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(71) Demandeur/Applicant:
PROCELLA THERAPEUTICS AB, SE

(72) Inventeurs/Inventors:
CHIEN, KENNETH R., US;
CLARKE, JONATHAN, SE;
LEUNG, CHUEN YAN, SE

(74) Agent: BORDEN LADNER GERVAIS LLP

(54) Titre : UTILISATION DE NEUROPILINE-1 (NRP1) EN TANT QUE MARQUEUR DE SURFACE CELLULAIRE POUR ISOLER DES CELLULES PROGENITRICES VENTRICULAIRES CARDIAQUES HUMAINES

(54) Title: USE OF NEUROPILIN-1 (NRP1) AS A CELL SURFACE MARKER FOR ISOLATING HUMAN CARDIAC VENTRICULAR PROGENITOR CELLS

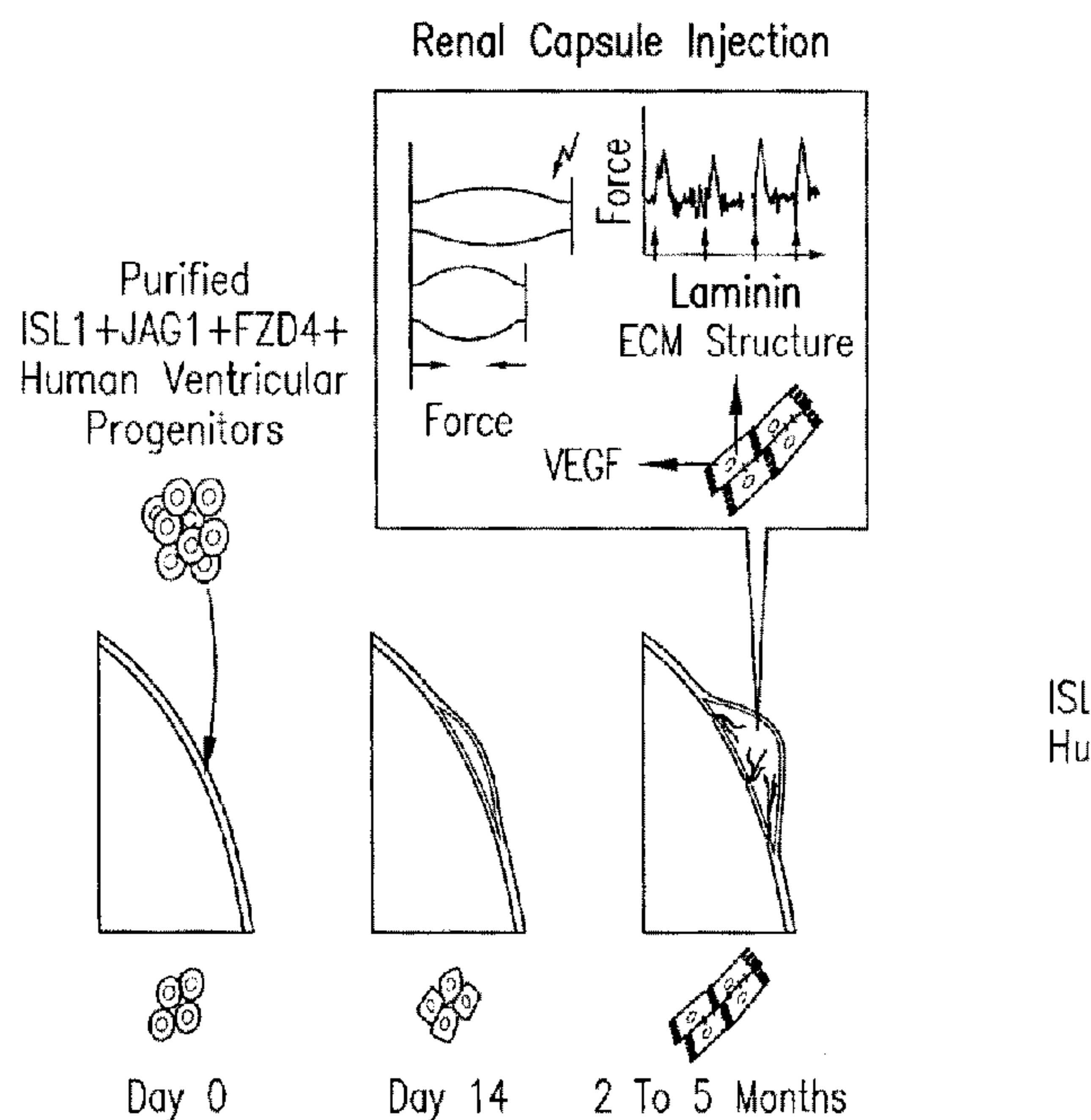


FIG.8A

(57) **Abrégé/Abstract:**

The present invention provides NRP1 as a cell surface marker for isolating human cardiomyogenic ventricular progenitor cells (HVPs), in particular progenitor cells that preferentially differentiate into cardiac ventricular muscle cells. Additional HVP cell surface markers identified by single cell sequencing are also provided. The invention provides in vitro methods of the separation of NRP1+ ventricular progenitor cells, and the large scale expansion and propagation thereof. Large clonal populations of isolated NRP1+ ventricular progenitor cells are also provided. Methods of in vivo use of NRP1+ ventricular progenitor cells for cardiac repair or to improve cardiac function are also provided. Methods of using the NRP1+ ventricular progenitor cells for cardiac toxicity screening of test compounds are also provided.

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(71) Applicant: PROCELLA THERAPEUTICS AB [—/SE];
c/o Bactiguard AB, Box 15, 146 21 Tullinge (SE).

(72) Inventors: CHIEN, Kenneth, R.; 19 Saville Street, Cambridge, MA 02138 (US). CLARKE, Jonathan; Bergsunds Strand 15, 11738 Stockholm (SE). LEUNG, Chuen, Yan; Vulcanusgatan 4, 113 21 Stockholm (SE).

(74) Agent: TUXWORTH, Pamela Mary et al.; J A Kemp, 14 South Square, Gray's Inn, London Greater London WC1R 5JJ (GB).

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(54) Title: USE OF NEUROPILIN-1 (NRP1) AS A CELL SURFACE MARKER FOR ISOLATING HUMAN CARDIAC VENTRICULAR PROGENITOR CELLS

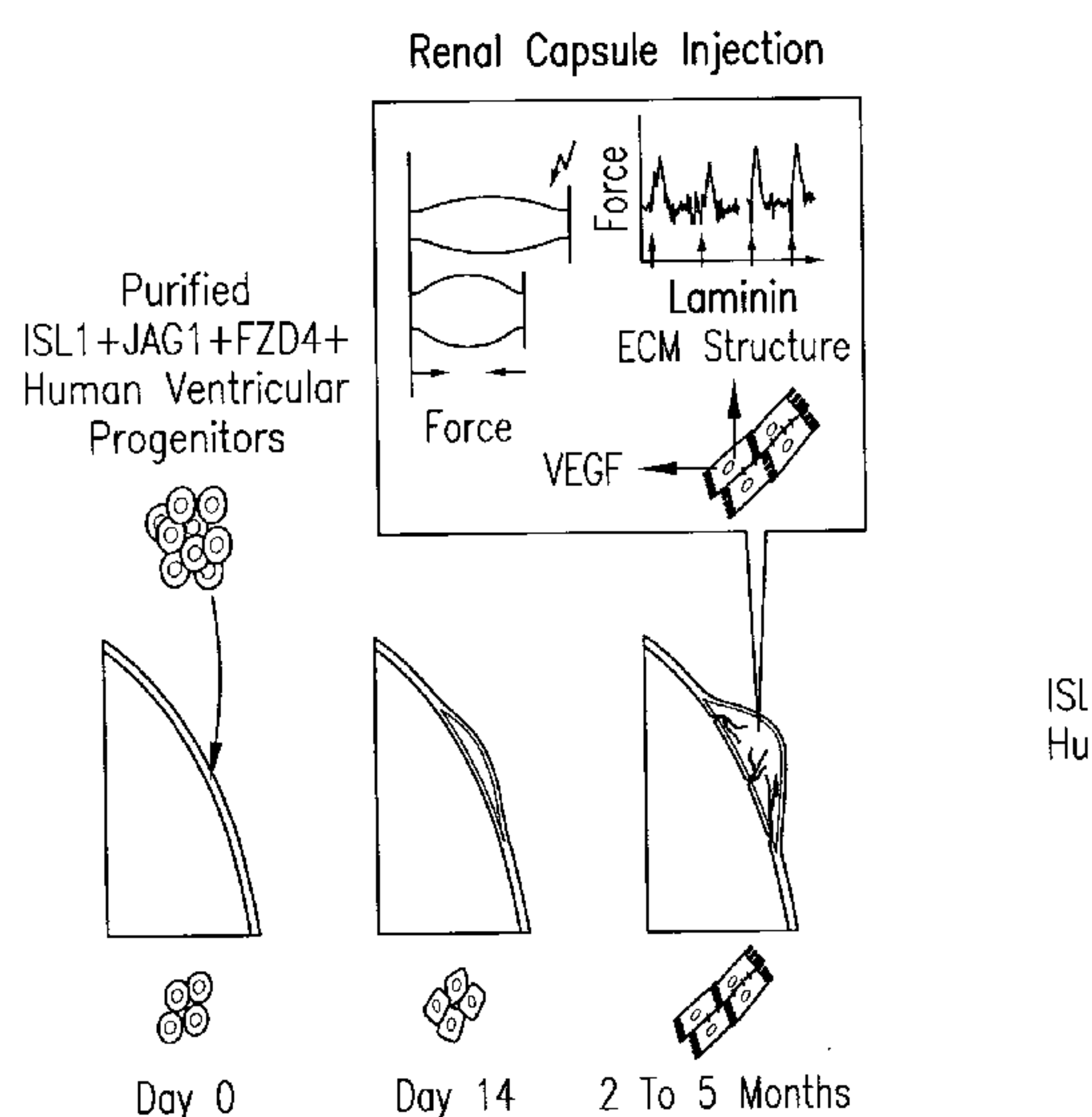


FIG.8A

(57) Abstract: The present invention provides NRP1 as a cell surface marker for isolating human cardiomyogenic ventricular progenitor cells (HVPs), in particular progenitor cells that preferentially differentiate into cardiac ventricular muscle cells. Additional HVP cell surface markers identified by single cell sequencing are also provided. The invention provides in vitro methods of the separation of NRP1+ ventricular progenitor cells, and the large scale expansion and propagation thereof. Large clonal populations of isolated NRP1+ ventricular progenitor cells are also provided. Methods of in vivo use of NRP1+ ventricular progenitor cells for cardiac repair or to improve cardiac function are also provided. Methods of using the NRP1+ ventricular progenitor cells for cardiac toxicity screening of test compounds are also provided.

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USE OF NEUROPILIN-1 (NRP1) AS A CELL SURFACE MARKER FOR ISOLATING HUMAN CARDIAC VENTRICULAR PROGENITOR CELLS

Related Application

5 This application claims the benefit of the priority date of U.S. Provisional Application No. 62/549,345, which was filed on August 23, 2017. The content of this provisional application is hereby incorporated by reference in its entirety.

Background of the Invention

10 Heart failure, predominantly caused by myocardial infarction, is the leading cause of death in both adults and children worldwide and is increasing exponentially worldwide (Bui, A.L. *et al.* (2011) *Nat. Rev. Cardiol.* 8:30-41). The disease is primarily driven by the loss of ventricular muscle that occurs during myocardial injury (Lin, Z. and Pu, W.T. (2014) *Sci. Transl. Med.* 6:239rv1) and is compounded by the negligible ability of the adult heart to
15 mount a regenerative response (Bergmann, O. *et al.* (2009) *Science* 324:98-102; Senyo, S.E. *et al.* (2013) *Nature* 493:433-436). Although heart transplantation can be curative, the markedly limited availability of human heart organ donors has led to a widespread unmet clinical need for a renewable source of pure, mature and functional human ventricular muscle tissue (Segers, V.F.M. and Lee, R.J. (2008) *Nature* 451:937-942; Später, D. *et al.* (2014)
20 *Development* 141:4418-4431).

 Human pluripotent stem cells (hPSCs) offer the potential to generate large numbers of functional cardiomyocytes for potential clinical restoration of function in damaged or diseased hearts. Transplantation of stem cells into the heart to improve cardiac function and/or to enrich and regenerate damaged myocardium has been proposed (see *e.g.*, U.S.
25 Patent Publication 20040180043). Combination therapy, in which adult stem cells are administered in combination with treatment with growth factor proteins has also been proposed (see *e.g.*, U.S. Patent Publication 20050214260).

 While cell transplantation into the heart offers a promising approach for improving cardiac function and regenerating heart tissue, the question of what type(s) of cells to
30 transplant has been the subject of much investigation. Types of cells investigated for use in regenerating cardiac tissue include bone marrow cells (see *e.g.*, Orlic, D. *et al.* (2001) *Nature* 410:701-705; Stamm, C. *et al.* (2003) *Lancet* 361:45-46; Perin, E.C. *et al.* (2003) *Circulation* 107:2294-2302), adult stem cells (see *e.g.*, Jackson, K.A. *et al.* (2001) *J. Clin. Invest.* 107:1395-1402), bone marrow-derived mesenchymal stem cells (see *e.g.*, Barbash, I.M. *et al.*

(2003) *Circulation* 108:863; Pettinger, M.F. and Martin, B.J. (2003) *Circ. Res.* 95:9-20), bone marrow stromal cells (Bittira, B. *et al.* (2003) *Eur. J. Cardiothorac. Surg.* 24:393-398), a combination of mesenchymal stem cells and fetal cardiomyocytes (see *e.g.*, Min, J.Y. *et al.* (2002) *Ann. Thorac. Surg.* 74:1568-1575) and a combination of bone marrow-derived mononuclear cells and bone marrow-derived mesenchymal stem cells (see *e.g.*, U.S. Patent Publication 20080038229). Dedifferentiation of adult mammalian cardiomyocytes *in vitro* to generate cardiac stem cells for transplantation has also been proposed (see *e.g.*, U.S. Patent Publication 20100093089).

A significant advancement in the approach of cell transplantation to improve cardiac function and regenerate heart tissue was the identification and isolation of a family of multipotent cardiac progenitor cells that are capable of giving rise to cardiac myocytes, cardiac smooth muscle and cardiac endothelial cells (Cai, C.L. *et al.* (2003) *Dev. Cell.* 5:877-889; Moretti, A. *et al.* (2006) *Cell* 127:1151-1165; Bu, L. *et al.* (2009) *Nature* 460:113-117; U.S. Patent Publication 20060246446). These cardiac progenitor cells are characterized by the expression of the LIM homeodomain transcription factor Islet 1 (Isl1) and thus are referred to as Isl1+ cardiac progenitor cells. (*Ibid*). In contrast, Isl1 is not expressed in differentiated cardiac cells. Additional markers of the Isl1+ cardiac progenitor cells that arise later in differentiation than Isl1 have been described and include Nkx2.5 and flk1 (see *e.g.*, U.S. Patent Publication 20100166714).

The renewal and differentiation of Isl1+ cardiac progenitor cells has been shown to be regulated by a Wnt/beta-catenin signaling pathway (see *e.g.*, Qyang, Y. *et al.* (2007) *Cell Stem Cell.* 1:165-179; Kwon, C. *et al.* (2007) *Proc. Natl. Acad. Sci. USA* 104:10894-10899). This has led to the development of methods to induce a pluripotent stem cell to enter the Isl1+ lineage and for expansion of the Isl1+ population through modulation of Wnt signaling (see *e.g.*, Lian, X. *et al.* (2012) *Proc. Natl. Acad. Sci. USA* 109:E1848-57; Lian, X. *et al.* (2013) *Nat. Protoc.* 8:162-175; U.S. Patent Publication 20110033430; U.S. Patent Publication 20130189785).

While human pluripotent stem cells hold great promise, a significant challenge has been the ability to move from simply differentiation of diverse cardiac cells to forming a larger scale pure 3D ventricular muscle tissue *in vivo*, which ultimately requires vascularization, assembly and alignment of an extracellular matrix, and maturation. Toward that end, a diverse population of cardiac cells (atrial, ventricular, pacemaker) has been coupled with artificial and decellurized matrices (Masumoto, H. *et al.* (2014) *Sci. Rep.* 4:5716; Ott, H.C. *et al.* (2008) *Nat. Med.* 14:213-221; Schaaf, S. *et al.* (2011) *PLoS One*

6:e26397), vascular cells and conduits (Tulloch, N.L. *et al.* (2011) *Circ. Res.* 109:47-59) and cocktails of microRNAs (Gama-Garvalho, M. *et al.* (2014) *Cells* 3:996-1026) have been studies, but the goal remains elusive.

While the identification of Isl1 as a marker of cardiac progenitor cells was a significant advance, since Isl1 is an intracellular protein it is not a suitable marker for use in isolating large quantities of viable cells. Rather, a cell surface marker(s) is still needed. Furthermore, Isl1 as a marker identifies a population that can differentiate into multiple cell types within the cardiac lineage, and thus there is still a need for markers that identify cardiac progenitor cells that are biased toward a particular cell type within the cardiac lineage, in particular for progenitor cells that differentiate into ventricular cells. Accordingly, there is still a great need in the art for additional markers of cardiac progenitor cells, in particular cell-surface markers of cardiac progenitor cells, that predominantly give rise to cardiomyocytes and that would allow for rapid isolation and large scale expansion of cardiomyogenic progenitor cells. Furthermore, there is still a great need in the art for methods and compositions for isolating cardiac ventricular progenitors, which differentiate into ventricular muscle cells *in vivo*, thereby allowing for transplantation of ventricular progenitors or ventricular muscle cells *in vivo* to enhance cardiac function.

Summary of the Invention

This invention describes the use of Neuropilin-1 (NRP1) as a cell surface marker for isolating human cardiac ventricular progenitor cells. Furthermore, additional cell surface markers suitable for use in isolating human cardiac ventricular progenitor cells are provided, as shown in Tables 1, 5 and 10. These human cardiac progenitor cells are biased toward the ventricular lineage such that they differentiate predominantly into ventricular muscle cells both *in vitro* and *in vivo*. That is, these cardiac progenitor cells can be cultured under conditions *in vitro* such that they are biased toward the ventricular lineage and thus are human ventricular progenitor (HVP) cells. Moreover, when introduced into the ventricular region of the heart in a subject, these progenitor cells differentiate almost exclusively into ventricular muscle cells that function according to their ventricular programming. In particular, the human ventricular progenitor cells provided herein utilize a cell autonomous pathway by which these cells can build a pure 3-D vascularized, functional and mature ventricular cell wall *in vivo* on the surface of normal murine kidney or heart, thereby allowing for organ-on-organ *in vivo* tissue engineering.

Using single cell sequencing of cardiac progenitors at different stages of differentiation, a panel of genes differentially expressed in HVPs was identified, as described in Example 14 and shown in Table 10. Within this panel, day 5 progenitor cells were shown to express both Islet 1 (Isl1) and NRP1. This identification of a key cell surface marker of cardiac ventricular progenitor cells allows for easy and rapid isolation of the cells. Furthermore, determination of culture conditions for expansion and ventricular lineage bias of the cells allows for the preparation of large cultures (a billion or more cells) of a clonal population of cardiac ventricular progenitor cells. These cells can be used, for example, to improve function in a damaged heart in a subject, particularly damage in the ventricular region. The progenitor cells can be transplanted *in vivo* for differentiation into ventricular cells *in situ* or, alternatively, a heart muscle patch, comprising ventricular muscle cells, can be prepared *in vitro* from the progenitors for subsequent transplantation *in vivo*. The cells also can be used, for example, in *in vitro* toxicity screening assays to evaluate the cardiac toxicity of test compounds, as well as for biochemical studies to identify relevant pathways used in cardiac maturation and differentiation.

Thus, the invention provides human cardiac ventricular progenitor cells in purified form. The human cardiac ventricular progenitors are capable of differentiation into ventricular muscle cells *in vitro* and *in vivo*. These progenitor cells can be expanded to large numbers of cells *in vitro* and when transplanted into the ventricular region of the heart *in vivo* they differentiate essentially exclusively into ventricular muscle cells. Still further, the cells have the capacity to migrate *in vivo* to different sites and, when transplanted *in vivo* the cells does what they are programmed to do as a ventricular cell (as opposed to a cardiac myocyte which simply contracts). Thus, the ventricular progenitor cells can be grafted to native tissue to enhance ventricular function and have the ability to call in vasculature into the new ventricular tissue.

Accordingly, in one aspect, the invention pertains to a method for isolating human cardiac ventricular progenitor cells, the method comprising:

contacting a culture of cells containing human cardiac progenitor cells with one or more agents reactive with NRP1; and

separating NRP1 reactive positive cells from non-reactive cells to thereby isolate human cardiac ventricular progenitor cells.

In one embodiment, the human cardiac progenitor cells are contacted both with an agent reactive with NRP1 and with at least one second agent that binds to an HVP marker(s)

(as described herein) to thereby separate NRP1/second agent reactive positive cells from non-reactive cells.

Preferably, the human cardiac progenitor cells are Islet 1+ human cardiac progenitor cells. Preferably, the human cardiac progenitor cells are JAG1+, FZD4+, LIFR+, FGFR3+ and/or TNFSF9+. In another embodiment, the culture of cells is also contacted with at least one second agent; and NRP1 reactive/second agent reactive positive cells are separated from non-reactive cells to thereby isolate cardiac ventricular progenitor cells. In one embodiment, the at least one second agent binds a marker selected from JAG1+, FZD4+, LIFR+, FGFR3+ and/or TNFSF9+. The culture of cells can be simultaneously contacted with the agent reactive with NRP1 and the at least one second agent. Alternatively, the culture of cells can be contacted with the at least one second agent before contacting with the agent reactive with NRP1. Alternatively, the culture of cells can be contacted with the agent reactive with NRP1 before contacting with the at least one second agent. In another embodiment, the culture of cells is also negatively selected for lack of expression of at least one marker of pluripotent stem cells, such as TRA-1-60. In another embodiment, the human cardiac ventricular progenitor cells are further cultured and differentiated such that they express the ventricular marker MLC2v. In certain embodiments, the starting culture of cells containing human cardiac progenitor cells is derived from human embryonic stem cells or human induced pluripotent cells.

In another aspect, the invention pertains to a method for isolating human cardiac ventricular progenitor cells, the method comprising:

culturing human pluripotent stem cells under conditions that generate cardiac progenitor cells to obtain a cultured cell population;
contacting the cultured cell population with one or more agents reactive with NRP1;
and
separating NRP1 reactive positive cells from non-reactive cells to thereby isolate human cardiac ventricular progenitor cells.

In another embodiment, the culture of cells is also contacted with at least one second agent; and NRP1 reactive/second agent reactive positive cells are separated from non-reactive cells to thereby isolate cardiac ventricular progenitor cells. In one embodiment, the at least one second agent binds a marker selected from JAG1+, FZD4+, LIFR+, FGFR3+ and/or TNFSF9+. The culture of cells can be simultaneously contacted with the agent reactive with NRP1 and the at least one second agent. Alternatively, the culture of cells can be contacted with the at least one second agent before contacting with the agent reactive with NRP1.

Alternatively, the culture of cells can be contacted with the agent reactive with NRP1 before contacting with the at least one second agent. In another embodiment, the culture of cells is also negatively selected for lack of expression of at least one marker of pluripotent stem cells, such as TRA-1-60. In another embodiment, the human cardiac ventricular progenitor cells are further cultured and differentiated such that they express the ventricular marker MLC2v. In certain embodiments, the starting culture of cells containing human cardiac progenitor cells is derived from human embryonic stem cells or human induced pluripotent cells.

In the methods for isolating human cardiac ventricular progenitor cells, various types of agents that bind to NRP1 can be used as the agent(s) reactive with NRP1. For example, in one embodiment, the agent reactive with NRP1 is an anti-NRP1 antibody, such as a monoclonal antibody. In another embodiment, the agent reactive with NRP1 is a soluble NRP1 ligand, such as a NRP1 ligand fusion protein. For example, the agent reactive with NRP1 can comprise the NRP1 ligand VEGF-A or Sema3A, such as a soluble fusion protein (e.g., an Ig fusion protein).

In the methods for isolating human cardiac ventricular progenitor cells, various types of separation methods can be used to separate NRP1 reactive positive cells from non-reactive cells. For example, in one embodiment, the reactive positive cells are separated from the non-reactive cells by fluorescence activated cell sorting (FACS). In another embodiment, the reactive positive cells are separated from the non-reactive cells by magnetic activated cells sorting (MACS).

In yet another aspect, the invention pertains to a method of obtaining a clonal population of human cardiac ventricular progenitor cells, the method comprising:

isolating a single NRP1+ human cardiac ventricular progenitor cell; and

culturing the single NRP1+ human cardiac ventricular progenitor cell under

conditions such that the cell is expanded to at least 1×10^9 cells to thereby obtain a clonal population of human cardiac ventricular progenitor cells.

In one embodiment, the single NRP1+ human cardiac ventricular progenitor cell is Islet 1 positive, Nkx2.5 negative and flk1 negative at the time of initial culture. The single NRP1+ human cardiac ventricular progenitor cell can be isolated by methods such as those described above (e.g., FACS or MACS). The single NRP1+ human cardiac ventricular progenitor cell can be isolated using a reagent(s) reactive with NRP1, such as those described above (e.g., anti-NRP1 antibodies, soluble NRP1 ligands, such as ligand fusion proteins). Upon further culture and differentiation, the clonal population of human cardiac ventricular progenitor cells can express the ventricular marker MLCV2.

In a preferred embodiment, the single NRP1+ human cardiac ventricular progenitor cell is cultured *in vitro* under conditions such that the cell is biased toward ventricular differentiation. For example, the single NRP1+ human cardiac ventricular progenitor cell can be cultured in Cardiac Progenitor Culture (CPC) medium (80% advanced DMEM/F12 supplemented with 20% KnockOut Serum Replacement, 2.5 mM GlutaMax and 100 µg/ml Vitamin C), which allows for differentiation of the cells into ventricular cells expressing the MLC2v ventricular marker. In various embodiments, the single NRP1+ human cardiac ventricular progenitor cell is expanded to a clonal population of, for example, at least 1×10^9 cells, at least 2×10^9 cells, at least 5×10^9 cells or at least 10×10^9 cells.

Accordingly in another aspect, the invention pertains to a clonal population of isolated NRP1+ human cardiac ventricular progenitor cells. In various embodiments, this clonal population comprises, for example, at least 1×10^9 cells, at least 2×10^9 cells, at least 5×10^9 cells or at least 10×10^9 cells. In a preferred embodiment, this clonal population comprises at least 1×10^9 NRP1+ human cardiac ventricular progenitor cells.

In yet another aspect, the invention pertains to a method of enhancing cardiac function in a subject using the NRP1+ human cardiac ventricular progenitor cells described herein. For example, in one embodiment, the invention provides a method of enhancing cardiac function in a subject, the method comprising administering a pharmaceutical composition comprising a clonal population NRP1+ human cardiac ventricular progenitor cells, such as a clonal population of at least at least 1×10^9 cells, at least 2×10^9 cells, at least 5×10^9 cells or at least 10×10^9 cells. In one embodiment, the clonal population is administered directly into the heart of the subject. For example, the clonal population can be administered directly into a ventricular region of the heart of the subject. In one embodiment, the pharmaceutical composition administered to the subject comprises the clonal population formulated onto a three dimensional matrix, such as a heart muscle patch comprising ventricular muscle cells. The subject is one in need of enhancement of cardiac function, for example someone who has suffered a myocardial infarction or someone who has a congenital heart disorder.

In yet another aspect, the invention pertains to a method for generating human ventricular tissue comprising:

transplanting NRP1+ human cardiac ventricular progenitor cells into an organ of a non-human animal; and

allowing the progenitor cells to grow *in vivo* such that human ventricular tissue is generated.

The non-human animal can be, for example, an immunodeficient mouse. The organ can be, for example, the kidney (e.g., the cells are transplanted under the kidney capsule) or the heart. In one embodiment, the cells are transplanted at a time when one, two, three, four or five of the following cell marker patterns are present: (i) after peak of cardiac mesoderm formation; (ii) at time of peak Islet-1 expression; (iii) before peak of NKX2.5 expression; (iv) before peak expression of downstream genes MEF-2 and TBX-1; and (v) before expression of differentiated contractile protein genes. In one embodiment, the cells are transplanted between day 5 and day 7 (inclusive) of *in vitro* culture of human pluripotent stem cells under conditions to generate human ventricular progenitor cells. In another embodiment, the cells are transplanted on day 6 of *in vitro* culture of human pluripotent stem cells under conditions to generate human ventricular progenitor cells. The method can further include harvesting the human ventricular tissue generated in the non-human animal.

In still another aspect of the invention, the human cardiac ventricular progenitor cells described herein can be used in screening assays to evaluate the cardiac toxicity of a test compound. Accordingly, the invention provides a method of screening for cardiac toxicity of test compound, the method comprising:

- providing NRP1+ cardiac ventricular progenitor cells;
- contacting the cells with the test compound; and
- measuring toxicity of the test compound for the cells,

wherein toxicity of the test compound for the cells indicates cardiac toxicity of the test compound. The toxicity of the test compound for the cells can be measured, for example, by assessing cell viability or other physiological parameters of the cell.

Culturing methods for generating human ventricular progenitor cells are also provided. For example, in one embodiment, the invention pertains to a method of generating human ventricular progenitors (HVPs) comprising:

- culturing human pluripotent stems cells (hPSCs) in a medium comprising CHIR98014 such that cells expressing cardiac mesodermal markers are generated; and
- culturing the cells expressing cardiac mesodermal markers in a medium comprising Wnt-C59 such that HVPs are generated.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

Brief Description of the Drawings

Figure 1 is a schematic diagram of an exemplary culturing protocol for generating human Isl1+cardiomyogenic progenitor cells from human pluripotent stem cells (hPSCs).

Figure 2 shows the results of Western blot analysis of protein expression during cardiac differentiation of hPSCs, showing expression of Isl1, Nkx2.5 and cTn1. GAPDH was used as a control.

Figure 3 shows the results of flow cytometry analysis of cardiomyogenic progenitor cells, showing expression of Isl1 on cells at day 6 of differentiation.

Figure 4 shows the results of double staining flow cytometry analysis of cardiomyogenic progenitor cells, showing coexpression of Isl1 and Jag1 on cells at day 6 of differentiation.

Figure 5 shows the results of Western blot analysis of protein expression during cardiac differentiation of hPSCs, showing expression of FZD4. GAPDH was used as a control.

Figure 6 shows the results of double staining flow cytometry analysis of cardiomyogenic progenitor cells, showing coexpression of Isl1 and FZD4 on cells at day 5 of differentiation.

Figure 7 is a schematic diagram of the generation of human ventricular progenitor (HVP) cells, their ultimate differentiation into ventricular myocytes, their antibody purification and their use in transplantation.

Figures 8A and 8B are schematic diagrams of the transplantation of HPVs into the renal capsule (Figure 8A) or intra-myocardially (Figure 8B) for organ-on-organ tissue engineering.

Figure 9 shows the results of double staining flow cytometry analysis of human ventricular progenitor (HVP) cells, showing coexpression of Isl1 and LIFR on the cells.

Figures 10A and 10B show the results of flow cytometry analysis of the expression of LIFR and FGFR3 on human ventricular progenitor cells (Fig. 10B) as compared to undifferentiated embryonic stem (ES) cells (Fig. 10A).

Figure 11 is a tSNE plot of day 5 cells from single cell sequencing, showing two clusters of cells based on differential gene expression, labeled 0 and 1.

Figures 12A and 12B are tSNE plots of Isl1 expression (Fig. 12A) and NRP1 expression (Fig. 12B) on day 5 cells from single cell sequencing. Dark grey denotes high expression, middle grey denotes low expression and light grey denotes no expression.

Figure 13 is a violin plot of expression levels of Isl1 and NRP1 in clusters of day 5 cells from single cell sequencing, showing that NRP1+ and ISL1+ cells are in the same cluster, since they have a similar gene expression profile.

Figures 14A, 14B and 14C show the results of flow cytometric analysis of the expression of NRP1 (Fig. 14A), TRA-1-60 (Fig. 14B) and both NRP1 and TRA-1-60 (Fig. 14C) on day 6 HVPs generated from H9 stem cells.

Figures 15A, 15B and 15C show the results of flow cytometric analysis of the expression of NRP1 (Fig. 15A), ISL1 (Fig. 15B) and both NRP1 and ISL1 (Fig. 15C) on day 6 HVPs generated from H9 stem cells.

Detailed Description of the Invention

The invention provides methods of isolating human cardiomyogenic progenitor cells, in particular cells that are biased to the ventricular lineage, as well as isolated clonal populations of such progenitor cells, based on the discovery that NRP1 is a cell surface marker for cardiac ventricular progenitor cells (HVPs). Additional suitable cells surface markers for HVPs are shown in, for example, Tables 1, 5 and 10. *In vitro* and *in vivo* uses for these cardiac ventricular progenitor cells are also provided.

HVPs have previously been shown to express cell surface markers such as JAG1, FZD4, LIFR, FGFR3 and/or TNFSF9, as well as the intracellular marker Islet 1 (see U.S. Serial Number 14/832,324, filed August 21, 2015, and U.S. Serial Number 14/984,783, filed December 30, 2015, the entire contents of each of which are expressly incorporated herein by reference). Furthermore, various positive and negative engraftment markers of HVPs have been identified (see U.S. Serial Number 15/433,713, filed February 15, 2017, the entire contents of which is expressly incorporated herein by reference).

In order that the present invention may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

As used herein, the terms "Neuropilin-1", "NRP1" are used interchangeably to refer to a protein known in the art that has been described in, for example, Soker, S. et al. (1998) *Cell* 92:735-745 and Rossignol, M. et al. (2000) *Genomics* 70:211-222. NRP1 is also referred to in the art as BDCA4, VEGF165R and CD304. A non-limiting example of an NRP1 protein is the human protein having the amino acid sequence set forth in Genbank Accession Number NP_003864.4.

As used herein, the term "stem cells" is used in a broad sense and includes traditional stem cells, progenitor cells, pre-progenitor cells, reserve cells, and the like. The term "stem

cell" or "progenitor" are used interchangeably herein, and refer to an undifferentiated cell which is capable of proliferation and giving rise to more progenitor cells having the ability to generate a large number of mother cells that can in turn give rise to differentiated, or differentiable daughter cells. The daughter cells themselves can be induced to proliferate and produce progeny that subsequently differentiate into one or more mature cell types, while also retaining one or more cells with parental developmental potential. The term "stem cell" refers then, to a cell with the capacity or potential, under particular circumstances, to differentiate to a more specialized or differentiated phenotype, and which retains the capacity, under certain circumstances, to proliferate without substantially differentiating. In one embodiment, the term progenitor or stem cell refers to a generalized mother cell whose descendants (progeny) specialize, often in different directions, by differentiation, e.g., by acquiring completely individual characters, as occurs in progressive diversification of embryonic cells and tissues. Cellular differentiation is a complex process typically occurring through many cell divisions. A differentiated cell may derive from a multipotent cell which itself is derived from a multipotent cell, and so on. While each of these multipotent cells may be considered stem cells, the range of cell types each can give rise to may vary considerably. Some differentiated cells also have the capacity to give rise to cells of greater developmental potential. Such capacity may be natural or may be induced artificially upon treatment with various factors. In many biological instances, stem cells are also "multipotent" because they can produce progeny of more than one distinct cell type, but this is not required for "stemness." Self-renewal is the other classical part of the stem cell definition, and it is essential as used in this document. In theory, self-renewal can occur by either of two major mechanisms. Stem cells may divide asymmetrically, with one daughter retaining the stem state and the other daughter expressing some distinct other specific function and phenotype. Alternatively, some of the stem cells in a population can divide symmetrically into two stems, thus maintaining some stem cells in the population as a whole, while other cells in the population give rise to differentiated progeny only. Formally, it is possible that cells that begin as stem cells might proceed toward a differentiated phenotype, but then "reverse" and re-express the stem cell phenotype, a term often referred to as "dedifferentiation".

The term "progenitor cell" is used herein to refer to cells that have a cellular phenotype that is more primitive (e.g., is at an earlier step along a developmental pathway or progression than is a fully differentiated cell) relative to a cell which it can give rise to by differentiation. Often, progenitor cells also have significant or very high proliferative potential. Progenitor cells can give rise to multiple distinct differentiated cell types or to a

single differentiated cell type, depending on the developmental pathway and on the environment in which the cells develop and differentiate.

The term "pluripotent" as used herein refers to a cell with the capacity, under different conditions, to differentiate to cell types characteristic of all three germ cell layers (endoderm, mesoderm and ectoderm). Pluripotent cells are characterized primarily by their ability to differentiate to all three germ layers, using, for example, a nude mouse and teratomas formation assay. Pluripotency is also evidenced by the expression of embryonic stem (ES) cell markers, although the preferred test for pluripotency is the demonstration of the capacity to differentiate into cells of each of the three germ layers. In some embodiments, a pluripotent cell is an undifferentiated cell.

The term "pluripotency" or a "pluripotent state" as used herein refers to a cell with the ability to differentiate into all three embryonic germ layers: endoderm (gut tissue), mesoderm (including blood, muscle, and vessels), and ectoderm (such as skin and nerve), and typically has the potential to divide in vitro for a long period of time, e.g., greater than one year or more than 30 passages.

The term "multipotent" when used in reference to a "multipotent cell" refers to a cell that is able to differentiate into some but not all of the cells derived from all three germ layers. Thus, a multipotent cell is a partially differentiated cell. Multipotent cells are well known in the art, and examples of multipotent cells include adult stem cells, such as for example, hematopoietic stem cells and neural stem cells. Multipotent means a stem cell may form many types of cells in a given lineage, but not cells of other lineages. For example, a multipotent blood stem cell can form the many different types of blood cells (red, white, platelets, etc.), but it cannot form neurons.

The term "embryonic stem cell" or "ES cell" or "ESC" are used interchangeably herein and refer to the pluripotent stem cells of the inner cell mass of the embryonic blastocyst (see U.S. Pat. Nos. 5,843,780, 6,200,806, which are incorporated herein by reference). Such cells can similarly be obtained from the inner cell mass of blastocysts derived from somatic cell nuclear transfer (see, for example, U.S. Pat. Nos. 5,945,577, 5,994,619, 6,235,970, which are incorporated herein by reference). The distinguishing characteristics of an embryonic stem cell define an embryonic stem cell phenotype.

Accordingly, a cell has the phenotype of an embryonic stem cell if it possesses one or more of the unique characteristics of an embryonic stem cell such that that cell can be distinguished from other cells. Exemplary distinguishing embryonic stem cell characteristics include, without limitation, gene expression profile, proliferative capacity, differentiation capacity,

karyotype, responsiveness to particular culture conditions, and the like. In some embodiments, an ES cell can be obtained without destroying the embryo, for example, without destroying a human embryo.

The term "adult stem cell" or "ASC" is used to refer to any multipotent stem cell
5 derived from non-embryonic tissue, including fetal, juvenile, and adult tissue. Stem cells have been isolated from a wide variety of adult tissues including blood, bone marrow, brain, olfactory epithelium, skin, pancreas, skeletal muscle, and cardiac muscle. Each of these stem cells can be characterized based on gene expression, factor responsiveness, and morphology in culture. Exemplary adult stem cells include neural stem cells, neural crest stem cells,
10 mesenchymal stem cells, hematopoietic stem cells, and pancreatic stem cells. As indicated above, stem cells have been found resident in virtually every tissue. Accordingly, the present invention appreciates that stem cell populations can be isolated from virtually any animal tissue.

The term "human pluripotent stem cell" (abbreviated as hPSC), as used herein, refers
15 to a human cell that has the capacity to differentiate into a variety of different cell types as discussed above regarding stem cells and pluripotency. Human pluripotent human stem cells include, for example, induced pluripotent stem cells (iPSC) and human embryonic stem cells, such as ES cell lines.

The term "human cardiac progenitor cell", as used herein, refers to a human
20 progenitor cell that is committed to the cardiac lineage and that has the capacity to differentiate into all three cardiac lineage cells (cardiac muscle cells, endothelial cells and smooth muscle cells). A culture of human cardiac progenitor cells can be obtained by, for example, culturing stem cells under conditions that bias the stem cells toward differentiation to the cardiac lineage. In certain embodiments, the stem cells that are cultured to generate
25 human cardiac progenitor cells are human embryonic stem cells or human induced pluripotent cells. In certain embodiments, c-kit⁺ adult progenitor cells are explicitly excluded for use in generating human cardiac progenitor cells.

The term "human cardiomyogenic progenitor cell", as used herein, refers to a human
progenitor cell that is committed to the cardiac lineage and that predominantly differentiates
30 into cardiac muscle cells (*i.e.*, more than 50% of the differentiated cells, preferably more than 60%, 70%, 80% or 90% of the differentiated cells, derived from the progenitor cells are cardiac muscle cells).

The term "cardiac ventricular progenitor cell", as used herein, refers to a progenitor cell that is committed to the cardiac lineage and that predominantly differentiates into cardiac

ventricular muscle cells (*i.e.*, more than 50% of the differentiated cells, preferably more than 60%, 70%, 80% or 90% of the differentiated cells, derived from the progenitor cells are cardiac ventricular muscle cells). This type of cell is also referred to herein as a human ventricular progenitor, or HVP, cell.

5 The term "cardiomyocyte" refers to a muscle cell of the heart (e.g. a cardiac muscle cell). A cardiomyocyte will generally express on its cell surface and/or in the cytoplasm one or more cardiac-specific marker. Suitable cardiomyocyte-specific markers include, but are not limited to, cardiac troponin I, cardiac troponin-C, tropomyosin, caveolin-3, GATA-4, myosin heavy chain, myosin light chain-2a, myosin light chain-2v, ryanodine receptor, and
10 atrial natriuretic factor.

 The term "derived from" used in the context of a cell derived from another cell means that a cell has stemmed (e.g. changed from or produced by) a cell that is a different cell type. The term "derived from" also refers to cells that have been differentiated from a progenitor cell.

15 The term "Isl1+ cardiac progenitor cell", as used herein, refers to a human progenitor cell that is committed to the cardiac lineage and that expresses Islet 1.

 The term "Isl1+ NRP1+ cardiac progenitor cell", as used herein, refers to a human progenitor cell that is committed to the cardiac lineage and that expresses both Islet 1 and NRP1.

20 With respect to cells in cell cultures or in cell populations, the term "substantially free of" means that the specified cell type of which the cell culture or cell population is free, is present in an amount of less than about 10%, less than about 9%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, less than about 4%, less than about 3%, less than about 2% or less than about 1% of the total number of cells present in the cell
25 culture or cell population.

 In the context of cell ontogeny, the adjective "differentiated", or "differentiating" is a relative term. A "differentiated cell" is a cell that has progressed further down the developmental pathway than the cell it is being compared with. Thus, stem cells can differentiate to lineage-restricted precursor cells (such as a mesodermal stem cell), which in
30 turn can differentiate into other types of precursor cells further down the pathway (such as an cardiomyocyte precursor), and then to an end-stage differentiated cell, which plays a characteristic role in a certain tissue type, and may or may not retain the capacity to proliferate further.

The term "differentiation" in the present context means the formation of cells expressing markers known to be associated with cells that are more specialized and closer to becoming terminally differentiated cells incapable of further differentiation. The pathway along which cells progress from a less committed cell, to a cell that is increasingly committed to a particular cell type, and eventually to a terminally differentiated cell is referred to as progressive differentiation or progressive commitment. Cells which are more specialized (e.g., have begun to progress along a path of progressive differentiation) but not yet terminally differentiated are referred to as partially differentiated. Differentiation is a developmental process whereby cells assume a specialized phenotype, e.g., acquire one or more characteristics or functions distinct from other cell types. In some cases, the differentiated phenotype refers to a cell phenotype that is at the mature endpoint in some developmental pathway (a so called terminally differentiated cell). In many, but not all tissues, the process of differentiation is coupled with exit from the cell cycle. In these cases, the terminally differentiated cells lose or greatly restrict their capacity to proliferate. However, we note that in the context of this specification, the terms "differentiation" or "differentiated" refer to cells that are more specialized in their fate or function than at a previous point in their development, and includes both cells that are terminally differentiated and cells that, although not terminally differentiated, are more specialized than at a previous point in their development. The development of a cell from an uncommitted cell (for example, a stem cell), to a cell with an increasing degree of commitment to a particular differentiated cell type, and finally to a terminally differentiated cell is known as progressive differentiation or progressive commitment. A cell that is "differentiated" relative to a progenitor cell has one or more phenotypic differences relative to that progenitor cell. Phenotypic differences include, but are not limited to morphologic differences and differences in gene expression and biological activity, including not only the presence or absence of an expressed marker, but also differences in the amount of a marker and differences in the co-expression patterns of a set of markers.

The term "differentiation" as used herein refers to the cellular development of a cell from a primitive stage towards a more mature (i.e. less primitive) cell.

As used herein, "proliferating" and "proliferation" refers to an increase in the number of cells in a population (growth) by means of cell division. Cell proliferation is generally understood to result from the coordinated activation of multiple signal transduction pathways in response to the environment, including growth factors and other mitogens. Cell proliferation may also be promoted by release from the actions of intra- or extracellular

signals and mechanisms that block or negatively affect cell proliferation.

The terms "renewal" or "self-renewal" or "proliferation" are used interchangeably herein, and refers to a process of a cell making more copies of itself (e.g. duplication) of the cell. In some embodiments, cells are capable of renewal of themselves by dividing into the
5 same undifferentiated cells (e.g. progenitor cell type) over long periods, and/or many months to years. In some instances, proliferation refers to the expansion of cells by the repeated division of single cells into two identical daughter cells.

The term "lineages" as used herein refers to a term to describe cells with a common ancestry or cells with a common developmental fate, for example cells that have a
10 developmental fate to develop into ventricular cardiomyocytes.

The term "clonal population", as used herein, refers to a population of cells that is derived from the outgrowth of a single cell. That is, the cells within the clonal population are all progeny of a single cell that was used to seed the clonal population.

The term "media" as referred to herein is a medium for maintaining a tissue or cell
15 population, or culturing a cell population (e.g. "culture media") containing nutrients that maintain cell viability and support proliferation. The cell culture medium may contain any of the following in an appropriate combination: salt(s), buffer(s), amino acids, glucose or other sugar(s), antibiotics, serum or serum replacement, and other components such as peptide growth factors, etc. Cell culture media ordinarily used for particular cell types are known to
20 those skilled in the art.

The term "phenotype" refers to one or a number of total biological characteristics that define the cell or organism under a particular set of environmental conditions and factors, regardless of the actual genotype.

A "marker" as used herein describes the characteristics and/or phenotype of a cell.
25 Markers can be used for selection of cells comprising characteristics of interest. Markers will vary with specific cells. Markers are characteristics, whether morphological, functional or biochemical (enzymatic) characteristics particular to a cell type, or molecules expressed by the cell type. Preferably, such markers are proteins, and more preferably, possess an epitope for antibodies or other binding molecules available in the art. However, a marker may
30 consist of any molecule found in a cell including, but not limited to, proteins (peptides and polypeptides), lipids, polysaccharides, nucleic acids and steroids. Examples of morphological characteristics or traits include, but are not limited to, shape, size, and nuclear to cytoplasmic ratio. Examples of functional characteristics or traits include, but are not limited to, the ability to adhere to particular substrates, ability to incorporate or exclude

particular dyes, ability to migrate under particular conditions, and the ability to differentiate along particular lineages. Markers may be detected by any method available to one of skill in the art.

5 The term "isolated cell" as used herein refers to a cell that has been removed from an organism in which it was originally found or a descendant of such a cell. Optionally the cell has been cultured *in vitro*, e.g., in the presence of other cells. Optionally the cell is later introduced into a second organism or re-introduced into the organism from which it (or the cell from which it is descended) was isolated.

10 The term "isolated population" with respect to an isolated population of cells as used herein refers to a population of cells that has been removed and separated from a mixed or heterogeneous population of cells. In some embodiments, an isolated population is a substantially pure population of cells as compared to the heterogeneous population from which the cells were isolated or enriched from.

15 The term "substantially pure", with respect to a particular cell population, refers to a population of cells that is at least about 75%, preferably at least about 85%, more preferably at least about 90%, and most preferably at least about 95% pure, with respect to the cells making up a total cell population.

20 The terms "subject" and "individual" are used interchangeably herein, and refer to an animal, for example a human, to whom cardiac ventricular progenitor cells as disclosed herein can be implanted into, for e.g. treatment, which in some embodiments encompasses prophylactic treatment or for a disease model, with methods and compositions described herein, is or are provided. For treatment of disease states that are specific for a specific animal such as a human subject, the term "subject" refers to that specific animal. The terms "non-human animals" and "non-human mammals" are used interchangeably herein, and
25 include mammals such as rats, mice, rabbits, sheep, cats, dogs, cows, pigs, and non-human primates. The term "subject" also encompasses any vertebrate including but not limited to mammals, reptiles, amphibians and fish. However, advantageously, the subject is a mammal such as a human, or other mammals such as a domesticated mammal, e.g. dog, cat, horse, and the like, or production mammal, e.g. cow, sheep, pig, and the like are also encompassed in
30 the term subject.

As used herein, the term "recipient" refers to a subject that will receive a transplanted organ, tissue or cell.

The term "three-dimensional matrix" or "scaffold" or "matrices" as used herein refers in the broad sense to a composition comprising a biocompatible matrix, scaffold, or the like.

The three-dimensional matrix may be liquid, gel, semi-solid, or solid at 25° C. The three-dimensional matrix may be biodegradable or non-biodegradable. In some embodiments, the three-dimensional matrix is biocompatible, or bioresorbable or bioreplacable. Exemplary three-dimensional matrices include polymers and hydrogels comprising collagen, fibrin, chitosan, MATRIGEL™, polyethylene glycol, dextrans including chemically crosslinkable or photocrosslinkable dextrans, processed tissue matrix such as submucosal tissue and the like. In certain embodiments, the three-dimensional matrix comprises allogeneic components, autologous components, or both allogeneic components and autologous components. In certain embodiments, the three-dimensional matrix comprises synthetic or semi-synthetic materials. In certain embodiments, the three-dimensional matrix comprises a framework or support, such as a fibrin-derived scaffold.

As used herein, the terms "administering," "introducing" and "transplanting" are used interchangeably and refer to the placement of cardiomyogenic progenitor cells and/or cardiomyocytes differentiated as described herein into a subject by a method or route which results in at least partial localization of the cells at a desired site. The cells can be administered by any appropriate route that results in delivery to a desired location in the subject where at least a portion of the cells remain viable. The period of viability of the cells after administration to a subject can be as short as a few hours, e.g. twenty-four hours, to a few days, to as long as several years.

The term "statistically significant" or "significantly" refers to statistical significance and generally means a two standard deviation (2SD) below normal, or lower, concentration of the marker. The term refers to statistical evidence that there is a difference. It is defined as the probability of making a decision to reject the null hypothesis when the null hypothesis is actually true. The decision is often made using the p-value. The term "substantially" or "predominantly" as used herein means a proportion of at least about 60%, or preferably at least about 70% or at least about 80%, or at least about 90%, at least about 95%, at least about 97% or at least about 99% or more, or any integer between 70% and 100%.

The term "disease" or "disorder" is used interchangeably herein, and refers to any alternation in state of the body or of some of the organs, interrupting or disturbing the performance of the functions and/or causing symptoms such as discomfort, dysfunction, distress, or even death to the person afflicted or those in contact with a person. A disease or disorder can also related to a distemper, ailing, ailment, malady, disorder, sickness, illness, complaint, indisposition or affection.

As used herein, the phrase "cardiovascular condition, disease or disorder" is intended

to include all disorders characterized by insufficient, undesired or abnormal cardiac function, e.g. ischemic heart disease, hypertensive heart disease and pulmonary hypertensive heart disease, valvular disease, congenital heart disease and any condition which leads to congestive heart failure in a subject, particularly a human subject. Insufficient or abnormal cardiac function can be the result of disease, injury and/or aging. By way of background, a response to myocardial injury follows a well-defined path in which some cells die while others enter a state of hibernation where they are not yet dead but are dysfunctional. This is followed by infiltration of inflammatory cells, deposition of collagen as part of scarring, all of which happen in parallel with in-growth of new blood vessels and a degree of continued cell death. As used herein, the term "ischemia" refers to any localized tissue ischemia due to reduction of the inflow of blood. The term "myocardial ischemia" refers to circulatory disturbances caused by coronary atherosclerosis and/or inadequate oxygen supply to the myocardium. For example, an acute myocardial infarction represents an irreversible ischemic insult to myocardial tissue. This insult results in an occlusive (e.g., thrombotic or embolic) event in the coronary circulation and produces an environment in which the myocardial metabolic demands exceed the supply of oxygen to the myocardial tissue.

As used herein, the term "treating" or "treatment" are used interchangeably herein and refers to reducing or decreasing or alleviating or halting at least one adverse effect or symptom of a cardiovascular condition, disease or disorder, i.e., any disorder characterized by insufficient or undesired cardiac function. Adverse effects or symptoms of cardiac disorders are well-known in the art and include, but are not limited to, dyspnea, chest pain, palpitations, dizziness, syncope, edema, cyanosis, pallor, fatigue and death. In some embodiments, the term "treatment" as used herein refers to prophylactic treatment or preventative treatment to prevent the development of a symptom of a cardiovascular condition in a subject.

Treatment is generally "effective" if one or more symptoms or clinical markers are reduced as that term is defined herein. Alternatively, a treatment is "effective" if the progression of a disease is reduced or halted. That is, "treatment" includes not just the improvement of symptoms or decrease of markers of the disease, but also a cessation or slowing of progress or worsening of a symptom that would be expected in absence of treatment. Beneficial or desired clinical results include, but are not limited to, alleviation of one or more symptom(s), diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable.

"Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. Those in need of treatment include those already diagnosed with a cardiac condition, as well as those likely to develop a cardiac condition due to genetic susceptibility or other factors such as weight, diet and health. In some embodiments, the term to treat also encompasses preventative measures and/or prophylactic treatment, which includes administering a pharmaceutical composition as disclosed herein to prevent the onset of a disease or disorder.

A therapeutically significant reduction in a symptom is, e.g. at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 100%, at least about 125%, at least about 150% or more in a measured parameter as compared to a control or non-treated subject. Measured or measurable parameters include clinically detectable markers of disease, for example, elevated or depressed levels of a biological marker, as well as parameters related to a clinically accepted scale of symptoms or markers for a disease or disorder. It will be understood, that the total daily usage of the compositions and formulations as disclosed herein will be decided by the attending physician within the scope of sound medical judgment. The exact amount required will vary depending on factors such as the type of disease being treated.

With reference to the treatment of a cardiovascular condition or disease in a subject, the term "therapeutically effective amount" refers to the amount that is safe and sufficient to prevent or delay the development of a cardiovascular disease or disorder. The amount can thus cure or cause the cardiovascular disease or disorder to go into remission, slow the course of cardiovascular disease progression, slow or inhibit a symptom of a cardiovascular disease or disorder, slow or inhibit the establishment of secondary symptoms of a cardiovascular disease or disorder or inhibit the development of a secondary symptom of a cardiovascular disease or disorder. The effective amount for the treatment of the cardiovascular disease or disorder depends on the type of cardiovascular disease to be treated, the severity of the symptoms, the subject being treated, the age and general condition of the subject, the mode of administration and so forth. Thus, it is not possible to specify the exact "effective amount". However, for any given case, an appropriate "effective amount" can be determined by one of ordinary skill in the art using only routine experimentation. The efficacy of treatment can be judged by an ordinarily skilled practitioner, for example, efficacy can be assessed in animal models of a cardiovascular disease or disorder as discussed herein, for example treatment of a rodent with acute myocardial infarction or ischemia-reperfusion injury, and any treatment or

administration of the compositions or formulations that leads to a decrease of at least one symptom of the cardiovascular disease or disorder as disclosed herein, for example, increased heart ejection fraction, decreased rate of heart failure, decreased infarct size, decreased associated morbidity (pulmonary edema, renal failure, arrhythmias) improved exercise
5 tolerance or other quality of life measures, and decreased mortality indicates effective treatment. In embodiments where the compositions are used for the treatment of a cardiovascular disease or disorder, the efficacy of the composition can be judged using an experimental animal model of cardiovascular disease, e.g., animal models of ischemia-reperfusion injury (Headrick J P, Am J Physiol Heart circ Physiol 285; H1797; 2003) and
10 animal models acute myocardial infarction. (Yang Z, Am J Physiol Heart Circ. Physiol 282:H949:2002; Guo Y, J Mol Cell Cardiol 33; 825-830, 2001). When using an experimental animal model, efficacy of treatment is evidenced when a reduction in a symptom of the cardiovascular disease or disorder, for example, a reduction in one or more symptom of dyspnea, chest pain, palpitations, dizziness, syncope, edema, cyanosis, pallor, fatigue and
15 high blood pressure which occurs earlier in treated, versus untreated animals. By "earlier" is meant that a decrease, for example in the size of the tumor occurs at least 5% earlier, but preferably more, e.g., one day earlier, two days earlier, 3 days earlier, or more.

As used herein, the term "treating" when used in reference to a treatment of a cardiovascular disease or disorder is used to refer to the reduction of a symptom and/or a
20 biochemical marker of a cardiovascular disease or disorder, for example a reduction in at least one biochemical marker of a cardiovascular disease by at least about 10% would be considered an effective treatment. Examples of such biochemical markers of cardiovascular disease include a reduction of, for example, creatine phosphokinase (CPK), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) in the blood, and/or a decrease in a
25 symptom of cardiovascular disease and/or an improvement in blood flow and cardiac function as determined by someone of ordinary skill in the art as measured by electrocardiogram (ECG or EKG), or echocardiogram (heart ultrasound), Doppler ultrasound and nuclear medicine imaging. A reduction in a symptom of a cardiovascular disease by at least about 10% would also be considered effective treatment by the methods as disclosed
30 herein. As alternative examples, a reduction in a symptom of cardiovascular disease, for example a reduction of at least one of the following; dyspnea, chest pain, palpitations, dizziness, syncope, edema, cyanosis etc. by at least about 10% or a cessation of such systems, or a reduction in the size one such symptom of a cardiovascular disease by at least about 10% would also be considered as affective treatments by the methods as disclosed herein. In some

embodiments, it is preferred, but not required that the therapeutic agent actually eliminate the cardiovascular disease or disorder, rather just reduce a symptom to a manageable extent.

Subjects amenable to treatment by the methods as disclosed herein can be identified by any method to diagnose myocardial infarction (commonly referred to as a heart attack) commonly known by persons of ordinary skill in the art are amenable to treatment using the methods as disclosed herein, and such diagnostic methods include, for example but are not limited to; (i) blood tests to detect levels of creatine phosphokinase (CPK), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and other enzymes released during myocardial infarction; (ii) electrocardiogram (ECG or EKG) which is a graphic recordation of cardiac activity, either on paper or a computer monitor. An ECG can be beneficial in detecting disease and/or damage; (iii) echocardiogram (heart ultrasound) used to investigate congenital heart disease and assessing abnormalities of the heart wall, including functional abnormalities of the heart wall, valves and blood vessels; (iv) Doppler ultrasound can be used to measure blood flow across a heart valve; (v) nuclear medicine imaging (also referred to as radionuclide scanning in the art) allows visualization of the anatomy and function of an organ, and can be used to detect coronary artery disease, myocardial infarction, valve disease, heart transplant rejection, check the effectiveness of bypass surgery, or to select patients for angioplasty or coronary bypass graft.

The terms "coronary artery disease" and "acute coronary syndrome" as used interchangeably herein, and refer to myocardial infarction refer to a cardiovascular condition, disease or disorder, include all disorders characterized by insufficient, undesired or abnormal cardiac function, e.g. ischemic heart disease, hypertensive heart disease and pulmonary hypertensive heart disease, valvular disease, congenital heart disease and any condition which leads to congestive heart failure in a subject, particularly a human subject. Insufficient or abnormal cardiac function can be the result of disease, injury and/or aging. By way of background, a response to myocardial injury follows a well-defined path in which some cells die while others enter a state of hibernation where they are not yet dead but are dysfunctional. This is followed by infiltration of inflammatory cells, deposition of collagen as part of scarring, all of which happen in parallel with in-growth of new blood vessels and a degree of continued cell death.

As used herein, the term "ischemia" refers to any localized tissue ischemia due to reduction of the inflow of blood. The term "myocardial ischemia" refers to circulatory disturbances caused by coronary atherosclerosis and/or inadequate oxygen supply to the myocardium. For example, an acute myocardial infarction represents an irreversible

ischemic insult to myocardial tissue. This insult results in an occlusive (e.g., thrombotic or embolic) event in the coronary circulation and produces an environment in which the myocardial metabolic demands exceed the supply of oxygen to the myocardial tissue.

The terms "composition" or "pharmaceutical composition" used interchangeably
5 herein refer to compositions or formulations that usually comprise an excipient, such as a pharmaceutically acceptable carrier that is conventional in the art and that is suitable for administration to mammals, and preferably humans or human cells. In some embodiments, pharmaceutical compositions can be specifically formulated for direct delivery to a target tissue or organ, for example, by direct injection or via catheter injection to a target tissue. In
10 other embodiments, compositions can be specifically formulated for administration via one or more of a number of routes, including but not limited to, oral, ocular parenteral, intravenous, intraarterial, subcutaneous, intranasal, sublingual, intraspinal, intracerebroventricular, and the like. In addition, compositions for topical (e.g., oral mucosa, respiratory mucosa) and/or oral administration can form solutions, suspensions, tablets, pills, capsules, sustained-release
15 formulations, oral rinses, or powders, as known in the art are described herein. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, University of the Sciences in Philadelphia (2005) Remington: The Science and Practice of Pharmacy with Facts and Comparisons, 21st Ed.

As used herein, the terms "administering," "introducing" and "transplanting" are used
20 interchangeably and refer to the placement of a pharmaceutical composition comprising cardiomyogenic progenitor cells, or a composition comprising a population of differentiated cardiomyocytes (e.g., ventricular cardiomyocytes) as described herein, into a subject by a method or route which results in at least partial localization of the pharmaceutical composition, at a desired site or tissue location. In some embodiments, the pharmaceutical
25 composition can be administered by any appropriate route which results in effective treatment in the subject, i.e. administration results in delivery to a desired location or tissue in the subject where at least a portion of the cells are located at a desired target tissue or target cell location.

The phrases "parenteral administration" and "administered parenterally" as used
30 herein mean modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intraventricular, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, sub capsular, subarachnoid, intraspinal, intracerebro spinal, and intrasternal injection and infusion. The

phrases "systemic administration," "administered systemically", "peripheral administration" and "administered peripherally" as used herein mean the administration of cardiovascular stem cells and/or their progeny and/or compound and/or other material other than directly into the cardiac tissue, such that it enters the animal's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous or intravenous administration.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically acceptable carrier" as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject agents from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation.

The term "drug" or "compound" or "test compound" as used herein refers to a chemical entity or biological product, or combination of chemical entities or biological products, administered to a subject to treat or prevent or control a disease or condition. The chemical entity or biological product is preferably, but not necessarily a low molecular weight compound, but may also be a larger compound, for example, an oligomer of nucleic acids, amino acids, or carbohydrates including without limitation proteins, oligonucleotides, ribozymes, DNazymes, glycoproteins, siRNAs, lipoproteins, aptamers, and modifications and combinations thereof.

The term "transplantation" as used herein refers to introduction of new cells (e.g. reprogrammed cells), tissues (such as differentiated cells produced from reprogrammed cells), or organs into a host (i.e. transplant recipient or transplant subject).

The term "agent reactive with NRP1", as used herein, refers to an agent that binds to or otherwise interacts with NRP1. Preferably, the agent "specifically" binds or otherwise interacts with NRP1 such that it does not bind or interact with other non-NRP1 proteins.

The term "antibody", as used herein, includes whole antibodies and any antigen binding fragment (*i.e.*, "antigen-binding portion") or single chain thereof. An "antibody" refers, in one preferred embodiment, to a glycoprotein comprising at least two heavy (H)

chains and two light (L) chains inter-connected by disulfide bonds, or an antigen binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The term “antigen-binding portion” of an antibody (or simply “antibody portion”), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen.

The term “monoclonal antibody,” as used herein, refers to an antibody that displays a single binding specificity and affinity for a particular epitope.

The term “human monoclonal antibody”, as used herein, refers to an antibody which displays a single binding specificity and which has variable and optional constant regions derived from human germline immunoglobulin sequences.

The term “humanized monoclonal antibody”, as used herein, refers to an antibody which displays a single binding specificity and which has heavy and light chain CDR1, 2 and 3 from a non-human antibody (e.g., a mouse monoclonal antibody) grafted into human framework and constant regions.

The term “chimeric monoclonal antibody”, as used herein, refers to an antibody which displays a single binding specificity and which has heavy and light chain variable regions from one species linked to constant regions from another species.

The term “fusion protein”, as used herein, refers to a composite protein, typically made using recombinant DNA technology, in which two different proteins, or portions thereof, are operatively linked together. A non-limiting example is an Fc fusion protein in which a non-immunoglobulin protein is operatively linked to an immunoglobulin Fc region.

Various aspects of the invention are described in further detail in the following subsections.

Methods of Isolating Human Cardiac Ventricular Progenitor Cells

In one aspect, the invention pertains to methods of isolating human cardiac ventricular progenitor cells. As described in the Examples, NRP1 has now been identified as a cell surface marker of human cardiac ventricular progenitor cells and thus this marker can be used to facilitate isolation of these progenitor cells. Alternative to NRP1, additional markers for human cardiac ventricular progenitor cells are provided herein, including the markers shown

in Tables 1, 5 and 10. Any of the proteins in these tables that is a cell surface protein can be used in the isolation of HVPs as described herein.

Accordingly, in one embodiment, the invention provides a method for isolating human cardiac ventricular progenitor cells, the method comprising:

5 contacting a culture of human cells containing cardiac progenitor cells with one or more agents reactive with NRP1; and

 separating NRP1 reactive positive cells from non-reactive cells to thereby isolate human cardiac ventricular progenitor cells.

10 Alternatively, after the contacting step, the method can comprise isolating NRP1 reactive positive cells from non-reactive cells to thereby isolate human cardiac ventricular progenitor cells.

 Also as described in the Examples, Islet 1 is a marker that is co-expressed with NRP1 by the cardiac ventricular progenitor cells and thus both markers can be used to facilitate isolation of these progenitor cells. Accordingly, in another embodiment of the above method,
15 the culture of human cells is also contacted with an agent reactive with Islet 1; and NRP1 reactive/Islet 1 reactive positive cells are separated from non-reactive cells to thereby isolate human cardiac ventricular progenitor cells. Alternatively or additionally, agents reactive with other HVP markers, including but not limited to JAG1, FZD4, LIFR, FGFR3 and/or TNFSF9, can be used in combination with NRP1 in the isolation of HVPs. Accordingly, the
20 culture of human cells can be simultaneously contacted with the agent(s) reactive with NRP1 and/or at least one second agent reactive with an HVP marker(s), including but not limited to JAG1, FZD4, LIFR, FGFR3 and/or TNFSF9. In one embodiment, the culture of human cells is contacted with the agent reactive with NRP1 before contacting with the second agent(s) reactive with an HVP marker(s). In another embodiment, the culture of human cells is
25 contacted with the second agent(s) reactive with an HVP marker(s) before contacting with the agent reactive with NRP1.

 In another embodiment, the method of isolating HVPs further comprises negatively selecting for at least one marker expressed on the surface of human pluripotent stem cells, such as TRA-1-60. The use of negative selection (in addition to positive selection for NRP1
30 expression, and/or expression of other positive HVP markers) ensures a rigorous definition of the HVP population as well as eliminating batch variation and potential teratoma-causing cells. Accordingly, in one embodiment, cardiac progenitor cells are selected for lack of expression of at least one marker expressed on the surface of human pluripotent stem cells (negative selection), such as TRA-1-60, to thereby isolate a highly purified population of

HVPs. Non-limiting examples of markers expressed on the surface of human pluripotent stem cells that can be used for negative selection include: TRA-1-60, TRA-1-81, TRA-2-54, SSEA1, SSEA3, SSEA4, CD9, CD24, E-cadherin and Podocalyxin, and combinations thereof.

In another embodiment, the invention provides a method for isolating human cardiac ventricular progenitor cells, the method comprising:

culturing human pluripotent stem cells under conditions that generate cardiac progenitor cells to obtain a culture of cells;

contacting the culture of cells with one or more agents reactive with NRP1; and

separating NRP1 reactive positive cells from non-reactive cells to thereby isolate

human cardiac ventricular progenitor cells.

Alternatively, after the culturing and contacting steps, the method can comprise isolating NRP1 from non-reactive cells to thereby isolate human cardiac ventricular progenitor cells.

Alternatively or additionally, second agents reactive with other HVP markers, including but not limited to JAG1, FZD4, LIFR, FGFR3 and/or TNFSF9, can be used in combination with NRP1 in the isolation of HVPs. Accordingly, the culture of human cells can be simultaneously contacted with the agent(s) reactive with NRP1 and/or at least one second agent reactive with an HVP marker(s), including but not limited to JAG1, FZD4, LIFR, FGFR3 and/or TNFSF9. In one embodiment, the culture of human cells is contacted with the agent reactive with NRP1 before contacting with the second agent(s) reactive with an HVP marker(s). In another embodiment, the culture of human cells is contacted with the second agent(s) reactive with an HVP marker(s) before contacting with the agent reactive with NRP1.

In another embodiment, the method of isolating HVPs further comprises negatively selecting for at least one marker expressed on the surface of human pluripotent stem cells, such as TRA-1-60. The use of negative selection (in addition to positive selection for NRP1 expression, and/or expression of other positive HVP markers) ensures a rigorous definition of the HVP population as well as eliminating batch variation and potential teratoma-causing cells. Accordingly, in one embodiment, cardiac progenitor cells are selected for lack of expression of at least one marker expressed on the surface of human pluripotent stem cells (negative selection), such as TRA-1-60, to thereby isolate a highly purified population of HVPs. Non-limiting examples of markers expressed on the surface of human pluripotent stem cells that can be used for negative selection include: TRA-1-60, TRA-1-81, TRA-2-54, SSEA1, SSEA3, SSEA4, CD9, CD24, E-cadherin and Podocalyxin, and combinations thereof.

In a preferred embodiment, the agent reactive with NRP1 is an anti-NRP1 antibody, such as a monoclonal antibody. Non-limiting examples include murine, rabbit, human, humanized or chimeric monoclonal antibodies with binding specificity for NRP1. Anti-NRP1 monoclonal antibodies are commercially available in the art. Moreover, anti-NRP1 antibodies can be prepared using standard techniques well established in the art using NRP1 as the antigen.

In another embodiment, the agent reactive with NRP1 is a NRP1 ligand, such as a soluble NRP1 ligand or a soluble NRP1 ligand fusion protein. Non-limiting examples of NRP1 ligands include VEGF-A and Sema3A. Soluble NRP1 ligands can be prepared using standard recombinant DNA techniques, for example by deletion of the transmembrane and cytoplasmic domains. A soluble ligand can be transformed into a soluble ligand fusion protein also using standard recombinant DNA techniques. A fusion protein can be prepared in which fusion partner can comprise a binding moiety that facilitates separation of the fusion protein.

In order to separate the NRP1 reactive positive cells from non-reactive cells, one of a variety of different cell separation techniques known in the art can be used. Preferably, the NRP1 reactive positive cells are separated from non-reactive cells by fluorescence activated cell sorting (FACS). The FACS technology, and apparatuses for carrying it out to separate cells, is well established in the art. When FACS is used for cell separation, preferably the agent(s) reactive with NRP1 that is used is a fluorescently-labeled anti-NRP1 monoclonal antibody. Alternatively, cell separation can be achieved by, for example, magnetic activated cell sorting (MACS). When MACS is used for cell separation, preferably the agent reactive with NRP1 that is used is magnetic nanoparticles coated with anti-NRP1 monoclonal antibody. Alternatively, other single cell sorting methodologies known in the art can be applied to the methods of isolating cardiac ventricular progenitor cells of the invention, including but not limited to IsoRaft array and DEPArray technologies.

Prior to contact with the agent(s) reactive with NRP1, and separation of NRP1 reactive cells, human pluripotent stem cells can be cultured under conditions that lead to the generation of cardiac progenitor cells. Culture conditions for generating cardiac progenitor cells have been described in the art (see *e.g.*, Lian, X. *et al.* (2012) *Proc. Natl. Acad. Sci. USA* 109:E1848-1857; U.S. Patent Publication No. 20130189785) and also are described in detail in Example 1 and Figure 1, as well as in Example 10. Typically, Wnt/ β -catenin signaling is first activated in the hPSCs, followed by an incubation period, followed by inhibition of

Wnt/ β -catenin signaling. Activation of Wnt/ β -catenin signaling is achieved by incubation with a Gsk3 inhibitor, preferably CHIR98014 (CAS 556813-39-9). Inhibition of Wnt/ β -catenin signaling is achieved by incubation with a Porcn inhibitor, preferably Wnt-C59 (CAS 1243243-89-1). Suitable hPSCs for use in the methods of the invention include induced pluripotent stem cells (iPSC), such as 19-11-1, 19-9-7 or 6-9-9 cells (Yu, J. *et al.* (2009) *Science* 324:797-801), and human embryonic stem cell lines, such as ES03 cells (WiCell Research Institute) or H9 cells (Thomson, J.A. *et al.* (1998) *Science* 282:1145-1147). Suitable culture media for generating cardiomyogenic progenitors include E8 medium, mTeSR1 medium and RPMI/B27 minus insulin, each described further in Example 1 and/or Example 10.

Preferably, the human cardiomyogenic progenitor cells are ventricular progenitor cells. Culture conditions have now been determined that bias the cardiomyogenic progenitor cells to the ventricular lineage. These ventricular cardiomyogenic progenitor cells can be cultured in RPMI/B27 medium and they can further differentiate into ventricular muscle cells. A preferred medium for culturing the cardiac ventricular progenitor cells *in vitro* such that they differentiate into ventricular cells *in vitro* (e.g., expressing the MLC2v marker described below) is the Cardiac Progenitor Culture (CPC) medium (advanced DMEM/F12 supplemented with 20% KnockOut Serum Replacement, 2.5 mM GlutaMAX and 100 μ g/ml Vitamin C).

Known markers of differentiated cardiac cells can be used to identify the type(s) of cells that are generated by differentiation of the cardiac progenitor cells. For example, cardiac troponin I (cTnI) can be used as a marker of cardiomyocyte differentiation. CD144 (VE-cadherin) can be used as a marker of endothelial cells. Smooth muscle actin (SMA) can be used as a marker of smooth muscle cells. MLC2v can be used as a marker of ventricular muscle cells. MLC2a, which is expressed on both immature ventricular muscle cells and atrial muscle cells, can be used as a marker for those cell types. Additionally, sarcolipin, which is specifically expressed in atrial muscle cells, can be used as a marker for atrial muscle cells. Phospholamban, which is expressed predominantly in the ventricles and, to a lesser extent, in the atria, can also be used as a marker. Hairy-related transcription factor 1 (HRT1), also called Hey1, which is expressed in atrial cardiomyocytes, can be used as a marker for atrial cardiomyocytes. HRT2 (Hey2), which is expressed in ventricular cardiomyocytes, can be used as a marker for ventricular cardiomyocytes. In addition, IRX4 has a ventricular-restricted expression pattern during all stages of development, and thus can

be used as a ventricular lineage marker. In summary, the genes expressed in the ventricles, and thus which are appropriate ventricular markers, are: MLC2v, IRX4 and HRT2, while genes expressed in the atria, and thus which are appropriate atrial markers are: MLC2a, HRT1, Sarcolipin and ANF (atrial natriuretic factor). The preferred marker of ventricular differentiation is MLC2v.

Clonal Populations of Human Cardiac Ventricular Progenitor Cells

In another aspect, the invention provides methods for obtaining a clonal population of human cardiac ventricular progenitor cells, as well as isolated clonal populations of such progenitors. The invention allows for the expansion and propagation of the cardiac ventricular progenitor cells such that a clonal population of a billion or more cells can be achieved. The ability to clonally expand the NRP1+ cardiac ventricular progenitor cells to such large numbers is a necessary feature for successful use of these cells *in vivo* to enhance cardiac function, since such a use requires on the order of a billion or more cells.

Accordingly, in another aspect, the invention provides a method for obtaining a clonal population of human cardiac ventricular progenitor cells, the method comprising:

isolating a single NRP1+ human cardiac ventricular progenitor cell; and
culturing the single NRP1+ human cardiac ventricular progenitor cell under conditions such that the cell is expanded to at least 1×10^9 cells to thereby obtain a clonal population of human cardiac ventricular progenitor cells.

In a preferred embodiment, the single NRP1+ human cardiac ventricular progenitor cell is Islet 1 positive, Nkx2.5 negative and flk1 negative at the time of initial culture. As described further in the Examples, such a single cell can be obtained at approximately day 6 of the culture under conditions that promote the generation of cardiomyogenic progenitors. The clonal population of human cardiac ventricular progenitors can be further cultured and differentiated *in vitro* such that the cells express the ventricular marker MLC2v.

Preferably, the single NRP1+ human cardiac ventricular progenitor cell is isolated by fluorescence activated cell sorting. Alternatively, the cell can be isolated by MACS or by other cell sorting methods known in the art and/or described herein.

Preferably, the single NRP1+ human cardiac ventricular progenitor cell is isolated using one or more agents reactive with NRP1, such as an anti-NRP1 antibody or other agent reactive with NRP1 as described hereinbefore.

In other embodiments, the clonal population of human cardiac ventricular progenitor cells is NRP1+ and positive for at least one second marker of HVPs (e.g., Isl1, FZD4, LIFR,

FGFR3, TNFSF9). Such double-positive cells can be isolated and clonally expanded as described herein before using both an agent reactive with NRP1 and an agent reactive with the second marker.

In a preferred embodiment, the single NRP1+ human cardiac ventricular progenitor
5 cell is cultured in Cardiac Progenitor Culture (CPC) medium, as described hereinbefore.

In a preferred embodiment, the single NRP1+ human cardiac ventricular progenitor cell is cultured under conditions such that the cell is biased toward ventricular differentiation. Preferred culture conditions include culture in CPC medium.

In various embodiments, the single NRP1+ human cardiac ventricular progenitor cell
10 can be expanded to at least 1×10^9 cells, at least 2×10^9 cells, at least 3×10^9 cells, at least 4×10^9 cells, at least 5×10^9 cells, at least 6×10^9 cells, at least 7×10^9 cells, at least 8×10^9 cells, at least 9×10^9 cells or at least 10×10^9 cells.

Accordingly, the invention also provides a clonal population of at least 1×10^9 NRP1+ human cardiac ventricular progenitor cells, which are obtainable or obtained by the
15 methods of the invention for obtaining a clonal population of human cardiac ventricular progenitor cells. In various embodiments, the clonal population of NRP1+ human cardiac ventricular progenitor cells comprises at least 1×10^9 cells, at least 2×10^9 cells, at least 3×10^9 cells, at least 4×10^9 cells, at least 5×10^9 cells, at least 6×10^9 cells, at least 7×10^9 cells, at least 8×10^9 cells, at least 9×10^9 cells or at least 10×10^9 cells. Differentiation of the
20 progenitor cells to the ventricular lineage *in vitro* can be achieved by culture under conditions described herein for biasing toward the ventricular lineage. Furthermore, transplantation of the cardiac ventricular progenitor cells *in vivo* leads to ventricular differentiation *in vivo*.

The invention also provides pharmaceutical compositions comprising the clonal population of cardiac ventricular progenitor cells. The pharmaceutical compositions typically
25 are sterile and can comprise buffers, media, excipients and the like suitable for pharmaceutical administration. In one embodiment, the pharmaceutical composition comprising the clonal population is formulated onto a three dimensional (3D) matrix. Compositions formulated onto a 3D matrix are particularly preferred for formation of a heart muscle cell patch that can be transplanted *in vivo* for heart muscle repair. Furthermore, the
30 compositions can be formulated into two dimensional (2D) sheets of cells, such as a muscular thin film (MTF) as described in Domian, I. J. *et al.* (2009) *Science* 326:426-429. Such 2D sheets of cell tissue also can be used in the formation of a heart muscle cell patch that can be transplanted *in vivo* for heart muscle repair.

Generation of Human Ventricular Progenitors (HVPs)

Prior to isolation by the aforementioned methods, and optionally obtaining a clonal population by the aforementioned methods, a non-clonal population of human ventricular progenitors (HVPs) can be obtained by culture of human pluripotent stem cells (hPSCs) under appropriate culture conditions to generate the HVPs. An exemplary set of culture conditions, and suitable starting cells, is described in detail in Example 1 and Example 10, also referred to herein as the Human Ventricular Progenitor Generation (HVPG) protocol. Suitable hPSC starting cells include induced pluripotent stem cells (iPSC) and human embryonic stem cells, such as ES cell lines. For the protocol, Wnt/ β -catenin signaling first is activated in the hPSCs, followed by an incubation period, followed by inhibition of Wnt/ β -catenin signaling. Wnt/ β -catenin signaling activation is achieved by incubation with a Gsk3 inhibitor, preferably CHIR98014 (CAS 556813-39-9; commercially available from, *e.g.*, Selleckchem). Wnt/ β -catenin signaling inhibition is achieved by incubation with a Porcn inhibitor, preferably Wnt-C59 (CAS 1243243-89-1; commercially available from, *e.g.*, Selleckchem or Tocris). The Gsk3 inhibitor is used to promote cardiac mesodermal differentiation, whereas the Porcn inhibitor is used to enhance ventricular progenitor differentiation from mesoderm cells.

Accordingly, in another aspect, the invention provides a method of generating human ventricular progenitors (HVPs) comprising culturing human pluripotent stems cells (hPSCs) in a medium comprising a Gsk3 inhibitor, preferably CHIR98014, for at least 24 hours, more preferably for 2 days or 3 days, followed by culturing the hPSCs in a medium comprising a Porcn inhibitor, preferably Wnt-C59 (and lacking the Gsk3 inhibitor), for at least 48 hours such that HVPs are generated. Experiments showed that after 24-hour treatment with CHIR-98014, more than 99% of hPSCs expressed the mesoderm marker Brachyury, and three days later after treatment with CHIR-98014, more than 95% of differentiated cells expressed Mesp1, which marks the cardiac mesoderm. Furthermore, 48-hour treatment with Wnt-C59 enhanced ventricular progenitor differentiation from mesoderm cells.

Accordingly, with regard to timing of the use of the Gsk3 and Porcn inhibitors, typically, at day 0 of culture, the hPSCs are cultured with the Gsk3 inhibitor, at day 3 of culture the medium is changed to remove the Gsk3 inhibitor and the cells are then cultured with media containing the Porcn inhibitor through day 5 of culture. HVP generation is optimal between days 5 and 7 (inclusive) in culture and peaks at day 6 of culture. Other non-

limiting, exemplary details on culture conditions and timing of the use of the Gsk3 and Porcn inhibitors are described in detail in Examples 1 and 10.

In Vivo Tissue Engineering

5 *In vivo* transplantation studies described in Example 6 and 7 in which the human ventricular progenitors (HVPs) were transplanted under the kidney capsule in nude mice document the ability of the HVPs to spontaneously assemble into a large wall of mature, functional, human ventricular muscle on the surface of the kidney capsule. Vascularization occurs via a paracrine pathway by calling the murine vasculature to the ventricular muscle
10 wall, while a matrix is generated via a cell autonomous pathway from the progenitors themselves. *In vivo* intra-myocardial transplantation studies described in Example 8 in which the HVPs were transplanted into the normal murine heart document that the HVPs spontaneously migrate to the epicardial surface, where they expand, subsequently differentiate, and mature into a wall of human ventricular muscle on the surface of the
15 epicardium. Taken together, these studies show that human ventriculogenesis can occur via a completely cell autonomous pathway *in vivo* via purified HVPs, thereby allowing their use in organ-on-organ *in vivo* tissue engineering.

The human ventricular myocardium has a limited capacity for regeneration, most of which is lost after 10 years of age (Bergmann, O. *et al.* (2015) *Cell* 161:1566-1575). As such,
20 new strategies to generate heart muscle repair, regeneration, and tissue engineering approaches during cardiac injury have been a subject of intense investigation in regenerative biology and medicine (Sahara, M. *et al.* (2015) *EMBO J.* 34:710-738; Segers, V.F.M. and Lee, R.T. (2008) *Nature* 451:937-942). Given the need to achieve coordinated vascularization and matrix formation during tissue engineering of any solid organ, the assumption has been
25 that the formation of an intact 3-D solid organ *in vivo* will ultimately require the addition of vascular cells and/or conduits, as well as biomaterials and/or decellularized matrix that will allow alignment and the generation of contractile force (Forbes, S.J. and Rosenthal, N. (2014) *Nature Med.* 20:857-869; Harrison, R.H. *et al.* (2014) *Tissue Eng. Part B Rev.* 20:1-16). The complexity of adding these various components to achieve the formation of a functional solid
30 organ has confounded attempts to reduce this to clinical practice (Webber, M.J. *et al.* (2014) *Ann. Biomed. Eng.* 43:641-656). Although hPSCs hold great promise, to date, it has not been possible to build a pure, vascularized, fully functional, and mature 3-D human ventricular muscle organ *in vivo* on the surface of a heart in any mammalian system (Vunjak-Novakovic, G. *et al.* (2011) *Annu. Rev. Biomed. Eng.* 13:245-267).

The ability of generate billions of purified HVPs from a renewable source of either human ES or iPS cell lines represent a new approach to the generation of functional ventricular muscle in the setting of advanced heart failure. The progenitors can be delivered by intramyocardial injection and then self-migrate to the epicardial surface where they expand and differentiate, losing progenitor markers. Over the course of several week, the cells exit the cell cycle, and proceed to form adult rod-shaped cells that display several independent markers of mature ventricular myocardium including the formation of T tubules, catecholamine responsiveness, loss of automaticity, adult rod shaped conformation with aligned sarcomeric structures, and the ability to generate force that is comparable to other heart muscle patches derived from hPSCs differentiated cardiomyocytes (Tulloch, N.L. *et al.* (2011) *Circ. Res.* 109:47-59). The scalability of this cell autonomous pathway has allowed the ectopic generation of human ventricular muscle that has a combined thickness in excess of 1.5 cm in thickness, approaching levels that correspond to the human ventricular free wall (Basavarajaiah, S. *et al.* (2007) *Br. J. Sports Med.* 41:784-788).

The ability to migrate to the epicardial niche, the site of most of the adult heart progenitors at later stages, is a unique feature of HVPs, and mimics the normal niche of these cells during expansion of the ventricular compact zone during ventriculogenesis. Previous studies have shown that the generation of acute ischemic injury and a breakdown in vascular permeability are a pre-requisite for the grafting of relatively small numbers of ES cell derived cardiomyocytes into injured myocardium (van Laake, L.W. *et al.* (2007) *Stem Cell Res.* 1:9-24; Laflamme, M.A. *et al.* (2007) *Nat. Biotechnol.* 25:1015-1024), and even then the survival rate is low (< 5%) (Laflamme, M.A. and Murry, C.E. (2011) *Nature* 473:326-335; Laflamme, M.A. *et al.* (2005) *Am. J. Pathol.* 167:663-671). The ability of intra-myocardial HVPs to form an extensive ventricular patch on the epicardial surface in the absence of acute ischemic injury provides a new therapeutic strategy for dilated cardiomyopathy without the need for additional biomaterials, cells, or transfer of exogenous genes and/or RNAs.

The ability to form a 3-D ventricular muscle wall on the epicardial surface of the *in vivo* normal heart is a unique feature of the ventricular progenitors as later stage progenitors do not display the ability for the formation of three-dimensional ventricular tissue in either the cardiac or non-cardiac context, emphasizing the importance of generating a committed ventricular lineage as well as purifying the specific ventricular progenitor at a specific stage of ventriculogenesis.

Accordingly, the invention provides methods for generating human ventricular tissue *in vivo* using the HVPs described herein. In one embodiment, the method comprises

transplanting the NRP1+ progenitors into an organ of a non-human animal and allowing the progenitors to grow *in vivo* such that human ventricular tissue is generated. Preferably, the non-human animal is immunodeficient such that it cannot mount an immune response against the human progenitor cells. In one embodiment, the non-human animal is a mouse, such as
 5 an immunodeficient NOD.Cg-*Prkdcscid Il2rgtm1 Wjl/SzJ* mouse or an immunodeficient SCID-beige mouse (commercially available from Charles River France). In one embodiment, the organ is a kidney (e.g., the cells are transplanted under the kidney capsule). In another embodiment, the organ is a heart. In various embodiments, at least 1×10^6 cells, at least 2×10^6 cells, at least 3×10^6 cells, at least 4×10^6 cells, at least 5×10^6 cells, at least 1×10^7 cells, at least 5×10^7 cells, at least 1×10^8 cells, at least 1×10^9 cells are transplanted.
 10

To obtain HVPs for transplantation, human pluripotent stem cells (hPSCs) can be cultured *in vitro* under conditions leading to the generation of HVPs, as described herein (referred to herein as the HVPG protocol). Regarding the timing of transplanting HVPs post *in-vitro* culture, for optimal ventricular tissue generation the cells should be transplanted at a
 15 stage that can be defined based on the cellular markers expressed by the HVPs at the time of transplantation, determined at days post the start of culture, which is defined as day 0 of the HVPG protocol. In one embodiment, the cells are transplanted after the peak of cardiac mesoderm formation, which can be defined as peak expression of the mesodermal marker MESP1. Typically, MESP1 expression is between day 2 and day 4 of culture (inclusive) and
 20 peaks at day 3. In one embodiment, the cells are transplanted at the time corresponding to peak Islet-1 expression. Typically, Islet 1 is expressed between day 4 to day 8 of culture (inclusive) and peaks at day 6 of culture. In one embodiment, the cells are transplanted before the peak of NKX2.5 expression. Typically, NKX2.5 expression starts at day 6 of culture, peaks at day 10 of culture and is then maintained afterwards. In one embodiment, the
 25 cells are transplanted prior to the peak expression of the downstream genes MEF-2 and TBX-1. Typically, these downstream genes are expressed between day 5 and day 15 of culture (inclusive) and peaks at day 8 of culture. In one embodiment, the cells are transplanted prior to the expression of differentiated contractile protein genes. Typically, the expression of contractile protein genes (including TNNT2 and MYH6) starts from day 10 of culture
 30 onward. In certain embodiments, the cells are transplanted at a time when two, three or four of the aforementioned marker patterns are present. In another embodiment, the cells are transplanted at a time when all five of the aforementioned marker patterns are present. In one embodiment, the cells are transplanted between day 4 to day 8 (inclusive) of culture. In a

more preferred embodiment, the cells are transplanted between day 5 to day 7 (inclusive) of culture. In the most preferred embodiment, the cells are transplanted on day 6 of culture.

The transplanted cells can be allowed to grow in the non-human animal for a suitable period time to allow for the generation of the desired size, amount or thickness of ventricular tissue. In various embodiments, the cells are allowed to grow for one week, two weeks, one month, two months, three months, four months, five months or six months. The method can further comprise harvesting ventricular tissue from the non-human animal after growth of the cells and differentiation into ventricular tissue.

10 Methods of Enhancing Cardiac Function

The cardiac ventricular progenitor cells of the invention can be used *in vivo* to enhance cardiac function by transplanting the cells directly into the heart. It has now been shown that the NRP1+ progenitors have the capacity to differentiate into all three types of cardiac lineage cells (cardiac myocytes, endothelial cells and smooth muscle cells) (see Example 3). Furthermore, when cultured under conditions that bias toward the ventricular lineage, the NRP1+ progenitors have now been shown to adopt a predominantly ventricular muscle phenotype when transplanted into the natural ventricle environment *in vivo*, demonstrating that these progenitor cells “recognize” the ventricular environment and respond and differentiate appropriately *in vivo*. Since damage to the ventricular environment is largely responsible for the impaired cardiac function in cardiac diseases and disorders, the ability to restore ventricular muscle cells using the ventricular progenitor cells of the invention represents a significant advance in the art.

Accordingly, in another aspect, the invention provides a method of enhancing cardiac function in a subject, the method comprising administering a pharmaceutical composition comprising the clonal population of NRP1+ cardiac ventricular progenitor cells of the invention to the subject. Preferably, the clonal population is administered directly into the heart of the subject. More preferably, the clonal population is administered directly into a ventricular region of the heart of the subject. In one embodiment, the pharmaceutical composition administered to the subject comprises the clonal population formulated onto a three dimensional matrix.

The methods of the invention for enhancing cardiac function in a subject can be used in a variety of clinical situations involving damage to the heart or reduced or impaired cardiac function. Non-limiting examples of such clinical situations include a subject who has suffered a myocardial infarction and a subject who has a congenital heart disorder.

Thus, in another aspect, the invention provides a method of treating a cardiovascular condition, disease or disorder in a subject, the method comprising administering a pharmaceutical composition comprising the clonal population of NRP1+ cardiac ventricular progenitor cells of the invention to the subject. A therapeutically effective amount of cardiac
5 ventricular progenitor cells can be administered for the treatment of a cardiovascular condition, disease or disorder. Examples of preferred cardiovascular conditions, diseases or disorders include coronary artery disease and acute coronary syndrome.

Methods of Use of Cardiac Ventricular Progenitor Cells *In Vitro*

10 The cardiac ventricular progenitor cells of the invention can be used *in vitro* in the study of various aspects of cardiac maturation and differentiation, in particular in identifying the cells signaling pathways and biological mediators involved in the process of cardiac maturation and differentiation.

Furthermore, since the NRP1+ cardiac ventricular progenitor cells of the invention are
15 committed to the cardiac lineage and, moreover, are biased toward ventricular differentiation, these progenitor cells also are useful for evaluating the cardiac toxicity of test compounds. All potential new drugs and therapeutics must be evaluated for their toxicity to cardiac cells, before they can be deemed safe for use in humans. Thus, the ability to assess cardiac toxicity in an *in vitro* culture system is very advantageous. Accordingly, in another aspect, the
20 invention provides a method of screening for cardiac toxicity of test compound, the method comprising:

providing NRP1+ human cardiac ventricular progenitor cells;
contacting the cells with the test compound; and
measuring toxicity of the test compound for the cells,

25 wherein toxicity of the test compound for the cells indicates cardiac toxicity of the test compound.

In a preferred embodiment, the NRP1+ human cardiac ventricular progenitor cells are provided by isolating the cells according to the methods described herein. In a particularly preferred embodiment, the cells are isolated by separating NRP1+ cells from a cell culture
30 comprising cardiac progenitor cells using an anti-NRP1 antibody. Preferably, the cells are isolated using FACS or MACS as described herein. In yet another embodiment, the NRP1+ human cardiac ventricular progenitor cells are further cultured and differentiation into MLC2v+ ventricular cells prior to contacting with the test compound.

The toxicity of the test compound for the cells can be measured by one or more of a variety of different methods for assessing cell viability or other physiological functions. Preferably, the effect of the test compound on cell viability is measured using a standard cell viability assay, wherein reduced cell viability in the presence of the test compound is
5 indicative of cardiac toxicity of the test compound. Additionally or alternatively, cell growth can be measured. Additionally or alternatively, other indicators of physiological functions can be measured, such as cell adhesion, cell signaling, surface marker expression, gene expression and the like. Similarly, a negative effect of the test compound on any of these indicators of physiological function is indicative of cardiac toxicity of the test compound.

10 The invention further provides a method of identifying a compound that modulates human cardiac ventricular progenitor cell differentiation, the method comprising:

providing NRP1+ human cardiac ventricular progenitor cells;

culturing the cells in the presence or absence of a test compound;

measuring differentiation of the cells in the presence or absence of the test compound;

15 and

selecting a test compound that modulates human cardiac ventricular progenitor cell differentiation, as compared to differentiation in the absence of the test compound, to thereby identify a compound that modulates human cardiac ventricular progenitor cell differentiation.

In one embodiment, the test compound stimulates human cardiac ventricular
20 progenitor cell differentiation. In another embodiment, the test compound inhibits human cardiac ventricular progenitor cell differentiation. Differentiation of the cells can be measured by, for example, measurement of the expression of differentiation markers appearing on the cultured cells over time, as described herein. In a preferred embodiment, the NRP1+ human cardiac ventricular progenitor cells are provided by isolating the cells
25 according to the methods described herein. In a particularly preferred embodiment, the cells are isolated by separating NRP1+ cells from a cell culture comprising cardiac progenitor cells using an anti-NRP1 antibody. Preferably, the cells are isolated using FACS or MACS as described herein.

The invention further provides a method of identifying a compound that modulates
30 human ventricular cardiomyocyte function, the method comprising:

providing NRP1+ human cardiac ventricular progenitor cells;

culturing the cells in the presence or absence of a test compound under conditions that generate human ventricular cardiomyocytes;

measuring function of the human ventricular cardiomyocytes in the presence or absence of the test compound; and

selecting a test compound that modulates human ventricular cardiomyocyte function, as compared to function in the absence of the test compound, to thereby identify a compound
5 that modulates human ventricular cardiomyocyte function.

In one embodiment, the test compound stimulates human ventricular cardiomyocyte function. In another embodiment, the test compound inhibits human ventricular cardiomyocyte function. Function of the cells can be measured by measurement of any suitable indicator of ventricular cell function, including but not limited to, for example,
10 formation of T tubules, acquisition of adult-rod shaped ventricular cardiomyocytes, and ability to generate force in response to electrical stimulation. Suitable assays for measuring such indicators of ventricular cell function are known in the art. In a preferred embodiment, the NRP1+ human cardiac ventricular progenitor cells are provided by isolating the cells according to the methods described herein. In a particularly preferred embodiment, the cells
15 are isolated by separating NRP1+ cells from a cell culture comprising cardiac progenitor cells using an anti-NRP1 antibody. Preferably, the cells are isolated using FACS or MACS as described herein.

In Vivo Animal Models Using Human Ventricular Progenitor Cells

20 The development of human iPS and ES cell based models of cardiac disease has opened new horizons in cardiovascular drug development and discovery. However, to date, these systems have had the limitations of being based on 2D structures in cultured cell systems. In addition, the fetal and immature properties of the cells limit their utility and fidelity to the adult heart. Human cardiac disease, in particular heart failure, is a complex,
25 multifactorial, multi-organ disease, that is influenced by environmental, hormonal, and other key organs that are known sites for therapeutic targets, such as the kidney. The ability to build a mature functional human ventricular organ either ectopically or on the surface of the intact normal murine heart opens up a new *in vivo* model system to allow studies that normally could only be assayed on a mature human ventricular muscle chamber, such as
30 ventricular arrhythmias, generation of contractile force, fibrosis, and the potential for regeneration. Accordingly, the option to study human cardiac disease outside of the *in vitro* tissue culture systems, and directly in the context of heart failure *in vivo*, is now clearly possible.

Thus, the human ventricular progenitor cells also can be used to create animal models that allow for *in vivo* assessment of human cardiac tissue function and for *in vivo* screening of compounds, such as to determine the cardiac toxicity of a test compound *in vivo* or to identify compounds that modulate human cardiac tissue differentiation or function *in vivo*.

5 Accordingly, the invention provides methods for testing the effects of test compounds on human ventricular tissue *in vivo* using the HVPs described herein. In one embodiment, the method comprises:

transplanting NRP1+ human ventricular progenitors into an organ of a non-human animal;

10 allowing the progenitors to grow *in vivo* such that human ventricular tissue is generated;

administering a test compound to the non-human animal; and

evaluating the effect of the test compound on the human ventricular tissue in the non-human animal.

15 In another embodiment, the method comprises:

administering a test compound to a non-human animal, wherein the non-human animal comprises NRP1+ human ventricular progenitors transplanted into an organ of the non-human animal; and

20 evaluating the effect of the test compound on the NRP1+ human ventricular progenitors in the non-human animal.

In one embodiment, the cardiac toxicity of the test compound is evaluated, for example by measuring the effect of the test compound on the viability of the human ventricular tissue or the NRP1+ human ventricular progenitors in the non-human animal (as compared to the viability of the tissue or progenitors in the absence of the test compound).

25 Cell viability can be assessed by standard methods known in the art.

In another embodiment, the ability of a test compound to modulate cardiac differentiation can be evaluated, for example by measuring the effect of the test compound on the differentiation of the human ventricular tissue or NRP1+ progenitors in the non-human animal (as compared to the differentiation of the tissue or progenitors in the absence of the test compound). Differentiation of the cells can be measured by, for example, measurement of the expression of differentiation markers appearing on the cells over time.

In another embodiment, the ability of a test compound to modulate cardiac function can be evaluated, for example by measuring the effect of the test compound on the function of the human ventricular tissue or NRP1+ human progenitors in the non-human animal (as

compared to the function of the tissue or progenitors in the absence of the test compound). Function of the tissue or progenitors can be measured by measurement of any suitable indicator of ventricular cell function, including but not limited to, for example, formation of T tubules, acquisition of adult-rod shaped ventricular cardiomyocytes, and ability to generate force in response to electrical stimulation. Suitable assays for measuring such indicators of ventricular cell function are known in the art.

Preferably, the non-human animal is immunodeficient such that it cannot mount an immune response against the human progenitor cells. In one embodiment, the non-human animal is a mouse, such as an immunodeficient NOD.Cg-*Prkdcscid Il2rgtm1Wjl/SzJ* mouse or an immunodeficient SCID-beige mouse (commercially available from Charles River France). In one embodiment, the organ is a kidney (e.g., the cells are transplanted under the kidney capsule). In another embodiment, the organ is a heart. In various embodiments, at least 1×10^6 cells, at least 2×10^6 cells, at least 3×10^6 cells, at least 4×10^6 cells, at least 5×10^6 cells, at least 1×10^7 cells, at least 5×10^7 cells, at least 1×10^8 cells, at least 1×10^9 cells are transplanted.

To create the animal models, HVPs for transplantation can be obtained as described above by culturing of hPSCs *in vitro* under conditions leading to the generation of HVPs. Regarding the timing of transplanting HVPs post *in-vitro* culture, for optimal ventricular tissue generation the cells should be transplanted at a stage that can be defined based on the cellular markers expressed by the HVPs at the time of transplantation, determined at days post the start of culture, which is defined as day 0 of the HVPG protocol. In one embodiment, the cells are transplanted after the peak of cardiac mesoderm formation, which can be defined as peak expression of the mesodermal marker MESP1. Typically, MESP1 expression is between day 2 and day 4 of culture (inclusive) and peaks at day 3. In one embodiment, the cells are transplanted at the time corresponding to peak Islet-1 expression. Typically, Islet 1 is expressed between day 4 to day 8 of culture (inclusive) and peaks at day 6 of culture. In one embodiment, the cells are transplanted before the peak of NKX2.5 expression. Typically, NKX2.5 expression starts at day 6 of culture, peaks at day 10 of culture and is then maintained afterwards. In one embodiment, the cells are transplanted prior to the peak expression of the downstream genes MEF-2 and TBX-1. Typically, these downstream genes are expressed between day 5 and day 15 of culture (inclusive) and peaks at day 8 of culture. In one embodiment, the cells are transplanted prior to the expression of differentiated contractile protein genes. Typically, the expression of contractile protein genes (including TNNT2 and MYH6) starts from day 10 of culture onward. In certain

embodiments, the cells are transplanted at a time when two, three or four of the
aforementioned marker patterns are present. In another embodiment, the cells are
transplanted at a time when all five of the aforementioned marker patterns are present. In one
embodiment, the cells are transplanted between day 4 to day 8 (inclusive) of culture. In a
5 more preferred embodiment, the cells are transplanted between day 5 to day 7 (inclusive) of
culture. In the most preferred embodiment, the cells are transplanted on day 6 of culture.

The transplanted cells can be allowed to grow in the non-human animal for a suitable
period time to allow for the generation of the desired size, amount or thickness of ventricular
tissue, prior to administration of the test compound(s). In various embodiments, the cells are
10 allowed to grow for one week, two weeks, one month, two months, three months, four
months, five months or six months.

The present invention is further illustrated by the following examples, which should
not be construed as further limiting. The contents of figures and all references, patents and
published patent applications cited throughout this application are expressly incorporated
15 herein by reference.

EXAMPLES

Example 1: Generation of Human Isl1+ Cardiomyogenic Progenitor Cells by 20 Modulation of Wnt Signaling in Human Pluripotent Stem Cells

Temporal modulation of canonical Wnt signaling has been shown to be sufficient to
generate functional cardiomyocytes at high yield and purity from numerous hPSC lines (Lian,
X. *et al.* (2012) *Proc. Natl. Acad. Sci. USA* 109:E1848-1857; Lian, X. *et al.* (2013) *Nat.*
Protoc. 8:162-175). In this approach, Wnt/ β -catenin signaling first is activated in the hPSCs,
25 followed by an incubation period, followed by inhibition of Wnt/ β -catenin signaling. In the
originally published protocol, Wnt/ β -catenin signaling activation was achieved by incubation
with the Gsk3 inhibitor CHIR99021 (GSK-3 α , IC₅₀ = 10 nM; GSK-3, β IC₅₀ = 6.7 nM) and
Wnt/ β -catenin signaling inhibition was achieved by incubation with the Porcn inhibitor IWP2
(IC₅₀ = 27 nM). Because we used Gsk3 inhibitor and Wnt production inhibitor for cardiac
30 differentiation, this protocol was termed GiWi protocol. To improve the efficiency of the
original protocol and reduce the potential side effects of the small molecules used in the
original protocol, a second generation protocol was developed that uses another set of small
molecules with higher inhibition potency. In this second generation GiWi protocol, Wnt/ β -

catenin signaling activation was achieved by incubation with the Gsk3 inhibitor CHIR98014 (CAS 556813-39-9; commercially available from, *e.g.*, Selleckchem) (GSK-3 α , IC_{50} = 0.65 nM; GSK-3, β IC_{50} = 0.58 nM) and Wnt/ β -catenin signaling inhibition was achieved by incubation with the Porcn inhibitor Wnt-C59 (CAS 1243243-89-1; commercially available from, *e.g.*, Selleckchem or Tocris) (IC_{50} = 74 pM). The Gsk3 inhibitor CHIR98014 was used to promote cardiac mesodermal differentiation, whereas the Porcn inhibitor Wnt-C59 was used to enhance ventricular progenitor differentiation from mesoderm cells.

For cardiomyocyte differentiation via the use of these small molecules, hPSCs were maintained on Matrigel (BD Biosciences) coated plates (Corning) in E8 medium (described in Chen, G. *et al.* (2011) *Nature Methods*, 8:424-429; commercially available; STEMCELL Technologies) or mTeSR1 medium (commercially available; STEMCELL Technologies). Suitable hPSCs include induced pluripotent stem cells (iPSCs) such as 19-11-1, 19-9-7 or 6-9-9 cells (Yu, J. *et al.* (2009) *Science*, 324:797-801) and human embryonic stem cells (hESCs), such as ES03 (WiCell Research Institute) and H9 cells (Thomson, J.A. *et al.* (1998) *Science*, 282:1145-1147).

hPSCs maintained on a Matrigel-coated surface in mTeSR1 medium were dissociated into single cells with Accutase (Life Technologies) at 37° C for 5 minutes and then seeded onto a Matrigel-coated cell culture dish at 100,000-200,000 cells/cm² in mTeSR1 medium supplemented with 5 μ M ROCK inhibitor Y-27632 (Selleckchem)(day -2) for 24 hours. Cells were then cultured in mTeSR1, changed daily. At day 0, cells were then treated with 1 μ M Gsk3 inhibitor CHIR98014 (Selleckchem) for 24 hours (day 0 to day 1) in RPMI/B27-ins (500 ml RPMI with 10 ml B27 supplement without insulin). The medium was then changed to the corresponding medium containing 2 μ M the Porcn inhibitor Wnt-C59 (Selleckchem) at day 3, which was then removed during the medium change on day 5. Cells were maintained in RPMI/B27 (stock solution: 500 ml RMPI medium + 10 ml B27 supplement) starting from day 7, with the medium changed every three days. This exemplary culturing protocol for generating cardiomyogenic progenitor cells is illustrated schematically in Figure 1.

Flow cytometry and immunostaining were performed to examine the expression of particular lineage markers. After 24 hour treatment with CHIR-98014, more than 99% of the hPSCs expressed the mesoderm marker Brachyury. Three days after treatment with CHIR-98014, more than 95% of differentiated cells expressed Mesp1, which marks the cardiac mesoderm. The culture protocol not only allowed the cells to synchronously differentiate

into the cardiac mesodermal lineage, but also reproducibly generated more than 90% of ventricular myocytes after 14 days of differentiation, as determined by cTnT flow cytometry and electrophysiology analysis.

To further assess cardiac differentiation of the hPSCs over time, Western blot analysis was performed on days 0-7 and d11 to examine the expression of Isl1 and Nkx2.5 (cardiomyogenic progenitor markers) and cTnI (a cardiac myocyte marker). Cells were lysed in M-PER Mammalian Protein Extraction Reagent (Pierce) in the presence of Halt Protease and Phosphatase Inhibitor Cocktail (Pierce). Proteins were separated by 10% Tris-Glycine SDS/PAGE (Invitrogen) under denaturing conditions and transferred to a nitrocellulose membrane. After blocking with 5% dried milk in TBST, the membrane was incubated with primary antibody overnight at 4° C. The membrane was then washed, incubated with an anti-mouse/rabbit peroxidase-conjugated secondary antibody at room temperature for 1 hour, and developed by SuperSignal chemiluminescence (Pierce). The results are shown in Figure 2. During cardiac differentiation of hPSCs, Isl1 expression started on day 4 and increased to its maximum expression on day 6, whereas NKx2.5 only started to express on day 6 and reached its maximum expression after day 10. Cardiomyocytes (cTnI+ cells) were not induced until day 11 of differentiation.

In addition, immunostaining of the day 6 cells was performed for Isl1 expression. Cells were fixed with 4% formaldehyde for 15 minutes at room temperature and then stained with primary (anti-Isl1) and secondary antibodies in PBS plus 0.4% Triton X-100 and 5% non-fat dry milk (Bio-Rad). Nuclei were stained with Gold Anti-fade Reagent with DAPI (Invitrogen). An epifluorescence microscope (Leica DM IRB) with a QImaging® Retiga 4000R camera was used for imaging analysis. The results showed substantial numbers of Isl1+ cells.

Flow cytometry analysis of day 6 cells for Isl1 expression also was performed. Cells were dissociated into single cells with Accutase for 10 minutes and then fixed with 1% paraformaldehyde for 20 minutes at room temperature and stained with primary and secondary antibodies in PBS 0.1% Triton X-100 and 0.5% BSA. Data were collected on a FACSCaliber flow cytometer (Beckton Dickinson) and analyzed using FloJo. The results, shown in Figure 3, showed that more than 95% of cells expressed Isl1 at this stage.

In summary, this example provides a protocol for human ventricular progenitor generation (HVPG protocol) that allows for the large-scale production of billions of Isl1+ human HPVs efficiently within 6 days.

Example 2: Identification of Jagged 1 as a Cell Surface Marker of Cardiac Progenitor Cells

To profile the transcriptional changes that occur during the cardiac differentiation process at a genome-scale level, RNA sequencing (RNA-seq) was performed at different time points following differentiation to build cardiac development transcriptional landscapes. We performed RNA-seq experiments on day 0 to day 7 samples, as well as day 19 and day 35 samples (two independent biological replicates per time point). Two batches of RNA-seq (100 bp and 50 bp read length) were performed using the illumine Hiseq 2000 platform. In total, 20 samples were examined. Bowtie and Tophat were used to map our reads into a reference human genome (hg19) and we calculate each gene expression (annotation of the genes according to Refseq) using RPKM method (Reads per kilobase transcript per million reads). Differentiation of hPSCs to cardiomyocytes involves five major cell types: pluripotent stem cells (day 0), mesoderm progenitors (day 1 to day 2), cardiac mesoderm cells (day 3 to day 4), heart field progenitors (day 5, day 6 and day 7), and cardiomyocytes (day 10 after).

Molecular mRNA analysis of cardiac differentiation from hPSCs using the HVPG protocol revealed dynamic changes in gene expression, with down-regulation of the pluripotency markers *OCT4*, *NANOG* and *SOX2* during differentiation. Induction of the primitive streak-like genes *T* and *MIXL1* occurred within the first 24 hours following CHIR-98014 addition, and was followed by upregulation of the cardiac mesodermal marker *MESPI* on day 2 and day 3. Expression of the cardiac muscle markers *TNNT2*, *TNNC1*, *MYL2*, *MYL7*, *MYH6*, *MYH7* and *IRX4* was detected at later stage of differentiation (after day 10).

By this analysis, genes enriched at each differentiation stage, including mesoderm cells, cardiac progenitors and cardiomyocytes, were identified. Mesoderm cells, which are related to day 1 differentiated cells, express brachyury. We identified potential surface markers for mesoderm cells, including: FZD10, CD48, CD1D, CD8B, IL15RA, TNFRSF1B, TNFSF13, ICOSLG, SEMA7A, SLC3A2, SDC1, HLA-A. Through similar analysis, we also identified surface markers for cardiac mesoderm mesp1 positive cells, including: CXCR4, ANPEP, ITGA5, TNFRSF9, FZD2, CD1D, CD177, ACVRL1, ICAM1, L1CAM, NGFR, ABCG2, FZD7, TNFRSF13C, TNFRSF1B.

Consistent with western blot analysis, ISL1 mRNA was expressed as early as day 4 and peaked on day 5, one day before its protein expression reached its peak. On day 5 of differentiation (the cardiac progenitor stage, isl1 mRNA expression maximum on day 5, isl1 protein expression maximum on day 6), the day 5 enriched genes were compared with an

anti-CD antibody array (a panel of 350 known CD antibodies) and a number of potential cell-surface protein markers were identified. We identified many cell-surface proteins expressed at this stage, including: FZD4, JAG1, PDGFRA, LIFR (CD118), TNFSF9, FGFR3.

The cell surface protein Jagged 1 (JAG1) and Frizzled 4 (FZD4) were selected for further analysis. Jagged 1 expression was further studied as described below and in Examples 3 and 4. Frizzled 4 expression was further studied as described in Example 5.

Firstly, the expression of Isl1 and Jag1 was profiled using the double staining flow cytometry technique. Flow cytometric analysis was carried out essentially as described in Example 1, using anti-Isl1 and anti-Jag1 antibodies for double staining. The results are shown in Figure 4. Jagged 1 expression was found to trace the expression of Islet 1 and on day 6 of differentiation, all of the Islet 1 positive cells also expressed Jagged 1, and vice versa. Because of the co-expression pattern of these two markers, a Jagged 1 antibody was used to enrich the 94.1% Islet 1+ cells differentiated population to 99.8% purity of Islet1+Jagged1+ cells.

It also was confirmed that Islet 1 is an earlier developmental gene than the Nkx2.5 gene using double immunostaining of ISL1 and NKX2.5 expression in HVPs. The purified HVPs uniformly express the ISL1 gene, but at this stage, only a few of the cells started to express Nkx2.5.

Furthermore, immunostaining with both anti-Isl1 and anti-Jag 1 was performed, essentially as described in Example 1, on week 4 human fetal heart tissue, neonatal heart tissue and 8-year old heart tissue. The results revealed that in the *in vivo* fetal heart, all of the Islet 1 positive cells also expressed Jagged 1. However, the neonatal heart and 8-year old heart did not express Islet 1 or Jagged 1. In the ventricle of week 4 human fetal heart, cardiac Troponin T (cTnT) staining revealed visible sarcomere structures. In addition, over 50% of ventricular cells in the week 4 fetal heart expressed both Islet1 and Jagged1, which was markedly decreased during subsequent maturation, with the loss of expression of both Islet1 and Jagged1 in the ventricular muscle cells of the human neonatal hearts.

The above-described experiments demonstrate that Jagged 1 is a cell surface marker for Islet 1 positive cardiomyogenic progenitor cells.

Example 3: Clonal Differentiation of Isl1+Jag1+ Cardiac Progenitor Cells

To characterize the clonal differentiation potential of Isl1+Jag1+ cells, cardiomyogenic progenitor cells were generated by the culturing protocol described in

Example 1, and one single Isl1+Jag1+ cell was seeded into one well of a Matrigel-coated 48-well plate. Cells were purified with antibody of Jag1 and then one single cell was seeded into one well. The single cells were then cultured for 3 weeks in Cardiac Progenitor Culture (CPC) medium (advanced DMEM/F12 supplemented with 2.5 mM GlutaMAX, 100 µg/ml Vitamin C, 20% Knockout Serum Replacement).

Immunostaining of the 3-week differentiation cell population was then performed with three antibodies: cardiac troponin I (cTnI) for cardiomyocytes, CD144 (VE-cadherin) for endothelial cells and smooth muscle actin (SMA) for smooth muscle cells. The results showed that the single cell-cultured, Isl1+Jag1+ cells gave rise to cTnI positive and SMA positive cells, but not VE-cadherin positive endothelial cells, indicating these generated Islet1+ cells are heart muscle progenitors that have limited differentiation potential to endothelial lineages. Purified Islet1+Jagged1+ cells differentiated with the HVPG protocol from human induced pluripotent stem cells (iPSC 19-9-11 line) also showed similar *in vitro* differentiation potential and predominantly differentiate to cTnI+SMA+ cells, but not VE-cadherin+ cells. Over the course of several weeks, the cells expressed the ventricular specific marker MLC2v, indicating that the initial ISL1+ subset was already committed to the ventricular cell fate. Because of the limited vascular differentiation potential of Islet1+ cells generated using the HVPG protocol, these generated Islet1+ cells might represent a distinct progenitor population from the previously reported KDR+ population (Yang, L. *et al.* (2008) *Nature* 453:524-528) or multipotent ISL1+ cells (Bu, L. *et al.* (2009) *Nature* 460:113-117; Moretti, A. *et al.* (2006) *Cell* 127:1151-1165), which can give rise to all three lineages of cardiovascular cells.

These results demonstrated that the Isl1+Jag1+ cardiomyogenic progenitor cells can be successfully cultured *in vitro* from a single cell to a significantly expanded cell population (1 x 10⁹ cells or greater) that contains all three types of cardiac lineage cells, with a predominance of cardiomyocytes. Furthermore, these cells can be cultured *in vitro* for extended periods of time, for at least 2-3 weeks, and even for months (*e.g.*, six months or more). Since the cardiomyogenic progenitor cells gradually differentiate into cardiomyocytes, which do not proliferate, a culture period of approximately 2-3 weeks is preferred.

Example 4: *In Vivo* Developmental Potential of Isl1+Jag1+ Cardiac Progenitor Cells

The ES03 human embryonic stem cell (hESC) line (obtained from WiCell Research Institute) expresses green fluorescent protein (GFP) driven by the cardiac-specific cTnT promoter. ES03 cells were used to generate Isl1+Jag1+ cardiomyogenic progenitor cells using the culturing protocol described in Example 1. The Isl1+Jag1+ cardiomyogenic progenitor cells were transplanted into the hearts of severe combined immunodeficient (SCID) beige mice to document their developmental potential *in vivo*.

Briefly, Isl1+Jag1+ cells were injected (1,000,000 cells per recipient) directly into the left ventricular wall of NOD/SCID-gamma mice in an open-chest procedure. Hearts were harvested 2–3 weeks post-surgery, fixed in 1% PFA and sectioned at 10 µm (n = 12). Histological analyses of the hearts of the transplanted mice revealed the presence of GFP+ donor cells, detected by epifluorescence and by staining with an anti-GFP antibody, demonstrating that the Isl1+Jag1+ cardiomyogenic progenitor cells were capable of differentiating into cardiomyocytes when transplanted *in vivo*.

The Isl1+Jag1+ cardiomyogenic progenitor cells were also transplanted directly into infarcted hearts of SCID beige mice (“injured mice”), as compared to similarly transplanted normal mice. When analyzed two weeks later, injured mice transplanted with the Isl1+Jag1+ cardiomyogenic progenitor cells had a larger graft size than the normal mice similarly transplanted, demonstrating the cardiomyocyte regeneration capacity of the Isl1+Jag1+ cardiomyogenic progenitor cells *in vivo*.

Example 5: Identification of Frizzled 4 as a Cell Surface Marker of Cardiac Progenitor Cells

As described in Example 2, Frizzled 4 (FZD4) was identified by RNA-seq analysis as being expressed in cardiac progenitor cells. Thus, to confirm FZD4 as a cell surface marker of cardiac progenitor cells, FZD4 expression was assessed during cardiac differentiation via Western blot analysis. The results, as shown in Figure 5, demonstrated that FZD4 was not express in pluripotent stem cells and the first 3 days differentiated cells. However, FZD4 started to express on day 4 and maximize its expression on day 5 of expression.

In order to quantify the co-expression pattern of FZD4 and Isl1 at the single cell level, FACS analysis was performed. As shown in Figure 6, on day 5 of differentiation, more than 83% of cells express both isl1 and FZD4, demonstrating that FZD4 is a cell surface marker for isl1 positive cells during cardiac progenitor differentiation using the GiWi protocol.

In order to confirm that both JAG1 and FZD4 were indeed co-expressed with ISL1 on the human ventricular progenitor cells, triple immunofluorescence analysis of day 6 differentiated cells from hPSCs was performed with antibodies to Islet 1, Jagged 1 and Frizzled 4. The triple staining experiment demonstrated that Isl1+ cells expressed both Jagged 1 and Frizzled 4.

Example 6: Human Ventricular Progenitors (HPVs) Generate a 3-D Ventricular Heart Muscle Organ *In Vivo*

The building of the ventricular heart muscle chamber is one of the most critical and earliest steps during human organogenesis, and requires a series of coordinated steps, including migration, proliferation, vascularization, assembly, and matrix alignment. To test the capacity of HVPs to drive ventriculogenesis *in vivo*, we transplanted purified HVPs or unpurified HVPs ($92.0 \pm 1.9\%$ ISL1+) under the kidney capsule of immunocompromised mice. After 2 months post-transplantation, animals transplanted with unpurified HVPs formed tumors, resulting in a tumor formation efficiency of 100% (100%, 4/4), whereas animals transplanted with purified HVPs did not form any tumors (0%, 0/10).

The engrafted kidneys with purified HVPs were further assayed for histological analysis. Hematoxylin and Eosin (H&E) staining revealed an organ that exceeded 0.5 cm in length with more than 1 mm thickness on the surface of the mouse kidney, and that uniformly expressed the ventricular specific marker MLC2v (O'Brien, T.X. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:5157-5161). The resulting human muscle organ was fully vascularized and red blood cells could be detected in the blood vessels. Analysis of cTnT, MLC2v, and MLC2a immunostaining further revealed that the transplanted HVPs not only differentiated into cardiac muscle cells (cTnT+ cells), but also further mature to become MLC2v+ ventricular myocytes that are negative for MLC2a expression. The resulting ventricular muscle organ is fully vascularized by murine derived vascular cells, consistent with the notion that its vascularization occurred via paracrine cues derived from the HVPs.

The blood vessel structured was revealed by immunostaining analysis of antibodies directed against VE-cadherin and smooth muscle actin expression. In addition, using a human specific monoclonal laminin antibody targeting laminin γ -1 chain, the HVPs secreted their own human laminin as their extracellular matrix (the mouse kidney region is negative for human laminin immunostaining). In addition, we found human fibronectin expression is restricted to areas near the blood vessels using a monoclonal human fibronectin antibody.

To assess the capacity of late stage cardiac cells to drive ventriculogenesis, NKX2.5+ cells (day 10 after differentiation) were transplanted under the kidney capsule of immunocompromised NSG mice. At three weeks post-transplantation, animals transplanted with NKX2.5+ cells did not form any visible human muscle graft, indicating that HVPs lose their ability for in vivo ventriculogenesis following peak Islet-1 expression.

Taken together, these studies indicate that the HVPs can synthesize and release their own cardiac laminin-derived matrix, as well as fibronectin which serves to stabilize the vasculature to the nascent ventricular organ.

10 **Example 7: HVPs Create a Mature, Functioning Ventricular Muscle Organ** ***In Vivo* via a Cell Autonomous Pathway**

One of the critical limitations for the utility of hPSCs for studies of human cardiac biology and disease is their lack of maturity and persistence of expression of fetal isoforms. To determine if the HVP derived organs could become functional mature ventricular muscle, long term transplantation studies were performed followed by detailed analyses of a panel of well accepted features of adult ventricular myocardium including formation of T tubules (Brette, F. and Orchard, C. (2003) *Circ. Res.* 92:1182-1192; Marks, A.R. (2013) *J. Clin. Invest.* 123:46-52), ability to generate force comparable to other studies of engineered ventricular tissue, loss of automaticity, and acquisition of adult-rod shaped ventricular cardiomyocytes.

After 5 months post-transplantation of purified HVPs, no tumors formed in all of our animals. Animals were sacrificed and the engrafted kidneys were removed for further analysis. The 5-month human graft was a hemisphere structure with the radius of 0.4 cm (diameter of 0.8 cm). The volume for the 5-month human graft was around 0.13 cm³ for one kidney, a volume that suggests feasibility for generating human ventricular muscle that achieves a thickness comparable to the *in vivo* human adult heart. Rod-shaped mature human ventricular myocytes were observed in the human muscle organ. In addition, muscle trips taken from our mature human muscle organ generated forces (0.36 ± 0.04 mN) in response to electric stimulation and increased their force generation after treatment with a β -adrenergic agonist isoprenaline (0.51 ± 0.02 mN, $p < 0.05$ compared to control). Taken together, these studies indicate that the HVPs are capable of generating a fully functional, mature human ventricular muscle organ *in vivo* via a cell autonomous pathway, i.e., without the addition of other cells, genes, matrix proteins, or biomaterials.

Example 8: HVPs Migrate Towards an Epicardial Niche and Spontaneously Form a Human Ventricular Muscle Patch on the Surface of a Normal Murine Heart *In Vivo*

The epicardium is a known niche for heart progenitors, driving the growth of the ventricular chamber during compact zone expansion, as well as serving as a home to adult epicardial progenitors that can expand after myocardial injury and that can drive vasculogenesis in response to known vascular cell fate switches, such as VEGF (Giordano, F.J. *et al.* (2001) *Proc. Natl. Acad. Sci. USA* 98:5780-5785; Masters, M. and Riley, P.R. (2014) *Stem Cell Res.* 13:683-692; Zangi, L. *et al.* (2013) *Nat. Biotechnol.* 31:898-907). To determine if the HVPs might migrate spontaneously to the epicardial surface of the normal heart, purified green fluorescent protein (GFP)-labeled HVPs were injected intra-myocardially into the hearts of immunocompromised mice. After one week or one month post-transplantation, animals were sacrificed and the engrafted hearts were removed for histology. After one week post-transplantation, the majority of GFP+ cells were retained in the myocardium. However, almost all the GFP+ cells migrated to the epicardium after one month post-transplantation. In addition, GFP+ cells were ISL1+ and Ki67+ after one week post-transplantation.

In order to trace the differentiation potential of Islet1+ cells, the purified ISL1+JAG1+ cells generated from a cTnT promoter driven green fluorescent protein (GFP)-expressing hESC line (H9-cTnT-GFP) were transplanted into the hearts of severe combined immunodeficient (SCID) beige mice to document their developmental potential *in vivo*. One month after transplantation of Isl1+Jag1+ cells directly into the ventricle of the hearts of SCID beige mice, Hematoxylin and eosin staining revealed a human muscle strip graft present in the epicardium of the murine heart. In addition, immunohistological analyses revealed the presence of GFP+ donor cells detected by epifluorescence and by staining with an anti-GFP antibody. More importantly, when analysed with antibodies of MLC2v and MLC2a, the grafted human muscle strip is positive for MLC2v (100% of cells +), and negative for the atrial marker MLC2a, indicating the transplanted ISL1+ cells not only further differentiated to cardiac muscle cells, but also became ventricular muscle cells.

Taken together, these studies indicate that the HVPs can migrate to an epicardial niche, where they expand, and subsequently differentiate in to a homogenous ventricular muscle patch, again without the addition of exogenous cells, genes, matrices, or biomaterials.

Example 9: Additional Experimental Materials and Methods

In this example, additional details on the experimental materials and methods used in Examples 1-8 are provided.

5 Maintenance of hPSCs

hESCs (ES03, H9) and human iPSCs (19-9-11) were maintained on Matrigel (BD Biosciences) coated plates in mTeSR1 medium (STEMCELL Technologies) according to previous published methods (Lian, X. *et al.* (2013) *Nat. Proc.* 8:162-175; Lian, X. *et al.* (2013) *Stem Cells* 31:447-457).

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Human Ventricular Progenitor Generation (HVPG) protocol

hPSCs maintained on a Matrigel-coated surface in mTeSR1 were dissociated into single cells with Accutase at 37°C for 10 min and then seeded onto a Matrigel-coated cell culture dish at 100,000–200,000 cell/cm² in mTeSR1 supplemented with 5 μM ROCK inhibitor Y-27632 (day -2) for 24 hours. At day -1, cells were cultured in mTeSR1. At day 0, cells were treated with 1 μM CHIR-98014 (Selleckchem) in RPMI supplemented with B27 minus insulin (RPMI/B27-ins) for 24 hours (day 0 to day 1), which was then removed during the medium change on day 1. At day 3, half of the medium was changed to the RPMI/B27-ins medium containing 2 μM Wnt-C59 (Selleckchem), which was then removed during the medium change on day 5. At day 6, cells were dissociated into single cells and purified with anti-JAG1 or anti-FZD4 antibody.

RNA-seq library construction

RNA was isolated (RNeasy Mini kit, Qiagen), quantified (Qubit RNA Assay Kit, Life Technologies) and quality controlled (BioAnalyzer 2100, Agilent). RNA (800 ng) from each sample was used as input for the Illumina TruSeq mRNA Sample Prep Kit v2 (Illumina) and sequencing libraries were created according to the manufacturer's protocol. Briefly, poly-A containing mRNA molecules were purified using poly-T oligo-attached magnetic beads. Following purification, the mRNA was fragmented and copied into first strand complementary DNA using random primers and reverse transcriptase. Second strand cDNA synthesis was then done using DNA polymerase I and RNase H. The cDNA was ligated to adapters and enriched with PCR to create the final cDNA library. The library was pooled and sequenced on a HiSeq 2000 (Illumina) instrument per the manufacturer's instructions.

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RNA-seq data processing

The RNA-seq reads were trimmed and mapped to the hg19 reference using Tophat 2. On average, approximately 23 million reads were generated per sample, and 76% of these reads were uniquely mapped. Expression levels for each gene were quantified using the python script rpkmforgenes and annotated using RefSeq. Genes without at least one sample with at least ten reads were removed from the analysis. Principle Component Analysis and heatmaps were constructed using the R and Gene-E respectively.

Transplantation

Aliquots of 2 million purified HVPs were collected into an eppendorf tube. Cells were spun down, and the supernatant was discarded. Each tube of cells was transplanted under the kidney capsule, or intra-myocardially injected into the heart of the immunodeficient mice, NOD.Cg-*Prkdcscid Il2rgtm1 Wjl/SzJ* or SCID-Beige respectively (Charles River France), following a previously described protocol (Shultz, L.D. et al. (2005) *J. Immunol.* 174:6477-6489). Engrafted Kidneys or hearts are harvested at various time intervals for histological and physiological analysis.

Flow cytometry

Cells were dissociated into single cells with Accutase for 10 min and then fixed with 1% paraformaldehyde for 20 min at room temperature and stained with primary and secondary antibodies in PBS plus 0.1% Triton X-100 and 0.5% BSA. Data were collected on a FACSCaliber flow cytometer (Beckton Dickinson) and analyzed using FlowJo.

Immunostaining

Cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then stained with primary and secondary antibodies in PBS plus 0.4% Triton X-100 and 5% non-fat dry milk (Bio-Rad). Nuclei were stained with Gold Anti-fade Reagent with DAPI (Invitrogen). An epifluorescence microscope and a confocal microscope (ZEISS, LSM 700) were used for imaging analysis.

Western Blot Analysis

Cells were lysed in M-PER Mammalian Protein Extraction Reagent (Pierce) in the presence of Halt Protease and Phosphatase Inhibitor Cocktail (Pierce). Proteins were separated by 10% Tris-Glycine SDS/PAGE (Invitrogen) under denaturing conditions and transferred to a nitrocellulose membrane. After blocking with 5% dried milk in TBST, the

membrane was incubated with primary antibody overnight at 4°C. The membrane was then washed, incubated with an anti-mouse/rabbit peroxidase-conjugated secondary antibody at room temperature for 1 hour, and developed by SuperSignal chemiluminescence (Pierce).

5 Electrophysiology (Patch Clamping)

Beating ventricular myocyte clusters were microdissected and replated onto glass coverslips before recording. Action potential activity was assessed using borosilicate glass pipettes (4-5 M Ohm resistance) filled with intracellular solution consisting of 120 mM K D-gluconate, 25 mM KCl, 4 mM MgATP, 2 mM NaGTP, 4 mM Na₂-phospho-creatin, 10 mM EGTA, 1 mM CaCl₂, and 10 mM HEPES (pH 7.4 adjusted with HCl at 25°C). Cultured cardiomyocytes seeded on coverslip dishes were submerged in extracellular solution (Tyrode's solution) containing 140 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 10 mM glucose, 1.8 mM CaCl₂, and 10 mM HEPES (pH 7.4 adjusted with NaOH at 25°C). Spontaneous action potentials were recorded at 37°C using patch clamp technique (whole-cell, current clamp configuration) performed using a Multiclamp 700B amplifier (Molecular Devices, CA, USA) software low-pass filtered at 1 kHz, digitized and stored using a Digidata 1322A and Clampex 9.6 software (Molecular Devices, CA, USA).

Statistics

Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical significance was determined by Student's t-test (two-tail) between two groups. $P < 0.05$ was considered statistically significant.

Example 10: Xeno-Free Human Ventricular Progenitor Differentiation Protocol

In this example, an alternative differentiation protocol for differentiation of human ventricular progenitors is provided, which utilizes a defined, xeno-free culture medium, Essential 8. The Essential 8 medium was developed for growth and expansion of human pluripotent stem cells (hPSCs) and is described further in Chen, G. *et al.* (2011) Nat. Methods 8:424-429 (referred to therein as "E8" medium).

hPSCs maintained on a Vitronectin (or Laminin 521)-coated surface in Essential 8 medium were dissociated into single cells with Versene solution at 37°C for 10 min and then seeded onto a Vitronectin (or Laminin 521)-coated cell culture dish at 100,000–200,000 cell/cm² in Essential 8 medium supplemented with 5 μ M ROCK inhibitor Y-27632 (day -2)

for 24 hours. At day -1, cells were cultured in Essential 8 medium. At day 0, cells were treated with 0.5 μ M CHIR-98014 in RPMI for 24 hours (day 0 to day 1), which was then removed during the medium change on day 1. At day 3, half of the medium was changed to the RPMI medium containing 0.5 μ M Wnt-C59, which was then removed during the medium change on day 5. At day 6, cells (human ventricular progenitors) were dissociated into single cells and purified with anti-JAG1 or anti-FZD4 antibody. Alternatively cells are purified with anti-LIFR or anti-FGFR3 antibody.

Example 11: Angiogenic Markers for Engraftable Human Ventricular Progenitor Cells

In this example, genes in the angiogenic family that are expressed in human ventricular progenitor cells (HVPs) were identified. HVPs were generated as described in Examples 1 or 10 and RNA sequencing (RNA-seq) was performed at different time points following differentiation as described in Example 1. Cluster analysis of gene expression profiles at different time points during HVP differentiation identified stage-specific signature genes. These genes were clustered hierarchically on the basis of the similarity of their expression profiles. First, genes showing expression in four different categories were identified: (i) cell surface expression; (ii) co-expression with Islet 1; (iii) high expression on day 5 of differentiation; and (iv) high d5/d0 ratio. This analysis confirmed the cell surface markers for HVPs of: JAG1, FZD4, FGFR3, LIFR (CD118) and TNFSF9. Next, from this same population of HVPs that identified the cell surface markers, gene ontology searches were performed to identify angiogenic family genes that were expressed in this population of HVPs, to thereby identify a gene fingerprint profile that identifies genes critical for cell engraftment.

Statistically, Pearson’s correlation with Isl1 expression was used to identify those angiogenic genes whose expression in the HVPs best correlated with Isl1 expression. Table 1 below lists the angiogenic genes that correlate with Isl1 expression with a Pearson’s correlation of 0.50 or higher.

Table 1: Angiogenic genes expressed in HVPs with a Pearson Correlation with Isl1 Expression of 0.50 or greater

Gene	Angiogenic genes (GO:0001525)	Pearson’s Correlation with Isl1 Expression
FGF10	fibroblast growth factor 10	0.98
PRKD1	protein kinase D1	0.95

Gene	Angiogenic genes (GO:0001525)	<u>Pearson's Correlation with Isl1 Expression</u>
CCBE1	collagen and calcium binding EGF domains 1	0.94
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	0.94
EPHB2	EPH receptor B2	0.92
GATA2	GATA binding protein 2	0.92
NTRK1	neurotrophic tyrosine kinase, receptor, type 1	0.92
PTGIS	prostaglandin I2 (prostacyclin) synthase	0.87
BMPER	BMP binding endothelial regulator	0.85
BMP4	bone morphogenetic protein 4	0.84
C1GALT1	core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1	0.84
MEIS1	Meis homeobox 1	0.83
TBX1	T-box 1	0.83
PKNOX1	PBX/knotted 1 homeobox 1	0.83
ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	0.82
TCF21	transcription factor 21	0.82
HEY1	hes-related family bHLH transcription factor with YRPW motif 1	0.80
HOXB3	homeobox B3	0.78
JAG1	jagged 1	0.75
HGF	hepatocyte growth factor (hepapoietin A; scatter factor)	0.74
IL6	interleukin 6	0.74
GHRL	ghrelin/obestatin prepropeptide	0.73
IHH	indian hedgehog	0.70
SRPK2	SRSF protein kinase 2	0.70
GATA6	GATA binding protein 6	0.69
HAND1	heart and neural crest derivatives expressed 1	0.69
AMOT	angiomotin	0.69
NRP2	neuropilin 2	0.65
PTEN	phosphatase and tensin homolog	0.65
SEMA3E	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3E	0.64
APOLD1	apolipoprotein L domain containing 1	0.62
SETD2	SET domain containing 2	0.62
DAB2IP	DAB2 interacting protein	0.61
KDR	kinase insert domain receptor	0.60
PGF	placental growth factor	0.60
EMP2	epithelial membrane protein 2	0.59
TAL1	T-cell acute lymphocytic leukemia 1	0.58
ACVR1	activin A receptor, type I	0.58
HIPK2	homeodomain interacting protein kinase 2	0.56
CSPG4	chondroitin sulfate proteoglycan 4	0.55
TNFAIP3	tumor necrosis factor, alpha-induced protein 3	0.55
NRP1	neuropilin 1	0.55
NFATC4	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	0.54

Gene	Angiogenic genes (GO:0001525)	<u>Pearson's Correlation with Isl1 Expression</u>
CDC42	cell division cycle 42	0.54
ANGPTL4	angiopoietin-like 4	0.53
BCAS3	breast carcinoma amplified sequence 3	0.53
HIPK1	homeodomain interacting protein kinase 1	0.53
NRXN3	neurexin 3	0.52
FZD5	frizzled class receptor 5	0.52
HHEX	hematopoietically expressed homeobox	0.50

Table 2 below lists the angiogenic genes that correlate with Isl1 expression with a Pearson's correlation of 0.49-0.00.

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Table 2: Angiogenic genes expressed in HVPs with a Pearson Correlation with Isl1 Expression of 0.49 to 0.00

Gene	Angiogenic genes (GO:0001525)	<u>Pearson's Correlation with Isl1 Expression</u>
ACVRL1	activin A receptor type II-like 1	0.49
ENPEP	glutamyl aminopeptidase (aminopeptidase A)	0.49
EFNA1	ephrin-A1	0.49
CHRNA7	cholinergic receptor, nicotinic, alpha 7 (neuronal)	0.49
TMEM100	transmembrane protein 100	0.48
NOS3	nitric oxide synthase 3 (endothelial cell)	0.47
LEF1	lymphoid enhancer-binding factor 1	0.47
NRXN1	neurexin 1	0.46
EPHB3	EPH receptor B3	0.44
ROCK1	Rho-associated, coiled-coil containing protein kinase 1	0.42
NF1	neurofibromin 1	0.42
CYSLTR2	cysteinyl leukotriene receptor 2	0.42
FGFR2	fibroblast growth factor receptor 2	0.41
GATA4	GATA binding protein 4	0.40
FMNL3	formin-like 3	0.40
C3	complement component 3	0.40
WASF2	WAS protein family, member 2	0.40
CALCRL	calcitonin receptor-like	0.39
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	0.39
VEGFA	vascular endothelial growth factor A	0.39
KRIT1	KRIT1, ankyrin repeat containing	0.39
CDH13	cadherin 13	0.39
COL18A1	collagen, type XVIII, alpha 1	0.39
STK4	serine/threonine kinase 4	0.38
C5	complement component 5	0.38

Gene	Angiogenic genes (GO:0001525)	<u>Pearson's Correlation with Isl1 Expression</u>
HDAC7	histone deacetylase 7	0.38
ANGPT2	angiopoietin 2	0.38
PLCG1	phospholipase C, gamma 1	0.37
EDNRA	endothelin receptor type A	0.35
TGFB2	transforming growth factor, beta 2	0.35
HAND2	heart and neural crest derivatives expressed 2	0.35
CD34	CD34 molecule	0.35
BTG1	B-cell translocation gene 1, anti-proliferative	0.34
TGFB1	transforming growth factor, beta receptor 1	0.33
FGFR1	fibroblast growth factor receptor 1	0.33
FN1	fibronectin 1	0.31
TWIST1	twist family bHLH transcription factor 1	0.31
ELK3	ELK3, ETS-domain protein (SRF accessory protein 2)	0.30
THSD7A	thrombospondin, type I, domain containing 7A	0.30
RGCC	regulator of cell cycle	0.30
PLCD1	phospholipase C, delta 1	0.29
SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	0.29
TBX20	T-box 20	0.28
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha	0.27
MMRN2	multimerin 2	0.27
FOXO4	forkhead box O4	0.26
RAMP2	receptor (G protein-coupled) activity modifying protein 2	0.25
FLT1	fms-related tyrosine kinase 1	0.25
ADRB2	adrenoceptor beta 2, surface	0.25
SLC12A6	solute carrier family 12 (potassium/chloride transporter), member 6	0.25
ADM	adrenomedullin	0.25
NPPB	natriuretic peptide B	0.24
SPINK5	serine peptidase inhibitor, Kazal type 5	0.24
MAPK14	mitogen-activated protein kinase 14	0.24
MMP2	matrix metalloproteinase 2	0.24
PTPRM	protein tyrosine phosphatase, receptor type, M	0.23
OVOL2	ovo-like zinc finger 2	0.23
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	0.22
OTULIN	OTU deubiquitinase with linear linkage specificity	0.21
B4GALT1	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	0.21
PDGFRB	platelet-derived growth factor receptor, beta polypeptide	0.20
F3	coagulation factor III (thromboplastin, tissue factor)	0.20
PRKCA	protein kinase C, alpha	0.20
LRP5	low density lipoprotein receptor-related protein 5	0.20
MAP3K7	mitogen-activated protein kinase kinase kinase 7	0.20
NRCAM	neuronal cell adhesion molecule	0.19
MAP2K5	mitogen-activated protein kinase kinase 5	0.18
S1PR1	sphingosine-1-phosphate receptor 1	0.18

Gene	Angiogenic genes (GO:0001525)	<u>Pearson's Correlation with Isl1 Expression</u>
NFATC3	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	0.18
TSPAN12	tetraspanin 12	0.18
LAMA5	laminin, alpha 5	0.17
LOXL2	lysyl oxidase-like 2	0.17
ANGPT1	angiopoietin 1	0.17
GTF2I	general transcription factor Iii	0.16
E2F8	E2F transcription factor 8	0.16
PDE3B	phosphodiesterase 3B, cGMP-inhibited	0.15
SHB	Src homology 2 domain containing adaptor protein B	0.14
MYH9	myosin, heavy chain 9, non-muscle	0.14
FZD8	frizzled class receptor 8	0.14
NOV	nephroblastoma overexpressed	0.14
SH2D2A	SH2 domain containing 2A	0.14
FGF8	fibroblast growth factor 8 (androgen-induced)	0.13
TIE1	tyrosine kinase with immunoglobulin-like and EGF-like domains 1	0.13
EGLN1	egl-9 family hypoxia-inducible factor 1	0.12
RORA	RAR-related orphan receptor A	0.11
MFGE8	milk fat globule-EGF factor 8 protein	0.11
ARHGAP24	Rho GTPase activating protein 24	0.10
ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	0.10
PARVA	parvin, alpha	0.10
ADIPOR2	adiponectin receptor 2	0.09
NPR1	natriuretic peptide receptor 1	0.09
ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	0.09
HIF3A	hypoxia inducible factor 3, alpha subunit	0.08
EPAS1	endothelial PAS domain protein 1	0.08
FOXC2	forkhead box C2	0.07
ANXA2	annexin A2	0.06
RBM15	RNA binding motif protein 15	0.06
PITX2	paired-like homeodomain 2	0.06
FOXC1	forkhead box C1	0.06
SRF	serum response factor	0.06
ECSCR	endothelial cell surface expressed chemotaxis and apoptosis regulator	0.05
SOX17	SRY (sex determining region Y)-box 17	0.04
HDAC5	histone deacetylase 5	0.04
LRG1	leucine-rich alpha-2-glycoprotein 1	0.04
ADAM8	ADAM metallopeptidase domain 8	0.03
UBP1	upstream binding protein 1 (LBP-1a)	0.02
VASH1	vasohibin 1	0.02
ANXA3	annexin A3	0.01
RRAS	related RAS viral (r-ras) oncogene homolog	0.01

Gene	<u>Angiogenic genes (GO:0001525)</u>	<u>Pearson's Correlation with Isl1 Expression</u>
TYMP	thymidine phosphorylase	0.01
PRCP	prolylcarboxypeptidase (angiotensinase C)	0.01
SEMA5A	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	0.00
GREM1	gremlin 1, DAN family BMP antagonist	0.00

Angiogenic genes whose expression negatively correlated with Isl1 expression in the HVPs were also identified. Table 3 below lists the angiogenic genes that negatively correlate with Isl1 expression with a Pearson's correlation of -0.50 or less.

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Table 3: Angiogenic genes expressed in HVPs with a Pearson Correlation with Isl1 Expression of -0.50 or less

Gene	<u>Angiogenic genes (GO:0001525)</u>	<u>Pearson's Correlation with Isl1 Expression</u>
ETS1	v-ets avian erythroblastosis virus E26 oncogene homolog 1	-0.50
BAX	BCL2-associated X protein	-0.50
XBP1	X-box binding protein 1	-0.52
TDGF1	teratocarcinoma-derived growth factor 1	-0.53
C5AR1	complement component 5a receptor 1	-0.53
EPHA1	EPH receptor A1	-0.53
HS6ST1	heparan sulfate 6-O-sulfotransferase 1	-0.56
SHC1	SHC (Src homology 2 domain containing) transforming protein 1	-0.56
SP100	SP100 nuclear antigen	-0.58
JAM3	junctional adhesion molecule 3	-0.58
CASP8	caspase 8, apoptosis-related cysteine peptidase	-0.60
FLT4	fms-related tyrosine kinase 4	-0.60
SFRP2	secreted frizzled-related protein 2	-0.61
HPSE	heparanase	-0.61
BAK1	BCL2-antagonist/killer 1	-0.65
GPX1	glutathione peroxidase 1	-0.65
VAV3	vav 3 guanine nucleotide exchange factor	-0.70
VAV2	vav 2 guanine nucleotide exchange factor	-0.72
EGF	epidermal growth factor	-0.72
ADAM15	ADAM metallopeptidase domain 15	-0.73
AGGF1	angiogenic factor with G patch and FHA domains 1	-0.76

10 Table 4 below lists the angiogenic genes that negatively correlate with Isl1 expression with a Pearson's correlation of -0.01 to -0.49.

Table 4: Angiogenic genes expressed in HVPs with a Pearson Correlation with Isl1 Expression of -0.01 to -0.49

Gene	Angiogenic genes (GO:0001525)	<u>Pearson's Correlation with Isl1 Expression</u>
EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3	-0.01
ROCK2	Rho-associated, coiled-coil containing protein kinase 2	-0.01
WNT5A	wingless-type MMTV integration site family, member 5A	-0.02
NR4A1	nuclear receptor subfamily 4, group A, member 1	-0.02
CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	-0.02
PTK2	protein tyrosine kinase 2	-0.03
SFRP1	secreted frizzled-related protein 1	-0.04
STAT1	signal transducer and activator of transcription 1, 91kDa	-0.04
ITGAV	integrin, alpha V	-0.04
EPHB4	EPH receptor B4	-0.05
CYR61	cysteine-rich, angiogenic inducer, 61	-0.05
TEK	TEK tyrosine kinase, endothelial	-0.06
COL15A1	collagen, type XV, alpha 1	-0.06
COL4A1	collagen, type IV, alpha 1	-0.07
ANG	angiogenin, ribonuclease, RNase A family, 5	-0.07
HSPB1	heat shock 27kDa protein 1	-0.07
PLXND1	plexin D1	-0.08
HSPG2	heparan sulfate proteoglycan 2	-0.09
VEGFC	vascular endothelial growth factor C	-0.09
SYNJ2BP	synaptojanin 2 binding protein	-0.09
THBS1	thrombospondin 1	-0.09
CTGF	connective tissue growth factor	-0.10
ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	-0.12
AAMP	angio-associated, migratory cell protein	-0.12
GJA5	gap junction protein, alpha 5, 40kDa	-0.12
PRKCB	protein kinase C, beta	-0.13
EGR3	early growth response 3	-0.13
JMJD6	jumonji domain containing 6	-0.13
TGFB1	transforming growth factor, beta-induced, 68kDa	-0.14
SIRT1	sirtuin 1	-0.14
ANGPTL3	angiopoietin-like 3	-0.14
ACKR3	atypical chemokine receptor 3	-0.14
SAT1	spermidine/spermine N1-acetyltransferase 1	-0.15
VEGFB	vascular endothelial growth factor B	-0.16
UTS2	urotensin 2	-0.16
JUN	jun proto-oncogene	-0.16
TNFSF12	tumor necrosis factor (ligand) superfamily, member 12	-0.16
EGFL7	EGF-like-domain, multiple 7	-0.17
MED1	mediator complex subunit 1	-0.17
SLIT2	slit guidance ligand 2	-0.17
SERPINF1	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	-0.18

Gene	Angiogenic genes (GO:0001525)	<u>Pearson's Correlation with Isl1 Expression</u>
NOTCH3	notch 3	-0.18
FGF9	fibroblast growth factor 9	-0.19
DLL4	delta-like 4 (Drosophila)	-0.19
CCL2	chemokine (C-C motif) ligand 2	-0.19
MMP14	matrix metalloproteinase 14 (membrane-inserted)	-0.19
TMPRSS6	transmembrane protease, serine 6	-0.19
EPGN	epithelial mitogen	-0.20
RBPJ	recombination signal binding protein for immunoglobulin kappa J region	-0.20
COL4A2	collagen, type IV, alpha 2	-0.20
PRKD2	protein kinase D2	-0.20
ALOX12	arachidonate 12-lipoxygenase	-0.21
RNH1	ribonuclease/angiogenin inhibitor 1	-0.21
APOH	apolipoprotein H (beta-2-glycoprotein I)	-0.21
CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	-0.21
ESM1	endothelial cell-specific molecule 1	-0.22
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	-0.22
ANPEP	alanyl (membrane) aminopeptidase	-0.22
LEMD3	LEM domain containing 3	-0.22
UTS2R	urotensin 2 receptor	-0.22
CIB1	calcium and integrin binding 1 (calmyrin)	-0.22
ITGB1BP1	integrin beta 1 binding protein 1	-0.22
AQP1	aquaporin 1 (Colton blood group)	-0.22
IL18	interleukin 18	-0.22
EPHA2	EPH receptor A2	-0.22
EPHB1	EPH receptor B1	-0.22
AGT	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	-0.22
PLAU	plasminogen activator, urokinase	-0.22
VEZF1	vascular endothelial zinc finger 1	-0.23
SPHK1	sphingosine kinase 1	-0.23
SRPX2	sushi-repeat containing protein, X-linked 2	-0.23
PDCL3	phosducin-like 3	-0.23
COL8A1	collagen, type VIII, alpha 1	-0.24
HDAC9	histone deacetylase 9	-0.24
CTSH	cathepsin H	-0.24
EDN1	endothelin 1	-0.24
CXCL8	chemokine (C-X-C motif) ligand 8	-0.24
ECM1	extracellular matrix protein 1	-0.24
BRCA1	breast cancer 1, early onset	-0.24
EFNB2	ephrin-B2	-0.25
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	-0.25
SASH1	SAM and SH3 domain containing 1	-0.25

Gene	Angiogenic genes (GO:0001525)	<u>Pearson's Correlation with Isl1 Expression</u>
WNT7B	wingless-type MMTV integration site family, member 7B	-0.25
RAMP1	receptor (G protein-coupled) activity modifying protein 1	-0.26
SCG2	secretogranin II	-0.26
COL8A2	collagen, type VIII, alpha 2	-0.26
SULF1	sulfatase 1	-0.26
CLIC4	chloride intracellular channel 4	-0.26
FGF1	fibroblast growth factor 1 (acidic)	-0.27
NODAL	nodal growth differentiation factor	-0.27
RASIP1	Ras interacting protein 1	-0.28
RLN2	relaxin 2	-0.28
POFUT1	protein O-fucosyltransferase 1	-0.28
FGF18	fibroblast growth factor 18	-0.28
AIMP1	aminoacyl tRNA synthetase complex-interacting multifunctional protein 1	-0.28
TGFBR2	transforming growth factor, beta receptor II (70/80kDa)	-0.28
RHOB	ras homolog family member B	-0.28
GBX2	gastrulation brain homeobox 2	-0.28
ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2	-0.29
MAPK7	mitogen-activated protein kinase 7	-0.30
PROK2	prokineticin 2	-0.30
E2F7	E2F transcription factor 7	-0.30
ERAP1	endoplasmic reticulum aminopeptidase 1	-0.31
MTDH	metadherin	-0.31
KLF5	Kruppel-like factor 5 (intestinal)	-0.31
DICER1	dicer 1, ribonuclease type III	-0.32
LECT1	leukocyte cell derived chemotaxin 1	-0.32
CX3CL1	chemokine (C-X3-C motif) ligand 1	-0.32
PTK2B	protein tyrosine kinase 2 beta	-0.33
SEMA4A	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4A	-0.34
ARHGAP22	Rho GTPase activating protein 22	-0.34
RSPO3	R-spondin 3	-0.34
KLF4	Kruppel-like factor 4 (gut)	-0.34
ROBO1	roundabout guidance receptor 1	-0.34
GPLD1	glycosylphosphatidylinositol specific phospholipase D1	-0.35
NUS1	NUS1 dehydrodolichyl diphosphate synthase subunit	-0.35
NRARP	NOTCH-regulated ankyrin repeat protein	-0.35
PDCD10	programmed cell death 10	-0.36
PF4	platelet factor 4	-0.36
PRKX	protein kinase, X-linked	-0.36
PML	promyelocytic leukemia	-0.36
ATP5B	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, beta polypeptide	-0.36
TNFRSF1	tumor necrosis factor receptor superfamily, member 12A	-0.36

Gene	Angiogenic genes (GO:0001525)	<u>Pearson's Correlation with Isl1 Expression</u>
2A		
ENG	endoglin	-0.37
THY1	Thy-1 cell surface antigen	-0.37
FGF2	fibroblast growth factor 2 (basic)	-0.37
CXCL12	chemokine (C-X-C motif) ligand 12	-0.37
CAV1	caveolin 1, caveolae protein, 22kDa	-0.38
PDGFA	platelet-derived growth factor alpha polypeptide	-0.38
PNPLA6	patatin-like phospholipase domain containing 6	-0.38
PLCD3	phospholipase C, delta 3	-0.38
DDAH1	dimethylarginine dimethylaminohydrolase 1	-0.39
GNA13	guanine nucleotide binding protein (G protein), alpha 13	-0.39
ADM2	adrenomedullin 2	-0.39
HMOX1	heme oxygenase 1	-0.40
MCAM	melanoma cell adhesion molecule	-0.41
RAPGEF3	Rap guanine nucleotide exchange factor (GEF) 3	-0.41
TNFAIP2	tumor necrosis factor, alpha-induced protein 2	-0.41
HTATIP2	HIV-1 Tat interactive protein 2, 30kDa	-0.42
NCL	nucleolin	-0.42
ERBB2	erb-b2 receptor tyrosine kinase 2	-0.43
NAA15	N(alpha)-acetyltransferase 15, NatA auxiliary subunit	-0.43
ATPIF1	ATPase inhibitory factor 1	-0.43
THBS4	thrombospondin 4	-0.43
SYK	spleen tyrosine kinase	-0.44
LIF	leukemia inhibitory factor	-0.44
THBS2	thrombospondin 2	-0.44
PPP1R16 B	protein phosphatase 1, regulatory subunit 16B	-0.44
NOTCH1	notch 1	-0.44
RUNX1	runt-related transcription factor 1	-0.45
PDCD6	programmed cell death 6	-0.45
VASH2	vasohibin 2	-0.45
GPI	glucose-6-phosphate isomerase	-0.46
ZC3H12A	zinc finger CCCH-type containing 12A	-0.46
WARS	tryptophanyl-tRNA synthetase	-0.46
HYAL1	hyaluronoglucosaminidase 1	-0.47
PIK3CB	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit beta	-0.47
TNMD	tenomodulin	-0.49

Example 12: Extracellular Matrix Markers for Engraftable Human Ventricular Progenitor Cells

5 In this example, genes in the extracellular matrix family that are expressed in human ventricular progenitor cells (HVPs) were identified. HVPs were generated as described in Examples 1 or 10 and RNA sequencing (RNA-seq) was performed at different time points

following differentiation as described in Example 1. Cluster analysis of gene expression profiles at different time points during HVP differentiation identified stage-specific signature genes. These genes were clustered hierarchically on the basis of the similarity of their expression profiles. First, genes showing expression in four different categories were identified: (i) cell surface expression; (ii) co-expression with Islet 1; (iii) high expression on day 5 of differentiation; and (iv) high d5/d0 ratio. This analysis confirmed the cell surface markers for HVPs of: JAG1, FZD4, FGFR3, LIFR (CD118) and TNFSF9. Next, from this same population of HVPs that identified the cell surface markers, gene ontology searches were performed to identify extracellular matrix family genes that were expressed in this population of HVPs, to thereby identify a gene fingerprint profile that identifies genes critical for cell engraftment.

Statistically, Pearson's correlation with Isl1 expression was used to identify those extracellular matrix genes whose expression in the HVPs best correlated with Isl1 expression. Table 5 below lists the extracellular matrix genes that correlate with Isl1 expression with a Pearson's correlation of 0.50 or higher.

Table 5: Extracellular matrix genes expressed in HVPs with a Pearson Correlation with Isl1 Expression of 0.50 or greater

Gene	Extracellular matrix genes (GO:0031012)	<u>Pearson's Correlation with Isl1 Expression</u>
FGF10	fibroblast growth factor 10	0.98
SMOC1	SPARC related modular calcium binding 1	0.97
CCBE1	collagen and calcium binding EGF domains 1	0.94
COL6A6	collagen, type VI, alpha 6	0.89
ADAMTS 12	ADAM metallopeptidase with thrombospondin type 1 motif, 12	0.85
COL19A1	collagen, type XIX, alpha 1	0.85
LAMA1	laminin, alpha 1	0.85
BMP4	bone morphogenetic protein 4	0.84
FBLN7	fibulin 7	0.81
FBLN2	fibulin 2	0.81
NDNF	neuron-derived neurotrophic factor	0.80
HTRA1	HtrA serine peptidase 1	0.80
HAPLN1	hyaluronan and proteoglycan link protein 1	0.79
EMILIN1	elastin microfibril interfacer 1	0.79
SPOCK3	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 3	0.76
PODNL1	podocan-like 1	0.73
IHH	indian hedgehog	0.70

Gene	Extracellular matrix genes (GO:0031012)	<u>Pearson's Correlation with Isl1 Expression</u>
ACAN	aggrecan	0.69
NID2	nidogen 2 (osteonidogen)	0.69
COL4A6	collagen, type IV, alpha 6	0.68
LAMC1	laminin, gamma 1 (formerly LAMB2)	0.65
FMOD	fibromodulin	0.65
MUC4	mucin 4, cell surface associated	0.64
EMID1	EMI domain containing 1	0.62
HMCN1	hemicentin 1	0.61
NID1	nidogen 1	0.60
VCAN	versican	0.58
CILP2	cartilage intermediate layer protein 2	0.57
SOD3	superoxide dismutase 3, extracellular	0.56
ADAMTS 3	ADAM metallopeptidase with thrombospondin type 1 motif, 3	0.54
ZP3	zona pellucida glycoprotein 3 (sperm receptor)	0.54
ANGPTL 4	angiopoietin-like 4	0.53
CRTAC1	cartilage acidic protein 1	0.52
LTBP4	latent transforming growth factor beta binding protein 4	0.50
FREM1	FRAS1 related extracellular matrix 1	0.50

Table 6 below lists the extracellular matrix genes that correlate with Isl1 expression with a Pearson's correlation of 0.49-0.00.

5 Table 6: Extracellular matrix genes expressed in HVPs with a Pearson Correlation with Isl1 Expression of 0.49 to 0.00

Gene	Extracellular matrix genes (GO:0031012)	<u>Pearson's Correlation with Isl1 Expression</u>
SSC5D	scavenger receptor cysteine rich family, 5 domains	0.49
GPC6	glypican 6	0.49
COL1A1	collagen, type I, alpha 1	0.49
ADAMTS L3	ADAMTS-like 3	0.48
FLRT3	fibronectin leucine rich transmembrane protein 3	0.48
FBLN1	fibulin 1	0.48
ADAMTS 9	ADAM metallopeptidase with thrombospondin type 1 motif, 9	0.48
COL27A1	collagen, type XXVII, alpha 1	0.47
RELN	reelin	0.46
COL9A2	collagen, type IX, alpha 2	0.46
EFEMP2	EGF containing fibulin-like extracellular matrix protein 2	0.45
AGRN	agrin	0.44
PCOLCE	procollagen C-endopeptidase enhancer	0.44

Gene	Extracellular matrix genes (GO:0031012)	<u>Pearson's Correlation with Isl1 Expression</u>
NTN4	netrin 4	0.44
CD248	CD248 molecule, endosialin	0.44
TGFB1	transforming growth factor, beta 1	0.43
ADAMTS 2	ADAM metallopeptidase with thrombospondin type 1 motif, 2	0.43
CTHRC1	collagen triple helix repeat containing 1	0.42
FGFR2	fibroblast growth factor receptor 2	0.41
APOE	apolipoprotein E	0.41
MMP11	matrix metallopeptidase 11	0.41
MMP15	matrix metallopeptidase 15 (membrane-inserted)	0.41
PODN	podocan	0.39
VEGFA	vascular endothelial growth factor A	0.39
COL18A1	collagen, type XVIII, alpha 1	0.39
GLG1	golgi glycoprotein 1	0.39
GPC2	glypican 2	0.37
DAG1	dystroglycan 1 (dystrophin-associated glycoprotein 1)	0.35
TGFB2	transforming growth factor, beta 2	0.35
PRELP	proline/arginine-rich end leucine-rich repeat protein	0.35
CHAD	chondroadherin	0.33
COL2A1	collagen, type II, alpha 1	0.33
FN1	fibronectin 1	0.31
SMC3	structural maintenance of chromosomes 3	0.31
COL4A5	collagen, type IV, alpha 5	0.30
FBN3	fibrillin 3	0.30
MMP23B	matrix metallopeptidase 23B	0.30
CCDC80	coiled-coil domain containing 80	0.29
SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	0.29
TNXB	tenascin XB	0.28
COL6A2	collagen, type VI, alpha 2	0.28
ADAMTS 13	ADAM metallopeptidase with thrombospondin type 1 motif, 13	0.28
LOXL1	lysyl oxidase-like 1	0.28
HAPLN2	hyaluronan and proteoglycan link protein 2	0.28
TNC	tenascin C	0.28
ENTPD2	ectonucleoside triphosphate diphosphohydrolase 2	0.28
TGFB3	transforming growth factor, beta 3	0.28
MFAP4	microfibrillar-associated protein 4	0.27
VWF	von Willebrand factor	0.27
WNT2	wingless-type MMTV integration site family member 2	0.27
MMRN2	multimerin 2	0.27
SPON1	spondin 1, extracellular matrix protein	0.26
ADAMTS 1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	0.26
F2	coagulation factor II (thrombin)	0.26
FLRT2	fibronectin leucine rich transmembrane protein 2	0.25

Gene	Extracellular matrix genes (GO:0031012)	<u>Pearson's Correlation with Isl1 Expression</u>
MMP2	matrix metalloproteinase 2	0.24
COL26A1	collagen, type XXVI, alpha 1	0.24
CASK	calcium/calmodulin-dependent serine protein kinase (MAGUK family)	0.24
NTN3	netrin 3	0.23
SLC1A3	solute carrier family 1 (glial high affinity glutamate transporter), member 3	0.22
F3	coagulation factor III (thromboplastin, tissue factor)	0.20
ADAMTS 6	ADAM metalloproteinase with thrombospondin type 1 motif, 6	0.20
COL5A2	collagen, type V, alpha 2	0.19
ERBB2IP	erb2 interacting protein	0.18
LAMB1	laminin, beta 1	0.18
COLQ	collagen-like tail subunit (single strand of homotrimer) of asymmetric acetylcholinesterase	0.18
LAMA5	laminin, alpha 5	0.17
LOXL2	lysyl oxidase-like 2	0.17
WNT11	wingless-type MMTV integration site family, member 11	0.17
LAMB2	laminin, beta 2 (laminin S)	0.17
COL5A1	collagen, type V, alpha 1	0.17
AEBP1	AE binding protein 1	0.17
COL9A3	collagen, type IX, alpha 3	0.16
CTSD	cathepsin D	0.16
COL21A1	collagen, type XXI, alpha 1	0.16
EGFLAM	EGF-like, fibronectin type III and laminin G domains	0.16
FBN2	fibrillin 2	0.15
NAV2	neuron navigator 2	0.15
EMILIN2	elastin microfibril interfacier 2	0.14
WNT9B	wingless-type MMTV integration site family, member 9B	0.14
NOV	nephroblastoma overexpressed	0.14
CHL1	cell adhesion molecule L1-like	0.13
DLG1	discs, large homolog 1 (Drosophila)	0.11
MFGE8	milk fat globule-EGF factor 8 protein	0.11
TIMP1	TIMP metalloproteinase inhibitor 1	0.11
CST3	cystatin C	0.10
APLP1	amyloid beta (A4) precursor-like protein 1	0.10
PRTN3	proteinase 3	0.10
ADAMTS 10	ADAM metalloproteinase with thrombospondin type 1 motif, 10	0.09
ILK	integrin-linked kinase	0.09
FRAS1	Fraser extracellular matrix complex subunit 1	0.09
ANXA2P 2	annexin A2 pseudogene 2	0.08
SMOC2	SPARC related modular calcium binding 2	0.07
ANXA2	annexin A2	0.06
ODAM	odontogenic, ameloblast associated	0.06

Gene	Extracellular matrix genes (GO:0031012)	<u>Pearson's Correlation with Isl1 Expression</u>
FREM2	FRAS1 related extracellular matrix protein 2	0.05
HAPLN3	hyaluronan and proteoglycan link protein 3	0.05
GPC3	glypican 3	0.03
LGALS1	lectin, galactoside-binding, soluble, 1	0.02
ADAMTS 8	ADAM metallopeptidase with thrombospondin type 1 motif, 8	0.02
LUM	lumican	0.01
HSP90B1	heat shock protein 90kDa beta (Grp94), member 1	0.00
HAPLN4	hyaluronan and proteoglycan link protein 4	0.00
MATN2	matrilin 2	0.00

Extracellular matrix genes whose expression negatively correlated with Isl1 expression in the HVPs were also identified. Table 7 below lists the extracellular matrix genes that negatively correlate with Isl1 expression with a Pearson's correlation of -0.50 or less.

5

Table 7: Extracellular matrix genes expressed in HVPs with a Pearson Correlation with Isl1 Expression of -0.50 or less

Gene	Extracellular matrix genes (GO:0031012)	<u>Pearson's Correlation with Isl1 Expression</u>
FKBP1A	FK506 binding protein 1A, 12kDa	-0.51
CLU	clusterin	-0.52
TFPI2	tissue factor pathway inhibitor 2	-0.52
PLSCR1	phospholipid scramblase 1	-0.53
FBLN5	fibulin 5	-0.53
VWA1	von Willebrand factor A domain containing 1	-0.54
ADAMTS 16	ADAM metallopeptidase with thrombospondin type 1 motif, 16	-0.55
MMP25	matrix metallopeptidase 25	-0.55
SFRP2	secreted frizzled-related protein 2	-0.61
SOD1	superoxide dismutase 1, soluble	-0.68

Table 8 below lists the extracellular matrix genes that negatively correlate with Isl1 expression with a Pearson's correlation of -0.01 to -0.49.

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Table 8: Extracellular matrix genes expressed in HVPs with a Pearson Correlation with Isl1 Expression of -0.01 to -0.49

Gene	Extracellular matrix genes (GO:0031012)	<u>Pearson's Correlation with Isl1 Expression</u>
PAPLN	papilin, proteoglycan-like sulfated glycoprotein	-0.01
SOST	sclerostin	-0.01

Gene	Extracellular matrix genes (GO:0031012)	<u>Pearson's</u> <u>Correlation with</u> <u>Isl1 Expression</u>
CDON	cell adhesion associated, oncogene regulated	-0.02
HMCN2	hemicentin 2	-0.02
WNT5A	wingless-type MMTV integration site family, member 5A	-0.02
PCSK6	proprotein convertase subtilisin/kexin type 6	-0.02
GSTO1	glutathione S-transferase omega 1	-0.02
LTBP1	latent transforming growth factor beta binding protein 1	-0.03
KAZALD 1	Kazal-type serine peptidase inhibitor domain 1	-0.03
LTBP2	latent transforming growth factor beta binding protein 2	-0.03
SFRP1	secreted frizzled-related protein 1	-0.04
ADAM11	ADAM metallopeptidase domain 11	-0.05
COL6A1	collagen, type VI, alpha 1	-0.05
COL22A1	collagen, type XXII, alpha 1	-0.05
CYR61	cysteine-rich, angiogenic inducer, 61	-0.05
ELN	elastin	-0.06
COL9A1	collagen, type IX, alpha 1	-0.06
VTN	vitronectin	-0.06
COL15A1	collagen, type XV, alpha 1	-0.06
COL4A1	collagen, type IV, alpha 1	-0.07
ANG	angiogenin, ribonuclease, RNase A family, 5	-0.07
HSPG2	heparan sulfate proteoglycan 2	-0.09
CRIP2	cysteine-rich protein 2	-0.09
CD151	CD151 molecule (Raph blood group)	-0.09
THBS1	thrombospondin 1	-0.09
ADAMTS 4	ADAM metallopeptidase with thrombospondin type 1 motif, 4	-0.09
CTGF	connective tissue growth factor	-0.10
CRISPLD 2	cysteine-rich secretory protein LCCL domain containing 2	-0.10
BMP7	bone morphogenetic protein 7	-0.11
COL6A3	collagen, type VI, alpha 3	-0.11
COL3A1	collagen, type III, alpha 1	-0.11
COL14A1	collagen, type XIV, alpha 1	-0.11
MATN3	matrilin 3	-0.11
CPZ	carboxypeptidase Z	-0.11
BMP1	bone morphogenetic protein 1	-0.11
WISP1	WNT1 inducible signaling pathway protein 1	-0.12
ADAMTS 18	ADAM metallopeptidase with thrombospondin type 1 motif, 18	-0.12
COL7A1	collagen, type VII, alpha 1	-0.12
IGFBP7	insulin-like growth factor binding protein 7	-0.12
COCH	cochlin	-0.13
ADAMTS 5	ADAM metallopeptidase with thrombospondin type 1 motif, 5	-0.13
COL11A2	collagen, type XI, alpha 2	-0.13
TGFBI	transforming growth factor, beta-induced, 68kDa	-0.14

Gene	Extracellular matrix genes (GO:0031012)	<u>Pearson's</u> <u>Correlation with</u> <u>Isl1 Expression</u>
COL16A1	collagen, type XVI, alpha 1	-0.14
ACHE	acetylcholinesterase (Yt blood group)	-0.14
THSD4	thrombospondin, type I, domain containing 4	-0.15
DGCR6	DiGeorge syndrome critical region gene 6	-0.15
TGFB1I1	transforming growth factor beta 1 induced transcript 1	-0.15
ADAMTS L1	ADAMTS-like 1	-0.15
SERPINA 1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	-0.16
MAMDC 2	MAM domain containing 2	-0.16
LAMA4	laminin, alpha 4	-0.17
LTBP3	latent transforming growth factor beta binding protein 3	-0.17
EGFL7	EGF-like-domain, multiple 7	-0.17
NPNT	nephronectin	-0.17
SERPINF 1	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	-0.18
ABI3BP	ABI family, member 3 (NESH) binding protein	-0.18
SERPINE 2	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	-0.18
WNT6	wingless-type MMTV integration site family, member 6	-0.19
TIMP3	TIMP metallopeptidase inhibitor 3	-0.19
SNCA	synuclein, alpha (non A4 component of amyloid precursor)	-0.19
PKM	pyruvate kinase, muscle	-0.19
FGF9	fibroblast growth factor 9	-0.19
VIT	vitrin	-0.19
WNT1	wingless-type MMTV integration site family, member 1	-0.19
LAMC3	laminin, gamma 3	-0.19
MMP14	matrix metallopeptidase 14 (membrane-inserted)	-0.19
PXDN	peroxidasin	-0.19
HNRNPM	heterogeneous nuclear ribonucleoprotein M	-0.19
FBN1	fibrillin 1	-0.20
ASPN	asporin	-0.20
ADAMTS L5	ADAMTS-like 5	-0.20
SPON2	spondin 2, extracellular matrix protein	-0.20
COL1A2	collagen, type I, alpha 2	-0.20
BGN	biglycan	-0.20
COL4A2	collagen, type IV, alpha 2	-0.20
ADAMTS L4	ADAMTS-like 4	-0.21
APOH	apolipoprotein H (beta-2-glycoprotein I)	-0.21
CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	-0.21
ADAMTS 7	ADAM metallopeptidase with thrombospondin type 1 motif, 7	-0.22
CALR	calreticulin	-0.22

Gene	Extracellular matrix genes (GO:0031012)	<u>Pearson's Correlation with Isl1 Expression</u>
MMP9	matrix metalloproteinase 9	-0.22
MMP24	matrix metalloproteinase 24 (membrane-inserted)	-0.22
SPOCK2	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2	-0.22
COL11A1	collagen, type XI, alpha 1	-0.23
MMP7	matrix metalloproteinase 7	-0.23
MMP16	matrix metalloproteinase 16 (membrane-inserted)	-0.23
MFAP2	microfibrillar-associated protein 2	-0.23
POSTN	periostin, osteoblast specific factor	-0.24
COL8A1	collagen, type VIII, alpha 1	-0.24
WNT2B	wingless-type MMTV integration site family, member 2B	-0.24
DCN	decorin	-0.24
EGFL6	EGF-like-domain, multiple 6	-0.24
MMP10	matrix metalloproteinase 10	-0.24
MGP	matrix Gla protein	-0.24
ECM1	extracellular matrix protein 1	-0.24
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	-0.25
MMP1	matrix metalloproteinase 1	-0.25
WNT10A	wingless-type MMTV integration site family, member 10A	-0.25
B4GALT7	xylosylprotein beta 1,4-galactosyltransferase, polypeptide 7	-0.25
COL12A1	collagen, type XII, alpha 1	-0.25
LAMA3	laminin, alpha 3	-0.25
LAMA2	laminin, alpha 2	-0.25
LAMB3	laminin, beta 3	-0.25
WNT7B	wingless-type MMTV integration site family, member 7B	-0.25
FLRT1	fibronectin leucine rich transmembrane protein 1	-0.25
ADAMTS15	ADAM metalloproteinase with thrombospondin type 1 motif, 15	-0.26
COL8A2	collagen, type VIII, alpha 2	-0.26
MFAP1	microfibrillar-associated protein 1	-0.26
TINAGL1	tubulointerstitial nephritis antigen-like 1	-0.26
FGF1	fibroblast growth factor 1 (acidic)	-0.27
OLFML2A	olfactomedin-like 2A	-0.27
CPA6	carboxypeptidase A6	-0.27
COL17A1	collagen, type XVII, alpha 1	-0.27
SPARCL1	SPARC-like 1 (hevin)	-0.27
MFAP5	microfibrillar associated protein 5	-0.27
COL4A4	collagen, type IV, alpha 4	-0.28
WNT8B	wingless-type MMTV integration site family, member 8B	-0.28
ADAMTS19	ADAM metalloproteinase with thrombospondin type 1 motif, 19	-0.29
CRTAP	cartilage associated protein	-0.29
WNT5B	wingless-type MMTV integration site family, member 5B	-0.30

Gene	Extracellular matrix genes (GO:0031012)	<u>Pearson's Correlation with Isl1 Expression</u>
WNT3	wingless-type MMTV integration site family, member 3	-0.30
UCMA	upper zone of growth plate and cartilage matrix associated	-0.30
GPC1	glypican 1	-0.30
TIMP2	TIMP metalloproteinase inhibitor 2	-0.30
ALPL	alkaline phosphatase, liver/bone/kidney	-0.30
LECT1	leukocyte cell derived chemotaxin 1	-0.32
GPC4	glypican 4	-0.32
SPOCK1	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1	-0.32
HSD17B1 2	hydroxysteroid (17-beta) dehydrogenase 12	-0.32
LGALS3	lectin, galactoside-binding, soluble, 3	-0.33
EMILIN3	elastin microfibril interfacer 3	-0.34
GFOD2	glucose-fructose oxidoreductase domain containing 2	-0.34
VWC2	von Willebrand factor C domain containing 2	-0.34
SERAC1	serine active site containing 1	-0.34
WNT8A	wingless-type MMTV integration site family, member 8A	-0.34
LMCD1	LIM and cysteine-rich domains 1	-0.34
CPXM2	carboxypeptidase X (M14 family), member 2	-0.34
ADAMTS 14	ADAM metalloproteinase with thrombospondin type 1 motif, 14	-0.34
GPLD1	glycosylphosphatidylinositol specific phospholipase D1	-0.35
FGFBP3	fibroblast growth factor binding protein 3	-0.35
BCAN	brevican	-0.35
ITGB4	integrin, beta 4	-0.35
LGALS3 BP	lectin, galactoside-binding, soluble, 3 binding protein	-0.36
LPL	lipoprotein lipase	-0.38
LAD1	ladinin 1	-0.39
WNT3A	wingless-type MMTV integration site family, member 3A	-0.39
TGFBR3	transforming growth factor, beta receptor III	-0.39
DST	dystonin	-0.40
WNT10B	wingless-type MMTV integration site family, member 10B	-0.40
LEFTY2	left-right determination factor 2	-0.41
TNFRSF1 1B	tumor necrosis factor receptor superfamily, member 11b	-0.41
WNT9A	wingless-type MMTV integration site family, member 9A	-0.41
TIMP4	TIMP metalloproteinase inhibitor 4	-0.42
WNT4	wingless-type MMTV integration site family, member 4	-0.42
NCAN	neurocan	-0.42
ADAMTS 20	ADAM metalloproteinase with thrombospondin type 1 motif, 20	-0.43
ITGA6	integrin, alpha 6	-0.43
LOX	lysyl oxidase	-0.43
THBS4	thrombospondin 4	-0.43
THBS2	thrombospondin 2	-0.44

<u>Gene</u>	<u>Extracellular matrix genes (GO:0031012)</u>	<u>Pearson's Correlation with Isl1 Expression</u>
ADAMTS L2	ADAMTS-like 2	-0.44
ENTPD1	ectonucleoside triphosphate diphosphohydrolase 1	-0.45
RUNX1	runt-related transcription factor 1	-0.45
VWA2	von Willebrand factor A domain containing 2	-0.45
RELL2	RELT-like 2	-0.46
PTPRZ1	protein tyrosine phosphatase, receptor-type, Z polypeptide 1	-0.46
LAMC2	laminin, gamma 2	-0.46
DST	dystonin	-0.40
WNT10B	wingless-type MMTV integration site family, member 10B	-0.40
LEFTY2	left-right determination factor 2	-0.41
TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b	-0.41
WNT9A	wingless-type MMTV integration site family, member 9A	-0.41
TIMP4	TIMP metalloproteinase inhibitor 4	-0.42

Example 13: Gene Expression Profile for Day 6 Islet 1 Negative Cells

In this example, the gene expression profile was determined for Islet 1 negative cells within the Day 6 HVP population to further characterize a subpopulation of cells within the Day 6 population that do not express the necessary markers to qualify as engraftable HVPs. Day 6 HVP populations were generated as described in Examples 1 or 10 and RNA sequencing (RNA-seq) was performed following differentiation as described in Example 2. Cells that were Islet 1 negative (Isl1-) were further analyzed with respect to their gene expression profile. Genes expressed in the Isl1- cells with an average RNA copy number of 2000 or higher are shown below in Table 9.

Table 9: Gene Expression Profile of Day 6 Islet 1 Negative Cells

<u>Gene</u>	<u>Sample #1</u>	<u>Sample #2</u>	<u>Avg. RNA Copy #</u>
ACTB	12288	28126	20207
MTRNR2L2	24511	9774	17142.5
MALAT1	14163	18092	16127.5
EEF1A1	11663	12456	12059.5
KRT8	8884	14087	11485.5
MTRNR2L8	12836	7688	10262
KRT18	5215	10552	7883.5
FN1	4900	10581	7740.5
MTRNR2L1	8550	5719	7134.5
TTN	3149	9601	6375
GAPDH	4907	6391	5649

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YWHAZ	5349	5414	5381.5
MTRNR2L9	5673	3850	4761.5
RPL3	3346	5911	4628.5
AHNAK	6727	2197	4462
KCNQ1OT1	5835	3064	4449.5
TUBB	4870	3936	4403
SLC2A3	3311	4301	3806
FTL	3484	4021	3752.5
HSP90B1	4173	2778	3475.5
KRT19	3502	3202	3352
HSPA8	3455	2903	3179
MYL6	1898	4375	3136.5
RPLP0	2319	3922	3120.5
BSG	2519	3593	3056
COL3A1	5312	695	3003.5
TPM1	2938	3059	2998.5
VCAN	2563	3422	2992.5
ENO1	2449	3535	2992
RPL4	2619	3328	2973.5
ACTG1	2687	3253	2970
MTRNR2L10	3409	2487	2948
HMGN2	2684	3153	2918.5
PRTG	2594	2980	2787
TPI1	2418	3113	2765.5
HMGB1	2577	2880	2728.5
VIM	2621	2704	2662.5
ATP5B	3000	2219	2609.5
HSP90AB1	2735	2419	2577
RPL7	2132	2896	2514
CBX5	2799	2219	2509
MYL7	1614	3382	2498
SERPINH1	2547	2327	2437
HNRNPK	2878	1932	2405
SRRM2	2758	2046	2402
PODXL	3683	1112	2397.5
EEF2	2579	2119	2349
SPARC	3026	1645	2335.5
ACTC1	437	4152	2294.5
HUWE1	2583	1977	2280
COL1A2	3544	941	2242.5
LINC00506	2965	1496	2230.5
HSPA5	2078	2356	2217
MDK	2223	2144	2183.5
HNRNPC	2292	2074	2183
HSP90AA1	2220	2138	2179
RGS5	2180	2150	2165

LAMC1	2757	1565	2161
APLNR	868	3246	2057
UGDH-AS1	2633	1457	2045
RPS3A	1601	2399	2000

Accordingly, the data shown in Table 9 provides a gene expression profile for Islet 1 negative, non-engraftable cells within a Day 6 HVP population that are not suitable for transplantation and thus are to be selected against when choosing cells for transplantation and engraftment.

5

Example 14: Single Cell Sequencing of HVPs

In this example, single cell sequencing was performed on HVPs from day 4, 5, 6, 8, 9 or 15 of differentiation to thereby identify expressed genes in individual cells at different stages of differentiation. Sequencing cell populations in bulk can mask cellular subtypes within that population. Single cell sequencing offers an unbiased approach to classify cell types within a population.

The single cell sequencing approach used herein comprised the three steps: library preparation, sequencing and analysis. For library preparation, a single cell was collected and lysed. The RNA transcriptome was captured, converted to DNA and prepped for sequencing. The captured transcriptome of a cell is termed a “library”. Sequencing was performed using a next-generation sequencer. For analysis, the sequences were read and mapped to genes. This created a transcriptome-wide expression profile for each cell. The expression profiles were then compared with each other. Similar cells were “clustered” together to represent a cell type.

To obtain the single cells used for single cell sequencing, the HVP differentiation was carried out as described previously (see Examples 1 and 10). Cells from Day 4, 5, 6, 8, 9 and 15 were collected following trypsin treatment, and picked manually with a mouth pipette. 128 cells were picked for each day. Library preparation for the single cells was performed using the Smart-seq2 protocol as previously described (Picelli, S. et al. (2013) *Nature Methods* 10:1096–1098). The single cell libraries were sequenced on an Illumina HiSeq 2500 Sequencing System. Reads were mapped to the human genome using BowTie (Langmead, B. et al. (2009) *Genome Biol.* 10(3):R25). Data analysis was carried out using the Seurat package (Sajita, R. et al. (2015) *Nature Biotechnology* 33:495–502).

Using t-SNE dimensionality reduction to visualise the data across Day 4, 5, 6, 8, 9 and 15, four major cell types were observed: a non-cardiac population, an epithelial-like population, cardiac progenitors and cardiomyocytes. Differentially expressed genes of the cardiac progenitors were extracted using the FindMarker function in the Seurat package. The parameters were the differentially expressed genes in the cardiac progenitor clusters that were expressed in at least 10% of the cells within the cardiac progenitor cluster. The list of differentially expressed genes in the cardiac progenitor cluster are shown below in Table 10.

Table 10: Differentially Expressed Genes in Cardiac Progenitor Cluster

	p_val	avg_diff
TMEM88	3.42E-82	1.560606117
COL3A1	3.79E-80	1.550451213
MIR3606	3.79E-80	1.550451213
APLNR	1.80E-51	1.342551564
HAS2	7.87E-55	1.302299387
MFAP4	1.89E-103	1.287366398
LIX1	2.20E-81	1.262443162
LUM	2.06E-48	1.121559334
PCOLCE	6.13E-80	1.096776495
HAND1	2.20E-31	1.090769383
HEY1	2.64E-56	1.052143244
PDGFRA	4.21E-102	1.029400192
S100A10	2.82E-41	0.99881898
IGF2	2.44E-62	0.945955427
MMP2	1.43E-71	0.910583615
COL1A1	9.55E-58	0.894758619
ART5	1.16E-23	0.883791494
LRRN4	3.99E-36	0.84972281
H19	2.73E-35	0.8408468
MIR675	2.73E-35	0.8408468
INS.IGF2	6.12E-46	0.756272517
S100A11	9.17E-53	0.745858163
SHISA3	3.45E-12	0.73714255
RGS2	8.20E-18	0.731496568
SLC9A3R1	4.75E-26	0.723600997
MIR3615	4.75E-26	0.723600997
ITM2C	1.77E-48	0.720322592
CYP27A1	2.14E-26	0.700002885
BMPER	3.56E-42	0.696442368
SPARC	4.07E-41	0.691895813
NID2	1.35E-39	0.688341218
PCOLCE.AS1	2.29E-72	0.684407943
PLAT	1.46E-69	0.677075789

	p_val	avg_diff
RGS5	1.66E-41	0.672985639
RGS4	4.78E-31	0.671536299
FBLN1	3.10E-46	0.664157944
GATA6.AS1	3.55E-24	0.659546503
EIF4EBP1	1.29E-36	0.656965269
HAPLN1	2.53E-20	0.65191049
COL1A2	1.99E-29	0.64867676
CDH3	5.74E-34	0.64562218
NXPH2	5.44E-23	0.630747775
BST2	2.63E-10	0.630640659
APOBEC3C	1.43E-38	0.627430539
SFRP5	2.01E-39	0.625478497
KRT19	3.22E-37	0.625378553
HOXB2	8.19E-33	0.602689448
CHIC2	6.53E-30	0.60132243
BMP5	5.89E-31	0.599081591
CALB2	6.82E-16	0.596591168
CFC1	1.44E-54	0.594202643
KRT18	2.71E-36	0.590624042
LRRTM1	9.36E-43	0.584576973
GYPC	1.22E-47	0.58078756
MEIS1	1.71E-57	0.58057826
KRT8	2.54E-43	0.577311366
TUBB6	2.02E-21	0.571891695
PBX3	3.17E-39	0.570615432
TNC	6.89E-36	0.570340016
CTSV	3.09E-17	0.56938514
ALX1	2.64E-14	0.552977549
FOXH1	5.22E-26	0.548734193
VIM.AS1	4.78E-28	0.542791752
TMEM141	6.48E-27	0.538575158
GATA6	1.63E-46	0.537437948
BMP4	8.51E-15	0.531659308
VIM	2.40E-29	0.530724516
ARL4D	2.48E-16	0.51292713
HBE1	2.21E-14	0.505457358
CD248	3.74E-19	0.503966584
GLIPR2	9.74E-30	0.502526018
TMEM185A	6.17E-39	0.49852561
KIAA1462	7.15E-75	0.497738542
FSHR	5.25E-45	0.497054658
HTRA1	3.27E-13	0.495987153
SPP1	8.45E-09	0.494008412
IGFBP4	7.48E-14	0.490252699
NRP1	8.31E-43	0.484466262

	p_val	avg_diff
SC5D	1.02E-22	0.482101626
FAM89A	5.27E-26	0.477661385
MIR1182	5.27E-26	0.477661385
LOC400043	3.49E-18	0.475045692
ASH2L	1.14E-46	0.474197679
PKP2	1.44E-25	0.474140269
FN1	5.68E-18	0.469988478
KDR	8.18E-30	0.463258104
BGN	2.90E-23	0.459541322
CREG1	7.00E-13	0.459398822
PTGIS	7.28E-39	0.456120126
DSP	6.66E-24	0.454180797
PKIG	5.19E-24	0.452582156
CTSB	7.02E-23	0.449928855
KLHL4	2.64E-40	0.449780298
CCDC34	2.05E-33	0.443083183
ATF7IP2	1.86E-59	0.440835688
KIAA1551	3.78E-27	0.439543958
SLC40A1	1.11E-23	0.439358025
SDC4	8.23E-17	0.437190914
IGFBP3	6.90E-07	0.436357742
SERINC3	1.64E-23	0.425115033
GUCY1A3	7.48E-28	0.424214515
CCDC3	1.41E-35	0.422569528
TGFB1	6.52E-16	0.41635348
LAMP5	8.54E-06	0.415693362
COL6A1	2.88E-25	0.407932515
PLS3	8.22E-12	0.406911474
LAPTM4A	1.16E-25	0.405037615
RUNX1T1	7.93E-78	0.403683684
FGF10	2.18E-13	0.401291848
LRIG3	1.89E-30	0.38790471
DSG2	6.23E-28	0.387712513
ARL2BP	7.58E-14	0.38444917
TCEAL9	1.70E-21	0.38384913
PAPSS1	1.40E-14	0.378653665
ANXA6	2.68E-21	0.378214393
LGALS1	2.64E-06	0.378105889
FSTL1	2.78E-24	0.376247551
MIR198	2.78E-24	0.376247551
TMEM98	8.37E-19	0.376170536
SARAF	4.16E-19	0.375950055
TP53I11	6.05E-17	0.373187071
CYB5D1	1.63E-23	0.372197782
IFITM3	7.56E-38	0.371288978

	p_val	avg_diff
LGR5	1.34E-16	0.369873765
WASF1	9.73E-17	0.368716853
ECE1	9.71E-35	0.364724949
MXRA8	2.10E-12	0.363862558
CSRP2	2.60E-17	0.362236698
SDCBP	6.33E-19	0.361772835
SLC16A1	9.31E-11	0.355359606
COL6A2	1.73E-24	0.353551929
EMP2	3.26E-30	0.352860372
LEPROTL1	2.29E-27	0.352310781
CA2	5.04E-08	0.349789326
CCBE1	9.52E-35	0.349748013
SMOC2	1.94E-17	0.347190203
HOXB1	2.81E-17	0.346717419
GATA5	7.66E-10	0.344901285
RBP1	8.58E-21	0.34419898
PHLDA1	3.77E-28	0.343778762
HAND2	2.82E-24	0.343032467
SLC30A3	2.09E-21	0.335148941
SERPINE2	5.79E-22	0.334593289
SYT10	1.62E-39	0.332905271
RHOC	1.16E-16	0.332380866
MPZL1	1.54E-20	0.330849688
LAPTM4B	1.72E-11	0.330782753
LRRC32	1.75E-22	0.327573069
CGNL1	4.43E-31	0.32754955
CXCL12	1.71E-14	0.326028623
NODAL	7.70E-06	0.324782701
DOK4	5.00E-10	0.322776109
ALDH2	1.15E-24	0.322496612
LOC101927497	5.25E-23	0.321216676
RAMP2	3.25E-12	0.320975405
PLSCR5	5.92E-24	0.320022623
COL5A2	4.08E-28	0.319401842
FAM114A1	7.04E-28	0.319200887
METTTL7A	1.05E-53	0.319011726
CYYR1	1.65E-18	0.318957318
TOMM34	1.96E-07	0.318041963
CDH11	2.13E-21	0.317968138
FLRT3	8.21E-08	0.316560332
LYPD6	9.56E-23	0.316391668
AFAP1L2	5.04E-32	0.315613744
SERPINB9	5.60E-12	0.315004476
DYNC1I1	1.11E-20	0.313338566
MGARP	1.62E-09	0.311406096

	p_val	avg_diff
PHGDH	1.17E-09	0.310776152
FHL3	8.79E-08	0.309585103
NPY	2.27E-05	0.309536196
PPP1R15A	2.34E-09	0.309484964
SPIN4	4.33E-24	0.309135856
FXVD6.FXVD2	5.50E-10	0.308833983
P3H2	9.86E-16	0.307905833
FMOD	1.98E-12	0.307428412
FAM213A	8.18E-22	0.305776171
DHDH	3.35E-08	0.303929476
ARSE	3.34E-11	0.302502476
BRINP1	2.18E-16	0.300841535
MYH10	6.61E-15	0.299656764
HSPA5	2.65E-11	0.29901021
ARF4	1.65E-12	0.297788482
CD74	2.28E-11	0.296915796
WNT5A	2.27E-44	0.29686904
IL6ST	1.62E-40	0.296859117
B2M	6.62E-25	0.296077624
MDK	1.00E-32	0.295677643
TIMP1	4.82E-11	0.294947926
PDIA4	1.34E-08	0.294630611
NPPB	0.012639596	0.29392058
RAB34	8.90E-13	0.293594025
TAX1BP3	2.28E-06	0.293246644
SVEP1	1.02E-62	0.291659157
CADM1	4.45E-19	0.29143391
EMP3	2.86E-12	0.291231978
ISL1	9.26E-08	0.289460012
CSNK2A2	1.33E-21	0.288992825
SERPING1	6.85E-12	0.287793225
TECRL	4.21E-05	0.286902439
SLC7A2	2.65E-37	0.286569819
GPR108	1.88E-10	0.284415977
MIR6791	1.88E-10	0.284415977
KDM6A	4.24E-13	0.283535404
RDH10	6.97E-33	0.283087084
PLOD1	3.49E-09	0.280933842
COPG1	5.98E-11	0.279866228
CASP6	6.03E-09	0.279773211
THBS1	8.65E-06	0.279660845
H2AFY2	1.19E-06	0.278216329
GOLGA7	1.10E-10	0.276228148
SELENOF	7.41E-11	0.274011316
FXVD6	3.31E-10	0.273563514

	p_val	avg_diff
POSTN	2.13E-28	0.273267932
EFEMP2	3.05E-20	0.273241569
TGFB2	2.05E-13	0.273044307
TPM4	1.38E-14	0.272429895
RAB38	0.000686908	0.271918483
NCCRP1	1.79E-11	0.271623914
SH3GLB1	1.51E-18	0.269224524
PMP22	1.57E-07	0.268878282
PLLP	4.96E-35	0.268507068
SCMH1	2.01E-09	0.267191985
MIR503HG	1.86E-07	0.265375187
MIR424	1.86E-07	0.265375187
KDEL2	9.12E-10	0.264599726
DUSP6	0.000293032	0.263830821
SERPINH1	3.02E-17	0.263654992
PTGES	2.95E-05	0.263223829
TMEM120A	4.23E-07	0.263152389
ADAM19	5.67E-22	0.261893827
CRB2	1.47E-12	0.261335033
ADA	2.80E-10	0.260801645
PGF	3.62E-22	0.259993454
NCAM1	1.73E-28	0.259741889
NKX3.1	1.80E-15	0.259560142
COLEC11	1.56E-26	0.259066004
LOC101927230	2.52E-09	0.257688783
RCN3	2.90E-06	0.256437487
ADAMTS12	1.28E-25	0.255820711
SNORD36B	0.01966799	0.255563423
HOXB.AS3	2.31E-58	0.254873973
STAR	9.39E-29	0.254111765
TMED2	8.28E-19	0.253051723
CAPG	2.11E-08	0.252316312
APOC1	1.79E-08	0.25228836
FKBP7	6.16E-14	0.25196672
PTPN13	2.10E-14	0.250842517
EHD2	4.27E-14	0.250458307
C1orf168	2.14E-15	0.250376891

From the list of genes, neuropilin 1 (NRP1) was selected as a candidate for positive sorting of HVPs. An anti-NRP1 antibody compatible for MACS sorting is commercially available.

5 The day 5 dataset was further examined, since RNA expressed on day 5 would be translated into protein on day 6, the key window where these cells could engraft. The tSNE

plot of the day 5 dataset, shown in Figure 11, reveals two clusters of cells with similar expression patterns, arbitrarily labeled 0 and 1. Cells within a cluster express similar genes. Cells in different clusters express different genes. The distance between each cell on the tSNE plot represents the degree of difference in their gene expression profile. The cluster labeled 1 has high levels of Isl1 expression.

Figure 12 shows the expression of Isl1 and NRP1 on day 5 cells, wherein dark grey denotes high expression, middle grey denotes low expression and light grey denotes no expression. Thus, Figure 12 demonstrates that many of the cells with high levels of Isl1 expression also have high levels of NRP1 expression.

This was confirmed by the violin plot of expression levels of Isl1 and NRP1 shown in Figure 13. Cells within cluster 1 (shown in Figure 11) have high levels of expression of Isl1. The violin plot of Figure 13 demonstrates that Isl1+ cells and NRP1+ cells are in the same cluster, since they have a similar gene expression profile. Accordingly, NRP1 is a suitable cell surface marker for positive selection of HVPs (e.g., day 5-6 Isl1+ HVPs).

Example 15: Further Characterization of NRP1+ HVPs

In this example, the expression of NRP1 on a human embryonic stem cell line was examined further. The H9 stem cell line (also known in the art as WA09; Thomson, J.A. *et al.* (1998) *Science* 282:1145-1147; WiCell Research Institute) was used for these experiments. H9 cells were cultured under cardiac differentiation conditions (e.g., as described herein in Examples 1 and 10) to generate day 6 human ventricular progenitor cells (HVPs).

In a first set of experiments, day 6 HVPs from H9 cells were stained with anti-NRP1, anti-TRA-1-60 or both anti-NRP1 and anti-TRA-160, followed by flow cytometry analysis. The results are shown in Figure 14A (single staining for NRP1+ cells), Figure 14B (single staining for TRA-1-60+ cells) and Figure 14C (double staining for NRP1+ TRA-1-60+ cells). The results determined that 92% of the day 6 HVPs were NRP1+ and 17% of the day 6 HVPs were TRA-1-60+. The double staining analysis determined that within the NRP1+ population, 15.5% of those cells are TRA-1-60+.

In a second set of experiments, day 6 HVPs from H9 cells were stained with anti-NRP1, anti-ISL1 or both anti-NRP1 and anti-ISL1, followed by flow cytometry analysis. The results are shown in Figure 15A (single staining for NRP1+ cells), Figure 15B (single staining for ISL1+ cells) and Figure 15C (double staining for NRP1+ ISL1+ cells). The

results determined that 78% of the day 6 HVPs were NRP1+, 80% of the day 6 HVPs were ISL1+ and 73% of the day 6 HVPs were NRP1+ ISL1+.

These results confirm the expression of NRP1 on the surface of a large majority of the day 6 HVPs, as well as coexpression with ISL1 and with TRA-1-60, thereby confirming the suitability of NRP1 as a positive selection, ISL1 as a positive co-selection marker, and TRA-1-60 as a negative selection marker.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A method for isolating human cardiac ventricular progenitor cells, the method comprising:
 - contacting a culture of human cells containing cardiac progenitor cells with one or more agents reactive with neuropilin-1 (NRP1); and
 - separating NRP1 reactive positive cells from non-reactive cells to thereby isolate human cardiac ventricular progenitor cells.
2. The method of claim 1, wherein the culture of human cells is further contacted with at least one second agent reactive with human cardiac ventricular progenitor cells; and NRP1 reactive/second agent reactive positive cells are separated from non-reactive cells to thereby isolate human cardiac ventricular progenitor cells.
3. The method of claim 2, wherein the at least one second agent is reactive with JAG1, FZD4, LIFR, FGFR3 and/or TNFSF9.
4. The method of claim 2, wherein the culture of human cells is contacted with the agent reactive with NRP1 before contacting with the at least one second agent.
5. The method of claim 2, wherein the culture of human cells is contacted with the at least one second agent before contacting with the agent reactive with NRP1.
6. The method of claim 2, wherein the culture of human cells is contacted simultaneously with the agent reactive with NRP1 and with the at least one second agent.
7. The method of claim 1, which further comprises contacting the human cardiac ventricular progenitor cells with one or more agents reactive with at least one marker that is expressed on the surface of human pluripotent stem cells and separating marker-nonreactive negative cells from reactive cells to thereby further isolate human cardiac ventricular progenitor cells.

8. The method of claim 7, wherein the at least one marker that is expressed on the surface of human pluripotent stem cells is selected from the group consisting of TRA-1-60, TRA-1-81, TRA-2-54, SSEA1, SSEA3, SSEA4, CD9, CD24, E-cadherin and Podocalyxin, and combinations thereof.
9. The method of claim 7, wherein the at least one marker that is expressed on the surface of human pluripotent stem cells is TRA-1-60.
10. The method of claim 9, wherein the agent reactive with TRA-1-60 is an anti-TRA-1-60 antibody.
11. The method of any one of claims 1-10, wherein the agent reactive with NRP1 is an anti-NRP1 antibody.
12. The method of any one of claims 1-10, wherein the agent reactive with NRP1 is a soluble NRP1 ligand fusion protein.
13. The method of claim 12, wherein the NRP1 ligand is VEGF-A or Sema3A.
14. The method of any one of claims 1-13, wherein the NRP1 reactive positive cells are separated from the non-reactive cells by fluorescent activated cell sorting (FACS) or magnetic activated cell sorting (MACS).
15. The method of any one of claims 1-14, wherein the human cardiac ventricular progenitor cells are further cultured and differentiated such that they are MLC2v positive.
16. The method of any one of claims 1-15, wherein the culture of human cells containing cardiac progenitor cells is derived from human embryonic stem cells of human induced pluripotent stem cells.
17. A method for isolating human cardiac ventricular progenitor cells, the method comprising:

culturing human pluripotent stem cells under conditions that generate cardiac progenitor cells to obtain a culture of cells;

contacting the culture of cells with one or more agents reactive with neuropilin-1 (NRP1); and

separating NRP1 reactive positive cells from non-reactive cells to thereby isolate human cardiac ventricular progenitor cells.

18. The method of claim 17, wherein the culture of human cells is further contacted with at least one second agent reactive with human cardiac ventricular progenitor cells; and NRP1 reactive/second agent reactive positive cells are separated from non-reactive cells to thereby isolate human cardiac ventricular progenitor cells.

19. The method of claim 18, wherein the at least one second agent is reactive with JAG1, FZD4, LIFR, FGFR3 and/or TNFSF9.

20. The method of claim 18, wherein the culture of human cells is contacted with the agent reactive with NRP1 before contacting with the at least one second agent.

21. The method of claim 18, wherein the culture of human cells is contacted with the at least one second agent before contacting with the agent reactive with NRP1.

22. The method of claim 18, wherein the culture of human cells is contacted simultaneously with the agent reactive with NRP1 and with the at least one second agent.

23. The method of claim 17, which further comprises contacting the human cardiac ventricular progenitor cells with one or more agents reactive with at least one marker that is expressed on the surface of human pluripotent stem cells and separating marker-nonreactive negative cells from reactive cells to thereby further isolate human cardiac ventricular progenitor cells.

24. The method of claim 23, wherein the at least one marker that is expressed on the surface of human pluripotent stem cells is selected from the group consisting of TRA-

1-60, TRA-1-81, TRA-2-54, SSEA1, SSEA3, SSEA4, CD9, CD24, E-cadherin and Podocalyxin, and combinations thereof.

25. The method of claim 23, wherein the at least one marker that is expressed on the surface of human pluripotent stem cells is TRA-1-60.

26. The method of claim 25, wherein the agent reactive with TRA-1-60 is an anti-TRA-1-60 antibody.

27. The method of any one of claims 17-26, wherein the agent reactive with NRP1 is an anti-NRP1 antibody.

28. The method of any one of claims 17-26, wherein the agent reactive with NRP1 is a soluble NRP1 ligand fusion protein.

29. The method of claim 28, wherein the NRP1 ligand is VEGF-A or Sema3A.

30. The method of any one of claims 17-29, wherein the NRP1 reactive positive cells are separated from the non-reactive cells by fluorescent activated cell sorting (FACS) or magnetic activated cell sorting (MACS).

31. The method of any one of claims 17-30, wherein the human cardiac ventricular progenitor cells are further cultured and differentiated such that they are MLC2v positive.

32. The method of any one of claims 17-31, wherein the culture of human cells containing cardiac progenitor cells is derived from human embryonic stem cells of human induced pluripotent stem cells.

33. A method for obtaining a clonal population of human cardiac ventricular progenitor cells, the method comprising:

isolating a single NRP1+ human cardiac ventricular progenitor cell by contacting a culture of human cardiac ventricular progenitor cells with one or more agents reactive with NRP1; and

culturing the single NRP1+ human cardiac ventricular progenitor cell under conditions such that the cell is expanded to at least 1×10^9 cells to thereby obtain a clonal population of human cardiac ventricular progenitor cells.

34. The method of claim 33, wherein the single NRP1+ human cardiac ventricular progenitor cell is Islet 1 positive, Nkx2.5 negative and flk1 negative at the time of initial culture.

35. The method of claim 33, wherein the single NRP1+ human cardiac ventricular progenitor cell is isolated by fluorescent activated cell sorting or magnetic activated cell sorting.

36. The method of claim 33, wherein the agent reactive with NRP1 is an anti-NRP1 antibody.

37. The method of claim 33, wherein the single NRP1+ human cardiac ventricular progenitor cell is cultured under conditions such that the cell is biased toward ventricular differentiation.

38. The method of claim 33, wherein the single NRP1+ human cardiac ventricular progenitor cell is expanded to at least 10×10^9 cells.

39. A clonal population of at least 1×10^9 NRP1+ human cardiac ventricular progenitor cells obtained by the method of claim 33.

40. A clonal population of at least 10×10^9 NRP1+ human cardiac ventricular progenitor cells obtained by the method of claim 38.

41. A method of enhancing cardiac function in a subject, the method comprising administering a pharmaceutical composition comprising the clonal population of claim 39 to the subject.

42. The method of claim 41, wherein the clonal population is administered directly into the heart of the subject.

43. The method of claim 41 or 42, wherein the subject has suffered a myocardial infarction.
44. The method of claim 41 or 42, wherein the subject has a congenital heart disorder.
45. The method of claim 42, wherein the clonal population is administered directly into a ventricular region of the heart of the subject.
46. The method of any one of claims 41-45, wherein the pharmaceutical composition comprises the clonal population formulated onto a two dimensional or three dimensional matrix.
47. A method for generating human ventricular tissue comprising:
transplanting NRP1+ human cardiac ventricular progenitor cells into an organ of a non-human animal; and
allowing the progenitor cells to grow *in vivo* such that human ventricular tissue is generated.
48. The method of claim 47, wherein the non-human animal is an immunodeficient mouse.
49. The method of claim 47, wherein the organ is a kidney or a heart.
50. The method of claim 47, wherein the cells are transplanted at a time when one, two, three, four or five of the following cell marker patterns are present: (i) after peak of cardiac mesoderm formation; (ii) at time of peak Islet-1 expression; (iii) before peak of NKX2.5 expression; (iv) before peak expression of downstream genes MEF-2 and TBX-1; and (v) before expression of differentiated contractile protein genes.
51. The method of claim 47, wherein the cells are transplanted between day 5 and day 7 (inclusive) of *in vitro* culture of human pluripotent stem cells under conditions to generate human ventricular progenitor cells.

52. The method of claim 51, wherein the cells are transplanted on day 6 of *in vitro* culture of human pluripotent stem cells under conditions to generate human ventricular progenitor cells.

53. A method of screening for cardiac toxicity of test compound, the method comprising:

providing NRP1+ human cardiac ventricular progenitor cells;

contacting the cells with the test compound; and

measuring toxicity of the test compound for the cells,

wherein toxicity of the test compound for the cells indicates cardiac toxicity of the test compound.

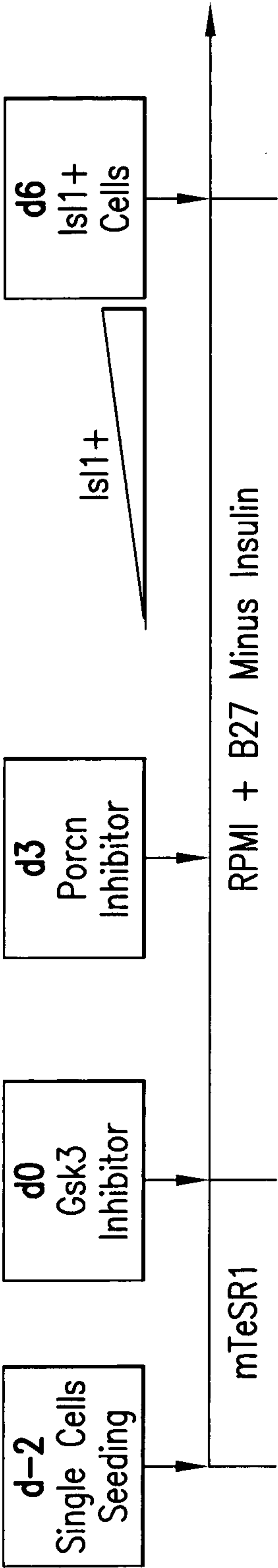


FIG.1

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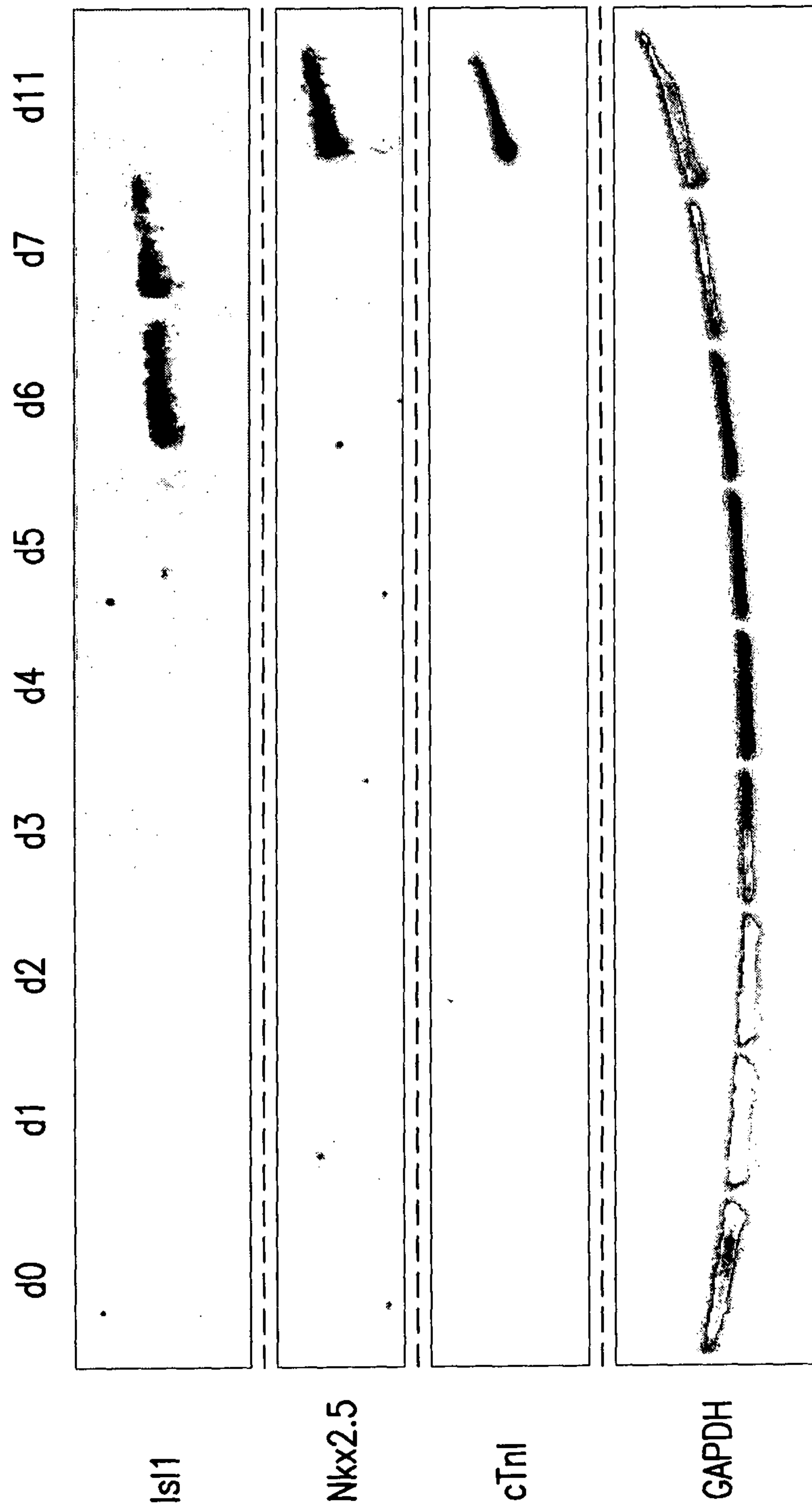


FIG.2

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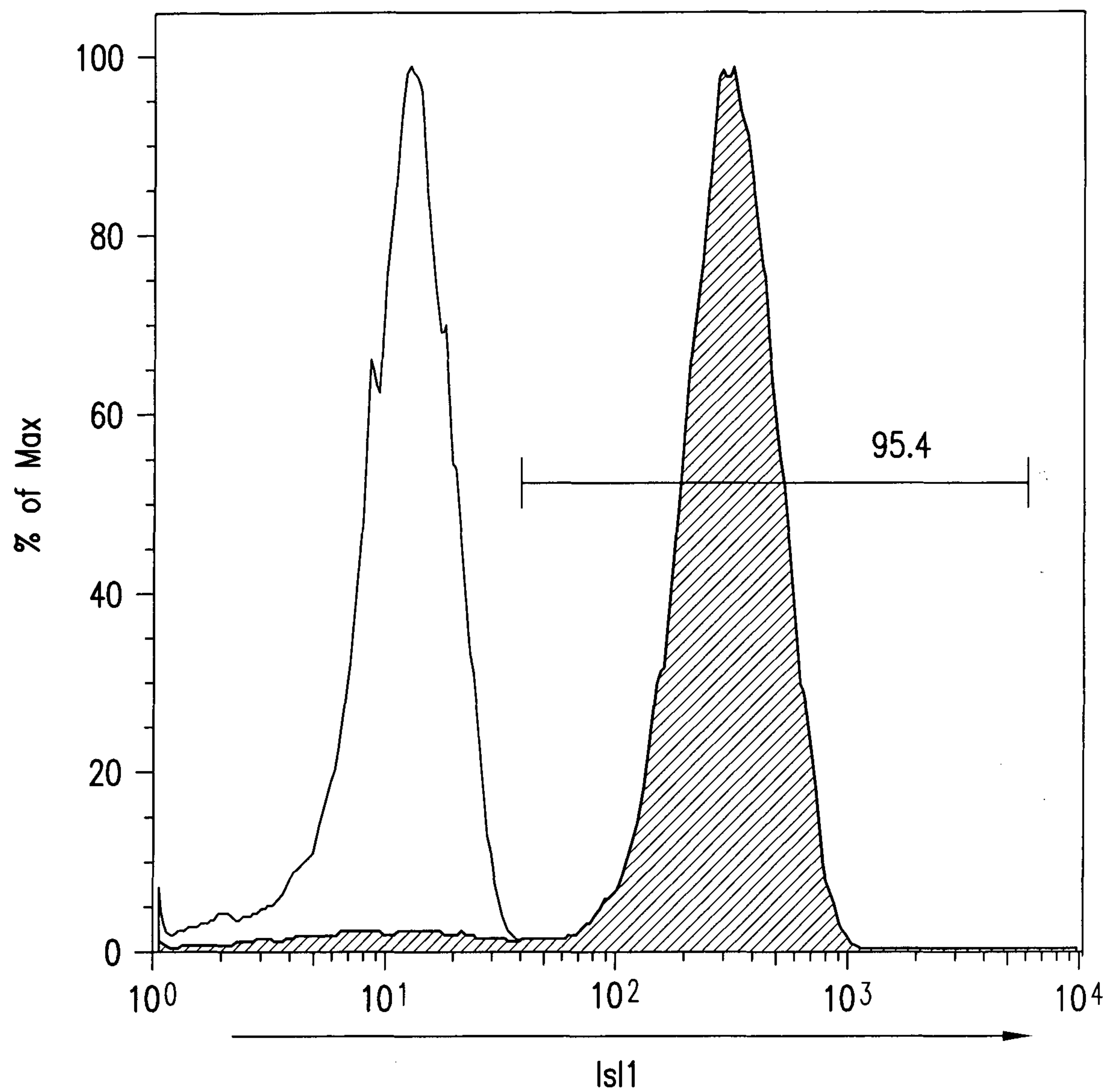
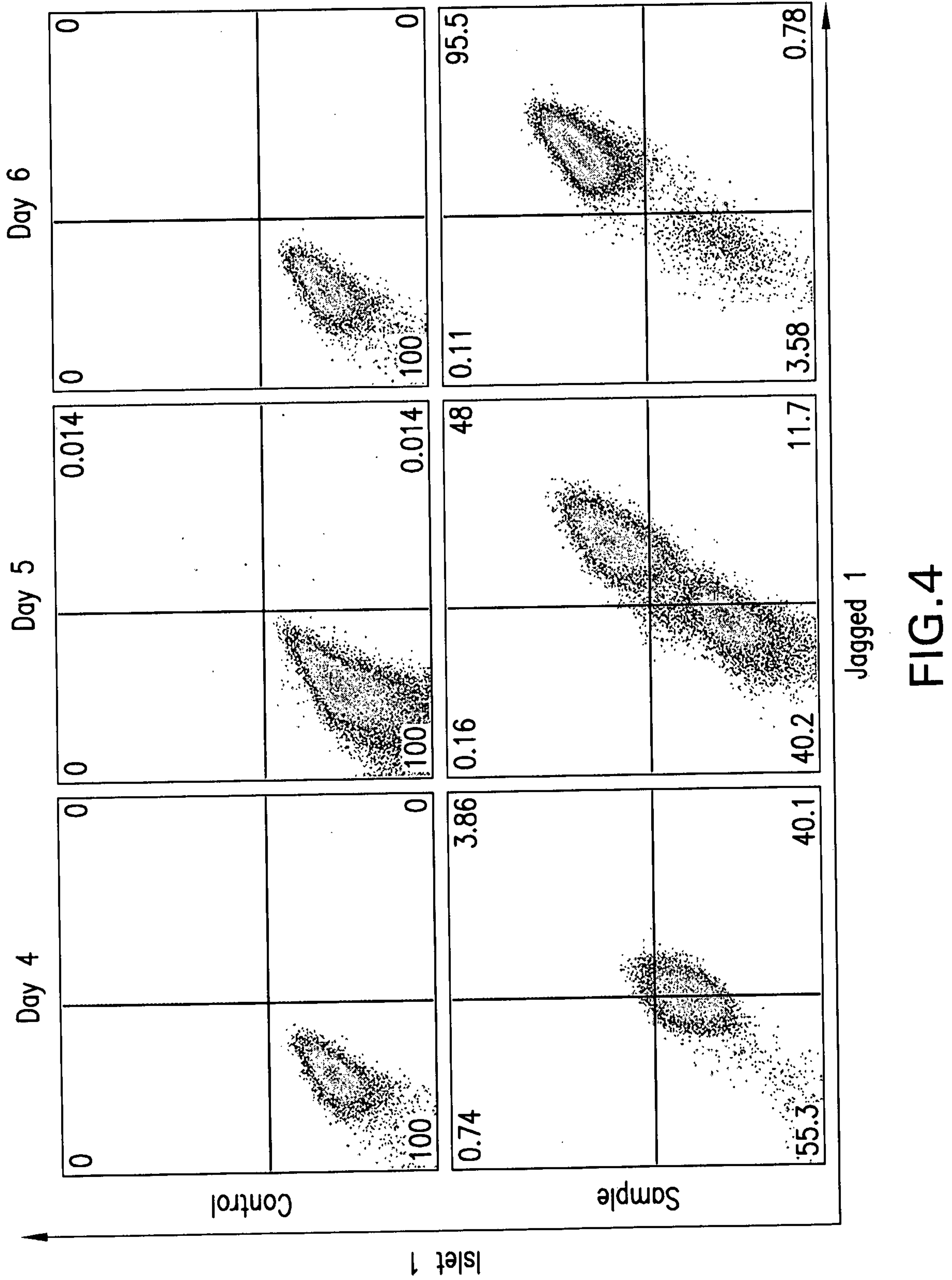


FIG.3

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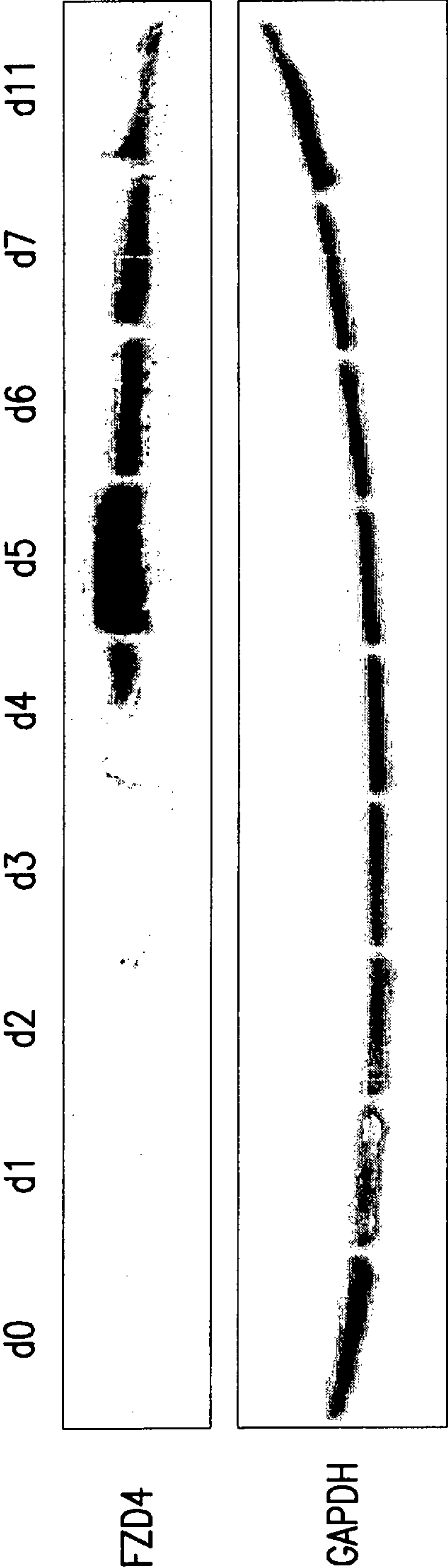


FIG.5

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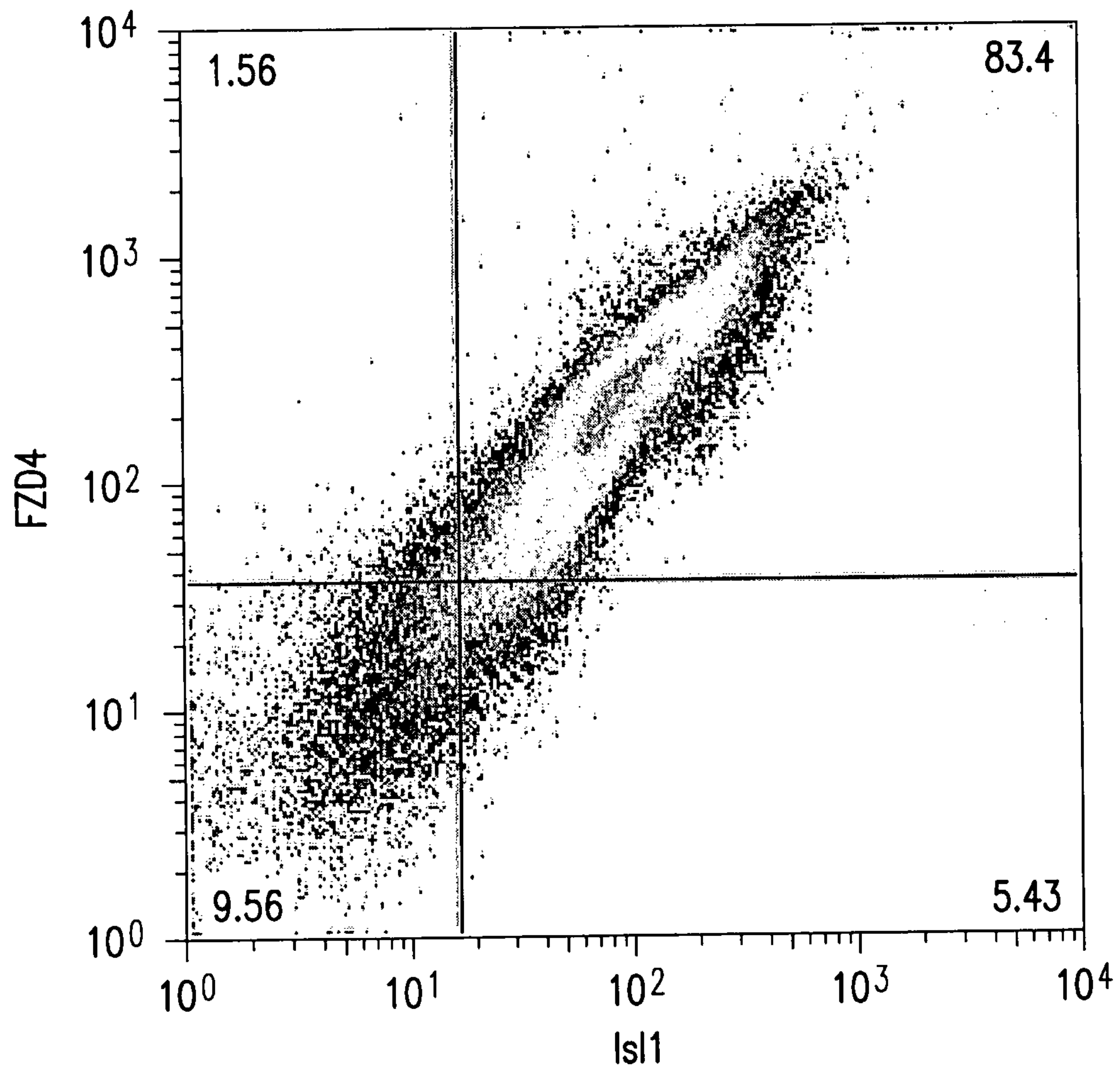


FIG.6

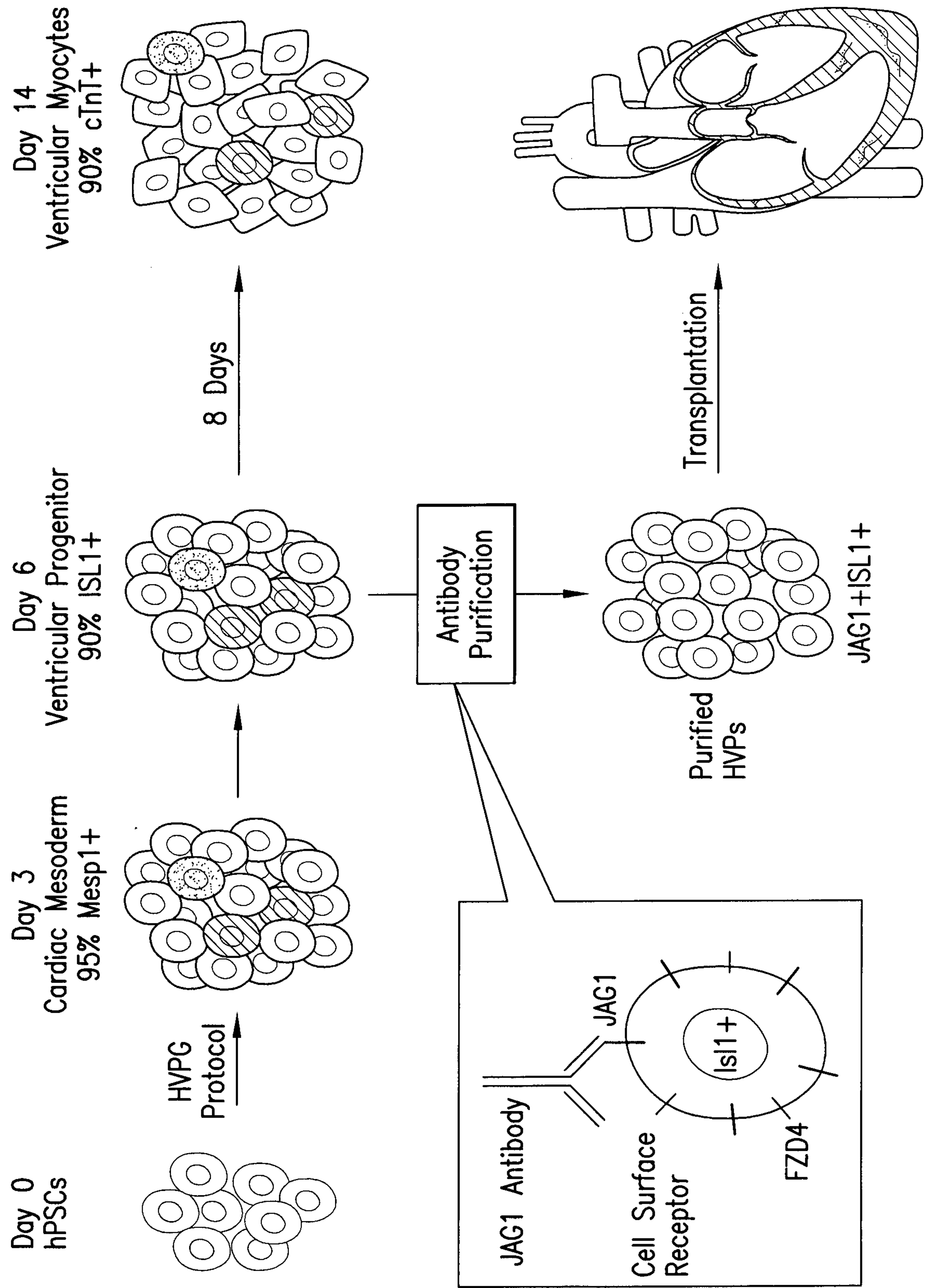


FIG.7

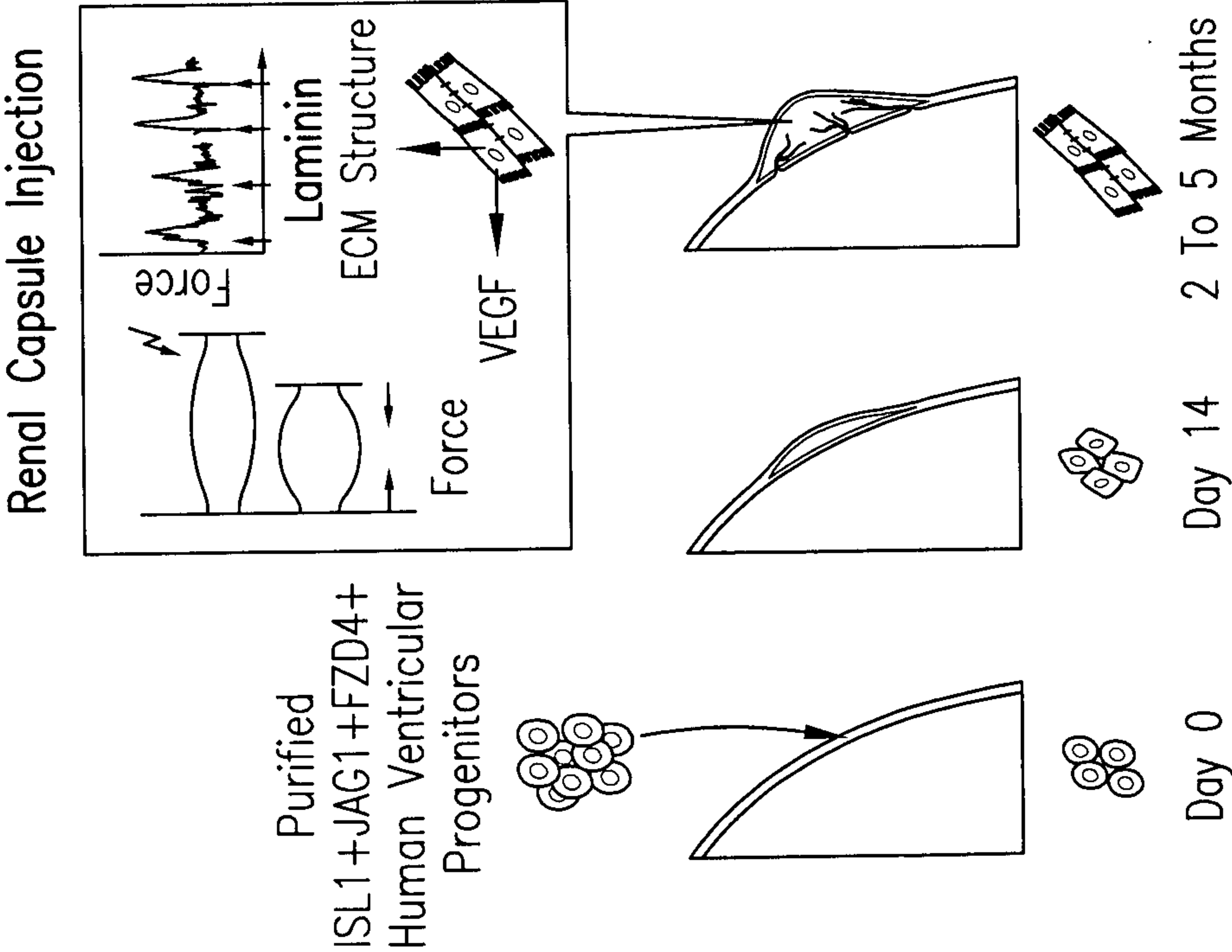


FIG.8A

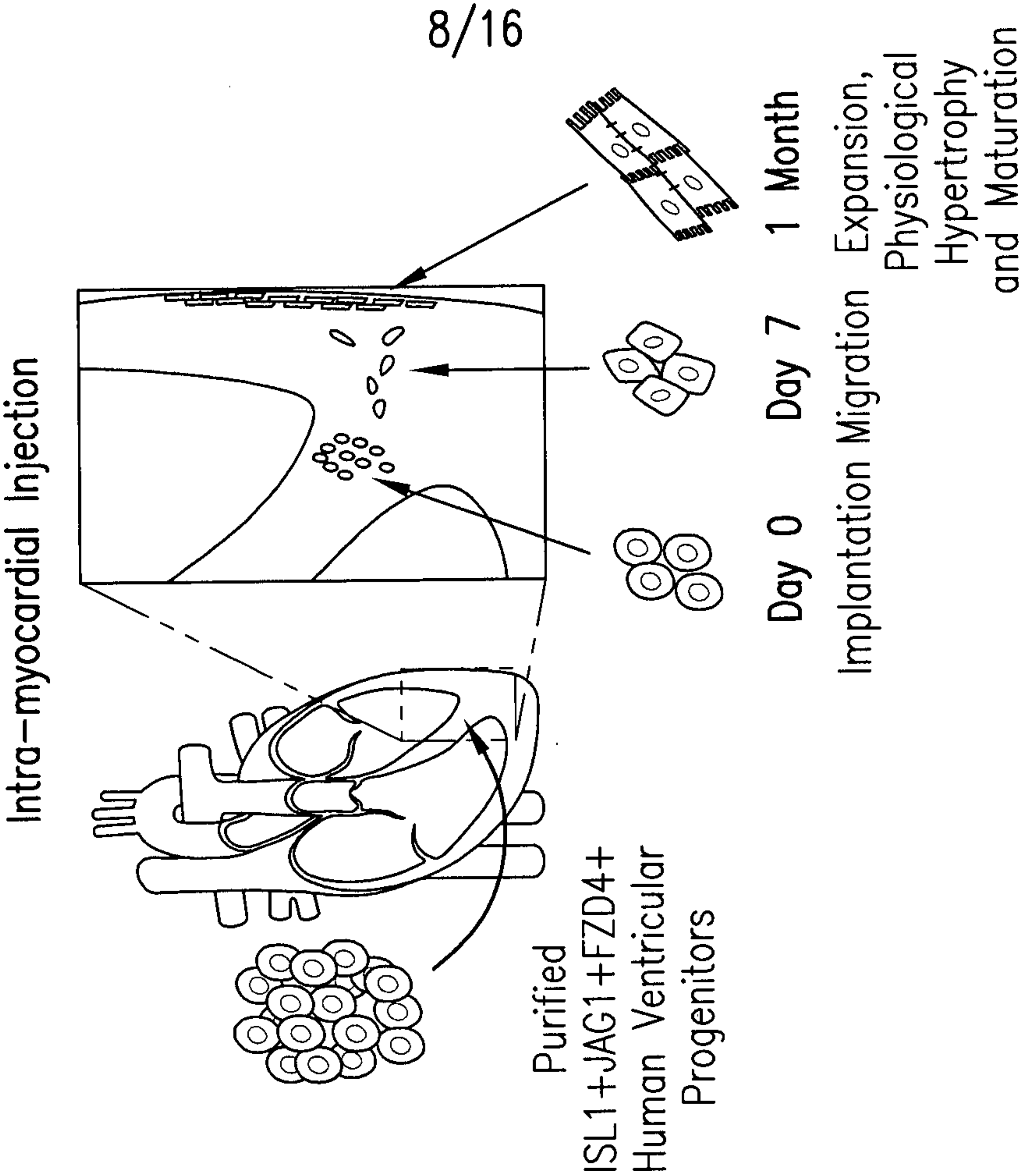


FIG.8B

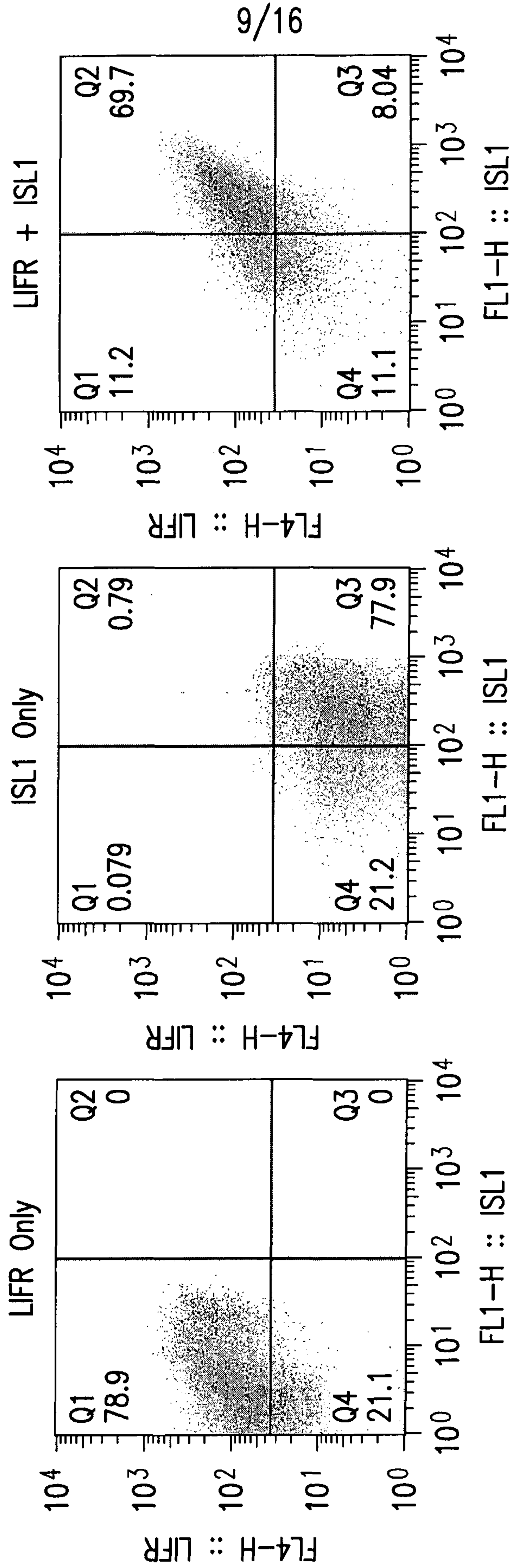
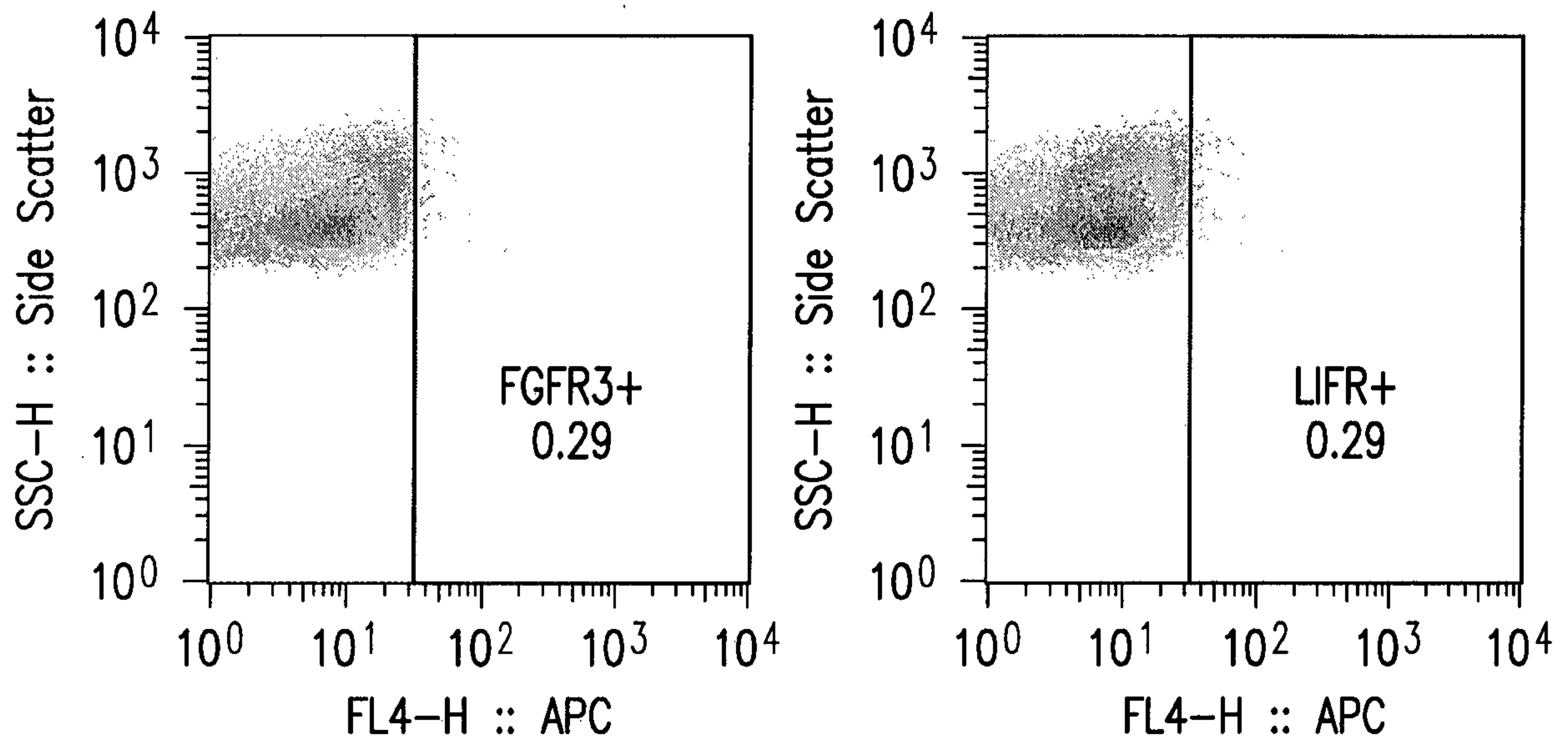


FIG.9

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Undifferentiated ES Cells

Negative Control



Antibody Stained

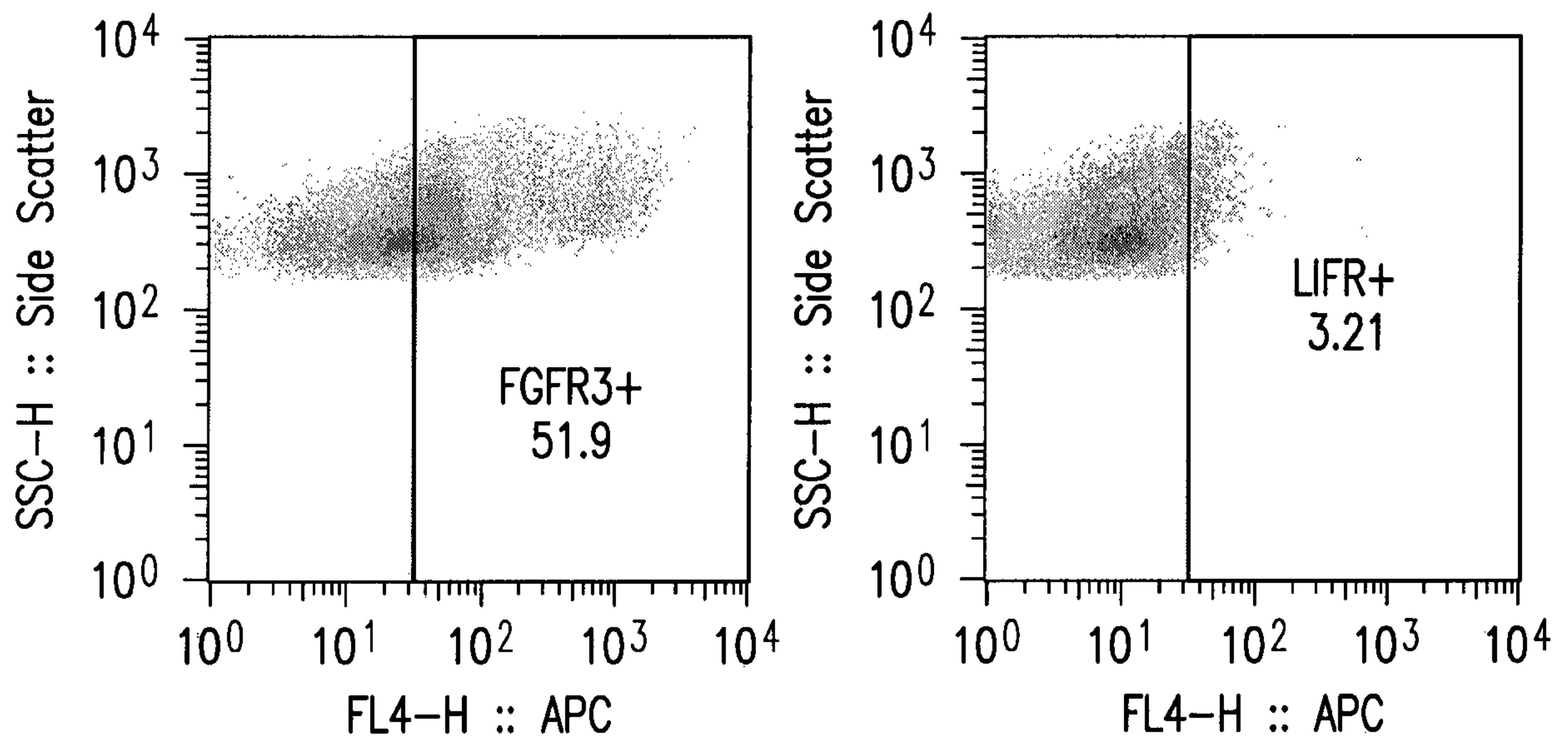
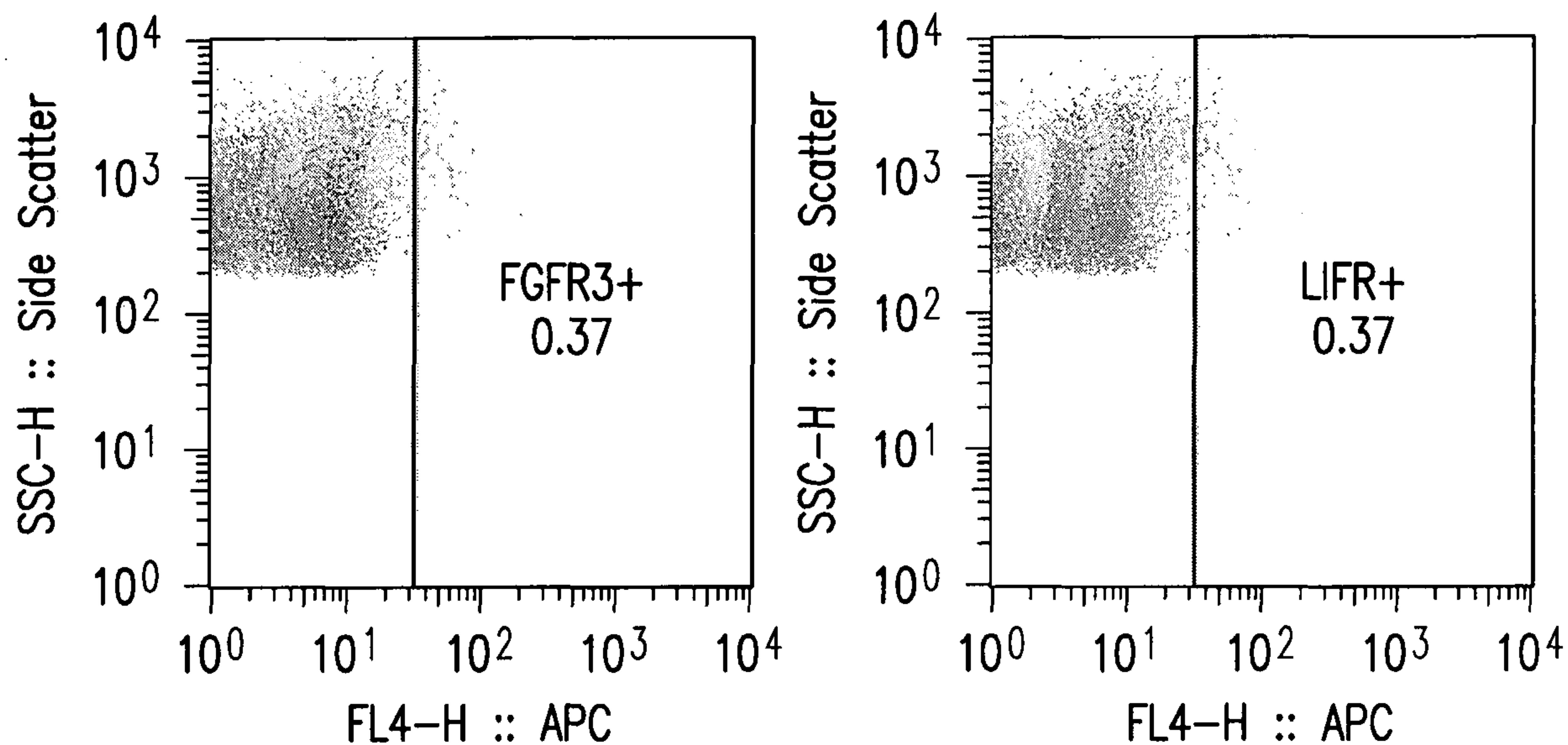


FIG.10A

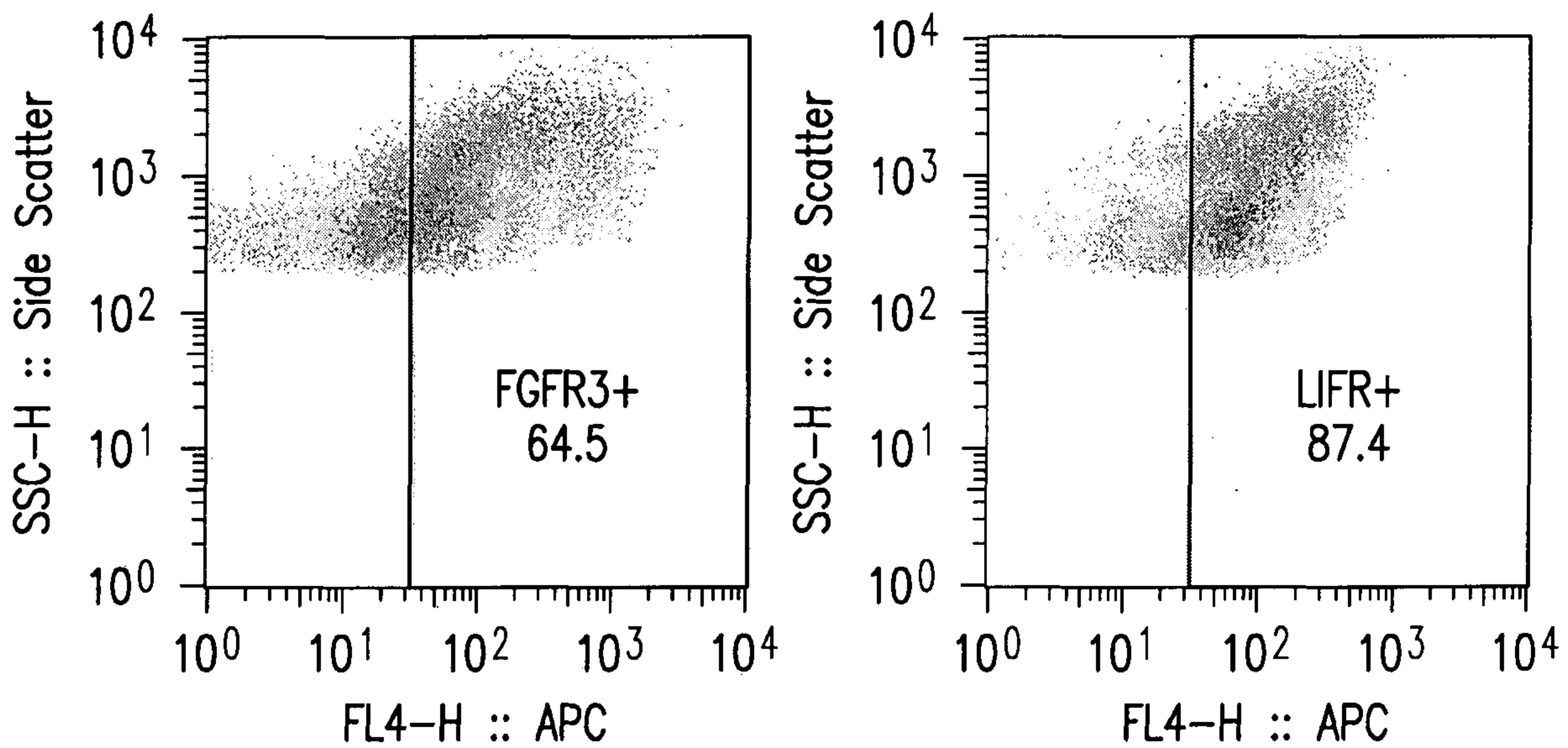
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Ventricular Progenitor Cells

Negative Control



Antibody Stained

**FIG. 10B**

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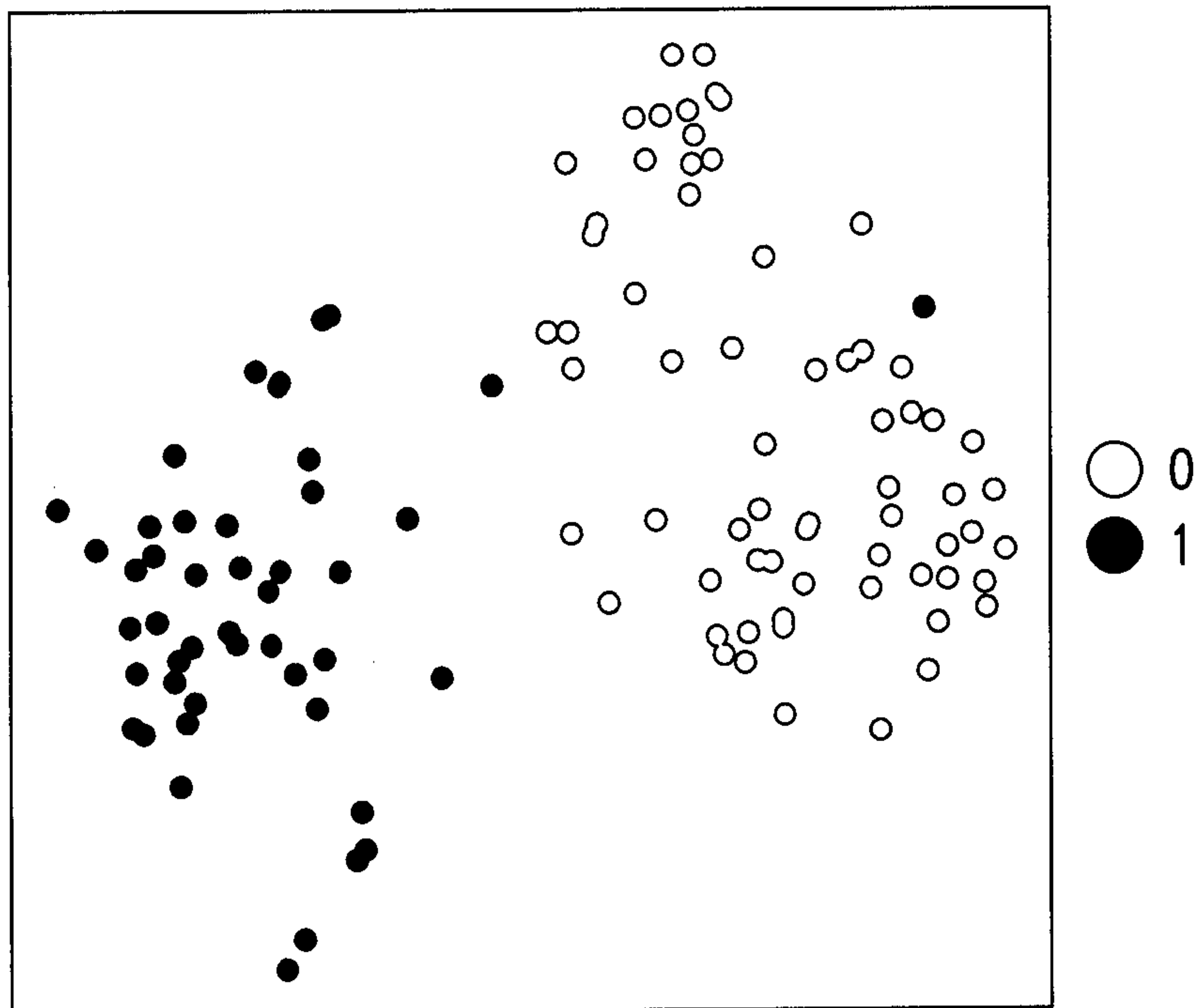


FIG. 11

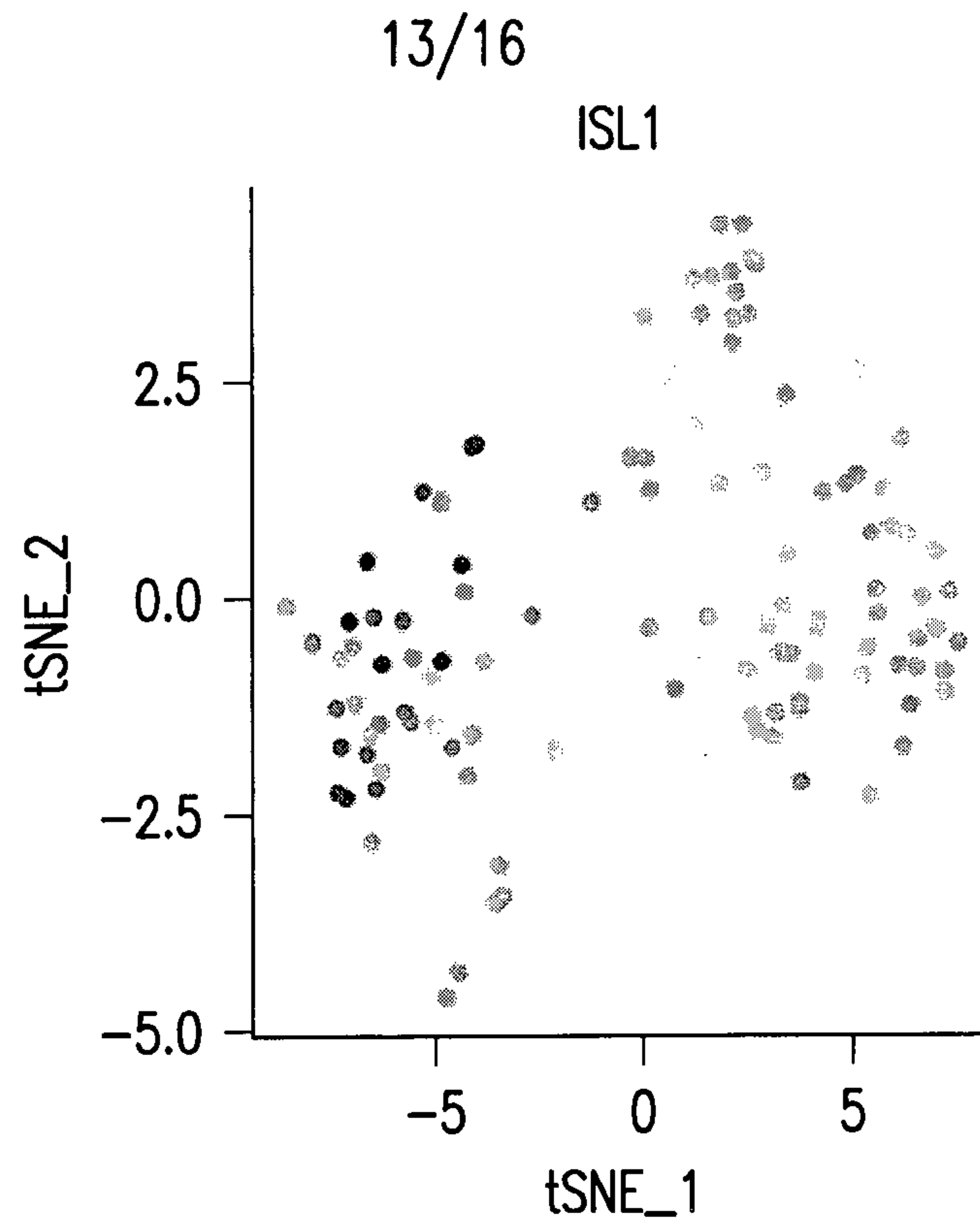


FIG. 12A

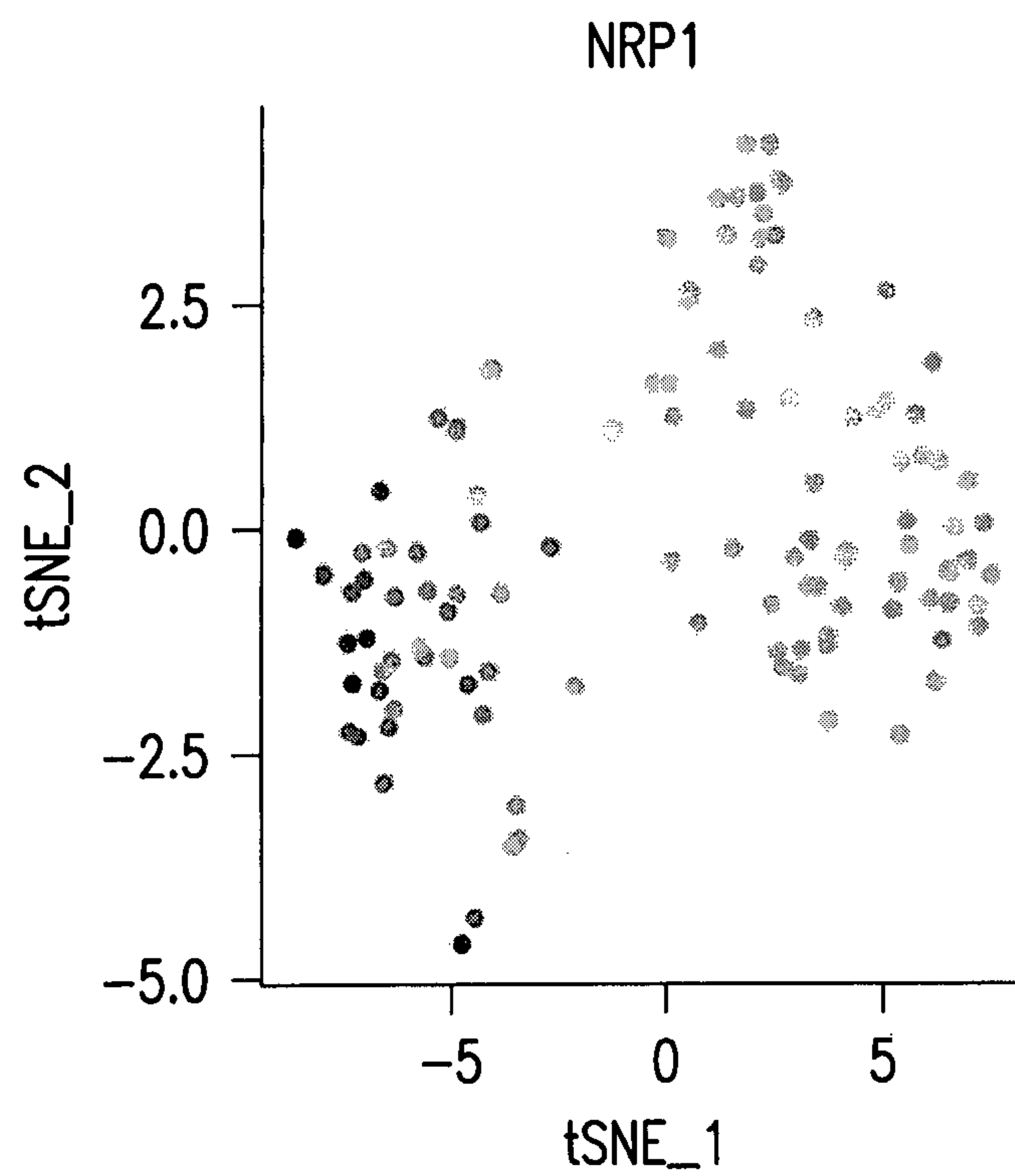
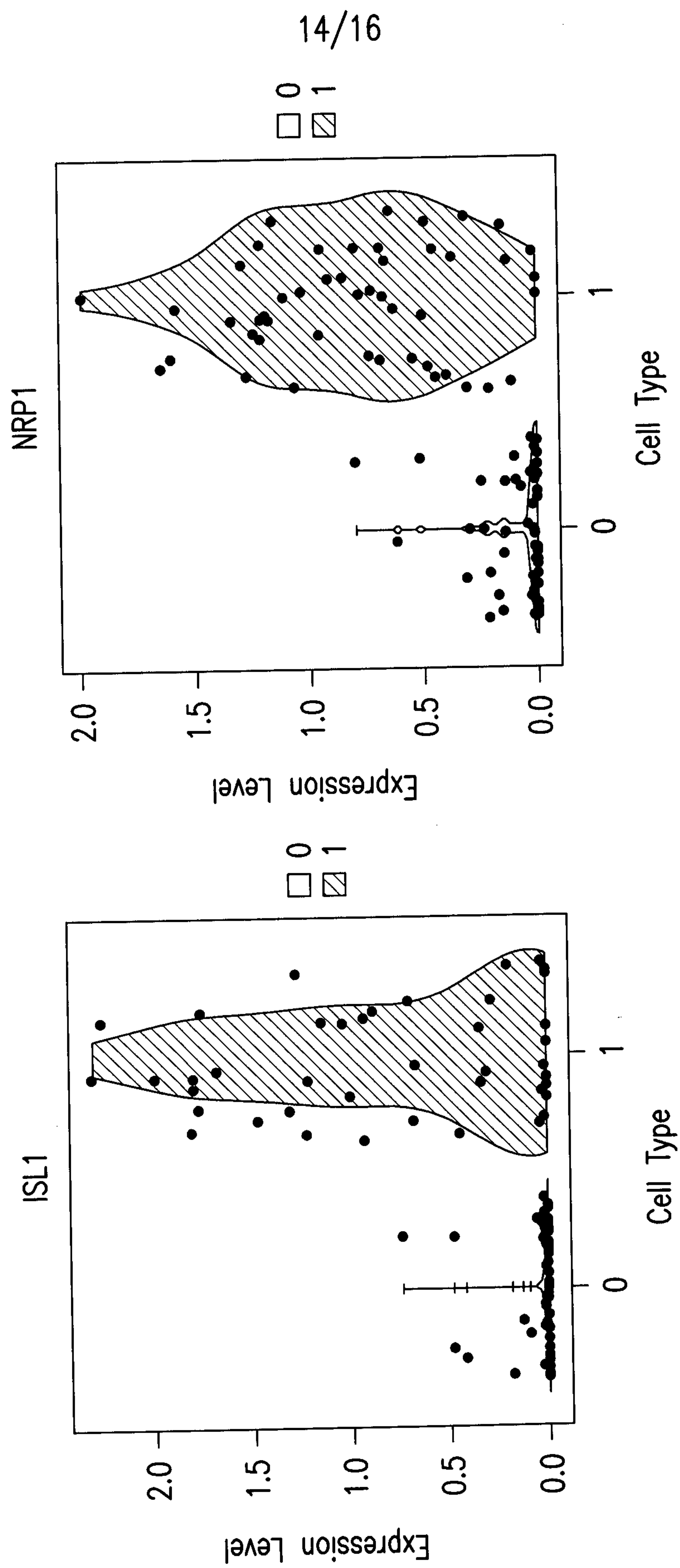


FIG. 12B



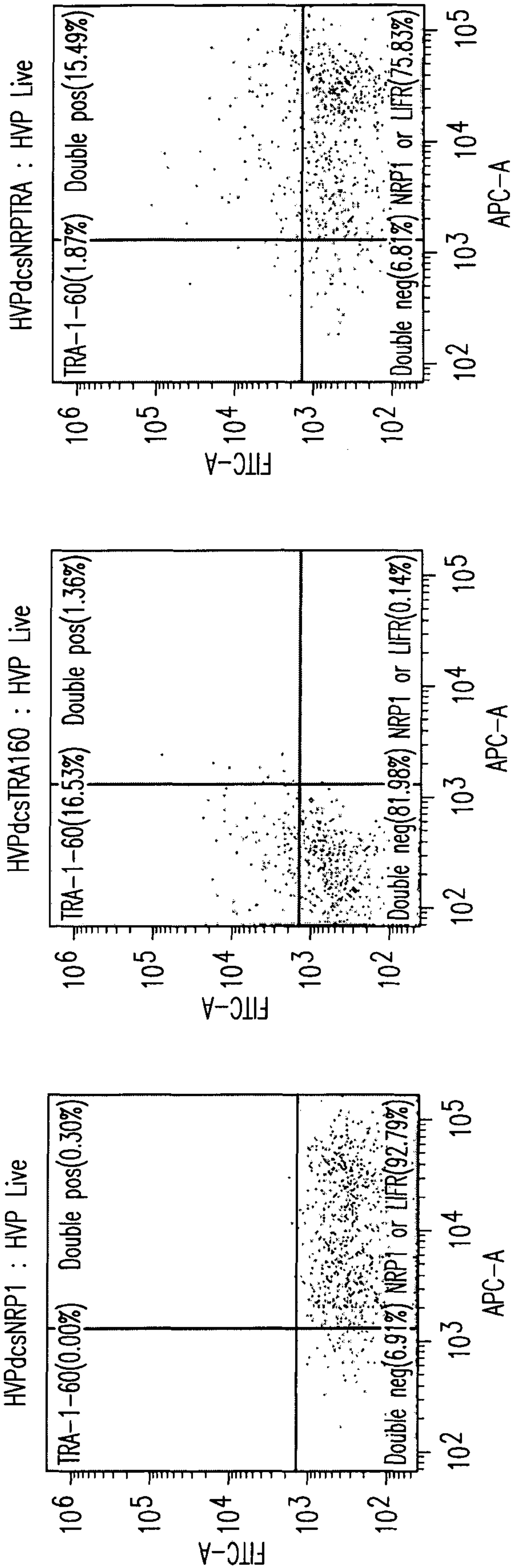


FIG.14A

FIG.14B

FIG.14C

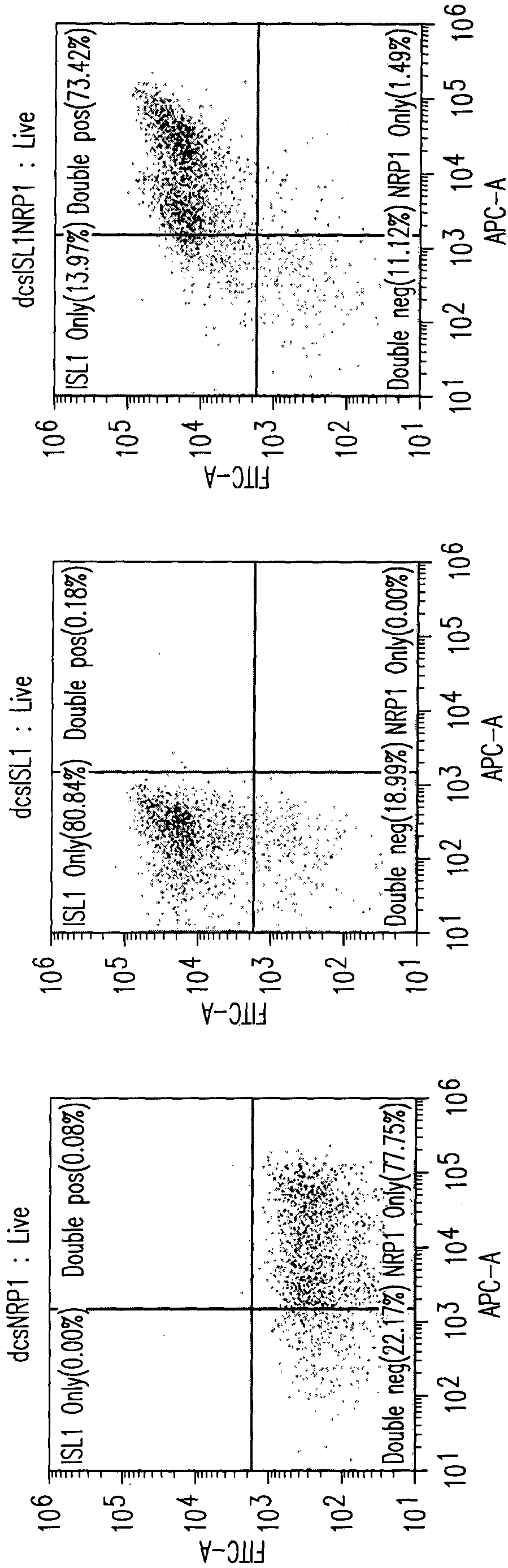


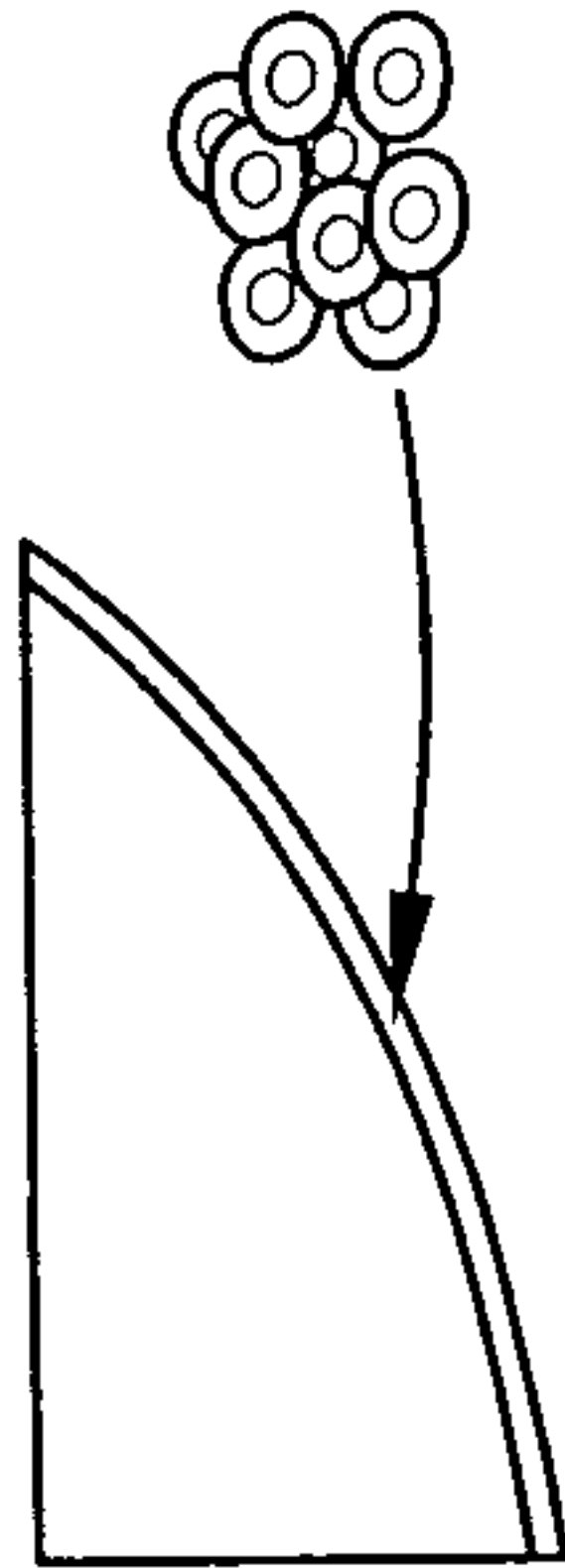
FIG.15A

FIG.15B

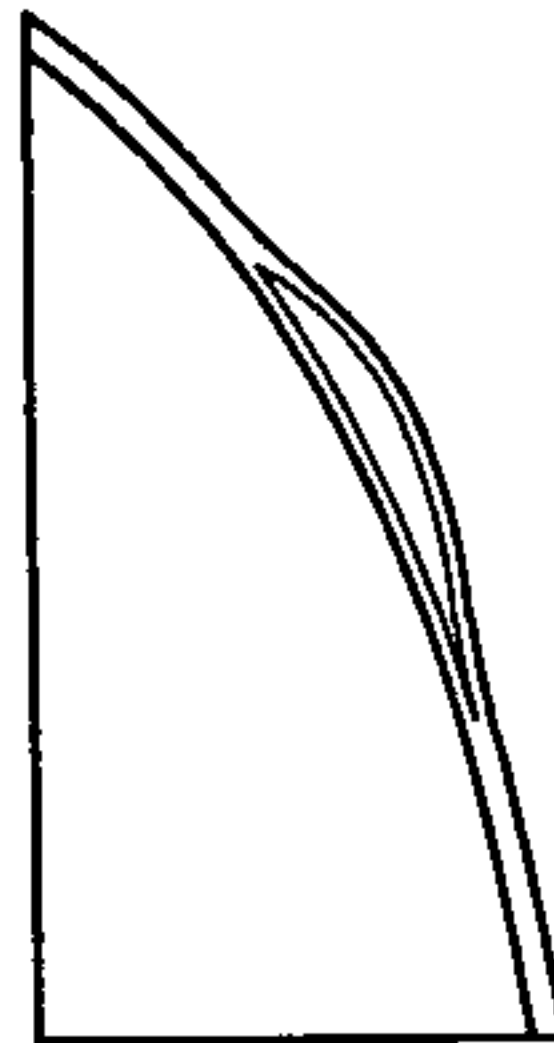
FIG.15C

Renal Capsule Injection

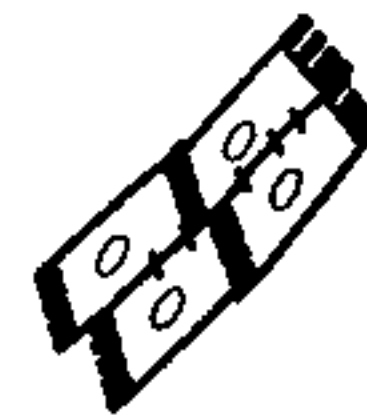
Purified
ISL1+JAG1+FZD4+
Human Ventricular
Progenitors



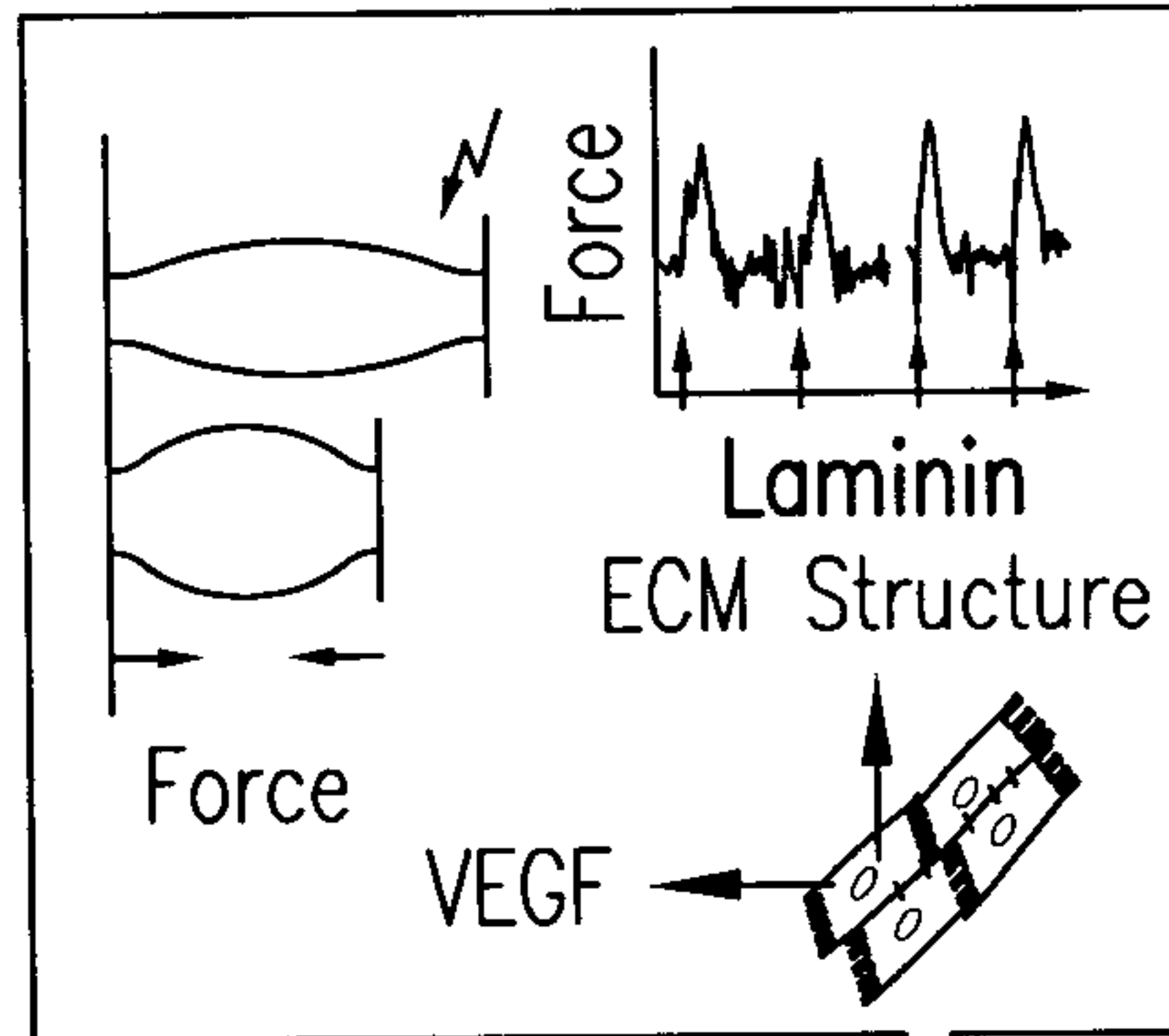
Day 0



Day 14



2 To 5 Months



ISL
Hu

FIG.8A