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(54) Title: METHODS AND COMPOSITIONS FOR INCREASED SAFETY OF STEM CELL-DERIVED POPULATIONS

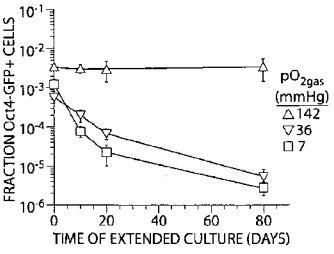

Fig. 1A



Fig. 1B

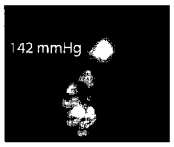

Fig. 1C

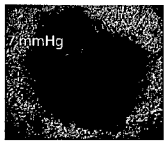

Fig. 1D

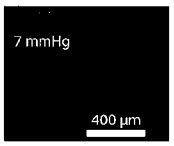

Fig. 1E

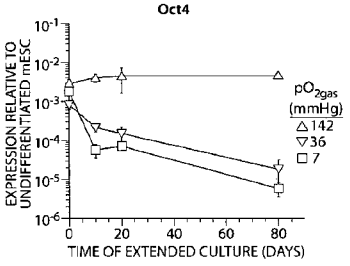

Fig. 1F

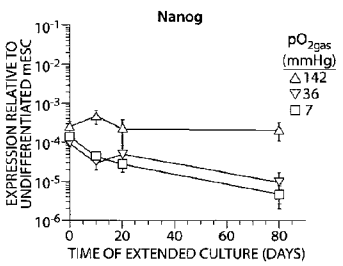

Fig. 1G

(57) Abstract: The invention provides methods and compositions relating to differentiated cell populations that derive from pluripotent stem cells. The methods relate to reducing the number of residual stem cells present in such populations. The compositions include differentiated cell populations that contain reduced number of stem cells or that contain no stem cells. Pluripotent stem cells may be reduced in number and/or function through exposure to low oxygen levels.

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METHODS AND COMPOSITIONS FOR INCREASED SAFETY OF STEM CELL-DERIVED POPULATIONS

FIELD OF INVENTION

The invention provides methods and compositions relating to differentiated cell populations that derive from pluripotent stem cells. The methods relate to reducing the number of residual stem cells present in such populations. The compositions include differentiated cell populations that contain reduced number of stem cells or that contain no stem cells. Pluripotent stem cells may be reduced in number and/or function through exposure to low oxygen levels.

BACKGROUND OF INVENTION

Much research and clinical investigation is currently focused on the use of stem cells and their differentiated progeny. As an example, there are a variety of therapeutic approaches contemplated and/or being developed that involve the use of embryonic (ES) stem cells and/or their progeny. ES cells are pluripotent cells that have the ability to generate any cell type in a mature organism. The clinical potential for stem cell based therapies is immense (Lawrenz et al. Cytotherapy 6, 212-22 (2004)), including treatments for heart disease, diabetes, Parkinson's disease, and leukemia (Hentze et al. Trends Biotechnol 25, 24-32 (2007); Pei et al. J Biol Chem 284, 3365-9 (2009); Jaenisch et al. Cell 132, 567-82 (2008)). However, stem cells such as ES cells pose the risk of tumor formation upon implantation. For example, as few as two ES cells have been shown to form tumors in severe combined immunodeficiency (SCID) mice (Lawrenz et al. Cytotherapy 6, 212-22 (2004)).

SUMMARY OF INVENTION

The invention provides a novel and unexpected approach to reducing the frequency of pluripotent stem cells, such as but not limited to ES cells, in a population of cells intended for in vivo administration or for in vitro uses such as drug testing and/or screening. Typically, the cell population intended for in vivo administration is derived from the differentiation of one or more pluripotent stem cells. It therefore at least contains differentiated progeny of pluripotent stem cells and also contains (or is likely to contain) residual pluripotent stem cells. The frequency of residual pluripotent stem cells is reduced by long-term exposure to an

oxygen partial pressure that is less than 142 mmHg (i.e., the oxygen partial pressure in a humidified incubator with an inlet gas containing 5% CO_2 and remainder air at 37°C, otherwise referred to herein as a normoxic condition). Usually this exposure will occur in vitro and will be referred to herein as culture at low oxygen partial pressure. The invention is therefore premised in part on the unexpected finding that exposure of a differentiated cell population to low oxygen partial pressure for extended periods of time does not compromise the quality or function of the differentiated cells in the population and rather serves to reduce residual pluripotent stem cells resident in the population, whether by further differentiation or by other means. This finding indicates that differentiated cell populations suspected of containing pluripotent stem cells can be treated in order to reduce and preferably eliminate such pluripotent stem cells without adverse effect on the differentiated cells within the population. The resultant cell population can then be used in vivo with a reduced risk, and preferably without risk, of tumor formation.

These findings also indicate that protocols for differentiating pluripotent stem cells may be modified to include at least a period of time in which the pluripotent stem cells and/or their progeny are exposed to low oxygen partial pressure. In this way, the pluripotent stem cells are differentiated as desired and in the process the numbers of such pluripotent stem cells are also reduced, thereby generating a differentiated cell population suitable for in vitro and/or in vivo use as described herein. In some embodiments, the pluripotent stem cells are differentiated towards non-cardiomyocyte lineages such as but not limited to neural cells, islet cells, and the like. In still other embodiments, the differentiation protocol is one carried out at normoxic conditions but for the findings of (and modifications provided by) the instant invention.

Thus, in one aspect, the invention provides a method comprising culturing a differentiated cell population at an oxygen partial pressure that is less than 142 mmHg for 20 days or more. In another aspect, the invention provides a method comprising culturing a differentiated cell population at an oxygen partial pressure that is less than 142 mmHg for 10 days or more, wherein the differentiated cell population is a non-cardiomyocyte differentiated cell population.

In another aspect, the invention provides a method comprising differentiating pluripotent stem cells towards a non-cardiomyocyte lineage in the presence of one or more differentiation stimuli at an oxygen partial pressure that is less than 142 mmHg. In another

aspect, the invention provides an improved method for differentiating pluripotent stem cells comprising differentiating pluripotent stem cells in the presence of one or more differentiation stimuli and at an oxygen partial pressure that is less than 142 mmHg, wherein the improvement relates to exposing the cells to an oxygen partial pressure that is less than 142 mmHg in order to reduce pluripotent stem cell number following sufficient differentiation. In some embodiments, the pluripotent stem cells are exposed to the one or more differentiation stimuli and an oxygen partial pressure that is less than 142 mmHg simultaneously. In some embodiments, the pluripotent stem cells are exposed to the one or more differentiation stimuli and an oxygen partial pressure that is less than 142 mmHg separately.

In another aspect, the invention provides a method comprising culturing an isolated in vitro differentiated cell population at an oxygen partial pressure that is less than 142 mmHg for 10 days or more. An in vitro differentiated cell population is a cell population that is differentiated in vitro, as described herein.

Various embodiments apply to the foregoing aspects and these are recited below.

In some embodiments, the differentiated cell population is isolated from a differentiation culture.

In some embodiments, the differentiated cell population is derived from an embryonic stem cell or an induced pluripotent stem cell. In some embodiments, the differentiated cell population is derived from a human embryonic stem cell or a human induced pluripotent stem cell.

In some embodiments, the differentiated cell population comprises cardiomyocyte lineage cells. In some embodiments, the differentiated cell population comprises cardiomyocyte precursor cells. As used herein, the terms precursors and progenitors are used interchangeably. In some embodiments, the differentiated cell population comprises neural cells. In some embodiments, the differentiated cell population comprises islet cells. In some embodiments, the differentiated cell population comprises hepatic cells.

In some embodiments, the differentiated cell population comprises non-stem cell precursor cells. As used herein, non-stem precursors are cells that are not pluripotent stem cells yet are not terminally differentiated cells. Non-stem precursors include cells that are committed to one or more lineages and that have proliferative capacity.

In some embodiments, the oxygen partial pressure is 4-10 mmHg. In some embodiments, the oxygen partial pressure is 7 mmHg. In some embodiments, the oxygen partial pressure is 10-50 mmHg. In some embodiments, the oxygen partial pressure is 36 mmHg. In some embodiments, the oxygen partial pressure is 50-100 mmHg. In some embodiments, the oxygen partial pressure is 100-140 mmHg.

In some embodiments, the differentiated cell population is cultured for 20 days. In some embodiments, the differentiated cell population is cultured for 30 days. In some embodiments, the differentiated cell population is cultured for 40 days.

In some embodiments, the culturing step reduces pluripotent stem cells in the differentiated cell population by at least 2-fold, or at least 5-fold, or at least 10-fold, or at least 20-fold, or at least 40-fold, or at least 100-fold, or at least 150-fold.

In some embodiments, the method further comprises performing a positive selection for differentiated cells prior to, during and/or after the culturing step. In some embodiments, the method further comprises performing a negative selection for pluripotent stem cells prior to, during and/or after the culturing step. In some embodiments, the positive or negative selection is based on cell surface expression of a marker. In some embodiments, the positive or negative selection is a fluorescent activated cell sorting (FACS) selection. In some embodiments, negative selection is based on expression of SSEA-4, TRA-60 and/or Oct4. In some embodiments, the positive or negative selection is based on a physical property. In some embodiments, the negative selection is based on buoyant density.

In some embodiments, the method further comprises harvesting the differentiated cell population after the culturing step.

In some embodiments, the method further comprises assaying for teratoma-causing cells in the cultured differentiated cell population. In some embodiments, the teratoma-causing cells are assayed by introducing the cultured differentiated cell population into an immunocompromised mouse. In some embodiments, 10^3 cells, 10^4 cells, 10^5 cells, 10^6 cells, or 10^7 cells from the cultured differentiated cell population are introduced into the immunocompromised mouse.

In some embodiments, the method further comprises measuring mRNA or protein expression of a stem cell marker in the cultured differentiated cell population. In some embodiments, the stem cell marker is Oct4.

In some embodiments, the method further comprises administering the differentiated cell population to a subject in need thereof.

In some embodiments, the differentiated cell population is cultured in a culture vessel comprising an oxygen permeable membrane. In some embodiments, the differentiated cell population is cultured in a culture vessel comprising a silicon rubber membrane.

In another aspect, the invention provides a differentiated cell population obtained according to any of the foregoing methods.

In another aspect, the invention provides an isolated differentiated cell population derived from isolated pluripotent stem cells and having less than 1 pluripotent stem cell per 10^3 cells. In some embodiments, the differentiated cell population comprises less than 1 pluripotent stem cell per 10^4 cells. In some embodiments, the differentiated cell population comprises less than 1 pluripotent stem cell per 10^5 cells. In some embodiments, the differentiated cell population comprises less than 1 pluripotent stem cell per 10^6 cells. In some embodiments, the differentiated cell population comprises less than 1 pluripotent stem cell per 10^7 cells. In some embodiments, the differentiated cell population comprises less than 1 pluripotent stem cell per 10^8 cells. In some embodiments, the differentiated cell population comprises less than 1 pluripotent stem cell per 10^9 cells.

In some embodiments, the pluripotent stem cells are embryonic stem cells or induced pluripotent stem cells. In some embodiments, the pluripotent stem cells are human embryonic stem cells or human induced pluripotent stem cells.

In some embodiments, the differentiated cell population comprises cardiomyocyte lineage cells. In some embodiments, the differentiated cell population comprises cardiomyocyte precursor cells. In some embodiments, the differentiated cell population comprises neural cells. In some embodiments, the differentiated cell population comprises islet cells. In some embodiments, the differentiated cell population comprises non-stem cell precursor cells.

In another aspect, the invention provides a pharmaceutical preparation comprising any of the foregoing differentiated cell populations. In some embodiments, the preparation comprises at least 10^7, at least 10^8, or at least 10^9 cells. In some embodiments, the preparation comprises a pharmaceutically acceptable carrier. In some embodiments, the differentiated cell population is isolated from its differentiation culture conditions. In some

embodiments, the differentiated cell population is encapsulated in a gel. In some embodiments, the preparation is formulated for parenteral administration.

In another aspect, the invention provides a method for treating a subject comprising administering to a subject in need thereof a differentiated cell population produced according to any of the foregoing methods in an effective amount to treat the subject.

In another aspect, the invention provides a method comprising exposing any of the foregoing differentiated cell populations or any of the differentiated cell populations produced according to any of the foregoing methods to an agent, and determining the effect of the agent on the differentiated cell population. In some embodiments, determining the effect of the agent comprises determining the cytotoxicity of the agent on the differentiated cell population.

It should be appreciated that all combinations of the foregoing concepts and additional concepts discussed in greater detail below (provided such concepts are not mutually inconsistent) are contemplated as being part of the inventive subject matter disclosed herein. In particular, all combinations of claimed subject matter appearing at the end of this disclosure are contemplated as being part of the inventive subject matter disclosed herein. It should also be appreciated that terminology explicitly employed herein that also may appear in any disclosure incorporated by reference should be accorded a meaning most consistent with the particular concepts disclosed herein.

BRIEF DESCRIPTION OF DRAWINGS

It is to be understood that the Figures are not necessarily to scale, emphasis instead being placed upon generally illustrating the various concepts discussed herein.

Figure 1. Expression of pluripotency markers in mESC having undergone differentiation and extended culture at various pO_{2gas}. mESC were differentiated at 142, 36, or 7 mmHg pO_{2gas} then subjected to 0, 10, 20, or 80 days of extended culture at the same pO_{2gas}. (A) Fraction of cells that are Oct4-GFP+ as measured with flow cytometry (n=3). (B and D) En face bright field images of cell aggregates after 20 days extended culture. (C and E) En face fluorescence images taken with a GFP filter corresponding with (B) and (D), respectively. (F-G) Relative expression of Oct4 and Nanog mRNA measured with real-time PCR (n=3).

Figure 2. The number of residual Oct4-GFP+ cells from mESC having undergone differentiation and extended culture at various pO_{2gas}. mESC were differentiated at 142, 36, or 7 mmHg pO_{2gas} then subjected to 0, 10, 20, or 80 days of extended culture at the same pO_{2gas}. The number of cells that are Oct4-GFP+ was determined by multiplying the fraction of Oct4-GFP+ cells, measured with flow cytometry, and the total cell number, measured by nuclei count (n=3).

Figure 3. The fraction of residual Oct4-GFP+ cells from mESC having undergone differentiation and extended culture at the same or different pO_{2gas}. (A) mESC differentiated at 142 mmHg pO_{2gas} and then exposed to extended culture for 20 days at either 142 mmHg (black bar) or 7 mmHg (white bar). (B) mESC differentiated at 7 mmHg pO_{2gas} and then exposed to extended culture for 20 days at either 7 mmHg (black bar) or 142 mmHg (white bar). Data acquired with flow cytometry. * indicates significant difference compared to the black bar of the same time (n=3).

Figure 4. Time-course development of tumors in nude mice after subcutaneous implantation of mESC. (A) Formation of tumors after implantation of undifferentiated mESC (n=6; positive control), mESC differentiated 10 days at 142, 36, or 7 mmHg followed by 20 days extended culture at the same pO_{2gas} (n=8) or only Matrigel (n=6; negative control). Tumor formation was determined by visual observation of the appearance of masses underneath the skin. (B) Representative H&E-stained tissue section of a tumor derived from mESC differentiated and having undergone 20 days of extended culture at 7 mmHg pO_{2gas}, with (i) small glandular structure, (ii) hyaline cartilage, and (iii) neuroepithelial structures highlighted.

Figure 5. Expression of Oct4-GFP and time-course development of tumors in nude mice after subcutaneous implantation of mESC sorted into GFP+ and GFP- populations. mESC were differentiated and cultured 20 extra days at either 142 or 7 mmHg pO_{2gas}. (A) Fraction of mESC that are Oct4-GFP+ as measured with flow cytometry before sorting (unsorted) and after being sorted into an Oct4-GFP- population (sorted GFP-) (n=3). (B-C) 10^5 cells were implanted without sorting (unsorted; n=8) or 10^5 cells were sorted into GFP+ and GFP- populations (n=6), then implanted.

Figure 6. Expression of pluripotency markers and time-course development of tumors in nude mice after subcutaneous implantation of mESC differentiated 10 days followed by 20 days extended culture at 142 or 7 mmHg pO_{2gas}, then sorted into SSEA-1+ and SSEA-1- populations. (A) Fraction of cells that are Oct4-GFP+, as measured with flow cytometry (n=3). (B) Formation of tumors after implantation of 10^5 unsorted cells or cells sorted into SSEA-1+ and SSEA-1- populations (n=6).

Figure 7. Expression of pluripotency markers and time-course development of tumors in nude mice after subcutaneous implantation of HIF-1$\alpha^{-/-}$ mESC cells differentiated 10 days, followed by 20 days extended culture at 142 or 7 mmHg pO_{2gas}. (A) Relative expression of Oct4 and Nanog mRNA measured with real-time PCR (n=3). (B) Formation of tumors after implantation of undifferentiated HIF-1$\alpha^{-/-}$ mESC (n=6; positive control) and HIF-1$\alpha^{-/-}$ mESC differentiated 10 days at 142 or 7 mmHg followed by 20 days extended culture at the same pO_{2gas} (n=6).

Figure 8. Characterization of (I) Oct4-GFP+ mESC that have never undergone differentiation and extended culture, (II) Oct4-GFP+ cells sorted with FACS from mESC that have undergone 10 days differentiation and 20 days extended culture at 142 mmHg, and (III) Oct4-GFP+ cells cultured similar to case II, but at 7 mmHg. (A) Histogram of the distribution of Oct4-GFP fluorescence intensity per cell, determined with flow cytometry. (B) Fraction of cells that are Oct4-GFP+ after 0 and 10 days of differentiation at 142 mmHg pO_{2gas} (n=3). (C-F) En face images of colonies from cell population II. (C) Bright field image. (D-F) Cells stained with DAPI (blue) and immunostained (red) for (D) Oct4, (E) Nanog, and (F) SSEA-1.

Figure 9. Differentiation of cases I, II, and III and assessment with real-time PCR. Expression of (A) Brachyury T, (B) Foxa2, and (C) Nestin after 4 days of differentiation and (D) cTnT, (E) cardiac-α-Actin, and (F) Nkx2.5 after 10 days of differentiation. (n=3).

Figure 10. Differentiation of cases I, II, and III and assessment with immunocytochemistry. En face images cells after (A-D) 4 days and (E-F) 10 days of differentiation stained with DAPI (A, C, E) and immunostained for (B) Foxa2, (D) Nestin, and (F) cTnT.

Figure 11. Differentiation of cases I, II, and III and assessment with flow cytometry. (A) Fraction and (B) number of Nestin+ cells after 4 days of differentiation. (C) Fraction and (D) number of MF-20+ cells after 10 days of differentiation. (n=3).

Figure 12. Expression of pluripotency markers and time-course development of tumors from hESC having undergone differentiation at various pO_{2gas}. hESC were differentiated at 142, 36, or 7 mmHg pO_{2gas} up to 90 days. (A-B) Relative expression of OCT4 and NANOG mRNA measured with real-time PCR (n=3). (C) Formation of tumors after subcutaneous implantation of undifferentiated hESC (positive control; n=6) and hESC differentiated 30 days at 142, 36, or 7 mmHg (n=6). This data was obtained through 55 days after implantation.

Figure 13. The fraction of residual Oct4-GFP+ cells from miPSC having undergone differentiation and extended culture at various pO_{2gas}. mESC were differentiated at 142, 36, or 7 mmHg pO_{2gas} then subjected to 0 or 20 days of extended culture at the same pO_{2gas}. The fraction of Oct4-GFP+ cells was acquired with flow cytometry (n=3).

Figure 14. The time required for tumor appearance after implantation of different numbers mESC and hESC. The number of implanted residual Oct4-GFP mESC, having undergone differentiation and extended culture under various conditions (Figures 1A, 4A, 5A), is plotted against the corresponding tumor formation data (Figures 3A, 4B-C, and 5B) (circles; n=68). 10^6, 10^4, and 10^2 undifferentiated hESC were implanted and the appearance of tumors observed (squares; n=6). The mouse (solid line) and human (dashed line) data fit the form $y = Ae^{Bx}$, where $A = 1.22 \times 10^4$ and 3.03×10^8 and B = -0.209 and -0.278, respectively.

Figures 15A and B. Schematic representation of a polystyrene (A) and silicone rubber membrane-based (B) culture dish.

DETAILED DESCRIPTION OF INVENTION

The invention provides, in part, methods for increasing the safety of cell populations intended for in vivo use. More specifically, the safety of such populations is increased by

reducing the number (and typically frequency) of tumor-causing cells in the population. These tumor-causing cells are pluripotent stem cells or their close or more immediate generational progeny. The population intended for use in vivo or in vitro, as provided herein, are more differentiated and committed progeny of such stem cells. It is well known that pluripotent stem cells, such as ES cells, are able to form tumors in vivo. These tumors may be teratomas or teratocarcinomas (Damjanov et al. Nat. Biotechnol. 25(11):1212 (2007); Lensch et al. Nat Biotechnol 25(11);1211 (2007)). These tumors typically comprised of all three germ layers (i.e., mesoderm, ectoderm and endoderm). For convenience and brevity, tumors generated by pluripotent stem cells are referred to primarily as teratomas however it is to be understood that such teachings equally apply to teratocarcinomas. As a result, the in vivo use of cell populations derived from the differentiation of stem cells is limited by the presence of residual stem cells. The invention provides methods for overcoming this limitation, as described below.

The invention therefore provides in part methods that involve exposure of a differentiated cell population to low oxygen partial pressure for extended periods of time. It has been found in accordance with the invention that subjecting a differentiated cell populations to low oxygen partial pressure for extended periods of time does not lead to cell death of the differentiated cells, and this approach is therefore a suitable manipulation of populations intended for in vivo use.

The invention further contemplates modification of protocols for differentiating pluripotent stem cells into one or more lineages by combining such protocols with low oxygen partial pressure exposure. Such protocols typically occur at normoxic conditions, as defined herein, and thus are not dependent on low oxygen partial pressure to differentiate pluripotent stem cells. Examples of such differentiation protocols are provided herein. In most instances, such differentiation protocols involve exposure of pluripotent stem cells to one or more differentiation stimuli. As used herein, a differentiation stimulus is a stimulus other than low oxygen partial pressure. Such differentiation stimuli may be chemical agents (e.g., retinoic acid, valproic acid, etc.), biological agents (e.g., growth factors, cytokines, interleukins, etc.), and the like. According to the invention, exposure to such stimuli may occur at the same time or at a different time as the exposure to low oxygen partial pressure. Thus, in one example, the differentiation protocol includes exposing pluripotent stem cells to one or more differentiation stimuli under a normoxic condition and then under a low oxygen

partial pressure condition. In another example, the differentiation protocol includes exposing pluripotent stem cells to a low oxygen partial pressure condition and then to a normoxic condition, all the while exposing the cells to one or more differentiation stimuli. The oxygen partial pressure may be cycled between normoxic and low oxygen partial pressure throughout the differentiation protocol in either example. In other examples, the differentiation protocol is performed under low oxygen partial pressure throughout. In some of these latter examples, the differentiation protocol does not differentiate pluripotent stem cells into the cardiomyocyte lineage. In other examples, the differentiation protocol may expose pluripotent stem cells (and their progeny) to a differentiation stimulus under normoxic conditions, followed by exposure to low oxygen partial pressure whether in the presence or absence of the differentiation stimulus. The invention contemplates a variety of such methods. Exposure to low oxygen partial pressure according to these various embodiments may occur for 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more contiguous or non-contiguous days, or it may occur for at least 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 75%, 80%, 90%, 95% or 100% of the culture time or the exposure time to the one or more differentiation stimuli.

Thus, in some aspects, the invention provides an improvement to the protocols used to differentiate pluripotent stem cells at normoxic conditions. The improvement comprises performing a part or all of the differentiation protocol at an oxygen partial pressure that is less than 142 mmHg. This improvement results in fewer residual pluripotent stem cells in the resultant differentiated cell population. The ability to overlay (or concurrently apply) differentiation stimuli and low oxygen partial pressure can preclude the need to perform two consecutive steps thereby saving time.

Although not intending to be bound by any particular theory or mechanism, one possible explanation for the effect of low oxygen partial pressure on residual stem cells is that low oxygen partial pressure causes loss of pluripotency by driving differentiation of any residual stem cells. Another possibility is that low oxygen partial pressure is selectively killing the residual stem cells while sparing the majority of differentiated cells in the population.

As will be understood in the art, upon cell division, pluripotent stem cells have the capacity to self-renew and/or differentiate. In its broadest sense, "differentiation" in this context implies that the daughter cell has lost its ability to self-renew and typically has started

to commit to one or more lineages (i.e., it has lost its pluripotent capacity). "Self-renewal" on the other hand implies that the daughter cell (like its stem cell parent) maintains the ability to self-renew and is pluripotent. Stem cell division may be symmetric or asymmetric. If symmetric, then the result is two daughter cells that are both stem cells or two daughter cells that are both committed. If asymmetric, then the result is one daughter cell that is a stem cell (i.e., it is pluripotent and can self-renew) and one daughter cell that committed. Differentiation (but not self-renewal) may also occur in the absence of cell division. Thus, the invention contemplates that any residual stem cells residing in a differentiated cell population may differentiate upon exposure to long-term reduced oxygen partial pressure whether in the presence or absence of proliferation.

Surprisingly, the low oxygen partial pressure exposure apparently has no deleterious effect on the viability of the majority of the differentiated cell population and no apparent deleterious effect on function. As shown in the Examples, a cardiomyocyte differentiated population derived from pluripotent stem cells demonstrate contractile function before and after exposure to low oxygen partial pressure. This is true regardless of the nature of the protocol used to generate the differentiated cells in the first instance. Thus while the Examples illustrate reduction in residual stem cell numbers in a population of cells that comprise cardiomyocytes and cardiomyocyte precursors derived from pluripotent stem cells in vitro using low oxygen conditions, the invention is not limited to these differentiated cell types or to cells differentiated using low oxygen.

The invention therefore provides methods for reducing the number and frequency of pluripotent stem cells in cell populations by exposing such populations to low oxygen partial pressure. As will be understood, the selective reduction in stem cell numbers observed in differentiated cell populations in accordance with the invention correlate with reduction in stem cell frequency since the remaining cells in the population are apparently spared. As a result, the invention may refer to reduced stem cell number or stem cell frequency, in various aspects or embodiments. These methods provided herein can be used to reduce the risk of tumor formation upon in vivo administration of the cell population. These methods will in turn allow for higher numbers of cells to be administered to subjects with reduced risk (or in some instances without risk) of tumor formation.

The invention also provides cell populations derived from pluripotent stem cells and having no pluripotent stem cells or reduced pluripotent stem cells (e.g., compared to a

differentiated cell population that is not exposed to a low oxygen partial pressure). The differentiated cells within this population will typically not be compromised, whether in number or function, by the exposure to low oxygen partial pressure. In other words, the differentiated cells will still be usable for one or more intended in vitro (e.g., drug screening or testing) or in vivo (e.g., tissue regeneration) purposes.

The invention further provides methods for using such "treated" cell populations in vivo (e.g., for preventative or therapeutic purposes), as well as uses of such cell populations in the making of medicaments for the prevention and/or treatment of certain disorders. The invention further contemplates methods for using the treated cell populations in vitro (e.g., in screening methods).

In still other aspects, the invention provides methods for monitoring reduction in stem cell number and/or function by subjecting a cell population to low oxygen partial pressure for an extended period of time by for example testing the tumorigenic content of the population by administering (typically by injection) all or part (i.e., an aliquot) of the population to a mouse such as but not limited to an immunocompromised mouse, and determining whether and when a tumor forms at the site of administration.

The invention provides alternative or additional methods for monitoring reduction in stem cell number and/or function after extended exposure to low oxygen partial pressure by monitoring the expression of early stem cell markers such as Oct4 (also known as POU5F1), Sox2, Nanog, SSEA-1 (for mouse), SSEA-4 (for human), Stat3, Hesx1, Zic3, Tra-1-60, Tra-1-80 and alkaline phosphatase. In some particular embodiments, Oct4, Nanog or Sox2, or some combination thereof are monitored (Jaenisch et al. Cell 132(4):567-82 (2008)). The target genes of Oct4, Sox2 and Nanog can also be monitored. Such target genes are described in Boheler J Cell Physiol 10.1002/jcp.21866 (2009) and are incorporated by reference herein. Such markers are indicative of the presence of pluripotent stem cells and thus may be used as surrogate markers for pluripotent stem cell content and/or tumorigenic activity in the population.

The invention further provides cell populations derived from the differentiation of pluripotent stem cells that have no or reduced numbers of residual pluripotent stem cells.

The invention contemplates performing the low oxygen partial pressure "tumor cell reduction step" on any differentiated cell population derived from and having (or suspected of having) residual pluripotent stem cells. In some instances, the nature of the differentiation

protocol used to generate the differentiated cell population is known. In other instances, it may not be known. The invention is not dependent on the nature of such protocol.

Based on the findings and teachings provided herein, those of ordinary skill in the art will understand that the extent of tumor-cell reduction (e.g., as controlled by the level of oxygen partial pressure and the time of exposure to low oxygen partial pressure) will depend on the nature of the differentiated cell population, the degree of tumor-cell reduction desired, and the ultimate use of the resultant population. The desired frequency may be 1 in 10^4, 1 in 10^5, 1 in 10^6, 1 in 10^7, 1 in 10^8, 1 in 10^9, or even lower. These frequencies will dictate in part the length of time of the low oxygen exposure.

Thus, the invention contemplates first generating a cell population from one or more pluripotent stem cells through the differentiation of such stem cells, and then exposing the generated cell population to low oxygen partial pressure for extended periods of time in order to reduce the number and/or pluripotent capacity of residual stem cells.

In some instances, the differentiated cell population is isolated from its differentiation culture conditions and then placed into low oxygen partial pressure conditions. In some instances, the differentiated cell population is not isolated from its differentiation culture conditions and rather the conditions are changed to low oxygen partial pressure conditions as taught herein. In some instances, differentiated cell populations treated in this latter manner may exclude those that are differentiated using only low oxygen partial pressure. If the differentiated cell population was generated using a protocol that involved low oxygen partial pressure exposure, then this population may be treated according to the invention in order to reduce residual pluripotent stem cells by exposure to low oxygen partial pressure for longer periods of time including for example 20, 25, 30, 35, 40, 45, 50 or more days. In some embodiments, populations differentiated using only low oxygen partial pressure may comprise cardiomyocyte and/or cardiomyocyte precursors. If the differentiated cell population was generated using a protocol that involved exposure to normoxic conditions as a last step, then this population may be treated according to the invention in order to reduce residual pluripotent stem cells.

Pluripotent Stem Cells

According to the invention, cell populations are exposed to low oxygen partial pressure in order to reduce the risk of tumor formation from pluripotent stem cells upon in

vivo administration. Pluripotent stem cells may be referred to herein as stem cells for brevity. Pluripotent stem cells, as discussed above, are cells that are both capable of self-renewal (i.e., generating, upon cell division, one or two cells with self-renewal and pluripotent capacity) and pluripotent (i.e., they have the ability to differentiate into mesoderm, ectoderm and endoderm lineages). It is to be understood that the invention contemplates and can be carried out using cell populations derived from pluripotent stem cells which in turn are derived from embryonic tissue (such as ES cells), pluripotent stem cells derived from the dedifferentiation of adult cells (such as induced pluripotent stem (iPS) cells), as well as other forms of pluripotent stem cells. ES cells are pluripotent stem cells derived from the inner cell mass of blastocysts and propagated in vitro. These cells have the capacity to differentiate into any cell type in the body. The invention contemplates other pluripotent stem cells including those resulting from somatic cell nuclear transfer (e.g., transfer of a nucleus from a somatic cell of a subject into an enucleated embryo), parthenogenesis, androgenesis or other asexual techniques. The invention contemplates the use of ES cells from these various sources although aspects, embodiments and exemplifications of the invention are discussed in the context of pluripotent stem cells that are ES cells, for the sake of brevity.

The invention contemplates the use of pluripotent stem cells from any species that may be treated using differentiated progeny of such stem cells. Such species include human, and various animal species including household species such as dogs and cats, agricultural species such as cows, pigs, and horses, laboratory species such as mice and rats, and the like.

The pluripotent stem cells may be genetically manipulated (e.g., they may be transfected) or they may not be genetically manipulated. Transfection refers to genetic manipulation of cells to introduce and typically express an exogenous nucleic acid. The exogenous nucleic acid may be a reporter such as green fluorescent protein (GFP) or it may be a selection marker such as thymidine kinase. It will be understood that in some instances reporters such as GFP may also serve as selection markers, particularly if their expression is controlled by pluripotent gene promoters such as an Oct4 promoter. The ES cells may be murine or human ES cells.

A number of ES cell lines currently exist. These include murine ES cell lines such as J1, R1, D3, CCE, SCC10, B6/Blu, EDJ22, and B6/GFP, and human ES cell lines such as BG01, BG02, BG03, SA01, SA02, ES01, ES02, ES03, ES04, ES05, ES06, H1, H9, TE03,

TE04, TE06, UC01, UC06, WA01, WA07, WA09, WA13 and WA14. Reference may be made to the NIH Human ES Cell Registry which lists various human ES cell lines and whether and from whom such lines are available.

In addition, protocols for generating ES cells and lines are known in the art. The generation of murine ES cells and lines has been described. See for example Teratocarcinomas and ES cells: a practical approach (1987). E.J. Robertson, editor. IRL Press. and Wernig et al. Nature. 2007 Jun 6 (online publication). U.S. Patent Nos. 5843780 and 6200806 assigned to WARF describe the generation of human ES cells.

iPS cells are stem cells generated by reprogramming somatic cells such as fibroblasts to an earlier developmental stage. This reprogramming may occur through the induced ectopic expression of gene combinations such as OCT4, SOX2, KLF4 and MYC, or OCT4, SOX2, NANOG and MYC, or OCT4, SOX2, NANOG and LIN28. Reprogramming may involve chemical stimuli such as valproic acid. (Huangfu et al., 2008, Nat. Biotechnol., 26(11):1269-1275.) Phenotypically, iPS cells are small round translucent cells that preferably grow in vitro in colonies that are themselves characterized as tightly packed and sharp-edged. Genetically, iPS cells express markers of pluripotency such as *OCT4* and *NANOG*, cell surface markers such as SSEA3, SSEA4, Tra-1-60, and Tra-1-80, and the intracellular enzyme alkaline phosphatase. These cells have a normal karyotype. Their cell cycle profile can be characterized by a short G1 phase, similar to that of hES cells. A number of iPS cell lines currently exist. These include iPS (Foreskin) (Clone 1), iPS(IMR90) (Clone 1), iPS(IMR90) (Clone 4), and the virus-vector free iPS – DF19-9 (Clone 7T), which can be purchased from WiCell Research Institute (Madison, WI). In addition, protocols for generating iPS cells and cell lines have been described. See for example Takahashi and Yamanaka, 2006, Cell 126(4):663-676; Wernig et al., 2007, Nature 448:7151; Okita et al., 2007 Nature 448:7151; Maherali et al., 2007 Cell Stem Cell 1:55-70; Lowry et al., 2008 PNAS 105:2883-2888; Park et al., 2008 Nature 451:141-146.; Takahashi et al., 2007 Cell 131, 861-872; Yu et al., 2007 Science 318:5858.

The invention refers to isolated pluripotent stem cells. As used herein, isolated pluripotent stem cells are cells which have been physically separated from their environment. If the cells are naturally occurring, then isolation implies that the cells are physically separated from the naturally occurring environment from which they derive. In some

instances, isolated stem cells are additionally or alternatively physically separated, in whole or in part, from an in vitro environment such as for example non-stem cells.

Thus, as used herein, the term isolated means that a molecule, cell, cell population and the like is physically separated from an environment in which it normally exists, or in which it originally or previously existed. Isolation may refer to physical separation of cells from a culture condition (e.g., a differentiation culture), from a naturally occurring environment or source, and the like. A differentiated cell population may be isolated from a differentiation culture condition, for example, by harvesting the cells and removing the culture medium (e.g., by centrifugation). Isolating may also involve washing the cells. Typically, the cells are resuspended in fresh medium. Isolation of the differentiated cell population from the differentiation culture therefore can serve to remove factors or stimuli used to differentiated the pluripotent stem cells towards one or more lineages.

The invention refers to tumor-forming cells which may be teratoma-forming cells or teratocarcinoma-forming cells. It is to be understood that these terms refer to cells that form (or have the capacity to form) tumors and in particular teratomas in vivo. Pluripotent stem cells are tumor-forming or teratoma-forming cells.

Differentiated Cell Population

The methods provided herein involve exposing a differentiated cell population to low oxygen partial pressure. As used herein, a differentiated cell population is a population of cells that is derived in vitro from pluripotent stem cells according to one or more differentiation protocols and that contains differentiated cells. Differentiated cells, as used herein, are cells that are not pluripotent stem cells, as described herein. Typically, a population of differentiated cells will have a phenotype or function associated with one or more cell lineages. For example, an example of a differentiated cell population is a population that comprises cells that are positive for a lineage specific marker. The marker may be associated with commitment to endoderm, mesoderm or ectoderm lineages, or it may be associated with trophoectoderm lineages, or it may be associated with commitment to specific lineages such as cardiomyocytes, islet cells, neuronal cells, and the like. An example of a cardiomyocyte specific marker is MF-20. Other markers for cardiomyocytes and other lineages are known in the art. The differentiated cells may comprise cells committed to one

or more lineages, including terminally differentiated cells and/or uni-, bi- and/or multilineage precursors.

Differentiated cells may comprise 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, or more of the cells in the differentiated cell population. The proportion of differentiated cells in the population may vary depending on the differentiation protocol. For example, the differentiated cells may represent 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more of the cells in the population. The remaining cells may be differentiated cells of unknown lineage, unidentified cells, and/or residual pluripotent stem cells. Pluripotent stem cells therefore must represent less than 100% of the cells in the differentiated cell population. (In other words, a cell population that is 100% pluripotent stem cells is not a differentiated cell population.) More typically, pluripotent stem cells represent 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10% or more (but less than 100%) of the cells in the differentiated cell population. In these latter instances, the invention seeks to reduce the frequency of pluripotent stem cells in the differentiated cell population to about 1 in 10^3 total cells (or 0.1%), or 1 in 10^4 total cells (or 0.01%), or 1 in 10^5 total cells (or 0.001%), or 1 in 10^6 total cells (or 0.0001%), or 1 in 10^7 total cells (or 0.00001%), or 1 in 10^8 total cells (or 0.000001%), or 1 in 10^9 total cells (or 0.0000001%), or less.

In some embodiments, pluripotent stem cells represent 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, or more (but less than 100%) of the differentiated cell population and the methods of the invention reduce the number (and accordingly frequency) of such pluripotent stem cells by 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold, 40-fold, 50-fold, 75-fold, 100-fold, 125-fold, 150-fold, or more.

It is to be understood that the methods of the invention may be applied to any differentiated cell population derived from pluripotent stem cells regardless of whether or not the presence of residual pluripotent stem cells has been confirmed in that population. That is, the invention does not require that the differentiated cell population be first tested for the presence of pluripotent stem cells prior to low oxygen exposure. Accordingly, the low oxygen methods provided herein may be applied to differentiated cell populations derived from pluripotent stem cells that are known to contain residual pluripotent stem cells or that may contain (including are likely to contain) pluripotent stem cells. As described in greater detail below, a person of ordinary skill in the art may assay the differentiated cell population

prior to, during and/or following low oxygen exposure in order to determine the extent of pluripotent stem cell reduction.

Low Oxygen Partial Pressure

The invention contemplates exposing cell populations comprising (or suspected of comprising or likely to comprise) pluripotent stem cells to low oxygen partial pressure. As used herein, low oxygen partial pressure refers to an oxygen partial pressure that is less than 142 mmHg if exposure such as culture occurs in the presence of CO_2 and a bicarbonate-based buffer, or less than 160 mmHg if exposure such as culture occurs in the absence of CO_2 and with another buffer such as HEPES. As used herein, low oxygen partial pressure (or low pO_2) refers to a pO_2 that is less than 142 mmHg for cultures in the presence of 5% CO_2 (inlet gas). This level of CO_2 represents typical culture conditions in the art. In some embodiments, low pO_2 may be a pO_2 that is less than 140 mmHg, less than 120 mmHg, less than 100 mmHg, less than 80 mmHg, less than 70 mmHg, less than 60 mmHg, less than 50 mmHg, less than 40 mmHg, less than 30 mmHg, less than 20 mmHg, less than 10 mmHg or lower. In some embodiments, low pO_2 may be zero mmHg. pO_2 in the methods of the invention may be in the range of 0-80 mmHg, 0-70 mmHg, 0-60 mmHg, 5-80 mmHg, 5-70 mmHg, or 5-60 mmHg. pO_2 in the methods of the invention may be in the range of 5-50 mmHg, 10-50 mmHg, 20-50 mmHg, and 20-40 mmHg, including every integer therebetween (i.e., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 and 50 mmHg). In some embodiments, the pO_2 is about 7 mmHg or about 36 mmHg. As used in the context of pO_2 measurements, the term "about" indicates a difference with the indicated value in the range of 0-15 mmHg.

Typically, the oxygen partial pressure referred to herein refers to the oxygen partial pressure sensed by cells in culture. The most common way of modulating oxygen levels in a culture is by modulating the oxygen partial pressure in the gas phase of a culture system. Such oxygen partial pressure is referred to herein as pO_{2gas}. pO_{2gas} can be regulated during culture using manual and automated devices. Examples of commercially available automated devices include but are not limited to OxyCycler C42 from BioSpherix (Redfield, NY), OWJ2720A from Queue Systems (Parkersburg, VA).

The oxygen partial pressure at the surface of a cell is referred to herein as pO_{2cell}. pO_{2cell} depends on several factors including medium depth, cell density, cellular oxygen consumption rate, diffusion characteristics of the medium, and pO_{2gas}. Thus, in some instances, pO_{2gas} is not indicative of or equivalent to the oxygen partial pressure experienced by cells in the liquid phase of the culture.

Cells grown in monolayers are more likely to be exposed to an oxygen partial pressure that approximates the gas phase oxygen partial pressure than are cells grown in a non-monolayer manner (e.g., in layers, spheres or aggregates). The difference between pO_{2cell} and pO_{2gas} for cells in a monolayer is typically due to diffusion gradients in the culture medium. Cells grown in a non-monolayer manner, particularly those buried within a sphere or aggregate, will have a pO_{2cell} that is less than the pO_{2gas} because of the internal oxygen gradients within the multiple layers of cells and/or aggregates.

Cells may be cultured under conditions in which pO_{2cell} approximates pO_{2gas} One way of accomplishing this is to enhance oxygen transport to cells in culture. For example, cells may be cultured under conditions where pO_{2cell} of a cell layer or at the surface of a 3-dimensional aggregate is approximately equal to pO_{2gas}. This can be accomplished in number of ways, as will be discussed in greater detail below.

Various modifications to the culture system may be performed in order to reduce the difference between pO_{2gas} and pO_{2cell}. Implementation of these methods therefore allow for reliance on pO_{2gas} as the readout for pO_{2cell}. For example, convective oxygen transport in mechanically mixed or perfused vessels may be used, including stirring of and/or bubbling of oxygen through a culture medium. The cultures may be subject to in situ generation of oxygen using electrochemical hydrolysis of water or other means. Alternatively or additionally, culture vessels having one or more sides, walls and/or bottom to which cells attach and grow that comprise an oxygen permeable membrane can also be used. It is to be understood that the invention contemplates various culture vessel configurations involving oxygen permeability. These include without limitation bags comprising oxygen permeable membranes. As used herein, an oxygen-permeable membrane is a membrane that has an oxygen permeability greater than that of a standard (e.g., polystyrene) culture dish. One example of an oxygen-permeable membrane is a fluoroethylene-propylene copolymer (FEP-Teflon) membrane. Culture vessels comprising this membrane are commercially available as Lumox dishes (Greiner Bio-One, Munich). Another example of an oxygen-permeable

membrane is a silicone rubber membrane, which is used in the Examples. The oxygen permeabilities of FEP Teflon and silicone rubber are 0.2–0.4 x 10^{-14} and 26 x 10^{-14} mol cm^{-1} $mmHg^{-1}$ sec^{-1}, respectively.

It is to be understood that although small oxygen gradients may exist across the diameter of a cell, generally such gradients will be small enough that the oxygen partial pressure at the cell surface and within the cell can be considered to be essentially the same.

Silicone rubber culture vessels, as used herein, are culture vessels that comprise a silicone rubber membrane bottom. In other words, the internal face of the vessel to which the ES cells attach is made of silicone rubber. The advantage of silicone rubber is its high permeability to gases such as oxygen. An example of a silicone rubber culture vessel is a silicone rubber dish, described in the Examples. Figure 15 provides an exemplary schematic of a culture dish having a silicone rubber membrane bottom. In still other embodiments, an insert of such oxygen permeable membranes is placed in a culture vessel. Inserts of silicone rubber membranes are available from for example Wilson Wolf. Oxygen control may also be accomplished by the use of a perfluorocarbon layer for growing cells, or by any other method known in the art.

A silicone rubber bottom culture vessel such as that illustrated in Figure 15 may be made according to the following protocol. Silicone rubber sheeting can be purchased from Specialty Manufacturing (Saginaw, MI). The sheet is optically clear (gloss finish) and is 0.005 inches (127 μm) thick. Prior to use the membrane material is cut to the desired shape with scissors and sterilized by autoclaving for 30 minutes at 121°C. The bottom surface of the 8 central wells of 24-well tissue culture plates (353047, Becton Dickinson) is removed using a 3/8 x 3 inch fixed handle nutdriver (12, Cooper Hand Tools, Apex, NC) heated in a Bunsen burner. A sterile scalpel is used to trim the edges of the holes so that they are flush with the rest of the plate bottom. A very thin layer of silicone adhesive (59530, Henkel Loctite Corp., Rocky Hill, CT) is spread on the base of the plate around each of the holes. A rectangular 8.5 x 4.5 cm piece of silicone rubber sheeting (non-reinforced vulcanized gloss/gloss 0.005 inch, Specialty Manufacturing, Saginaw, MI), previously sterilized by autoclaving, is placed over the holes, and manually pressed/stretched so that the silicone sheet is flat (no wrinkles) and sealed onto the plate bottom. After allowing the adhesive to set for 24 hr, the plates are completely filled with a 70% ethanol solution for 1 hr and dried overnight under a germicidal UV lamp in a biological safety cabinet.

Oxygen partial pressure is generally referred to herein in units of mmHg. However many oxygen control devices associated with culture incubators express oxygen levels as a percentage. Generally pO_{2gas} in mmHg can be determined based on knowledge of a percent oxygen measurement in the culture incubator using the following formula:

$$pO_2 = [\ (\%\ \text{oxygen}) / (100\ \%)\ ] \times (760\ \text{mmHg})$$

In this equation, 760 mmHg is the atmospheric pressure.

For a humidified environment at 37°C (which is generally the case with culture incubators), pO_{2gas} can be determined based on the composition of the oxygen at the incubator inlet measured as percent oxygen (usually specified by the content of a premixed compressed gas tank) using the following formula:

$$pO_2 = [\ (\%\ \text{oxygen}) / (100\ \%)\ ] \times (760 - 47.1\ \text{mmHg})$$

In this equation, 760 mmHg is the atmospheric pressure and 47.1 mmHg is the vapor pressure of water at 37 °C. Inlet oxygen levels of 1%, 5%, 20%, and 40% correspond to pO_{2gas} of 7.13 mmHg, 35.6 mmHg, 142 mmHg, and 285 mmHg in the humidified incubator. Inlet gas for a standard culture incubator is 19.9% oxygen (95% air and 5% CO_2), and pO_{2gas} is therefore 142 mmHg.

In many cases, during cell culture in bioreactors, the % oxygen is actually given as % of air saturation. The equation to convert between the two is

$$\%\ \text{absolute} = \%\ \text{air saturation} \times 0.209$$

In this equation, 0.209 is the volume fraction or mole fraction of oxygen under atmospheric conditions.

Culture Conditions and Times

Differentiated cell populations comprising (or suspected of comprising or likely to comprise) pluripotent stem cells are exposed to low oxygen partial pressure. This typically occurs while such populations are being cultured in vitro. Such cultures are usually performed at about 37°C, in incubators, as described herein. As described herein, the differentiated cell population is derived from the differentiation of pluripotent stem cells. Thus, it is to be understood that the low oxygen culture of the present invention is applied to a population of cells that already comprises a proportion of differentiated cells rather than a starting population of pluripotent stem cells. The methods of the invention may involve transferring cells to a new culture vessel although this is not essential (e.g., in some instances

the differentiation step and the "pluripotent stem cell reduction" step may be carried out in the same vessel, and in other instances the two steps may be carried out in different vessels). Separate vessels and optionally wash of cells may be involved if the culture conditions are different between the two steps.

The exposure time (e.g., the culture period) may be 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 15 days, 20 days, 25 days, 30 days, 35 days, 40 days or more.

In other instances, the invention contemplates isolating a cell population that has been previously cultured in low oxygen partial pressure in order to form a differentiated cell population from its low oxygen partial pressure "differentiation" culture environment and then exposing it to low oxygen partial pressure for the purpose of reducing residual stem cell number. In still other instances, the invention contemplates culturing pluripotent stem cells (and their progeny) under low oxygen partial pressure followed by a period of normoxic oxygen partial pressure in order to differentiate the stem cells towards one or more lineages, and then culturing the resultant population under low oxygen partial pressure again in order to reduce the number of residual pluripotent stem cells in the population. In this latter instance, the cells may be isolated from the differentiation culture or they may be continuously maintained in the same culture environment with only the oxygen partial pressure changing. In these instances, the period of low oxygen partial pressure required to reduce pluripotent stem cell numbers may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, or more days. As discussed herein, the length of culture will depend in part on the desired stem cell frequency ultimately desired by the end user of the differentiated cell population. As an example, some end users contemplating in vitro culture and/or testing of lower numbers of the differentiated population (e.g., 10^3 or 10^4 cells) may not need to culture as long as some end users contemplating in vivo administration of higher numbers of the population (e.g., 10^8 or 10^9 cells). As another example, some in vitro applications may require longer culture periods at low oxygen partial pressure than some in vivo applications. The invention contemplates these various possibilities.

In still other instances, pluripotent stem cells are differentiated under low oxygen partial pressure and then cultured under low oxygen partial pressure in order to reduce pluripotent stem cell number and/or function. In these instances, the cultures may be continuous and may be a total of 20, 25, 30, 35, 40, 45, 50, 55, 60 or more days. By

continuous, it is meant that the cells are not isolated from the differentiation culture conditions and then recultured.

The culture may occur in culture vessels having oxygen-permeable bottom membranes (e.g., oxygen-permeable silicone rubber membrane). These vessels may be constructed so as to replace their bottoms with these membranes as shown in Figure 15. These membranes are preferably coated with substrates (e.g., proteins) that promote cell adhesion such as but not limited to gelatin, fibronectin, laminin, or Matrigel (i.e., a gelatinous protein mixture secreted by mouse tumor cells, commercially available from BD Biosciences). Combinations of these substrates may also be employed. The Examples demonstrate the use of culture vessels having silicone rubber bottom membranes. Importantly, the use of oxygen-permeable membranes such as those of the Examples eliminates the need for stirred or perfused culture systems such as those used in the prior art. (Bauwens et al., Biotechnol Bioengin, 90(4):453-461, 2005).

The culture conditions for the low oxygen partial pressure exposure (i.e., in order to reduce pluripotent stem cell number and/or function) may vary depending on the nature of the differentiated cell population. In some instances, the culture may be supplemented with growth factors such as cytokines, interleukins or small molecules. In other instances, the culture does not comprise any exogenous growth factors. In some embodiments, the culture conditions used for the low oxygen partial pressure exposure are similar or identical to those used to culture primary cells of the same lineage(s). For example, the culture conditions for a differentiated cell population that comprises neural cells may be similar or identical to that used to culture (e.g., maintain) freshly explanted neural cells from in vivo sources. In some embodiments, the culture conditions used for low oxygen partial pressure exposure are similar or identical to the differentiation culture conditions used to generate the differentiated cell population in the first instance. In some embodiments relating to differentiated cell populations that comprise cardiomyocytes, the differentiated cell population may be cultured in insulin, transferring and selenium containing medium (ITS). In some instances, such medium is serum-free. In some instances, such medium comprises serum.

The differentiated cell population is typically harvested following low oxygen exposure (for the purpose of reducing pluripotent stem cell number). Thus, in some instances, this population is referred to as being "harvested" or as being the "harvested differentiated cell population". Once harvested, the population may be further manipulated

or it may used in vitro or in vivo as described herein. Similarly, even if the population is not harvested and/or further manipulated, it may be referred to as the "cultured differentiated cell population" in order to distinguish it from its starting differentiated cell population (i.e., the differentiated cell population prior to low oxygen exposure for the purpose of reducing pluripotent stem cell numbers).

Assay for Pluripotent Stem Cells

The number of pluripotent stem cells in the cell population may be monitored prior to, during and/or after low oxygen exposure. Pluripotent stem cells may be monitored by in vivo (preferably non-human) assays and/or in vitro assays. In vivo assays may involve administration of a cell population into a non-human subject in order to determine if teratoma-causing cells are present. A well-known assay is the injection of cells into an immunocompromised animal, such as an immunocompromised mouse. Immunocompromised mice such as SCID mice are a suitable recipient in these assays, although it is to be understood that other mouse immunocompromised or wild type mouse strains may also be used. In other instances, immunocompromised or wild type preferably non-human animals other than mice can be used as recipients. In this assay, the site of injection is observed over a period of time for the appearance of a tumor. The time it takes for tumors to develop and be observable is dependent on the number of administered pluripotent stem cells and the site of injection. For example, a population having more pluripotent stem cells will form an observable tumor faster than will a population having few pluripotent stem cells. As shown in the Examples, populations exposed to low oxygen conditions exhibited delayed visual appearance of tumors at the site of injection as compared to untreated controls, indicating that the number of pluripotent stem cells in the treated population was reduced compared to the untreated control.

An exemplary protocol for assaying pluripotent stem cells by teratoma formation is described by Keller et al. Genes Dev 19:1129-1155, 2005; Spagnoli et al. Curr Opin Genet Different 16:469-475, 2006. Briefly, a cell population is injected subcutaneously or intramuscularly into preferably an immunocompromised mouse. The administration route is preferably one that maintains the administered cells locally in order to ensure easy access, observation and harvest of any resulting tumor mass. The number of cells to be administered will depend upon the population being tested and the expected frequency of pluripotent stem

cells in the population. If one aim of the assay is to determine the number or frequency of the stem cells in the population, a limiting dilution assay may be carried out in which a range of cell numbers are injected. Another measure of stem cell number may be obtained by performing a time course of tumor development for a number of populations having known stem cell number, thereby generating a standard curve, and then comparing the readout from the test population (or various aliquots of the test population) to the standard curve. A biopsy of any resulting tumor may also be performed to confirm the pluripotent nature of the teratoma-causing cells. The presence of endodermal, mesodermal and ectodermal lineages can be determined via immunohistochemical staining, microscopy (e.g., transmission electron microscopy, TEM), Northern blot analysis, reverse transcriptase polymerase chain reaction (RT-PCR), real-time PCR, RNA fluorescence in situ hybridization (FISH), or rapid amplification of cDNA ends (RACE) techniques. Further reference can be made to Human Embryonic Stem Cells: The Practical Handbook, Eds. Sullivan, Cowan and Eggan, Harvard University, John Wiley & Sons (Publishers), 2007.

Additionally or alternatively, the presence of pluripotent stem cells may be determined in vitro by detecting mRNA and/or protein expression of stem cell markers. Stem cell markers are genes that are preferentially (and in some instances exclusively) expressed by pluripotent stem cells. Typically, the markers are chosen such that they are not expressed by non-stem cells in the differentiated cell population. Thus, the presence and number of pluripotent stem cells may be determined by detecting or measuring mRNA or protein expression levels of these markers. These markers include Oct4 (also known as POU5F1), Sox2, Nanog, SSEA-1 (for mouse), SSEA-4 (for human), Stat3, Hesx1, Zic3, Tra-1-60, Tra-1-80, alkaline phosphatase, Klf4, Lin28, and hTERT. In some important embodiments, the marker is Oct4.

mRNA levels can be monitored using Northern blot analysis, reverse transcriptase polymerase chain reaction (RT-PCR), real-time PCR, RNA fluorescence in situ hybridization (FISH), or rapid amplification of cDNA ends (RACE) techniques. In some instances, real time and reverse transcriptase PCR are combined. PCR primers and/or probes specific for Oct4 and other stem cell markers are known in the art and reference may be made to Powers et al. Biotechnol Bioeng 101(2):241-54 (2008) for exemplary sequences. Protein levels may be monitored using Western analysis, enzyme assay, immunocytochemistry, flow cytometry and other techniques. Antibodies specific for pluripotency proteins such as Oct4 are known

in the art and commercially available from sources such as R&D Systems (Minneapolis, MN).

As discussed herein, the invention contemplates reducing the pluripotent stem cell content of a cell population using the low oxygen methods provided herein by 2-fold, more preferably by 5-fold, more preferably by 10-fold, and even more preferably by 20-fold. In still other instances, the pluripotent stem cell content of the cell population may be reduced by 50-fold, 75-fold, 100-fold, 125-fold, or 150-fold, or more. As an example, a 10-fold decrease in stem cell numbers may be indicated by an Oct4 mRNA expression level that is 10-fold lower than the expression level prior to low oxygen exposure. Fold decreases can be measured in vivo by comparing the time course of a test population with that of control populations having known numbers of pluripotent stem cells, as described herein.

Thus, the invention contemplates monitoring stem cell function in a differentiated cell population during low oxygen exposure. As an example, the differentiated cell population is exposed to low oxygen culture for a period of time and aliquots are taken from the population at various times and tested for teratoma activity and/or mRNA or protein expression. Such a time course assay may reveal the duration of low oxygen exposure required to eliminate pluripotent stem cells. In this way, the differentiated cell population need not be cultured at low oxygen unnecessarily beyond the point at which no pluripotent stem cells exist in the population. It is expected that any cell population intended for in vivo use in humans will be tested in this manner prior to actual administration into a subject.

Differentiation Protocols

It is to be understood that the invention contemplates reducing pluripotent stem cell numbers in any differentiated population regardless of the type of differentiated cell lineage and/or the manner of differentiating. Mesodermal cells and progenitors (or tissues) include bone, muscle such as cardiac muscle, skeletal muscle and smooth muscle (e.g., of the gut), connective tissue such as the dermis and cartilage, kidneys, the urinoogenital system, blood (or hematopoietic), heart and vasculature. Endodermal cells and progenitors (or tissues) include epithelial cells such as those lining the digestive tube, liver, pancreas including the beta cells in islets of Langerhans, trachea, bronchi, alveoli, urinary bladder, urethra, thyroid and thymus. Ectodermal cells and progenitors (or tissues) include nerve cells (and nervous

tissues) such as astrocytes, neurons including spinal motor neurons, glia, skin, hair, and epidermis.

Various methods for differentiating pluripotent stem cells are known in the art. These include differentiation protocols described in U.S. Patent Nos. 7,326,572 (endoderm differentiation), 7,282,366 (hepatocyte differentiation), 7,250,294 (neural differentiation), 7,033,831 (islet cell differentiation), and in published PCT application WO2008/156708.

Differentiation of ES cells may be achieved via induction of embryoid body (EB) formation. As an example, differentiation towards the cardiomyocyte lineage can be achieved using EB formation. Briefly, this method involves dispersing ES cells into a single cell suspension of ES cell medium lacking LIF, followed by culture in hanging drops for 2 days. EB generation via the hanging drop method may employ DMEM with 10% FBS either in the presence or absence of ascorbic acid. The hanging drop method is preferred to the extent that it provides better control and uniformity in size and shape. After 2 days of culture, the EBs are transferred into a culture vessel (e.g., a flask, a plate, or a well (e.g., from a multi-well dish)). Preferably, the culture vessel is coated with a substrate that promotes cell adhesion such as but not limited to gelatin, fibronectin, or laminin. In some embodiments, combinations of these substrates may be used. For example, in some instances it may be useful to coat the vessel with fibronectin followed by gelatin. The EBs are allowed to differentiate for 2, 3, 4, or more days with daily medium changes, followed by a change to a serum-free medium supplemented with insulin, transferrin and selenium (ITS; see Methods and Materials in the Examples section below) with or without 0.2 mM ascorbic acid. The cells may be cultured for an additional 5, 6, 7 or more days in these latter conditions. Differentiation of human ES cells can be achieved using the same hanging drop method procedure and the same medium as used for maintenance of undifferentiated ES cells except that bFGF is omitted.

Although many aspects and embodiments of the invention are described in the context of cardiomyocytes, it is to be understood that the invention is not so limited and that other mesodermal, endodermal and ectodermal cell types, including progenitors and more differentiated cells such as terminally differentiated end stage cells, may be similarly be treated to low oxygen conditions in order to reduce pluripotent stem cell number.

A variety of differentiation factors that can act on pluripotent stem cells and their precursor progeny are known in the art. For example, members of the BMP family of factors

have been used to differentiate stem cells such as ES cells. These include the use of BMP-4 and BMP-7 to generate endoderm-like differentiation. (Xu et al. Nat Biotechnol 20:1261-1264, 2002; Pera et al. J Cell Sci 117:1269-1280, 2004.) Activin A can be used to differentiate pluripotent stem cells such as ES cells into definitive endoderm using monolayers or three dimensional (e.g., EB) culture systems. (D'Amour et al. Nat Biotechnol 23:1534-1541, 2005.)

Nervous system cells have been observed as a result of culture with epidermal growth factor and fibroblast growth factor (resulting in the generation of neurospheres that comprise neural stem cells), subsequent removal of these factors (resulting in the generation of astrocyte-like cells) or supplementation with nerve growth factor (resulting in the generation of neurons and glial cells). (Kim et al. Nature 418:50-6, 2002; Lee et al. Nat Biotechnol 18:675-9, 2000.) Dopaminergic neurons, useful in Parkinson's disease, may be formed through culture or contact with FGF20 and FGF2. Bjorklund et al. (PNAS 2002, 99:2344-2349) provides additional methods for differentiating ES cells into dopaminergic neurons.

Hepatic cell differentiation may be induced through contact and/or culture with an insulin, dexamethasone, and collagen type I (via EB formation) combination; a sodium butyrate and DMSO combination; an FGF4, HGF and collagen type I combination; an aFGF, HGF, oncostatin M, dexamethasone and collagen type I combination; and a bFGF, variant HGF, DMSO and dexamethasone combination in the presence of poly-amino-urethane coated non-woven polytetrafluoroethylene fabric. (Shirahashi et al. Cell Transplant 13:197-211, 2004; Rambhatla et al. Cell Transplant 12:1-11, 2003; Schwartz et al. Stem Cells Dev 14:643-655, 2005; Baharvand et al. Int J Dev Biol 50:645-652, 2006; Soto-Gutierrez et al. Cell Transplant 15:335-341, 2006.) Hepatic differentiation may also occur spontaneously. (Lavon et al. Differentiation 72:230-238, 2004.)

Pancreatic differentiation, including differentiation towards beta-islet cells, can be induced using Activin A, retinoic acid, FGF2 and FGF10, betacellulin, HGF, Exendin 4, DKK1 and DKK3. (Gu et al. Mech Dev 120:35-43, 2003; Grapin-Botton et al. Trends Genet 16:124-130, 2000; D'Amour et al. Nat Biotechnol 23:1534-1541, 2005a; D'Amour et al. published US application US2005-0266554A1.)

Endothelial differentiation may be induced in the presence of ECM proteins such as collagen type IV, optionally in the presence of VEGF and bFGF. (Gerecht-Nir et al. Lab Invest 83:1811-1820, 2003.)

Further reference may be made to published PCT application WO2009/007852 for a review of various differentiative procedures known in the art and applicable to the differentiation of the immature and precursor cells of the invention. Such teachings, and in particular those found on pages 57-61 (under the subheading "Cell Differentiation") of WO2009/007852, are incorporated by reference herein. Still other references include West and Daley, 2004, Curr Opin Cell Biol 16:688-692; US Pat. No. 6,534,052 Bl; Kehat and Gepstein, 2003, 8:229-236; Nir et al., 2003, 58:313-323; and U.S. Patent Nos. 6,613,568 and 6,833,269.

Cardiomyocytes, Cardiomyocyte Progenitor Cells, and Other Differentiated Cells

In various aspects of the invention, the differentiated cell population comprises cardiomyocytes and/or cardiomyocyte progenitor cells. As used herein, cardiomyocyte progenitors are cells that have committed to the cardiomyocyte lineage but which still exhibit substantial proliferative and optionally differentiative capacity. One population of cardiomyocyte progenitors has an isl1+/Nkx2.5/flk-1+ phenotype. This population can be counted or extracted using flow cytometry and sorting. Another population of cardiomyocyte progenitors has a brachyury+/flk-1+ phenotype.

Flow cytometry can be used to detect and count cardiomyocytes and their progenitors using one or more cardiac lineage markers. The cardiomyocyte lineage marker preferably is specific to the cardiomyocyte lineage (i.e., expressed only in cells committed to the cardiomyocyte lineage). Non-specific markers may also be used in some instances. Typical cardiomyocyte lineage markers include but are not limited to brachyury, nkx2.5, cardiac troponin I, or α-myosin heavy chain (α-MHC), gata-4, atrial natriuretic protein (ANP), myosin light chain (MLC)-2v, β-myosin heavy chain (β-MHC), and connexin 43. MF-20, an anti-sarcomeric myosin heavy chain antibody (commercially available from DBHB, Iowa City, IA) can also be used to identify cardiomyocyte committed cells. It is to be understood that these methods can be readily applied to any other marker, provided a probe for such marker is available.

Cardiomyocyte and/or cardiomyocyte progenitor presence in the cultures of the invention may also be detected according to mRNA expression profiles. For example, the presence of these cells may be determined by the presence of brachyury, nkx2.5, cardiac troponin I, or α-myosin heavy chain (α-MHC), gata-4, atrial natriuretic protein (ANP),

myosin light chain (MLC)-2v, β-myosin heavy chain (β-MHC), and connexin 43 mRNA transcripts within cultured cell populations. Methods for detecting mRNA transcripts from differentiated cell populations (including cultured populations) are known in the art.

Endoderm progenitors that contribute to pancreatic development may be characterized by their gene expression profiles also. Stage 1 (definitive endoderm (DE) progenitor cells) express SOX17, CER, FOXA2, and CXCR4 and cells transitioning from ES cells to DE cells express BRA, FDF4, WNT3 and NCAD. Stage 2 endoderm progenitors express HNF1B and HNF4A. Stage 3 endoderm progenitors express PDX1, HNF6 and HLXB9. Stage 4 endoderm progenitors express NKX6-1, NGN3, PAX4 and NKX2-2. And finally stage 5 endoderm progenitors express INS, CGC, GHRL, SST and PPY.

Negative and/or Positive Selection Steps

The invention further contemplates that negative and/or positive selection steps may be used in addition to low oxygen exposure to reduce the number of pluripotent stem cells in a differentiated cell population. The selection steps, whether negative or positive, may be performed prior to and/or after low oxygen exposure. Selection methods may be based on, for example, cell surface phenotype, cell size and/or granularity, cell cycle status, reporter gene expression (e.g., a detectable marker, such as GFP, tagged to a protein under the control of mesoderm or endoderm–associated transcription factor(s)), and the like. These selection methods may be applied to cells harvested at any stage of culture. These methods are known in the art and the invention is not to be limited in this regard.

As used herein, a negative selection step is one that selects for, and preferably removes, pluripotent stem cells in order to reduce their numbers. Conversely, a positive selection step is one that selects for, and preferably retains, the differentiated cells of interest.

Negative and positive selection may be performed based on any criteria known to distinguish pluripotent stem cells from differentiated cells. In some instances, the selection is performed based on cell surface markers that are preferentially (or exclusively) expressed by one cell type but not the other. These markers can therefore be those expressed by pluripotent stem cells or they may be those expressed by differentiated cells. It will be understood that the markers expressed by differentiated cells will vary based on the nature of the differentiated cells (e.g., cardiomyocyte markers will differ from hepatic cell markers, etc.), and in some instances on the developmental stage of the differentiated cells. For

example, markers for pluripotent stem cells include SSEA-4, TRA-60, CD9, and Oct4. A marker for cardiomyocytes is ALCAM (CD166). Selection, and thus separation, based on cell surface markers can be effected using antibodies to the cell surface markers of choice combined with fluorescence activated cell sorting (FACS), panning, magnetic bead separation, and the like. Selection, and thus separation, based on transcription factor expression can be effected using temporally controlled suicide genes or reporter genes, the products of which can be observed and used as a physical sorting parameter (e.g., GFP expression).

Physical parameters that can be used to separate pluripotent stem cells from differentiated cells include buoyant density (e.g., using a Ficoll or Percoll gradient), size, granularity, and the like.

In Vivo Uses

The differentiated cell populations of the invention may be used in a variety of in vivo methods including but not limited to therapeutic or cosmetic applications. The differentiated cell populations can be used in transplant settings in the treatment or prevention of various conditions including but not limited to Parkinson's disease (dopaminergic neurons), Alzheimer's disease (neural precursors), Huntington's disease (GABAergic neurons), blood disorders such as leukemia, lymphoma myeloma and anemia (hematopoietic cells), side-effects of radiation e.g., in transplant patients (hematopoietic precursors), cardiovascular disease, myocardial infarction, ischemic cardiac tissue or heart-failure (partially- or fully-differentiated cardiomyocytes), muscular dystrophy (skeletal muscle cells), liver cirrhosis or failure (hepatocytes), chronic hepatitis (hepatocytes), diabetes including type I diabetes (insulin-producing cells such as islet cells), ischemic brain damage (neurons), spinal cord injury (glial progenitor cells and motor neurons), amyotrophic lateral sclerosis (ALS) (motor neurons), orthopedic tissue injury (osteoblasts), kidney disease (kidney cells), corneal scarring (corneal stem cells), cartilage damage (chondrocytes), bone damage (osteogenic cells including osteocytes), osteoarthritis (chondrocytes), myelination disorders such as Pelizaeus-Merzbacher disease, multiple sclerosis, adenoleukodystrophies, neuritis and neuropathies (oligodendrocytes), and hair loss.

The differentiated cell populations may be provided as pharmaceutical compositions that are sterile and appropriate for in vivo use, optionally together with a pharmaceutically

acceptable carrier. As used herein, a pharmaceutically-acceptable carrier means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. Pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers and other materials which are well-known in the art. Such preparations may routinely contain salt, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. The differentiated cell population may be formulated for local or systemic administration including as part of an implant.

The cells may be used alone or together with another agent, whether active or inactive, including but not limited to a scaffold, a matrix, and the like. These cells may further be included in a kit that additionally comprises at a minimum instructions for use of the cells, and optionally comprises one or more other agents whether active or inactive. The cells may be provided as a frozen aliquot of cells, a culture of cells, or a liquid suspension of cells.

The differentiated cells may be administered in numbers effective to produce a desired result, including but not limited to a short-term or long-term therapeutic result. Such result may include an improvement in or complete eradication of symptoms associated with a particular condition. The cell numbers to be administered will depend on a number of factors including the weight and age of the subject, the type of condition being treated, the desired effect (e.g., short-term or long-term), and the like. Some treatments therefore may require as few as 10^3 cells, while others may require 10^4, 10^5, 10^6, 10^7, 10^8, 10^9 or more cells.

In Vitro Uses

The differentiated cell populations produced according to the invention may also be studied for gene expression profiles and responses to various external stimuli in order to understand differentiation more fully and independently of effects at the pluripotent stem cell level or stage since the population would be essentially devoid of such cells.

The invention further provides methods for screening and/or identifying agents (or compounds, as the terms are used interchangeably herein) being used or to be used clinically. These assays may measure the therapeutic efficacy and/or toxicity of the candidate agent, among other things. The readouts from such in vitro assays are correlative of the in vivo toxicity or efficacy such agents would exhibit in subjects. Thus, the effect of the agent on the differentiated cells generated according to the invention in vitro is a form of surrogate marker

or readout for how the agent will function in vivo in a subject. The agents to be tested include those used clinically as well as experimental agents. In some more common embodiments, such testing will focus on the toxicity of agents including drugs in particular differentiated progeny. Accordingly, in these assays, the readout would be cell death (or conversely cell viability). These in vitro assays may employ suspensions of differentiated cells, adherent populations of differentiated cells, or three dimensional structures comprised of differentiated cells (e.g., in vitro organ tissues, matrices and architectures).

Drugs that can be tested according to these methods particularly for whether they are toxic to cells include but are not limited to adrenergic agent; adrenocortical steroid; adrenocortical suppressant; aldosterone antagonist; anabolic; analeptic; analgesic; androgen; anesthesia, adjunct to; anesthetic; anorectic; anterior pituitary suppressant; anti-acne agent; anti-adrenergic; anti-allergic; anti-androgen; antianemic; anti-anginal; anti-arthritic; anti-asthmatic; anti-atherosclerotic; anticholelithic; anticholelithogenic; anticholinergic; anticoagulant; anticoccidal; anticonvulsant; antidepressant; antidiabetic; antidiarrheal; antidiuretic; anti-emetic; anti-epileptic; anti- estrogen; antifibrinolytic; antiglaucoma agent; antihemophilic; antihemorrhagic; antihistamine; antihyperlipidemia; antihyperlipoproteinemic; antihypertensive; antihypotensive; anti-inflammatory; antikeratinizing agent; antimigraine; antimitotic; antimycotic, antinauseant, antineoplastic, antineutropenic, antiparkinsonian; antiperistaltic, antipneumocystic; antiproliferative; antiprostatic hypertrophy; antipruritic; antipsychotic; antirheumatic; antiseborrheic; antisecretory; antispasmodic; antithrombotic; antitussive; anti-ulcerative; anti-urolithic; benign prostatic hyperplasia therapy agent; blood glucose regulator; bone resorption inhibitor; bronchodilator; carbonic anhydrase inhibitor; cardiac depressant; cardioprotectant; cardiotonic; cardiovascular agent; choleretic; cholinergic; cholinergic agonist; cholinesterase deactivator; coccidiostat; cognition adjuvant; cognition enhancer; depressant; diagnostic aid; diuretic; dopaminergic agent; ectoparasiticide; emetic; enzyme inhibitor; estrogen; fibrinolytic; free oxygen radical scavenger; gastrointestinal motility effector; glucocorticoid; gonad-stimulating principle; hair growth stimulant; hemostatic; histamine H2 receptor antagonists; hormone; hypocholesterolemic; hypoglycemic; hypolipidemic; hypotensive; immunomodulator; immunoregulator; immunostimulant; immunosuppressant; impotence therapy adjunct; keratolytic; LHRH agonist; liver disorder treatment; luteolysin; mental performance enhancer; mood regulator; mucolytic; mucosal protective agent; mydriatic; nasal

decongestant; neuromuscular blocking agent; neuroprotective; NMDA antagonist; non-hormonal sterol derivative; oxytocic; plasminogen activator; platelet activating factor antagonist; platelet aggregation inhibitor; post-stroke and post-head trauma treatment; progestin; prostaglandin; prostate growth inhibitor; prothyrotropin; psychotropic; pulmonary surface; relaxant; repartitioning agent; scabicide; sclerosing agent; sedative; sedative-hypnotic; selective adenosine Al antagonist; serotonin antagonist; serotonin inhibitor; serotonin receptor antagonist; steroid; symptomatic multiple sclerosis; thyroid hormone; thyroid inhibitor; thyromimetic; tranquilizer; amyotrophic lateral sclerosis agent; cerebral ischemia agent; Paget' s disease agent; unstable angina agent; uricosuric; vasoconstrictor; vasodilator; wound healing agent; xanthine oxidase inhibitor. Those of ordinary skill in the art will know or be able to identify agents that fall within any of these categories, particularly with reference to the Physician's Desk Reference.

The following Examples are provided to illustrate specific instances of the practice of the present invention and are not intended to limit the scope of the invention. As will be apparent to one of ordinary skill in the art, the present invention will find application in a variety of compositions and methods.

EXAMPLES

Methods and Materials

Undifferentiated mouse embryonic stem cell (mESC) and mouse induced pluripotent stem cell (miPSC) culture. Undifferentiated R1 mESC and miPSC, both with an inserted GFP coding sequence driven by an inserted Oct4 promoter (Oct4-GFP mESC) [1] (generously donated by Professor Douglas Melton of Harvard University, Cambridge, MA, USA), and R1 mESC with a homozygous HIF-1α gene knockout (HIF-1$\alpha^{-/-}$ mESC) [2] (generously donated by Professor Peter Carmeliet of Katholieke Universiteit Leuven, Leuven, Belgium) were propagated as described previously [3] in Dulbecco's modified eagles medium (DMEM; SCRR-2010; American Type Culture Collection (ATCC), Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS; SCRR 30-2020; ATCC), 1x L-alanyl-L-glutamine (SCRR 20-2115; ATCC), 1x penicillin streptomycin (P/S; 15070; Invitrogen,

Carlsbad, CA, USA), 1x MEM non-essential amino acids (SCRR 20-2116; ATCC), 100 μM 2-mercaptoethanol (M7522; Sigma-Aldrich; St. Louis, MO, USA), and 10^3 units/mL leukemia inhibitory factor (LIF2005; Millipore, Billerica, MA, USA). Briefly, cells were grown in 25 cm^2 cell culture flasks (Becton Dickinson) using 4 mL of medium (Table 1) exchanged daily. Cells were detached with 0.25% trypsin every two days and placed in a new culture flask at a density of 1.2 x 10^4 cell/cm^2.

Gas phase pO_2 control. Cell culture vessels were placed inside polystyrene chambers (MIC-101, Billups-Rothenburg, Del Mar, CA) contained within a standard incubator (OWJ2720A, Queue Systems, Parkersburg, WV) maintained at 37 °C. An open dish of deionized water in each chamber provided humidification. pO_{2gas} was maintained by flowing premixed gas containing 5% CO_2 and 20%, 5%, or 1% O_2 with a balance of N_2 (certified medical gas from Airgas, Hingham, MA, USA), which corresponded, under humidified conditions, to 142, 36, and 7 mmHg pO_{2gas} inside chambers maintained at 37 °C, as described previously [3]. The flow rate of this gas to the chambers was 2 L/min for 15 min for an initial purge following closure of the chamber (after cell medium exchange or passage); and was 30 mL/min at all other times.

Differentiation of mESC and miPSC. mESC and miPSC were differentiated as previously described [3]. In brief, embryoid bodies (EBs) were formed with 500 cells per 20-μL in propagation medium without LIF in hanging drops. Detached undifferentiated mES cells were centrifuged, supernatant medium removed, resuspended in medium without LIF (Table 1), and diluted to 25,000 membrane-intact cells/ml. Single 20-μl drops of this cell suspension were placed on the inside surface of the lids of 10 x 10 cm Petri dishes (Becton Dickinson) using an 8-channel pipette. The lids were inverted and placed onto dish bottoms filled with 15 ml of pre-warmed (37 °C) solution containing 75% DPBS, 25% water, and 0.002% (w/v) type A gelatin. After 2 days, 30 EBs were transferred per well of fibronectin-coated (F1141; Sigma) 24-well silicone rubber plates filled with 2 mL of propagation medium without LIF. After 3 additional days, medium was replaced with 2.5 mL of 50% DMEM (90-133-PB; Mediatech, Manassas, VA, USA) and 50% Ham's F-12 (10-080-CV; Mediatech) supplemented with 0.75% (w/v) sodium bicarbonate (25-035-CI; Mediatech), 9 mM glucose (G8769; Sigma-Aldrich), 5 μg/mL human insulin (I9278; Sigma-Aldrich), 50 μg/mL holo

transferring (T1283; Sigma-Aldrich), 31.2 nM sodium selenite (S9133; Sigma-Aldrich), and 1x P/S (see Table 2). Cells were differentiated for a total of ten days. In some conditions in Table 3, 0.2 mM ascorbic acid (A4034; Sigma-Aldrich) was also included. This protocol produces a final population of approximately 10% and 30% cardiomyocytes at 142 and 7 mmHg, respectively.

Extended culture of differentiated mESC and miPSC. After the ten day differentiation protocol, cells were subjected to extended culture for up to 80 extra days. In some experiments, the pO_{2gas} was identical to the pO_{2gas} during differentiation, while in others the pO_{2gas} was changed. Cell samples were acquired by detachment with a 5 minute incubation of 0.25% trypsin (25-053-CI; Mediatech) at 37 °C.

Silicone rubber membrane-based plate. Differentiation and extended culture was performed on custom-made silicone rubber plates, having high oxygen permeability, as described previously [3]. In brief, the bottoms of 24-well polystyrene plates (353047; Becton Dickinson) were replaced with silicone rubber sheets (nonreinforced vulcanized gloss/gloss 0.005" thick; Specialty Manufacturing, Saginaw, MI, USA) and sterilized by filling with 70% (v/v) ethanol (111000200; Pharmco-AAPER, Brookfield, CT, USA) in water for 1 hr and drying overnight under a germicidal UV lamp in a biological safety cabinet (SterilGARD III Advance; The Baker Company, Sanford, MA, USA). In use, the plated sat on top of a 150x15 mm Petri dish (35384-326; VWR, West Chester, PA, USA) such that only the corners of the plates was supported by the Petri dish and the silicone rubber membrane was exposed directly to the gas phase. By using silicone rubber membranes, pO_{2cell} at the cell-membrane interface was maintained equal to pO_{2gas}, and rapid equilibration of pO_{2cell} after a change in pO_{2gas} was achieved. Cell attachment was achieved by incubating silicone rubber membranes at 37 °C with, for mESC, 2 μg/mL fibronectin (F1141; Sigma-Aldrich) in PBS (21-040; Mediatech) for 24 hr or, for human embryonic stem cells (hESC), Matrigel (354234, Becton Dickinson) diluted 1:20 in serum-free medium for 1 hr.

Flow cytometric analysis of Oct4-GFP expression. Medium was removed from the wells of the 24-well silicone rubber membrane-based culture dish, the cells were washed with PBS, and 200 μl of trypsin solution was added to each well. After 5-min incubation at 37 °C, 800

μl of ES cell differentiation medium was added to each well. The contents of the well were vigorously pipetted using a 1 ml pipette to dislodge and disperse the cells and were then transferred to a 1 ml tube. Detached cell Oct4-GFP expression was measured with a flow cytometer (FACScan; Becton Dickinson). A negative control was prepared using R1 mESC without the Oct4-GFP reporter differentiated under the same conditions as the Oct4-GFP cells and gating set so that all cells were counted as negative for Oct4-GFP. The fraction of cells expressing Oct4-GFP (Oct4-GFP+) was measured with flow cytometry and calculated as the number of cells above this threshold divided by the number of cells in the entire population at each specific measurement time and condition.

Real-time polymerase chain reaction (PCR). Total RNA was isolated using the RNeasy Kit (74104, Qiagen, Valencia, CA) and RNase-Free DNase Set (79254, Qiagen), cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems, Foster City, CA), and real-time polymerase chain reaction (PCR) was performed on a Fast Real-Time PCR System (7900HT, Applied Biosystems), using Power SYBR Green PCR Master Mix (4367659, Applied Biosystems). 28S ribosomal RNA (rRNA) was used as an endogenous control. Primer sequences for 28S rRNA, Oct4 (mouse), Nanog (mouse), Nestin, and Nkx2.5 have been previously reported [4]. The following additional primers sequences were used (in order of forward and reverse primer and 5' to 3'): Brachyury T TCCCGGTGCTGAAGGTAAAT (SEQ ID NO:1) and CCGTCACGAAGTCCAGCAA (SEQ ID NO:2), Foxa2 TCAAGGCCTACGAACAGGTCAT (SEQ ID NO:3) and GCCCGCTTTGTTCGTGACT (SEQ ID NO:4), cTnT AGATGCTGAAGAAGGTCCAGTAGAG (SEQ ID NO:5) and CACCAAGTTGGGCATGAAGA (SEQ ID NO:6), cardiac-α-Actin GCTTCCGCTGTCCAGAGACT (SEQ ID NO:7) and TGCCAGCAGATTCCATACCA (SEQ ID NO:8), OCT4 (human) TGGGCTCGAGAAGGATGTG (SEQ ID NO:9) and GCATAGTCGCTGCTTGATCG (SEQ ID NO:10), and NANOG (human) GCAAATGTCTTCTGCTGAGATGC (SEQ ID NO:11) and CCATGGAGGAGGGAAGAGGA (SEQ ID NO:12). A standard calibration curve was constructed using pooled cDNA from all conditions, serial diluted.

Cell processing for nuclei counts and immunostaining. Medium was removed from each

well, attached cells were washed with DPBS, and 200 µl of trypsin solution was added. After 5 min at 37 °C, 800 µl of differentiation medium was added, and the contents were vigorously pipetted up and down using a 1-ml pipette to dislodge and disperse the cells, which were transferred to a 1 ml tube and allowed to settle for 2 min. The bottom 50 µl, which contained any remaining large clumps of cells and extra-cellular matrix, was removed and discarded (or saved for later analysis). The cell sample was mixed by vortexing, and an aliquot saved for nuclei counting. The remaining cell sample was centrifuged, and the supernatant, discarded. Cells were resuspended in 750 µl DPBS, 250 µl of 4% (w/v) paraformaldehyde (Alfa Aesar, Ward Hill, MA) in DPBS was added to fix the cells, and each sample was incubated for 20 min at room temperature. Samples were then centrifuged, supernatant removed, and 1 ml of DPBS added. Samples of nuclei and of fixed cells were stored at 4 °C prior to analysis.

Cell enumeration. Detached cells were lysed by vigorous vortexing in 1% Triton X-100 (T-9284; Sigma-Aldrich) and 0.1 M citric acid (0627; Mallinckrodt Specialty Chemicals Co., Paris, KY, USA) in deionized water to liberate nuclei. Nuclei were stained with Guava ViaCount (4000-0041; Millipore) and counted using a Guava PCA flow cytometer (Millipore), as previous described [3].

Cell implantation into mice. SCID mice and 43-56 day old female nude mice (Charles River Laboratories International, Wilmington, MA, USA) were housed and cared for at MIT under the supervision of the committee on animal care, and experiments were performed using an approved protocol (#1007-082-10). The protocol for implanting cells was based on one designed to be a reliable animal model for biosafety testing of stem cell products. Using this protocol, implantation of only two ES cells was sufficient to form detectable tumors [5]. 10^5 cells (for mESC and miPSC) and 10^6 cells (for hESC) in 200 µl of 50% Matrigel (354234; BD Biosciences, Bedford, MA, USA) and 50% serum-free media were injected subcutaneously into the lower left flank of isoflurane-anaesthetized mice using a 23G needle in a laminar-flow hood. Mice were observed every 3-4 days for the appearance of masses under the skin.

Histology. Tumors were excised from sacrificed mice, fixed overnight in 4%

paraformaldehyde (36606; Alfa Aesar, Ward Hill, MA, USA) in PBS, paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E) at the Division of Comparative Medicine at MIT.

Flow activated cell sorting (FACS). Before sorting, Oct4-GFP mESC underwent 10 days differentiation, 20 days extended culture, and were detached trypsin. Cells were then either sorted with an Aria 2 FACS (Becton Dickinson) into Oct4-GFP+ and Oct4-GFP- populations or sorted based on expression of the mESC surface marker SSEA-1. For SSEA-1 sorting, cells were treated with a phycoerythrin (PE)-conjugated anti-SSEA-1 antibody (12-8813; eBioscience, San Diego, CA, USA) before sorting into SSEA-1+ and SSEA-1- populations.

Flow cytometric analysis of immunostained cells. The fraction of Nestin+ and MF-20+ cells was measured with flow cytometry, as previously described [3]. In brief, detached cells were fixed in 1% (w/v) paraformaldehyde in PBS for 20 min. $3x10^5$ fixed cells were incubated 10 min in 0.5% (w/v) saponin (S-4521; Sigma-Aldrich) in PBS to permeabilize cell membranes, and then incubated in 2% (v/v) FBS in PBS for 30 min to block. Cells were incubated with primary anti-Nestin (MAB2736; R&D Systems, Minneapolis, MN, USA) diluted 1:50 or anti-sarcomeric myosin heavy chain (MF-20; MF-20 supernatant; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA) diluted 1:10 antibody for 1 hour, washed 1x, and incubated with secondary goat anti-mouse PE-conjugated antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:250 in 2% FBS in the dark. Stained cells were washed thrice with 2% FBS, and fluorescence intensity data were acquired using the Guava PCA flow cytometer with the Express software module.

Immunocytochemistry. Cells attached to silicone rubber were fixed in 4% (w/v) parafromaldehyde in PBS for 20 min and then permeabilized and blocked with a 45 min incubation in 3% donkey serum (DS; D9663; Sigma-Aldrich) and 0.1% Triton X-100 in PBS. Cells were then incubated overnight at 4 °C with primary anti-Oct4 (sc-9081; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100, anti-Nanog (IHC-00205; Bethyl Laboratories, Montogomery, TX, USA) diluted 1:100, anti-SSEA-1 (MC-480; DSHB) diluted 1:10, anti-Foxa2 (sc-6554; Santa Cruz Biotechnology) diluted 1:200, anti-Nestin diluted 1:50, or anti-cardiac troponin T (cTnT; MS-295-P1; NeoMarkers, Fremont, CA,

USA) diluted 1:50 antibody in 3% DS, washed 1x, and incubated with secondary donkey Alexa Fluor 488 (green) or 594 (red) antibodies (Invitrogen) against the appropriate species diluted 1:250 for 2 hr in the dark. DNA was stained with a 10 min incubation with 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI; 32670, Sigma-Aldrich) in the dark. Stained cells were then washed thrice with 3% DS and images acquired with an Axiovert 200 fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Differentiation of hESC. CyT49 hESC (generously provided by ViaCyte, Inc, San Diego, CA, USA) were plated on Matrigel-coated silicone rubber plates at $1.5x10^5$ cells cm^{-1}, propagated 4 days in self-renewal media, and then switched to DMEM/F12 (15-090-CV; Mediatech) with 10% Knockout^{TM} serum replacement (A1099202; Invitrogen) for up to 90 days.

Statistics. Statistical analysis was performed using unpaired t-tests, with significant results requiring $p<0.05$. Data is presented as mean ± standard deviation.

Example 1: Low pO_2 Reduces Residual mESC

We differentiated and performed extended culture on Oct4-GFP+ mESC at various pO_2 to reduce residual mESC. Before differentiation, greater than 95% of the mESC expressed Oct4-GFP (Oct4-GFP+) (data not shown). This fraction decreased drastically during the 10 day differentiation, and, by the start of the extended low oxygen culture, Oct4-GFP+ cells represented a small fraction ($3x10^{-3}$) of the total cell population at 142 mmHg (Figure 1A). Differentiation under 36 and 7 mmHg resulted in even greater reduction in the fraction, to $6x10^{-4}$ and $1x10^{-3}$, respectively. These residual Oct4-GFP+ subpopulation had GFP intensity values similar to that of undifferentiated mESC (data not shown), and we hypothesized that these cells were residual pluripotent cells that could develop into teratomas upon implantation into animals.

After differentiation, culture was extended for up to 80 additional days to further reduce the fraction of Oct4-GFP+ cells. The differentiation process was largely complete by about 10 days of culture, and we considered the subsequent days in culture to represent a teratoma reduction phase of culture. In these experiments, cells were cultured at a constant oxygen partial pressure throughout differentiation and extended culture. Extended culture at

142 mmHg did not significantly change the fraction of Oct4-GFP+ cells but at 36 and 7 mmHg resulted in a dramatic decrease in the fraction (Figure 1A). The greatest difference was observed after 80 days of extended culture, at which point the fraction of Oct4-GFP+ cells was a factor of 1260 lower at 7 mmHg as compared to 142 mmHg. The low oxygen reduction in the fraction of Oct4-GFP in Figure 1A can be modeled assuming first order kinetics with a rate constant of 0.102±0.006 and 0.19950±0.00002 day^{-1} for 36 and 7 mmHg, respectively.

When observed with fluorescence microscopy, cells cultured at 142 mmHg that expressed GFP resided in clusters within aggregates (Figure 1B-C). However, at 7 mmHg, no cells expressing GFP were visually observed anywhere in the dish, including in aggregates (Figure 1D-E). No GFP regions were observed for cells cultured at 36 mmHg also (data not shown). The inability to visually observe Oct4-GFP+ cells at 36 or 7 mmHg with microscopy is likely due to the low fraction of cells expressing high enough levels of GFP to be detected by eye, not because of a complete absence of GFP-expressing cells.

Real-time PCR measurements demonstrated a reduction in the gene expression of Oct4 and Nanog at 36 and 7 mmHg compared to 142 mmHg (Figure 1F-G), correlating with the trend of a reduction in pluripotency at low compared to high pO_2.

Low oxygen culture reduction the number of Oct4-GFP+ cells in addition to the fraction (Figure 2). At the start of differentiation, each well started with $1.5x10^4$ Oct4-GFP+ cells. After 10 days of differentiation at 142 mmHg, this number remained unchanged. However, after 10 days of differentiation at 36 and 7 mmHg, the number of residual Oct4-GFP+ was much lower, $3x10^3$ and $4x10^3$, respectively. During 20 days of extended culture, the number of Oct4-GFP+ cells decreased with culture at 142 mmHg to $5x10^3$ and remained at this value until day 80. This reduction in the number of Oct4-GFP+ cells was proportional to the reduction in the total cell number (Figure 1A). However, extended culture at 36 and 7 drastically decreased the number of Oct4-GFP+ cells, resulting in only 10 and 4 remaining Oct4-GFP+ cells per well by day 80, respectively. This reduction was substantially faster than the reduction in the total cell number, as demonstrated by the rapid decrease in the fraction of Oct4-GFP+ cells (Figure 1A).

Example 2: Long-term hypoxic culture of differentiated mES cells leads to a greatly reduced rate of teratoma formation following implantation in SCID mice.

mES cells having undergone different pO_{2gas} culture conditions and injection subcutaneously into SCID mice formed tumors at different rates. 4/4 animals injected with 10^5 undifferentiated mES cells in Matrigel (positive control) formed visible tumors by day 14. Tumor formation was determined by visual observation of the appearance of masses underneath the skin. Differentiated cells cultured 35 extra days before implantation had slower tumor formation rates. 10^5 mES cells were differentiated 10 days at 142 mmHg (n=4) or 36 mmHg (n=6) in Matrigel, followed by 35 days of extended culture at the same pO_{2gas} to reduce the number and frequency of pluripotent cells, or only Matrigel (n=4; negative control). Visible tumors appeared in 4/4 animals after 17 days for cells cultured at 142 mmHg. Culture at lower pO_2 (36 mmHg) had an even greater effect. Tumors were first observed in 2/6 animals on day 31, and by day 38, 5/6 animals had developed tumors. 0/4 animals injected with only Matrigel (negative control) formed tumors. These *in vivo* data are consistent with the *in vitro* Oct4-GFP and real-time PCR data and confirm that long-term hypoxic culture of mES cells greatly reduces the rate of teratoma formation *in vivo*.

Example 3: The effect of a step change in pO_{2gas} on the fraction of Oct4-GFP+ cells

To investigate the effects of using different pO_2 values during differentiation and extended culture on the fraction of Oct4-GFP+ cells, mESC were initially differentiated for 10 days at either 142 or 7 mmHg pO_{2gas}, and then subjected to 20 days extended culture at the same or the other value of pO_{2gas} (Figure 3). After 10 days of differentiation at 142 mmHg (Figure 3A), the fraction of Oct4-GFP+ cells was $1.4x10^{-2}$. Extended culture for 20 days at 142 mmHg did not significantly change the fraction of Oct4-GFP+ cells. However, extended culture at 7 mmHg decreased the fraction to $5x10^{-4}$. 10 days of differentiation at 7 mmHg resulted in a fraction of Oct4-GFP+ cells of $6x10^{-4}$, a factor of 22 lower than at 142 mmHg (Figure 3B). Extended culture for 20 days at 7 mmHg further decreased the fraction to $3x10^{-5}$. However, extended culture at 142 mmHg increased the mean fraction to $3x10^{-3}$.

Example 4: Long-term hypoxic culture of differentiated mES cells does not limit cell survival.

If differentiated cells that have been previously cultured under normoxic conditions are to be cultured under hypoxic conditions in order to reduce the fraction of cells that are pluripotent, it is possible that the cells will die if they are unable to use anaerobic metabolism.

To investigate this possibility, we differentiated the cells and cultured the cells for extended time with three different sequences of pO_{2gas}: (A) Differentiate at 142 mmHg for 10 days, then extended culture at 142 mmHg for 20 days; (B) Differentiate at 142 mmHg for 10 days, then extended culture at 7 mmHg for 20 days; (C) Differentiate at 7 mmHg for 6 days, then differentiate at 142 mmHg for 4 days, then extended culture at 7 mmHg for 20 days. With all sequences, spontaneously-contracting cells were observed. The total number of cells and the number of MF-20+ (cardiomyocyte marker) cells is tabulated in Table 3 for days 0 and 20 of extended culture, along with the relative decrease from each number from 0 to 20 days culture. At day 0 of extended culture, sequence C maximized the number and fraction of cardiomyocytes, corresponding with our previous findings. With all sequences, the total cell number and number of MF-20+ cells decreased from 0 to 20 days. The decreases were smallest with sequences B and C, which involved extended culture at 7 mmHg, compared to sequence A, which used extended culture at 142 mmHg.

Regardless of whether differentiation was carried out for the first 10 days at 142 or 7 mmHg, if the 20 day extended culture was performed at 7 mmHg, the fraction of Oct4-GFP+ cells decreased approximately by a factor of 25 compared to day 10 cells. Additionally, if the 20 day extended culture was performed at 142 mmHg, the fraction of Oct4-GFP+ cells either increased or stayed the same compared to day 0. These results demonstrate that differentiation can be performed at any pO_2, followed by an extended culture period at low pO_2 to reduce the fraction of Oct4-GFP+ cells without negative affecting cell number. This combination of pO_2 control strategies allows for the production of the desired differentiated cell type at the best pO_2 for producing that cell type while also reducing the fraction of potentially-tumorigenic residual pluripotent cells.

Example 5: Low pO_2 Delays Appearance of Tumors after Implantation of Cultured mESC

mESC having undergone different pO_{2gas} culture conditions and injection subcutaneously into nude mice formed tumors at different rates (Figure 4A). 6/6 animals injected with undifferentiated mESC (positive control) formed visible tumors by day 14. Differentiated cells cultured 20 extra days before implantation had slower tumor formation rates. Visible tumors appeared in 7/8 animals by day 17 and 8/8 by day 21 for cells cultured at 142 mmHg. Culture at lower pO_2 (36 and 7mmHg) had an even greater effect. Tumors

were first observed in 3/8 animals by day 31, 5/8 by day 35, and 7/8 by day 38 for cells cultured at 36 mmHg and in 5/8 animals by day 35, 6/8 animals by day 38, and 7/8 animals by day 42 for 7 mmHg. 0/6 animals injected with only Matrigel (negative control) formed tumors. All animals with no tumors were monitored for a total of 210 days after implantation, during which no tumors were observed.

The resulting tumors were excised from sacrificed mice, fixed, embedded, sectioned, and H&E stained, and the resulting tissue sections histopathologically analyzed (Figure 4B). The tumors contained derivatives of all three germ layers and were identified as teratomas. These *in vivo* data are consistent with the *in vitro* Oct4-GFP and real-time PCR data and confirm that long-term hypoxic culture of mESC greatly reduces the rate of teratoma formation *in vivo*.

Example 6: Combination of Low pO_2 Culture with Fluorescence Activated Cell Sorting (FACS)

mESC after differentiation and 20 days of extended culture were sorted with FACS based on GFP expression into Oct4-GFP- and Oct4-GFP+ populations before implantation to investigate Oct4-GFP+ cells as the tumor cell source (Figure 5). FACS was unable to remove all the Oct4-GFP+ cells from the 142 mmHg Oct4-GFP- population, instead decreasing the fraction of Oct4-GFP+ cells by a factor of 50, as determined by subsequent flow cytometric analysis of the sorted population, but was able to remove all Oct4-GFP+ cells from the 7 mmHg Oct4-GFP+ population (Figure 5A). After FACS, unsorted, Oct4-GFP+ populations, and the Oct4-GFP- populations from 142 and 7 mmHg were implanted into nude mice (Figure 5B). The 142 and 7 mmHg Oct4-GFP+ populations resulted in tumors that appeared at approximately the same time as 142 and 7 mmHg unsorted cells, respectively. The 142 mmHg Oct4-GFP- population resulted in an 11 day delay in tumor appearance compared to unsorted cells, but the 7 mmHg Oct4-GFP- population resulted in no tumors during 210 days of observation. The complete absence of tumor formation with Oct4-GFP- populations verified to contain no Oct4-GFP- cells demonstrates that only Oct4-GFP+ pluripotent stem cells are responsible for the tumor formation in this system, confirming our approach of focusing on this subpopulation.

The data in Figure 5 also shows that low pO_2 culture can be combined with FACS to further decrease and eliminate residual pluripotent cells. However, sorting based on GFP

expression requires cells containing genetic modifications, which creates another major hurdle to clinical application. Therefore, we investigated combining low pO_2 culture with sorting based on staining with a PE-conjugated anti-SSEA-1 antibody (Figure 6). mESC, after differentiation and 20 days of extended culture, were sorted into SSEA-1+ and SSEA-1- populations and subsequently analyzed with flow cytometry for Oct4-GFP expression (Figure 6A). The SSEA-1- populations had a fraction of Oct4-GFP+ cells that were a factor of 31 and 16 lower than unsorted cells from 142 and 7 mmHg, respectively. Culture at 7 mmHg combined with FACS removal of SSEA-1+ cells resulted in a reduction in the fraction of Oct4-GFP+ cells by a factor of 1020 compared to cells culture at 142 mmHg without sorting. After FACS, unsorted, SSEA-1+ populations, and the SSEA-1- populations from 142 and 7 mmHg were implanted into nude mice (Figure 6B). The 142 and 7 mmHg SSEA-1+ populations resulted in tumors that appeared at approximately the same time as 142 and 7 mmHg unsorted cells, respectively. The 142 mmHg SSEA-1- population resulted in a 14-day delay in tumor appearance compared to unsorted cells, but the 7 mmHg SSEA-1- population resulted in no tumors during 210 days of observation. Low pO_2 culture can be used in combination with FACS removal based on surface markers to eliminate the risk of tumor formation.

Example 7: HIF-1α is Necessary for the Low pO_2 Reduction in Residual mESC and Delay in Appearance of Tumors after Implantation of Cultured mESC

To determine if HIF-1α is necessary for the low pO_2 reduction in residual mESC and delay in appearance of tumors after implantation of cultured mESC, a HIF-$1\alpha^{-/-}$ mESC line was differentiated and subjected to extended culture (Figure 7). After 20 days of extended culture, Oct4 and Nanog gene expression was the same between 142 and 7 mmHg (Figure 7A). The relative loss of Oct4 and Nanog gene expression for HIF-$1\alpha^{-/-}$ mESC at both pO_{2gas} conditions was similar to the relative loss of expression of wild type mESC at 142 mmHg (Figure 1F-G). Upon implantation into nude mice, all animals injected with undifferentiated HIF-$1\alpha^{-/-}$ mESC formed tumors by day 21, 7 days later than wild type mESC (Figure 4A). Differentiated HIF-$1\alpha^{-/-}$ mESC cultured 20 extra days before implantation had slower tumor formation rates, with all animals forming tumors by day 31, regardless of culture pO_{2gas} (7 or 142 mmHg), 14 days later than wild type mESC cultured at 142 mmHg. Without HIF-1α, pluripotency gene expression and tumor formation rates are the same between low and high

pO_2, showing that HIF-1α is necessary for the positive benefits of low pO_2 culture.

Example 8: Residual Oct4-GFP+ mESC In Vitro Phenotype Is the Same as Undifferentiated mESC

Oct4-GFP+ cells separated from Oct4-GFP- cells after 10 days of differentiation and 35 days of extended culture at 7 mmHg were plated onto gelatin-coated polystyrene in DMEM with 10% FBS and 10^3 U/ml LIF (data not shown). The Oct4-GFP+ cells proliferated once separated from Oct4-GFP- cells. Their morphology resembled that of undifferentiated ES cells.

We further investigated residual Oct4-GFP+ mESC by removing Oct4-GFP+ cells from differentiated mixtures, plating them in LIF-supplemented media, and characterizing their *in vitro* phenotype. Specifically, we compared three cases: (I) Oct4-GFP+ mESC that have never undergone differentiation and extended culture, (II) Oct4-GFP+ mESC sorted with FACS from mESC that have undergone differentiation and 20 days extended culture at 142 mmHg, and (III) Oct4-GFP+ mESC cultured similar to case II, but at 7 mmHg (Figures 8-11). The distribution of Oct4-GFP intensity was similar among the three cases (Figure 8A), and Oct4-GFP expression was lost in greater than 99% of resulting cells when differentiated (Figure 8B). Oct4-GFP+ mESC colonies among all three cases were morphologically similar and contained Oct4, Nanog, and SSEA-1 protein (Figure 8C-F). Upon differentiation, all three cases expressed markers for all three germ layers (Figures 9-11). All three cases (1) expressed Brachyury T, Foxa2, Nestin, cardiac Troponin T (cTnT), cardiac-α-Actin, and Nkx2.5 mRNA (Figure 9), (2) stained positive for Foxa2, Nestin, and cTnT protein (Figure 10), and (3) contained the same fraction and number of Nestin+ and MF-20+ cells (Figure 11). According to all the *in vitro* characterization assays we formed, residual Oct4-GFP+ mESC are the same as undifferentiated mESC.

Example 9: Low pO_2 Reduces in Residual hESC and Delays the Appearance of Tumors after Implantation of Cultured Hesc

We extended our investigation to hESC by differentiating CyT49 hESC at various pO_2 to reduce residual hESC (Figure 12). Before differentiation, more than 95% of the hESC stained positive for OCT4 protein (data not shown). During differentiation, OCT4 and NANOG gene expression decreased drastically and was lowest under low pO_2 (Figure 12A-

B). For culture at 142 mmHg, OCT4 and NANOG gene expression decreased until day 30, then remained constant until day 90. For culture at 36 and 7 mmHg, expression decreased during the entire 90 day period and was lower at all time points compared to 142 mmHg. After 90 days of differentiation, OCT4 expression was 357 and 102 times lower at 36 and 7 mmHg, respectively, and NANOG expression was 2844 and 456 times lower at 36 and 7 mmHg, respectively, compared to 142 mmHg. These results are qualitatively similar to data with mESC, but the loss of expression compared to undifferentiated cells is lower in hESC compared to mESC (Figure 1F-G). These *in vitro* results demonstrate that differentiation under low pO_2 can reduce pluripotency gene expression.

hESC having undergone 30 days of differentiation at different pO_{2gas} culture conditions and injection subcutaneously into nude mice formed tumors at different rates (Figure 12C). All animals injected with undifferentiated hESC (positive control) formed visible tumors by day 17. Cells differentiated 30 days before implantation had slower tumor formation rates. Visible tumors appeared in 2/6 animals by day 31 and 6/6 by day 38 for cells cultured at 142 mmHg. Culture at lower pO_2 (36 and 7mmHg) had an even greater effect. Tumors were observed in all animals by day 52 for cells cultured at 7 mmHg. As of day 55, no animals with cells from 36 mmHg have formed tumors. These *in vivo* data are consistent with the *in vitro* real-time PCR data and confirm that hypoxic culture of hESC greatly reduces the rate of teratoma formation *in vivo*.

Example 10: Low pO_2 Reduces Residual miPSC

We extended our investigation to miPSC.by differentiating Oct4-GFP+ miPSC at various pO_2 to reduce residual miPSC (Figure 13). Before differentiation, greater than 95% of the miPSC were Oct4-GFP+ (data not shown). This fraction decreased drastically during the 10 day differentiation, and, by the start of the extended low oxygen culture, Oct4-GFP+ cells represented a small fraction ($4x10^{-2}$) of the total cell population at 142 mmHg. Differentiation under 36 and 7 mmHg resulted in even greater reduction in the fraction, to $2x10^{-4}$ and $3x10^{-4}$, respectively. These residual Oct4-GFP+ subpopulation had GFP intensity values similar to that of undifferentiated miPSC (data not shown). After differentiation, culture was extended for 20 additional days to further reduce the fraction of Oct4-GFP+ cells. Extended culture at 142 mmHg did not significantly change the fraction of Oct4-GFP+ cells but at 36 and 7 mmHg resulted in a dramatic decrease in the fractions. The greatest

difference was observed after 20 days of extended culture, at which point the fraction of Oct4-GFP+ cells was a factor of 13203 lower at 7 mmHg compared to 142 mmHg. These results are qualitatively similar to data with mESC, but the loss of expression compared to undifferentiated cells is higher at low pO_2 and lower at high pO_2 in miPSC compared to mESC (Figure 1A). These *in vitro* results demonstrate that differentiation and extended culture under low pO_2 can reduce residual miPSC.

Example 11: Number of Implanted Pluripotent Cells Correlates with the Appearance of Tumors

To better assess our nude mouse model for detecting residual pluripotent cells, we compared the number of implanted Oct4-GFP+ mESC and hESC to the time required to form a tumor (Figure 14). The mouse and human data correlated and fit an exponential equation, with $R^2 = 0.90$ and $R^2 = 0.99$, respectively. Undifferentiated mESC data was not included, because we observed that implanting 10^5 and 10^6 undifferentiated mESC both formed tumors on day 14, suggesting there is an upper limit on the sensitivity of this animal model. This data shows that our animal model is very sensitive to small numbers of and could be used in the estimation of residual pluripotent cells.

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Table 1. Composition of undifferentiated mES cell maintenance and mES cell differentiation medium[a]

Component	Manufacturer and Catalogue Number	Volume per liter of medium (ml)	Notes
Dulbecco's modified eagles medium (DMEM)	ATCC SCRR 2010	868	ES cell qualified
Fetal bovine serum	ATCC SCRR 30-2020	100	ES cell qualified
L-alanyl-L-glutamine	ATCC SCRR 20-2115	10	200 mM stock solution
MEM non-essential amino acid solution	ATCC SCRR 20-2116	10	
2-mercaptoethanol solution	Sigma-Aldrich M7522	10	10 mM stock solution prepared in DMEM
Leukemia inhibitory factor[a]	Chemicon ESG 1106	1	10^6 unit/ml stock solution

[a] Leukemia inhibitory factor was added only to undifferentiated ES cell maintenance medium. Ascorbic acid was added only to ES cell differentiation medium.

Table 2. Formulation of serum-free medium

Component	Manufacturer and Catalogue Number	Volume per liter of medium (ml)	Notes
Dulbecco's modified eagles medium (DMEM)	Mediatech 90-133-PB	485	8.4 mg/ml stock solution prepared in deionized water
Sodium bicarbonate	Mediatech 25-035-CI	10	7.5% (w/v) solution
F12 nutrient mixture	Invitrogen 31765-035	496	
25M glucose solution	Sigma-Aldrich G8769	1.6	Final medium concentration of 9mM
Human insulin solution	Sigma-Aldrich I9278	0.5	10 mg/ml solution
Holo transferrin solution	Sigma-Aldrich T1283	5	10 mg/ml stock solution prepared in DPBS
Sodium selenite solution	Sigma-Aldrich S9133	0.26	0.12 mM stock solution prepared in DPBS

Table 3. Comparison of changes in pO_{2gas} on total cell number and number of MF-20+ cells

pO_{2gas} (mmHg)	Ascorbic Acid	Total cell number ($x10^6$)			Number MF-20+ cells ($x10^5$)		
		Time (days)[a]		Change (%)	Time (days)[a]		Change (%)
		0	20		0	20	
142	No	1.8±0.1	0.7±0.2	63	1.4±0.1	0.5±0.01	66
142-7[b]	No	1.8±0.1	0.9±0.1	50	1.4±0.1	1.2±0.1	17
7-142-7[c]	Yes	2.1±0.4	1.0±0.2	54	3.2±0.3	2.6±0.6	19

[a] Time in extended culture

[b] Differentiation 10 d at 142 mmHg, then 20 d extended culture at 7 mmHg

[c] Differentiation 6 d at 7 mmHg followed by 4 d at 142 mmHg, then 20 d extended culture at 7 mmHg

EQUIVALENTS

While several inventive embodiments have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the function and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the inventive embodiments described herein. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the inventive teachings is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific inventive embodiments described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, inventive embodiments may be practiced otherwise than as specifically described and claimed. Inventive embodiments of the present disclosure are directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the inventive scope of the present disclosure.

All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

All references, patents and patent applications disclosed herein are incorporated by reference with respect to the subject matter for which each is cited, which in some cases may encompass the entirety of the document.

The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

The phrase "and/or," as used herein in the specification and in the claims, should be understood to mean "either or both" of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with "and/or" should be construed in the same fashion, i.e., "one or more" of

the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the "and/or" clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to "A and/or B", when used in conjunction with open-ended language such as "comprising" can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

As used herein in the specification and in the claims, "or" should be understood to have the same meaning as "and/or" as defined above. For example, when separating items in a list, "or" or "and/or" shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as "only one of" or "exactly one of," or, when used in the claims, "consisting of," will refer to the inclusion of exactly one element of a number or list of elements. In general, the term "or" as used herein shall only be interpreted as indicating exclusive alternatives (i.e. "one or the other but not both") when preceded by terms of exclusivity, such as "either," "one of," "only one of," or "exactly one of." "Consisting essentially of," when used in the claims, shall have its ordinary meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase "at least one," in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase "at least one" refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, "at least one of A and B" (or, equivalently, "at least one of A or B," or, equivalently "at least one of A and/or B") can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one,

optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

In the claims, as well as in the specification above, all transitional phrases such as "comprising," "including," "carrying," "having," "containing," "involving," "holding," "composed of," and the like are to be understood to be open-ended, i.e., to mean including but not limited to. Only the transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

What is claimed is:

CLAIMS

1. A method comprising

culturing a differentiated cell population at an oxygen partial pressure that is less than 142 mmHg for 20 days or more.

2. A method comprising

culturing an isolated in vitro differentiated cell population at an oxygen partial pressure that is less than 142 mmHg for 10 days or more.

3. A method comprising

culturing a differentiated cell population at an oxygen partial pressure that is less than 142 mmHg for 10 days or more, wherein the differentiated cell population is a non-cardiomyocyte differentiated cell population.

4. The method of any one of claims 1-3, wherein the differentiated cell population is isolated from a differentiation culture.

5. The method of any one of claims 1-4, wherein the differentiated cell population is derived from an embryonic stem cell or an induced pluripotent stem cell.

6. The method of any one of claims 1-5, wherein the differentiated cell population is derived from a human embryonic stem cell or a human induced pluripotent stem cell.

7. The method of any one of claims 1, 2, and 4-6, wherein the differentiated cell population comprises cardiomyocyte lineage cells.

8. The method of any one of claims 1, 2, and 4-7, wherein the differentiated cell population comprises cardiomyocyte precursor cells.

9. The method of any one of claims 1-6, wherein the differentiated cell population comprises neural cells.

10. The method of any one of claims 1-6, wherein the differentiated cell population comprises islet cells.

11. The method of any one of claims 1-10, wherein the differentiated cell population comprises non-stem cell precursor cells.

12. The method of any one of claims 1-11, wherein the oxygen partial pressure is 4-10 mmHg.

13. The method of any one of claims 1-11, wherein the oxygen partial pressure is 7 mmHg.

14. The method of any one of claims 1-11, wherein the oxygen partial pressure is 10-50 mmHg.

15. The method of any one of claims 1-11, wherein the oxygen partial pressure is 36 mmHg.

16. The method of any one of claims 1-11, wherein the oxygen partial pressure is 50-100 mmHg.

17. The method of any one of claims 1-11, wherein the oxygen partial pressure is 100-140 mmHg.

18. The method of any one of claims 1-17, wherein the differentiated cell population is cultured for 20 days.

19. The method of any one of claims 1-17, wherein the differentiated cell population is cultured for 30 days.

20. The method of any one of claims 1-19, wherein the culturing step reduces pluripotent stem cells in the differentiated cell population by at least 2-fold.

21. The method of any one of claims 1-19, wherein the culturing step reduces pluripotent stem cells in the differentiated cell population by at least 5-fold.

22. The method of any one of claims 1-19, wherein the culturing step reduces pluripotent stem cells in the differentiated cell population by at least 10-fold.

23. The method of any one of claims 1-19, wherein the culturing step reduces pluripotent stem cells in the differentiated cell population by at least 20-fold.

24. The method of any one of claims 1-19, wherein the culturing step reduces pluripotent stem cells in the differentiated cell population by at least 40-fold.

25. The method of any one of claims 1-19, wherein the culturing step reduces pluripotent stem cells in the differentiated cell population by at least 100-fold.

26. The method of any one of claims 1-19, wherein the culturing step reduces pluripotent stem cells in the differentiated cell population by at least 150-fold.

27. The method of any one of claims 1-26, further comprising performing a positive selection for differentiated cells prior to, during and/or after the culturing step.

28. The method of any one of claims 1-27, further comprising performing a negative selection for pluripotent stem cells prior to, during and/or after the culturing step.

29. The method of claim 27 or 28, wherein the positive or negative selection is based on cell surface expression of a marker.

30. The method of claim 27 or 28, wherein the positive or negative selection is a fluorescent activated cell sorting (FACS) selection.

31. The method of claim 29 or 30, wherein negative selection is based on expression of SSEA-4, TRA-60 and/or Oct4.

32. The method of claim 27 or 28, wherein the positive or negative selection is based on a physical property.

33. The method of claim 32, wherein the negative selection is based on buoyant density.

34. The method of any one of claims 1-33, further comprising harvesting the differentiated cell population after the culturing step.

35. The method of any one of claims 1-33, further comprising assaying for teratoma-causing cells in the cultured differentiated cell population.

36. The method of claim 35, wherein the teratoma-causing cells are assayed by introducing the cultured differentiated cell population into an immunocompromised mouse.

37. The method of claim 36, wherein 10^3 cells, 10^4 cells, 10^5 cells, 10^6 cells, or 10^7 cells from the cultured differentiated cell population are introduced into the immunocompromised mouse.

38. The method of any one of claims 1-37, further comprising measuring mRNA or protein expression of a stem cell marker in the cultured differentiated cell population.

39. The method of claim 38, wherein the stem cell marker is Oct4.

40. The method of any one of claims 1-39, further comprising administering the cultured differentiated cell population to a subject in need thereof.

41. The method of any one of claims 1-40, wherein the differentiated cell population is cultured in a culture vessel comprising an oxygen permeable membrane.

42. The method of any one of claims 1-40, wherein the differentiated cell population is cultured in a culture vessel comprising a silicon rubber membrane.

43. A method comprising

differentiating pluripotent stem cells towards a non-cardiomyocyte lineage in the presence of one or more differentiation stimuli and under low oxygen partial pressure.

44. The method of claim 43, wherein low oxygen partial pressure is 4-10 mmHg.

45. The method of claim 43, wherein low oxygen partial pressure is 7 mmHg.

46. The method of claim 43, wherein low oxygen partial pressure is 10-50 mmHg.

47. The method of claim 43, wherein low oxygen partial pressure is 36 mmHg.

48. The method of claim 43, wherein low oxygen partial pressure is 50-100 mmHg.

49. The method of claim 43, wherein low oxygen partial pressure is 100-140 mmHg.

50. The method of claim 43-48 or 49, wherein the pluripotent stem cells are embryonic stem cells or induced pluripotent stem cells.

51. The method of claim 43-48 or 49, wherein the pluripotent stem cells are human embryonic stem cells or human induced pluripotent stem cells.

52. The method of claim 43-50 or 51, wherein the pluripotent stem cells are exposed to the one or more differentiation stimuli and low oxygen partial pressure simultaneously.

53. The method of claim 43-51 or 52, wherein the pluripotent stem cells are exposed to the one or more differentiation stimuli and low oxygen partial pressure separately.

54. A differentiated cell population obtained according to the method of any one of claims 1-52 or 53.

55. An isolated differentiated cell population derived from isolated pluripotent stem cells and having less than 1 pluripotent stem cell per 10^3 cells.

56. The differentiated cell population of claim 54 or 55, having less than 1 pluripotent stem cell per 10^4 cells.

57. The differentiated cell population of claim 54 or 55, having less than 1 pluripotent stem cell per 10^5 cells.

58. The differentiated cell population of claim 54 or 55, having less than 1 pluripotent stem cell per 10^6 cells.

59. The differentiated cell population of claim 54 or 55, having less than 1 pluripotent stem cell per 10^7 cells.

60. The differentiated cell population of claim 54 or 55, having less than 1 pluripotent stem cell per 10^8 cells.

61. The differentiated cell population of claim 54 or 55, having less than 1 pluripotent stem cell per 10^9 cells.

62. The differentiated cell population of any one of claims 54-61, wherein the isolated pluripotent stem cells are embryonic stem cells or induced pluripotent stem cells.

63. The differentiated cell population of any one of claims 54-61, wherein the isolated pluripotent stem cells are human embryonic stem cells or human induced pluripotent stem cells.

64. The differentiated cell population of any one of claims 54-63, wherein the isolated differentiated cell population comprises cardiomyocyte lineage cells.

65. The differentiated cell population of any one of claims 54-64, wherein the isolated differentiated cell population comprises cardiomyocyte precursor cells.

66. The differentiated cell population of any one of claims 54-63, wherein the isolated differentiated cell population comprises neural cells.

67. The differentiated cell population of any one of claims 54-63, wherein the isolated differentiated cell population comprises islet cells.

68. The differentiated cell population of any one of claims 54-67, wherein the isolated differentiated cell population comprises non-stem cell precursor cells.

69. A pharmaceutical preparation comprising the differentiated cell population of any one of claims 54-68.

70. The pharmaceutical preparation of claim 69, wherein the preparation comprises at least 10^7, at least 10^8, or at least 10^9 cells.

71. The pharmaceutical preparation of claim 69 or 70, wherein the preparation comprises a pharmaceutically acceptable carrier.

72. The pharmaceutical preparation of claim 69, 70 or 71, wherein the differentiated cell population is encapsulated in a gel.

73. The pharmaceutical preparation of claim 69, 70 or 71, wherein the preparation is formulated for parenteral administration.

74. A method for treating a subject comprising

administering to a subject in need thereof a differentiated cell population of any one of claims 54-68 or produced according to a method of any one of claims 1-53 in an effective amount to treat the subject.

75. A method comprising

exposing the differentiated cell population of any one of claims 54-68 or produced by the method of any one of claims 1-53 to an agent, and

determining the effect of the agent on the differentiated cell population.

5

76. The method of claim 75, wherein determining the effect of the agent comprises determining the cytotoxicity of the agent on the differentiated cell population.

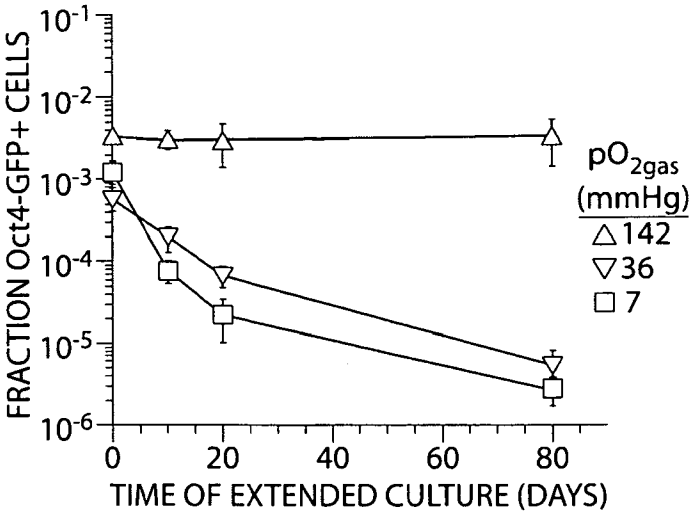

Fig. 1A

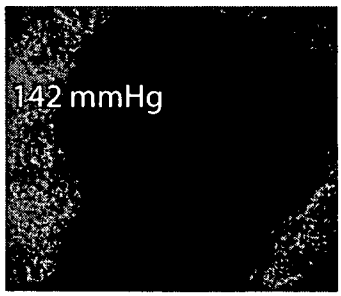

Fig. 1B

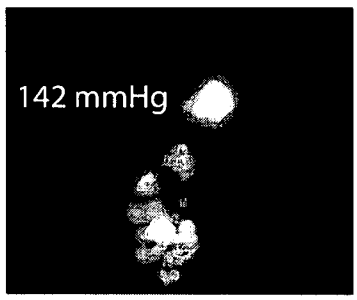

Fig. 1C

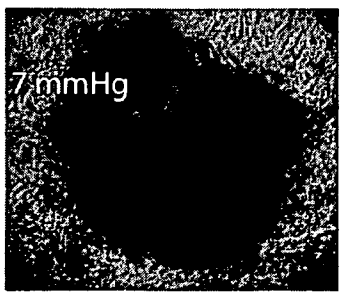

Fig. 1D

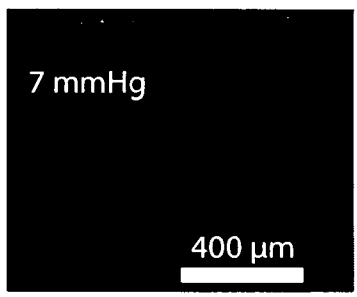

Fig. 1E

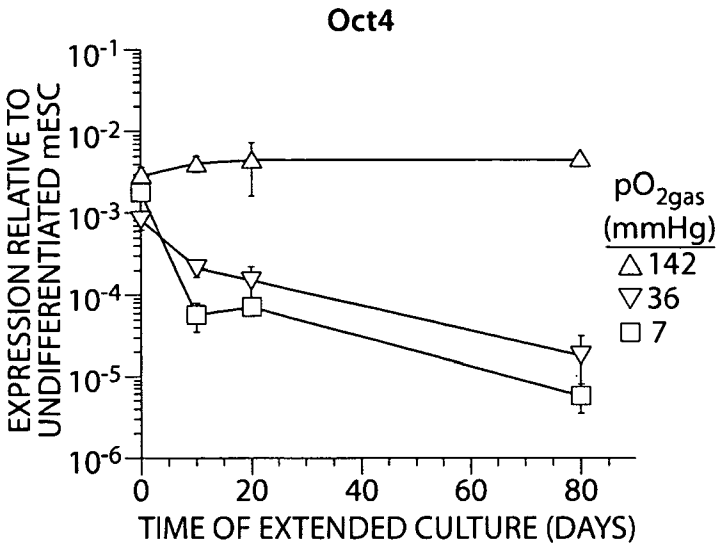
Oct4
EXPRESSION RELATIVE TO UNDIFFERENTIATED mESC
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
0
20
40
60
80
TIME OF EXTENDED CULTURE (DAYS)
pO_{2gas} (mmHg)
△142
▽36
□7

Fig. 1F

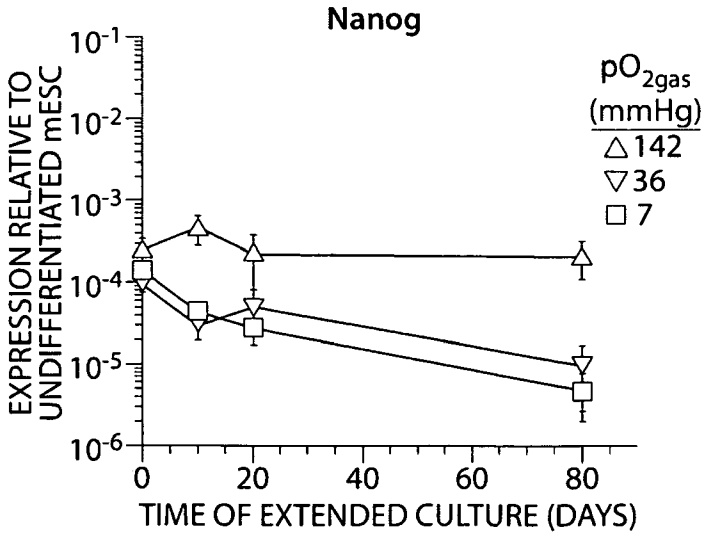
Nanog
EXPRESSION RELATIVE TO UNDIFFERENTIATED mESC
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
0
20
40
60
80
TIME OF EXTENDED CULTURE (DAYS)
pO_{2gas} (mmHg)
△142
▽36
□7

Fig. 1G

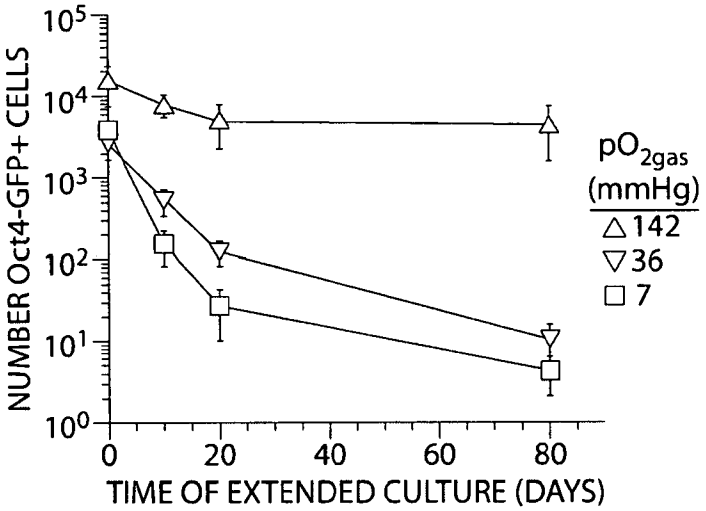
NUMBER Oct4-GFP+ CELLS
10^5
10^4
10^3
10^2
10^1
10^0
0
20
40
60
80
TIME OF EXTENDED CULTURE (DAYS)
pO_{2gas}
(mmHg)
△142
▽36
□ 7

Fig. 2

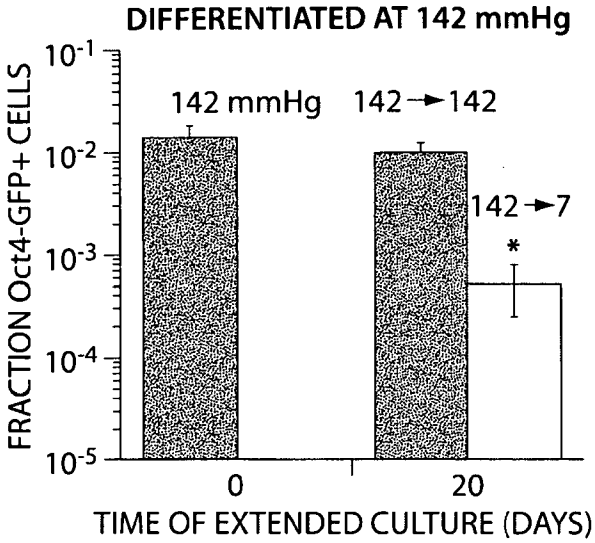
DIFFERENTIATED AT 142 mmHg
FRACTION Oct4-GFP+ CELLS
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
142 mmHg
142→142
142→7
*
0
20
TIME OF EXTENDED CULTURE (DAYS)

Fig. 3A

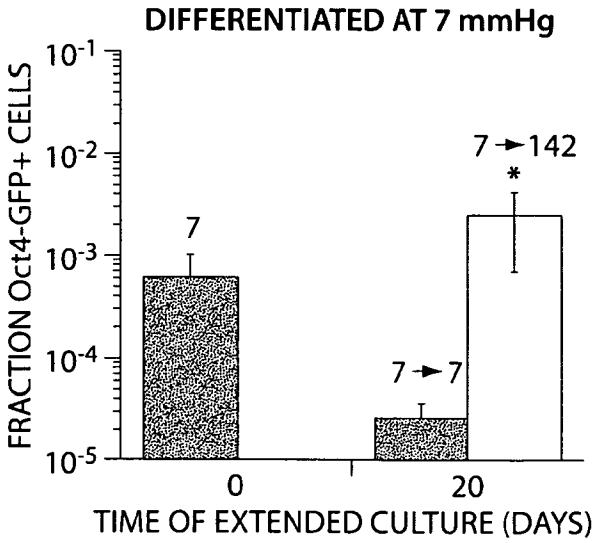
DIFFERENTIATED AT 7 mmHg
FRACTION Oct4-GFP+ CELLS
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
7
7→7
7→142
*
0
20
TIME OF EXTENDED CULTURE (DAYS)

Fig. 3B

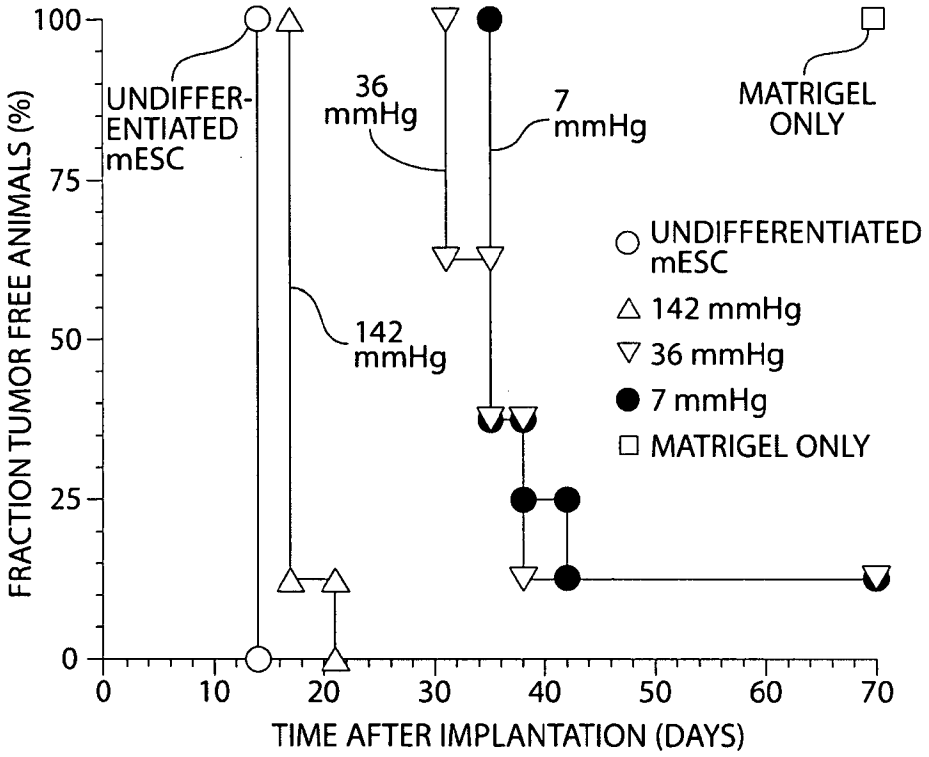
100
75
50
25
0
FRACTION TUMOR FREE ANIMALS (%)
0
10
20
30
40
50
60
70
TIME AFTER IMPLANTATION (DAYS)
UNDIFFER-
ENTIATED
mESC
36
mmHg
7
mmHg
MATRIGEL
ONLY
142
mmHg
UNDIFFERENTIATED
mESC
142 mmHg
36 mmHg
7 mmHg
MATRIGEL ONLY

Fig. 4A

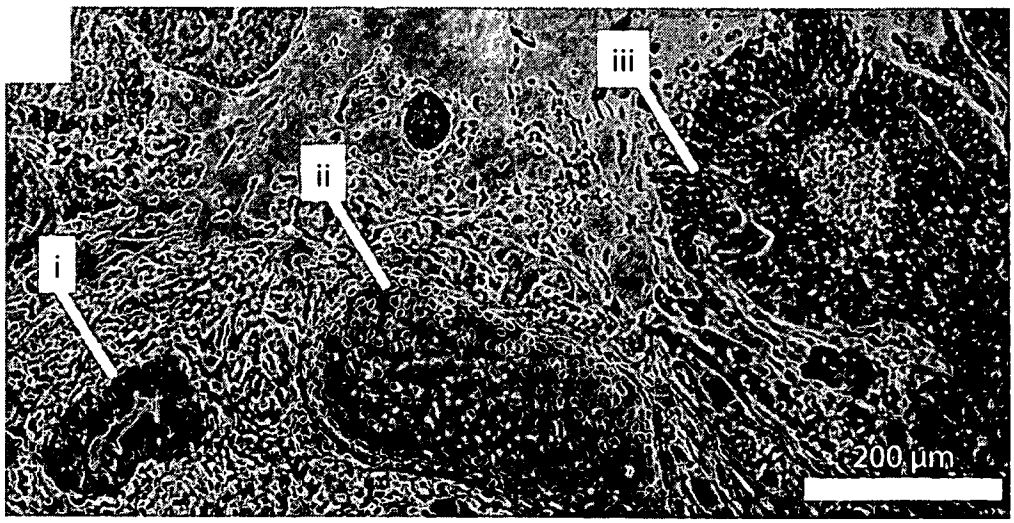
i
ii
iii
200 µm

Fig. 4B

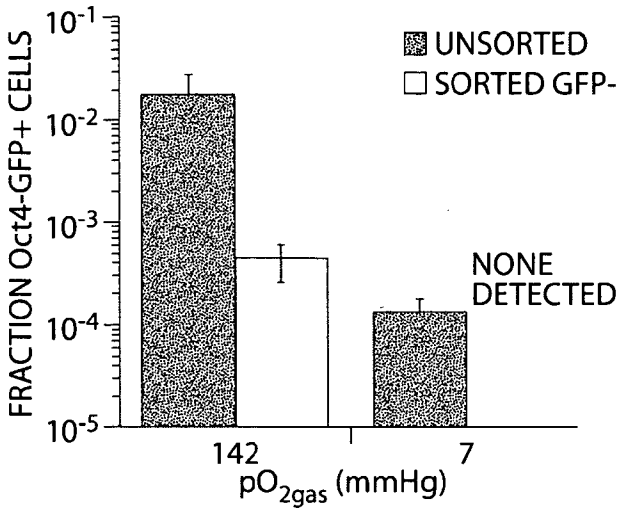
FRACTION Oct4-GFP+ CELLS
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
UNSORTED
SORTED GFP-
NONE DETECTED
142
7
pO_{2gas} (mmHg)

Fig. 5A

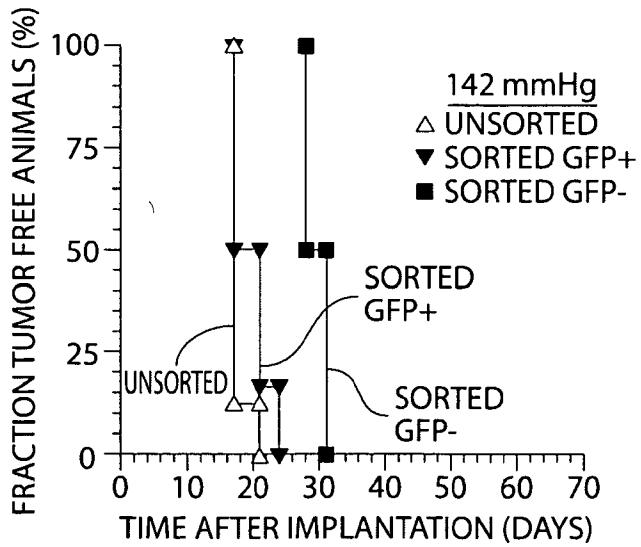
FRACTION TUMOR FREE ANIMALS (%)
100
75
50
25
0
142 mmHg
△ UNSORTED
▼ SORTED GFP+
■ SORTED GFP-
SORTED GFP+
UNSORTED
SORTED GFP-
0 10 20 30 40 50 60 70
TIME AFTER IMPLANTATION (DAYS)

Fig. 5B

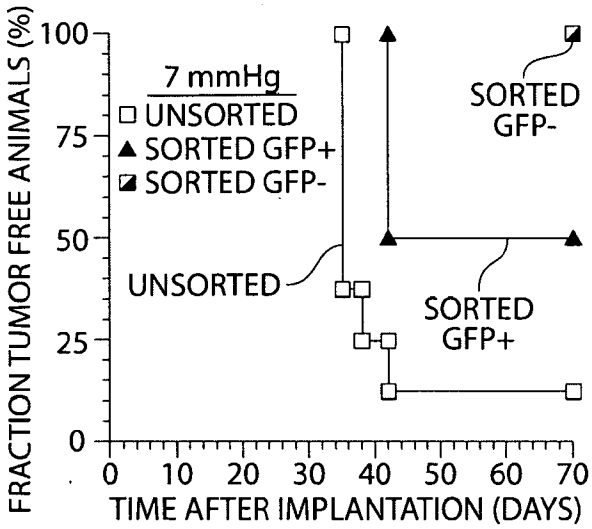
FRACTION TUMOR FREE ANIMALS (%)
100
75
50
25
0
7 mmHg
UNSORTED
SORTED GFP+
SORTED GFP-
UNSORTED
SORTED GFP-
SORTED GFP+
0 10 20 30 40 50 60 70
TIME AFTER IMPLANTATION (DAYS)

Fig. 5C

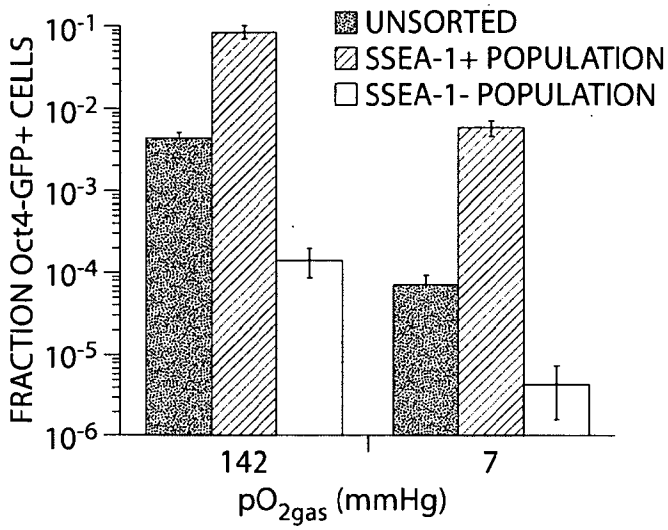
UNSORTED
SSEA-1+ POPULATION
SSEA-1- POPULATION
FRACTION Oct4-GFP+ CELLS
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
142
7
pO_{2gas} (mmHg)

Fig. 6A

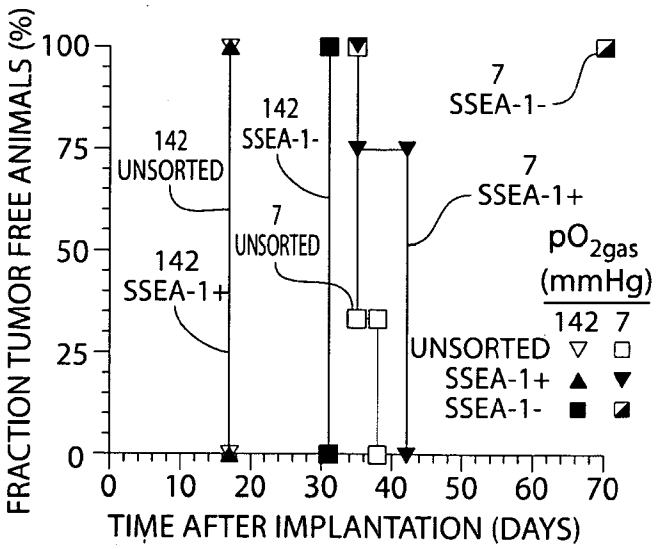
FRACTION TUMOR FREE ANIMALS (%)
100
75
50
25
0
142 UNSORTED
142 SSEA-1+
142 SSEA-1-
7 UNSORTED
7 SSEA-1+
7 SSEA-1-
pO_{2gas} (mmHg)
142 7
UNSORTED
SSEA-1+
SSEA-1-
0 10 20 30 40 50 60 70
TIME AFTER IMPLANTATION (DAYS)

Fig. 6B

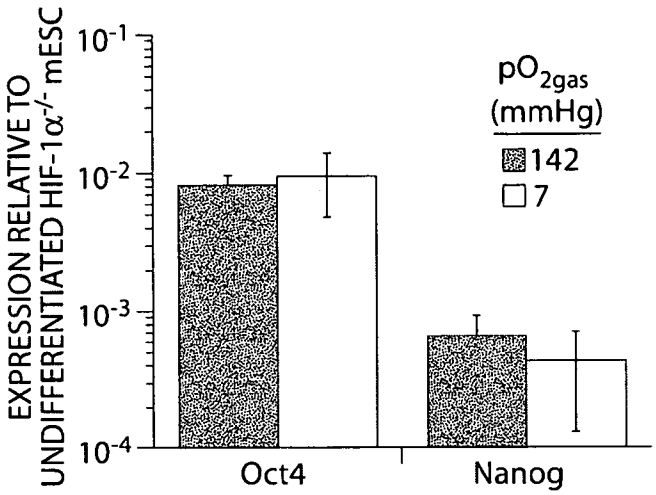
EXPRESSION RELATIVE TO UNDIFFERENTIATED HIF-1α-/- mESC
10-1
10-2
10-3
10-4
pO2gas (mmHg)
142
7
Oct4
Nanog

Fig. 7A

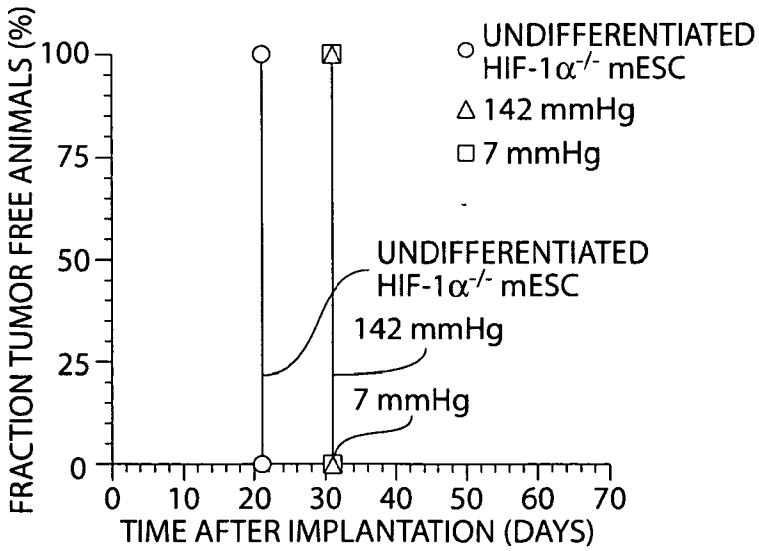
FRACTION TUMOR FREE ANIMALS (%)
100
75
50
25
0
0 10 20 30 40 50 60 70
TIME AFTER IMPLANTATION (DAYS)
UNDIFFERENTIATED HIF-1α-/- mESC
142 mmHg
7 mmHg
UNDIFFERENTIATED HIF-1α-/- mESC
142 mmHg
7 mmHg

Fig. 7B

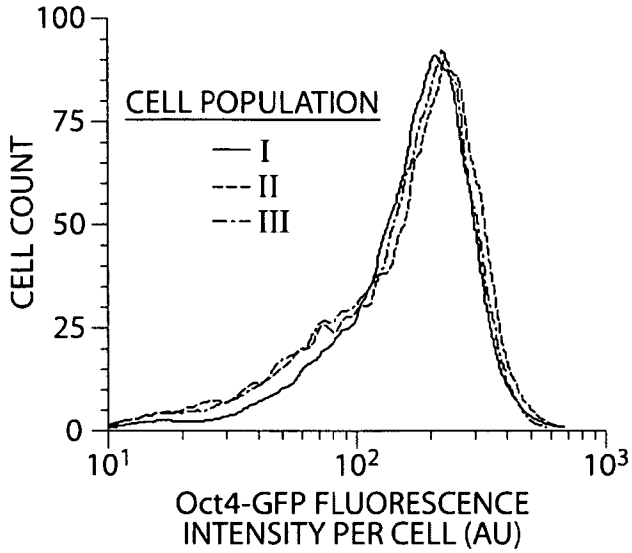
100
75
50
25
0
CELL COUNT
CELL POPULATION
I
II
III
10^1
10^2
10^3
Oct4-GFP FLUORESCENCE
INTENSITY PER CELL (AU)

Fig. 8A

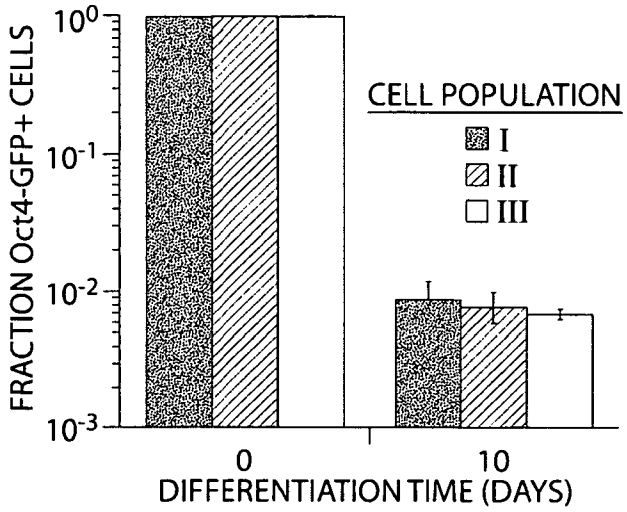
10^0
10^{-1}
10^{-2}
10^{-3}
FRACTION Oct4-GFP+ CELLS
CELL POPULATION
I
II
III
0
10
DIFFERENTIATION TIME (DAYS)

Fig. 8B

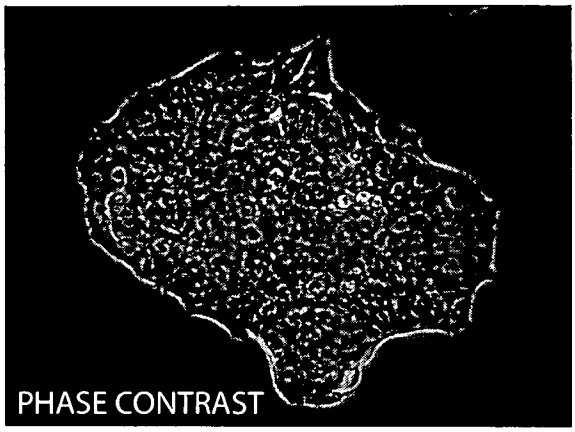

Fig. 8C

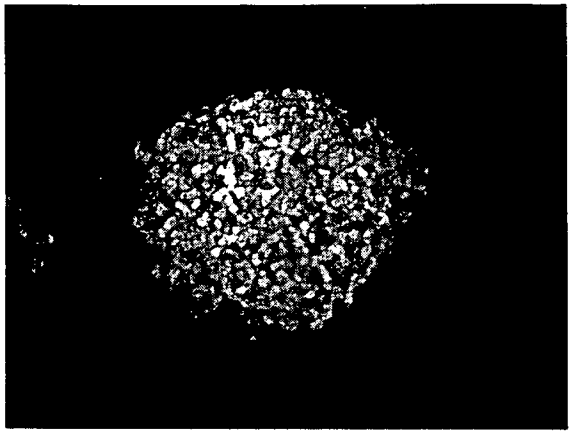

Fig. 8D

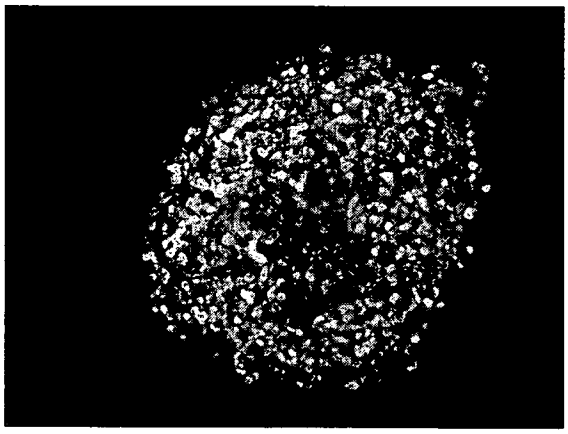

Fig. 8E

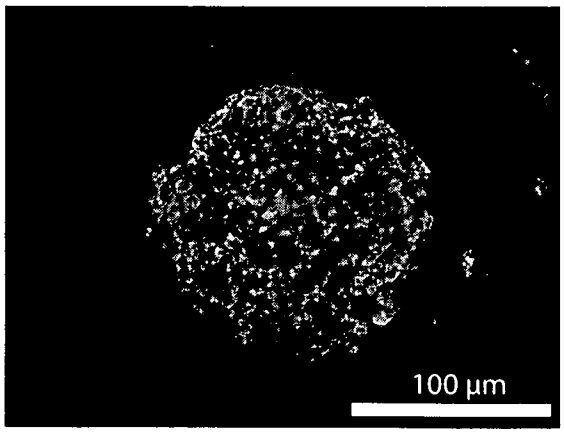

Fig. 8F

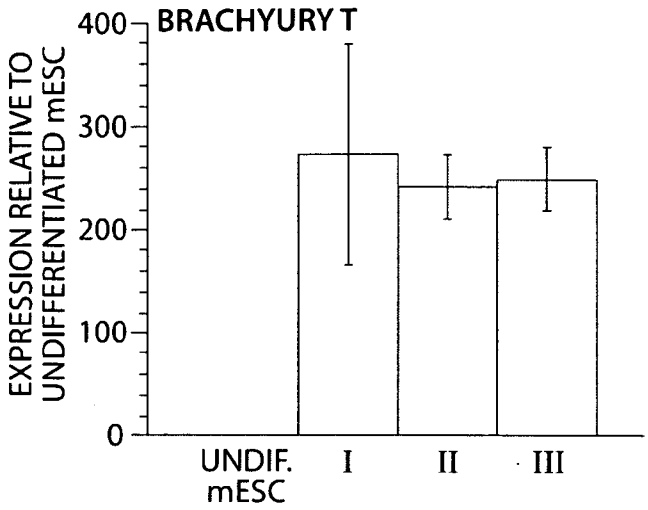
BRACHYURY T
EXPRESSION RELATIVE TO UNDIFFERENTIATED mESC
400
300
200
100
0
UNDIF. mESC
I
II
III

Fig. 9A

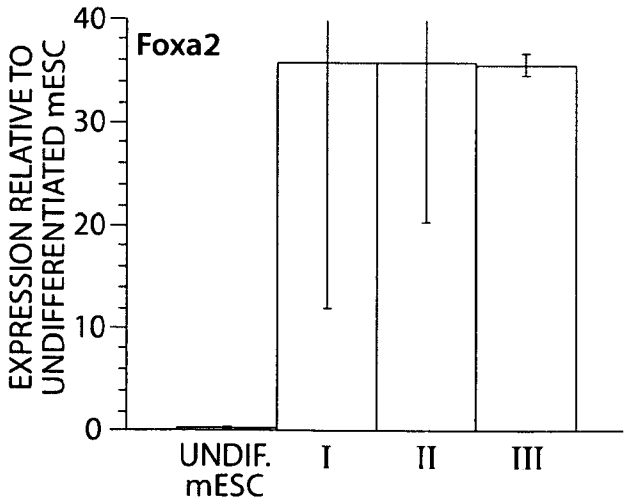
Foxa2
EXPRESSION RELATIVE TO UNDIFFERENTIATED mESC
40
30
20
10
0
UNDIF. mESC
I
II
III

Fig. 9B

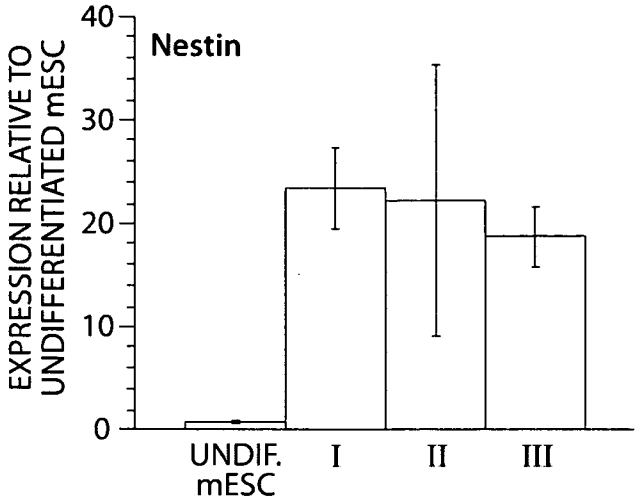
Nestin
EXPRESSION RELATIVE TO UNDIFFERENTIATED mESC
40
30
20
10
0
UNDIF. mESC
I
II
III

Fig. 9C

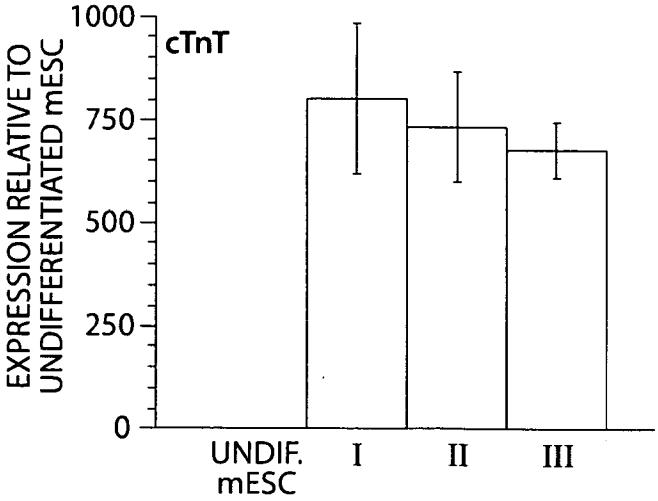
cTnT
EXPRESSION RELATIVE TO UNDIFFERENTIATED mESC
1000
750
500
250
0
UNDIF. mESC
I
II
III

Fig. 9D

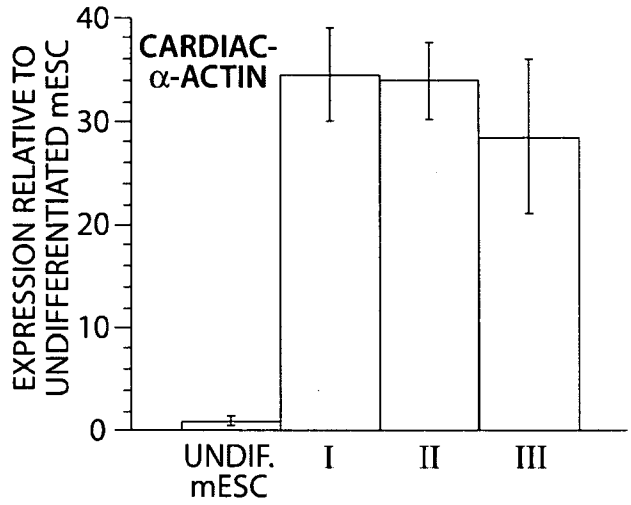
CARDIAC-
α-ACTIN
EXPRESSION RELATIVE TO UNDIFFERENTIATED mESC
40
30
20
10
0
UNDIF. mESC
I
II
III

Fig. 9E

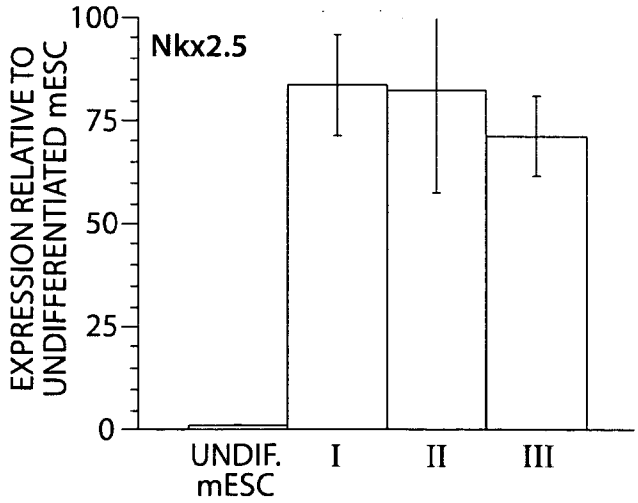
Nkx2.5
EXPRESSION RELATIVE TO UNDIFFERENTIATED mESC
100
75
50
25
0
UNDIF. mESC
I
II
III

Fig. 9F

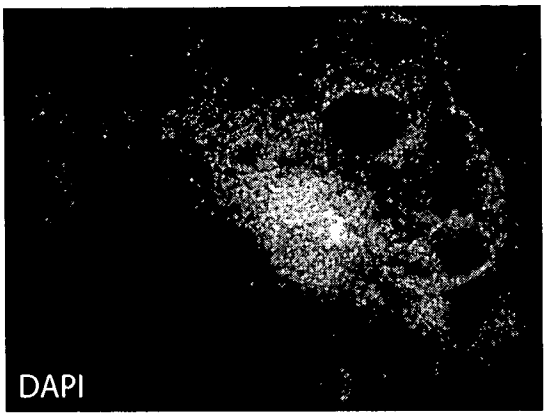

Fig. 10A



Fig. 10B



Fig. 10C

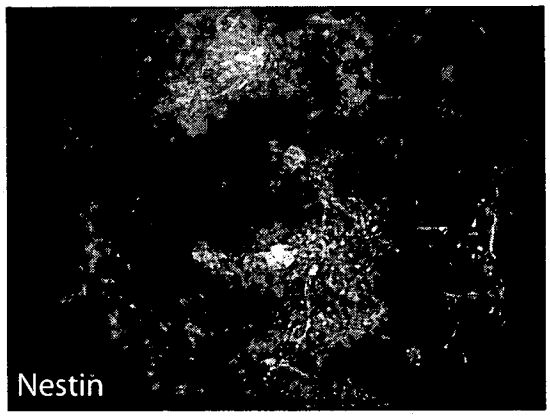

Fig. 10D

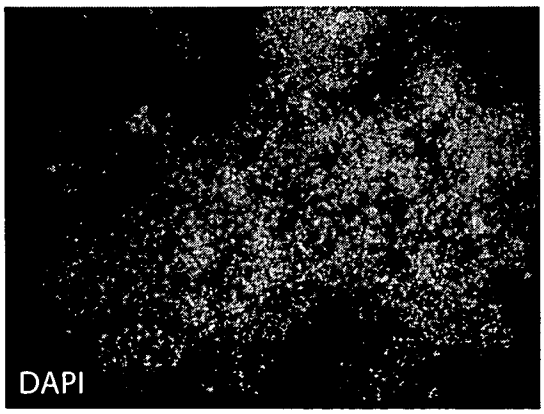

Fig. 10E

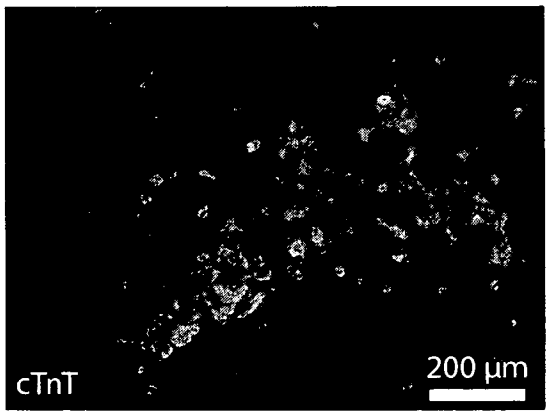

Fig. 10F

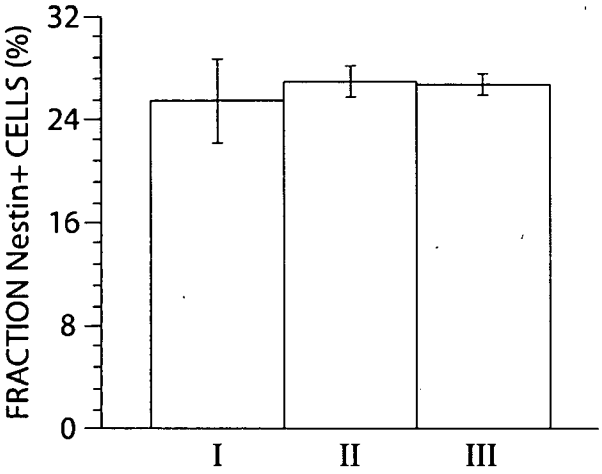

Fig. 11A

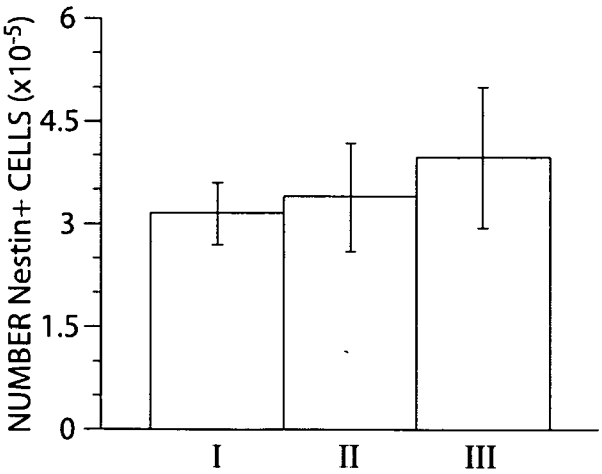

Fig. 11B

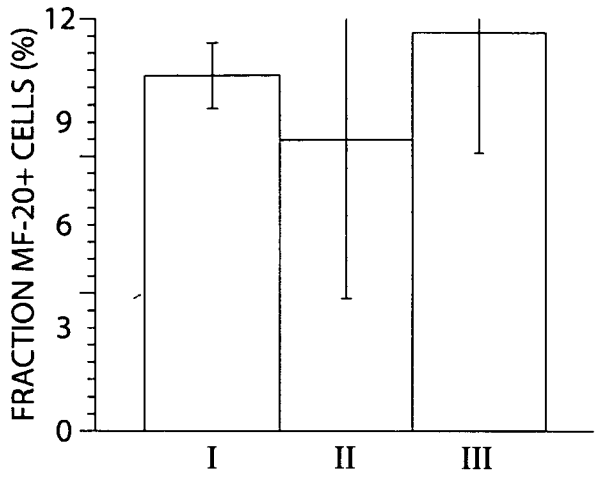

Fig. 11C

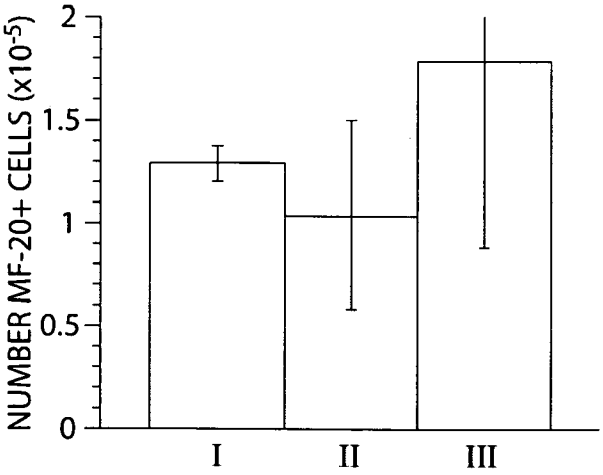

Fig. 11D

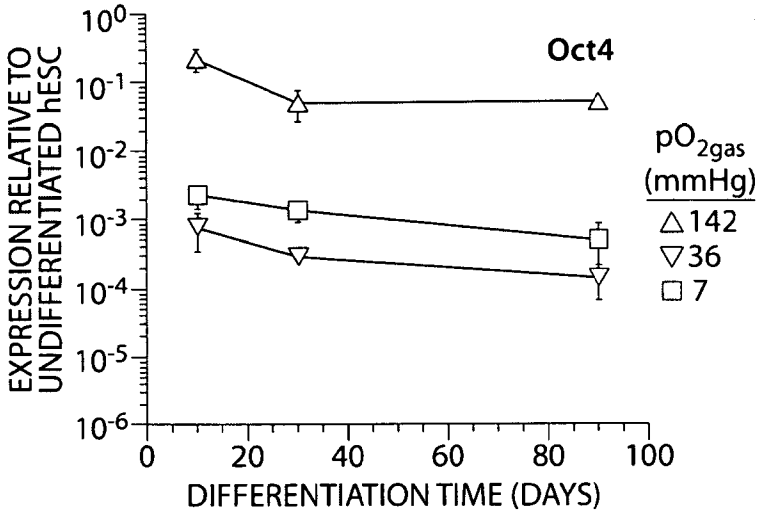

Fig. 12A

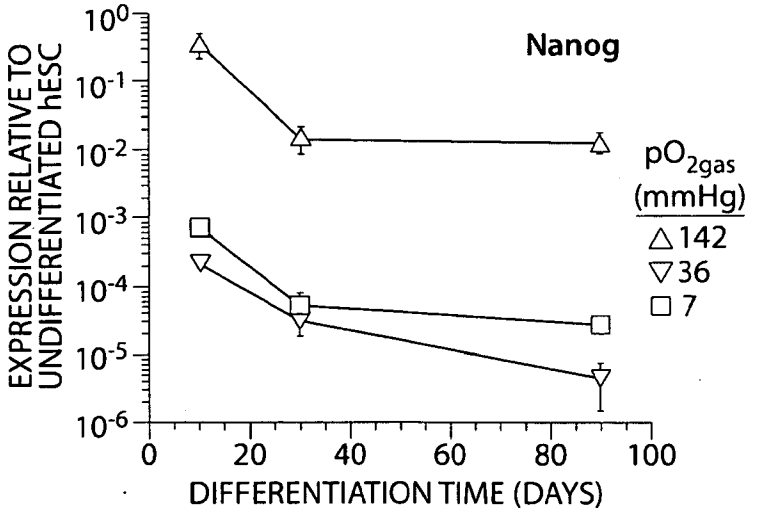

Fig. 12B

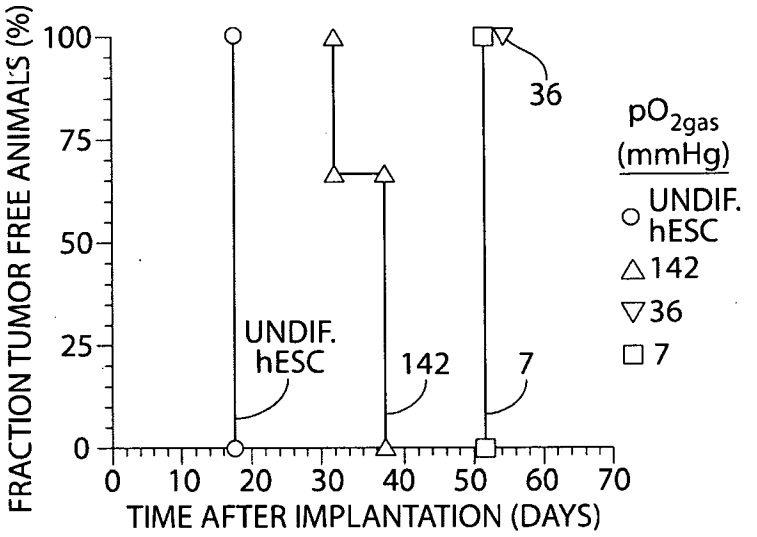
FRACTION TUMOR FREE ANIMALS (%)
100
75
50
25
0
0 10 20 30 40 50 60 70
TIME AFTER IMPLANTATION (DAYS)
UNDIF. hESC
142
7
36
pO2gas (mmHg)
UNDIF. hESC
142
36
7

Fig. 12C

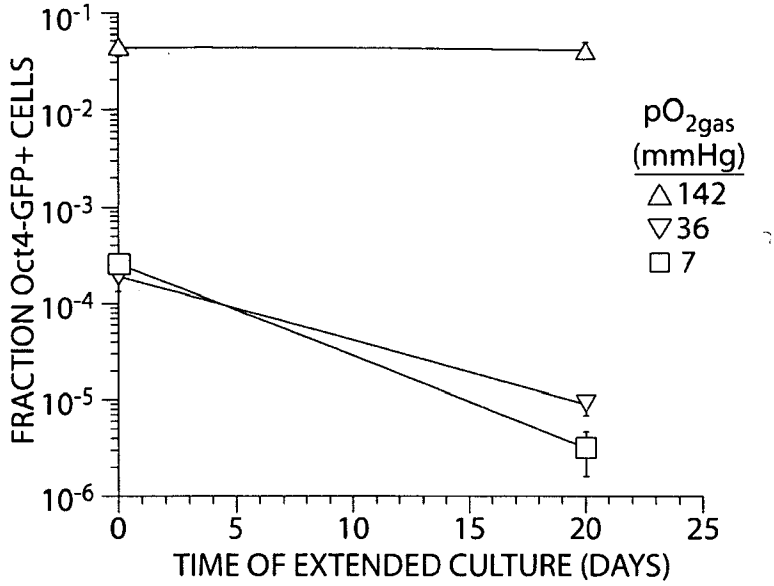
FRACTION Oct4-GFP+ CELLS
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
0
5
10
15
20
25
TIME OF EXTENDED CULTURE (DAYS)
pO_{2gas} (mmHg)
△142
▽36
□7

Fig. 13

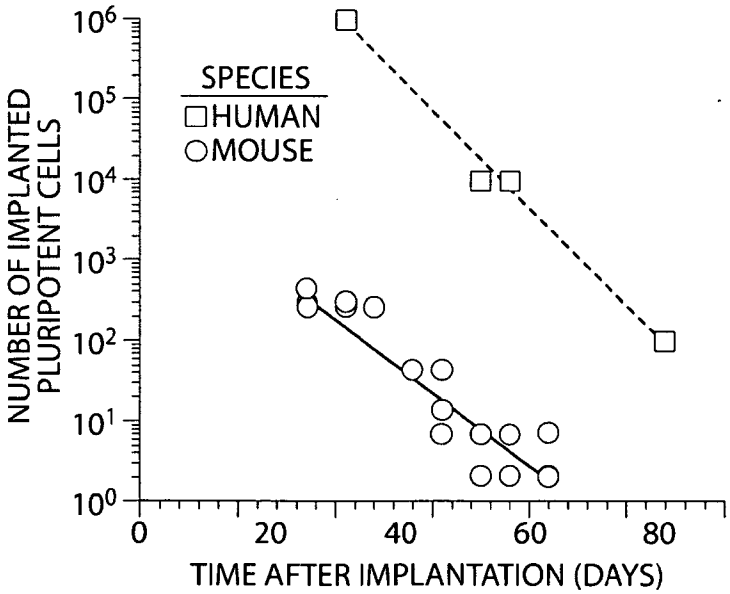
NUMBER OF IMPLANTED PLURIPOTENT CELLS
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
0
20
40
60
80
TIME AFTER IMPLANTATION (DAYS)
SPECIES
□HUMAN
○MOUSE

Fig. 14

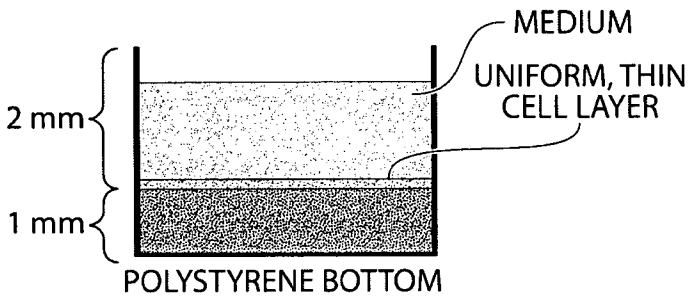

Fig. 15A

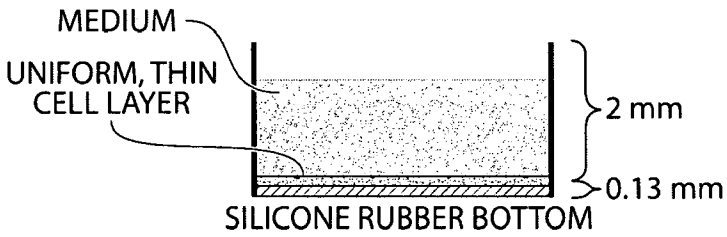

Fig. 15B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/001934

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/0735 C12N5/074
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MONDRAGON-TERAN PAUL ET AL: "Lowering oxygen tension enhances the differentiation of mouse embryonic stem cells into neuronal cells.", BIOTECHNOLOGY PROGRESS 2009 SEP-OCT LNKD-PUBMED:19455654, vol. 25, no. 5, 19 May 2009 (2009-05-19), pages 1480-1488, XP002607342, ISSN: 1520-6033 * abstract ----- -/--	1-76

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 October 2010	15/11/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Fax: (+31–70) 340–3016	Loubradou-Bourges, N

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/001934

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KIM T-S ET AL: "Increase in dopaminergic neurons from mouse embryonic stem cell-derived neural progenitor/stem cells is mediated by hypoxia inducible factor-1alpha", JOURNAL OF NEUROSCIENCE RESEARCH, WILEY-LISS, US, vol. 86, no. 11, 15 August 2008 (2008-08-15), pages 2353-2362, XP002555196, ISSN: 0360-4012, DOI: DOI:10.1002/JNR.21687 [retrieved on 2008-04-25] * abstract	1-76
X	WO 00/29550 A2 (CALIFORNIA INST OF TECHN [US]; CESTE MARIE [US]; DOYLE JOHN [US]; WOLD) 25 May 2000 (2000-05-25) examples 3,5	1-76
X	BAUWENS C ET AL: "DEVELOPMENT OF A PERFUSION FED BIOREACTOR FOR EMBRYONIC STEM CELL-DERIVED CARDIOMYOCYTE GENERATION: OXYGEN-MEDIATED ENHANCEMENT OF CARDIOMYOCYTE OUTPUT", BIOTECHNOLOGY AND BIOENGINEERING, WILEY & SONS, HOBOKEN, NJ, US, vol. 90, no. 4, 1 May 2005 (2005-05-01), pages 452-461, XP009063637, ISSN: 0006-3592, DOI: DOI:10.1002/BIT.20445 * abstract	1-51, 54-76
X	NIEBRUEGGE S ET AL: "Generation of human embryonic stem cell-derived mesoderm and cardiac cells using size-specified aggregates in an oxygen-controlled bioreactor", BIOTECHNOLOGY AND BIOENGINEERING 20090201 JOHN WILEY AND SONS INC. USA LNKD-DOI:10.1002/BIT.22065, vol. 102, no. 2, 1 February 2009 (2009-02-01), pages 493-507, XP002607343, ISSN: 0006-3592 * abstract	1-69, 74-76
X	WO 2009/035217 A1 (CHABIOTECH CO LTD [KR]; CHUNG HYUNG-MIN [KR]; KIM JU-MI [KR]; LEE SOO-) 19 March 2009 (2009-03-19) claims 1-7; examples 1-5	1-76

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/001934

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KOAY E J ET AL: "Hypoxic chondrogenic differentiation of human embryonic stem cells enhances cartilage protein synthesis and biomechanical functionality", OSTEOARTHRITIS AND CARTILAGE 200812 GB LNKD- DOI:10.1016/J.JOCA.2008.04.007, vol. 16, no. 12, December 2008 (2008-12), pages 1450-1456, XP002607344, ISSN: 1063-4584 * abstract	1-76
X	PURPURA KELLY A ET AL: "Soluble Flt-1 Regulates Flk-1 Activation to Control Hematopoietic and Endothelial Development in an Oxygen-Responsive Manner", STEM CELLS (MIAMISBURG), vol. 26, no. 11, 2008, pages 2832-2842, XP002607465, ISSN: 1066-5099 * abstract figure 1	1,3-76
X,P	MILLMAN J R ET AL: "The effects of low oxygen on self-renewal and differentiation of embryonic stem cells", CURRENT OPINION IN ORGAN TRANSPLANTATION 2009 LIPPINCOTT WILLIAMS AND WILKINS USA LNKD- DOI:10.1097/MOT.0B013E3283329D53, vol. 14, no. 6, December 2009 (2009-12), pages 694-700, XP009133588, ISSN: 1087-2418 * abstract table 2	1-76
X,P	HORTON R E ET AL: "Engineering microenvironments for embryonic stem cell differentiation to cardiomyocytes", REGENERATIVE MEDICINE 2009 FUTURE MEDICINE LTD. GBR LNKD- DOI:10.2217/RME.09.48, vol. 4, no. 5, 2009, pages 721-732, XP009140541, ISSN: 1746-0751 page 728	1-76
X,P	FERNANDES T G ET AL: "Different stages of pluripotency determine distinct patterns of proliferation, metabolism, and lineage commitment of embryonic stem cells under hypoxia", STEM CELL RESEARCH 2010 ELSEVIER NLD LNKD- DOI:10.1016/J.SCR.2010.04.003, vol. 5, no. 1, July 2010 (2010-07), pages 76-89, XP27106373, ISSN: 1873-5061 * abstract	1-76

-/--

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/001934

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	SILVAN U ET AL: "Hypoxia and pluripotency in embryonic and embryonal carcinoma stem cell biology", DIFFERENTIATION 2009 ELSEVIER LTD GBR LNKD- DOI:10.1016/J.DIFF.2009.06.002, vol. 78, no. 2-3, 14 July 2009 (2009-07-14), pages 159-168, XP26601260, ISSN: 0301-4681 * abstract page 161	1-76
X	POWERS D E ET AL: "Effects of oxygen on mouse embryonic stem cell growth, phenotype retention, and cellular energetics", BIOTECHNOLOGY AND BIOENGINEERING 20081001 US LNKD- DOI:10.1002/BIT.21986, vol. 101, no. 2, 1 October 2008 (2008-10-01), pages 241-254, XP002607347, ISSN: 0006-3592 * abstract	1-76
A	MA TENG ET AL: "Hypoxia and stem cell-based engineering of mesenchymal tissues.", BIOTECHNOLOGY PROGRESS 2009 JAN-FEB LNKD- PUBMED:19198002, vol. 25, no. 1, January 2009 (2009-01), pages 32-42, XP002607348, ISSN: 1520-6033 the whole document	1-76
A	KUROSAWA H ET AL: "Effect of oxygen on in vitro differentiation of mouse embryonic stem cells", JOURNAL OF BIOSCIENCE AND BIOENGINEERING, ELSEVIER, AMSTERDAM, NL, vol. 101, no. 1, 1 January 2006 (2006-01-01), pages 26-30, XP025182966, ISSN: 1389-1723, DOI: DOI:10.1263/JBB.101.26 [retrieved on 2006-01-01] the whole document	1-76

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2010/001934

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   [X] on paper

   [X] in electronic form

   b. (time)

   [X] in the international application as filed

   [ ] together with the international application in electronic form

   [ ] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2010/001934

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0029550 A2	25-05-2000	AU 1824600 A	05-06-2000
		AU 2154200 A	05-06-2000
		CA 2351556 A1	25-05-2000
		EP 1144591 A2	17-10-2001
		EP 1131406 A2	12-09-2001
		JP 2002530067 T	17-09-2002
		JP 2002530068 T	17-09-2002
		WO 0029549 A2	25-05-2000
		US 2004005704 A1	08-01-2004
WO 2009035217 A1	19-03-2009	EP 2188370 A1	26-05-2010
		KR 20090028104 A	18-03-2009
		US 2010221833 A1	02-09-2010