ml FGF2 and additionally supplemented either with 20 ng/ml VEGF or with 1 μm A83-01 (TGFβ inhibitor). Gradual transition of ERG-EC to mesenchymal cells defined by CD31-CD73+CD105+ phenotype was observed in cultures containing FGF+A83-01, but not FGF+VEGF. Although ETV2-EC cells undergo a similar mesenchymal transition, efficiency was lower than in ERG-EC.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0065] Endothelial cells comprise the lining of the blood vessels and are important for a variety of processes in the body. For example, endothelial cells play roles in angiogenesis, regulation of blood pressure, blood clotting, inflammation, and filtration. Endothelial cells are a heterogeneous group of cells and may have a variety of characteristics depending upon vessel size, specification to a specific organ, and morphology. Some characteristics of endothelial cells include expression of CD31, CD105 (endoglin), and Willebrand factor (also called Factor VIII), as well as the ability to take up acetylated low density lipoprotein (ac-LDL).

[0066] The present invention overcomes several major problems with current technologies by providing methods and compositions for endothelial cell production by forward programming or transdifferentiation. In contrast to previous methods using step-wise differentiation protocols, certain aspects of these methods increase the level of endothelial programming transcription factors in non-endothelial cells to provide endothelial cells by forward programming or transdifferentiation. Extra steps, such as adding different growth factors during various intermediate developmental stages may be unnecessary in certain aspects of the present methods. Therefore, certain aspects of the present methods may be more time- and cost-efficient and may enable manufacture of endothelial cells or endothelial progenitor cells for therapeutics from a renewable source, such as, for example, stem cells or somatic cells. Further embodiments and advantages of the invention are described below.

I. Definitions

[0067] "Programming" is a process that alters the type of progeny a cell can produce. For example, a cell has been programmed when it has been altered so that it can form progeny of at least one new cell type, either in culture or *in vivo*, as compared to what it would have been able to form under the same conditions without programming. This means that after sufficient proliferation, a measurable proportion of progeny having phenotypic characteristics of the new cell type are observed, if essentially no such progeny could form before programming; alternatively, the proportion having characteristics of the new cell type is measurably more than before programming. This process includes differentiation, dedifferentiation and transdifferentiation. "Differentiation" is the process by which a less specialized cell becomes a more specialized cell type. "Dedifferentiation" is a cellular process in which a partially or terminally differentiated cell reverts to an earlier developmental stage, such as pluripotency or multipotency. "Transdifferentiation" is a process of transforming one differentiated cell type into another differentiated cell type. Under certain conditions, the proportion of progeny with characteristics of the new cell type may be at least about 1%, 5%, 25% or more in order of increasing preference.

[0068] The term "endothelial programming factor" is a gene that, when expressed alone or in combination with another programming factor gene, is capable of causing direct differentiation of pluripotent cells or non-endothelial somatic cells into endothelial cells or endothelial precursor cells.

[0069] The term "forward programming" refers to the programming of a multipotent or pluripotent cell, as opposed to a differentiated somatic cell that has no pluripotency, by the provision of one or more specific lineage-determining genes or gene products to the multipotent or pluripotent cell. For example, forward programming may describe the process of programming ESCs or iPSCs to endothelial cells, endothelial precursor cells, other precursor cells, or other differentiated somatic cells.

[0070] The term "exogenous," when used in relation to a protein, gene, nucleic acid, or polynucleotide in a cell or organism refers to a protein, gene, nucleic acid, or polynucleotide that has been introduced into the cell or organism by artificial or natural means; or in relation to a cell, refers to a cell that was isolated and subsequently introduced to other cells or to an organism by artificial or natural means. An exogenous nucleic acid may be from a different organism or cell, or it may be one or more additional copies of a nucleic acid that occurs naturally within the organism or cell. An exogenous cell may be from a different organism, or it may be from the same organism. By way of a non-limiting example, an exogenous nucleic acid is one that is in a chromosomal location different from that of natural cells, or is otherwise flanked by a different nucleic acid sequence than that found in nature. An exogenous nucleic acid may also be extra-chromosomal, such as an episomal vector.

[0071] By "expression construct" or "expression cassette" is meant a nucleic acid molecule that is capable of directing transcription. An expression construct includes, at a minimum, one or more transcriptional control elements (such as promoters, enhancers or a structure functionally equivalent thereof) that direct gene expression in one or more desired cell types, tissues or organs. Additional elements, such as a transcription termination signal, may also be included.

[0072] A "vector" or "construct" (sometimes referred to as a gene delivery system or gene transfer "vehicle") refers to a macromolecule or complex of molecules comprising a polynucleotide to be delivered to a host cell, either *in vitro* or *in vivo*.

[0073] A "plasmid," a common type of a vector, is an extra-chromosomal DNA molecule separate from the chromosomal DNA that is capable of replicating independently of the chromosomal DNA. In certain cases, it is circular and double-stranded.

[0074] An "origin of replication" ("ori") or "replication origin" is a DNA sequence, *e.g.*, in a lymphotrophic herpes virus, that when present in a plasmid in a cell is capable of maintaining linked sequences in the plasmid, and/or a site at or near where DNA synthesis initiates. An ori for EBV includes FR sequences (20 imperfect copies of a 30 bp repeat), and preferably DS sequences; however, other sites in EBV bind EBNA-1, *e.g.*, Rep* sequences can substitute for DS as an origin of replication (Kirshmaier and Sugden, 1998). Thus, a replication origin of EBV includes FR, DS or Rep* sequences or any functionally equivalent sequences through nucleic acid modifications or synthetic combination derived therefrom. For example, the present invention may also use genetically engineered replication origin of EBV, such as by insertion or mutation of individual elements, as specifically described in Lindner, *et. al.*, 2008.

[0075] The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous (*i.e.*, is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA."

[0076] A "gene," "polynucleotide," "coding region," "sequence," "segment," "fragment," or "transgene" that "encodes" a particular protein, is a nucleic acid molecule that is transcribed and optionally also translated into a gene product, *e.g.*, a polypeptide, *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The coding region may be present in either a cDNA, genomic DNA, or RNA form. When present in a DNA form, the nucleic acid molecule may be single-stranded (*i.e.*, the sense strand) or double-stranded. The boundaries of a coding region are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A gene can include, but is not limited to, cDNA from prokaryotic or eukaryotic mRNA, genomic DNA sequences from prokaryotic or eukaryotic DNA, and synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the gene sequence.

[0077] The term "control elements" refers collectively to promoter regions, polyadenylation signals, transcription termination sequences, upstream regulatory domains,

origins of replication, internal ribosome entry sites (IRES), enhancers, splice junctions, and the like, which collectively provide for the replication, transcription, post-transcriptional processing, and translation of a coding sequence in a recipient cell. Not all of these control elements need be present so long as the selected coding sequence is capable of being replicated, transcribed, and translated in an appropriate host cell.

[0078] The term "promoter" is used herein in its ordinary sense to refer to a nucleotide region comprising a DNA regulatory sequence, wherein the regulatory sequence is derived from a gene that is capable of binding RNA polymerase and initiating transcription of a downstream (3' direction) coding sequence.

[0079] By "enhancer" is meant a nucleic acid sequence that, when positioned proximate to a promoter, confers increased transcription activity relative to the transcription activity resulting from the promoter in the absence of the enhancer domain.

[0080] By "operably linked" with reference to nucleic acid molecules is meant that two or more nucleic acid molecules (*e.g.*, a nucleic acid molecule to be transcribed, a promoter, and an enhancer element) are connected in such a way as to permit transcription of the nucleic acid molecule. "Operably linked" with reference to peptide and/or polypeptide molecules means that two or more peptide and/or polypeptide molecules are connected in such a way as to yield a single polypeptide chain, *i.e.*, a fusion polypeptide, having at least one property of each peptide and/or polypeptide component of the fusion. The fusion polypeptide is preferably chimeric, *i.e.*, composed of heterologous molecules.

[0081] "Homology" refers to the percent of identity between two polynucleotides or two polypeptides. The correspondence between one sequence and another can be determined by techniques known in the art. For example, homology can be determined by a direct comparison of the sequence information between two polypeptide molecules by aligning the sequence information and using readily available computer programs. Alternatively, homology can be determined by hybridization of polynucleotides under conditions that promote the formation of stable duplexes between homologous regions, followed by digestion with single strand-specific nuclease(s), and size determination of the digested fragments. Two DNA, or two polypeptide, sequences are "substantially homologous" to each other when at least about 80%, preferably at least about 90%, and most preferably at least

about 95% of the nucleotides, or amino acids, respectively match over a defined length of the molecules, as determined using the methods above.

[0082] The term "cell" is herein used in its broadest sense in the art and refers to a living body that is a structural unit of tissue of a multicellular organism, is surrounded by a membrane structure that isolates it from the outside, has the capability of self-replicating, and has genetic information and a mechanism for expressing it. Cells used herein may be naturally-occurring cells or artificially modified cells (*e.g.*, fusion cells, genetically modified cells, *etc.*).

[0083] As used herein, the term "stem cell" refers to a cell capable of giving rising to at least one type of a more specialized cell. A stem cells has the ability to self-renew, *i.e.*, to go through numerous cycles of cell division while maintaining the undifferentiated state, and has potency, *i.e.*, the capacity to differentiate into specialized cell types. Typically, stem cells can regenerate an injured tissue. Stem cells herein may be, but are not limited to, embryonic stem (ES) cells, induced pluripotent stem cells, or tissue stem cells (also called tissue-specific stem cells, or somatic stem cells). Any artificially produced cell having the above-described abilities (*e.g.*, fusion cells, reprogrammed cells, or the like used herein) may be a stem cell.

[0084] "Embryonic stem (ES) cells" are pluripotent stem cells derived from early embryos. An ES cell was first established in 1981, which has also been applied to production of knockout mice since 1989. In 1998, a human ES cell was established, which is currently becoming available for regenerative medicine.

[0085] Unlike ES cells, tissue stem cells have a limited differentiation potential. Tissue stem cells are present at particular locations in tissues and have an undifferentiated intracellular structure. Therefore, the pluripotency of tissue stem cells is typically low. Tissue stem cells have a higher nucleus/cytoplasm ratio and have few intracellular organelles. Most tissue stem cells have low pluripotency, a long cell cycle, and proliferative ability beyond the life of the individual. Tissue stem cells are separated into categories, based on the sites from which the cells are derived, such as the dermal system, the digestive system, the bone marrow system, the nervous system, and the like. Tissue stem cells in the dermal system include epidermal stem cells, hair follicle stem cells, and the like. Tissue stem cells in the digestive system include pancreatic (common) stem cells, liver stem cells, and the like. Tissue stem cells in the bone marrow system include hematopoietic stem cells, mesenchymal stem cells,

and the like. Tissue stem cells in the nervous system include neural stem cells, retinal stem cells, and the like.

[0086] "Induced pluripotent stem cells," commonly abbreviated as iPS cells or iPSCs, refer to a type of pluripotent stem cell artificially prepared from a non-pluripotent cell, typically an adult somatic cell, or terminally differentiated cell, such as a fibroblast, a hematopoietic cell, a myocyte, a neuron, an epidermal cell, or the like, by inserting certain genes, referred to as reprogramming factors.

[0087] "Pluripotency" refers to a stem cell that has the potential to differentiate into all cells constituting one or more tissues or organs, or preferably, any of the three germ layers: endoderm (interior stomach lining, gastrointestinal tract, the lungs), mesoderm (muscle, bone, blood, urogenital), or ectoderm (epidermal tissues and nervous system). "Pluripotent stem cells" used herein refer to cells that can differentiate into cells derived from any of the three germ layers, for example, direct descendants of totipotent cells or induced pluripotent cells.

[0088] As used herein "totipotent stem cells" refers to cells having the ability to differentiate into all cells constituting an organism, such as cells that are produced from the fusion of an egg and sperm cell. Cells produced by the first few divisions of the fertilized egg are also totipotent. These cells can differentiate into embryonic and extraembryonic cell types. Pluripotent stem cells can give rise to any fetal or adult cell type. However, alone they cannot develop into a fetal or adult animal because they lack the potential to contribute to extraembryonic tissue, such as the placenta.

[0089] In contrast, many progenitor cells are multipotent stem cells, *i.e.*, they are capable of differentiating into a limited number of cell fates. Multipotent progenitor cells can give rise to several other cell types, but those types are limited in number. An example of a multipotent stem cell is a hematopoietic cell — a blood stem cell that can develop into several types of blood cells, but cannot develop into brain cells or other types of cells. At the end of the long series of cell divisions that form the embryo are cells that are terminally differentiated, or that are considered to be permanently committed to a specific function.

[0090] As used herein, the term "somatic cell" refers to any cell other than a germ cell, such as an egg, a sperm, or the like, that does not directly transfer its DNA to the next

generation. Typically, somatic cells have limited or no pluripotency. Somatic cells used herein may be naturally-occurring or genetically modified.

[0091] Cells are "substantially free" of certain undesired cell types, as used herein, when they have less that 10% of the undesired cell types, and are "essentially free" of certain cell types when they have less than 1% of the undesired cell types. However, even more desirable are cell populations wherein less than 0.5% or less than 0.1% of the total cell population comprise the undesired cell types. Thus, cell populations wherein less than 0.1% to 1% (including all intermediate percentages) of the cells of the population comprise undesirable cell types are essentially free of these cell types. A medium is "essentially free" of certain reagents, as used herein, when there is no external addition of such agents. More preferably, these agents are absent or present at an undetectable amount.

II. Cells involved in endothelial cell programming

[0092] In certain embodiments of the invention, there are disclosed methods and compositions for providing endothelial cells by forward programming of cells that are not endothelial cells. There may be also provided cells that comprise exogenous expression cassettes including one or more endothelial programming factor genes and/or reporter expression cassettes specific for endothelial cell identification. In some embodiments, the cells may be stem cells, including but not limited to, embryonic stem cells, fetal stem cells, or adult stem cells. In further embodiments, the cells may be any somatic cells.

B. Stem Cells

[0093] Stem cells are cells found in most, if not all, multi-cellular organisms. They are characterized by the ability to renew themselves through mitotic cell division and the ability to differentiate into a diverse range of specialized cell types. The two broad types of mammalian stem cells are: embryonic stem cells that are found in blastocysts, and adult stem cells that are found in adult tissues. In a developing embryo, stem cells can differentiate into all of the specialized embryonic tissues. In adult organisms, stem cells and progenitor cells act as a repair system for the body, replenishing specialized cells, and also maintain the normal turnover of regenerative organs, such as blood, skin or intestinal tissues.

[0094] Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are capable of long-term proliferation *in vitro*, while retaining the potential to differentiate into all cell types of the body, including endothelial cells. Thus these cells could

potentially provide an unlimited supply of patient-specific functional endothelial cells for both drug development and therapeutic uses. The differentiation of human ESCs/iPSCs to endothelial cells *in vitro* recapitulates normal *in vivo* development; *i.e.* they undergo the normal sequential developmental stages including mesoderm differentiation and angiogenic specification (**FIG. 1**). That sequential developmental process requires the addition of different growth factors at different stages of differentiation. Certain aspects of the invention provide fully functional endothelial cells by forward programming from human ESCs/iPSCs via expression of a combination of transcription factors important for endothelial cell differentiation/function, similar to the generation of iPSCs, bypassing most, if not all, normal developmental stages (**FIG. 1**). This approach may be more time- and cost-efficient, and generate endothelial cells with functions highly similar, if not identical, to human primary adult endothelial cells. In addition, human ESC/iPSCs, with their unlimited proliferation ability, have a unique advantage over somatic cells as the starting cell population for endothelial cell differentiation.

2. Embryonic stem cells

[0095] Embryonic stem cell lines (ES cell lines) are cultures of cells derived from the epiblast tissue of the inner cell mass (ICM) of a blastocyst or earlier morula stage embryos. A blastocyst is an early stage embryo—approximately four to five days old in humans and consisting of 50–150 cells. ES cells are pluripotent and give rise during development to all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm. In other words, they can develop into each of the more than 200 cell types of the adult body when given sufficient and necessary stimulation for a specific cell type. They do not contribute to the extra-embryonic membranes or the placenta.

[0096] Nearly all research to date has taken place using mouse embryonic stem cells (mES) or human embryonic stem cells (hES). Both have the essential stem cell characteristics, yet they require very different environments in order to maintain an undifferentiated state. Mouse ES cells may be grown on a layer of gelatin and require the presence of Leukemia Inhibitory Factor (LIF). Human ES cells could be grown on a feeder layer of mouse embryonic fibroblasts (MEFs) and often require the presence of basic Fibroblast Growth Factor (bFGF or FGF-2). Without optimal culture conditions or genetic manipulation (Chambers *et al.*, 2003), embryonic stem cells will rapidly differentiate.

[0097] A human embryonic stem cell may also be defined by the presence of several transcription factors and cell surface proteins. The transcription factors Oct-4, Nanog, and Sox-2 form the core regulatory network that ensures the suppression of genes that lead to differentiation and the maintenance of pluripotency (Boyer *et al.*, 2005). The cell surface antigens most commonly used to identify hES cells include the glycolipids SSEA3 and SSEA4 and the keratan sulfate antigens Tra-1-60 and Tra-1-81.

[0098] Methods for obtaining mouse ES cells are well known. In one method, a preimplantation blastocyst from the 129 strain of mice is treated with mouse antiserum to remove the trophoectoderm, and the inner cell mass is cultured on a feeder cell layer of chemically inactivated mouse embryonic fibroblasts in medium containing fetal calf serum. Colonies of undifferentiated ES cells that develop are subcultured on mouse embryonic fibroblast feeder layers in the presence of fetal calf serum to produce populations of ES cells. In some methods, mouse ES cells can be grown in the absence of a feeder layer by adding the cytokine leukemia inhibitory factor (LIF) to serum-containing culture medium (Smith, 2000). In other methods, mouse ES cells can be grown in serum-free medium in the presence of bone morphogenetic protein and LIF (Ying *et al.*, 2003).

[0099] Human ES cells can be obtained from blastocysts using previously described methods (Thomson *et al.*, 1995; Thomson *et al.*, 1998; Thomson and Marshall, 1998; Reubinoff *et al,* 2000.) In one method, day-5 human blastocysts are exposed to rabbit anti-human spleen cell antiserum, then exposed to a 1:5 dilution of Guinea pig complement to lyse trophectoderm cells. After removing the lysed trophectoderm cells from the intact inner cell mass, the inner cell mass is cultured on a feeder layer of gamma-inactivated mouse embryonic fibroblasts and in the presence of fetal bovine serum. After 9 to 15 days, clumps of cells derived from the inner cell mass can be chemically (*i.e.* exposed to trypsin) or mechanically dissociated and replated in fresh medium containing fetal bovine serum and a feeder layer of mouse embryonic fibroblasts. Upon further proliferation, colonies having undifferentiated morphology are selected by micropipette, mechanically dissociated into clumps, and replated (see U.S. Patent No. 6,833,269). ES-like morphology is characterized as compact colonies with apparently high nucleus to cytoplasm ratio and prominent nucleoli. Resulting ES cells can be routinely passaged by brief trypsinization or by selection of individual colonies by micropipette. In some methods, human ES cells can be grown without serum by culturing the ES cells on a feeder layer of fibroblasts in the presence of basic

fibroblast growth factor (Amit *et al.*, 2000). In other methods, human ES cells can be grown without a feeder cell layer by culturing the cells on a protein matrix such as Matrigel™ or laminin in the presence of "conditioned" medium containing basic fibroblast growth factor (Xu *et al.*, 2001). The medium is previously conditioned by coculturing with fibroblasts.

[0100] Methods for the isolation of rhesus monkey and common marmoset ES cells are also known (Thomson, and Marshall, 1998; Thomson *et al.*, 1995; Thomson and Odorico, 2000).

[0101] Another source of ES cells are established ES cell lines. Various mouse cell lines and human ES cell lines are known and conditions for their growth and propagation have been defined. For example, the mouse CGR8 cell line was established from the inner cell mass of mouse strain 129 embryos, and cultures of CGR8 cells can be grown in the presence of LIF without feeder layers. As a further example, human ES cell lines H1, H7, H9, H13 and H14 were established by Thompson *et al.* In addition, subclones H9.1 and H9.2 of the H9 line have been developed. It is anticipated that virtually any ES or stem cell line known in the art may be used with the present invention, such as, *e.g.*, those described in Yu and Thompson, 2008.

[0102] The source of ES cells for use in connection with the present invention can be a blastocyst, cells derived from culturing the inner cell mass of a blastocyst, or cells obtained from cultures of established cell lines. Thus, as used herein, the term "ES cells" can refer to inner cell mass cells of a blastocyst, ES cells obtained from cultures of inner mass cells, and ES cells obtained from cultures of ES cell lines.

3. Induced pluripotent stem cells

[0103] Induced pluripotent stem (iPS) cells are cells that have the characteristics of ES cells but are obtained by the reprogramming of differentiated somatic cells. Induced pluripotent stem cells have been obtained by various methods. In one method, adult human dermal fibroblasts are transfected with transcription factors Oct4, Sox2, c-Myc and Klf4 using retroviral transduction (Takahashi *et al.*, 2007). The transfected cells are plated on SNL feeder cells (a mouse cell fibroblast cell line that produces LIF) in medium supplemented with basic fibroblast growth factor (bFGF). After approximately 25 days, colonies resembling human ES cell colonies appear in culture. The ES cell-like colonies are picked and expanded on feeder cells in the presence of bFGF.

[0104] Based on cell characteristics, cells of the ES cell-like colonies are induced pluripotent stem cells. The induced pluripotent stem cells are morphologically similar to human ES cells, and express various human ES cell markers. Also, when grown under conditions that are known to result in differentiation of human ES cells, the induced pluripotent stem cells differentiate accordingly. For example, the induced pluripotent stem cells can differentiate into cells having endothelial cell structures and endothelial cell markers. It is anticipated that virtually any iPS cells or cell lines may be used with the present invention, including, *e.g.*, those described in Yu and Thompson, 2008.

[0105] In another method, human fetal or newborn fibroblasts are transfected with four genes, Oct4, Sox2, Nanog and Lin28 using lentivirus transduction (Yu *et al.*, 2007). At 12-20 days post infection, colonies with human ES cell morphology become visible. The colonies are picked and expanded. The induced pluripotent stem cells making up the colonies are morphologically similar to human ES cells, express various human ES cell markers, and form teratomas having neural tissue, cartilage, and gut epithelium after injection into mice.

[0106] Methods of preparing induced pluripotent stem cells from mouse are also known (Takahashi and Yamanaka, 2006). Induction of iPS cells typically require the expression of or exposure to at least one member from Sox family and at least one member from Oct family. Sox and Oct are thought to be central to the transcriptional regulatory hierarchy that specifies ES cell identity. For example, Sox may be Sox-1, Sox-2, Sox-3, Sox-15, or Sox-18; Oct may be Oct-4. Additional factors may increase the reprogramming efficiency, like Nanog, Lin28, Klf4, or c-Myc; specific sets of reprogramming factors may be a set comprising Sox-2, Oct-4, Nanog and, optionally, Lin-28; or comprising Sox-2, Oct4, Klf and, optionally, c-Myc.

[0107] iPS cells, like ES cells, have characteristic antigens that can be identified or confirmed by immunohistochemistry or flow cytometry, using antibodies for SSEA-1, SSEA-3 and SSEA-4 (Developmental Studies Hybridoma Bank, National Institute of Child Health and Human Development, Bethesda Md.), and TRA-1-60 and TRA-1-81 (Andrews *et al.*, 1987). Pluripotency of embryonic stem cells can be confirmed by injecting approximately 0.5-10 X 10^6 cells into the rear leg muscles of 8-12 week old male SCID mice. Teratomas develop that demonstrate at least one cell type of each of the three germ layers.

[0108] In certain aspects of the present invention, iPS cells are made from reprogramming somatic cells using reprogramming factors comprising an Oct family member and a Sox family member, such as Oct4 and Sox2 in combination with Klf or Nanog as described above. The somatic cell for reprogramming may be any somatic cell that can be induced to pluripotency, such as a fibroblast, a keratinocyte, a hematopoietic cell, a mesenchymal cell, a liver cell, a stomach cell, or a β cell. In a certain aspect, T cells may also be used as source of somatic cells for reprogramming (see U.S. Application No. 61/184,546).

[0109] Reprogramming factors may be expressed from expression cassettes comprised in one or more vectors, such as an integrating vector or an episomal vector, *e.g.*, an EBV element-based system (see U.S. Application No. 61/058,858; Yu *et al.*, 2009). In a further aspect, reprogramming proteins could be introduced directly into somatic cells by protein transduction (see U.S. Application No. 61/172,079).

4. Embryonic Stem Cells Derived by Somatic Cell Nuclear Transfer

[0110] Pluripotent stem cells can be prepared by means of somatic cell nuclear transfer, in which a donor nucleus is transferred into a spindle-free oocyte. Stem cells produced by nuclear transfer are genetically identical to the donor nuclei. In one method, donor fibroblast nuclei from skin fibroblasts of a rhesus macaque are introduced into the cytoplasm of spindle-free, mature metaphase II rhesus macaque ooctyes by electrofusion (Byrne *et al.*, 2007). The fused oocytes are activated by exposure to ionomycin, then incubated until the blastocyst stage. The inner cell mass of selected blastocysts are then cultured to produce embryonic stem cell lines. The embryonic stem cell lines show normal ES cell morphology, express various ES cell markers, and differentiate into multiple cell types both *in vitro* and *in vivo*. As used herein, the term "ES cells" refers to embryonic stem cells derived from embryos containing fertilized nuclei. ES cells are distinguished from embryonic stem cells produced by nuclear transfer, which are referred to as "embryonic stem cells derived by somatic cell nuclear transfer."

5. Other stem cells

[0111] Fetal stem cells are cells with self-renewal capability and pluripotent differentiation potential. They can be isolated and expanded from fetal cytotrophoblast cells

(European Patent EP0412700) and chorionic villi, amniotic fluid and the placenta (WO/2003/042405). Cell surface markers of fetal stem cells include CDl17/c-kit^{+}, SSEA3^{+}, SSEA4^{+} and SSEAl^{-}.

[0112] Somatic stem cells have been identified in most organ tissues. The best characterized is the hematopoietic stem cell. This is a mesoderm-derived cell that has been purified based on cell surface markers and functional characteristics. The hematopoictic stem cell, isolated from bone marrow, blood, cord blood, fetal liver and yolk sac, is the progenitor cell that reinitiates hematopoiesis for the life of a recipient and generates multiple hematopoietic lineages (see U.S. Pat. No. 5,635,387; 5,460,964; 5,677,136; 5,750,397; 5,759,793; 5,681,599; 5,716,827; Hill *et al.*, 1996). When transplanted into lethally irradiated animals or humans, hematopoietic stem cells can repopulate the erythroid, neutrophil-macrophage, megakaryocyte and lymphoid hematopoietic cell pool. *In vitro*, hematopoietic stem cells can be induced to undergo at least some self-renewing cell divisions and can be induced to differentiate to the same lineages as is seen *in vivo*. Therefore, this cell fulfills the criteria of a stem cell.

[0113] The next best characterized is the mesenchymal stem cells (MSC), originally derived from the embryonic mesoderm and isolated from adult bone marrow, can differentiate to form muscle, bone, cartilage, fat, marrow stroma, and tendon. During embryogenesis, the mesoderm develops into limb-bud mesoderm, tissue that generates bone, cartilage, fat, skeletal muscle and possibly endothelium. Mesoderm also differentiates to visceral mesoderm, which can give rise to cardiac muscle, smooth muscle, or blood islands consisting of endothelium and hematopoietic progenitor cells. Primitive mesodermal or mesenchymal stem cells, therefore, could provide a source for a number of cell and tissue types. A number of mesenchymal stem cells have been isolated (see, for example, U.S. Pat. No. 5,486,359; 5,827,735; 5,811,094; 5,736,396; U.S. Pat. No. 5,837,539; 5,837,670; 5,827,740; Jaiswal *et al.*, 1997; Cassiede *et al.*, 1996; Johnstone *et al.*, 1998; Yoo *et al.*, 1998; Gronthos, 1994; Makino *et al.*, 1999).

Of the many mesenchymal stem cells that have been described, all have demonstrated limited differentiation to form only those differentiated cells generally considered to be of mesenchymal origin. To date, the most multipotent mesenchymal stem

cell expresses the $SH2^+$ $SH4^+$ $CD29^+$ $CD44^+$ $CD71^+$ $CD90^+$ $CD106^+$ $CD120a^+$ $CD124^+$ $CD14^-$ $CD34^-$ $CD45^-$ phenotype.

[0114] Other stem cells have been identified, including gastrointestinal stem cells, epidermal stem cells, neural and hepatic stem cells, also termed oval cells (Potten, 1998; Watt, 1997; Alison *et al*, 1998).

[0115] In some embodiments, the stem cells useful for methods described herein include, but are not limited to, embryonic stem cells, induced plurpotent stem cells, mesenchymal stem cells, bone-marrow derived stem cells, hematopoietic stem cells, chrondrocyte progenitor cells, epidermal stem cells, gastrointestinal stem cells, neural stem cells, hepatic stem cells, adipose-derived mesenchymal stem cells, pancreatic progenitor cells, hair follicular stem cells, endothelial progenitor cells, and smooth muscle progenitor cells.

[0116] In some embodiments, the stem cells used for methods described herein are isolated from umbilical cord, placenta, amniotic fluid, chorion villi, blastocysts, bone marrow, adipose tissue, brain, peripheral blood, the gastrointestinal tract, cord blood, blood vessels, skeletal muscle, skin, liver, and menstrual blood. Stem cells prepared in the menstrual blood are called endometrial regenerative cells (available from Medistem, Inc.).

[0117] One ordinarily skilled in the art can locate, isolate, and expand such stem cells. The detailed procedures for the isolation of human stem cells from various sources are described in Current Protocols in Stem Cell Biology (2007). Alternatively, commercial kits and isolation systems can be used—*e.g.*, the BD FACSAria cell sorting system, BD IMag magnetic cell separation system, and BD IMag mouse hematopoietic progenitor cell enrichment set from BD Biosciences. Methods of isolating and culturing stem cells from various sources are also described in 5,486,359, 6,991,897, 7,015,037, 7,422,736, 7,410,798, 7,410,773, 7,399,632.

C. Somatic cells

[0118] In certain aspects of the invention, there may also be provided methods of transdifferentiation, *i.e.*, the direct conversion of one somatic cell type into another, *e.g.*, deriving endothelial cells from other somatic cells. However, the human somatic cells may

be limited in supply, especially those from living donors. In certain aspects, to provide an unlimited supply of starting cells for programming, somatic cells may be immortalized by introduction of immortalizing genes or proteins, such as hTERT or oncogenes. The immortalization of cells may be reversible (*e.g.*, using removable expression cassettes) or inducible (*e.g.*, using inducible promoters).

[0119] Somatic cells in certain aspects of the invention may be primary cells (non-immortalized cells), such as those freshly isolated from an animal, or may be derived from a cell line (immortalized cells). The cells may be maintained in cell culture following their isolation from a subject. In certain embodiments, the cells are passaged once or more than once (*e.g.*, between 2-5, 5-10, 10-20, 20-50, 50-100 times, or more) prior to their use in a method of the invention. In some embodiments the cells will have been passaged no more than 1, 2, 5, 10, 20, or 50 times prior to their use in a method of the invention. They may be frozen, thawed, *etc.*

[0120] The somatic cells used or described herein may be native somatic cells, or engineered somatic cells, *i.e.*, somatic cells which have been genetically altered. Somatic cells of the present invention are typically mammalian cells, such as, for example, human cells, primate cells or mouse cells. They may be obtained by well-known methods and can be obtained from any organ or tissue containing live somatic cells, *e.g.*, blood, bone marrow, skin, lung, pancreas, liver, stomach, intestine, heart, reproductive organs, bladder, kidney, urethra and other urinary organs, *etc.*

[0121] Mammalian somatic cells useful in the present invention include, but are not limited to, Sertoli cells, endothelial cells, granulosa cells, neurons, pancreatic islet cells, epidermal cells, epithelial cells, hepatocytes, hair follicle cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, cardiac muscle cells, and other muscle cells, *etc.*

[0122] In some embodiments, cells are selected based on their expression of an endogenous marker known to be expressed only or primarily in a desired cell type. For example, vimentin is a fibroblast marker. Other useful markers include various keratins, cell adhesion molecules such as cadherins, fibronectin, CD molecules, *etc.* The population of somatic cells may have an average cell cycle time of between 18 and 96 hours, *e.g.*, between

24-48 hours, between 48-72 hours, *etc.* In some embodiments, at least 90%, 95%, 98%, 99%, or more of the cells would be expected to divide within a predetermined time such as 24, 48, 72, or 96 hours.

[0123] Methods described herein may be used to program one or more somatic cells, *e.g.*, colonies or populations of somatic cells into endothelial cells. In some embodiments, a population of cells of the present invention is substantially uniform in that at least 90% of the cells display a phenotype or characteristic of interest. In some embodiments at least 95%, 96%, 97%, 98%, 99%, 99.5%, 99.8%, 99.9, 99.95% or more of the cells display a phenotype or characteristic of interest. In certain embodiments of the invention the somatic cells have the capacity to divide, *i.e.*, the somatic cells are not post-mitotic.

[0124] Somatic cells may be partially or completely differentiated. Differentiation is the process by which a less specialized cell becomes a more specialized cell type. Cell differentiation can involve changes in the size, shape, polarity, metabolic activity, gene expression and/or responsiveness to signals of the cell. For example, hematopoietic stem cells differentiate to give rise to all the blood cell types including myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells) and lymphoid lineages (T-cells, B-cells, NK-cells). During progression along the path of differentiation, the ultimate fate of a cell becomes more fixed. As described herein, both partially differentiated somatic cells and fully differentiated somatic cells can be programmed as described herein to produce desired cell types such as endothelial cells.

III. Endothelial programming factors

[0125] Certain aspects of the invention provide endothelial programming factors for endothelial programming. The endothelial cells could be produced directly from other cell sources by increasing the level of endothelial programming factors in cells. The numerous functions of endothelial cells could be controlled at the transcriptional level by the concerted actions of a limited number of endothelial cell-enriched transcription factors. Any transcription factors important for endothelial cell differentiation or function may be used herein, like endothelial cell-enriched transcription factors, particularly the genes thereof listed in this section. The inventors also contemplate that all the isoforms and variants of the genes listed in this section are included in this invention, and non-limiting examples of accession numbers for certain isoforms or variants are provided.

[0126] For example, by effecting expression of a combination of transcription factors disclosed herein, the differentiation into endothelial cells from pluripotent stem cells may bypass most, if not all, normal developmental stages.

[0127] In certain embodiments, the endothelial programming factor is ERG, which is also known as: transcriptional regulator ERG, ets-related transforming protein ERG, TMPRSS2-ERG prostate cancer specific, v-ets erythroblastosis virus E26 oncogene like, v-ets avian erythroblastosis virus E26 oncogene related, transforming protein ERG. In some embodiments, the endothelial programming factor may be an isoform of ERG, such as ERG-1 (accession number: NM_182918.3; GI:209954798) (SEQ ID NOS:13 and 14), ERG-2 (SEQ ID NOS:9 and 10), ERG isoform 3 (SEQ ID NOS:11 and 12), or ERG-4 (accession number: NM_001136155.1; GI:209954807) (SEQ ID NOS:15 and 16). In particular embodiments, the endothelial programming factor is ERG-3. In other particular embodiments, the endothelial programming factor is ETV2 (also called ER71, ETSRP71) (NCBI Accession No. NM_014209, Version NM_014209.2, GI: 153791177) (SEQ ID NOS:17 and 18).

[0128] In other embodiments, the one or more endothelial programming factors is v-ets erythroblastosis virus E26 oncogene homolog 1 (avian) (ETS1) isoform 1 (NCBI Accession No. NM_001143820, Version NM_001143820.1, GI:219689117), ETS1 isoform 3 (NCBI Accession No. NM_001162422, Version NM_001162422.1, GI:241666445), ETS1 isoform 2 (NCBI Accession No. NM_005238, Version NM_005238.3, GI:219689116), V-ets erythroblastosis virus E26 oncogene homolog 2 (avian) (ETS2) (NCBI Accession No. NM_005239, Version NM_005239.4, GI:56119171), E74-like factor 1 (ELF-1) isoform b (NCBI Accession No. NM_001145353, Version NM_001145353.1, GI: 223941928), ELF-1 isoform a (NCBI Accession No. NM_172373, Version NM_172373.3, GI: 223941931), ELK-4 isoform a (NCBI Accession No. NM_001973, Version NM_001973.2, GI: 41872447), ELK-4 isoform b (NCBI Accession No. NM_021795, Version NM_021795.2, GI: 41872461), friend leukemia virus integration 1 (FLI-1) isoform 2 (NCBI Accession No. NM_001167681, Version NM_001167681.1, GI: 264681553), FLI-1 isoform 1 (NCBI Accession No. NM_002017, Version NM_002017.3, GI: 194018460), ETV6 (also called TEL or TEL1) (NCBI Accession No. NM_001987, Version NM_001987.4, GI: 153267458), T-cell acute lymphocytic leukemia 1 (TAL1, also called SCL) (NCBI Accession No. NM_003189, Version NM_003189.2, GI: 197927279), GATA binding protein 2 (GATA2)

isoform 1 variant 1 (NCBI Accession No. NM_001145661, Version NM_001145661.1, GI: 224611698), GATA2 isoform 1 variant 2 (NCBI Accession No. NM_032638, Version NM_032638.4, GI: 224611697), GATA2 isoform 2 (NCBI Accession No. NM_001145662, Version NM_001145662.1, GI: 224611700), or a Forkhead (FOX) transcription factors (*e.g.*, a member of the FoxC, FoxF, FoxH, or FoxO family).

[0129] In yet other embodiments, the one or more endothelial programming factors is BMP-4, which is important for the modulation of the proliferative and differentiative potential of hematopoietic progenitor cells (Bhardwaj *et al.*, 2001; Bhatia *et al.*, 1999; Chadwick 2003). Additionally, BMP-4 can modulate early hematopoietic cell development in human fetal, neonatal, and adult hematopoietic progenitor cells (Davidson and Zon, 2000; Huber *et al.*, 1998; Marshall *et al.*, 2000). For example, BMP-4 can regulate the proliferation and differentiation of highly purified primitive human hematopoietic cells from adult and neonatal sources (Bhatia *et al.*, 1999), and BMP-4 can promote hematopoietic differentiation in human embryonic stem cells (Chadwick, 2003). BMP-4 can also promote differentiation of endothelial cells from endothelial progenitor cells (Wang *et al.*, 2007).

[0130] In further embodiments, the one or more endothelial programming factors is vascular endothelial growth factor (VEGF), which is an important signaling protein that is involved in formation of the embryonic circulatory system and angiogenesis. VEGF can affect a variety of cell types including vascular endothelium and other cell types (*e.g.*, neurons, cancer cells, kidney epithelial cells). *In vitro*, VEGF can stimulate endothelial cell mitogenesis and cell migration. VEGF function has also been shown to be important in a variety of disease states including cancer, diabetes, autoimmune diseases, and ocular vascular diseases.

[0131] In still further embodiments, the one or more endothelial programming factors may be Vezf1/DB1, endothelial PAS domain-containing protein 1 (EPAS1), FOXO3a, hypoxia-inducible transcription factor-2, FoxF1, FoxH1, FoxC1, FoxC2, Kruppel-like factor 2, or Kruppel-like factor 6.

[0132] Forward programming to provide endothelial cells may be accomplished by increasing the expression of any one or more of the endothelial cell factors described in this section.

IV. Delivery of gene or gene products

[0133] In certain embodiments, vectors for delivery of nucleic acids encoding endothelial programming or differentiation factors may be constructed to express those factors in cells. Details of components of such vectors and delivery methods are disclosed below. In addition, protein transduction compositions or methods may be used to effect expression of the endothelial programming factors.

[0134] In a further aspect, the following systems and methods may also be used in delivery of a reporter expression cassette for identification of desired cell types, such as endothelial cells. In particular, an endothelial cell-specific regulatory element may be used to drive expression of a reporter gene. Therefore endothelial cells derived from programming may be characterized, selected or enriched via use of the reporter.

B. Nucleic acid delivery systems

[0135] One of skill in the art would be well-equipped to construct a vector through standard recombinant techniques (see, for example, Sambrook et al., 2001 and Ausubel et al., 1996). Vectors include but are not limited to, plasmids, cosmids, viruses (bacteriophage, animal viruses, and plant viruses), and artificial chromosomes (e.g., YACs), such as retroviral vectors (e.g. derived from Moloney murine leukemia virus vectors (MoMLV), MSCV, SFFV, MPSV, SNV etc), lentiviral vectors (e.g. derived from HIV-1, HIV-2, SIV, BIV, FIV etc.), adenoviral (Ad) vectors including replication competent, replication deficient and gutless forms thereof, adeno-associated viral (AAV) vectors, simian virus 40 (SV-40) vectors, bovine papilloma virus vectors, Epstein-Barr virus vectors, herpes virus vectors, vaccinia virus vectors, Harvey murine sarcoma virus vectors, murine mammary tumor virus vectors, Rous sarcoma virus vectors.

2. Viral Vectors

[0136] In generating recombinant viral vectors, non-essential genes are typically replaced with a gene or coding sequence for a heterologous (or non-native) protein. A viral vector is a kind of expression construct that utilizes viral sequences to introduce nucleic acid and possibly proteins into a cell. The ability of certain viruses to infect cells or enter cells via receptor-mediated endocytosis, and to integrate into host cell genomes and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign nucleic acids into cells (*e.g.*, mammalian cells). Non-limiting examples of virus vectors that

may be used to deliver a nucleic acid of certain aspects of the present invention are described below.

[0137] Retroviruses have promise as gene delivery vectors due to their ability to integrate their genes into the host genome, transfer a large amount of foreign genetic material, infect a broad spectrum of species and cell types, and be packaged in special cell-lines (Miller, 1992).

[0138] In order to construct a retroviral vector, a nucleic acid is inserted into the viral genome in place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes—but without the LTR and packaging components—is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences, is introduced into a special cell line (*e.g.*, by calcium phosphate precipitation), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture medium (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The medium containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

[0139] Lentiviruses are complex retroviruses, which, in addition to the common retroviral genes *gag, pol*, and *env*, contain other genes with regulatory or structural function. Lentiviral vectors are well known in the art (see, for example, Naldini *et al.*, 1996; Zufferey *et al.*, 1997; Blomer et al., 1997; U.S. Patents 6,013,516 and 5,994,136).

[0140] Recombinant lentiviral vectors are capable of infecting non-dividing cells and can be used for both *in vivo* and *ex vivo* gene transfer and expression of nucleic acid sequences. For example, recombinant lentivirus capable of infecting a non-dividing cell—wherein a suitable host cell is transfected with two or more vectors carrying the packaging functions, namely gag, pol and env, as well as rev and tat—is described in U.S. Patent 5,994,136.

3. Episomal Vectors

[0141] The use of plasmid- or liposome-based extra-chromosomal (*i.e.*, episomal) vectors may be also provided in certain aspects of the invention. Such episomal vectors may include, *e.g.*, oriP-based vectors, and/or vectors encoding a derivative of EBNA-1. These vectors may permit large fragments of DNA to be introduced unto a cell and maintained extra-chromosomally, replicated once per cell cycle, partitioned to daughter cells efficiently, and elicit substantially no immune response.

[0142] In particular, EBNA-1, the only viral protein required for the replication of the oriP-based expression vector, does not elicit a cellular immune response because it has developed an efficient mechanism to bypass the processing required for presentation of its antigens on MHC class I molecules (Levitskaya *et al.*, 1997). Further, EBNA-1 can act in *trans* to enhance expression of the cloned gene, inducing expression of a cloned gene up to 100-fold in some cell lines (Langle-Rouault *et al.*, 1998; Evans *et al.*, 1997). Finally, the manufacture of such oriP-based expression vectors is inexpensive.

[0143] Other extra-chromosomal vectors include other lymphotrophic herpes virus-based vectors. Lymphotrophic herpes virus is a herpes virus that replicates in a lymphoblast (*e.g.*, a human B lymphoblast) and becomes a plasmid for a part of its natural life-cycle. Herpes simplex virus (HSV) is not a "lymphotrophic" herpes virus. Exemplary lymphotrophic herpes viruses include, but are not limited to EBV, Kaposi's sarcoma herpes virus (KSHV); Herpes virus saimiri (HS) and Marek's disease virus (MDV). Other sources of episome-based vectors are also contemplated, such as yeast ARS, adenovirus, SV40, or BPV.

[0144] One of skill in the art would be well-equipped to construct a vector through standard recombinant techniques (*see*, for example, Maniatis *et al.*, 1988 and Ausubel *et al.*, 1994).

[0145] Vectors can also comprise other components or functionalities that further modulate gene delivery and/or gene expression, or that otherwise provide beneficial properties to the targeted cells. Such other components include, for example, components that influence binding or targeting to cells (including components that mediate cell-type or tissue-specific binding); components that influence uptake of the vector nucleic acid by the cell; components that influence localization of the polynucleotide within the cell after uptake (such

as agents mediating nuclear localization); and components that influence expression of the polynucleotide.

[0146] Such components also may include markers, such as detectable and/or selection markers that can be used to detect or select for cells that have taken up and are expressing the nucleic acid delivered by the vector. Such components can be provided as a natural feature of the vector (such as the use of certain viral vectors that have components or functionalities mediating binding and uptake), or vectors can be modified to provide such functionalities. A large variety of such vectors are known in the art and are generally available. When a vector is maintained in a host cell, the vector can either be stably replicated by the cells during mitosis as an autonomous structure, incorporated within the genome of the host cell, or maintained in the host cell's nucleus or cytoplasm.

4. Transposon-based system

[0147] According to a particular embodiment the introduction of nucleic acids may use a transposon - transposase system. The used transposon - transposase system could be the well known Sleeping Beauty, the Frog Prince transposon - transposase system (for a description of the latter, *see*, *e.g.*, EP1507865), or the TTAA-specific transposon piggyBac system.

[0148] Transposons are sequences of DNA that can move around to different positions within the genome of a single cell, a process called transposition. In the process, they can cause mutations and change the amount of DNA in the genome. Transposons were also once called jumping genes, and are examples of mobile genetic elements.

[0149] There are a variety of mobile genetic elements, and they can be grouped based on their mechanism of transposition. Class I mobile genetic elements, or retrotransposons, copy themselves by first being transcribed to RNA, then reverse transcribed back to DNA by reverse transcriptase, and then being inserted at another position in the genome. Class II mobile genetic elements move directly from one position to another using a transposase to "cut and paste" them within the genome.

5. mRNA Delivery

[0150] One of skill in the art would be well-equipped to deliver to a cell any mRNA useful in the invention. For example, such techniques are provided in Yamamoto *et al.*, 2009.

6. Homologous Recombination

[0151] n certain aspects of the invention, nucleic acid molecules can be introduced into cells in a specific manner for genome engineering, for example, via homologous recombination. As discussed above, some approaches to express genes in cells involve the use of viral vectors or transgenes that integrate randomly in the genome. These approaches, however, have the drawback of integration occurring either at sites that are unable to effectively mediate expression from the integrated nucleic or that result in the disruption of native genes. Problems associated with random integration could be partially overcome by homologous recombination to a specific locus in the target genome, *e.g.*, Rosa26 locus.

[0152] Homologous recombination (HR), also known as general recombination, is a type of genetic recombination used in all forms of life in which nucleotide sequences are exchanged between two similar or identical strands of DNA. The technique has been the standard method for genome engineering in mammalian cells since the mid 1980s. The process involves several steps of physical breaking and the eventual rejoining of DNA. This process is most widely used to repair potentially lethal double-strand breaks in DNA. In addition, homologous recombination produces new combinations of DNA sequences during meiosis, the process by which eukaryotes make germ cells like sperm and ova. These new combinations of DNA represent genetic variation in offspring which allow populations to evolutionarily adapt to changing environmental conditions over time. Homologous recombination is also used in horizontal gene transfer to exchange genetic material between different strains and species of bacteria and viruses. Homologous recombination is also used as a technique in molecular biology for introducing genetic changes into target organisms.

[0153] Homologous recombination can be used as targeted genome modification. The efficiency of standard HR in mammalian cells is only 10^{-6} to 10^{-9} of cells treated (Capecchi, 1990). The use of meganucleases, or homing endonucleases, such as I-SceI have been used to increase the efficiency of HR. Both natural meganucleases as well as engineered meganucleases with modified targeting specificities have been utilized to increase HR efficiency (Pingoud and Silva, 2007; Chevalier et al., 2002).

[0154] On the path toward increasing the efficiency of HR has been to engineer chimeric endonucleases with programmable DNA specificity domains (Silva et al., 2011). Zinc-finger nucleases (ZFN) are one example of such a chimeric molecule in which Zinc-

finger DNA binding domains are fused with the catalytic domain of a Type IIS restriction endonuclease such as FokI (as reviewed in Durai *et al.,* 2005; PCT/US2004/030606).

[0155] Another class of such specificity molecules includes Transcription Activator Like Effector (TALE) DNA binding domains fused to the catalytic domain of a Type IIS restriction endonuclease such as FokI (Miller *et al.,* 2011; PCT/IB2010/000154). TALENs can be designed for site-specific genome modification at virtually any given site of interest (Cermak *et al.,* 2011; Christian *et al.,* 2010; Li *et al.,* 2011; Miller *et al.,* 2011; Weber *et al.,* 2011; Zhang *et al.,* 2011). The site-specific DNA binding domain is expressed as a fusion protein with a DNA cleavage enzyme such as Fok I. The DNA binding domain is a scaffold of repeating amino acids; linking each of the repeats are two variable amino acids that bind to a single nucleotide in the DNA. For example, Asn-Asn binds guanosine, Asn-Ile binds adenosine, Asn-Gly bind thymidine, and His-Asp binds Cytosine. These two amino acids are known as the Repeat Variable Diresidue or RVD. There are many different RVD's and they can be engineered into the TAL Effector/Fok1 protein construct to create a specific TALEN. The RNA encoding the recombinant TALEN can then be purified and transfected into a cell for site-specific genome modification. Once the TALEN introduces the double strand DNA break, the DNA can be modified by non-homologous end joining (NHEJ) or by homologous directed repair (HDR). This allows DNA mutagenesis, deletions, or additions depending on what additional sequences are present during the DNA repair.

C. Regulatory Elements:

[0156] Eukaryotic expression cassettes included in vectors useful in the invention preferably contain (in a 5'-to-3' direction) a eukaryotic transcriptional promoter operably linked to a protein-coding sequence, splice signals including intervening sequences, and a transcriptional termination/polyadenylation sequence.

2. Promoter/Enhancers

[0157] A "promoter" is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind, such as RNA polymerase and other transcription factors, to initiate the specific transcription of a nucleic acid sequence. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that a promoter is in a correct functional location and/or

orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence.

[0158] A promoter generally comprises a sequence that functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as, for example, the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation. Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. To bring a coding sequence "under the control of" a promoter, one positions the 5′ end of the transcription initiation site of the transcriptional reading frame "downstream" of (*i.e.*, 3′ of) the chosen promoter. The "upstream" promoter stimulates transcription of the DNA and promotes expression of the encoded RNA.

[0159] The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription. A promoter may or may not be used in conjunction with an "enhancer," which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

[0160] A promoter may be one naturally associated with a nucleic acid sequence, as may be obtained by isolating the 5′ non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other

genes, and promoters or enhancers isolated from any other virus, or prokaryotic or eukaryotic cell, and promoters or enhancers not "naturally occurring," *i.e.,* containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. For example, promoters that are most commonly used in recombinant DNA construction include the β-lactamase (penicillinase), lactose and tryptophan (trp) promoter systems. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in connection with the compositions disclosed herein (*see* U.S. Patent Nos. 4,683,202 and 5,928,906). Furthermore, it is contemplated that the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

[0161] Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the organelle, cell type, tissue, organ, or organism chosen for expression. Those of skill in the art of molecular biology generally know the use of promoters, enhancers, and cell type combinations for protein expression, (*see*, for example Sambrook *et al.* 1989). The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

[0162] Additionally any promoter/enhancer combination (as per, for example, the Eukaryotic Promoter Data Base EPDB, through world wide web at epd.isb-sib.ch/) could also be used to drive expression. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

[0163] Non-limiting examples of promoters include early or late viral promoters, such as, SV40 early or late promoters, cytomegalovirus (CMV) immediate early promoters, Rous Sarcoma Virus (RSV) early promoters; eukaryotic cell promoters, such as, *e. g.*, beta actin promoter (Ng, 1989; Quitsche *et al.*, 1989), GADPH promoter (Alexander *et al.*, 1988, Ercolani *et al.*, 1988), metallothionein promoter (Karin *et al.*, 1989; Richards *et al.*, 1984);

and concatenated response element promoters, such as cyclic AMP response element promoters (cre), serum response element promoter (sre), phorbol ester promoter (TPA) and response element promoters (tre) near a minimal TATA box. It is also possible to use human growth hormone promoter sequences (*e.g.*, the human growth hormone minimal promoter described at Genbank, accession no. X05244, nucleotide 283-341) or a mouse mammary tumor promoter (available from the ATCC, Cat. No. ATCC 45007). A specific example could be a phosphoglycerate kinase (PGK) promoter.

[0164] Endothelial cells can be readily identified and purified based on cell-surface antigen expression of, for example, CD31 and/or VE-cadherin. In some embodiments, endothelial cells produced by forward programming may be identified based on expression of a reporter genc. To increase both specificity and activity, the use of cis-acting regulatory elements has been contemplated. For example, an endothelial cell-specific promoter may be used. Many endothelial cell-specific promoters are known in the art. (*See, e.g.*, DeVal and Black, 2009). Examples include, but are not limited to a promoter of Mef2c, Flk1, Tal1, endoglin, LMO2, Fli1, Tie2, Tie1, Flt1, Gata2, Prox1, ECE1, FLT4, PDGFR-beta, FOXP1, NRP1, NOTCH4, LYL1, EPCR, von Willebrand factor, factor VIII–related antigen, CD31/PECAM-1, angiotensin-converting enzyme, vascular endothelial cadherin (Cdh5), CD34, CD102/ICAM-2, CD51/61 (vitronectin receptor), CD105/endoglin, CD36, CD73/VAP-2, or Sca-1.

[0165] In certain aspects, methods of the invention also concern enhancer sequences, *i.e.* nucleic acid sequences that increase a promoter's activity and that have the potential to act in cis, and regardless of their orientation, even over relatively long distances (up to several kilobases away from the target promoter). However, enhancer function is not necessarily restricted to such long distances as they may also function in close proximity to a given promoter.

[0166] Many endothelial cell promoter and enhancer sequences have been identified, and may be useful in methods of the invention. *See, e.g.*, U.S. Patent App. 20100081193; DeVal and Black, 2009; Liu *et al.* 1995; Collins *et al.* 1995; Schlaeger *et al.*, 1997.

3. Initiation Signals and Internal Ribosome Binding Sites

[0167] A specific initiation signal also may be used for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences.

Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

[0168] In certain embodiments of the invention, the use of internal ribosome entry sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5' methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message (see U.S. Patent Nos. 5,925,565 and 5,935,819).

4. Origins of Replication

[0169] In order to propagate a vector in a host cell, it may contain one or more origins of replication sites (often termed "ori"), for example, a nucleic acid sequence corresponding to oriP of EBV as described above or a genetically engineered oriP with a similar or elevated function in programming, which is a specific nucleic acid sequence at which replication is initiated. Alternatively a replication origin of other extra-chromosomally replicating virus as described above or an autonomously replicating sequence (ARS) can be employed.

5. Selection and Screenable Markers

[0170] In certain embodiments of the invention, cells containing a nucleic acid construct of the present invention may be identified *in vitro* or *in vivo* by including a marker in the expression vector. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression vector. Generally, a selection

marker is one that confers a property that allows for selection. A positive selection marker is one in which the presence of the marker allows for its selection, while a negative selection marker is one in which its presence prevents its selection. An example of a positive selection marker is a drug resistance marker.

[0171] Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selection markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable markers such as GFP, whose basis is colorimetric analysis, are also contemplated. Alternatively, screenable enzymes as negative selection markers such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be utilized. One of skill in the art would also know how to employ immunologic markers, possibly in conjunction with FACS analysis. The marker used is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selection and screenable markers are well known to one of skill in the art. One feature of the present invention includes using selection and screenable markers to select for endothelial cells after the programming factors have effected a desired programming change in those cells.

D. Nucleic acid Delivery

[0172] Introduction of a nucleic acid, such as DNA or RNA, into cells to be programmed with the current invention may use any suitable methods for nucleic acid delivery for transformation of a cell, as described herein or as would be known to one of ordinary skill in the art. Such methods include, but are not limited to, direct delivery of DNA such as by ex vivo transfection (Wilson *et al.*, 1989, Nabel *et al*, 1989), by injection (U.S. Patent Nos. 5,994,624, 5,981,274, 5,945,100, 5,780,448, 5,736,524, 5,702,932, 5,656,610, 5,589,466 and 5,580,859), including microinjection (Harland and Weintraub, 1985; U.S. Patent No. 5,789,215); by electroporation (U.S. Patent No. 5,384,253; Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984); by calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990); by using DEAE-dextran followed by polyethylene glycol (Gopal, 1985); by direct sonic loading (Fechheimer *et al.*, 1987); by

liposome mediated transfection (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987; Wong *et al.*, 1980; Kaneda *et al.*, 1989; Kato *et al.*, 1991) and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988); by microprojectile bombardment (PCT Application Nos. WO 94/09699 and 95/06128; U.S. Patent Nos. 5,610,042; 5,322,783 5,563,055, 5,550,318, 5,538,877 and 5,538,880); by agitation with silicon carbide fibers (Kaeppler *et al.*, 1990; U.S. Patent Nos. 5,302,523 and 5,464,765); by *Agrobacterium*-mediated transformation (U.S. Patent Nos. 5,591,616 and 5,563,055); by desiccation/inhibition-mediated DNA uptake (Potrykus *et al.*, 1985), and any combination of such methods. Through the application of techniques such as these, organelle(s), cell(s), tissue(s) or organism(s) may be stably or transiently transformed.

2. Liposome-Mediated Transfection

[0173] In a certain embodiment of the invention, a nucleic acid may be entrapped in a lipid complex such as, for example, a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated is a nucleic acid complexed with Lipofectamine (Gibco BRL) or Superfect (Qiagen). The amount of liposomes used may vary based upon the nature of the liposome as well as the cell used, for example, about 5 to about 20 μg vector DNA per 1 to 10 million of cells may be contemplated.

[0174] Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987). The feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells has also been demonstrated (Wong *et al.*, 1980).

[0175] In certain embodiments of the invention, a liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell

membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.,* 1989). In other embodiments, a liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.,* 1991). In yet further embodiments, a liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In other embodiments, a delivery vehicle may comprise a ligand and a liposome.

3. Electroporation

[0176] In certain embodiments of the present invention, a nucleic acid is introduced into an organelle, a cell, a tissue or an organism *via* electroporation. Electroporation involves the exposure of a suspension of cells and DNA to a high-voltage electric discharge. Recipient cells can be made more susceptible to transformation by mechanical wounding. Also the amount of vectors used may vary upon the nature of the cells used, for example, about 5 to about 20 μg vector DNA per 1 to 10 million of cells may be contemplated.

[0177] Transfection of eukaryotic cells using electroporation has been quite successful. Mouse pre-B lymphocytes have been transfected with human kappa-immunoglobulin genes (Potter *et al.,* 1984), and rat hepatocytes have been transfected with the chloramphenicol acetyltransferase gene (Tur-Kaspa *et al.,* 1986) in this manner.

[0178] One type of electroporation is nucleofection, in which nucleic acid is transferred to a cell through the use of a device called a Nucleofector and in combination with cell specific reagents (such as the Amaxa system; Lonza Cologne AG).

4. Calcium Phosphate

[0179] In other embodiments of the present invention, a nucleic acid is introduced to the cells using calcium phosphate precipitation. Human KB cells have been transfected with adenovirus 5 DNA (Graham and Van Der Eb, 1973) using this technique. Also in this manner, mouse L(A9), mouse C127, CHO, CV-1, BHK, NIH3T3 and HeLa cells were transfected with a neomycin marker gene (Chen and Okayama, 1987), and rat hepatocytes were transfected with a variety of marker genes (Rippe *et al.,* 1990).

5. DEAE-Dextran

[0180] In another embodiment, a nucleic acid is delivered into a cell using DEAE-dextran followed by polyethylene glycol. In this manner, reporter plasmids were introduced into mouse myeloma and erythroleukemia cells (Gopal, 1985).

6. Sonication Loading

[0181] Additional embodiments of the present invention include the introduction of a nucleic acid by direct sonic loading. LTK⁻fibroblasts have been transfected with the thymidine kinase gene by sonication loading (Fechheimer *et al.*, 1987).

7. Microprojectile Bombardment

[0182] Microprojectile bombardment techniques can be used to introduce a nucleic acid into at least one, organelle, cell, tissue or organism (U.S. Patent No. 5,550,318; U.S. Patent No. 5,538,880; U.S. Patent No. 5,610,042; and PCT Application WO 94/09699). This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). There are a wide variety of microprojectile bombardment techniques known in the art, many of which are applicable to the invention.

[0183] In this microprojectile bombardment, one or more particles may be coated with at least one nucleic acid and delivered into cells by a propelling force. Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold particles or beads. Exemplary particles include those comprised of tungsten, platinum, and preferably, gold. It is contemplated that in some instances DNA precipitation onto metal particles would not be necessary for DNA delivery to a recipient cell using microprojectile bombardment. However, it is contemplated that particles may contain DNA rather than be coated with DNA. DNA-coated particles may increase the level of DNA delivery via particle bombardment but are not, in and of themselves, necessary.

[0184] For the bombardment, cells in suspension are concentrated on filters or solid culture medium. Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate.

E. Protein Transduction

[0185] In certain aspects of the present invention, the cells to be programmed into endothelial cells may be contacted with endothelial programming factors comprising polypeptides of endothelial cell transcription factor genes at a sufficient amount for forward programming. Protein transduction has been used as a method for enhancing the delivery of macromolecules into cells. Protein transduction domains may be used to introduce endothelial programming polypeptides or functional fragments thereof directly into cells. Research by many groups has shown that a region of the TAT protein which is derived from the HIV Tat protein can be fused to a target protein allowing the entry of the target protein into the cell. A particular exemplary protein sequence of this domain is RKKRRQRRR (SEQ ID NO:1) where R encodes Arginine, K encodes Lysine and Q encodes Glutamine. This sequence has been shown to enable the entry of a protein fusion both as an N-terminal or C-terminal fusion. The mechanism of TAT mediated entry is thought to be by macropinocytosis (Gump and Dowdy).

[0186] A "protein transduction domain" or "PTD" is an amino acid sequence that can cross a biological membrane, particularly a cell membrane. When attached to a heterologous polypeptide, a PTD can enhance the translocation of the heterologous polypeptide across a biological membrane. The PTD is typically covalently attached (*e.g.*, by a peptide bond) to the heterologous DNA binding domain. For example, the PTD and the heterologous DNA binding domain can be encoded by a single nucleic acid, *e.g.*, in a common open reading frame or in one or more exons of a common gene. An exemplary PTD can include between 10-30 amino acids and may form an amphipathic helix. Many PTDs are basic in character. For example, a basic PTD can include at least 4, 5, 6 or 8 basic residues (*e.g.*, arginine or lysine). A PTD may be able to enhance the translocation of a polypeptide into a cell that lacks a cell wall or a cell from a particular species, *e.g.*, a mammalian cell, such as a human, simian, murine, bovine, equine, feline, or ovine cell.

[0187] A PTD can be linked to an artificial transcription factor, for example, using a flexible linker. Flexible linkers can include one or more glycine residues to allow for free rotation. For example, the PTD can be spaced from a DNA binding domain of the transcription factor by at least 10, 20, or 50 amino acids. A PTD can be located N- or C-terminal relative to a DNA binding domain. Being located N- or C-terminal to a particular domain does not require being adjacent to that particular domain. For example, a PTD N-

terminal to a DNA binding domain can be separated from the DNA binding domain by a spacer and/or other types of domains. A PTD can be chemically synthesized then conjugated chemically to a separately prepared DNA binding domain with or without a linker peptide. An artificial transcription factor can also include a plurality of PTDs, *e.g.*, a plurality of different PTDs or at least two copies of one PTD.

[0188] Several proteins and small peptides have the ability to transduce or travel through biological membranes independent of classical receptor- or endocytosis-mediated pathways. Examples of these proteins include the HIV-1 TAT protein, the herpes simplex virus 1 (HSV-1) DNA-binding protein VP22, and the Drosophila Antennapedia (Antp) homeotic transcription factor. The small protein transduction domains (PTDs) from these proteins can be fused to other macromolecules, peptides, or proteins to successfully transport them into a cell. Sequence alignments of the transduction domains from these proteins show a high basic amino acid content (Lys and Arg) which may facilitate interaction of these regions with negatively charged lipids in the membrane. Secondary structure analyses show no consistent structure between all three domains.

[0189] The advantages of using fusions of these transduction domains is that protein entry is rapid, concentration-dependent, and appears to work with difficult cell types.

[0190] The Tat protein from human immunodeficiency virus type I (HIV-1) has the remarkable capacity to enter cells when added exogenously (Frankel and Pabo, 1988; Mann and Frankel, 1991; Fawell *et al.*, 1994). A particular example of a Tat PTD may include residues 47-57 of the human immunodeficiency virus Tat protein: YGRKKRRQRRR (SEQ ID NO:2). This peptide sequence is referred to as "TAT" herein. This peptide has been shown to successfully mediate the introduction of heterologous peptides and proteins in excess of 100 kDa into mammalian cells *in vitro* and *in vivo* (Ho *et al.*, 2001). Schwarze *et al.* showed that when the 120 kDa β-galactosidase protein fused with TAT was injected into mouse intraperitoneally, the fusion proteins were found in all types of cells and tissues even including brain, which has been thought to be difficult because of the blood-brain-barrier (Schwarze *et al.*, 1999).

[0191] The antennapedia homeodomain also includes a peptide that is a PTD (Derossi *et al.*, 1994). This peptide, also referred to as "Penetratin", includes the amino acid sequence: AKIWFQNRRMKWKKENN (SEQ ID NO:3).

[0192] The HSV VP22 protein also includes a PTD. This PTD is located at the VP22 C-terminal 34 amino acid residues: DAATATRGRSAASRPTERPRAPARSASRPRRPVE (SEQ ID NO:4). *See, e.g.*, Elliott and O'Hare (1997) and U.S. Pat. No. 6,184,038.

[0193] In one embodiment, the PTD is obtained from a human or other mammalian protein. Exemplary mammalian PTDs are described in WO 03/059940 (human SIM-2) and WO 03/059941 (Mph). In certain embodiments, the PTD could be a synthetic PTD. The minimal Tat PTD (aa 47-57) was modified to optimize protein transduction potential (Ho *et al.*, 2001). A FITC coupled with series of synthetic PTDs was tested with cultured T lymphocytes. Some synthetic PTDs showed enhanced protein transduction compared to Tat PTD. These PTD include: YARKARRQARR (SEQ ID NO:5); YARAARRAARR (SEQ ID NO:6); YARAARRAARA (SEQ ID NO:7); YARAAARQARA (SEQ ID NO:8). Especially, the FITC conjugated with synthetic PTD YARAAARQARA (SEQ ID NO:8); showed enhanced uptake by whole blood cells when the mice were i.p. injected.

[0194] The poly-arginine peptides composed of about 6-12 arginine residues also can mediate protein transduction in some cases. For additional information about poly-arginine, see, *e.g.*, Rothbard *et al.* (2000); Wender *et al.* (2000).

[0195] For additional information about PTDs, *see also* U.S. 2003/0082561; U.S. 2002/0102265; U.S. 2003/0040038; Schwarze *et al.* (1999); Derossi *et al.* (1996); Hancock *et al.* (1991); Buss *et al.* (1988); Derossi *et al.* (1998); Lindgren *et al.* (2000); Kilic *et al.* (2003); Asoh *et al.* (2002); and Tanaka *et al.* (2003).

[0196] In addition to PTDs, cellular uptake signals can be used. Such signals include amino acid sequences that are specifically recognized by cellular receptors or other surface proteins. Interaction between the cellular uptake signal and the cell causes internalization of the artificial transcription factor that includes the cellular uptake signal. Some PTDs may also function by interaction with cellular receptors or other surface proteins.

[0197] A number of assays are available to determine if an amino acid sequence can function as a PTD. For example, the amino acid sequence can be fused to a reporter protein such as β-galactosidase to form a fusion protein. This fusion protein is contacted with culture cells. The cells are washed and then assayed for reporter activity. Another assay detects the presence of a fusion protein that includes the amino acid sequence in question and another detectable sequence, *e.g.*, an epitope tag. This fusion protein is contacted with culture cells.

The cells are washed and then analyzed by Western or immunofluorescence to detect presence of the detectable sequence in cells. Still other assays can be used to detect transcriptional regulatory activity of a fusion protein that includes the putative PTD, a DNA binding domain, and optionally an effector domain. For example, cells contacted with such fusion proteins can be assayed for the presence or amount of mRNA or protein, *e.g.*, using microarrays, mass spectroscopy, and high-throughput techniques.

V. Cell culturing

[0198] Generally, cells of the present invention are cultured in a culture medium, which is a nutrient-rich buffered solution capable of sustaining cell growth.

[0199] Culture media suitable for isolating, expanding and differentiating stem cells into endothelial cells according to the method described herein include but not limited to high glucose Dulbecco's Modified Eagle's Medium (DMEM), DMEM/F-15, Liebovitz L-15, RPMI 1640, Iscove's modified Dubelcco's media (IMDM), and Opti-MEM SFM (Invitrogen Inc.). Chemically Defined Medium comprises a minimum essential medium such as Iscove's Modified Dulbecco's Medium (IMDM) (Gibco), supplemented with human serum albumin, human Ex Cyte lipoprotein, transferrin, insulin, vitamins, essential and non essential amino acids, sodium pyruvate, glutamine and a mitogen is also suitable. As used herein, a mitogen refers to an agent that stimulates division of a cell. An agent can be a chemical, usually some form of a protein that encourages a cell to commence cell division, triggering mitosis. In one embodiment, serum free media such as those described in U.S. Ser. No. 08/464,599 and WO96/39487, and the "complete media" as described in U.S. Pat. No. 5,486,359 are contemplated for use with the method described herein. In some embodiments, the culture medium is supplemented with 10% Fetal Bovine Serum (FBS), human autologous serum, human AB serum or platelet rich plasma supplemented with heparin (2U/mL). Cell cultures may be maintained in a CO_2 atmosphere, *e.g.*, 5% to 12%, to maintain pH of the culture fluid, incubated at 37°C in a humid atmosphere and passaged to maintain a confluence below 85%.

[0200] Pluripotent stem cells to be differentiated into endothelial cells may be cultured in a medium sufficient to maintain the pluripotency. Culturing of induced pluripotent stem (iPS) cells generated in certain aspects of this invention can use various medium and techniques developed to culture primate pluripotent stem cells, more specially, embryonic stem cells, as described in U.S. Pat. App. 20070238170 and U.S. Pat. App. 20030211603. For example, like human embryonic stem (hES) cells, iPS cells can be maintained in 80%

DMEM (Gibco #10829-018 or #11965-092), 20% defined fetal bovine serum (FBS) not heat inactivated, 1% non-essential amino acids, 1 mM L-glutamine, and 0.1 mM .beta.-mercaptoethanol. Alternatively, ES cells can be maintained in serum-free medium, made with 80% Knock-Out DMEM (Gibco #10829-018), 20% serum replacement (Gibco #10828-028), 1% non-essential amino acids, 1 mM L-glutamine, and 0.1 mM .beta.-mercaptoethanol. Just before use, human bFGF may be added to a final concentration of .about 4 ng/mL (WO 99/20741).

[0201] Endothelial cells of this invention can be made by culturing pluripotent stem cells or other non-endothelial cells in a medium under conditions that increase the intracellular level of endothelial programming factors to be sufficient to promote programming of the cells into endothelial cells. The medium may also contain one or more endothelial cell differentiation and maturation agents, like various kinds of growth factors. However, by increasing the intracellular level of endothelial programming transcription factors, aspects of the present invention bypass most stages toward mature endothelial cells without the need to change the medium for each of the stages. Therefore, in view of the advantages provided by the present invention, in particular aspects, the medium for culturing cells under endothelial programming may be essentially free of one or more of the endothelial cell differentiation and maturation agents, or may not undergo serial change with media containing different combination of such agents.

[0202] These agents may either help induce cells to commit to a more mature phenotype—or preferentially promote survival of the mature cells—or have a combination of both these effects. Endothelial cell differentiation and maturation agents illustrated in this disclosure may include soluble growth factors (peptide hormones, cytokines, ligand-receptor complexes, and other compounds) that are capable of promoting the growth of cells of the endothelial cell lineage. Non-limiting examples of such agents include but are not limited to endothelial growth factors such as basic FGF (bFGF), BMP-4, and VEGF, or isoforms or variants thereof.

VI. Endothelial cell characteristics

[0203] Cells can be characterized according to a number of phenotypic criteria. The criteria include but are not limited to the detection or quantitation of expressed cell markers, enzymatic activity, and the characterization of morphological features and intercellular signaling. In other aspects, cells to be programmed may comprise reporter gene expression

cassette comprising tissue- or cell-specific transcriptional regulatory element, like endothelial cell-specific promoters for endothelial cell identification.

[0204] Endothelial cells embodied in certain aspects of this invention have morphological features characteristic of endothelial cells in the nature. The features are readily appreciated by those skilled in evaluating such things, and include a squamous appearance and a large central nucleus. One or more such features present in a single cell are consistent with the cell being a member of the endothelial cell lineage. Unbiased determination of whether cells have morphologic features characteristic of endothelial cells can be made by coding micrographs of programming progeny cells, adult or fetal endothelial cells, and one or more negative control cells, such as a fibroblast, or RPE (Retinal pigment epithelial) cells—then evaluating the micrographs in a blinded fashion, and breaking the code to determine if the endothelial cells from programming are accurately identified.

[0205] Cells of this invention can also be characterized according to whether they express phenotypic markers characteristic of cells of the endothelial cell lineage. Non-limiting examples of cell markers useful in distinguishing endothelial cells include: 7B4 antigen, ACE (angiotensin-converting enzyme), BNH9/BNF13, CD31, CD34, CD54 (ICAM-1), CD62P (p-Selectin GMP140), CD105 (Endoglin), CD144, CD146, Endocan (also called ESM-1), Endoglin (CD105), Endoglyx-1, Endomuci, Eotaxin-3, EPAS1 (Endothelial PAS domain protein 1), Factor VIII related antigen, FLI-1, Flk-1 (VEGFR-2), Flt-1 (VEGFR-1), GATA2, GBP-1 (guanylate-binding protein-1), GRO-alpha, Hex, ICAM-2 (intercellular adhesion molecule 2), LMO2, LYVE-1, MRB (magic roundabout), Nucleolin, PAL-E (pathologische anatomie Leiden-endothelium), RTKs, sVCAM-1, TAL1, TEM1 (Tumor endothelial marker 1), TEM5 (Tumor endothelial marker 5), TEM7 (Tumor endothelial marker 7), Thrombomodulin (TM, CD141), VCAM-1 (vascular cell adhesion molecule-1) (CD106), VE-cadherin (CD144), and vWF (von Willebrand factor, also called Factor VIII).

[0206] Assessment of the level of expression of such markers can be determined in comparison with other cells. Positive controls for the markers of mature endothelial cells include adult endothelial cells of the species of interest, and established endothelial cell lines. The reader is cautioned that permanent cell lines or long-term endothelial cells cultures may be metabolically altered, and fail to express certain characteristics of primary endothelial cells. Negative controls include cells of a separate lineage, such as an adult fibroblast cell line, or retinal pigment epithelial (RPE) cells. Undifferentiated stem cells are positive for

some of the markers listed above, but negative for markers of mature endothelial cells, as illustrated in the examples below.

[0207] Tissue-specific (*e.g.*, endothelial cell-specific) protein and oligosaccharide determinants listed in this disclosure can be detected using any suitable immunological technique—such as flow immunocytochemistry for cell-surface markers, immunohistochemistry (for example, of fixed cells or tissue sections) for intracellular or cell-surface markers, Western blot analysis of cellular extracts, and enzyme-linked immunoassay, for cellular extracts or products secreted into the medium. Expression of an antigen by a cell is said to be "antibody-detectable" if a significantly detectable amount of antibody will bind to the antigen in a standard immunocytochemistry or flow cytometry assay, optionally after fixation of the cells, and optionally using a labeled secondary antibody or other conjugate (such as a biotin-avidin conjugate) to amplify labeling.

[0208] The expression of tissue-specific (*e.g.*, endothelial cell-specific) markers can also be detected at the mRNA level by Northern blot analysis, dot-blot hybridization analysis, or by real time polymerase chain reaction (RT-PCR) using sequence-specific primers in standard amplification methods (U.S. Pat. No. 5,843,780). Sequence data for the particular markers listed in this disclosure can be obtained from public databases such as GenBank. Expression at the mRNA level is said to be "detectable" according to one of the assays described in this disclosure if the performance of the assay on cell samples according to standard procedures in a typical controlled experiment results in clearly discernable hybridization or amplification product within a standard time window. Unless otherwise required, expression of a particular marker is indicated if the corresponding mRNA is detectable by RT-PCR. Expression of tissue-specific markers as detected at the protein or mRNA level is considered positive if the level is at least 2-fold, and preferably more than 10- or 50-fold above that of a control cell, such as an undifferentiated pluripotent stem cell, a fibroblast, or other unrelated cell type.

[0209] Cells can also be characterized according to whether they display enzymatic activity that is characteristic of cells of the endothelial lineage. For example, assays that detect uptake of acetylated low density lipoprotein, bradykinin degradation, angiotensin I conversion, or nitric oxide production may be useful. *See, e.g.*, Voyta *et al.*, 1984; King, *et al.*, 1989; Graf *et al.*, 1992; Ming *et al.*, 2002. In other embodiments, cells of the invention are assayed for the ability to form tube-like structures or to respond to pro-inflammatory

stimuli (*e.g.*, TNF and/or IL-1) by upregulating the expression of one or more cell-adhesion molecules such (*e.g.*, CD54 and/or CD62E). *See, e.g.*, Chalupowicz *et al.*, 1995.

[0210] The skilled reader will readily appreciate that an advantage of programming-derived endothelial cells is that they will be essentially free of other cell types that typically contaminate primary endothelial cell cultures isolated from adult or fetal tissue, such as fibroblasts, immune cells, pericytes, Kupffer cells, and other stromal cells. Programming-derived endothelial cells can be characterized as essentially free of some or all of contaminant cell types if less than 0.1% (preferably less than 100 or 10 ppm) bear markers or other features of the undesired cell type, as determined by immunostaining and fluorescence-activated quantitation, or other appropriate techniques. Moreover, programming-derived endothelial cells may be free or essentially free of mesenchymal cells or hematopoietic cells.

[0211] Endothelial cells provided by programming according to this invention can have a number of the features of the stage of cell they are intended to represent. The more of these features that are present in a particular cell, the more it can be characterized as a cell of the endothelial cell lineage. Cells having at least 2, 3, 5, 7, or 9 of these features are increasingly more preferred. In reference to a particular cell population as may be present in a culture vessel or a preparation for administration, uniformity between cells in the expression of these features is often advantageous. In this circumstance, populations in which at least about 40%, 60%, 80%, 90%, 95%, or 98% of the cells have the desired features are increasingly more preferred.

VII. Use of endothelial cells

[0212] The endothelial cells provided by methods and compositions of certain aspects of the invention can be used in a variety of applications. These include but are not limited to transplantation or implantation of the endothelial cells *in vivo*; screening cytotoxic compounds, carcinogens, mutagens growth/regulatory factors, pharmaceutical compounds, *etc.*, *in vitro*; elucidating the mechanism of cardiovascular diseases and injuries; studying the mechanism by which drugs and/or growth factors operate; diagnosing and monitoring cancer in a patient; gene therapy; and the production of biologically active products, to name but a few.

B. Test compound screening

[0213] Programming-derived endothelial cells of this invention can be used to screen for factors (such as solvents, small molecule drugs, peptides, and polynucleotides) or environmental conditions (such as culture conditions or manipulation) that affect the characteristics of endothelial cells provided herein.

[0214] In some applications, stem cells (differentiated or undifferentiated) are used to screen factors that promote maturation of cells along the endothelial cell lineage, or promote proliferation and maintenance of such cells in long-term culture. For example, candidate endothelial cell maturation factors or growth factors are tested by adding them to stem cells in different wells, and then determining any phenotypic change that results, according to desirable criteria for further culture and use of the cells.

[0215] Particular screening applications of this invention relate to the testing of pharmaceutical compounds in drug research. The reader is referred generally to the standard textbook *In vitro Methods in Pharmaceutical Research,* Academic Press, 1997, and U.S. Pat. No. 5,030,015). In certain aspects of this invention, cells programmed to the endothelial lineage play the role of test cells for standard drug screening and toxicity assays, as have been previously performed on endothelial cell lines or primary endothelial cells in short-term culture. Assessment of the activity of candidate pharmaceutical compounds generally involves combining the endothelial cells provided in certain aspects of this invention with the candidate compound, determining any change in the morphology, marker phenotype, or metabolic activity of the cells that is attributable to the compound (compared with untreated cells or cells treated with an inert compound), and then correlating the effect of the compound with the observed change. The screening may be done either because the compound is designed to have a pharmacological effect on endothelial cells, or because a compound designed to have effects elsewhere may have unintended endothelial cell side effects. Two or more drugs can be tested in combination (by combining with the cells either simultaneously or sequentially), to detect possible drug-drug interaction effects.

[0216] In some applications, compounds are screened for toxicity to endothelial cells. *See, e.g.*, Kuzuya *et al.*, 2001. In other applications, endothelial cells derived from the programming methods disclosed herein are used to test the vascular permeability of a compound.

C. Endothelial cell therapy and transplantation

[0217] This invention also provides for the use of endothelial cells provided herein to restore a degree of liver function to a subject needing such therapy, perhaps due to a cardiovascular disease, cardiovascular injury, or tissue injury. For example, endothelial cells and endothelial progenitor cells derived by methods disclosed here may be used to treat vascular diseases, cardiovascular diseases , ischemic diseases, vascular or other tissue injury (such as, e.g., by engineering of grafts), or hypertension, as disclosed in, for example, Dzau *et al.*, 2005 and Li *et al.*, 2009.

[0218] To determine the suitability of endothelial cells provided herein for therapeutic applications, the cells can first be tested in a suitable animal model. At one level, cells are assessed for their ability to survive and maintain their phenotype *in vivo*. Endothelial cells provided herein are administered to immunodeficient animals (such as SCID mice, or animals rendered immunodeficient chemically or by irradiation) at a site amenable for further observation, such as under the kidney capsule, into the spleen, or into a liver lobule. Tissues are harvested after a period of a few days to several weeks or more, and assessed as to whether starting cell types such as pluripotent stem cells are still present. This can be performed by providing the administered cells with a detectable label (such as green fluorescent protein, or β-galactosidase); or by measuring a constitutive marker specific for the administered cells. Where endothelial cells provided herein are being tested in a rodent model, the presence and phenotype of the administered cells can be assessed by immunohistochemistry or ELISA using human-specific antibody, or by RT-PCR analysis using primers and hybridization conditions that cause amplification to be specific for human polynucleotide sequences. Suitable markers for assessing gene expression at the mRNA or protein level are provided in elsewhere in this disclosure.

[0219] Endothelial cells and endothelial progenitor cells provided by methods of the invention may be tested in various animal models for their ability to treat cardiovascular diseases, vascular disease, vascular injuries, tissue injuries, and the like. Various such animal models that may find use in certain aspects of the present invention are discussed in, for example, Dzau *et al.*, 2005 and Li *et al.*, 2009.

[0220] Endothelial cells and endothelial progenitor cells provided in certain aspects of this invention that demonstrate desirable functional characteristics according to their profile of enzymes, or efficacy in animal models, may also be suitable for direct administration to

human subjects in need thereof. For purposes of hemostasis, the cells can be administered at any site that has adequate access to the circulation. Endothelial cells may also be delivered at a site of injury or disease.

[0221] The endothelial cells or endothelial precursors provided in certain aspects of this invention can be used for therapy of any subject in need thereof. Human conditions that may be appropriate for such therapy include cardiovascular disease, vascular disease, ischemia, vascular injury, tissue injury, diabetes, coronary artery disease, atherosclerosis, peripheral artery disease, aneurysm, or hypertension. For human therapy, the dose is generally between about 10^9 and 10^{12} cells, and typically between about 5×10^9 and 5×10^{10} cells, making adjustments for the body weight of the subject, nature and severity of the affliction, and the replicative capacity of the administered cells. The ultimate responsibility for determining the mode of treatment and the appropriate dose lies with the managing clinician.

[0222] Certain aspects of the invention include endothelial cells or endothelial progenitor cells provided herein that form part of a bioengineered tissue graft. Such a tissue graft may be a heart tissue graft (*see, e.g.*, U.S. Patent App. 20080199843), a vascularized tissue graft (*see, e.g.*, U.S. Patent App. 20070299508), or any other tissue graft known in the art (*see, e.g.*, U.S. Patent App. Nos. 20080063627; 20070184122; 20070141037; 20100145444; 20090324683; 20090149569; 20070122388).

D. Distribution for Commercial, Therapeutic, and Research Purposes

[0223] For purposes of manufacture, distribution, and use, the endothelial lineage cells of this invention are typically supplied in the form of a cell culture or suspension in an isotonic excipient or culture medium, optionally frozen to facilitate transportation or storage.

[0224] This invention also includes different reagent systems, comprising a set or combination of cells that exist at any time during manufacture, distribution, or use. The cell sets comprise any combination of two or more cell populations described in this disclosure, exemplified but not limited to programming-derived cells (endothelial lineage cells, their precursors and subtypes), in combination with undifferentiated stem cells, somatic cell-derived endothelial cells, or other differentiated cell types. The cell populations in the set sometimes share the same genome or a genetically modified form thereof. Each cell type in the set may be packaged together, or in separate containers in the same facility, or at different

locations, at the same or different times, under control of the same entity or different entities sharing a business relationship.

VIII. Cells and methods for testing candidate genes in programming

[0225] The ability of a particular candidate gene or a combination of candidate genes to act as programming factors for a specific cell type, such as endothelial cells, can be tested using the methods and cells provided in this disclosure. Efficacy of particular candidate genes or combinations of candidate genes in programming can be assessed by their effect on cell morphology, marker expression, enzymatic activity, proliferative capacity, or other features of interest, which is then determined in comparison with parallel cultures that did not include the candidate genes or combinations. Candidate genes may be transcription factors important for differentiation into desired cell types or for function of the desired cell types.

[0226] In certain embodiments, starting cells, such as pluripotent stem cells, comprising at least one expression cassette for expression of a candidate gene or a combination of candidate genes may be provided. The expression cassette may comprise an externally controllable transcriptional regulatory element, such as an inducible promoter. The activity of these promoters may be induced by the presence or absence of biotic or abiotic factors. Inducible promoters are a very powerful tool in genetic engineering because the expression of genes operably linked to them can be turned on or off at certain stages of development of an organism or in a particular tissue. Tet-On and Tet-Off inducible gene expression systems based on the essential regulatory components of the *E. coli* tetracycline-resistance operon may be used. Once established in the starting cells, the inducer doxycycline (Dox, a tetracycline derivative) could controls the expression system in a dose-dependent manner, allowing to precisely modulate the expression levels of candidate genes.

[0227] To aid identification of desired cell types, the starting cells may further comprise a cell-specific or tissue-specific reporter expression cassette. The reporter expression cassette may comprise a reporter gene operably linked to a transcriptional regulatory element specific for the desired cell types. For example, the reporter expression cassette may comprise a endothelial cell-specific promoter for endothelial cell production, isolation, selection, or enrichment. The reporter gene may be any selectable or screenable marker gene known in the art and exemplified in the preceding disclosure.

IX. Examples

[0228] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1 - Forward programming into endothelial cells

[0229] Alternative approaches for endothelial cell differentiation from human ESC/iPSCs are shown in **FIG.** 1. Endothelial cells can likely be efficiently induced from human ESC/iPSCs via expression of an appropriate transgene or transgene combination (top box), bypassing most, if not all, developmental stages required during normal differentiation (bottom box).

[0230] The strategy employed for identifying transgenes that could directly convert human ESC/iPSCs to mature endothelial cells (**FIG. 2**). Human ESC/iPSCs were engineered to constitutively express rtTET protein for inducible gene expression. Transgenes under the control of the inducible promoter Ptight were introduced into the engineered hESC/iPSCs by electroporation. Upon doxycycline (Dox) addition, transgene expression is induced, and EC differentiation is monitored by the characteristic EC morphology along with expression of definitive EC markers (e.g., CD31, CD144 (VE-cadherin)) by flow cytometry. Endothelial cells thus programmed are purified for in vitro and in vivo functional assays.

[0231] The establishment of human ESC/iPSC inducible (R/I) lines for endothelial cell differentiation (**FIG. 3**). The human Rosa26 locus on chromosome 3 was selected to allow the expression of rtTET, while minimizing the chromosome location-dependent silencing effect. First, the LoxP recombination sites (LOX71 and LOX2272) were introduced into a site between exon 1 and exon 2 of the human ROSA 26 gene via homologous recombination. The targeting construct (KI construct) used the phosphoglycerate kinase promoter (PGK)-driven expression of diphtheria toxin A fragment gene (DTA) for negative selection, and contains a ~ 2.0 kb 5' arm and a 4.5 kb 3' arm. A splicing acceptor signal from

the human BCL2 gene (SA) was placed in front of the LOX71 site to allow the expression of selection markers from the endogenous human ROSA26 promoter. The coding region for thymidine kinase (TK) was included to enable negative selection against incorrect Cre/LoxP recombination events at step 2 using ganciclovir. The neomycin phosphotransferase (Neo) was used for positive selection during homologous recombination (step 1). The foot-and-mouth disease virus peptide (2A) was used to co-express the TK and Neo genes from the endogenous human ROSA26 promoter. BGHpA is a polyadenylation signal derived from bovine growth hormone gene. The homologous recombination yielded parental human ESC/iPSC lines for efficient cassette exchange via Cre/LoxP recombination.

[0232] To establish inducible cell lines for endothelial cell differentiation, rtTET driven by the constitutively active eukaryotic elongation factor 1α promoter (pEF) was introduced into the Rosa 26 locus by lipid-mediated cotransfection of the recombination mediated cassette exchange (RMCE) vector and a Cre-expressing plasmid. The puromycin N-acetyl-transferase (Puro) was used to select for recombination events. The correctly recombined inducible cells are resistant to puromycin (Puro+) and ganciclovir (TK-), and sensitive to geneticin selection (Neo-).

[0233] Confirmation of the Tet-On inducible gene expression in human H1 ESC inducible lines (**FIG. 4**). **FIG. 4A** shows a two-vector PiggyBac stable gene expression system. Ptight is an rtTET-responsive inducible promoter; pEF is the eukaryotic elongation factor 1α promoter; and hPBase is the coding region for the PiggyBac transposase with codons optimized for expression in human cells.

[0234] EGFP driven by the Ptight promoter was introduced into human ESC inducible lines using Fugene HD-mediated transfection of both shown in **FIG. 4A**. Human ESCs with stable *PiggyBac* transposon integration were selected with geneticin (100 μg/mL). The cells were observed after 2 days induction with or without Doxycycline (at 1 μg/mL), and EGFP expression in the Doxycycline-induced cells was confirmed microscopically. **FIG. 4B** shows flow cytometric analysis of EGFP expression in human ESC inducible lines after 4 days induction with or without Doxycycline (1 μg/mL). Gray lines are Human ESC inducible lines with transfection of the EGFP vector; black lines are Human ESC R/I lines with stable PiggyBac transposon integration after 4 days induction with or without Doxycycline.

[0235] Forward programming of endothelial cells (ECs) from human embryonic stem cell (ESC) inducible lines through ERG-3 expression (**FIG. 5**). ERG-3 was cloned into the PiggyBac vector (**FIG. 4A**) under the control of the Ptight promoter and introduced into the human ESC inducible line along with an hPBase-expressing vector by electroporation. Transfected cells were cultured in TeSR medium on matrigel in the presence of geneticin (100 μg/ml) for selection of transformants having stable genomic transgene integration. Doxycycline (0.2 μg/ml) was added to induce ERG expression, and the TeSR was replaced with endothelial serum-free medium (ESFM; Invitrogen) supplemented with 10 ng/ml basic FGF and 20 ng/ml VEGF (both from Peprotech). Differentiated cells acquire the EC morphology on day 2-3 of ERG induction. As shown in **FIG. 5**, bright-field images of forward programmed ECs showed EC morphology.

[0236] Although ERG-3 was selected for these experiments, the other ERG isoforms (including ERG-1, ERG-2, and ERG-4) provided similar results. Thus, although ERG isoform 3 was selected for the experiments because it was consistently the more efficient isoform, all other isoforms can be used as well because all isoforms provide endothelial cells by forward programming.

[0237] Forward programming of endothelial cells (ECs) from human embryonic stem cell (ESC) inducible lines through ETV2 expression. ETV2 was cloned into the PiggyBac vector (**FIG. 4A**) under the control of the Ptight promoter and then introduced into the human ESC inducible line by electroporation along with the hPBase-expressing vector. Transfected cells were cultured in TeSR medium on matrigel in the presence of geneticin (100 μg/ml) for selection of transformants having stable genomic transgene integration. Doxycycline (0.2 μg/ml) was added to induce ETV2 expression, and the TeSR was replaced with endothelial serum-free medium (ESFM; Invitrogen) supplemented with 10 ng/ml basic FGF and 20 ng/ml VEGF (both from Peprotech). Differentiated cells acquire EC morphology on day 2-3 of ETV2 induction. As can be seen in **FIG. 6**, bright-field images of forward programmed ECs showed EC morphology.

[0238] Flow cytometric expression analysis of markers in forward programmed ECs. The ERG-3-induced differentiated cells up-regulated the expression of the EC markers (CD144 and CD31), while down-regulating the expression of the human pluripotent stem cell marker TRA-1-60 (**FIG. 7**). The ETV2-induced differentiated cells up-regulated the expression of the EC markers (CD144 and CD31), while down-regulated the expression of

the human pluripotent stem cell marker TRA-1-60. (**FIG. 8**). Over time ERG-3-induced ECs and ETV2-induced ECs increased expression of EC markers (CD31, CD144, ESAM, CD34) but not hematopoietic markers (CD43, CD45, CD41a, CD235a) (**FIG. 9**).

[0239] Comparison of forward programming to normal differentiation. Cell cultures on day 3 of induction were dissociated into single-cell suspension by Accutase treatment (Invitrogen) and plated on gelatin-coated plastic in ESFM supplemented with 10 ng/ml basic FGF. After 2 hours of plating, medium containing non-adherent cells was removed and attached cells were cultured in ESFM supplemented with 10 ng/ml basic FGF and 5 μg/ml human fibronectin (Invitrogen). The morphology of ERG-3-ECs and ETV2-ECs was highly similar to that of HUVEC and EECs (**FIG. 10**).

[0240] Flow cytometric analysis of arterial EC markers. The expression of all three markers for arterial ECs (CD304/NRP1, CD184 and DLL4) in ERG-3-ECs and ETV2-ECs suggests an arterial fate of these induced ECs, different from HUVEC and EECs (**FIG. 11**).

[0241] Analysis of markers found in HUVEC. ERG-3-ECs and ETV2-ECs show a similar staining pattern for CD144, vWF, and eNOS as compared to HUVEC (**FIG. 12**). DAPI was used to counterstain the nuclei. Although the staining was generally weaker than in HUVEC, vWF was clearly expressed in both ERG-3-ECs and ETV2-ECs.

[0242] Analysis of proteins found in tight junctions. Tight junction proteins Claudin 5 and ZO-1 are expressed in ERG-3-ECs and ETV2-ECs (**FIG. 13**).

[0243] Forward programmed ECs exhibit EC functional characteristics. Ac-LDL was incorporated by ERG-3-ECs and ETV2-ECs (**FIG. 14**). ECs were incubated with AcLDL-Dil conjugate (Invitrogen, 2 μg/ml) for 4 hours at 37°C, followed by incubation with 0.5 μg/ml Hoechst 33258 for 5 minutes for counterstaining. For flow cytometric analysis, the Ac-LDL-treated cultures were cultured in fresh medium overnight prior to accutase dissociation. Non-treated ECs were used as control. A barrier function test revealed that ERG-3-ECs are similar to HUVEC (**FIG. 15**). In particular, ERG-3-ECs showed similar kinetics in barrier function recovery as compared to HUVEC, while the ETV2-ECs were slower, suggesting that the ERG-3-ECs and ETV2-ECs are different with regard to barrier function. ERG-3-ECs were also similar to HUVEC in their ability to form tubes when plated on solidified matrigel (**FIG. 16**).

[0244] Hemogenic function of forward programmed ECs. ETV2 and ERG-3 induction was performed in medium containing 50% ESFM, 50% StemLine HSC medium (Sigma), 10 ng/ml FGF, 5 ng/ml VEGF, 50 ng/ml SCF, 20 ng/ml SCF, 10 ng/nl TPO, 10 ng/ml IL3 and 20 ng/ml IL6. Hematopoietic cells (defined by CD31+CD43+ phenotype) were detected in ETV2, but not in ERG-3-induced cultures (**FIG. 18**). The majority of the hematopoietic cells in the day 9 ETV2 culture were also CD235a/CD41a-CD45+, suggesting definitive hematopoiesis.

[0245] Mesenchymogenic potential of forward programmed ECs. ERG-3-ECs were cultured in ESFM containing 10 ng/ml FGF2 and additionally supplemented either with 20 ng/ml VEGF or with 1 μm A83-01 (TGFβ inhibitor). Gradual transition of ERG-3-EC to mesenchymal cells (defined by CD31-CD73+CD105+ phenotype) was observed in cultures containing FGF+A83-01, but not FGF+VEGF (**FIG. 19**). Although ETV2-EC cells undergo a similar mesenchymal transition, efficiency was lower than in ERG-3-EC.

Example 2 – Transdifferentiation into endothelial cells or endothelial precursor cells

[0246] Similar to forward programming, endothelial cells or endothelial precursors may also be obtained via transdifferentiation from human somatic cells such as skin fibroblasts, adipose tissue-derived cells, keratinocytes, and blood cells. To identify genes that can convert somatic cells to endothelial cells or endothelial precursor cells, a lentiviral transgene delivery system will be used for the inducible expression of candidate genes (called the TET-ON system). Briefly, the cytomegalovirus (CMV) promoter will be used to drive the expression of the rtTET protein, and the candidate genes will be placed under the control of the rtTET-responsive inducible promoter (called Ptight). Both the rtTET and transgene-expressing lentivirus will be used to cotransduce cells. Doxycycline (0.2 – 1 μg/mL) will be added to the transduced cells to induce transgene expression, and the cell culture medium will be replaced with endothelial cell culture medium to support programming. Alternatively, the piggyBac vector system (rather than a lentiviral delivery system) may be used for the inducible expression of candidate genes.

[0247] The confirmation of endothelial cells or endothelial precursors will be carried out similarly to forward programming from hESC/iPSCs and may include morphological characteristics, cell-surface marker expression, and functional characteristics. Genes

identified from forward programming from hESC/iPSCs, such as ERG and ETV2 are strong candidates for use in the transdifferentiation of human somatic cells to endothelial cells or endothelial precursors, although additional programming genes (*e.g.*, iPSC reprogramming genes, such as OCT4) may be needed to achieve optimal programming efficiency by destabilizing the established differentiated state in the somatic cells.

* * *

[0248] All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references provide exemplary procedural or other details supplementary to those set forth herein.

U.S. Patent 4,683,202

U.S. Patent 5,030,015

U.S. Patent 5,302,523

U.S. Patent 5,322,783

U.S. Patent 5,384,253

U.S. Patent 5,460,964

U.S. Patent 5,464,765

U.S. Patent 5,486,359

U.S. Patent 5,486,359

U.S. Patent 5,538,877

U.S. Patent 5,538,880

U.S. Patent 5,550,318

U.S. Patent 5,563,055

U.S. Patent 5,580,859

U.S. Patent 5,589,466

U.S. Patent 5,591,616

U.S. Patent 5,610,042

U.S. Patent 5,635,387

U.S. Patent 5,656,610

U.S. Patent 5,677,136

U.S. Patent 5,681,599

U.S. Patent 5,702,932

U.S. Patent 5,716,827

U.S. Patent 5,736,396

U.S. Patent 5,736,524

U.S. Patent 5,750,397

U.S. Patent 5,759,793

U.S. Patent 5,780,448

U.S. Patent 5,789,215
U.S. Patent 5,811,094
U.S. Patent 5,827,735
U.S. Patent 5,827,740
U.S. Patent 5,837,539
U.S. Patent 5,837,670
U.S. Patent 5,843,780
U.S. Patent 5,925,565
U.S. Patent 5,928,906
U.S. Patent 5,935,819
U.S. Patent 5,945,100
U.S. Patent 5,981,274
U.S. Patent 5,994,136
U.S. Patent 5,994,624
U.S. Patent 6,013,516
U.S. Patent 6,184,038
U.S. Patent 6,833,269
U.S. Patent 6,991,897
U.S. Patent 7,015,037
U.S. Patent 7,399,632
U.S. Patent 7,410,773
U.S. Patent 7,410,798
U.S. Patent 7,422,736
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WHAT IS CLAIMED IS:

1. A method of providing endothelial cells by forward programming of pluripotent stem cells, comprising:

providing the endothelial cells by culturing the pluripotent stem cells under conditions to increase the expression level of one or more endothelial programming factor genes capable of causing forward programming of the pluripotent cells into endothelial cells, thereby forward programming the pluripotent stem cells into endothelial cells, wherein at least one endothelial programming factor gene comprises v-ets avian erythroblastosis virus E26 oncogene homolog (ERG), ets variant 2 (ETV2), or any isoform thereof, and

wherein the endothelial cells and the pluripotent stem cells are human cells.

2. The method of claim 1, wherein the at least one endothelial programming factor gene comprises v-ets avian erythroblastosis virus E26 oncogene homolog (ERG), or any isoform thereof.

3. The method of claim 1, wherein the at least one endothelial programming factor gene comprises ets variant 2 (ETV2), or any isoform thereof.

4. The method of claim 1, wherein the pluripotent stem cells are induced pluripotent stem cells.

5. The method of any one of claims 1-4, wherein the pluripotent stem cells comprise at least one exogenous expression cassette, wherein the expression cassette comprises one or more endothelial programming factor genes.

6. The method of any one of claims 1-5, comprising contacting the pluripotent stem cells with one or more endothelial programming factors comprising gene products of the one or more endothelial programming factor genes in an amount sufficient to cause forward programming of the pluripotent cells into endothelial cells.

7. The method of claim 6, wherein the one or more gene products are polypeptides of one or more endothelial programming factor genes or RNA transcripts of one or more endothelial programming factor genes.

8. The method of any one of claims 1-7, wherein the v-ets avian erythroblastosis virus E26 oncogene homolog (ERG) is selected from the group consisting of ERG-1, ERG-2, ERG-3, and ERG-4.

9. The method of any one of claims 1-8, wherein the pluripotent stem cells or progeny cells thereof further comprise a reporter expression cassette comprising an endothelial cell-specific transcriptional regulatory element operably linked to a reporter gene.

10. The method of any one of claims 1-9, wherein the endothelial cells comprise one or more characteristics of endothelial cells comprising:

(i) expression of one or more endothelial cell markers;

(ii) the ability to take up acetylated low density lipoprotein (ac-LDL), form tube-like structures in a three dimensional matrix, upregulate expression of one or more cell adhesion molecules in response to one or more pro-inflammatory stimuli, or a combination of two or more of these abilities; or

(iii) endothelial cell morphological features.

11. The method of claim 10, wherein the one or more endothelial cell markers are selected from the group consisting of: CD144, CD31, CD34, ESAM, CD102, CD143, CD93, CD184, CD105, CD146, von Willebrand factor, ZO-1, CD304, and DLL4.

12. The method of claim 10, wherein the one or more pro-inflammatory stimuli are selected from the group consisting of TNF and IL-1, and the one or more cell adhesion molecules are selected from the group consisting of CD54, CD62E, and CD106.

13. The method of any one of claims 1-12, wherein the provided endothelial cells are mature endothelial cells.

14. The method of claim 10, further comprising selecting or enriching for endothelial cells, wherein the selected or enriched endothelial cells comprise the one or more endothelial cell characteristics of claim 10.

15. The method of any one of claims 1-14, wherein the pluripotent stem cells or progeny cells thereof are cultured in a medium comprising one or more growth factors.

16. The method of claim 15, wherein the one or more growth factors comprise basic fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), or both.

17. The method of any one of claims 1-16, wherein the provided endothelial cells are obtained after up to 10 days of the increased expression or after up to 4 days of the increased expression.

18. The method of any one of claims 1-17, further comprising dispersing cell groupings into individual cells, wherein the dispersing is performed at least 24 hours after the increased expression.

19. The method of claim 18, further comprising plating the individual cells onto a surface coated with a matrix component.

20. The method of claim 19, further comprising: (a) culturing the plated cells for at least 12 hours; (b) removing the unattached cells; and (c) culturing the remaining attached cells for at least two days.

21. The method of any one of claims 1-20, further comprising culturing the provided endothelial cells in a hematopoietic culture medium to generate hematopoietic cells or cultivating the provided endothelial cells in a mesenchymal culture medium to generate mesenchymal cells.

22. An endothelial cell provided by a process in accordance with any one of claims 1-21, wherein the cell comprises at least one exogenous expression cassette, wherein the at least one expression cassette comprises one or more endothelial programming factor genes, and wherein the one or more endothelial programming factor genes comprise v-ets avian erythroblastosis virus E26 oncogene homolog (ERG), ets variant 2 (ETV2), or any isoform thereof.

23. An endothelial cell provided by a process in accordance with any one of claims 1-21, for use in the treatment of a subject having or at risk of developing a disease or injury, wherein the cell comprises at least one exogenous expression cassette, wherein the at least one expression cassette comprises one or more endothelial programming factor genes, wherein the one or more endothelial programming factor genes comprise v-ets avian erythroblastosis virus E26 oncogene homolog (ERG), ets variant 2 (ETV2), or any isoform

thereof, and wherein the disease is ischemia or cardiovascular disease, or the injury is a tissue injury, in need of a tissue graft or a cardiovascular injury.

24. Use of an endothelial cell provided by a process in accordance with any one of claims 1-21 in the preparation of a medicament for treating a subject having or at risk of developing a disease or injury, wherein the cell comprises at least one exogenous expression cassette, wherein the at least one expression cassette comprises one or more endothelial programming factor genes, wherein the one or more endothelial programming factor genes comprise v-ets avian erythroblastosis virus E26 oncogene homolog (ERG), ets variant 2 (ETV2), or any isoform thereof, and wherein the disease is ischemia or a cardiovascular disease, or the injury is a tissue injury, in need of a tissue graft or a cardiovascular injury.

25. A cell population comprising endothelial cells, wherein at least 80% of the endothelial cells comprise an exogenous expression cassette that comprises one or more endothelial programming factor genes, wherein at least one endothelial programming factor gene comprises v-ets avian erythroblastosis virus E26 oncogene homolog (ERG), ets variant 2 (ETV2), or any isoform thereof, and wherein the endothelial cells are human cells.

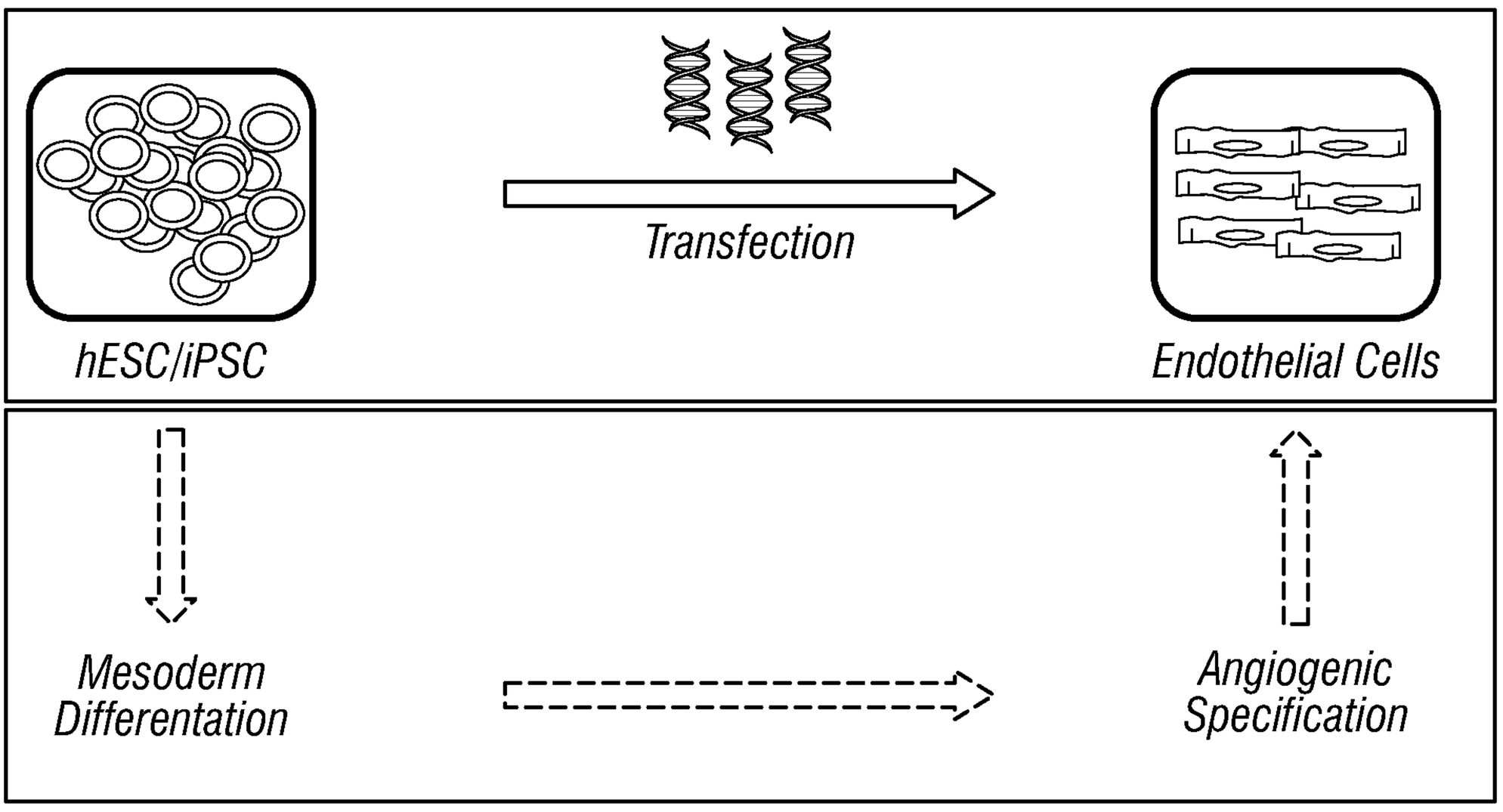

Forward Programming
Transfection
hESC/iPSC
Endothelial Cells
Mesoderm Differentation
Angiogenic Specification
Normal differentiation

FIG. 1

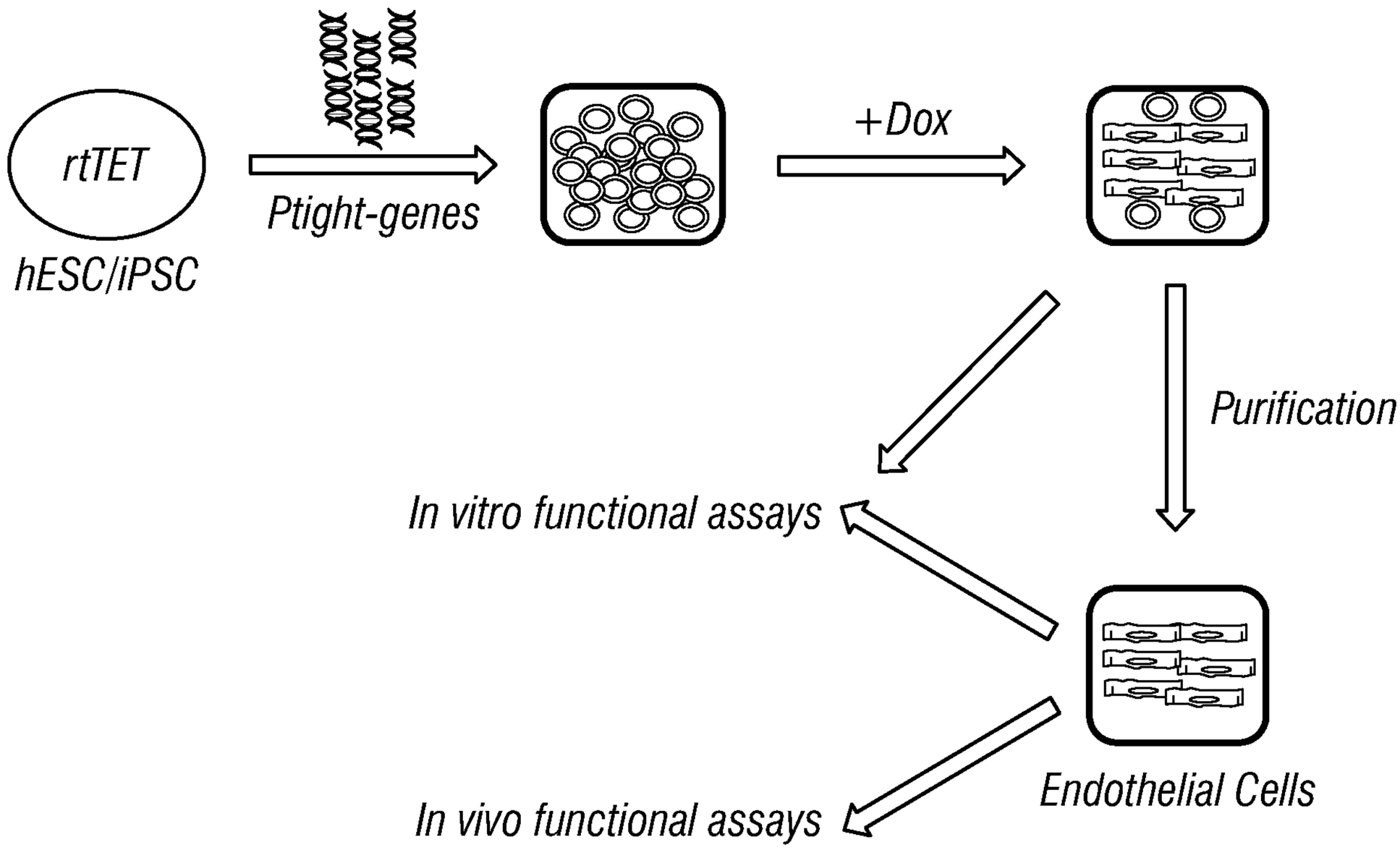

rtTET
hESC/iPSC
Ptight-genes
+Dox
Purification
In vitro functional assays
In vivo functional assays
Endothelial Cells

FIG. 2

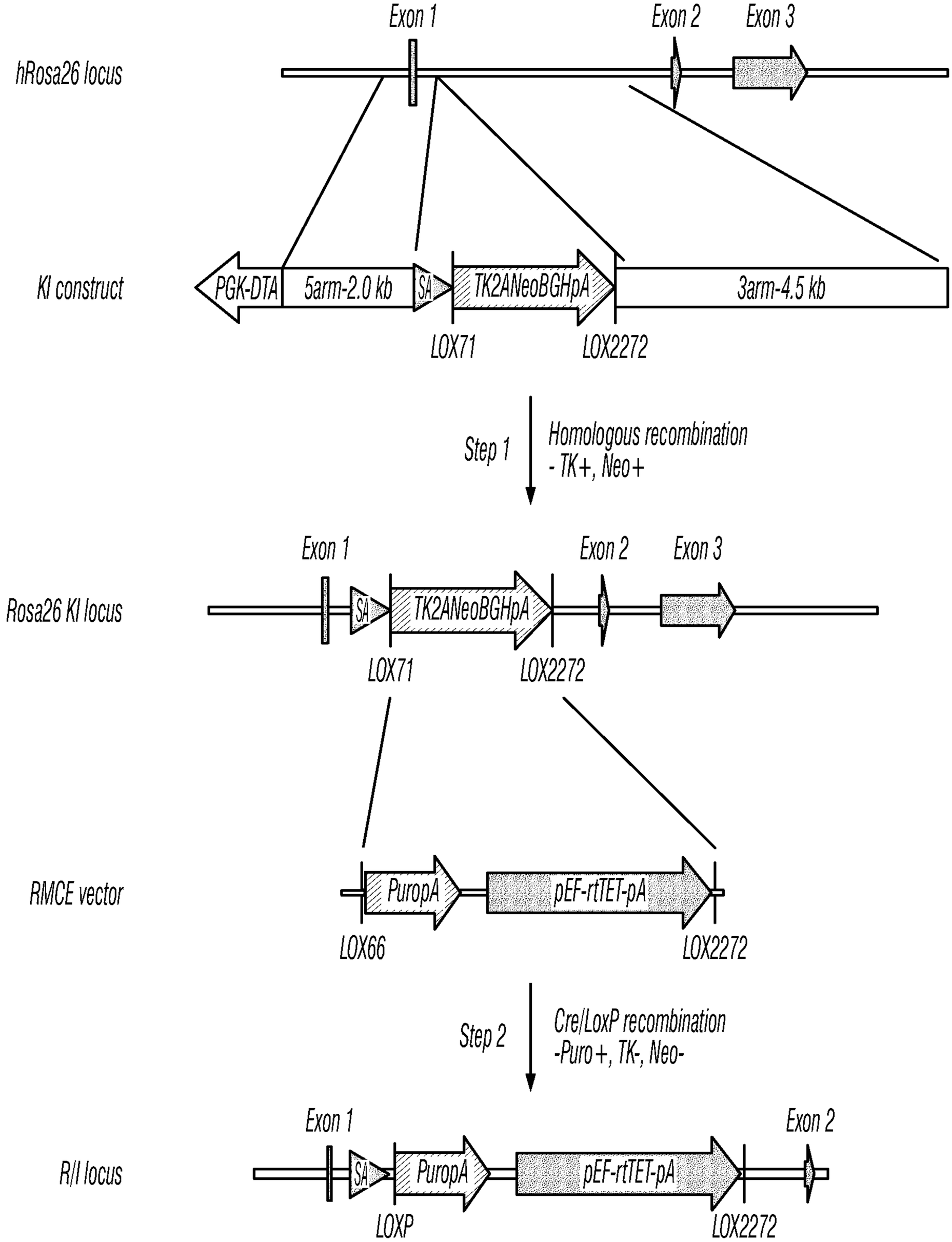

Exon 1
Exon 2
Exon 3
hRosa26 locus
KI construct
PGK-DTA
5arm-2.0 kb
SA
TK2ANeoBGHpA
3arm-4.5 kb
LOX71
LOX2272
Step 1
Homologous recombination
- TK+, Neo+
Exon 1
Exon 2
Exon 3
Rosa26 KI locus
SA
TK2ANeoBGHpA
LOX71
LOX2272
RMCE vector
PuropA
pEF-rtTET-pA
LOX66
LOX2272
Step 2
Cre/LoxP recombination
-Puro+, TK-, Neo-
Exon 1
Exon 2
R/I locus
SA
PuropA
pEF-rtTET-pA
LOXP
LOX2272

FIG. 3

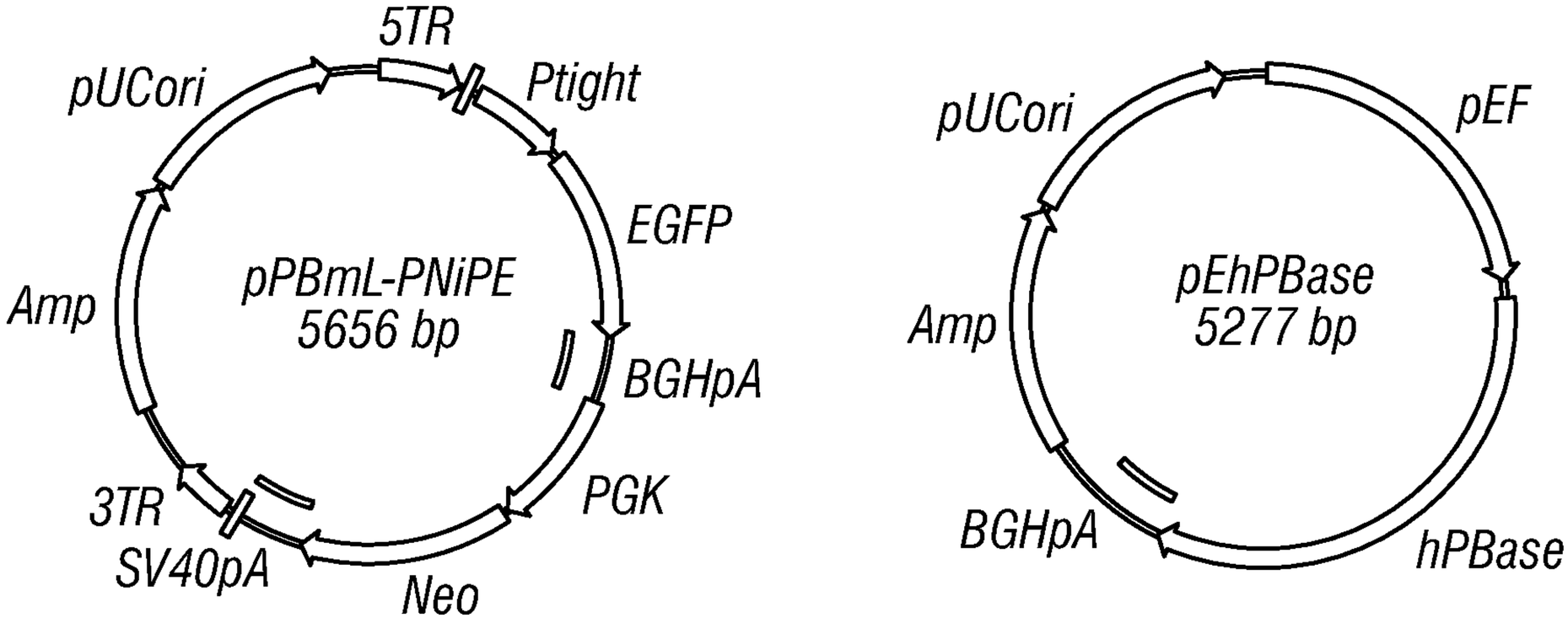

5TR
pUCori
Ptight
EGFP
Amp
pPBmL-PNiPE
5656 bp
BGHpA
PGK
3TR
SV40pA
Neo
pUCori
pEF
Amp
pEhPBase
5277 bp
BGHpA
hPBase

FIG. 4A

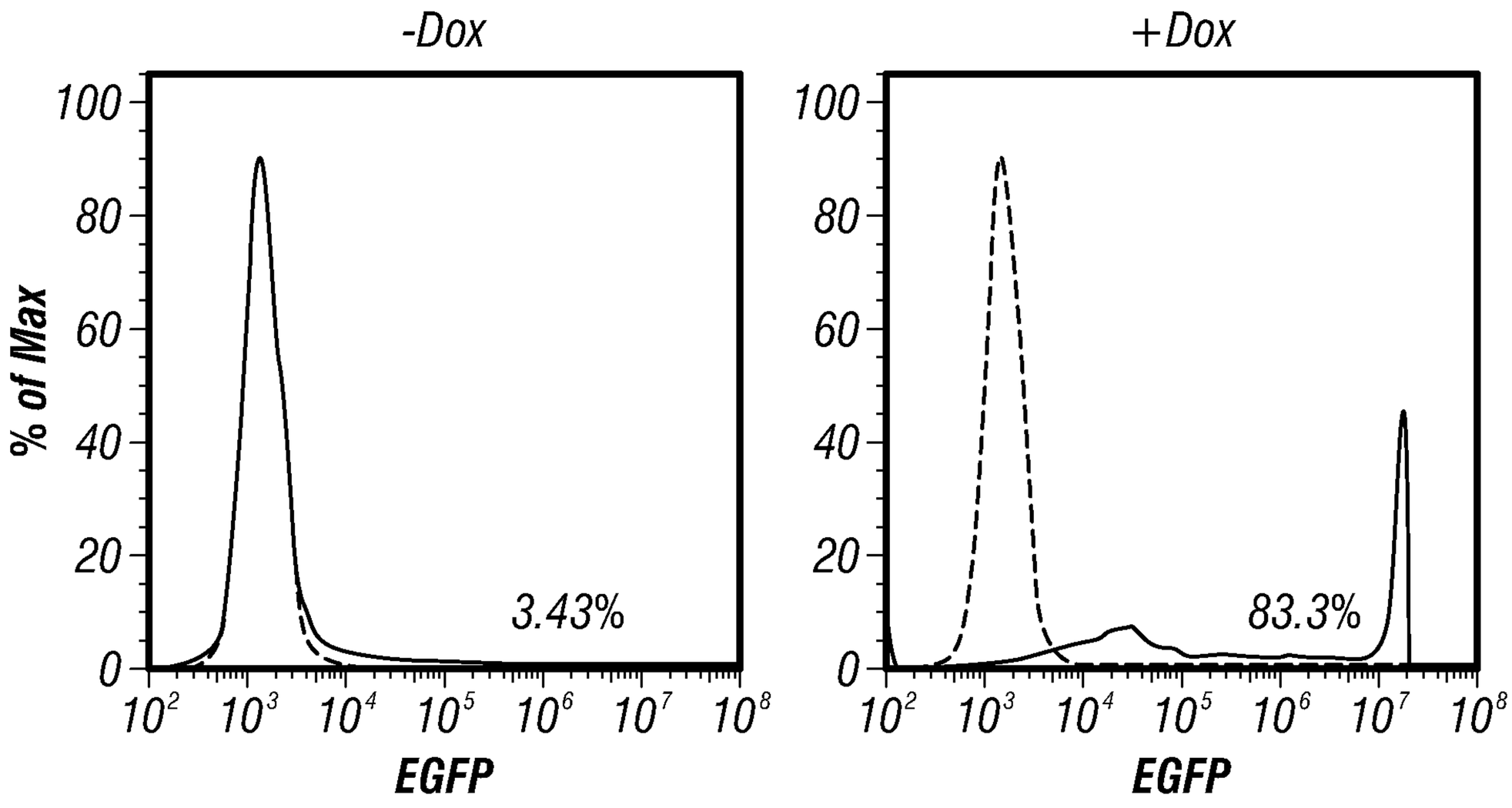

-Dox
+Dox
% of Max
100
80
60
40
20
0
3.43%
83.3%
10^2 10^3 10^4 10^5 10^6 10^7 10^8
EGFP
EGFP

FIG. 4B

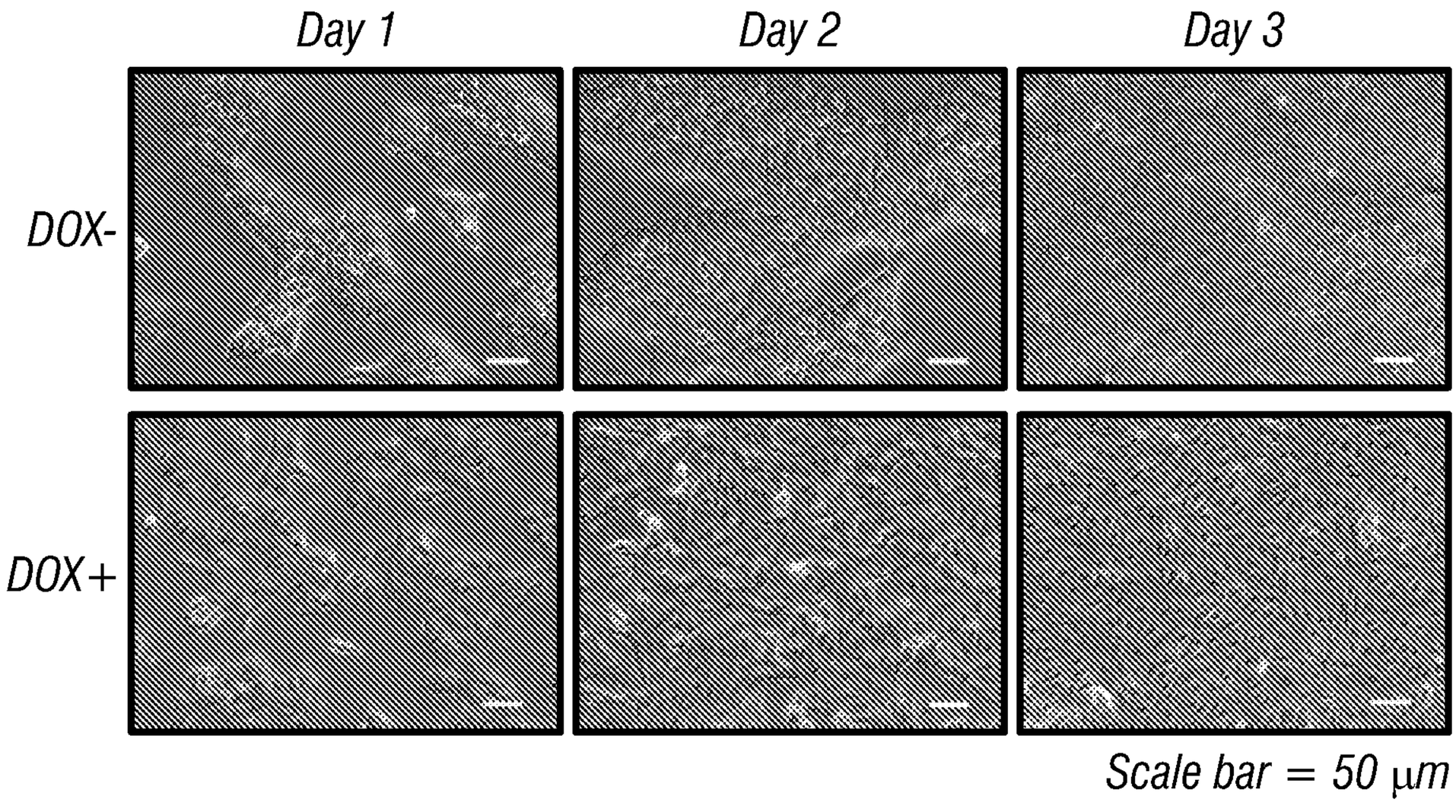

Day 1
Day 2
Day 3
DOX-
DOX+
Scale bar = 50 μm

FIG. 5

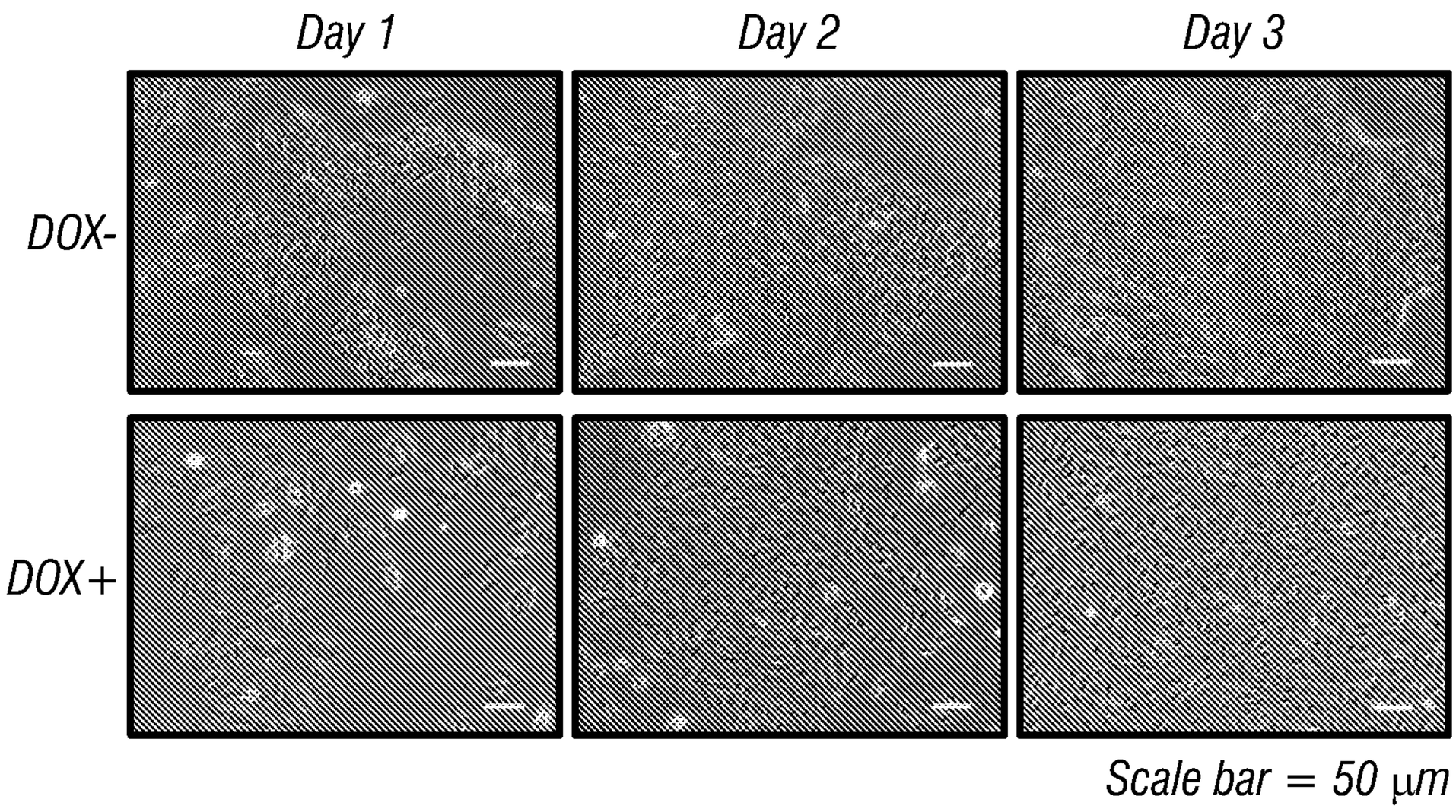

Day 1
Day 2
Day 3
DOX-
DOX+
Scale bar = 50 μm

FIG. 6

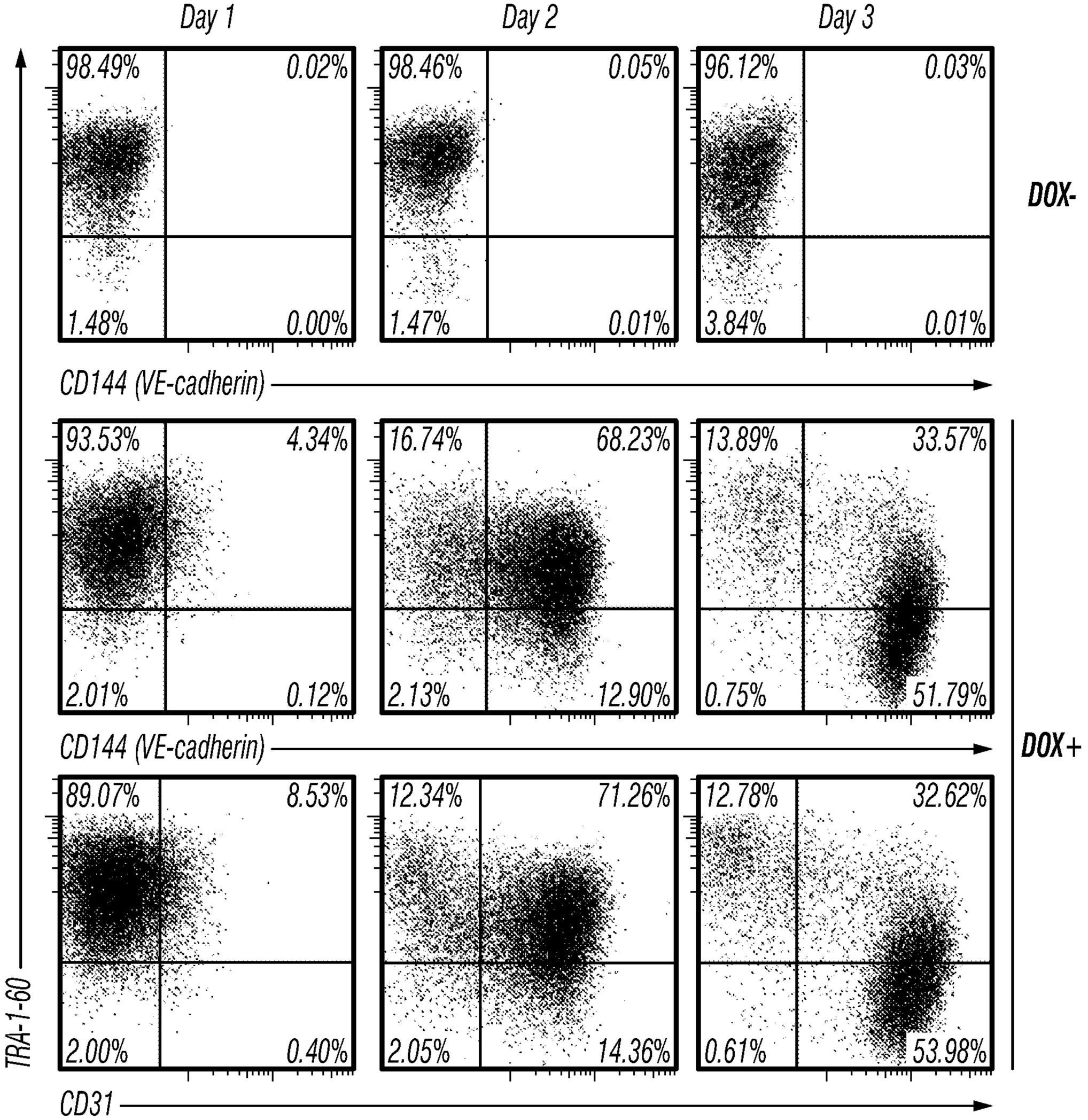

Day 1
Day 2
Day 3
98.49%
0.02%
1.48%
0.00%
98.46%
0.05%
1.47%
0.01%
96.12%
0.03%
3.84%
0.01%
DOX-
CD144 (VE-cadherin)
93.53%
4.34%
2.01%
0.12%
16.74%
68.23%
2.13%
12.90%
13.89%
33.57%
0.75%
51.79%
CD144 (VE-cadherin)
DOX+
89.07%
8.53%
2.00%
0.40%
12.34%
71.26%
2.05%
14.36%
12.78%
32.62%
0.61%
53.98%
TRA-1-60
CD31

FIG. 7

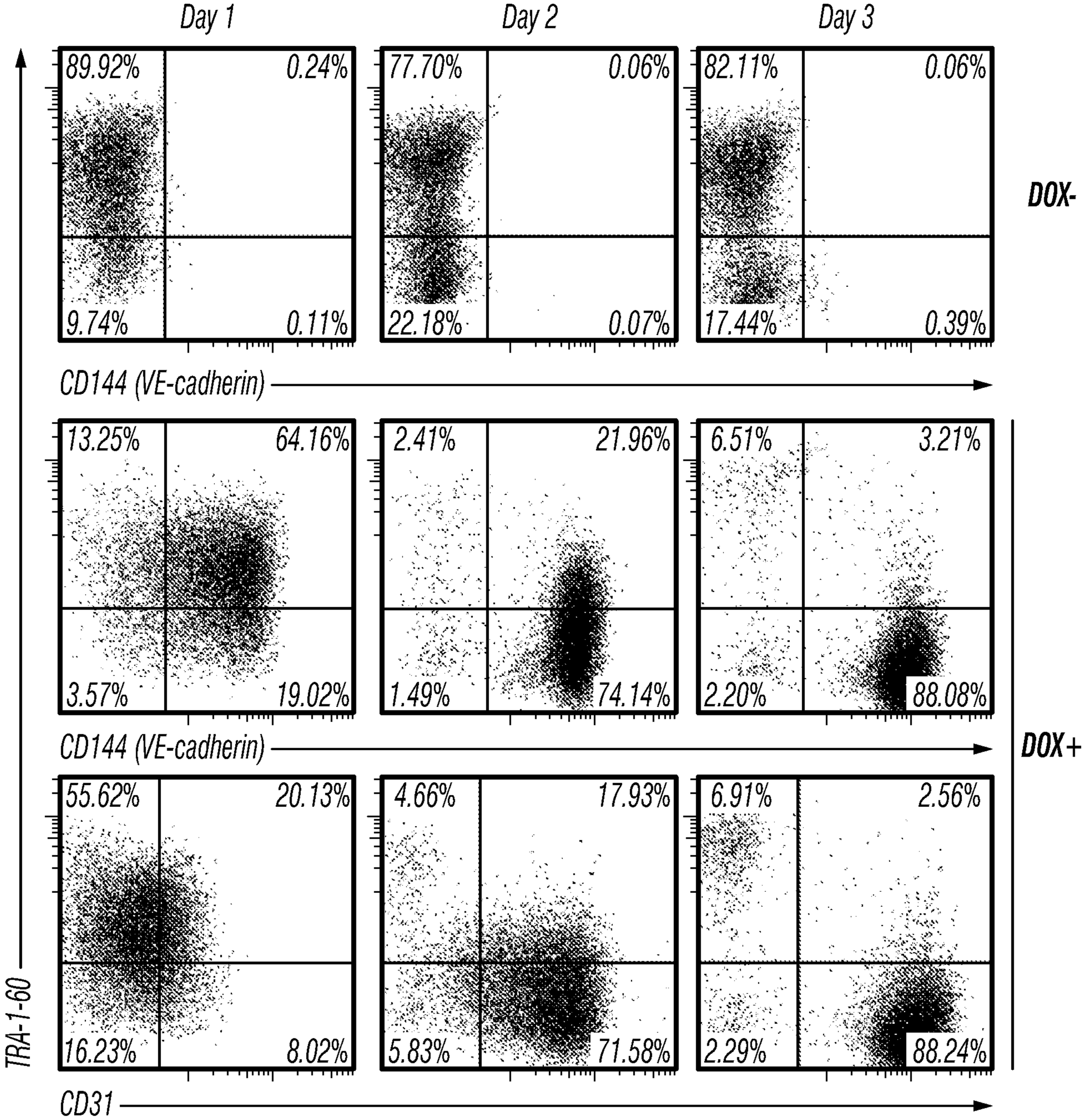

Day 1
Day 2
Day 3
89.92%
0.24%
9.74%
0.11%
77.70%
0.06%
22.18%
0.07%
82.11%
0.06%
17.44%
0.39%
DOX-
CD144 (VE-cadherin)
13.25%
64.16%
3.57%
19.02%
2.41%
21.96%
1.49%
74.14%
6.51%
3.21%
2.20%
88.08%
CD144 (VE-cadherin)
DOX+
55.62%
20.13%
16.23%
8.02%
4.66%
17.93%
5.83%
71.58%
6.91%
2.56%
2.29%
88.24%
TRA-1-60
CD31

FIG. 8

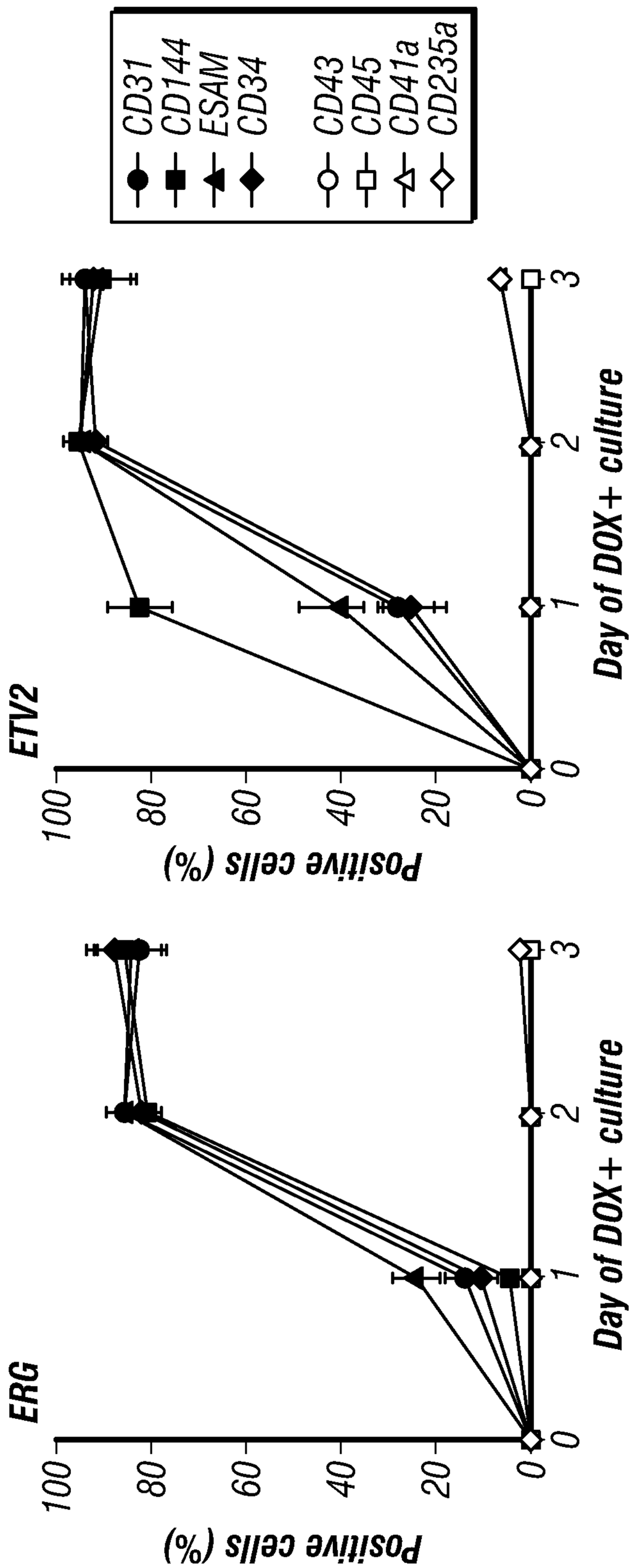

CD31
CD144
ESAM
CD34
CD43
CD45
CD41a
CD235a
ETV2
ERG
Positive cells (%)
100
80
60
40
20
0
0
1
2
3
Day of DOX+ culture

FIG. 9

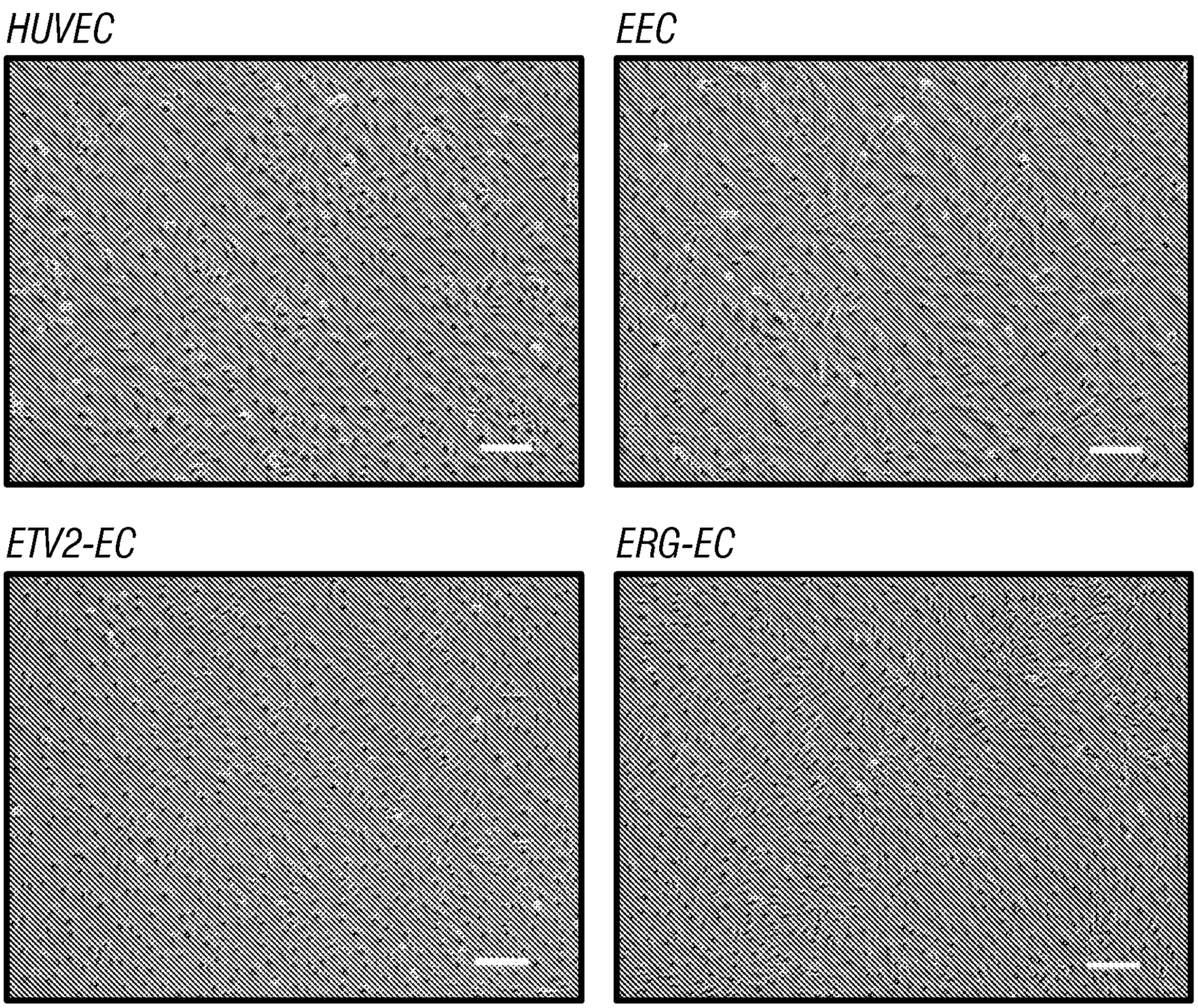


FIG. 10

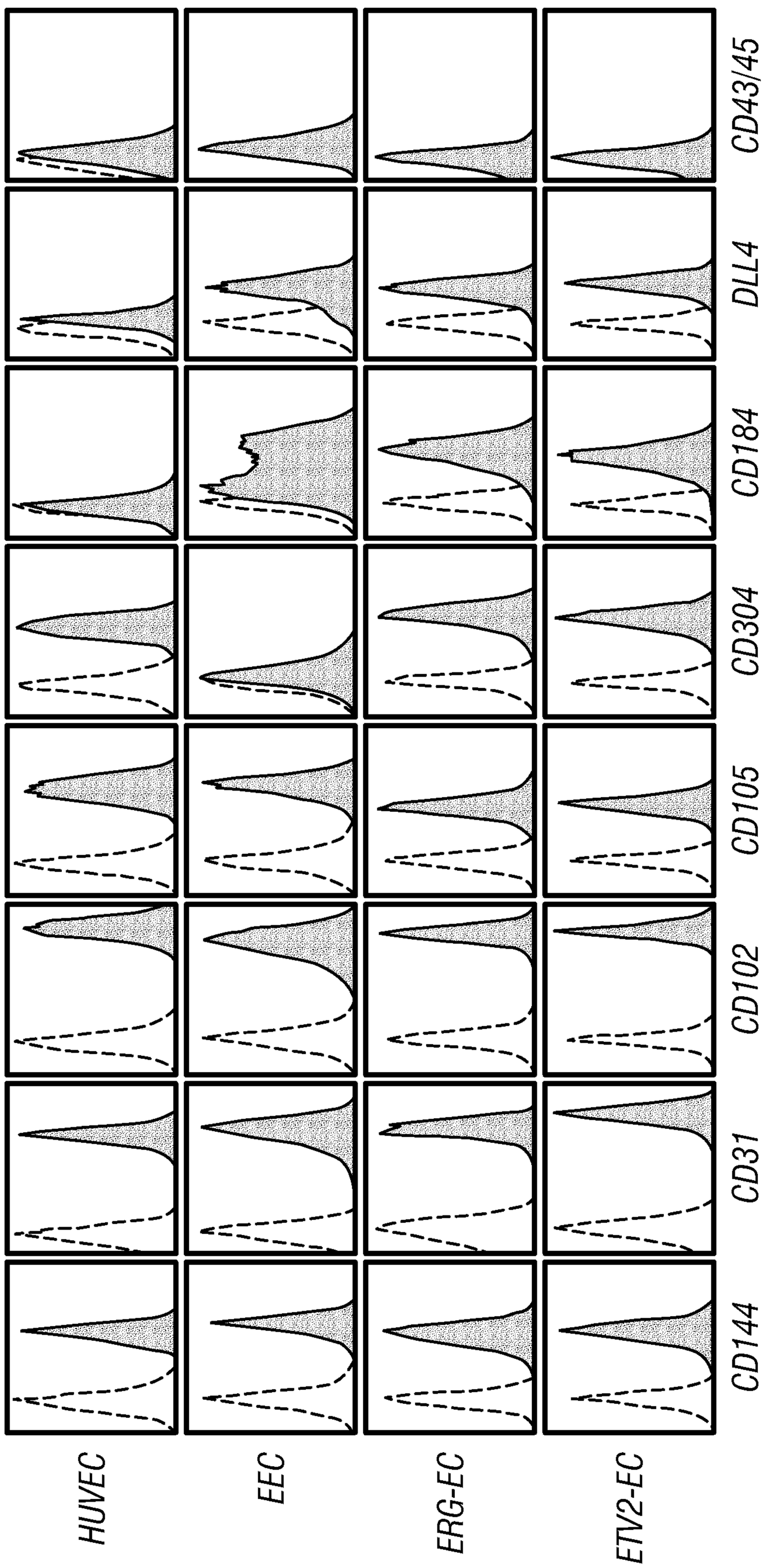

CD43/45
DLL4
CD184
CD304
CD105
CD102
CD31
CD144
HUVEC
EEC
ERG-EC
ETV2-EC

FIG. 11

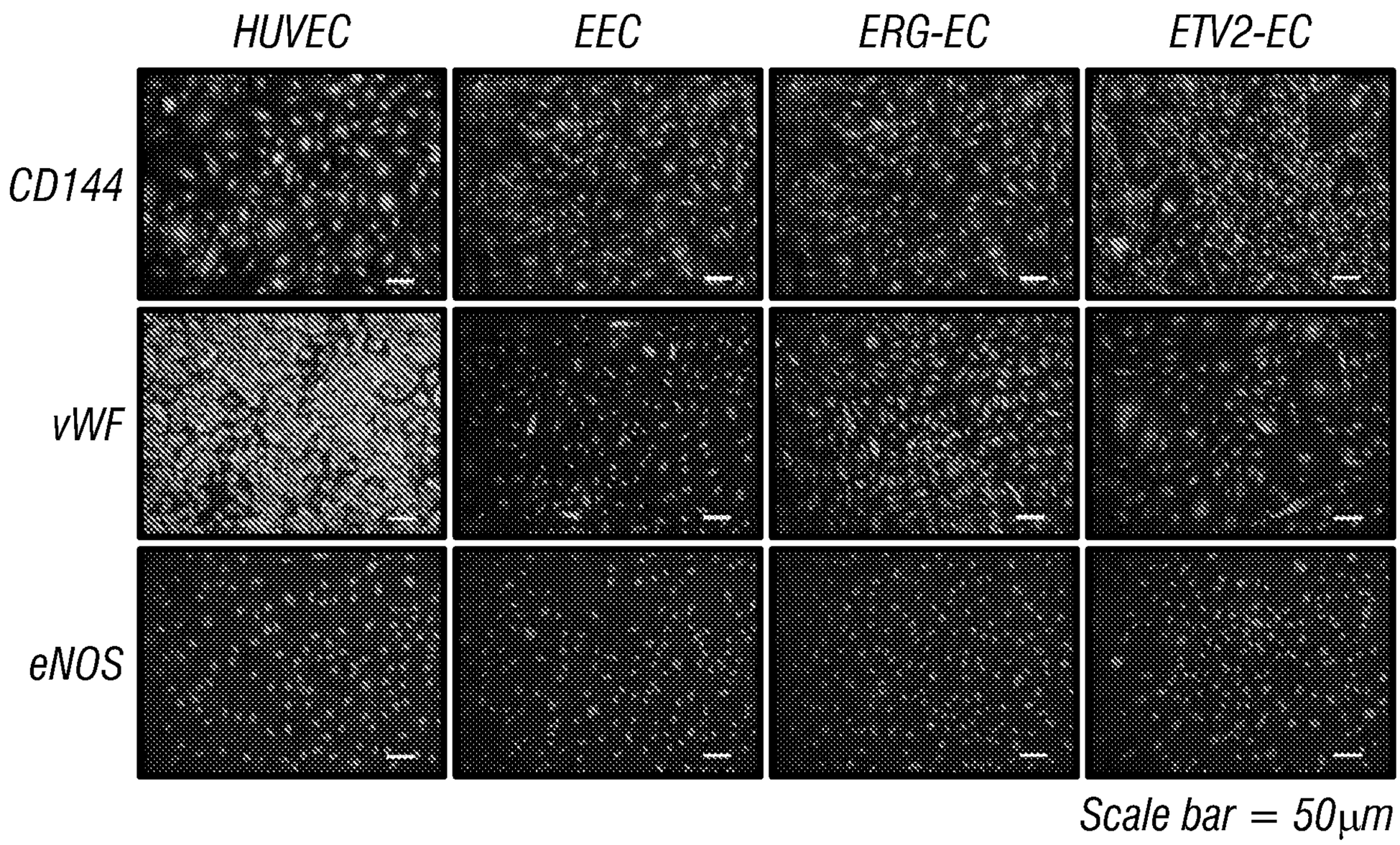


FIG. 12

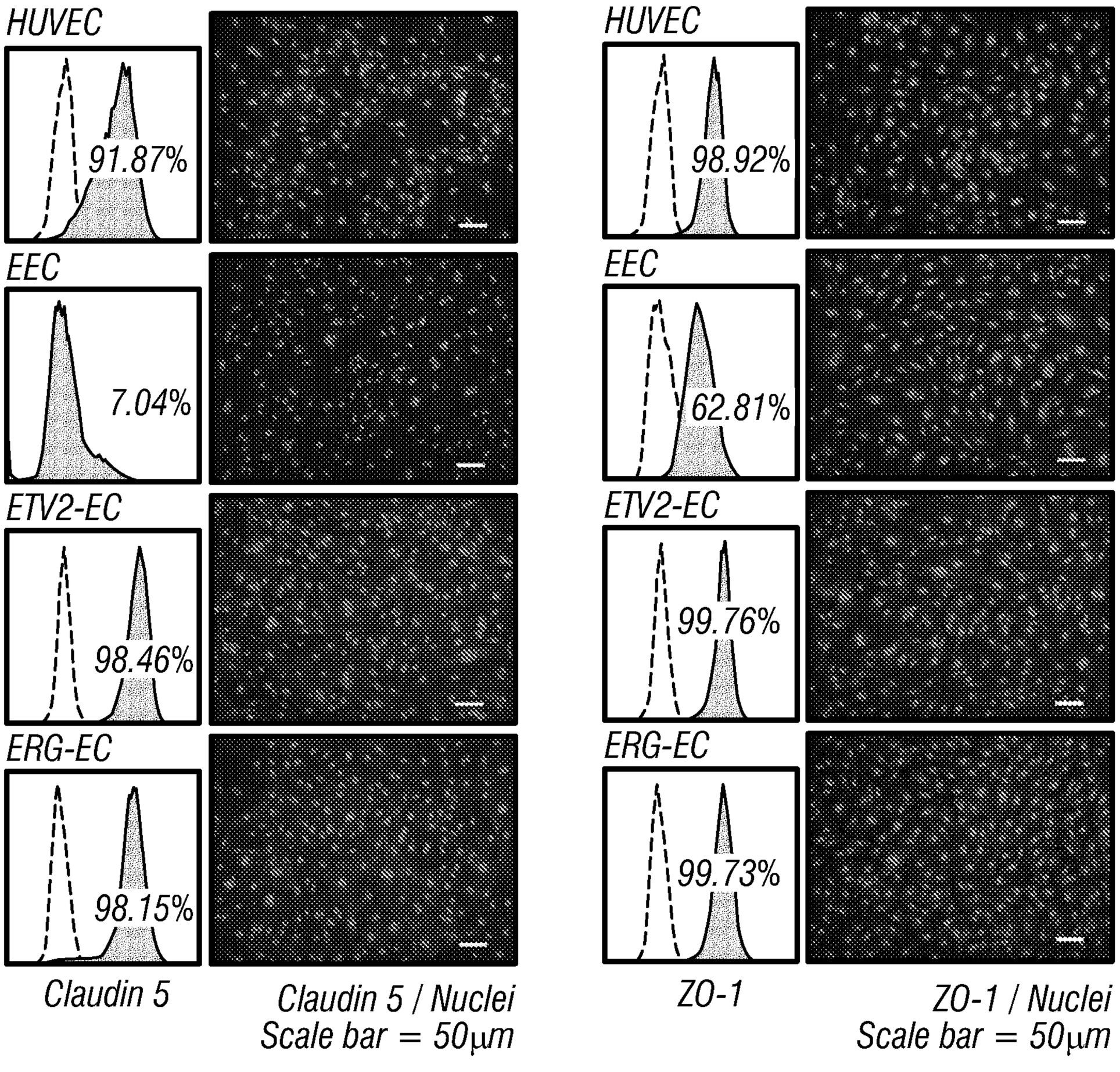


FIG. 13A FIG. 13B

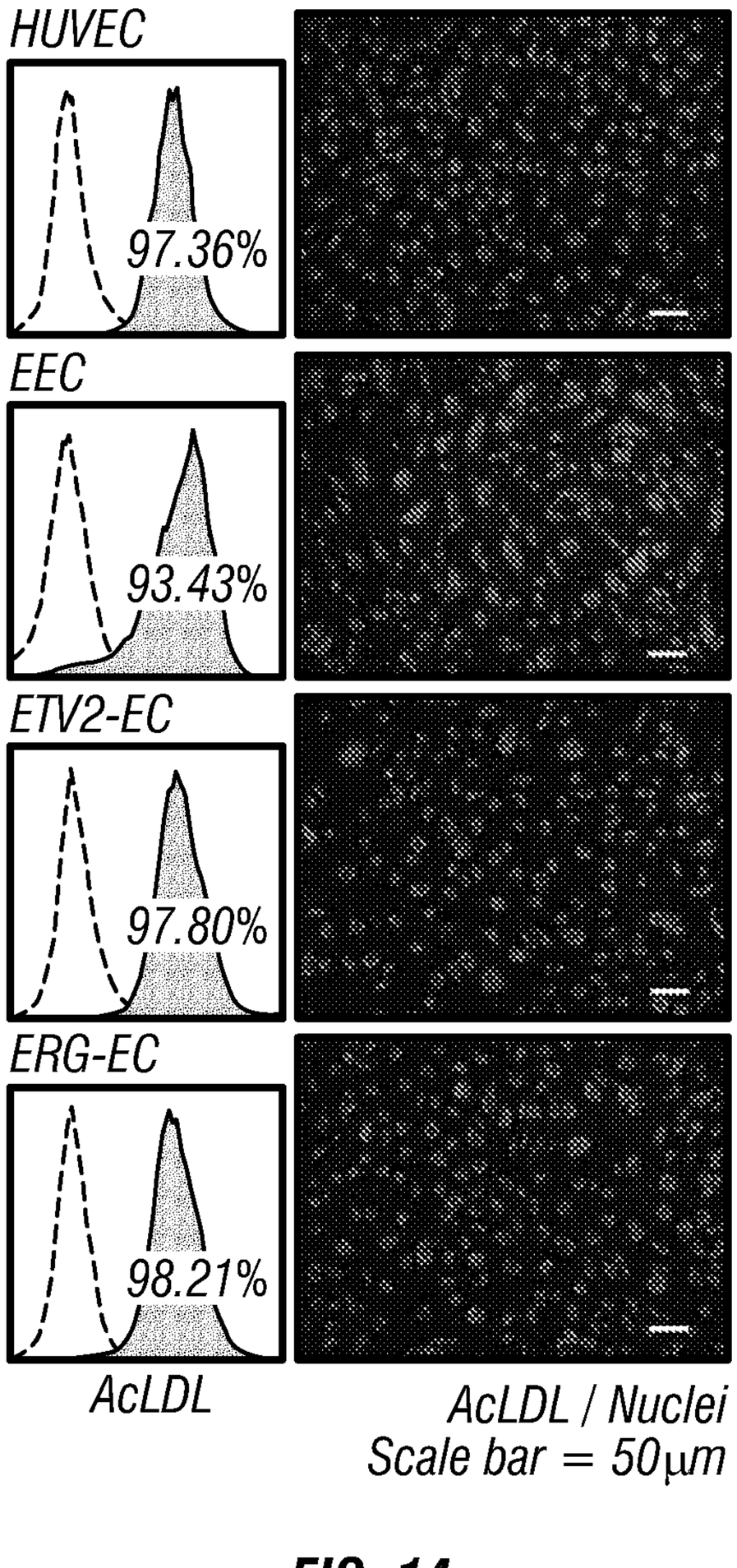

HUVEC
97.36%
EEC
93.43%
ETV2-EC
97.80%
ERG-EC
98.21%
AcLDL
AcLDL / Nuclei
Scale bar = 50μm

FIG. 14

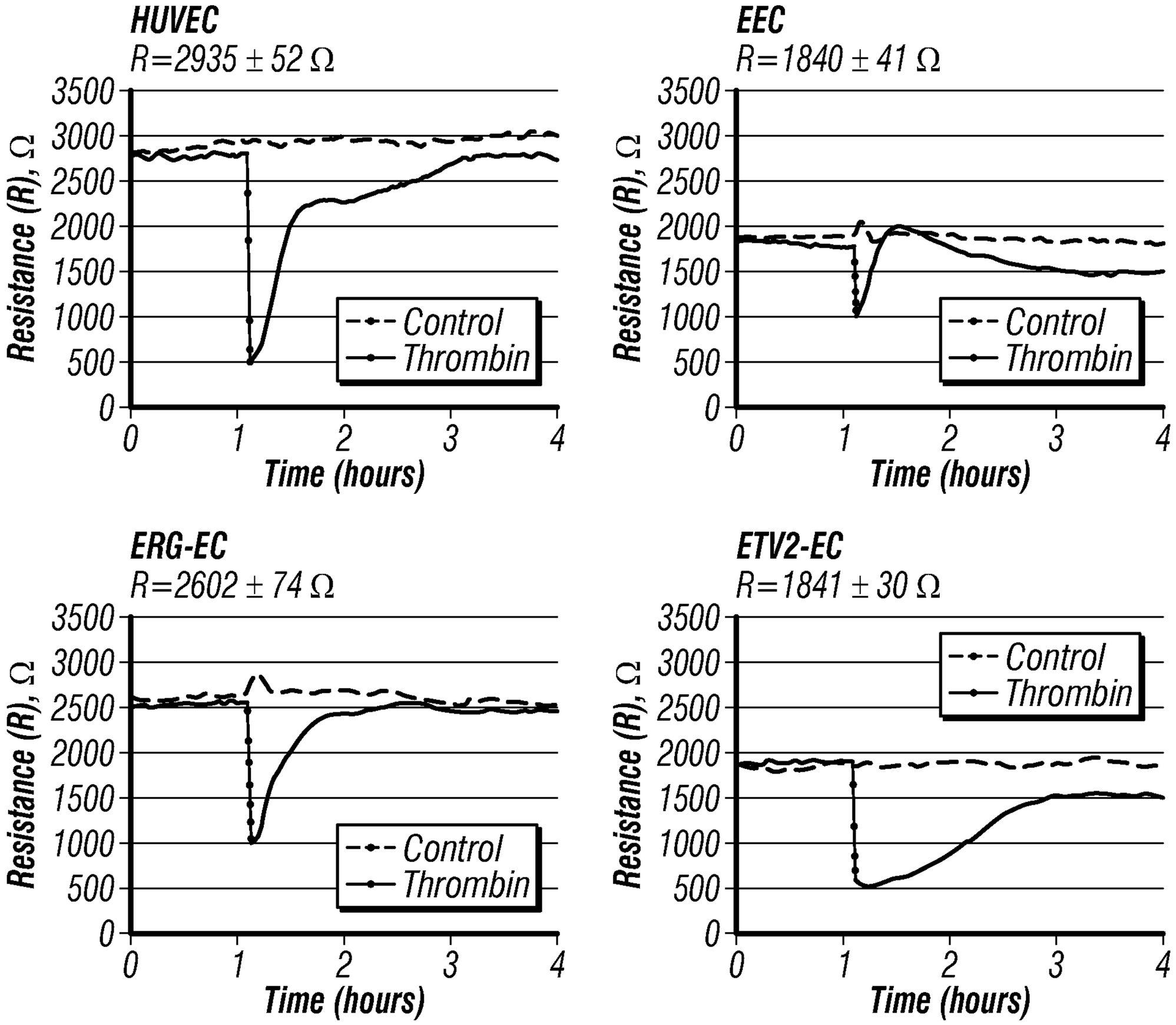

HUVEC
R=2935 ± 52 Ω
EEC
R=1840 ± 41 Ω
ERG-EC
R=2602 ± 74 Ω
ETV2-EC
R=1841 ± 30 Ω
Resistance (R), Ω
3500
3000
2500
2000
1500
1000
500
0
0
1
2
3
4
Time (hours)
Control
Thrombin

FIG. 15

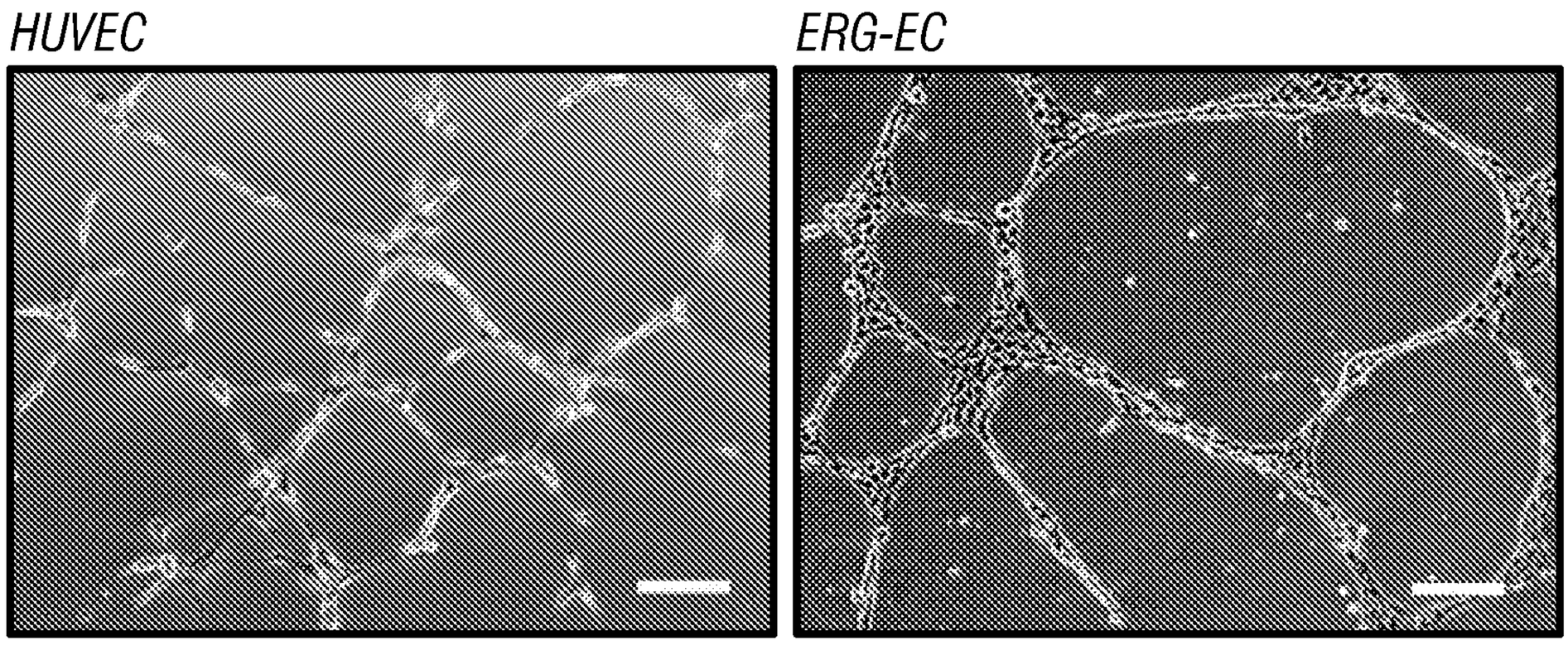

HUVEC
ERG-EC

FIG. 16

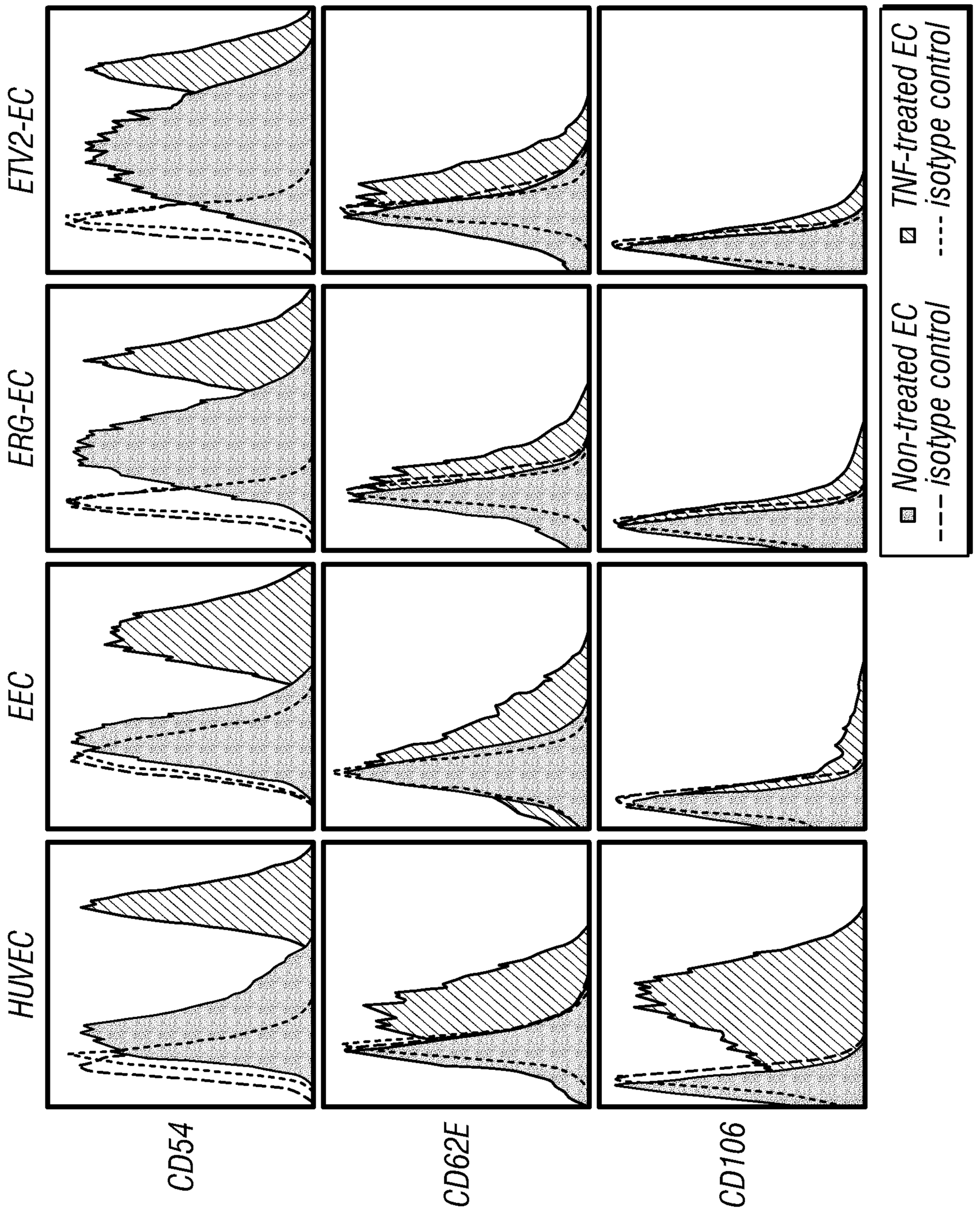

ETV2-EC
ERG-EC
EEC
HUVEC
CD54
CD62E
CD106
Non-treated EC
isotype control
TNF-treated EC
isotype control

FIG. 17

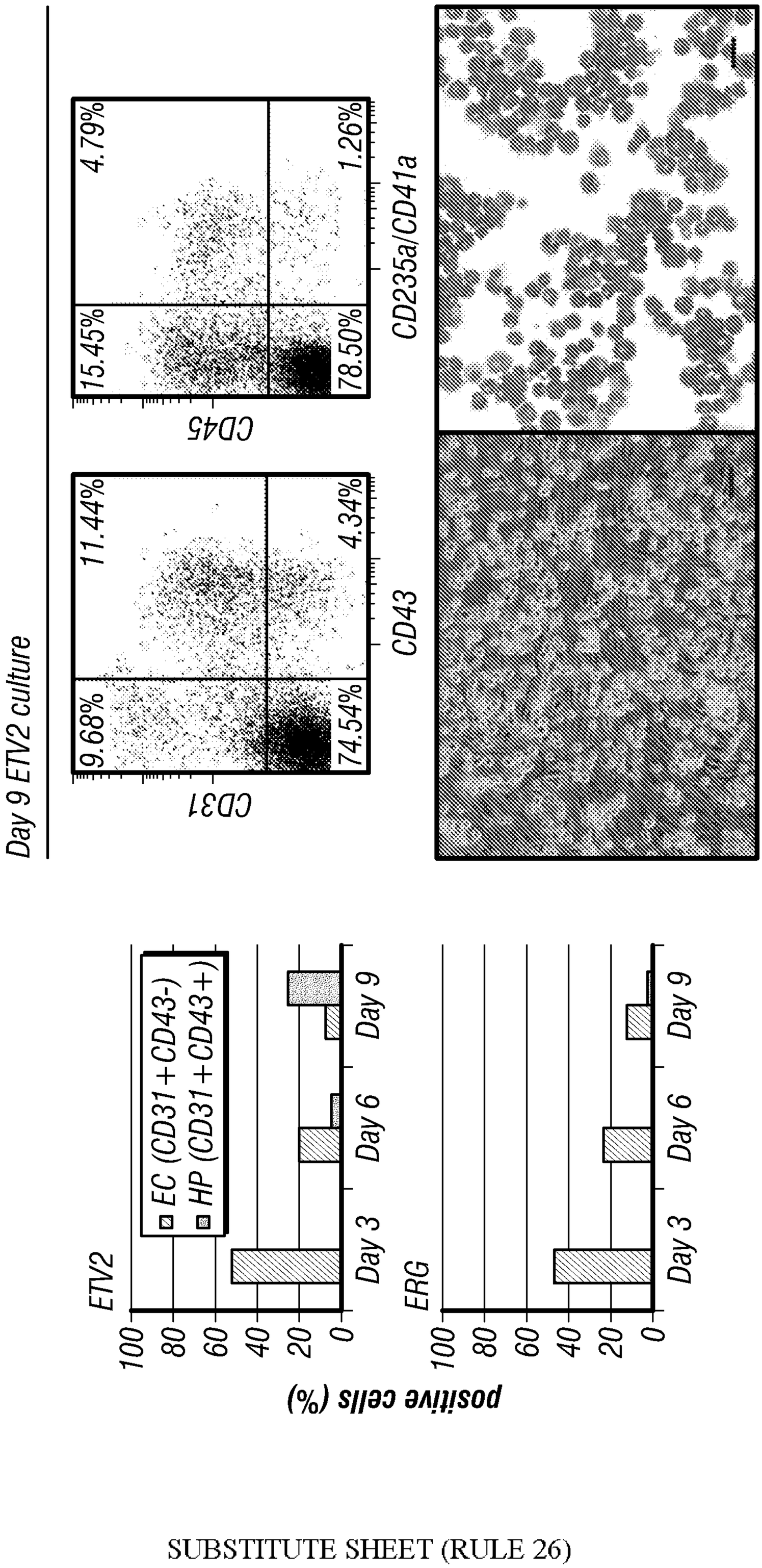

Day 9 ETV2 culture
4.79%
1.26%
15.45%
78.50%
CD45
CD235a/CD41a
11.44%
4.34%
9.68%
74.54%
CD31
CD43
ETV2
ERG
EC (CD31+CD43-)
HP (CD31+CD43+)
Day 3
Day 6
Day 9
100
80
60
40
20
0
positive cells (%)

FIG. 18

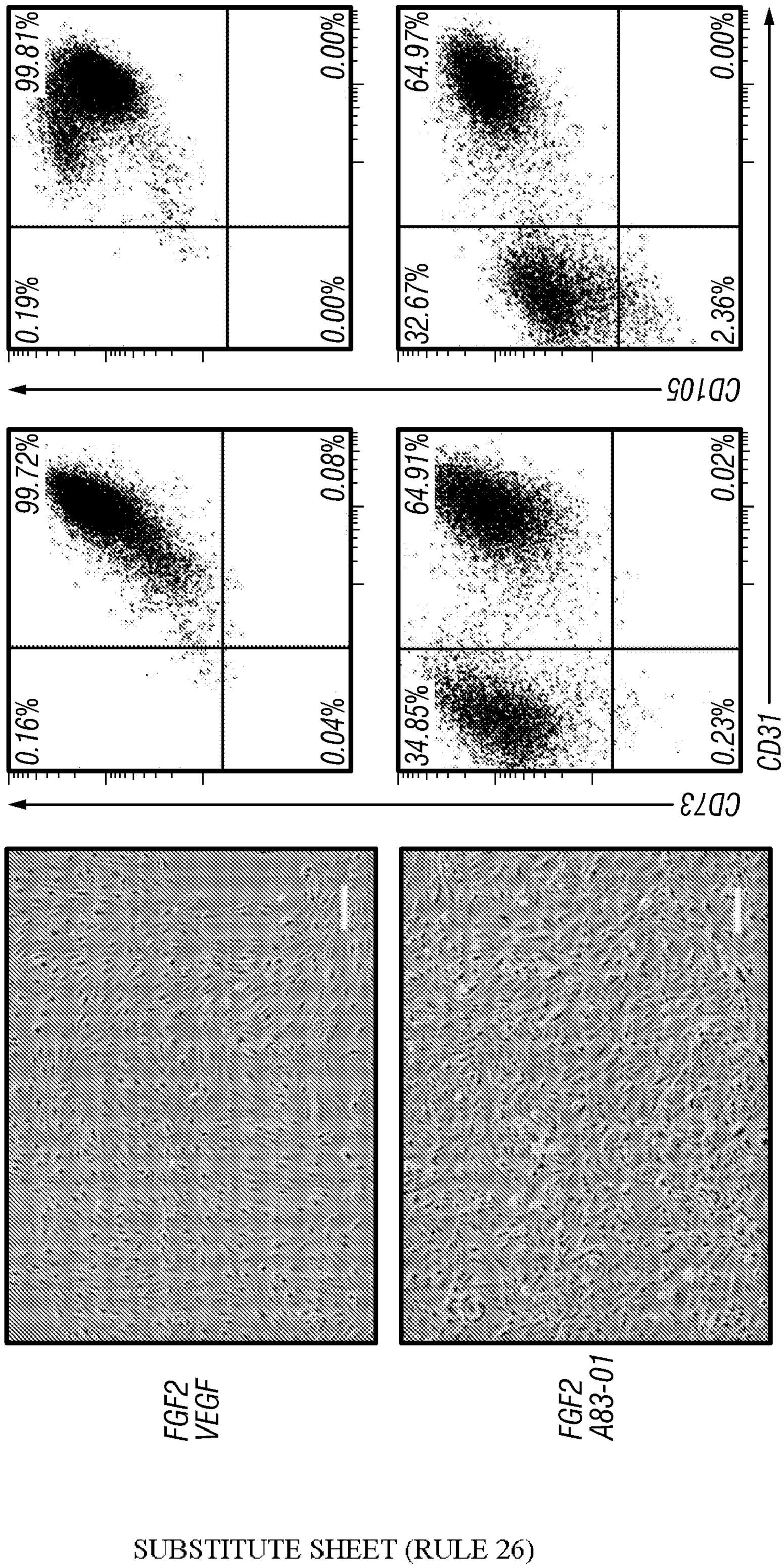
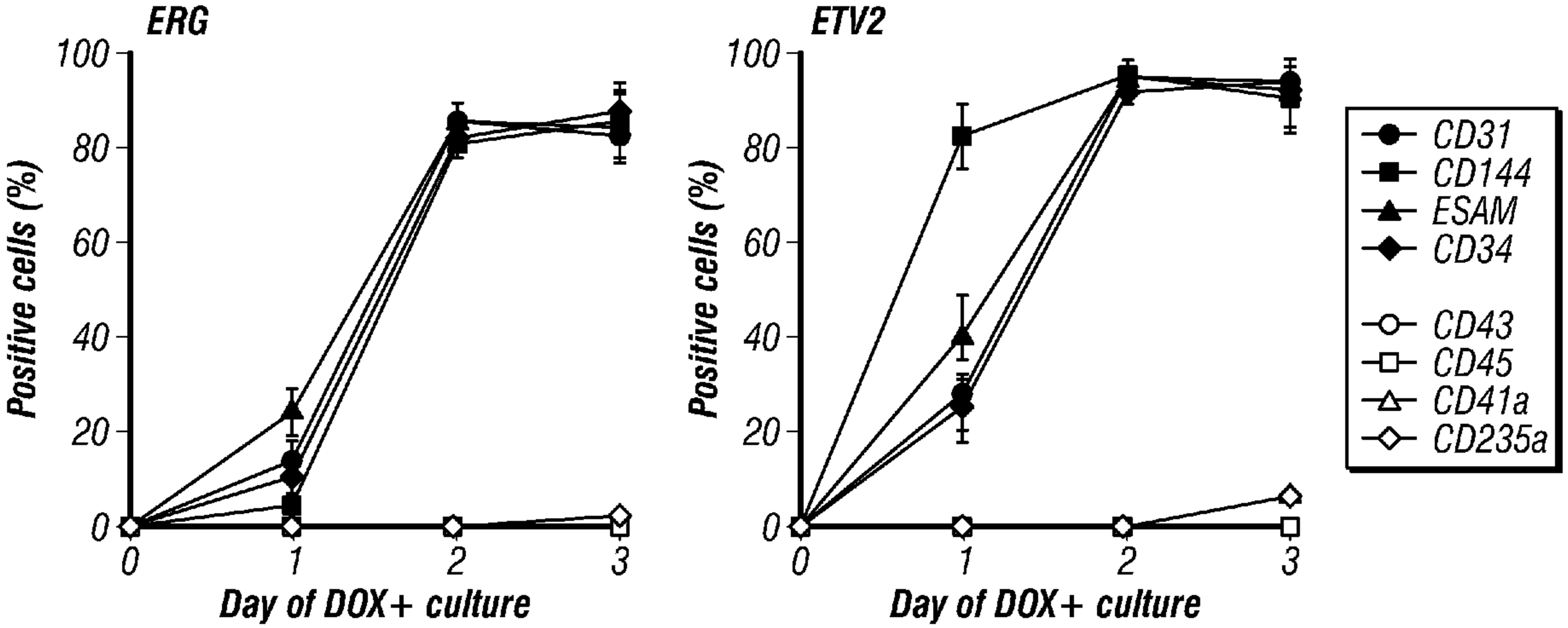

99.81%
0.00%
0.19%
0.00%
64.97%
0.00%
32.67%
2.36%
CD105
99.72%
0.08%
0.16%
0.04%
64.91%
0.02%
34.85%
0.23%
CD73
CD31
FGF2
VEGF
FGF2
A83-01

FIG. 19


ERG
ETV2
Positive cells (%)
100
80
60
40
20
0
0
1
2
3
Day of DOX+ culture
CD31
CD144
ESAM
CD34
CD43
CD45
CD41a
CD235a