

**(12) STANDARD PATENT  
(19) AUSTRALIAN PATENT OFFICE**

**(11) Application No. AU 2009228354 B2**

**(54) Title  
Human cardiovascular progenitor cells**

**(51) International Patent Classification(s)**  
**C12N 5/00** (2006.01)      **C12N 5/077** (2010.01)  
**A01N 65/00** (2006.01)

**(21) Application No:** **2009228354**      **(22) Date of Filing:** **2009.03.25**

**(87) WIPO No:** **WO09/120762**

**(30) Priority Data**

**(31) Number** **61/040,049**      **(32) Date** **2008.03.27**      **(33) Country** **US**

**(43) Publication Date:** **2009.10.01**  
**(44) Accepted Journal Date:** **2015.01.22**

**(71) Applicant(s)**  
**Mount Sinai School of Medicine of New York University**

**(72) Inventor(s)**  
**Keller, Gordon;Kattman, Steven;Yang, Lei**

**(74) Agent / Attorney**  
**Davies Collison Cave, Level 15 1 Nicholson Street, MELBOURNE, VIC, 3000**

**(56) Related Art**  
**US2005/0214939 A1 (GOLD et al.) 29/09/2005**  
**Kattman SJ et al. Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. Dev Cell. 2006 Nov;11(5):723-32.**

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

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
1 October 2009 (01.10.2009)

(10) International Publication Number  
WO 2009/120762 A3

(51) International Patent Classification:  
C12N 5/00 (2006.01) A01N 65/00 (2009.01)

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

(21) International Application Number:  
PCT/US2009/038220

(22) International Filing Date:  
25 March 2009 (25.03.2009)

(25) Filing Language:  
English

(26) Publication Language:  
English

(30) Priority Data:  
61/040,049 27 March 2008 (27.03.2008) US

(71) Applicant (for all designated States except US):  
**MOUNT SINAI SCHOOL OF MEDICINE OF NEW YORK UNIVERSITY [US/US]**; One Gustave L. Levy Place, Box 1675, New York, NY 10029 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **KELLER, Gordon** [CA/CA]; 1 Douglas Crescent, Toronto, Ontario M4W 2E6 (CA). **YANG, Lei** [US/US]; Mt. Sinai School of Medicine, 1425 Madison Avenue, New York, NY 10029 (US). **KATTMAN, Steven** [CA/CA]; 83 Elm Avenue, Apt. 206, Toronto, Ontario M4W 1P1 (CA).

(74) Agent: **MACLEOD, Janet, M.**; Intellectual Property Group, Crowell & Moring LLP, P.O. Box 14300, Washington, DC 20044-4300 (US).

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

Published:

— with international search report (Art. 21(3))

(88) Date of publication of the international search report:  
30 December 2009

(54) Title: HUMAN CARDIOVASCULAR PROGENITOR CELLS

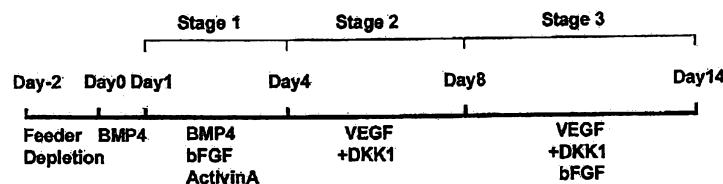


Fig. 1

(57) **Abstract:** The present invention provides populations of human cardiovascular progenitor cells, methods of making such cells, and methods of using the cells for production of populations of cardiovascular colonies and populations of cardiomyocytes. Methods of cardiomyocytes replacement therapy are also provided.

## HUMAN CARDIOVASCULAR PROGENITOR CELLS

### CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U. S. Provisional Application  
5 No. 61/040,049 filed March 27, 2008.

### STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under Grant Nos. R01 HL 71800-01 and P20-GM07501 awarded by the National Institutes of Health. The government may have certain rights in the invention.

### 10 BACKGROUND OF THE INVENTION

During embryonic development, the tissues of the body are formed from three major cell populations: ectoderm, mesoderm and definitive endoderm. These cell populations, also known as primary germ cell layers, are formed through a process known as gastrulation. Following gastrulation, each primary germ cell layer 15 generates a specific set of cell populations and tissues. Mesoderm gives rise to blood cells, endothelial cells, cardiac and skeletal muscle, and adipocytes. Definitive endoderm generates liver, pancreas and lung. Ectoderm gives rise to the nervous system, skin and adrenal tissues. The process of tissue development from these germ cell layers involves multiple differentiation steps, reflecting complex molecular 20 changes. With respect to mesoderm and its derivatives, three distinct stages have been defined. The first is the induction of mesoderm from cells within a structure known as the epiblast. The newly formed mesoderm, also known as nascent mesoderm, migrates to different positions that will be sites of future tissue development in the early embryo. This process, known as patterning, entails some molecular changes that 25 are likely reflective of the initial stages of differentiation towards specific tissues. The final stage, known as specification, involves the generation of distinct tissues from the patterned mesodermal subpopulations.

Evidence suggests that mesoderm is induced in successive waves which represent subpopulations with distinct developmental potential. The mesoderm 30 that is formed first migrates to the extraembryonic region and gives rise to hematopoietic and endothelial cells, whereas the next population migrates anteriorly in the developing embryo and contributes to the heart and cranial mesenchyme. These

lineage relationships were defined initially through histological analysis and have been largely confirmed by cell tracing studies. With respect to hematopoietic commitment, there is now compelling evidence from studies with the ES cell differentiation model and on the mouse embryo that the earliest identifiable 5 progenitor is a cell that also displays vascular potential, a cell that is known as the hemangioblast (Choi et al. (1998); Development 125:725-732; Huber et al. (2004) Nature 432:625-30). Analysis of this progenitor revealed that it co-expresses the mesoderm gene brachyury and the receptor tyrosine kinase Flk-1, indicating that it represents a subpopulation of mesoderm undergoing commitment to the 10 hematopoietic and vascular lineages (Fehling et al. (2003) Development 130:4217-4227). Lineage-tracing studies have demonstrated that the heart develops from a Flk-1<sup>+</sup> population, suggesting that a comparable multipotential cell may exist for the cardiovascular system (Ema et al. (2006) Blood 107:111-117). Analyses of ES cell differentiation cultures have provided evidence for the existence of a Flk-1<sup>+</sup> 15 progenitor with cardiac and endothelial potential (Yamashita et al. (2005) FASEB 19:1534-1536). Recent studies also support the existence of murine cardiovascular progenitors that may give rise to multiple cardiovascular lineages. (Kattman et al. (2006) Dev. Cell 11:723-732; Moretti et al. (2006) Cell 127:1151-1165; Wu et al. (2006) Cell 127:1137-1150). A human cardiovascular progenitor population has not 20 heretofore been identified.

## SUMMARY OF THE INVENTION

The present invention provides enriched populations of human cardiovascular progenitor cells. The human cardiovascular progenitor cells are characterized by the presence of the cell surface marker KDR and the absence of the 25 cell surface marker C-KIT. The human cardiovascular progenitor cells are capable of differentiating into cardiomyocytes, endothelial cells and vascular smooth muscle cells *in vitro* and *in vivo*.

In another embodiment, the present invention provides methods of producing human cardiovascular progenitor cells from human embryoid bodies (EBs) 30 comprising culturing the EBs in the presence of an activin and a bone morphogenic factor (BMP) and optionally basic Fibroblast Growth Factor (bFGF), and subsequently culturing in the presence of an inhibitor of the Wnt growth factor and optionally vascular endothelial growth factor (VEGF) to provide human

cardiovascular progenitor cells, and harvesting the cells from the cell culture.

In another embodiment, the present invention provides a method of generating a population of cells containing at least about 30% and preferably at least about 40%, and more preferably at least about 50% human cardiomyocytes

5 comprising culturing the human cardiovascular progenitor cells in the presence of an inhibitor of Wnt and optionally VEGF. In one embodiment, the cells are cultured as monolayers. In another embodiment, the cells are cultured as aggregates. The presence of cardiomyocytes may be determined by assessing contractile activity of cells or by measuring expression of genes indicative of cardiomyocytes, such as for example cardiac troponin T (CTNT).

10 The present invention further provides a method of generating human cardiovascular colonies containing cardiomyocytes, endothelial cells and vascular smooth muscle cells comprising culturing the human cardiovascular progenitor cells in the presence of VEGF, bFGF, and an inhibitor of Wnt. In a preferred embodiment, 15 the cells are cultured in methylcellulose.

The human cardiovascular precursor cells and the differentiated cells derived therefrom are useful in methods of screening for agents that affect cardiovascular progenitors, cardiomyocytes, endothelial cells, vascular smooth muscle cells, and cardiovascular tissue.

20 The human cardiovascular precursor cells are also useful for expansion and storage and as a source of cardiomyocytes, endothelial cells and vascular smooth muscle cells.

25 The human cardiovascular precursor cells and the differentiated cells derived therefrom are also useful in methods of improving or reconstituting cardiac function in a mammal, and for engineering cardiovascular tissue *in vitro* for transplantation.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is an outline of a protocol used to direct differentiation of human embryonic stem cells (hESC) to the cardiac lineage in accordance with one 30 embodiment of the present invention.

Fig. 2 shows the kinetics of CTNT<sup>+</sup> cell development in EBs induced with a combination of BMP4, bFGF and activin A. EBs were harvested at the indicated time points and analyzed by intracellular flow cytometry for the frequency

of cells expressing CTNT.

Fig. 3 shows the frequency of CTNT<sup>+</sup> cells in day 14 EBs following manipulation of the Wnt signaling pathway at different stages.

5 Fig. 4 shows the frequency of CTNT<sup>+</sup> cells in day 14 EBs following culture in combinations of BMP4, activin A and bFGF during the induction stage.

Fig. 5 shows the total number of CTNT<sup>+</sup> cells per well of 24 well plates for day 14 EBs treated with the indicated combinations of VEGF and DKK1. For Figs. 1-5, bars represent the standard error or the means of three independent experiments. Statistical analysis was performed with the unpaired t-test. \*P = 0.07, 10 \*\*P<0.01, \*\*\*P<0.001.

Fig. 6 shows gene expression analysis of EBs at different stages of development.

Fig. 7 shows flow cytometric analysis of different aged EBs.

15 Fig. 8 shows quantitative RT-PCR gene analysis of the three populations isolated from day 6 EBs.

Fig. 9 shows cardiac potential of the three day 6 populations.

Fig. 10 shows the cardiac potential of the day 6 KDR<sup>low</sup>/C-KIT<sup>neg</sup> population.

20 Fig. 11 shows quantitative RT-PCR expression analysis of adherent populations generated from the day 6 EB-derived KDR<sup>low</sup>/C-KIT<sup>neg</sup> fraction.

Fig. 12 shows an analysis for the presence of CTNT<sup>+</sup>, CD31<sup>+</sup> and SMA<sup>+</sup> cells following 10-12 days of culture of the KDR<sup>low</sup>/C-KIT<sup>neg</sup> population.

Fig. 13 depicts an expression analysis of 4-day-old cardiovascular colonies isolated from mixed RFP/GFP cultures.

25 Fig. 14 depicts a cell dose response showing the relationship between the number of KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived cells plated and the number of cardiovascular colonies that develop.

Fig. 15 depicts exemplar traces showing whole-cell voltage clamp recordings of transient outward K<sup>+</sup> current (I<sub>to</sub>) natively expressed in KDR<sup>low</sup>/C-30 KIT<sup>neg</sup>- derived cardiomyocytes.

Fig. 16 shows mean current density-voltage relationship for cells as in Fig. 15. From a batch of 10 cells, 8 showed the I<sub>to</sub> current and the mean +/- SEM current densities were plotted using traces from these 8 cells.

Fig. 17 shows mean time-to-peak current (solid squares) and inactivation  $\tau$  (open squares) for cells as in Fig. 16 (n = 8).

Fig. 18 shows electrical activity for KDR<sup>low</sup>/C-KIT<sup>neg</sup> cells isolated from day 6 EBs that were cultured in the MEA (Multi ChannelSystems) dish for 2-5 weeks. Extracellular electrical activity was recorded with or without 1  $\mu$ M quinidine (Sigma).

Fig. 19 is a model depicting development of the cardiovascular progenitors in hESC cultures.

Fig. 20 shows images of undifferentiated hES2 cells, day 4 EBs and 10 day 14 EBs with contracting cardiomyocytes (magnification x 200).

Fig. 21 shows the kinetics of the development of CTNT<sup>+</sup> cells in EBs.

Fig. 22 shows the kinetics of the development of contracting EBs induced with the protocol in Fig. 1. Between 150-200 EBs were scored at each 15 time point for the presence of contracting EBs. Bars represent standard error of the mean of three independent experiments.

Fig. 23 shows the total number of cells per well of 24 well plates in day 14 EBs following induction with the combinations of factors indicated below the graph.

20 Fig. 24 shows the total number of CTNT<sup>+</sup> cells per well of 24 well plates in day 14 EBs following induction with the combinations of factors indicated below the graph.

Fig. 25 shows the frequency of CTNT<sup>+</sup> cells from day 14 EBs treated with the indicated combinations of VEGF and DKK.

25 Fig. 26 shows the total number of CTNT<sup>+</sup> cells per well of 24 well plates for day 14 EBs treated with the indicated combinations of VEGF and DKK. For Figs. 23-26, bars represent standard error of the mean of three independent experiments. Statistical analysis was performed with unpaired t-test. \*P<0.05, \*\*P<0.01

30 Fig. 27 shows quantitative RT-PCR analysis of three populations isolated from day 6 EBs. Average expression normalized to cyclophilin is shown. Bars represent standard error of the mean of three independent experiments.

Fig. 28 is a graph of relative cell numbers after 10-14 days in

monolayer culture of KDR<sup>low</sup>/C-KIT<sup>neg</sup> populations isolated from day 6 EBs. 40,000 cells per well of 96 well plates were induced with the combinations of factors shown below the graph. Bars represent standard error of the mean of three independent experiments.

5 Fig. 29 shows flow cytometric analysis demonstrating the emergence of the three different KDR/C-KIT populations in EBs generated from H1 hESC.

Fig. 30 shows the development of CTNT+ cells from the H1 hESC-derived KDR<sup>low</sup>/C-KIT<sup>neg</sup> fraction following 7 days of culture.

10 Fig. 31 shows quantitative RT-PCR expression analysis of KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived adherent populations cultured in the presence of VEGF (10 ng/mL), DKK1 (150 ng/mL) and bFGF (10 ng/mL). Cultures were harvested at the specified times and analyzed for the expression of the indicated genes. Average expression normalized to cyclophilin is shown.

15 Fig. 32 shows expression analysis of individual 4-day-old cardiovascular colonies generated from the hES2-derived KDR<sup>low</sup>/C-KIT<sup>neg</sup> population.

## DETAILED DESCRIPTION OF THE INVENTION

20 The present invention provides populations of human cardiovascular progenitor cells. The human cardiovascular progenitor cells of the present invention are capable of differentiation, under appropriate conditions, into cardiomyocytes, endothelial cells, and vascular smooth muscle cells. The human cardiovascular progenitor cells are characterized by the expression of the tyrosine kinase receptor KDR and by the lack of expression of the tyrosine kinase receptor C-KIT.

25 In one embodiment of the invention, the human cardiovascular progenitor cells are obtained from embryoid bodies (EBs). EBs are three dimensional colonies that contain developing populations from a broad spectrum of lineages. Conditions for formation of EBs from embryonic stem cells (ESC) are known in the art. For example, human ESC maintained on embryonic feeder cells may be depleted of feeders, for example by culturing on a layer of basement membrane matrix such as Matrigel<sup>TM</sup> (BD Biosciences, Bedford, MA), dissociated to form small clusters and plated in serum free media in the presence of a BMP. In one preferred embodiment of the invention, feeder-depleted human ESC are cultured in serum free media in the

presence of BMP4 for about one day to form EBs. In another preferred embodiment, the concentration of BMP4 is from about 0.1 ng/mL to about 1.0 ng/mL, and more preferably about 0.5 ng/mL.

Human ESC may be obtained commercially or by methods known in the art. For example, human ESC may be prepared from blastocysts by methods known in the art and disclosed for example in U.S. Patent No. 5,843,780 to Thomson et al. and by Reubinoff et al. (2000) *Nature Biotech* 18:399.

EBs may also be formed from induced pluripotent stem (iPS) cells by methods known in the art and disclosed, for example, by Takahashi et al. (2007) *Cell* 131:861 and Mali et al. (2008) *Stem Cells* 26:1998.

The human cardiovascular precursor cells of the present invention may be obtained by culturing human EBs in serum free media in the presence of an activin and a BMP, subsequently culturing in the presence of an inhibitor of Wnt to provide a population of cardiovascular progenitor cells, and optionally harvesting the cells from culture and further optionally enriching the harvested cell population for cardiovascular progenitor cells. In preferred embodiments the activin is activin A, the BMP is BMP4, and the inhibitor of Wnt is Dickkopf-1 (DKK-1).

Another embodiment of the present invention provides a method for producing human cardiovascular precursor cells comprising culturing human EBs in serum free media in the presence of activin A and BMP4 and optionally bFGF for about one to about four days, and subsequently culturing the cells in serum free media in the presence of DKK-1 and optionally VEGF for about one to two days, harvesting the population of cells, and optionally enriching the population for human cardiovascular progenitor cells.

In a preferred embodiment the EBs are cultured in the presence of activin A and BMP4 and optionally bFGF for about four days. The concentrations of activin A, BMP4 and optionally bFGF are sufficient to induce a primitive streak-like population of cells characterized by the upregulation of expression of *T* and *WNT3A*. In the subsequent step, the concentrations of DKK-1 and optionally VEGF are sufficient to provide a population of cardiovascular progenitor cells.

In a preferred embodiment, the concentration of activin A is from about 0.5 ng/mL to about 30 ng/mL, and more preferably from about 1.0 ng/mL to about 5.0 ng/mL. In a most preferred embodiment the concentration of activin A is

about 3.0 ng/mL.

In a preferred embodiment, the concentration of BMP4 is from about 1.0 ng/mL to about 20.0 ng/mL, and more preferably from about 5.0 ng/mL to about 15 ng/mL. In a most preferred embodiment, the concentration of BMP4 is about 10 5 ng/mL.

In a preferred embodiment, the concentration of bFGF is from about 1.0 ng/mL to about 10.0 ng/mL, and more preferably from about 3.0 ng/mL to about 6.0 ng/mL. In a most preferred embodiment, the concentration of bFGF is about 5.0 ng/mL.

10 In a preferred embodiment, the concentration of DKK1 is from about 100 ng/mL to about 200 ng/mL, and more preferably from about 125 ng/mL to about 175 ng/mL. In a most preferred embodiment, the concentration of DKK1 is about 150 ng/mL.

15 In another preferred embodiment, the concentration of VEGF is from about 1.0 ng/mL to about 50.0 ng/mL, and more preferably from about 1.0 ng/mL to about 25.0 ng/mL. In a most preferred embodiment, the concentration of VEGF is about 10.0 ng/mL.

20 In another preferred embodiment of the present invention, human EBs are cultured in serum free media for about four days in the presence of about 3.0 ng/mL of activin A, about 10.0 ng/mL of BMP4 and about 5.0 ng/mL of bFGF, at which time about 150 ng/mL of DKK1 and about 10.0 ng/mL of VEGF are added and cells are cultured for about one to two days to produce cardiovascular progenitor cells. In another preferred embodiment, the population is enriched for cardiovascular 25 progenitor cells, for example by sorting for cells that express KDR and do not express C-KIT. In a preferred embodiment, the sorting is by immunoselection, for example by flow cytometry.

30 In another embodiment, the present invention provides a method of generating a population of human cardiomyocytes comprising culturing the human cardiovascular progenitor cells in the presence of an inhibitor of Wnt and optionally VEGF for about seven to about 10 days, and harvesting a population of human cardiomyocytes from culture. In a preferred embodiment, the inhibitor of Wnt is DKK1, and the preferred concentrations of DKK1 and VEGF are as described hereinabove. In one preferred embodiment, the cells are plated as monolayers in

gelatin-coated wells. In another embodiment, the cells are cultured in low cluster dishes as aggregates. The populations of human cardiomyocytes contain at least about 30%, and preferably at least about 40%, and more preferably at least about 50% cardiomyocytes. In other embodiments, the populations of human cardiomyocytes 5 contain at least about 70%, and preferably at least about 80% and more preferably at least about 90% and up to 100% cardiomyocytes. The cardiomyocytes may be identified by assessing contractile activity or by measuring expression of genes indicative of cardiomyocytes, such as for example *CTNT*.

The human cardiovascular progenitor cells of the present invention are 10 also useful for generating subpopulations of cardiomyocytes including, for example, atrial, ventricular, and pacemaker cells, using differentiation conditions known to those of skill in the art.

In another embodiment, the present invention provides a method of generating cardiovascular colonies containing cardiomyocytes, endothelial cells, and 15 vascular smooth muscle cells comprising culturing the human cardiovascular precursor cells in the presence of VEGF, bFGF and an inhibitor of Wnt, preferably DKK1, for about three to about seven days. In a preferred embodiment, the cells are plated in methylcellulose. The preferred concentrations of VEGF, bFGF and DKK are as described hereinabove. The presence of cardiomyocytes, endothelial cells, and 20 vascular smooth muscle cells can be determined by measuring expression of genes indicative of cardiomyocytes, such as *CTNT*, and genes indicative of endothelial cells, such as *CD31*, *VE-CADHERIN* and genes indicative of vascular smooth muscle cells, such as *SMA* and *CALPONIN*.

The present invention further provides methods for screening for 25 agents that have an effect on human cardiovascular progenitor cells, cardiovascular colonies, cardiomyocytes, endothelial cells and vascular smooth muscle cells. The method comprises contacting cells from one of the cell populations described hereinabove with a candidate agent, and determining whether the agent has an effect on the cell population. The agent to be tested may be natural or synthetic, one 30 compound or a mixture, a small molecule or polymer including polypeptides, polysaccharides, polynucleotides and the like, an antibody or fragment thereof, a compound from a library of natural or synthetic compounds, a compound obtained from rational drug design, a condition such as a cell culture condition, or any agent

the effect of which on the cell population may be assessed using assays known in the art. The effect on the cell population may be determined by any standard assay for phenotype or activity, including for example an assay for marker expression, receptor binding, contractile activity, electrophysiology, cell viability, survival, morphology, 5 or DNA synthesis or repair. Standard proliferation and differentiation assays are described in U.S. Patent No. 6,110,739. Such agents are useful for the control of cell growth, differentiation and survival *in vivo* and *in vitro*, and tissue maintenance, regeneration and repair.

The present invention further provides compositions comprising 10 populations of human cardiovascular precursor cells and compositions comprising populations of human cardiovascular colonies. The compositions may comprise pharmaceutically acceptable carriers and diluents. The compositions may further comprise components that facilitate engraftment. Compositions comprising these populations are useful for cell and tissue replacement and repair, and for generating 15 populations of cardiomyocytes, endothelial cells, and vascular smooth muscle cells *in vitro* and *in vivo*. Compositions comprising human cardiovascular progenitor cells are useful for expansion of the progenitor populations. The compositions may be formulated as a medicament or delivery device for treating a cardiac condition.

In another embodiment, the present invention provides methods of cell 20 replacement and methods of tissue replacement useful for treatment of disorders characterized by insufficient cardiac function including, for example, congenital heart disease, coronary heart disease, cardiomyopathy, endocarditis and congestive heart failure. Both the differentiated cells and the cardiovascular progenitor cells are useful for replacement therapy, since the progenitor populations are capable of 25 differentiation to the cardiomyocyte, endothelial and vascular smooth muscle lineages *in vivo*. The cells are also useful for generating cardiovascular tissue *in vitro*. Methods for engineering cardiac tissue are known in the art and reviewed for example by Birla in "Stem Cell Therapy and Tissue Engineering for Cardiovascular Repair" Springer, 2006.

30 Accordingly, in one embodiment the present invention provides a method of cardiomyocyte replacement therapy comprising administering to a subject in need of such treatment a composition comprising cardiomyocytes isolated from a cell population enriched for human cardiovascular progenitor cells obtained in

accordance with the present invention. In another embodiment, the present invention provides a method of treating a disorder characterized by insufficient cardiac function comprising administering to a subject in need of such treatment a composition comprising human cardiovascular progenitor cells. In a preferred embodiment, the 5 subject is a human. The composition may be administered by a route that results in delivery to or migration to cardiac tissue including, for example, injection or implantation, and under conditions that result in a reduction of at least one adverse effect or symptom or the disorder.

10 All references cited herein are incorporated herein in their entirety.  
The following examples serve to further illustrate the present invention.

### **Example 1**

#### **Materials and Methods**

15 **Maintenance of human ES cells**

H1(NIH code WA01) from WiCell Research Institute (Madison, WI) and hES2 (NIH code ES02) from ESI International (Singapore) were maintained on irradiated mouse embryonic feeder cells in hESC media consisting of Dulbecco's Modified Eagle Medium (DMEM)/F12 (50:50; Mediatech, Herndon, VA) 20 supplemented with 20% knock-out serum replacement (SR), 100 µM nonessential amino acids, 2 mM glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin (Invitrogen, Grand Island, NY), 10<sup>-4</sup> M β-mercaptoethanol (Sigma, St Louis, MO) and 20 ng/mL hbFGF (R&D Systems, Minneapolis, MN) in 6-well tissue culture plates. The AAVS1-targeted hES2 cell line was generated by co-infection of parental 25 hES2 cells with 10<sup>6</sup> viral particles of both, AAV2-TRUF11 (CAG-GFP-TK-neo) and wild type AAV2. After G418 selection, GFP positive cells were sorted and subclones were isolated. Targeted integration of the transgenes was confirmed by ligation-mediated polymerase chain reaction (LM PCR). Wild type AAV sequences were not detected in GFP positive clones. RFP expressing hES cells were generated 30 by targeting the RFP cDNA to the Rosa26 locus of hES2 cells as described by Irion et al. (2007) Nat. Biotechnol. 12:1477-1482.

**Differentiation of human ES cells**

Prior to the generation of EBs, hESCs were cultured on a layer of Matrigel (BD Biosciences, Bedford, MA) to deplete the feeders for 48 hours. Cells from the Matrigel culture were dissociated to small clusters by 20-minute treatment 5 with collagenase B (1 mg/mL; Roche, Indianapolis, IN) followed by a 2-minute treatment with trypsin-EDTA (0.05%). From day 0-1, EBs were plated in 2 mL basic media (StemPro-34 (Invitrogen), 2 mM glutamine,  $4 \times 10^{-4}$  M monothioglycerol (MTG), and 50  $\mu$ g/mL ascorbic acid (Sigma) plus 0.5 ng/mL BMP4 (R&D Systems). The following concentrations of factors were used for EB formation, mesoderm 10 induction and cardiac specification: BMP4:10 ng/mL, hbFGF: 5 ng/mL, ActivinA: 3 ng/ml, hDKK1:150 ng/mL, hVEGF: 10 ng/mL. The factors were added with the following sequence: day 1-4, BMP4, hbFGF and ActivinA; day 4-8, VEGF and DKK1; following day 8, VEGF, DKK1, and bFGF. The medium was changed every 4 days beyond day 8. All human factors and inhibitors were purchased from R&D 15 Systems (Minneapolis, MN). Cultures were maintained in a 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub> environment for the first 10-12 days of culture and then in 5% CO<sub>2</sub>/air environment for the following days.

**Flow cytometry sorting and tube formation on Matrigel**

EBs were harvested and dissociated to single cells with trypsin (0.25% 20 trypsin-EDTA). Following trypsinization, analyses were carried out using FacsCalibur flow cytometer (Becton Dickinson). Cells were sorted using a MoFlo (Dako Cytomation, Fort Collins, CO) cell sorter. Flow cytometric data were analyzed using the FlowJo (Treestar, San Carlos, CA) software programs. Anti-KDR-PE, anti-C-KIT-APC were purchased from R&D Systems. Sorted KDR<sup>low</sup>/C-KIT<sup>neg</sup> 25 population was differentiated in Stempro34 medium supplemented with VEGF (25 ng/ml) and bFGF (25 ng/ml). Tube-like structures were formed within 24 hours after transferred to Matrigel-coated glass coverslips.

**Immunofluorescence**

Dissociated cells were cultured on glass cover slips for 2 days. At this 30 point the cells were fixed with 4% PFA and then stained. The following antibodies were used for immunostaining: Anti-human CD31, anti-human VE-Cadherin from R&D Systems, anti-mouse Troponin T and anti-human Smooth Muscle Actin from

Lab Vision (Freemont, CA), anti-human ANP, Connexin 43 and anti-human  $\alpha$  / $\beta$  MHC antibodies from Chemicon (Temecula, CA), and anti-human  $\alpha$ -Actinin from Sigma (St. Louis, MO). Anti-human smooth muscle myosin heavy chain (SMHC), Caldesmon, Von Willebrand Factor antibodies were purchased from DakoCytomation (Carpinteria, CA). The Cy2, Cy3 and Cy5 conjugated second antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Fluorescence was visualized using a Leica DMRA2 fluorescence microscope (Leica, Wetzlar, Germany), and images were recorded using a digital Hamamatsu CCD camera (Hamamatsu City, Japan).

## 10 Colony Assays

To generate cardiovascular colonies, KDR<sup>low</sup>/C-KIT<sup>neg</sup> cells isolated from day 6 EBs were aggregated in the presence of VEGF (25 ng/mL), bFGF (10 ng/ml) and DKK1 (150 ng/ml) for 2-3 days. At this stage, the aggregates were dissociated and the cells were cultured in methylcellulose containing VEGF (25 ng/mL), bFGF (25 ng/ml) and DKK1 (150 ng/ml) in a 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub> environment. Colonies were scored following 4-6 days of cultures for the colony frequency analysis.

## RT-PCR

For expression studies, individual colonies were isolated from the methylcellulose cultures and analyzed using a modified version of the protocol previously described by Brady et al. (1993) Methods Enzymol. 225:611-623. The amplified cDNA was then subjected to normal PCR. Real-time quantitative PCR was performed on the MasterCycler EP RealPlex (Eppendorf). Experiments were done in triplicate using Platinum SYBR GreenER qPCR SuperMix (Invitrogen). All primers are described in Table I. All annealing reactions were carried out at 60°C.

**Table 1**  
**Primers for Normal PCR**

GENE	FORWARD PRIMER	REVERSE PRIMER
<i>T(Brachyury)</i>	tgt ccc agg tgg ctt aca gat gaa (SEQ ID NO:1)	ggg gtg cca aag ttg cca ata cac (SEQ ID NO:2)
<i>WNT3A</i>	aat gcc act gca tct tcc act ggt (SEQ ID NO:3)	tgg tga cag ttc ctt gct gtc tga (SEQ ID NO:4)
<i>DKK1</i>	tct cag tgt ggc act tac ctg t (SEQ ID NO:5)	cca aga gat cct tgc gtt cta gac t (SEQ ID NO:6)
<i>KDR</i>	cct cta ctc cag taa acc tga ttg gg	tgt tcc cag cat ttc aca cta tgg

	(SEQ ID NO:7)	(SEQ ID NO:8)
<i>ISL1</i>	cac aag cgt ctc ggg att gtg ttt (SEQ ID NO:9)	agt ggc aag tct tcc gac aa (SEQ ID NO:10)
<i>NKX2.5</i>	gcg att atg cag cgt gca atg agt (SEQ ID NO:11)	aac ata aat acg ggt ggg tgc gtg (SEQ ID NO:12)
<i>TBX20</i>	gaa aga cca cac agc ctc att gct (SEQ ID NO:13)	tca atg tca gtg agc ctg gag gaa (SEQ ID NO:14)
<i>GFP</i>	ttc tcg gcc aca agc tgg aat aca (SEQ ID NO:15)	act ggg tgg aca ggt aat ggt tgt (SEQ ID NO:16)
<i>RFP</i>	acc tgg tgg agt tca aga cca tct (SEQ ID NO:17)	acc tct aca aat gtg gta tgg ctg (SEQ ID NO:18)
<i>CD31</i>	atc att tct agc gca tgg cct ggt (SEQ ID NO:19)	att tgt gga ggg cga ggt cat aga (SEQ ID NO:20)
$\beta$ - <i>ACTIN</i>	ttt gaa tga tga gcc ttc gtc ccc (SEQ ID NO:21)	gtt ctc aag tca gtg tac agg taa gc (SEQ ID NO:22)

#### Primers for both Normal PCR and QPCR

<i>OCT4</i>	aac ctg gag ttt gtg cca ggg ttt (SEQ ID NO:23)	tga act tca cct tcc ctc caa cca (SEQ ID NO:24)
<i>C-KIT</i>	att ccc ag ccc atg agt cct tga (SEQ ID NO:25)	aca cgt gga aca cca aca tcc t (SEQ ID NO:26)
<i>CALPONIN</i>	aag gac gca ctg agc aac gct att (SEQ ID NO:27)	acg cca ctg tca cat cca cat agt (SEQ ID NO:28)
<i>TBX5</i>	aaa tga aac cca gca tag gag ctg gc (SEQ ID NO:29)	aca ctc agc ctc aca tct tac cct (SEQ ID NO:30)
<i>CTNT</i>	ttc acc aaa gat ctg ctc ctc gct (SEQ ID NO:31)	tta tta ctg gtg tgg agt ggg tgt gg (SEQ ID NO:32)
<i>MLC2A</i>	aca tca tca ccc acg gag aag aga (SEQ ID NO:33)	att gga aca tgg cct ctg gat gga (SEQ ID NO:34)
<i>VE-CAD</i>	tgg aga agt ggc atc agt caa cag (SEQ ID NO:35)	tct aca atc cct tgc agt gtg ag (SEQ ID NO:36)
<i>SMA</i>	aat act ctg tct gga tcg gtg gct (SEQ ID NO:37)	acg agt cag agc ttt ggc tag gaa (SEQ ID NO:38)
<i>NFATC</i>	ttg acc tga act cgt gcc tta gga (SEQ ID NO:39)	ggc ctt cag gtt tct ttc cgt (SEQ ID NO:40)
<i>NEUD</i>	ccc atg gtg ggt tgt cat ata ttc atg t (SEQ ID NO:41)	cca gca tca cat ctc aaa cag cac (SEQ ID NO:42)

#### Primers for QPCR

<i>CD31</i>	tct atg acc tcg ccc tcc aca aa (SEQ ID NO:43)	gaa cgg tgt ctt cag gtt ggt att tca (SEQ ID NO:44)
<i>ISL1</i>	ttg tac ggg atc aaa tgc gcc aag (SEQ ID NO:45)	agg cca cac agc gga aac a (SEQ ID NO:46)
<i>NKX2.5</i>	acc tca aca gct ccc tga ctc t (SEQ ID NO:47)	ata atc gcc gcc aca aac tct cc (SEQ ID NO:48)
<i>TBX20</i>	gtc tct cag ctc ctg ggt atc atc tt (SEQ ID NO:49)	tgt tgc tat gga tgc tgt gct ggt (SEQ ID NO:50)
<i>MEOX1</i>	tgg aag cgt gtg aag gga ggt (SEQ ID NO:51)	aag gaa gag ggt gaa ggt ggg att g (SEQ ID NO:52)
<i>NRG1</i>	agt tca gct cct tcc acc aca ac (SEQ ID NO:53)	cgt ttc ata ctc ctc atc ctc cac tat cc

		(SEQ ID NO:54)
<b>PAX6</b>	tcg aag ggc caa atg gag aag aga ag (SEQ ID NO:55)	ggt ggg ttg tgg aat tgg ttg gta ga (SEQ ID NO:56)
<b>SOX1</b>	cct gtg tgt acc ctg gag ttt ctg t (SEQ ID NO:57)	tgc acg aag cac ctg caa taa gat g (SEQ ID NO:58)
<b>GATA1</b>	tgt cag taa acg ggc agg tac tca (SEQ ID NO:59)	ata cca tcc ttc cgc atg gtc agt (SEQ ID NO:60)
<b>SOX17</b>	agg aaa tcc tca gac tcc tgg gtt (SEQ ID NO:61)	ccc aaa ctg ttc aag tgg cag aca (SEQ ID NO:62)
<b>FOXA2</b>	gca ttc cca atc ttg aca cgg tga (SEQ ID NO:63)	gcc ctt gca gcc aga ata cac att (SEQ ID NO:64)
<b>FOXA3</b>	ttg gcc atg tcg tca cca ttc tct (SEQ ID NO:65)	ccc aca ccc taa cca gcc ttt (SEQ ID NO:66)
<b>KDR</b>	act ttg gaa gac aga acc aaa tta tct c (SEQ ID NO:67)	tgg gca cca ttc cac ca (SEQ ID NO:68)
<b>T</b>	cag tgg cag tct cag gtt aag aag ga (SEQ ID NO:69)	cgc tac tgc agg tgt gag caa (SEQ ID NO:70)
<b>CYCLOPHININ</b>	gaa gag tgc gat caa gaa ccc atg ac (SEQ ID NO:71)	gtc tct cct cct tct cct cct atc ttt act t (SEQ ID NO:72)

***In vivo* analyses of KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived populations**

KDR<sup>low</sup>/C-KIT<sup>neg</sup> cells derived from GFP expressing hESC were cultured in the presence of VEGF (10 ng/mL), bFGF (10 ng/ml) and DKK1 (150 ng/ml) for 5-10 days before transplantation. NOD/SCID-gamma mice were anesthetized, intubated, and 100,000 donor cells were injected directly into the left ventricular wall in an open-chest procedure. Hearts were harvested 2-11 weeks post surgery, fixed in 1% paraformaldehyde in cacodylate buffer, and vibrotome-sectioned at 300um. Grafted regions were identified and cryo-sectioned at 10um. Immunohistochemistry was done for GFP antibody (Chemicon, AB3080, 1:100; Vector ABC and DAB kits),  $\alpha$ -actinin antibody (Sigma, A7811, 1:500; Chemicon AQ300R secondary antibody, 1:20), CD31 antibody (Dako, M0823, 1:50; Vector ABC and DAB kits), and smooth muscle MHC antibody (Biomedical Technologies, BT-562, 1:300; Alexa 555 secondary antibody, 1:200). Confocal images were analyzed for colocalization using ImageJ and Pierre Bourdoncle's plugin with default settings. For evaluation in the murine infarct model, myocardial infarction was induced in SCID beige mice via direct coronary ligation, using techniques described by Rubert et al. (2007) *Nature Biotechnol.* 25:993-994. Ten to twenty minutes later the mice were injected with 500,000 KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived cells (n=9) or an

equivalent volume of serum free cell media (n=12). All injections were attempted in the border zone of the infarct. Two weeks later assessment of ventricular function was performed using 9.4 Tesla Magnetic Resonance Imaging.

### **Patch Clamp**

5 Whole cell patch clamp recordings were performed at room temperature using an IX50 inverted microscope (Olympus), a Multiclamp 700A Amplifier, a Digidata 1300 Analogue/Digital converter and a PC with pClamp9.1 software (Axon Instruments, Foster City, CA). Bath solution was (in mM): NaCl 136, KCl 4, CaCl<sub>2</sub> 1, MgCl<sub>2</sub> 2, CoCl<sub>2</sub> 5, HEPES 10, glucose 10, and tetrodotoxin (TTX) 10 0.02 (pH 7.4). Pipettes were of 3-5 MΩ resistance when filled with intracellular solution containing (in mM): KCl 135, EGTA 10, HEPES 10, and glucose 5 (pH 7.2). Cells were stepped from a holding potential of -80 mV to test potentials from -80 mV to +40 mV in 20 mV increments, before a -30 mV tail pulse (durations as in Figure 4). Data were analyzed using pClamp9.1 software (Axon Instruments). Current 15 amplitudes were normalized to cell size (whole-cell membrane capacitance). Inactivation τ values were calculated using a single exponential fit of current decay.

### **Field Potential Recording**

KDR<sup>low</sup>/C-KIT<sup>neg</sup> cells isolated from day 6 EBs were cultured in the MEA (Multi ChannelSystems) dish with StemPro-34 plus 10 ng/mL VEGF and 20 150 ng/mL DKK1 for 2-4 weeks. 2 days before recording, cells were changed to DMEM (Cell grow) with 15% FBS. Extracellular electrical activity was simultaneously recorded from 60 channels and analyzed with software MC Rack (Multi Channel Systems).

25

### **Example 2**

#### **Regulation of cardiac development in hESC differentiation cultures**

To direct the differentiation of hESC to the cardiac lineage, a staged protocol was designed that involves the induction of a primitive streak-like population, the patterning of cardiac mesoderm and the specification of the 30 cardiovascular lineages using combinations of BMP4, activin A, VEGF, bFGF and DKK1, factors known to play a role in mesoderm induction and cardiac specification in different model systems. An outline of the protocol is shown in Fig. 1. With the

protocol exemplified here, hESC were first differentiated in the presence of BMP4 for 24 hours to allow the formation of EBs. The combination of activin and BMP4 at stage 1 induced a primitive streak-like population and mesoderm as demonstrated by the upregulation and transient expression of *T* (Brachyury) and 5 *WNT3a*, genes known to be expressed in these populations in the mouse (Figure 6). At stage 2, VEGF and the Wnt inhibitor DKK1 further patterned the mesoderm and promoted the expansion and maturation of KDR<sup>+</sup> progenitor population. bFGF was added again at day 8 of differentiation to support the continued expansion of the developing cardiovascular lineages. This protocol 10 supports cardiac development within the EBs as demonstrated by the emergence of contracting cells and cells that expressed cardiac troponin T (CTNT) (Figure 2),  $\alpha$ -actinin,  $\alpha/\beta$  myosin heavy chain, ANP and connexin 43 (Figures 20, 21). The highest frequency of CTNT<sup>+</sup> cells was routinely observed between days 14 and 15 16 of culture (Figures 2, 21) although this population could be maintained within the EBs over a two-month period. The kinetics of development of contracting 15 EBs paralleled the emergence of the CTNT<sup>+</sup> population (Figure 22).

### Example 3

20 **Role of Wnt signaling on emergence of cardiac lineage from human ES cells**

The role of Wnt signaling pathway on emergence of the cardiac lineage from the human ES cells was investigated, specifically focusing on that stage beyond primitive streak induction (Stage 2). EBs were induced in the presence of 25 BMP4, bFGF and activin A as outlined in the protocol. Either Wnt3A or DKK1 were added to the cultures at the days indicated in Fig. 3. These factors were maintained in the cultures throughout the 14-day period. VEGF and bFGF were added at the indicated times. At day 14, the EBs were harvested and analyzed as above. The control was cultures that did not receive WNT or DKK1. Addition of DKK1 to the 30 EBs at day 4 of differentiation led to a more than two-fold increase in the frequency of CTNT<sup>+</sup> cells (up to 40%) at day 14 of culture (Figure 3). The effect of DKK1 was less pronounced if added at day 6 or 8 and there was no enhancement of the frequency of CTNT<sup>+</sup> cells if the addition was delayed to day 10 of differentiation. Wnt3A had the opposite effect and completely suppressed development of CTNT<sup>+</sup>

cells if added to the cultures at days 4 or 6. These findings indicate that stage-specific inhibition of the canonical Wnt pathway is necessary to promote cardiac specification of the BMP4/activin induced PS population.

5

#### Example 4

##### **Role of BMP4, activin A and bFGF in induction of the cardiac lineage**

To evaluate the role of BMP4, activin A and bFGF in the induction of 10 the cardiac lineage in hESC differentiation cultures, single factors as well as different combinations were tested during the induction stage (days 1-4). At day 4 the factors were removed and the EBs treated as outlined in Fig. 1. Day 4-14 EBs were treated with combinations of VEGF and DKK1 indicated in Figs. 5, 25, and 26, with addition of bFGF after day 8. EBs were harvested at day 14 and analyzed for frequency of 15 CTNT<sup>+</sup> cells for each combination and for total number of CTNT<sup>+</sup> cells per well of 24 well plates. BMP4, bFGF or activin A alone or the combinations of BMP4 and FGF or activin A and bFGF were poor inducers of cardiac development as demonstrated by the low frequency (Figure 4) and total number of CTNT<sup>+</sup> cells generated (Figures 23, 24). While BMP4 and activin A did induce significant numbers of CTNT<sup>+</sup> cells, the 20 combination of the 3 factors was the most potent and generated the highest frequency and total number of cardiac cells, routinely yielding cultures consisting of between 40% and 50% CTNT<sup>+</sup> cells. While VEGF was not essential for cardiac development, the addition of 10 ng/ml of this factor did increase the total number of CTNT<sup>+</sup> cells that developed. Increasing concentrations of VEGF above this concentration reduced 25 the cell number in the cultures, resulting in a significant decrease in the total number of CTNT<sup>+</sup> cells generated (Figures 5, 25 and 26).

Molecular analysis of the developing EBs revealed dynamic changes in expression patterns following the establishment of a primitive streak-like population. Together with *T* and *WNT3A*, expression of *DKK1* was upregulated early and 30 persisted through the 18-day time course (Figure 6). Expression of endogenous DKK1, between days 2 and 4 of differentiation, may function to specify cardiac mesoderm, resulting in the establishment of the cardiac lineage in the absence of the addition of exogenous inhibitor (Figure 3). *KDR* was expressed in undifferentiated ES cells. The levels of expression appeared to increase between days 4 and 6 and then

persisted for the next 12 days. *ISL1*, a gene that marks progenitors of the secondary heart field in the early embryo, was expressed in the EBs between days 6 and 8, preceding the expression of the cardiac transcription factor *Nkx2.5* that was first detected at day 8 of differentiation. Expression of two TBX transcription factors 5 required for proper cardiac development *TBX5*, and *TBX20*, as well as the contractile proteins *MLC2A* and *CTNT* was upregulated between days 8 and 10 of differentiation, reflecting the onset of cardiac development.

### Example 5

10 **Identification of the KDR<sup>low</sup>/C-KIT<sup>neg</sup> EB population with cardiovascular potential**

Developing EBs were analyzed for expression of KDR and C-KIT. C-KIT was used as a potential marker to define distinct populations as its expression in mouse EBs identifies the earliest hemangioblast-derived hematopoietic and 15 vascular progenitors as well as the anterior primitive streak and the developing endoderm. Figure 7 shows flow cytometric analysis of different aged EBs. As shown in Figure 7, three distinct populations, KDR<sup>high</sup>/C-KIT<sup>+</sup>, KDR<sup>low</sup>/C-KIT<sup>neg</sup> and KDR<sup>neg</sup>/C-KIT<sup>+</sup> can be resolved within the day 6 EBs. Development of the three populations was dependent on induction with both BMP4 and activin A (not shown). 20 The three populations were isolated by cell sorting and analyzed for gene expression patterns and cardiac potential. Figure 8 shows quantitative RT-PCR gene expression analysis of the three populations isolated from day 6 EBs. Average expression was normalized to cyclophilin. The KDR<sup>high</sup>/C-KIT<sup>+</sup> population expressed *CD31*, *VE-CADHERIN* and *SMA*, genes associated with vascular 25 development, as well as *GATA-1*, a gene indicative of hematopoietic commitment (Figures 8, 27). Genes involved in cardiac development, including *NKX2.5*, *ISL1* and *TBX5* were expressed at highest levels in the KDR<sup>low</sup>/C-KIT<sup>neg</sup> fraction. This fraction also expressed *SMA*, very low levels of *GATA-1*, but no detectable *VE-CADHERIN* or *CD31*. As *SMA* is expressed early in the cardiac lineage as well as in the VSM 30 lineage, this expression could reflect the onset of cardiac development. The KDR<sup>neg</sup>/C-KIT<sup>+</sup> cells expressed the highest levels of *OCT4* and *T* of the 3 fractions, indicating the presence of residual undifferentiated ES cells as well as primitive streak-like cells. The relatively high levels of *FOXA2* and *SOX17* in this fraction

suggest that it also contains cells undergoing commitment to the endoderm lineage. *SOX1* and *PAX6* were only detected at very low levels, suggesting little, if any, differentiation to the neuroectoderm lineage in the EBs at this stage of development (Figure 27). Taken together, these expression patterns demonstrate that the

5 KDR<sup>high</sup>/C-KIT<sup>+</sup> population contains hematopoietic and vascular progenitors, the KDR<sup>low</sup>/C-KIT<sup>neg</sup> population contains cardiac progenitors and the KDR<sup>neg</sup>/C-KIT<sup>+</sup> population consists of a mixture of undifferentiated ES cells, primitive streak cells and endodermal cells.

To monitor cardiac potential, the cells were plated either as

10 monolayers on gelatin coated wells or allowed to reaggregate in low cluster dishes and cultured as aggregates. Specifically, cells were isolated from day 6 EBs and cultured on gelatin-coated wells as monolayers with VEGF (10 ng/ml), DKK1 (150 ng/ml) and bFGF (10 ng/ml). CTNT<sup>+</sup> cells were analyzed after 10 days culture. Results are shown in Fig. 9. KDR<sup>low</sup>/C-KIT<sup>neg</sup> cells from day 6 EBs were sorted and

15 cultured as a monolayer or as aggregates in low cluster wells with VEGF (10 ng/ml) and DKK1 (150 ng/ml). Cultures were harvested and analyzed for the presence of CTNT<sup>+</sup> cells following 7-10 days of differentiation. Results are shown in Fig. 10. The numbers (%) represent the mean +/- standard error of three independent experiments. Bars represent standard error of the mean of three independent

20 experiments. Statistic analysis was performed with unpaired t-test. \*P<0.01, \*\*\*P<0.001. The KDR<sup>low</sup>/C-KIT<sup>neg</sup> population displayed the greatest cardiomyocyte potential (Figure 9) and readily generated CTNT<sup>+</sup> cells and populations of contracting cells in both the aggregates in suspension cultures as well as in the adherent monolayers (Figure 10). Approximately 40% of the cells within the aggregates were

25 CTNT<sup>+</sup> whereas greater than 50% of the monolayer cultures consistently expressed this marker. The high frequency of cardiomyocytes in the monolayer cultures routinely led to the development of sheets of cells contracting as a synchronous mass. The isolated KDR<sup>low</sup>/C-KIT<sup>neg</sup> cells expanded approximately 1.5-fold as aggregates (data not shown) and 3-fold in the monolayer cultures (Figure 28). With the induction

30 protocol and sorting strategy outlined, an output of 1 cardiomyocyte per 4 input hESC was estimated. In a typical experiment,  $2 \times 10^6$  hESC generated  $1 \times 10^6$  day 6 EB cells of which 30% (3.0x9 05 cells) represented the KDR<sup>low</sup>/C-KIT<sup>neg</sup> fraction. This population expanded 3-fold ( $1 \times 10^6$  cells) in the monolayer culture format to yield a

population that consisted of approximately 50% cardiomyocytes ( $5 \times 10^5$ ). Kinetic analysis of EBs generated from a second hESC line, H1 demonstrated the development of the 3 KDR/C-KIT populations at day 5 rather than day 6 of differentiation. Analysis of the day 5 KDR<sup>low</sup>/C-KIT<sup>neg</sup> population indicated that it 5 also displayed cardiac potential (Figures 29, 30).

### Example 6

#### Characterization of KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived lineages

10 Quantitative RT-PCR expression analysis of adherent populations generated from the day 6 EB-derived KDR<sup>low</sup>/C-KIT<sup>neg</sup> fraction plated with VEGF (10 ng/ml), DKK1 (150 ng/ml) and bFGF (10 ng/ml) was performed. Cultures were harvested at the indicated days in Fig. 11 and the cells were analyzed for expression 15 (D7 represents populations one day following plating). Average expression normalized to *CYCLOPHININ* is shown. Bars represent standard error of the mean of three independent experiments.

16 Expression analysis (qPCR) of KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived adherent populations at different days following plating demonstrated the upregulation of genes 20 associated with endothelial (*CD31*, *VE-CADHERIN*), VSM (*CALPONIN*, *SMA*), cardiac development (*NKX2.5*, *ISL1*, *TBX5*, *TBX20*) and cardiac maturation (*CTNT*, *MLC2A*) (Figures 11, 31). Expression of *NFATC* and Neuregulin 1 (*NRG1*) suggest 25 the presence of endocardium in the cultures. The low levels of *NEUD*, *PAX6*, *SOX1*, *FOXA2*, *FOXA3*, *SOX17* and *MEOX1* expression indicate that the cultures are not contaminated with neuroectoderm, endoderm or somatic mesoderm (Figures 11, 31).

26 KDR<sup>low</sup>/C-KIT<sup>neg</sup> cells were isolated from day 6 EBs and cultured as a monolayer with the different combinations of factors indicated below the graph in 30 Fig. 12. Cells were harvested and analyzed for the presence of CTNT+, CD31+ and SMA+ cells following 10-12 days culture. SMA antibody can stain both cardiomyocytes and smooth muscle cells. The number (%) represent standard error of the mean of three independent experiments.

31 Flow cytometric analysis of KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived adherent cells cultured for 10-12 days in VEGF and DKK1 revealed that almost 90% expressed SMA, 50% expressed CTNT and 4% expressed CD31 (Figure 12). Addition of bFGF 35 to the cultures reduced the proportion of CTNT+ cells to 30% and increased the

CD31+ population to 30%. The proportion of SMA+ cells decreased modestly to an average of 80% in the presence of bFGF. The addition of bFGF did not significantly influence the cell number in the monolayer cultures (Figure 28). These findings clearly indicate that the majority of cells within KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived population 5 are of the cardiovascular lineages. They also show that bFGF can influence the proportion of cardiomyocytes and endothelial cells that develop in this population.

Immunostaining analysis of the KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived population cultured in VEGF, DKK1 and bFGF demonstrated the presence of CD31+, VE-Cadherin+ and von Willebrand Factor+ endothelial cells, of CTNT+ cardiomyocytes 10 and of SMA+, SMHC+ and Caldesmon+ cells indicative of VSM development. The immature cardiomyocytes within the population expressed both CTNT and SMA, whereas the VSM cells expressed only SMA. To further characterize the KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived endothelial cells, the population was expanded in the presence of VEGF and bFGF and then cultured on Matrigel-coated cover slips. Under these 15 conditions the cells formed a lattice indicative of endothelial cell organization into tube-like structures. The cells within these structures expressed CD31 and displayed the capacity to take up Dil-AC-LDL confirming their endothelial phenotype. The findings from the immunostaining analysis are consistent with those from the flow cytometric studies and demonstrate that the KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived population 20 consists of cells of the cardiac, endothelial and vascular smooth muscle lineages.

KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived cells were also transplanted into the hearts of NOD/SCID mice to document their developmental potential *in vivo*. A GFP expressing version of the hES2 cell line was used for these studies. Histological analyses revealed the presence of GFP+ cells in the hearts following transplantation. 25 Detection of GFP with an antibody identified the same population as visualized by epifluorescence, indicating that the GFP signal represents the presence of donor cells and does not result from background fluorescence. GFP+ populations co-expressing either  $\alpha$ -actinin, CD31 or SMHC were detected in the grafts, indicating differentiation to the cardiac, endothelial and vascular smooth muscle lineages *in vivo*. Teratomas 30 were not detected in any of the transplanted animals ( $N > 10$ ). To further evaluate the *in vivo* potential of this population, KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived cells were transplanted directly into infarcted hearts of SCID beige mice. When analyzed 2 weeks later, animals transplanted with the KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived cardiovascular population

had a 31 % higher ejection fraction than those injected with media alone (56%+/-3.6% vs 39%+/-4.8%, p= .008). These findings are consistent with previous reports and demonstrate that transplantation of hESC-derived cardiomyocytes leads to improvement in cardiac function in rodent models of myocardial infarction.

5

### Example 7

#### Identification and characterization of cardiovascular colony-forming cells

10 When plated in methylcellulose in the presence of VEGF, bFGF and DKK1, KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived cells generated small compact colonies within 4 days of culture. PCR analysis of individual 4-day-old colonies demonstrated co-expression of markers indicative of cardiac (*CTNT*), vascular (*CD31* and/or *VE-CADHERIN*) and VSM (*SMA* and/or *CALPONIN*) development (Figures 13, 32).

15 When maintained in culture for a further 6 days, a portion of these colonies generated contracting cells, confirming that they contain cardiomyocytes. *ISLI* and *TBX5* were typically not expressed in the same colonies, suggesting that their expression may define colonies that contain distinct subpopulations of developing cardiac cells from different heart fields. Immunostaining of adherent populations from individual

20 colonies grown on cover slips revealed the presence of SMA+, CTNT+ and VE-CAD+ cells, confirming that these colonies do have cardiac, endothelial and VSM potentials.

Two different approaches were used to determine if the cardiovascular colonies are clonal. First, KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived cells from GFP expressing hESC 25 were mixed in the methylcellulose assay with KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived cells from hESC engineered to express red fluorescent protein (RFP) in the methylcellulose colony assay. Colonies analyzed 4 days later were found to express either GFP or RFP but not both (Fig. 13), a finding consistent with the interpretation that they arise from a single cell and do not form as a result of cell aggregation. As a second

30 approach, a cell dose response experiment was performed, in which different numbers of KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived cells were plated in the cardiac methylcellulose cultures. The relationship between the number of colonies that develop and the number of cells plated was linear, with a slope approaching one, further supporting the notion that the colonies are derived from a single cell (Fig. 14). Taken together,

these findings indicate that these colonies represent clones of cardiovascular cells derived from a cardiovascular colony-forming cell (hCV-CFC).

Functional potential of KDR<sup>low</sup>/C-KIT<sup>neg</sup>-derived cardiomyocytes was evaluated with whole-cell current and field potential measurements. In whole-cell voltage clamp analysis, 80% of cells studied expressed a predominant voltage-gated, transient outward potassium current (Figs. 15, 16). The voltage dependence, density and gating kinetics of this current (Figs. 16, 17) resembled that of the I<sub>to</sub> potassium current found in human atrial and ventricular myocytes. Field potentials recorded from cultured cells using microelectrodes revealed that the human cardiovascular progenitors derived cardiac cells were electrically coupled to one another. In addition, as expected, the Vaughn Williams class Ia agent quinidine decreased the measured T wave amplitude and increased the QT interval (Fig. 19).

Throughout this specification, unless the context requires otherwise, the word "comprise" or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or method step or group of elements or integers or method steps but not the exclusion of any element or integer or method step or group of elements or integers or method steps.

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

**CLAIMS:**

1. A population of human cardiovascular progenitor cells, wherein the progenitor cells express KDR and do not express C-KIT and wherein the progenitor cells are progenitors of cardiomyocytes, endothelial cells, and vascular smooth muscle cells.
2. The population of human cardiovascular progenitor cells of Claim 1 obtained by culturing human embryoid bodies in serum free media in the presence of activin and BMP and subsequently adding an inhibitor of Wnt to the culture.
3. The population of Claim 2 wherein activin is activin A, BMP is BMP4, and the inhibitor of Wnt is DKK-I .
4. A method for producing human cardiovascular progenitor cells which express KDR and do not express C-KIT and which are progenitors of cardiomyocytes, endothelial calls and vascular smooth muscle cells, comprising:
  - (a) culturing human embryoid bodies in serum free media in the presence of activin and BMP; and
  - (b) adding an inhibitor of Wnt to the media and culturing to provide a population of human cardiovascular progenitor cells.
5. The method of Claim 4 further comprising:
  - (c) harvesting the human cardiovascular progenitor cells from culture to provide a harvested cell population; and optionally
  - (d) enriching the harvested cell population for cardiovascular progenitor cells.
6. The method of Claim 4 wherein activin is activin A, BMP is BMP4, and the inhibitor of Wnt is DKKI.
7. The method of Claim 4 wherein step (a) further comprises the presence of bFGF; and step (b) further comprises adding VEGF.

8. A method for producing human cardiovascular progenitor cells which express KDR and do not express C-KIT and which are progenitors of cardiomyocytes, endothelial cells and vascular smooth muscle cells, comprising:

- (a) culturing human embryoid bodies in serum free media in the presence of activin A and BMP4 for about one to four days;
- (b) adding DKK1 to the serum free media and culturing for about one to two days; and
- (c) harvesting a population of human cardiovascular progenitor cells.

9. The method of Claim 8 wherein step (a) further comprises the presence of bFGF; and step (b) further comprises adding VEGF.

10. The method of Claim 9 wherein the concentration of activin A is from about 0.5 ng/ml to about 30 ng/ml; the concentration of BMP4 is from about 1.0 ng/ml to about 20 ng/ml; the concentration of bFGF is from about 1.0 ng/ml to about 10 ng/ml; the concentration of DKK1 is from about 100 ng/ml to about 200 ng/ml; and the concentration of VEGF is from about 1.0 ng/ml to about 50.0 ng/ml.

11. A method for producing human cardiovascular progenitor cells which express KDR and do not express C-KIT and which are progenitors of cardiomyocytes, endothelial cells and vascular smooth muscle cells, comprising:

- (a) culturing human embryoid bodies in serum free media for about four days in the presence of about 3.0 ng/ml of activin A, about 10.0 ng/ml of BMP4 and about 5.0 ng/ml of bFGF; and
- (b) adding about 150 ng/ml of DKK1 and about 10.0 ng/ml of VEGF to the serum free media and culturing for about one to two days to produce cardiovascular progenitor cells.

12. The method of Claim 11 further comprising:
  - (c) sorting for cells that express KDR and do not express C-KIT.
13. The method of Claim 12 wherein the sorting is by immunoselection or by flow cytometry.
14. A method of generating a population of human cardiomyocytes comprising culturing the population of human cardiovascular progenitor cells of Claim 1 in the presence of an inhibitor of Wnt for about seven to about ten days, and harvesting a population of human cardiomyocytes.
15. The method of Claim 14 comprising culturing said human cardiovascular progenitor cells in the presence of VEGF and an inhibitor of Wnt.
16. The method of Claim 14 or Claim 15 wherein the inhibitor of Wnt is DKK1 .
17. The method of Claim 15 wherein the concentration of VEGF is from about 1.0 ng/ml to about 50.0 ng/ml; and the concentration of DKK1 is from about 100 ng/ml to about 200 ng/ml.
18. The method of Claim 14 wherein the cells are cultured as monolayers or as aggregates.
19. A method of generating cardiovascular colonies containing cardiomyocytes, endothelial cells, and vascular smooth muscle cells comprising culturing the population of human cardiovascular progenitor cells of Claim 1 in the presence of VEGF, bFGF and an inhibitor of Wnt for about three to about seven days.
20. The method of Claim 19 wherein the inhibitor of Wnt is DKK1 .
21. The method of Claim 20 wherein the concentration of DKK1 is from about 100 ng/ml to about 200 ng/ml; the concentration of VEGF is from about 1.0 ng/ml to about 50.0 ng/ml; and the concentration of bFGF is from about 1.0 ng/ml to about 10.0 ng/ml.

22. A composition comprising the population of human cardiovascular precursor cells of Claim 1.
23. A composition comprising the population of human cardiovascular colonies produced by the method of Claim 19.
24. A method of cardiomyocytes replacement comprising administering to a subject in need of such replacement a composition comprising cardiomyocytes produced by the method of Claim 14.
25. A method of treating a disorder characterized by insufficient cardiac function comprising administering to a subject in need of such treatment a composition comprising human cardiovascular progenitor cells which express KDR and do not express C-KIT and which are progenitors of cardiomyocytes, endothelial cells and vascular smooth muscle cells.
26. A population of human cardiovascular progenitor cells according to any one of Claims 1 to 3 or a method according to any one of Claims 4 to 21 or 24 and 25 or a composition according to Claims 22 or 23 substantially as herein described with reference to the Figures and/or Examples.

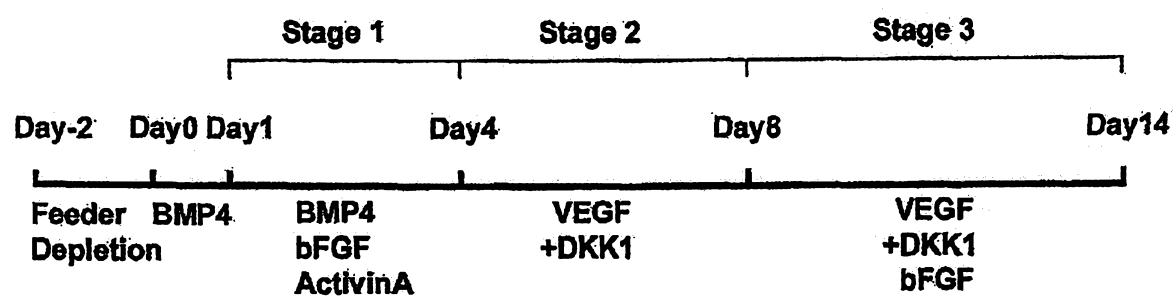


Fig. 1

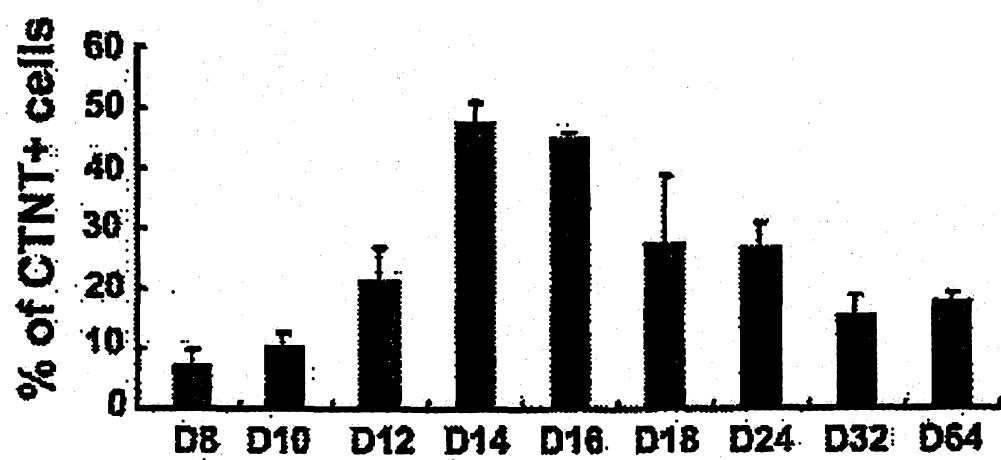


Fig. 2

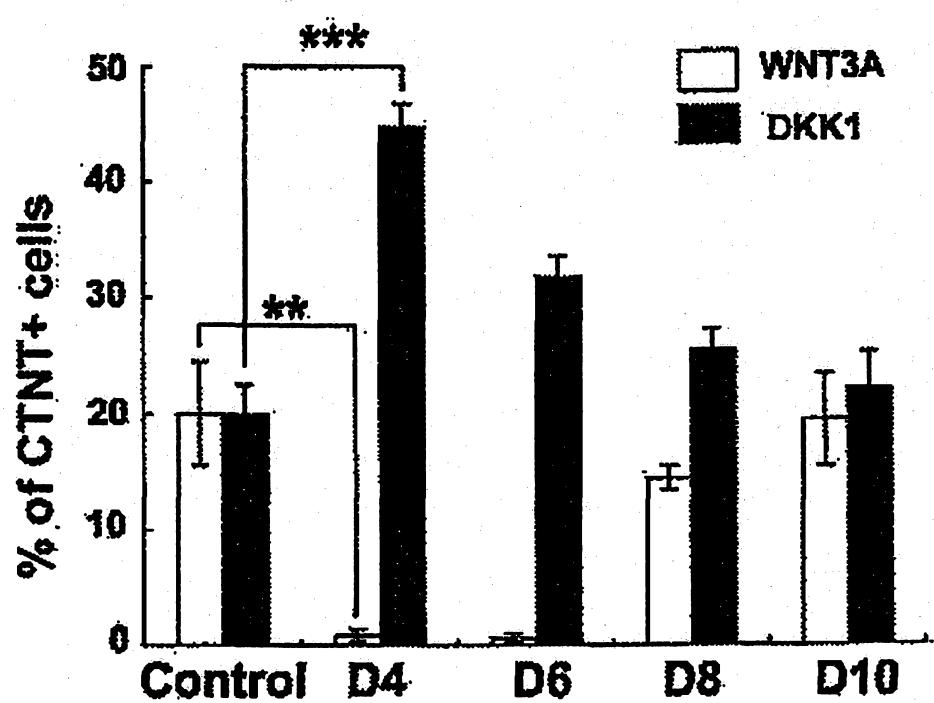


Fig. 3

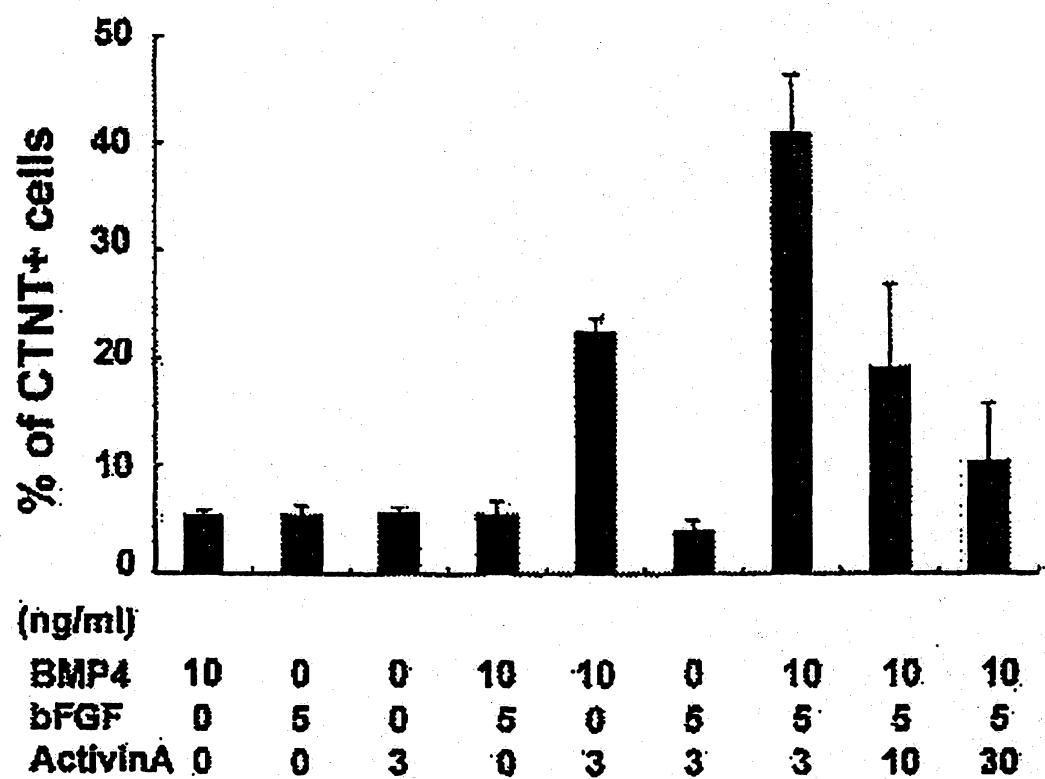


Fig. 4

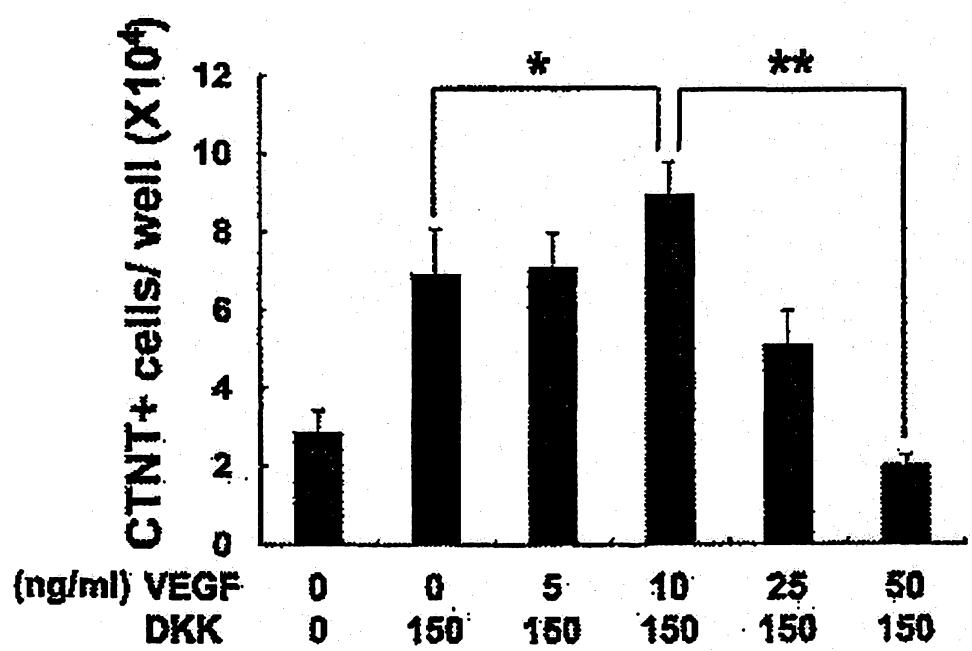


Fig. 5

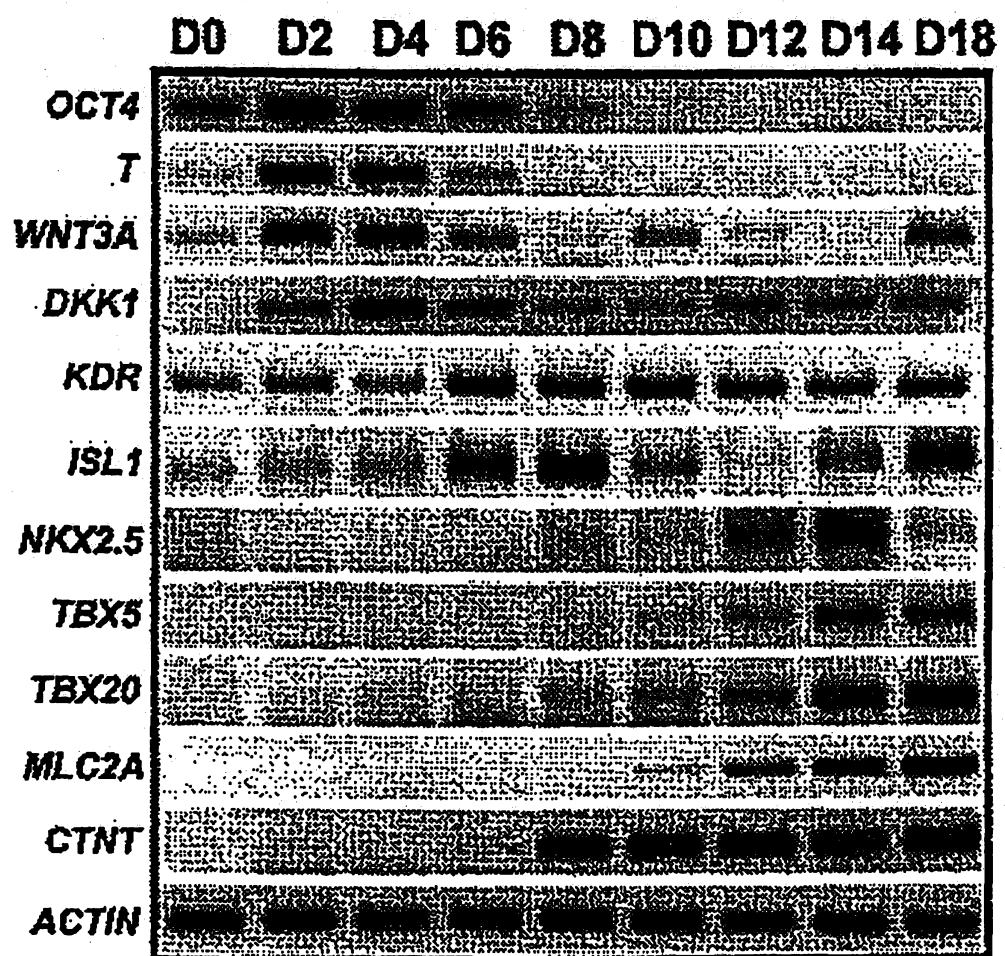


Fig. 6

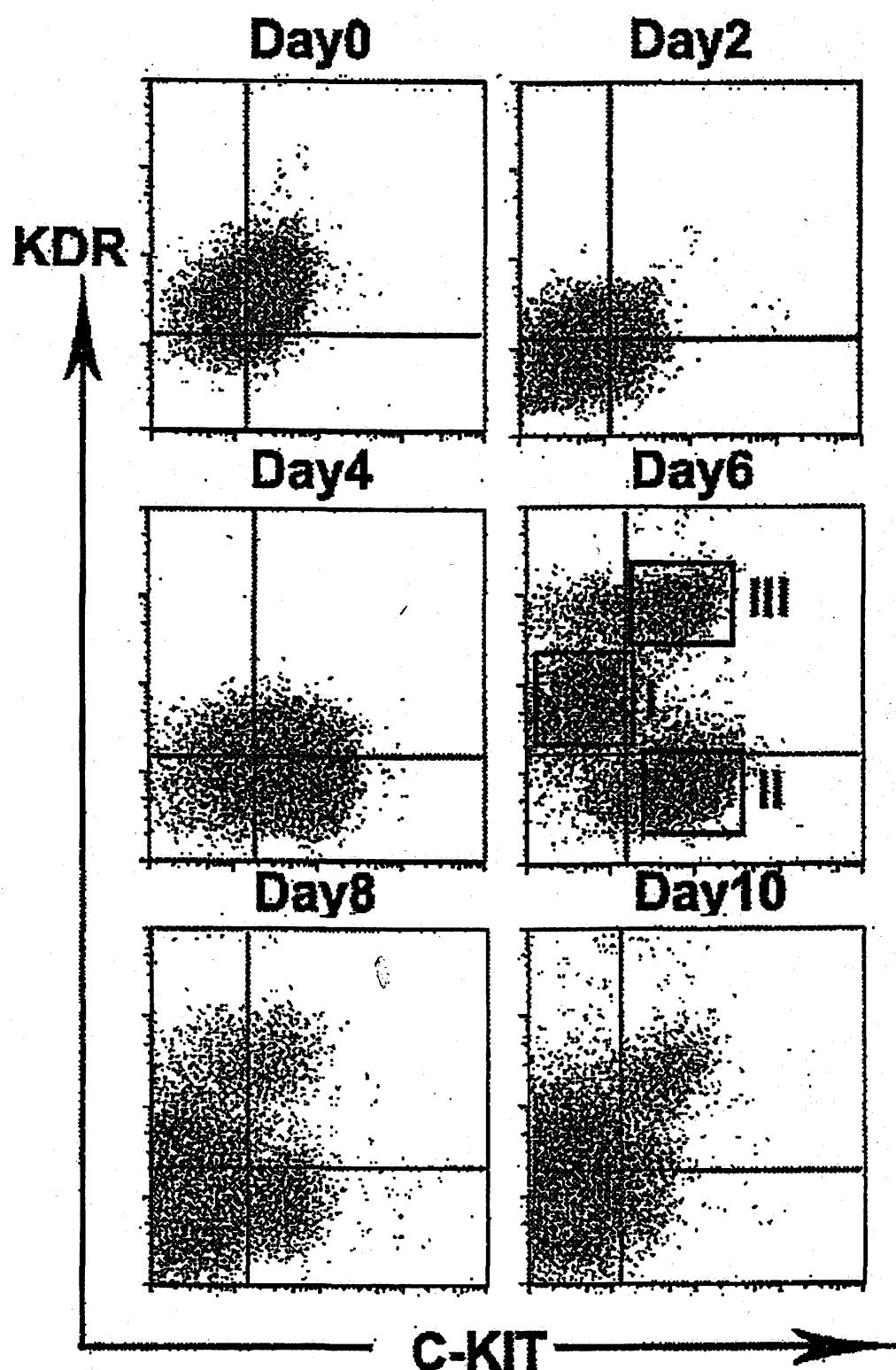


Fig. 7

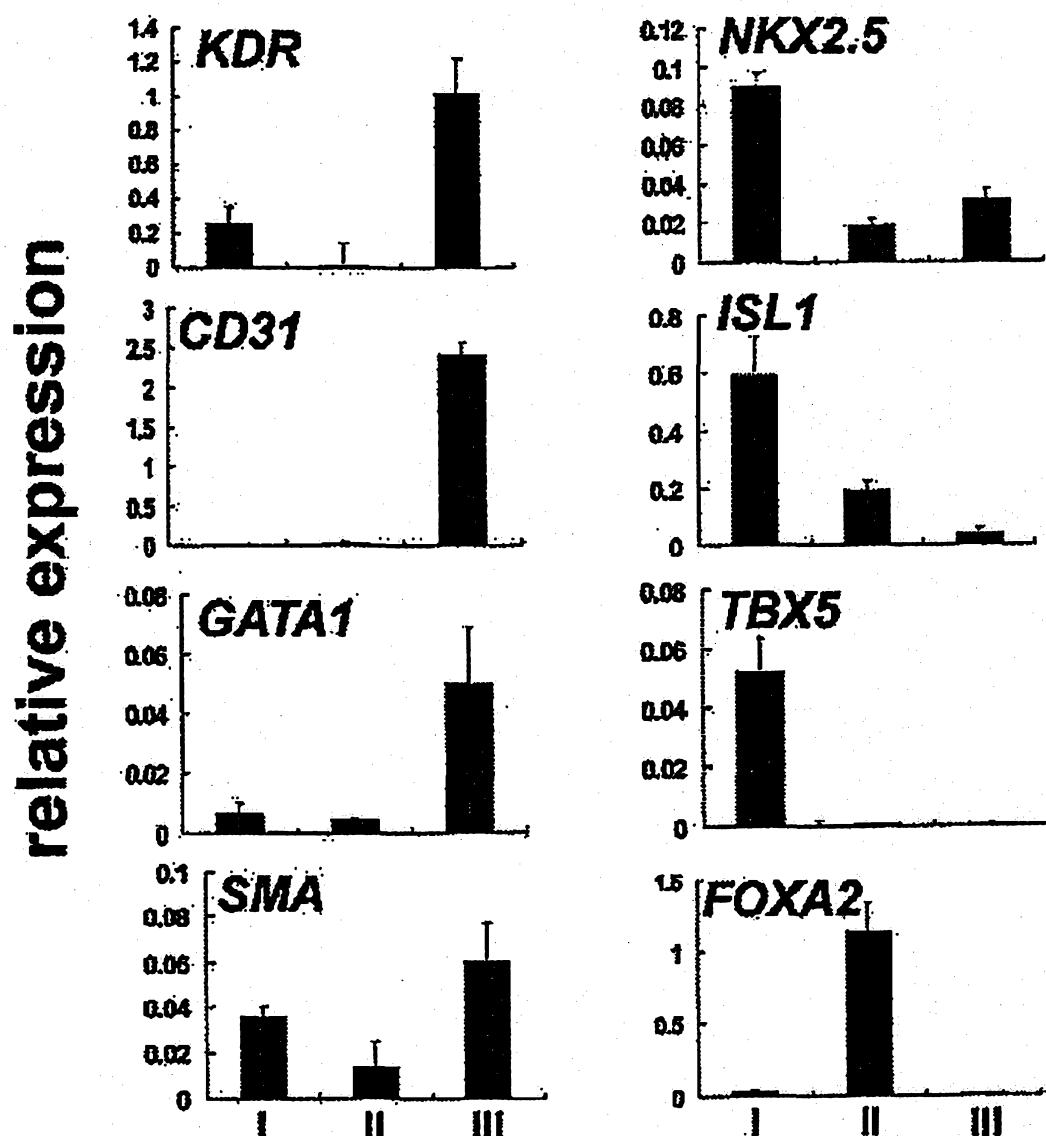


Fig. 8

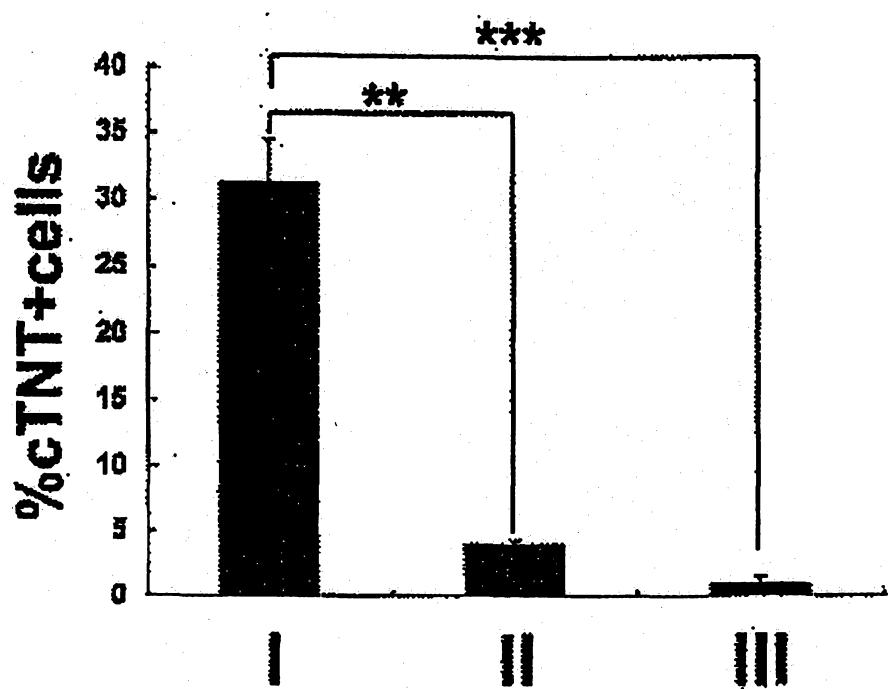


Fig. 9

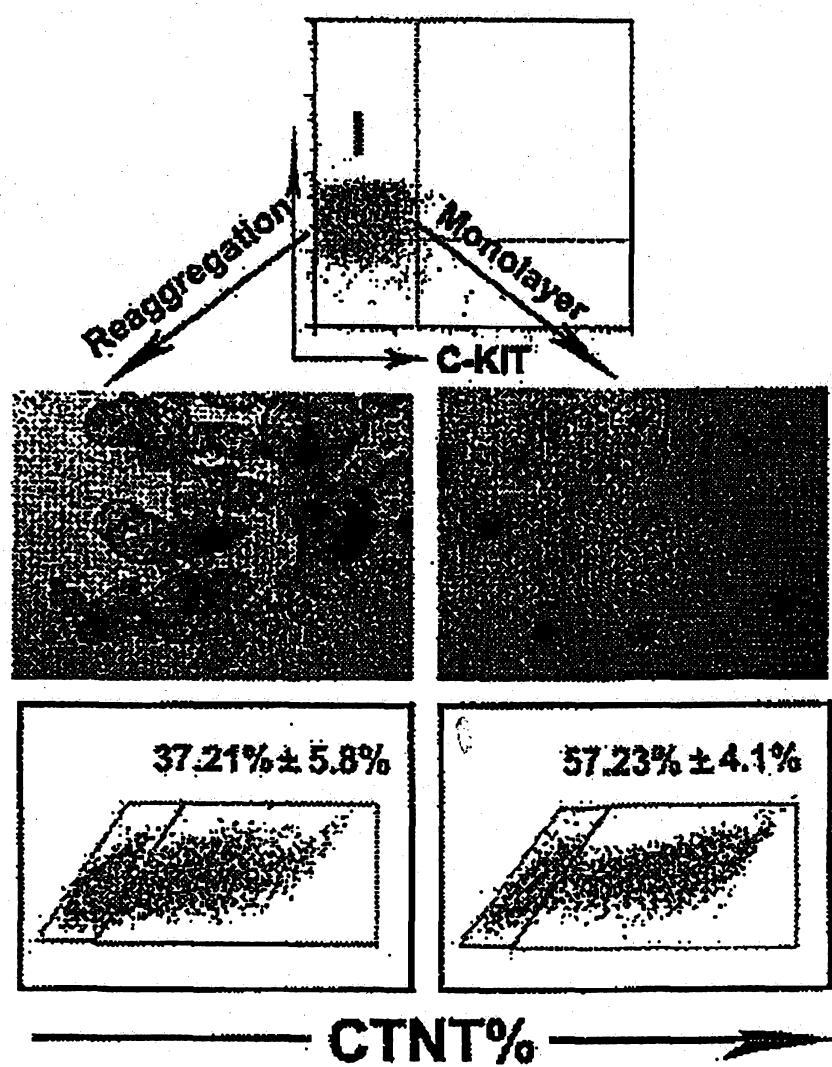


Fig. 10

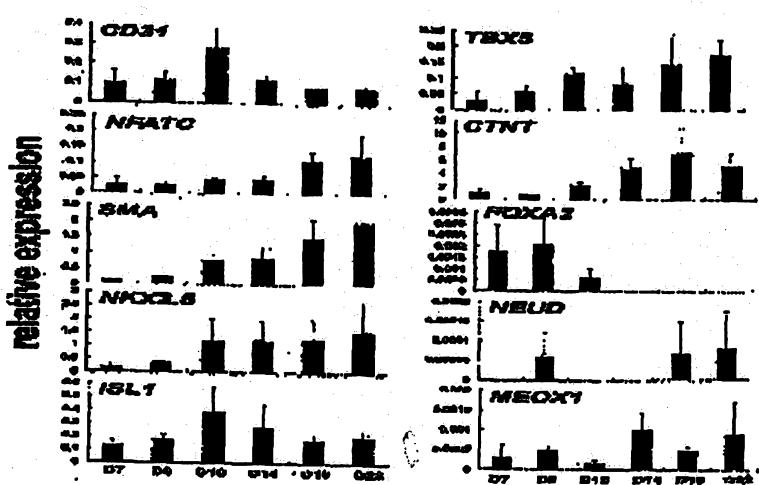


Fig. 11

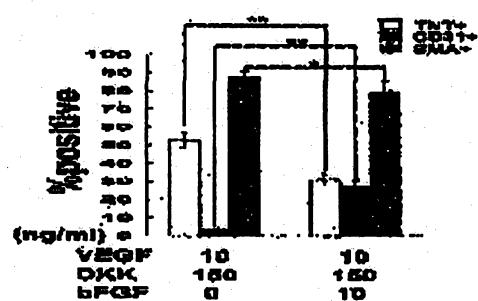


Fig. 12

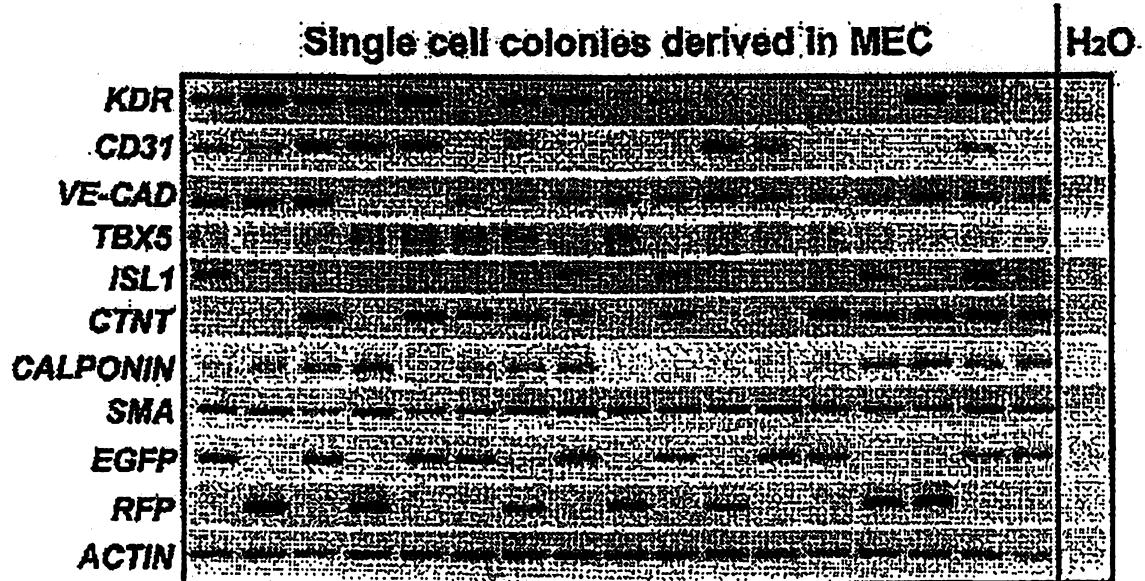


Fig. 13

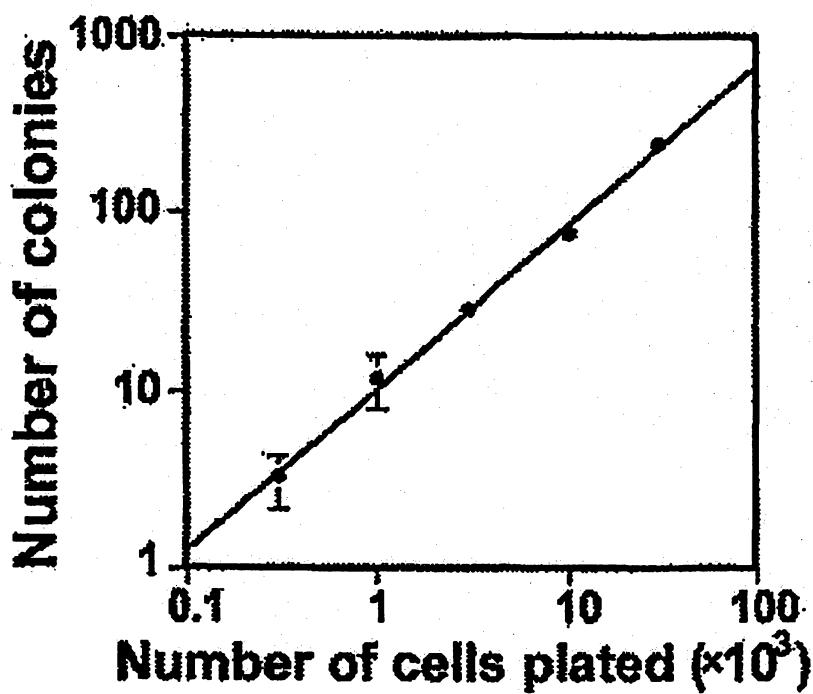


Fig. 14

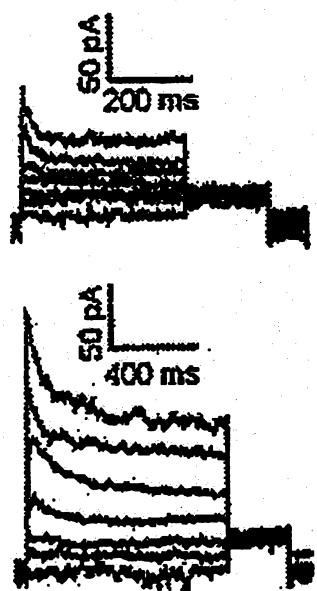


Fig. 15

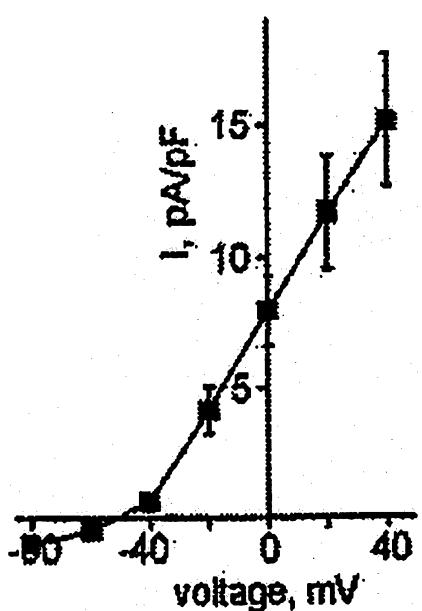


Fig. 16

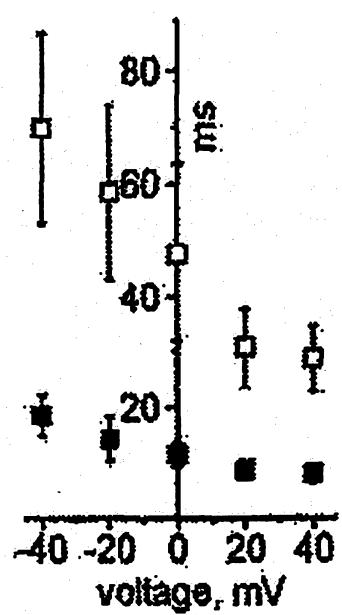


Fig. 17

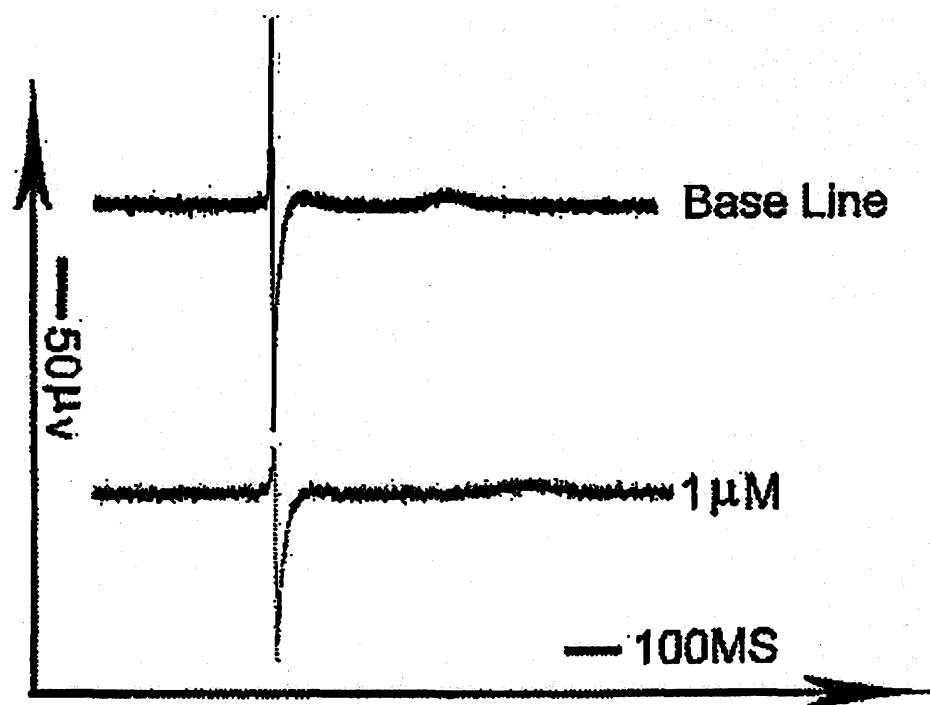


Fig. 18

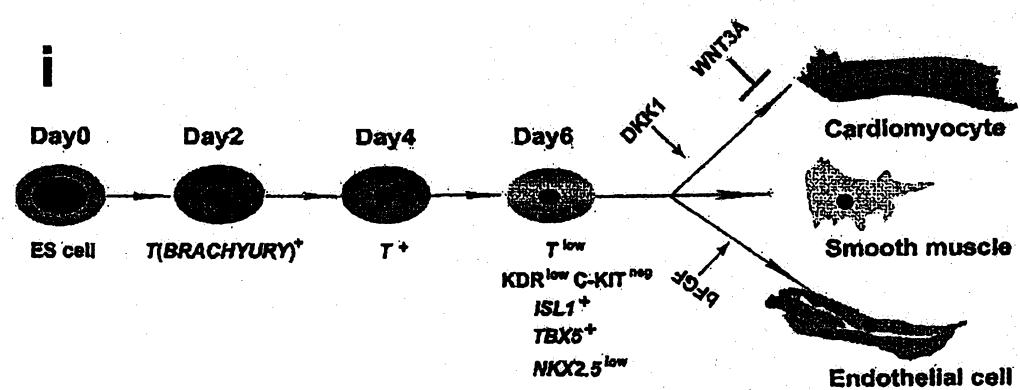


Fig. 19

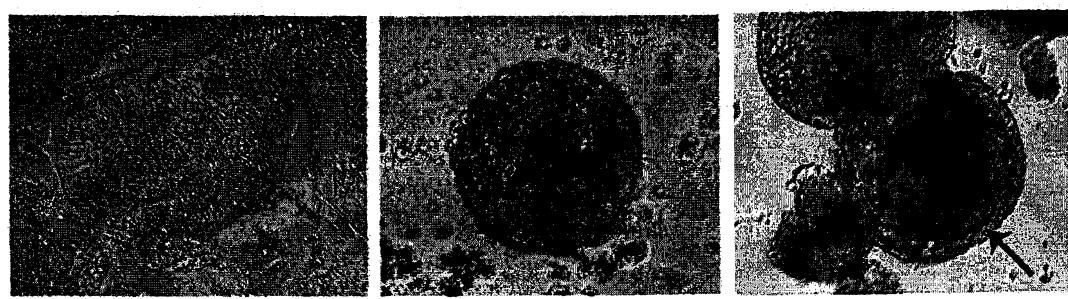


Fig. 20

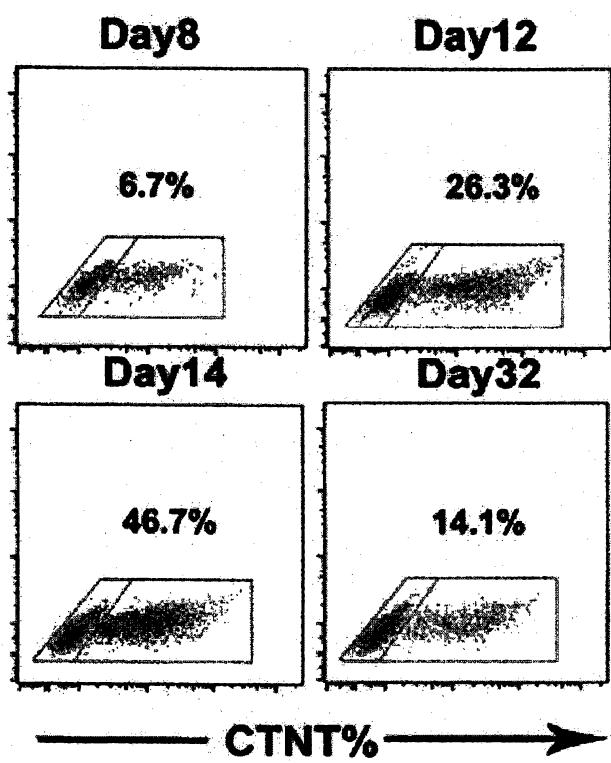


Fig. 21

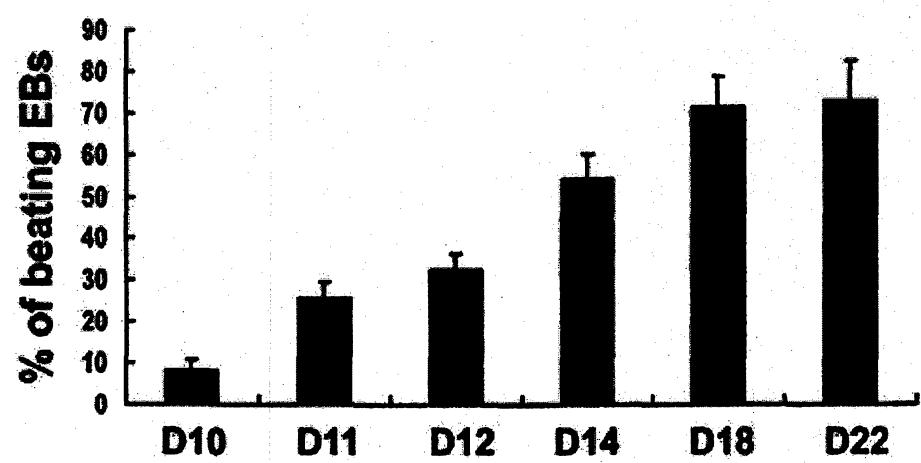


Fig. 22

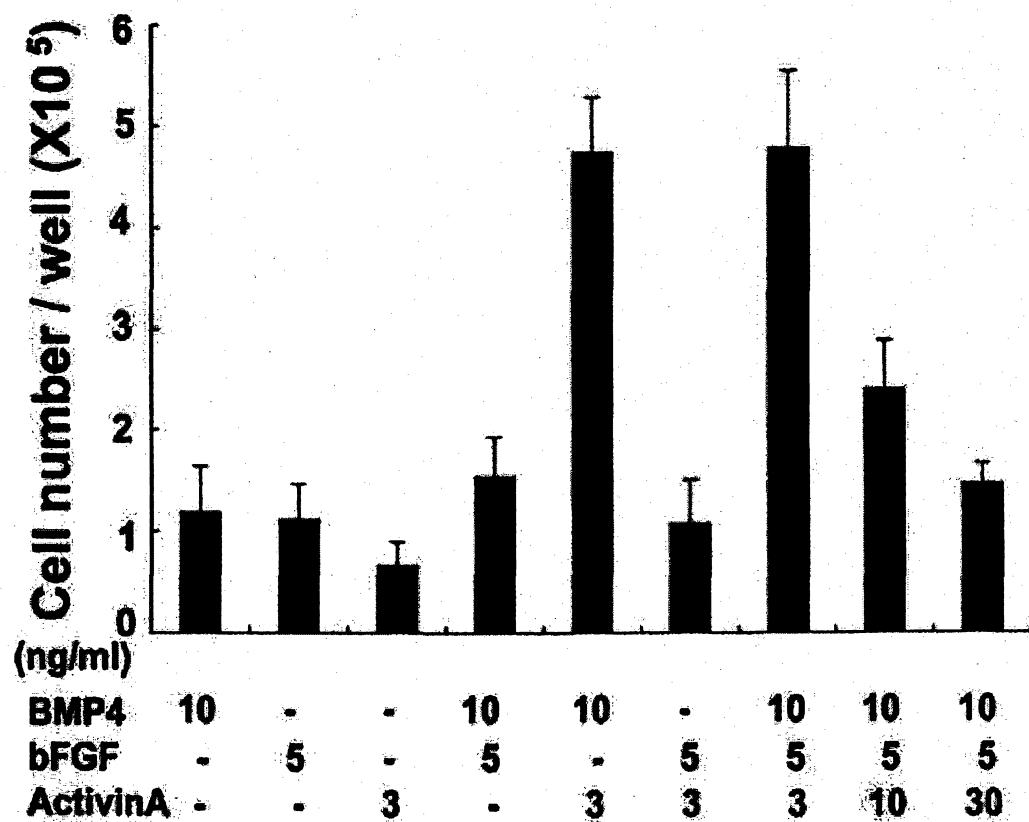


Fig. 23

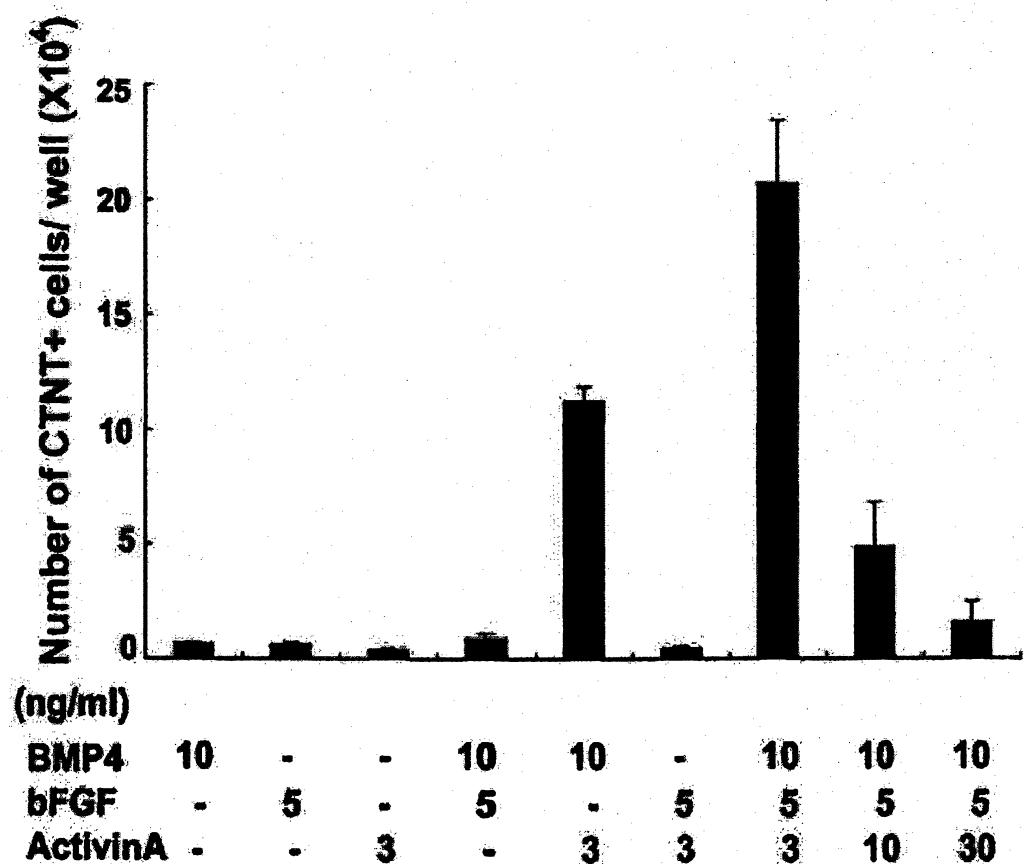


Fig. 24

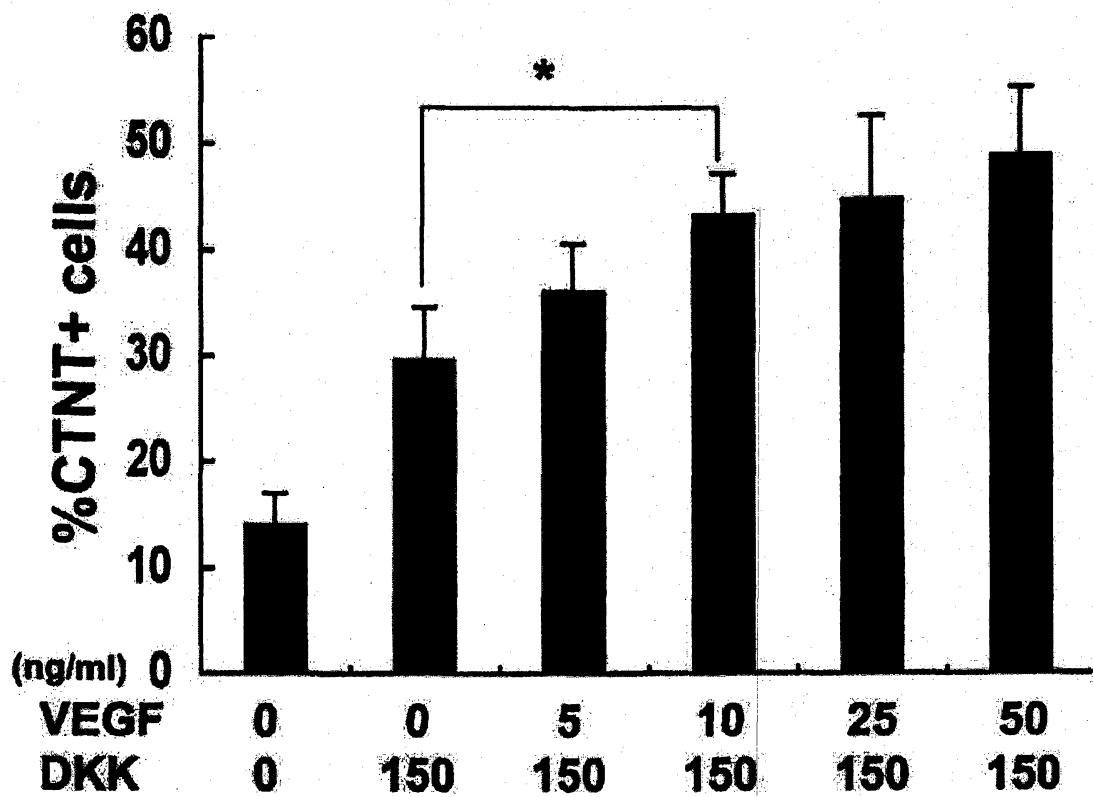


Fig. 25

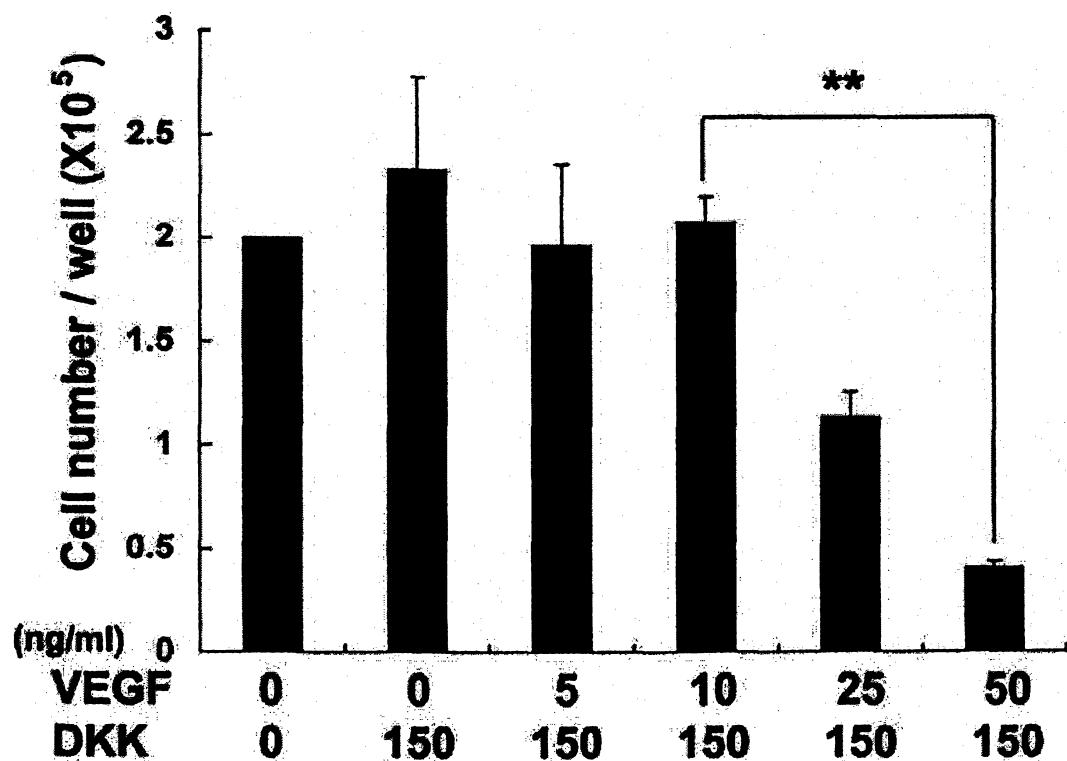


Fig. 26

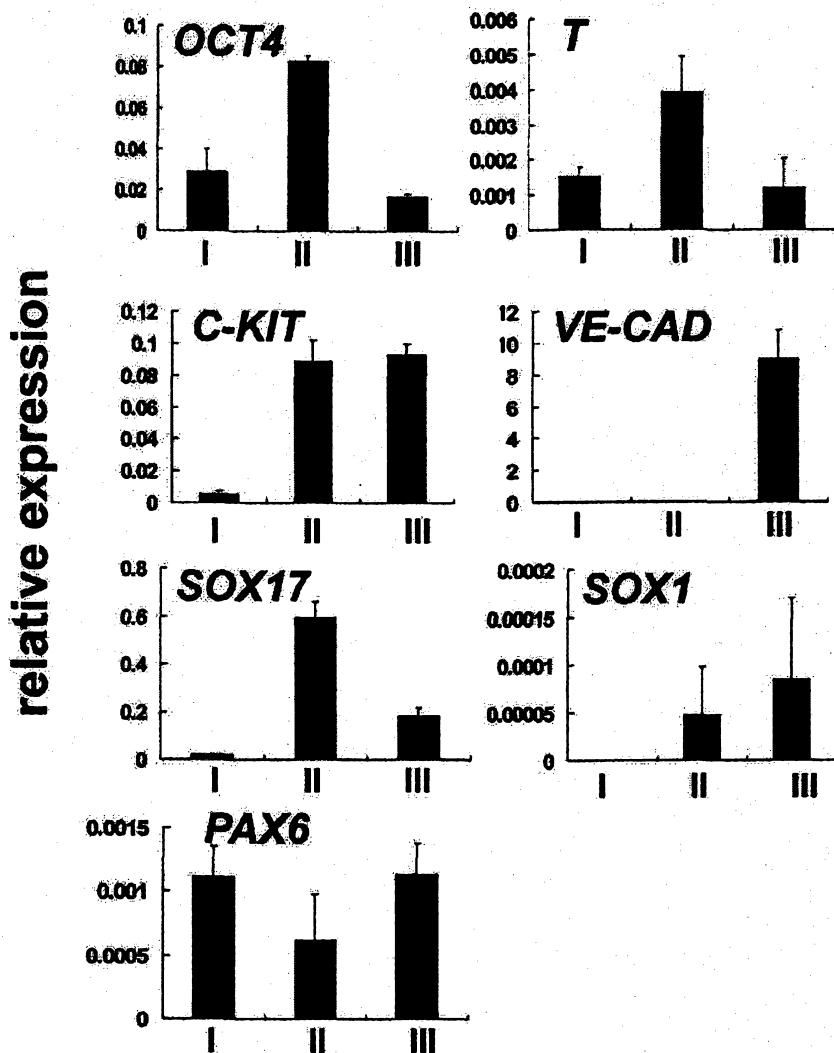


Fig. 27

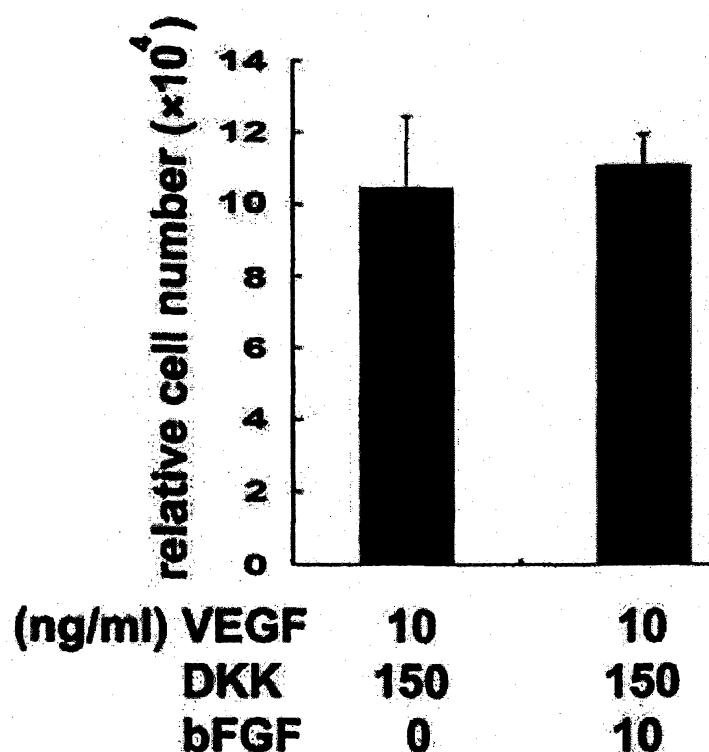


Fig. 28

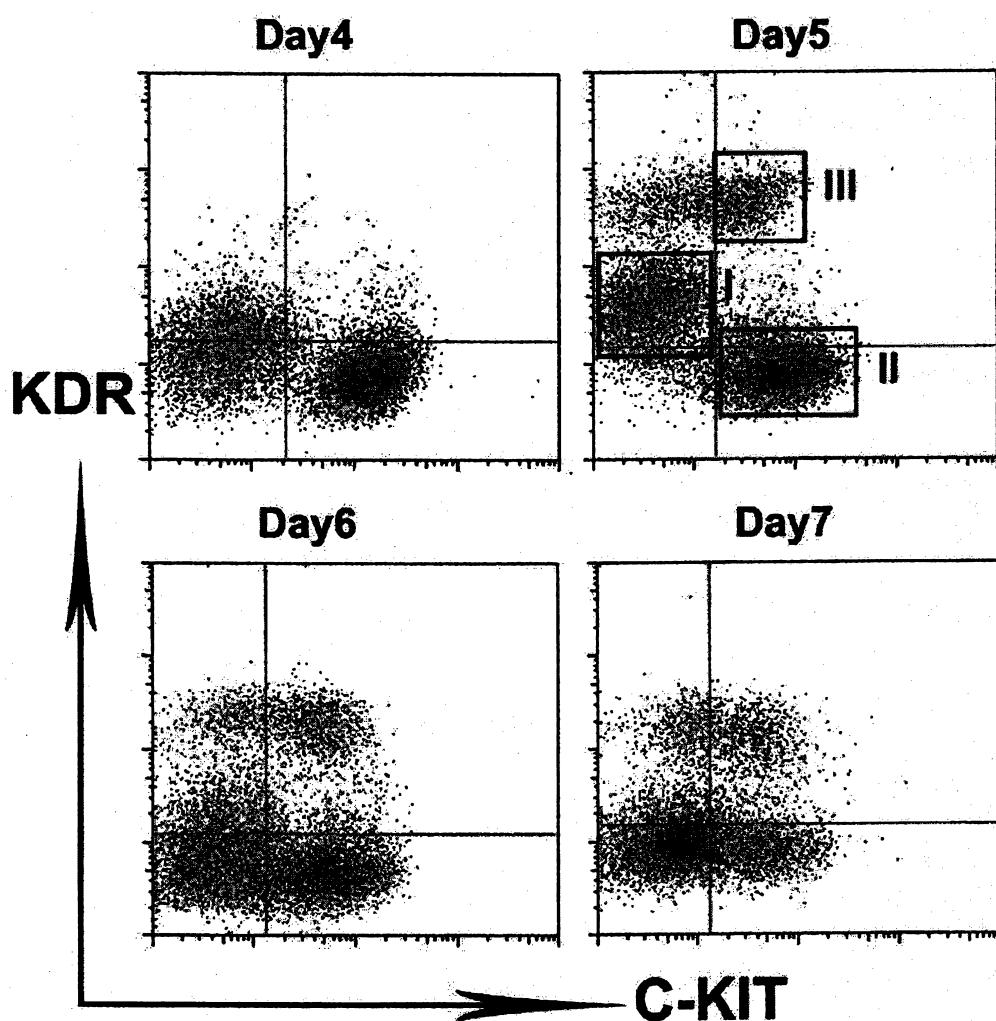


Fig. 29

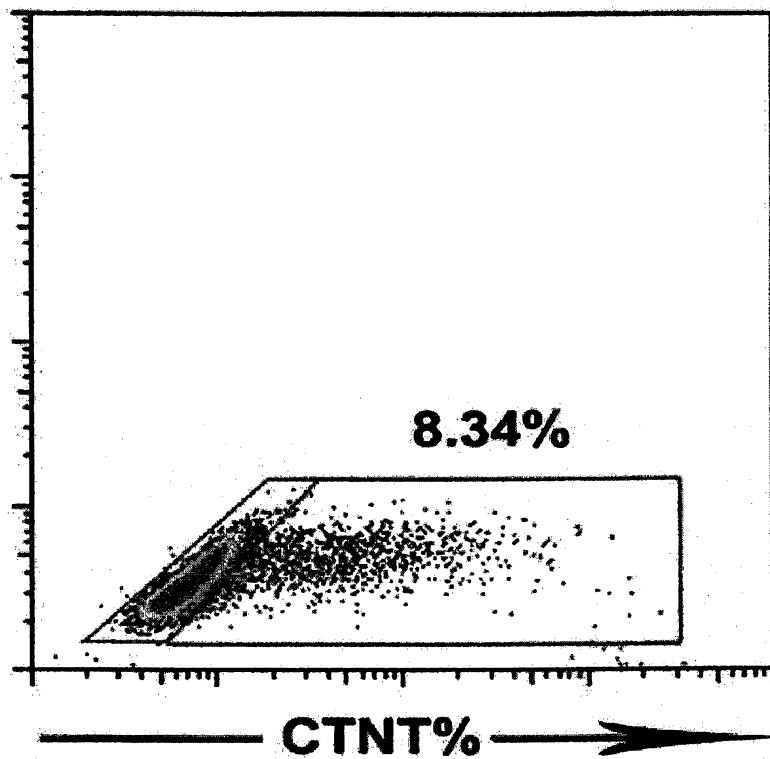


Fig. 30

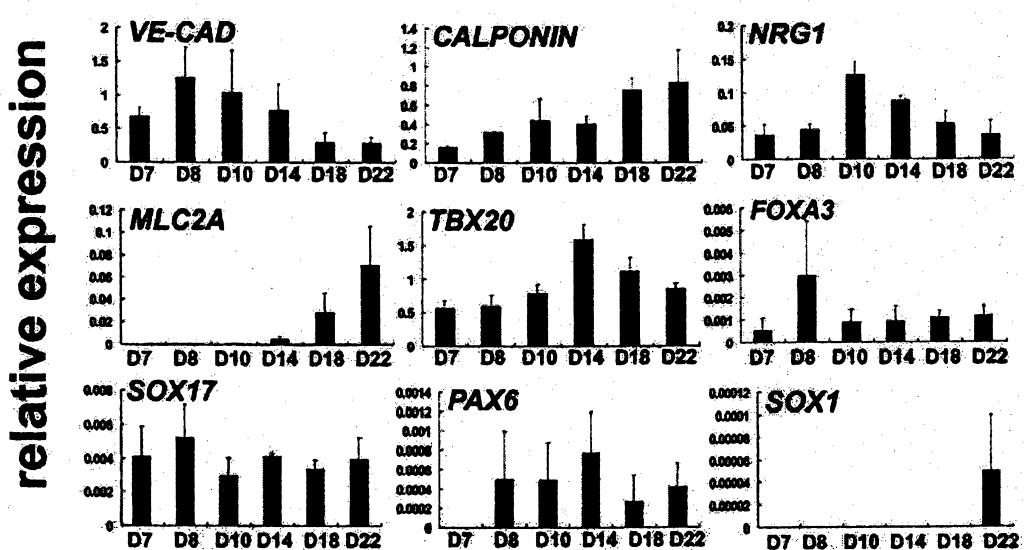


Fig. 31



Fig. 32

17 Nov 2010  
2009228354

- 1 -

SEQUENCE LISTING

<110> MOUNT SINAI SCHOOL OF MEDICINE OF NEW YORK UNIVERSITY  
KELLER, GORDON  
YANG, LEI  
KATTMAN, STEVEN

<120> HUMAN CARDIOVASCULAR PROGENITOR CELLS

<130> 31018256/EJH

<140> AU 2009228354  
<141> 2010-09-27

<150> PCT/US09/38220  
<151> 2009-03-25

<150> US 61/040,049  
<151> 2008-03-27

<160> 72

<170> PatentIn version 3.5

<210> 1  
<211> 24  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic primer

<400> 1  
tgtcccgagg ggcttacaga tgaa 24

<210> 2  
<211> 24  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic primer

<400> 2  
ggtgtgccaa agttgccaat acac 24

<210> 3  
<211> 24  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic primer

<400> 3  
aatgccactg catcttccac tggt 24

2009228354 17 Nov 2010

C:\WRP\orbNDCT\AAR\330XSNY\_1.DOC-1\11/2010

- 2 -

```
<210> 4
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 4
tggtgacagt tccttgctgt ctga 24

<210> 5
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 5
tctcagtggt gcacttacct gt 22

<210> 6
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 6
ccaagagatc cttgcgttct agact 25

<210> 7
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 7
cctctactcc agtaaacctg attggg 26

<210> 8
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 8
tgttcccaagc atttcacact atgg 24
```

17 Nov 2010  
2009228354

- 3 -

<210> 9  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 9  
cacaaggcgtc tcgggattgt gttt 24  
  
<210> 10  
<211> 20  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 10  
agtggcaagt cttccgacaa 20  
  
<210> 11  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 11  
gcgattatgc agcgtgcaat gagt 24  
  
<210> 12  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 12  
aacataaataa cgggtgggtg cgtg 24  
  
<210> 13  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 13  
gaaagaccac acagcctcat tgct 24

17 Nov 2010  
2009228354

C:\NRPon\NDCC\AA\REV10\KSK9\_1.DOC-16/11/2010

- 4 -

```
<210> 14
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 14
tcaatgtcag tgagcctgga ggaa 24

<210> 15
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 15
ttctcgccca caagctggaa taca 24

<210> 16
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 16
actgggtgga caggtaatgg ttgt 24

<210> 17
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 17
acctgggtgga gttcaagacc atct 24

<210> 18
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 18
acctctacaa atgtggatg gctg 24
```

17 Nov 2010  
2009228354

- 5 -

<210> 19  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 19  
atcatttcta gcgcatggcc tggt 24  
  
<210> 20  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 20  
atttgtggag ggcgaggtca taga 24  
  
<210> 21  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 21  
tttgaatgat gagccttcgt cccc 24  
  
<210> 22  
<211> 26  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 22  
ggtctcaagt cagtgtacag gtaagc 26  
  
<210> 23  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 23  
aacctggagt ttgtgccagg gttt 24

17 Nov 2010  
2009228354

C:\NRP\orbNDCl\AAR\1310589\_1.DOC-16/11/2010

- 6 -

<210> 24  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 24  
tgaacttcac cttccctcca acca 24  
  
<210> 25  
<211> 23  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 25  
attcccaagcc catgagtcct tga 23  
  
<210> 26  
<211> 22  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 26  
acacgtggaa caccaacatc ct 22  
  
<210> 27  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 27  
aaggacgcac tgagcaacgc tatt 24  
  
<210> 28  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 28  
acgccactgt cacatccaca tagt 24

2009228354 17 Nov 2010

- 7 -

<210> 29  
<211> 26  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 29  
aatgaaacc cagcatagga gctggc 26  
  
<210> 30  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 30  
acactcagcc tcacatctta ccct 24  
  
<210> 31  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 31  
ttcaccaaag atctgctcct cgct 24  
  
<210> 32  
<211> 26  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 32  
ttattactgg tgtggagtgg gtgtgg 26  
  
<210> 33  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 33  
acatcatcac ccacggagaa gaga 24

17 Nov 2010  
2009228354

- 8 -

```
<210> 34
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 34
attggaacat ggcctctgga ttga 24

<210> 35
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 35
tggagaagtgcatcgtca acag 24

<210> 36
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 36
tctacaatcc cttgcagtgt gag 23

<210> 37
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 37
aatactctgt ctggatcggt ggct 24

<210> 38
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 38
acgagtcaga gctttggcta ggaa 24
```

- 9 -

```
<210> 39
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 39
ttgacctgaa ctcgtgcctt agga 24

<210> 40
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 40
ggccttcagg ttgtttcttt ccgt 24

<210> 41
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 41
cccatgggtgg gttgtcatat attcatgt 28

<210> 42
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 42
ccagcatcac atctcaaaca gcac 24

<210> 43
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 43
tctatgacct cgccctccac aaa 23
```

2009228354 17 Nov 2010

- 10 -

<210> 44  
<211> 27  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic primer

<400> 44  
gaacgggtgtc ttcaggttgg tatttca 27

<210> 45  
<211> 24  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic primer

<400> 45  
ttgtacggga tcaaattgcgc caag 24

<210> 46  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic primer

<400> 46  
aggccacacaca gcggaaaca 19

<210> 47  
<211> 22  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic primer

<400> 47  
acctcaacacag ctccctgact ct 22

<210> 48  
<211> 23  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic primer

<400> 48  
ataatcgccg ccacaaaactc tcc 23

2009228354 17 Nov 2010

- 11 -

<210> 49  
<211> 26  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 49  
gtctctcagc tcctggat catctt 26  
  
<210> 50  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 50  
tggatgtatg gatgtgtgc tggt . 24  
  
<210> 51  
<211> 21  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 51  
tggaaacgtg tgaaggagg t 21  
  
<210> 52  
<211> 25  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 52  
aaggaagagg gtgaaggtgg gattg 25  
  
<210> 53  
<211> 23  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 53  
agttcagctc cttccaccac aac 23

17 Nov 2010  
2009228354

- 12 -

<210> 54  
<211> 29  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 54  
cgtttcatac tcctcatcct ccactatcc 29  
  
<210> 55  
<211> 26  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 55  
tcgaaggggcc aaatggagaa gagaag 26  
  
<210> 56  
<211> 26  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 56  
ggtgtgggttgtt ggaattggtt ggtaga 26  
  
<210> 57  
<211> 25  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 57  
cctgtgtgtta ccctggagtt tctgt 25  
  
<210> 58  
<211> 25  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 58  
tgcacgaagc acctgcaata agatg 25

2009228354 17 Nov 2010

- 13 -

<210> 59  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 59  
tgtcagtaaa cgggcaggta ctca 24  
  
<210> 60  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 60  
ataccatcct tccgcattgt cagt 24  
  
<210> 61  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 61  
aggaaatcct cagactcctg ggtt 24  
  
<210> 62  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 62  
cccaaactgt tcaagtggca gaca 24  
  
<210> 63  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 63  
gcattcccaa tcttgacacg gtga 24

- 14 -

<210> 64  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 64  
gcccttgcag ccagaataca catt 24  
  
<210> 65  
<211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 65  
ttggccatgt cgtcaccatt ctct 24  
  
<210> 66  
<211> 21  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 66  
cccacacccct aaccagcctt t 21  
  
<210> 67  
<211> 28  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 67  
acttttggaaag acagaaccaa attatctc 28  
  
<210> 68  
<211> 17  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic primer  
  
<400> 68  
tgggcaccat tccacca 17

2009228354 17 Nov 2010

- 15 -

```
<210> 69
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 69
cagtggcagt ctcaggttaa gaagga 26

<210> 70
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 70
cgctactgca ggtgtgagca a 21

<210> 71
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 71
gaagagtgcg atcaagaacc catgac 26

<210> 72
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic primer

<400> 72
gtctctctc cttctctcc tatcttact t 31
```