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GENERATING PLURIPOTENT CELLS DE NOVO

Technical Field
[0001] The technology described herein relates to the production of pluripotent cells.

Background
[0002] Current methods of obtaining pluripotent cells rely primarily upon tissues of limited availability (e.g. embryonic tissue or cord blood) or the addition of reprogramming factors (Hanna, J. et al. Cell 2008 133, 250-264; Hockemeyer, D. et al. Cell stem cell 2008 3, 346-353; Kim, D. et al. Cell stem cell 2009 4, 472-476; Kim, J. B. Nature 2009 461, 649-643; Okabe, M. et al. Blood 2009 114, 1764-1767), which involves introduction of exogenous nucleic acids. Methods of readily producing stem cells, particularly autologous stem cells, without the complications introduced by the addition of exogenous reprogramming factors, would accelerate research into cellular differentiation and the development of stem-cell based therapies. While it is hypothesized that damage to cells as a result of exposure to irritants, such as burns, chemical injury, trauma and radiation, may alter normal somatic cells to become cancer cells, there is no direct evidence that healthy adult somatic cells can be converted to other states without the specific manipulation of reprogramming factors.

has been hypothesized that these cells represent either a population of adult stem cells or are merely an artifact of the techniques being used. In either case, they remain rare and do not represent an adequate source of pluripotent cells for research and therapeutic purposes.

**Summary**

[0004] Described herein are methods of generating or producing pluripotent cells de novo, from differentiated or adult cells. Aspects of the technology described herein which relate to the production of pluripotent cells are based upon the inventors’ recognition that environmental stresses, for example, those which reduce the amount of cytoplasm and/or mitochondria in a somatic cell can induce the cell to convert to a pluripotent phenotype. In some embodiments, the environmental stress can be an external physical and/or chemical stress. In some embodiments of the methods described herein, the pluripotent cells are produced without utilizing gene induction. The pluripotent cells produced according to the methods described herein are capable of differentiation into each of the three germ layers both *in vitro* and *in vivo*, generate teratomas *in vivo*, and can generate embryos and chimeric mice.

[0005] In one aspect, the technology described herein relates to a method to generate a pluripotent cell, comprising subjecting a somatic cell to a stress, and selecting cells exhibiting pluripotency, wherein the somatic cell is in not present as part of a tissue. In some embodiments, the stress comprises removing at least about 40% of the cytoplasm from the somatic cell. In some embodiments, the stress comprises removing at least about 40% of the mitochondria from the somatic cell.

[0006] In some embodiments, the somatic cell is an isolated somatic cell. In some embodiments, the somatic cell is present in a heterogeneous population of somatic cells. In some embodiments, the somatic cell is present in a homogenous population of somatic cells.

[0007] In some embodiments, selecting the cells exhibiting pluripotency comprises selecting cells expressing Oct4 or Nanog, or Oct4 and Nanog expression. In some embodiments, selecting cells exhibiting pluripotency comprises selecting cells which are not adherent.

[0008] In some embodiments, at least about 50% of the cytoplasm is removed from the somatic cell. In some embodiments, at least about 60% of the cytoplasm is removed from the somatic cell. In some embodiments, between 60-80% of the cytoplasm is removed from the somatic cell. In some embodiments, at least about 80% of the cytoplasm is removed from the somatic cell. In some embodiments, at least about 90% of the cytoplasm is removed from the somatic cell.
somatic cell.

In some embodiments, the stress comprises exposure of the somatic cell to at least one environmental stimulus selected from: trauma, mechanical stimuli, chemical exposure, ultrasonic stimulation, oxygen-deprivation, radiation, and exposure to extreme temperatures. In some embodiments, the exposure to extreme temperatures comprises exposing the somatic cell to temperatures below 35°C or above 42°C. In some embodiments, the exposure to extreme temperatures comprises exposing the somatic cell to temperatures at, or below freezing or exposure of the somatic cell to temperatures at least about 85°C.

In some embodiments, the mechanical stimulus comprises passing the somatic cell through at least one device with a smaller aperture than the size of the somatic cell. In some embodiments, the mechanical stimulus comprises passing the somatic cell through several devices having progressively smaller apertures.

In some embodiments, the removal of a portion of the cytoplasm removes at least about 50% of the mitochondria from the cytoplasm. In some embodiments, the removal of cytoplasm or mitochondria removes about 50%-90% of the mitochondria from the cytoplasm. In some embodiments, the removal of cytoplasm or mitochondria removes more than 90% of the mitochondria from the cytoplasm.

In some embodiments, the method further comprises culturing the pluripotent cell to allow propagation of the stem cell.

In some embodiments, the pluripotent stem cell expresses one or more pluripotent stem cell markers selected from the group consisting of: Oct4 and Nanog.

In some embodiments, the somatic cell is a mammalian somatic cell. In some embodiments, the somatic cell is a human somatic cell. In some embodiments, the somatic cell is an adult somatic cell or a neonatal somatic cell.

In some embodiments, the method further comprises maintaining the pluripotent cell in vitro.

In one aspect, the technology described herein relates to an assay comprising; contacting a pluripotent stem cell produced by the method described herein with a candidate agent. In some embodiments, this assay is used to identify agents which affect one or more of the viability, differentiation, proliferation of the pluripotent stem cell.

In one aspect, the technology described herein relates to the use of a pluripotent
stem cell produced by the method described herein in a method of cell therapy for a subject.

[0018] In one aspect, the technology described herein relates to a method of autologous cell therapy in a subject in need of cell therapy, comprising (a) generating a pluripotent stem cell from a somatic cell according to the methods described herein, wherein the somatic cell is obtained from the subject, and (b) administering a composition comprising the pluripotent stem cell or a differentiated progeny thereof to the subject.

[0019] In some embodiments, the method further comprises differentiating the pluripotent stem cell along a pre-defined cell lineage prior to administering the composition comprising the differentiated cell or progeny thereof to the subject.

[0020] In one aspect, the technology described herein relates to a composition comprising a pluripotent stem cell, wherein the pluripotent stem cell is generated from a somatic cell by the methods described herein.

**Brief Description of the Drawings**

[0021] This patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0022] Figures 1A-1E depict Oct4 expressing cell generation from CD45 positive somatic cells. Figure 1A depicts Oct4-GFP expression of stress treated cells. Stress- treated cells express Oct4-GFP, while untreated controls did not. Magnification of an Oct4-expressing colony is shown in the upper right in the stress-treated group. Scale bar indicates 100 μm. Figure 1B depicts population analysis of stress-treated cells and non-stress treated control. A GFP expressing cell population is observed only in the stress treated group at day 5. Figure 1C depicts single cell images of CD45 positive cells before and after the stress treatment. Cells were stained with CD45 antibody and DAPI. Scale bar indicates 10 μm. Figure 1D depicts cell-size analysis of CD45 positive cells before and after the stress treatment at day 7. Figure 1E depicts chronological change of CD45 positive cells after the stress treatment.

[0023] Figures 2A-2C depict characterization of animal callus cells (ACCs). Figure 2A depicts immunohistochemistry with stem cell markers E-cadherin, Nanog, SSEA-1, and PCAM-1. Nuclei were stained with DAPI. Scale bar indicates 50μm. Figure 2B depicts chronological gene expression change of pluripotent marker genes. The messenger RNA levels were normalized to GAPDH. (n=3, the average+S.D.) Figure 2C depicts methylation analysis of
Oct4 and Nanog promoter genes.

Figures 3A-3E depict cellular modifications after stress treatment. Figure 3A depicts relative gene expression of stress defense genes during the ACCs generation phase. Samples were collected at day 3 and day 7 and compared with CD45 positive cells. Blue graphs indicate the gene expression of heat shock proteins. Green graph indicates DNA repair gene expression. Red graphs indicate the gene expression of redox genes. (n=3, the average+S.D.) Figure 3B depicts mitochondrial morphological changes of ACCs. The panel marked (i) depicts GFP expression of ACs at day10. ACs at day 10 contained GFP expressing central cells and non-GFP differentiated peripheral cells. The panel marked (ii) depicts mitochondrial morphology of ACCs. The panel marked (iii) depicts mitochondrial morphology of differentiated cells. Mitochondria were stained with Mitotracker (Red) and nuclei were stained with DAPI. GFP shows Oct4 expression. Scale bar indicates 10 μm. Figure 3 C depicts total cellular ATP measurement. (n=3, the average+S.D.) Figure 3D depicts ROS measurement. Error bars indicate SD. Figure 3E depicts relative gene expression of mtDNA replication factors. (n=3, the average+S.D.)

Figures 4A-4E depict chimera mouse generation from ACCs. Figure 4A depicts a scheme of chimera mouse generation. Panel (i) demonstrates that ACs were dissociated into single cells with trypsin or (panel ii) ACs were cut into small pieces then injected into blastocysts. Figure 4B depicts E13.5 Chimera fetuses derived from ACCs from CD45 positive cells. GFP shows tissues generated from ACCs. Figure 4C depicts adult chimera mice generated with ACCs. Figure 4D depicts chimera contribution analysis. Tissues from 9 pups were analyzed by FACS. Figure 4E depicts 4N embryos at E10.5 generated with ACCs.

Figures 5A-5C experiments with ACC-generating conditions. Figure 5A demonstrates that CD45 positive cells were exposed to various stresses and Oct4-GFP expression was analyzed by FACS. Percentage of Oct4-GFP expressing cells in survived cells after stress treatment. (n=3, the average+S.D.) Figure 5B depicts the determination of pH condition. CD45 positive cells were exposed to different pH solutions. At 3 days after stress treatment, Oct4-GFP expression was analyzed by FACS. Figure 5C depicts the determination of culture condition. Stress treated cells were cultured in various mediums. The number of GFP-expressing ACs was counted at day 14. (n=3, the average+S.D.)

Figures 6A-6B depict ACCs generation from CD45 positive cells derived from ICR mice. Figure 6A depicts chronological change of CD45 positive cells after stress treatment. The
expression of E-cadherin and SSEA-1 was analyzed by FACS. Figure 6B demonstrates that Oct4 gene expression of E-Cadherin/SSEA1 double positive cells was confirmed by RT-PCR. (n=3, the average±S.D.)

Figures 7A-7C depict ACC generation from various tissues derived from GOF mice. Figure 7A depicts the ratio of Oct4-GFP expressing cells after stress treatment. Somatic cells were isolated from various tissues, and exposed to various stresses. Oct4-GFP expression was analyzed by FACS. Figure 7B depicts E-cadherin, Nanog, PCAM-1 and AP expression of ACs. Pictured are fat ACs derived from GOF mice. Figure 7C depicts embryonic gene expression of ACCs derived from various tissues. Gene expressions were normalized by GAPDH. (n=3, the average±S.D.)

Figure 8 depicts relative gene expression of stress defense genes during the first 7 days. After stress treatment, cells were collected at day 1, 3 and 7, and gene expression was compared with native CD45 positive cells. Blue graphs indicate the gene expressions of heat shock proteins. Green graph indicates DNA repair gene expression. Red graphs indicate the gene expression of redox genes. Y-axis indicates relative folds of expression.

Figures 9A-9E depict differentiation of ACCs. Figure 9A depicts an in vitro differentiation assay. ACCs were sorted and plated into differentiation medium. ACCs differentiated into cells from three germ layers, and expressed ectoderm specific marker βIII tubulin and GFAP, mesenchymal marker, α-smooth muscle actin, and endoderm marker, α-fetoprotein and cytokeratin 7. Figure 9B depicts an in vivo differentiation assay. Transplanted cells were histologically analyzed. ACCs generated tissues representing all three germ layers. Figure 9C depicts 2N chimeras generated with ACCs derived from GOF mice (top) and 129F1 mice (bottom). Figure 9D depicts a chimera contribution analysis. Chimera fetuses generated with ACCs derived from various somatic cells were analyzed by FACS. Graph shows the average of 5 chimera fetuses at E13.5 to 15.5. Figure 9E depicts 4N embryos at E9.5 generated with ACCs derived from F1 GFP (C57BL/6×DBA/2 or 129/SvxC57BL/6GFP).

Figure 10 demonstrates that stress treatment caused reprogramming to somatic cells via Mesenchymal-Epithelial Transition (MET). The expression of MET-related genes is shown in native cells, and in cells 3 and 7 days after stress treatment was begun.

Figure 11 depicts FACS analysis of cell populations before and after stress. GFP expression was evident, indicating generation of pluripotent cells, in post-stressed cell
populations from each tested tissue type.

**Detailed Description**

[0033] Aspects of the technology described herein relate to the production or generation of pluripotent cells from somatic cells. The aspects of the technology described herein are based upon the inventors’ discovery that stress can induce the production of pluripotent stem cells from somatic cells without the need to introduce exogenous nucleic acids or proteins to the cell. In some embodiments, the stress induces a reduction in the amount of cytoplasm and/or mitochondria in a somatic cell; triggering a dedifferentiation process and resulting in pluripotent cells. These pluripotent cells are characterized by one or more of, the ability to differentiate into each of the three germ layers (*in vitro* and/or *in vivo*), the generation of teratoma-like cell masses *in vivo*, and the ability to generate viable embryos and/or chimeric mice.

[0034] Described herein are experiments demonstrating that treatment of somatic cells with certain environmental stresses, including, but not limited to stresses which reduce the amount of cytoplasm and/or mitochondria in the somatic cell, can reduce mitochondrial activity, demethylate regions of the genome associated with dedifferentiation, cause the cells to display markers of known dedifferentiation pathways. Accordingly, in some embodiments, provided herein are methods of generating pluripotent cells from somatic cells, the methods comprising removing at least about 40% of the cytoplasm and/or mitochondria from a somatic cell, and selecting pluripotency or cells exhibiting pluripotency markers, wherein the somatic cell is not present in a tissue. Also described herein are other stress treatments that can generate pluripotent cells from somatic cells.

[0035] For convenience, certain terms employed herein, in the specification, examples and appended claims are collected here. Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. Unless explicitly stated otherwise, or apparent from context, the terms and phrases below do not exclude the meaning that the term or phrase has acquired in the art to which it pertains. The definitions are provided to aid in describing particular embodiments, and are not intended to limit the claimed invention, because the scope of the invention is limited only by the claims. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0036] As used herein the term "comprising" or "comprises" is used in reference to
compositions, methods, and respective component(s) thereof, that are essential to the method or composition, yet open to the inclusion of unspecified elements, whether essential or not.

[0037] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment.

[0038] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[0039] As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural references unless the context clearly dictates otherwise. Thus for example, references to "the method" includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The abbreviation, "e.g." is derived from the Latin exempli gratia, and is used herein to indicate a non-limiting example. Thus, the abbreviation "e.g." is synonymous with the term "for example."


[0041] Unless otherwise stated, the present invention was performed using standard procedures, as described, for example in Sambrook et al., Molecular Cloning: A Laboratory Manual (3 ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (2001); Davis et al., Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York,
The terms “decrease,” “reduce,” “reduced,” and “reduction” are all used herein generally to mean a decrease by a statistically significant amount relative to a reference. However, for avoidance of doubt, “reduce,” “reduction”, or “decrease” typically means a decrease by at least 10% as compared to the absence of a given treatment and can include, for example, a decrease by at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99%, up to and including, for example, the complete absence of the given entity or parameter as compared to the absence of a given treatment, or any decrease between 10-99% as compared to the absence of a given treatment.

The terms “increased”, “increase”, or “enhance” are all used herein generally to mean an increase by a statistically significant amount; for the avoidance of any doubt, the terms “increased”, “increase”, or “enhance” means an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level.

As used herein, the terms “treat,” “treatment,” “treating,” or “amelioration” when used in reference to a disease, disorder or medical condition, refer to therapeutic treatments for a condition, wherein the object is to reverse, alleviate, ameliorate, inhibit, slow down or stop the progression or severity of a symptom or condition. The term “treating” includes reducing or alleviating at least one adverse effect or symptom of a condition. Treatment is generally “effective” if one or more symptoms or clinical markers are reduced. Alternatively, treatment is “effective” if the progression of a condition is reduced or halted. That is, “treatment” includes not
just the improvement of symptoms or markers, but also a cessation or at least slowing of progress or worsening of symptoms that would be expected in the absence of treatment. Beneficial or desired clinical results include, but are not limited to, alleviation of one or more symptom(s), diminishment of extent of the deficit, stabilized (i.e., not worsening) state of health, delay or slowing of the disease progression, and amelioration or palliation of symptoms. Treatment can also include the subject surviving beyond when mortality would be expected statistically.

[0045] As used herein, the term "administering," refers to the placement of a pluripotent cell produced according to the methods described herein and/or the at least partially differentiated progeny of such a pluripotent cell into a subject by a method or route which results in at least partial localization of the cells at a desired site. A pharmaceutical composition comprising a pluripotent cell produced according to the methods described herein and/or the at least partially differentiated progeny of such a pluripotent cell can be administered by any appropriate route which results in an effective treatment in the subject.

[0046] As used herein, a "subject" means a human or animal. Usually the animal is a vertebrate such as a primate, rodent, domestic animal or game animal. Primates, for example, include chimpanzees, cynomologous monkeys, spider monkeys, and macaques, e.g., Rhesus monkeys. Rodents include mice, rats, woodchucks, ferrets, rabbits and hamsters. Domestic and game animals include cows, horses, pigs, deer, bison, buffalo, feline species, e.g., domestic cat, canine species, e.g., dog, fox, wolf, avian species, e.g., chicken, emu, ostrich, and fish, e.g., trout, catfish and salmon. Patient or subject includes any subset of the foregoing, e.g., all of the above. In certain embodiments, the subject is a mammal, e.g., a primate, e.g., a human.

[0047] Preferably, the subject is a mammal. The mammal can be a human, non-human primate, mouse, rat, dog, cat, horse, or cow, but is not limited to these examples. Mammals other than humans can be advantageously used as subjects that represent animal models of a disease associated with a deficiency, malfunction, and/or failure of a given cell or tissue or a deficiency, malfunction, or failure of a stem cell compartment. In addition, the methods described herein can be used to treat domesticated animals and/or pets. A subject can be male or female. A subject can be one who has been previously diagnosed with or identified as suffering from or having a deficiency, malfunction, and/or failure of a cell type, tissue, or stem cell compartment or one or more diseases or conditions associated with such a condition, and optionally, but need not have already undergone treatment for such a condition. A subject can also be one who has been
diagnosed with or identified as suffering from a condition including a deficiency, malfunction, 
and/or failure of a cell type or tissue or of a stem cell compartment, but who shows improvements 
in known risk factors as a result of receiving one or more treatments for such a condition. 
Alternatively, a subject can also be one who has not been previously diagnosed as having such a 
condition. For example, a subject can be one who exhibits one or more risk factors for such a 
condition or a subject who does not exhibit risk factors for such conditions.

[0048] As used herein, the term “select”, when used in reference to a cell or population of 
cells, refers to choosing, separating, segregating, and/or selectively propagating one or more cells 
having a desired characteristic. The term “select” as used herein does not necessarily imply that 
cells without the desired characteristic are unable to propagate in the provided conditions.

[0049] As used herein, “maintain” refers to continuing the viability of a cell or population 
of cells. A maintained population will have a number of metabolically active cells. The number of 
these cells can be roughly stable over a period of at least one day or can grow.

[0050] The term “statistically significant” or “significantly” refers to statistical 
significance and generally means a two standard deviation (2SD) difference above or below a 
reference, e.g. a concentration or abundance of a marker, e.g. a stem cell marker or differentiation 
marker. The term refers to statistical evidence that there is a difference. It is defined as the 
probability of making a decision to reject the null hypothesis when the null hypothesis is actually 
true. The decision is often made using the p-value.

[0051] Other than in the operating examples, or where otherwise indicated, all numbers 
expressing quantities of ingredients or reaction conditions used herein should be understood as 
modified in all instances by the term “about.” The term “about” when used in connection with 
percentages can mean ±1%.

[0052] Other terms are defined herein within the description of the various aspects of the 
technology described herein.

[0053] The aspects of the technology described herein relate to methods of generating a 
pluripotent cell from a somatic cell as well as uses and methods of using those pluripotent cells. In 
contrast with existing methods of generating pluripotent cells (i.e. induced pluripotent stem cells 
or iPS cells) which rely upon increasing the expression of reprogramming factors, for example, by 
introducing nucleic acid constructs encoding one or more reprogramming factors (e.g. Oct4), the 
methods described herein subject the somatic cells to a stress but do not require introduction of
foreign reprogramming actors.

[0054] In some embodiments, the stress reduces the volume of the cell's cytoplasm and/or the number of the cell’s mitochondria. The reduction of the volume of the cell’s cytoplasm or the number of the cell’s mitochondria induces a stress response during which the cell acquires at least pluripotent capabilities. In one aspect, described herein is a method to generate a pluripotent cell, comprising removing at least about 40% of the cytoplasm from a somatic cell, and selecting cells exhibiting pluripotency, wherein the somatic cell is not present in a tissue. In one aspect, the invention as described herein relates to a method to generate a pluripotent cell, comprising removing at least about 40% of the mitochondria from a somatic cell, and selecting cells exhibiting pluripotency, wherein the somatic cell is not present in a tissue.

[0055] As used herein, the term “pluripotent” refers to a cell with the developmental potential, under given sets of conditions, to differentiate to cell types characteristic of all three germ cell layers, i.e., endoderm (e.g., gut tissue), mesoderm (e.g., blood, muscle, and vessels), and ectoderm (e.g., skin and nerve).

[0056] As used herein, the term “somatic cell” refers to any cell other than a germ cell, a cell present in or obtained from a pre-implantation embryo, or a cell resulting from proliferation of such a cell in vitro. Stated another way, a somatic cell refers to any cells forming the body of an organism, as opposed to germline cells. In mammals, germline cells (also known as "gametes") are the spermatozoa and ova which fuse during fertilization to produce a cell called a zygote, from which the entire mammalian embryo develops. Every other cell type in the mammalian body—a part from the sperm and ova, the cells from which they are made (gametocytes) and undifferentiated stem cells—is a somatic cell: internal organs, skin, bones, blood, and connective tissue are all made up of somatic cells. In some embodiments the somatic cell is a "non-embryonic somatic cell," by which is meant a somatic cell that is not present in or obtained from an embryo and does not result from proliferation of such a cell in vitro. In some embodiments the somatic cell is an "adult somatic cell," by which is meant a cell that is present in or obtained from an organism other than an embryo or a fetus or results from proliferation of such a cell in vitro. It is noted that adult and neonatal or embryonic cells can be distinguished by structural differences, e.g., epigenetic organization such as methylation patterns. In some embodiments, the somatic cell is a mammalian somatic cell. In some embodiments, the somatic cell is a human somatic cell. In some embodiments, the somatic cell is an adult somatic cell. In some embodiments, the somatic cell is a
neonatal somatic cell.

[0057] The somatic cell used in the methods described herein can be a cell which is not present in a tissue. As used herein, a “tissue” refers to an organized biomaterial (e.g. a group, layer, or aggregation) of similarly specialized cells united in the performance of at least one particular function. When cells are removed from an organized superstructure, or otherwise separated from an organized superstructure which exists in vivo, they are no longer present in a tissue. For example, when a blood sample is separated into two or more non-identical fractions, or a spleen is minced and mechanically-dissociated with Pasteur pipettes, the cells are no longer present in a tissue. In some embodiments, somatic cells which are not present in a tissue are isolated somatic cells. The term “isolated” as used herein in reference to cells refers to a cell that is mechanically or physically separated from another group of cells with which they are normally associated in vivo. Methods for isolating one or more cells from another group of cells are well known in the art. See, e.g., Culture of Animal Cells: a manual of basic techniques (3rd edition), 1994, R. I. Freshney (ed.), Wiley-Liss, Inc.; Cells: a laboratory manual (vol. 1), 1998, D. L. Spector, R. D. Goldman, L. A. Leinwand (eds.), Cold Spring Harbor Laboratory Press; Animal Cells: culture and media, 1994, D. C. Darling, S. J. Morgan, John Wiley and Sons, Ltd. Optionally the isolated cell has been cultured in vitro, e.g., in the presence of other cells.

[0058] In some embodiments, a somatic cell, while not present in a tissue, is present in a population of cells. In some embodiments, the population of cells is a population of somatic cells. As used herein, a “population of cells” refers to a group of at least 2 cells, e.g. 2 cells, 3 cells, 4 cells, 10 cells, 100 cells, 1000 cells, 10,000 cells, 100,000 cells or any value in between, or more cells. Optionally, a population of cells can be cells which have a common origin, e.g. they can be descended from the same parental cell, they can be clonal, they can be isolated from or descended from cells isolated from the same tissue, or they can be isolated from or descended from cells isolated from the same tissue sample. A population of cells can comprise 1 or more somatic cell types, e.g. 1 cell type, 2 cell types, 3 cell types, 4 cell types or more cell types. A population of cells can be heterogeneous or homogeneous. A population of cells can be substantially homogeneous if it comprises at least 90% of the same somatic cell type, e.g. 90%, 92%, 95%, 98%, 99%, or more of the cells in the population are of the same somatic cell type. A population of cells can be heterogeneous if less than 90% of the cells present in the population are of the same somatic cell type.
The methods of generating a pluripotent cell described herein can comprise, for example, removing part of the cytoplasm from a somatic cell and/or removing mitochondria from a somatic cell. In some embodiments, the removal of part of the cytoplasm or mitochondria from a somatic cell removes partial epigenetic control of the cell. In some embodiments, at least about 40% of the cytoplasm is removed, e.g. at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or more of the cytoplasm of a somatic cell is removed. In some embodiments, between 60% and 80% of the cytoplasm of a somatic cell is removed. In some embodiments, at least about 40% of the mitochondria are removed, e.g. at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or more of the mitochondria of a somatic cell are removed. In some embodiments, between 50% and 90% of the mitochondria of a somatic cell are removed.

The method of subjecting the somatic cell to stress and/or removing part of the cytoplasm or mitochondria from a somatic cell can be any environmental stimulus that will cause pores and/or ruptures in the membrane of a somatic cell below the threshold of lethality. Non-limiting examples of suitable environmental stimuli include trauma, mechanical stimuli, chemical exposure, ultrasonic stimulation, oxygen-deprivation, nutrient-deprivation, radiation, and/or exposure to extreme temperatures. In some embodiments, one environmental stimulus can be applied to a somatic cell. In some embodiments, multiple environmental stimuli can be applied to a somatic cell, e.g. 2 stimuli, 3 stimuli, 4 stimuli or more stimuli can be applied. Multiple environmental stimuli can be applied concurrently or separately.

The length of time for which the cells are exposed to stress can vary depending upon the stimulus being used. For example, when using low nutrition conditions to stress cells according to the methods described herein, the cells can be cultured under low nutrition conditions for 1 week or more, e.g. 1 week, 2 weeks, or 3 weeks or longer. In some embodiments, the cells are cultured under low nutrition conditions for about 3 weeks. In another non-limiting example, cells exposed to low pH or hypoxic conditions according to the methods described herein can be exposed for minutes or long, e.g. including for several hours, e.g. for at least 2 minutes, for at least 5 minutes, for at least 20 minutes, for at least 1 hour, for at least 2 hours, for at least 6 hours or longer.

Mechanical stimuli that induce the generation of pluripotent cells can include any form of contact of a substance or surface with the cell membrane which will mechanically disrupt
the integrity of the membrane. An exemplary form of mechanical stimulus is trituration. Trituration is a process of grinding and/or abrading the surface of a particle via friction. A non-limiting example of a process for trituration of a cell is to cause the cell to pass through a device wherein the device has an aperture smaller than the size of the cell. For example, a cell can be caused, by vacuum pressure and/or the flow of a fluid, to pass through a pipette in which at least part of the interior space of the pipette has a diameter smaller than the diameter of the cell. In some embodiments, the cell is passed through at least one device with a smaller aperture than the size of the cell. In some embodiments, the cell is passed through several devices having progressively smaller apertures. In some embodiments, cells can be tritured for 5 or more minutes, e.g. 5 minutes, 10 minutes, 20 minutes, 30 minutes, or 60 minutes. In some embodiments, the cells can be tritured by passing them through a Pasteur pipette with an internal diameter of 50 μm. In some embodiments, the cells can be tritured by passing them through a Pasteur pipette with an internal diameter of 50 μm for 20 minutes.

[0063] Other methods of applying stress necessary to induce somatic cells to generate pluripotent cells include, for example, exposure to certain chemicals, or physico-chemical conditions (e.g. high or low pH, osmotic shock, temperature extremes, oxygen deprivation, etc). Treatments of this kind and others that induce the generation of pluripotent cells are discussed further below. Chemical exposure can include, for example, any combination of pH, osmotic pressure, and/or pore-forming compounds that disrupt or compromise the integrity of the cell membrane. By way of non-limiting example, the cells can be exposed to low pH, streptolysin O, or distilled water (i.e. osmotic shock). Low pH can include a pH lower than 6.8, e.g. 6.7, 6.5, 6.3, 6.0, 5.8, 5.4, 5.0, 4.5, 4.0, or lower. In some embodiments, the low pH is from 5.4 to 5.6. In some embodiments, the low pH is about 5.5. In some embodiments, the cells can be exposed to low pH conditions for up to several days, e.g. for 6 days or less, for 4 days or less, for 3 days or less, for 2 days or less, for 1 day or less, for 12 hours or less, for 6 hours or less, for 3 hours or less, for 2 hours or less, for 1 hour or less, for 30 minutes or less, for 20 minutes or less, or less than 10 minutes. In some embodiments, the cells can be exposed to a pH from 5.4 to 5.6 for 3 days or less. In some embodiments, the cells can be exposed to a pH of from about 5.6 to 6.8 for 3 days or less. In some embodiments, the cells can be exposed of a pH of from about 5.6 to 6.8 for 1 hour or less. In some embodiments, the cells can be exposed of a pH of from about 5.6 to 6.8 for about 20 minutes.

[0064] In some embodiments, cells can be exposed to ATP to induce the generation of
pluripotent cells. In some embodiments, cells can be exposed to ATP at concentrations from about 20 μM to about 200 mM. In some embodiments, cells can be exposed to ATP at concentrations from about 200 μM to about 20 mM. In some embodiments, cells can be exposed to ATP at concentrations of about 2.4 mM. In some embodiments, cell can be exposed to ATP diluted in HBSS. In some embodiments, cells can be exposed to ATP for 1 minute or longer, e.g. at least 1 minute, at least 2 minutes, at least 5 minutes, at least 15 minutes, at least 30 minutes, at least 45 minutes, at least 1 hour or longer. In some embodiments, the cells can be exposed to ATP for from about 5 minutes to about 30 minutes. In some embodiments, the cells can be exposed to ATP for about 15 minutes. In some embodiments, the cells can be exposed to about 2.4 mM ATP for about 15 minutes.

[0065] In some embodiments, cells can be exposed to CaCl₂ to induce the generation of pluripotent cells. In some embodiments, cells can be exposed to CaCl₂ at concentrations from about 20 μM to about 200 mM. In some embodiments, cells can be exposed to CaCl₂ at concentrations from about 200 μM to about 20 mM. In some embodiments, cells can be exposed to CaCl₂ at concentrations of about 2 mM. In some embodiments, cells can be exposed to CaCl₂ diluted in HBSS. In some embodiments, cells can be exposed to CaCl₂ for 1 day or longer, e.g. at least 1 day, at least 2 days, at least 1 week, at least 2 weeks, at least 3 weeks or longer. In some embodiments, the cells can be exposed to CaCl₂ for from about 1 week to 3 weeks. In some embodiments, the cells can be exposed to CaCl₂ for about 2 weeks. In some embodiments, the cells can be exposed to about 2 mM CaCl₂ for about 2 weeks.

[0066] Examples of pore-forming compounds include streptolysin O (SLO), saponin, digitonin, filipin, Ae I, cytolysin of sea anemone, aerolysin, amatoxin, amoebapore, amoebapore homolog from Entamoeba dispar, brevinin-1E, brevinin-2E, barbatolysin, cytolysin of Enterococcus faecalis, delta hemolysin, diphtheria toxin, El Tor cytolysin of Vibrio cholerae, equinatoxin, enterotoxin of Aeromonas hydrophila, esculetin, granulysin, haemolysin of Vibrio parahaemolyticus, intermediysin of Streptococcus intermedins, the lentivirus lytic peptide, leukotoxin of Actinobacillus actinomycetemcomitans, magainin, melittin, membrane-associated lymphotxin, Met-enkephalin, neokyotorphin, neokyotorphin fragment 1, neokyotorphin fragment 2, neokyotorphin fragment 3, neokyotorphin fragment 4, NKlysin, paradaxin, alpha cytolysin of Staphylococcus aureus, alpha cytolysin of Clostridium septicum, Bacillus thuringiensis toxin, colicin, complement, defensin, histolysin, listeriolysin, magainin,
melittin, pneumolysin, yeast killer toxin, valinomycin, Peterson's crown ethers, perforin, perfringolysin O, theta-toxin of Clostridium perfringens, phallolysin, phallotoxin, and other molecules, such as those described in Regen et al. Biochem Biophys Res Commun 1989 159:566-571; which is incorporated herein by reference in its entirety. Methods of purifying or synthesizing pore-forming compounds are well known to one of ordinary skill in the art. Further, pore-forming compounds are commercially available, e.g. streptolysin O (Cat No. S5265; Sigma-Aldrich, St. Louis, MO). By way of non-limiting example, cells can be exposed to SLO for about 5 minutes or more, e.g. at least 5 minutes, at least 10 minutes, at least 20 minutes, at least 30 minutes, at least 45 minutes, at least 1 hour, at least 2 hours, at least 3 hours, or longer. In some embodiments, cells are exposed to SLO for from about 30 minutes to 2 hours. In some embodiments, cells are exposed to SLO for about 50 minutes. By way of non-limiting example, cells can be exposed to SLO at concentrations of from about 10 ng/mL to 1 mg/mL. In some embodiments, cells can be exposed to SLO at concentrations of from about 1 μg/mL to 100 μg/mL. In some embodiments, cells can be exposed to SLO at about 10 μg/mL. In some embodiments, cells can be exposed to SLO at about 10 μg/mL for about 50 minutes.

[0067] Oxygen-deprivation conditions that induce the generation of pluripotent cells can include culturing cells under reduced oxygen conditions, e.g. culturing cells in 10% oxygen or less. In some embodiments, the cells are cultured under 5% oxygen or less. The length of culturing under reduced oxygen conditions can be 1 hour or longer, e.g. 1 hour, 12 hours, 1 day, 2 days, 1 week, 2 weeks, 3 weeks, 1 month, 2 months or longer. In some embodiments, the cells can be cultured under reduced oxygen conditions for from 1 week to 1 month. In some embodiments, the cells can be cultured under reduced oxygen conditions for about 3 weeks.

[0068] Nutrient-deprivation conditions that induce the generation of pluripotent cells can include the lack of any factor or nutrient that is beneficial to cell growth. In some embodiments, nutrient-deprivation conditions comprise culturing the cells in basal culture medium, e.g. F12 or DMEM without further supplements such as FBS or growth factors. The length of culturing in nutrient-deprivation conditions can be 1 hour or longer, e.g. 1 hour, 12 hours, 1 day, 2 days, 1 week, 2 weeks, 3 weeks, 1 month, 2 months or longer. In some embodiments, the cells can be cultured under nutrient-deprivation conditions for from 1 week to 1 month. In some embodiments, the cells can be cultured under nutrient-deprivation conditions for about 2 weeks. In some embodiments, the cells can be cultured under nutrient-deprivation conditions for about 3 weeks.
Exposure to extreme temperatures that induces the generation of pluripotent cells can include exposure to either low temperatures or high temperatures. For a mammalian somatic cell, an extreme low temperature can be a temperature below 35°C, e.g. 34°C, 33°C, 32°C, 31°C, or lower. In some embodiments, an extreme low temperature can be a temperature below freezing. Freezing of cells can cause membrane perforations by ice crystals and provides an avenue for reducing cytoplasm. For a mammalian somatic cell, an extreme high temperature can be a temperature above 42°C, e.g. 43°C, 44°C, 45°C, 46°C or higher. In some embodiments, the extreme high temperature can be a temperature of about 85°C or higher. The length of culturing under extreme temperatures can be 20 minutes or longer, e.g. 20 minutes, 30 minutes, 1 hour, 12 hours, 1 day, 2 days, 1 week, 2 weeks, 3 weeks, 1 month, 2 months or longer. Clearly, the higher the temperature, the shorter the exposure that will generally be tolerated to permit the generation of pluripotent cells.

Further examples of stresses that can be used in the methods described herein include, but are not limited to, ultrasonic stimulation and radiation treatment.

In some embodiments, after being exposed to a stress, the cells can be cultured prior to selection according to the methods described below herein. The cells can be cultured for at least 1 hour prior to selection, e.g. the stressful stimulus is removed and the cells are cultured for at least 1 hour, at least 2 hours, at least 6 hours, at least 12 hours, at least 1 day, at least 2 days, at least 7 days or longer prior to selecting as described herein. By way of non-limiting example, cells can be exposed to SLO for about 50 minutes and then cultured in culture medium without SLO for about 7 days prior to selection. In some embodiments, the culture medium used to culture the cells prior to selection does not contain differentiation factors or promote differentiation. In some embodiments, the culture medium is one suitable for the culture of stem cells and/or pluripotent cells. Examples of such media are described below herein.

In some embodiments, the amount of cytoplasm in a somatic cell is reduced. The reduction of cytoplasm in a cell can be determined by monitoring the size of the cell. Methods of determining cell size are well known to one of ordinary skill in the art and include, by way of non-limiting example, cytofluorimetric analysis. In brief, single cells are stained with propidium iodide filtered and measured, for example, on a DAKO GALAXY™ (DAKO) analyzer using FLOMAX™ software. Cytofluorimetric analysis can then be performed to establish cell size. Microbeads of predefined sizes are re-suspended in isotonic phosphate saline (pH 7.2) and used as
a standard for which to compare size of cells contained in spheres using cytofluorimetric analysis. Both cells and beads are analyzed using the same instrument setting (forward scatter, representing cell and bead size, and side scatter, representing cellular granularity). Cell size can be calculated on a curve employing bead size on the x-axis and forward scatter values on the y-axis.

[0073] In some embodiments, the amount of mitochondria in a somatic cell is reduced. Methods of determining the number of mitochondria in a cell are well known to one of ordinary skill in the art and include staining with a mitochondria-specific dye and counting the number of mitochondria visible per cell when viewed under a microscope. Mitochondria-specific dyes are commercially available, e.g. MITOTRACKER™ (Cat No M7512 Invitrogen; Grand Island, NY). In some embodiments, the number of mitochondria or the intensity of the signal from mitochondria-specific dyes can be decreased by at least 40% following treatment with the methods described above herein. In some embodiments, cells are selected in which the number of mitochondria or the intensity of the signal from mitochondria-specific dyes decreased by at least 40% following treatment with the methods described above herein.

[0074] In some aspects, after removing a portion of the cytoplasm and/or mitochondria of a somatic cell, the method further comprises selecting cells exhibiting pluripotency. Pluripotent cells can be selected by selecting cells which display markers, phenotypes, or functions of pluripotent cells. Selecting cells can comprise isolating and propagating cells displaying the desired characteristics or culturing a population of cells with unknown characteristics under conditions such that cells with the desired characteristic(s) will survive and/or propagate at a higher rate than those cells not having the desired characteristic(s). Non-limiting examples of markers and characteristics of pluripotent cells are described herein below. In some embodiments, selecting the cells for pluripotency comprises, at least in part, selecting cells which express Oct4. In some embodiments, selecting the cells for pluripotency comprises, at least in part, selecting cells which express Nanog. In some embodiments, selecting the cells for pluripotency comprises, at least in part, selecting cells which express Oct4 and Nanog. In some embodiments, pluripotent cells can be selected by selecting cells expressing SSEA-1 and E-cadherin using antibodies specific for those markers and FACS. In some embodiments cells can be selected on the basis of size using FACS or other cell sorting devices as known in the art and/or described herein. Cells can also be selected by their inability to adhere to culture dishes.

[0075] Cells can also be selected on the basis of smaller size after being subjected to stress.
That is, stressed cells that progress to pluripotency are smaller than their non-pluripotent somatic precursors. In some embodiments, cells with a diameter of less than 8 µm are selected, e.g. cells with a diameter of 8 µm or less, 7 µm or less, 6 µm or less, 5 µm or less, or smaller. Cells can be selected on the basis of size after being cultured for a brief period (e.g. several minutes to several days) or after being allowed to rest following the stress treatment. In some embodiments, the cells can be selected on the basis of size immediately following the stress treatment. Cells can be selected on the basis of size by any method known in the art, e.g. the use of a filter or by FACS.

In some embodiments of the methods described herein, a pluripotent cell generated according to the methods described herein can be cultured to allow propagation of that pluripotent cell (i.e. propagation of a stem cell). In some embodiments, a pluripotent cell generated according to the methods described herein can be maintained in vitro. Conditions suitable for the propagation and or maintaining of stem and/or pluripotent cells are known in the art. Propagation of stem cells permits expansion of cell numbers without substantially inducing or permitting differentiation. By way of non-limiting example, conditions suitable for propagation of pluripotent cells include plating cells at 1x10^6 cells/cm^2 in F12/DMEM (1:1, v/v) supplemented with 2% B27, 20 ng/mL basic fibroblast growth factor, and 10 ng/mL epidermal growth factor. About 50% of the medium can be replaced every 2-3 days for the duration of the culture. In some embodiments, the conditions suitable for the propagation of stem and/or pluripotent cells comprise culturing the cells in B27-LIF (i.e. serum-free medium containing LIF (1 x 10^3 units/mL, Chemicon; Cat No: ESG1107 EMD Millipore, Billerica, MA) and B27 supplement (Cat No: 0080085-SA; Invitrogen; Grand Island, NY) as described in Hitoshi, S. et al. Genes & development 2004 18, 1806-1811; which is incorporated by reference herein in its entirety. Other media suitable for culturing the cells described herein are described in the Examples herein, e.g. ES establishment culture medium, 3i and ACTH, ES culture condition, ES-LIF, embryonic neural stem cell culture condition, and EpiSCs culture condition.

During propagation, the pluripotent cell generated according to the methods described herein will continue to express the same pluripotent stem cell marker(s). Non-limiting examples of pluripotent stem cell markers include AP, SSEA-1, E-cadherin antigen, Oct4, Nanog, Ecat1, Rex1, Zfp296, GDF3, Dppa3, Dppa4, Dppa5, Sox2, Esrbb, Dnmt3b, Dnmt3l, Utf1, Tc11, Bat1, Fgf4, Neo, Cripto, Cdx2, and S1c2a3. Methods of determining if a cell is expressing a pluripotent stem cell marker are well known to one of ordinary skill in the art and include, for
example, RT-PCR, the use of reporter gene constructs (e.g. expression of the Oct4-GFP construct described herein coupled with FACS or fluorescence microscopy), and FACS or fluorescence microscopy using antibodies specific for cell surface markers of interest.

[0078] Pluripotent cell markers also include elongated telomeres, as compared to somatic cells. Telomere length can be determined, for example, by isolating genomic DNA, digesting the gDNA with restriction enzymes such as Hinfl and Rsal, and detecting telomeres with a telomere length assay reagent. Such reagents are known in the art and are commercially available, e.g. the TELOTAGGG™ TELOMERE LENGTH ASSAY kit (Cat No. 12209136001 Roche; Indianapolis, IN).

[0079] Assays

[0080] Some aspects of the technology described herein relate to assays using a pluripotent stem cell produced by the methods described herein. For example, a pluripotent stem cell produced by the methods described herein can be used to screen and/or identify agents which modulate the viability, differentiation, or propagation of pluripotent stem cells. Such assays can comprise contacting a pluripotent cell produced according to the methods described herein with a candidate agent and determining whether the viability, differentiation and/or propagation of the pluripotent cell contacted with the candidate agent varies from the viability, differentiation and/or propagation of a pluripotent cell not contacted with the candidate agent. In some embodiments, an agent can increase the viability, differentiation, and/or propagation of the pluripotent stem cell. In some embodiments, an agent can decrease the viability, differentiation, and/or propagation of the pluripotent stem cell. In some embodiments, the pluripotent stem cell can be contacted with multiple candidate agents, e.g. to determine synergistic or antagonistic effects or to screen candidate agents in pools.

[0081] A candidate agent is identified as an agent that modulates the viability of a pluripotent cell produced if the number of pluripotent cells which are viable, i.e. alive is higher or lower in the presence of the candidate agent relative to its absence. Methods of determining the viability of a cell are well known in the art and include, by way of non-limiting example determining the number of viable cells at at least two time points, by detecting the strength of a signal from a live cell marker, or the number or proportion of cells stained by a live cell marker. Live cell markers are available commercially, e.g. PRESTO BLUE™ (Cat No A-13261; Life Technologies; Grand Island, NY). A candidate agent is identified as an agent that modulates the
propagation of a pluripotent cell produced if the rate of propagation of the pluripotent cell is altered, i.e. the number of progeny cells produced in a given time is higher or lower in the presence of the candidate agent. Methods of determing the rate of propagation of a cell are known in the art and include, by way of non-limiting example, determining an increase in live cell number over time.

[0082] A candidate agent is identified as an agent that modulates the differentiation of a pluripotent cell if the rate or character of the differentiation of the pluripotent cell is higher or lower in the presence of the candidate agent. Methods of determing the rate or character of differentiation of a cell are known in the art and include, by way of non-limiting example, detecting markers or morphology of a particular lineage and comparing the number of cells and/or the rate of appearance of cells with such markers or morphology in the population contacted with a candidate agent to a population not contacted with the candidate agent. Markers and morphological characterisitics of various cell fate lineages and mature cell types are known in the art. By way of non-limiting example, mesodermal cells are distinguished from pluripotent cells by the expression of actin, myosin, and desmin. Chondrocytes can be distinguished from their precursor cell types by staining with safranin-O and or FASTGREEN™ dyes (Fisher; Pittsburg, PA; F99). Osteocytes can be distinguished from their precursor cell types by staining with Alizarin Red S (Sigma; St. Louis, MO; Cat No A5533).

[0083] In some embodiments, the pluripotent cells are contacted with one or more candidate agents and cultured under conditions which promote differentiation to a particular cell lineage or mature cell type. Conditions suitable for differentiation are known in the art. By way of non-limiting example, conditions suitable for differentiation to the mesoderm lineage include DMEM supplemented with 20% fetal calf serum (FCS), with the medium exchanged every 3 days. By way of further non-limiting example, conditions suitable for differentiation to the neural lineage include plating cells on ortinin-coated chamber slides in F12/DMEM (1:1, v/v) supplemented 2% B27, 10% FCS, 10 ng/mL bFGF, and 20 ng/m LEGF. The medium can be exchanged every 3 days.

[0084] As used herein, a “candidate agent” refers to any entity which is normally not present or not present at the levels being administered to a cell, tissue or subject. A candidate agent can be selected from a group comprising: chemicals; small organic or inorganic molecules; nucleic acid sequences; nucleic acid analogues; proteins; peptides; aptamers; peptidomimetic, peptide
derivative, peptide analogs, antibodies; intrabodies; biological macromolecules, extracts made from biological materials such as bacteria, plants, fungi, or animal cells or tissues; naturally occurring or synthetic compositions or functional fragments thereof. In some embodiments, the candidate agent is any chemical entity or moiety, including without limitation synthetic and naturally-occurring non-proteinaceous entities. In certain embodiments the candidate agent is a small molecule having a chemical moiety. For example, chemical moieties include unsubstituted or substituted alkyl, aromatic, or heterocyclyl moieties including macrolides, leptomycins and related natural products or analogues thereof. Candidate agents can be known to have a desired activity and/or property, or can be selected from a library of diverse compounds.

[0085] Candidate agents can be screened for their ability to modulate the viability, propagation, and/or differentiation of a pluripotent cell. In one embodiment, candidate agents are screened using the assays for viability, differentiation, and/or propagation described above and in the Examples herein.

[0086] Generally, compounds can be tested at any concentration that can modulate cellular function, gene expression or protein activity relative to a control over an appropriate time period. In some embodiments, compounds are tested at concentrations in the range of about 0.1nM to about 1000mM. In one embodiment, the compound is tested in the range of about 0.1μM to about 20μM, about 0.1μM to about 10μM, or about 0.1μM to about 5μM.

[0087] Depending upon the particular embodiment being practiced, the candidate or test agents can be provided free in solution, or can be attached to a carrier, or a solid support, e.g., beads. A number of suitable solid supports can be employed for immobilization of the test agents. Examples of suitable solid supports include agarose, cellulose, dextran (commercially available as, e.g., Sephadex, Sepharose) carboxymethyl cellulose, polystyrene, polyethylene glycol (PEG), filter paper, nitrocellulose, ion exchange resins, plastic films, polyaminemethylvinylether maleic acid copolymer, glass beads, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. Additionally, for the methods described herein, test agents can be screened individually, or in groups or pools. Group screening is particularly useful where hit rates for effective test agents are expected to be low, such that one would not expect more than one positive result for a given group.

obtained from, e.g., ArQule (Woburn, MA), Invitrogen (Carlsbad, CA), Ryan Scientific (Mt. Pleasant, SC), and Enzo Life Sciences (Farmingdale, NY). These libraries can be screened for the ability of members to modulate the viability, propagation, and/or differentiation of pluripotent stem cells. The candidate agents can be naturally occurring proteins or their fragments. Such candidate agents can be obtained from a natural source, e.g., a cell or tissue lysate. Libraries of polypeptide agents can also be prepared, e.g., from a cDNA library commercially available or generated with routine methods. The candidate agents can also be peptides, e.g., peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred and from about 7 to about 15 being particularly preferred. The peptides can be digests of naturally occurring proteins, random peptides, or "biased" random peptides. In some methods, the candidate agents are polypeptides or proteins. Peptide libraries, e.g. combinatorial libraries of peptides or other compounds can be fully randomized, with no sequence preferences or constants at any position. Alternatively, the library can be biased, i.e., some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, in some cases, the nucleotides or amino acid residues are randomized within a defined class, for example, of hydrophobic amino acids, hydrophilic residues, sterically biased (either small or large) residues, towards the creation of cysteines, for cross-linking, prolines for SH-3 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, or to purines.

[0089] The candidate agents can also be nucleic acids. Nucleic acid candidate agents can be naturally occurring nucleic acids, random nucleic acids, or "biased" random nucleic acids. For example, digests of prokaryotic or eukaryotic genomes can be similarly used as described above for proteins.

[0090] In some embodiments, the candidate agent that is screened and identified to modulate viability, propagation and/or differentiation of a pluripotent cell according to the methods described herein, can increase viability, propagation and/or differentiation of a pluripotent cell by at least 5%, preferably at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 1-fold, 1.1-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 50-fold, 100-fold or more relative to an untreated control. In some embodiments, the candidate agent that is screened and identified to modulate viability, propagation and/or differentiation of a pluripotent cell according to the methods described herein, can decrease viability, propagation and/or differentiation of a pluripotent cell by at least 5%, preferably at least 10%, 20%, 30%, 40%, 50%, 50%, 70%, 80%,
90%, 95%, 97%, 98%, 99% or more, up to and including complete reduction (i.e., zero viability, growth, propagation, or differentiation) relative to an untreated control.

[0091] In some embodiments, the candidate agent functions directly in the form in which it is administered. Alternatively, the candidate agent can be modified or utilized intracellularly to produce a form that modulates the desired activity, e.g., introduction of a nucleic acid sequence into a cell and its transcription resulting in the production of an inhibitor or activator of gene expression or protein activity within the cell.

[0092] Therapy

[0093] Some aspects of the technology described herein relate to methods of cell therapy comprising administering a pluripotent cell, produced by the methods described herein, or the at least partially differentiated progeny of such a cell to a subject in need of cell therapy. In some embodiments, a therapeutically effective amount of pluripotent cells or the at least partially differentiated progeny of the pluripotent cell is provided. In some embodiments, the pluripotent cells and/or their progeny are autologous. In some embodiments, the pluripotent cells and/or their progeny are allogenic. In some embodiments, the pluripotent cells and/or their progeny are syngeneic. In some embodiments, the pluripotent cells and/or their progeny are xenogenic. In some embodiments, the cell therapy can be autologous therapy, e.g., a somatic cell from a subject can be used to generate a pluripotent cell according to the methods described herein and the pluripotent cell and/or at least partially differentiated progeny of that pluripotent cell can be administered to the subject. As used herein, a “subject in need of cell therapy” refers to a subject diagnosed as having, or at risk of having or developing a disease associated with the failure of a naturally occurring cell or tissue type or a naturally occurring pluripotent and/or multipotent cell (e.g., stem cell).

[0094] Stem cells generated according to the methods described herein can be used in cancer therapy. For example, high dose chemotherapy plus hematopoietic stem cell transplantation to regenerate the bone marrow hematopoietic system can benefit from the use of stem cells generated as described herein.

[0095] Non-limiting examples of diseases associated with the failure of a naturally occurring cell or tissue type or a naturally occurring pluripotent and/or multipotent cell include aplastic anemia, Fanconi anemia, and paroxysmal nocturnal hemoglobinuria (PNH). Others include, for example: acute leukemias, including acute lymphoblastic leukemia (ALL), acute
myelogenous leukemia (AML), acute biphenotypic leukemia and acute undifferentiated leukemia; chronic leukemias, including chronic myelogenous leukemia (CML), chronic lymphocytic leukemia (CLL), juvenile chronic myelogenous leukemia (JCML) and juvenile myelomonocytic leukemia (JMML); myeloproliferative disorders, including acute myelofibrosis, angiogenic myeloid metaplasia (myelofibrosis), polycythemia vera and essential thrombocytemia; lysosomal storage diseases, including mucopolysaccharidoses (MPS), Hurler's syndrome (MPS-IH), Scheie syndrome (MPS-IS), Hunter's syndrome (MPS-II), Sanfilippo syndrome (MPS-III), Morquio syndrome (MPS-IV), Maroteaux-Lamy Syndrome (MPS-VI), Sly syndrome, beta-glucuronidase deficiency (MPS-VII), adrenoleukodystrophy, mucolipidosis II (I-cell Disease), Krabbe disease, Gaucher's disease, Niemann-Pick disease, Wolman disease and metachromatic leukodystrophy; histiocytic disorders, including familial erythrophagocytic lymphohistiocytosis, histiocytosis-X and hemophagocytosis; phagocyte disorders, including Chediak-Higashi syndrome, chronic granulomatous disease, neutrophil actin deficiency and reticular dysgenesis; inherited platelet abnormalities, including amegakaryocytosis/congenital thrombocytopenia; plasma cell disorders, including multiple myeloma, plasma cell leukemia, and Waldenstrom's macroglobulinemia. Other malignancies treatable with stem cell therapies include but are not limited to breast cancer, Ewing sarcoma, neuroblastoma and renal cell carcinoma, among others. Also treatable with stem cell therapy are: lung disorders, including COPD and bronchial asthma; congenital immune disorders, including ataxia-telangiectasia, Kostmann syndrome, leukocyte adhesion deficiency, DiGeorge syndrome, bare lymphocyte syndrome, Omenn's syndrome, severe combined immunodeficiency (SCID), SCID with adenosine deaminase deficiency, absence of T & B cells SCID, absence of T cells, normal B cell SCID, common variable immunodeficiency and X-linked lymphoproliferative disorder; other inherited disorders, including Lesch-Nyhan syndrome, cartilage-hair hypoplasia, Glanzmann thrombasthenia, and osteopetrosis; neurological conditions, including acute and chronic stroke, traumatic brain injury, cerebral palsy, multiple sclerosis, amyotrophic lateral sclerosis and epilepsy; cardiac conditions, including atherosclerosis, congestive heart failure and myocardial infarction; metabolic disorders, including diabetes; and ocular disorders including macular degeneration and optic atrophy. Such diseases or disorders can be treated either by administration of pluripotent cells themselves, permitting in vivo differentiation to the desired cell type with or without the administration of agents to promote the desired differentiation, and/or by administering pluripotent cells.
differentiated to, or at least partially differentiated towards the desired cell type *in vitro*. Methods of diagnosing such conditions are well known to medical practitioners of ordinary skill in the art. In some embodiments, the subject can be one who was treated with radiation therapy or other therapies which have ablated a population of cells or stem cells, e.g. the subject can be a subject with cancer whose bone marrow has been ablated by radiation therapy.

[0096] In some embodiments, pluripotent cells are administered to the subject. In some embodiments, an at least partially differentiated cell is administered to the subject. In some embodiments, the method of cell therapy can further comprise differentiating the pluripotent cell along a pre-defined cell lineage prior to administering the cell. Methods of differentiating stem cells along desired cell lineages are known in the art and examples are described herein.

[0097] In some embodiments, a composition comprising a pluripotent cell obtained according to the methods described herein or an at least partially differentiated cell which is the progeny of the pluripotent cell is administered to the subject.

[0098] In some embodiments, administration of the pluripotent cells and/or their at least partially differentiated progeny can occur within a relatively short period of time following production of the pluripotent cell in culture according to the methods described herein (e.g. 1, 2, 5, 10, 24 or 48 hours after production). In some embodiments, administration of the at least partially differentiated progeny can occur within a relatively short period of time following differentiation of the pluripotent cell in culture according to the methods described herein (e.g. 1, 2, 5, 10, 24 or 48 hours after production). In some embodiments, the pluripotent cells and/or their at least partially differentiated progeny can be cryogenically preserved prior to administration.

[0099] **Compositions**

[0100] In some aspects, the technology described herein relates to a composition comprising a pluripotent cell generated according to the methods described herein and/or the at least partially differentiated progeny of the pluripotent cell. In some embodiments, a pharmaceutical composition comprises a pluripotent cell generated according to the methods described herein and/or the at least partially differentiated progeny of the pluripotent cell, and optionally a pharmaceutically acceptable carrier. The compositions can further comprise at least one pharmaceutically acceptable excipient.

[0101] The pharmaceutical composition can include suitable excipients, or stabilizers, and can be, for example, solutions, suspensions, gels, or emulsions. Typically, the composition will
contain from about 0.01 to 99 percent, preferably from about 5 to 95 percent of cells, together with the carrier. The cells, when combined with pharmaceutically or physiologically acceptable carriers, excipients, or stabilizer, can be administered parenterally, subcutaneously, by implantation or by injection. For most therapeutic purposes, the cells can be administered via injection as a solution or suspension in liquid form. The term “pharmaceutically acceptable carrier” refers to a carrier for administration of the pluripotent cell generated according to the methods described herein and/or the at least partially differentiated progeny of the pluripotent cell. Such carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, and combinations thereof. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation, for example the carrier does not decrease the impact of the agent on the subject. In other words, a carrier is pharmaceutically inert and compatible with live cells.

Suitable formulations also include aqueous and non-aqueous sterile injection solutions which can contain anti-oxidants, buffers, bacteriostats, bactericidal antibiotics and solutes which render the formulation isotonic with the bodily fluids of the intended recipient. Aqueous and non-aqueous sterile suspensions can include suspending agents and thickening agents. The formulations can be presented in unit-dose or multi-dose containers.

Examples of parenteral dosage forms include, but are not limited to, solutions ready for injection, suspensions ready for injection, and emulsions. Parenteral dosage forms can be prepared, e.g., using bioreposable scaffold materials to hold pluripotent cells generated according to the methods described herein and/or the at least partially differentiated progeny of the pluripotent cell.

Administration

As used herein, the term “administer” or “transplant” refers to the placement of cells into a subject by a method or route which results in at least partial localization of the cells at a desired site such that a desired effect is produced.

The pluripotent stem cells described herein, and/or their at least partially differentiated progeny, can be administered in any manner found appropriate by a clinician and can include local administration, e.g. by injection of a suspension of cells or, for example, by implantation of a preparation of cells deposited or grown on or within an implantable scaffold or support. Implantable scaffolds can include any of a number of degradable or resorbable polymers, or, for example, a silk scaffold, among others. Suitable routes for administration of a
pharmaceutical composition comprising pluripotent stem cells described herein, and/or their at least partially differentiated progeny include but are not limited to local administration, e.g. intraperitoneal, parenteral, intracavity or subcutaneous administration. The phrases “parenteral administration” and “administered parenterally” as used herein, refer to modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intraperitoneal, intradermal, subcutaneous injection and infusion. Administration can involve the use of needles, catheters and syringes suitable for injection, or surgical implantation. The use of a combination of delivery means and sites of delivery are contemplated to achieve the desired clinical effect.

[00107] In one embodiment, a therapeutically effective amount of pluripotent stem cells described herein, and/or their at least partially differentiated progeny is administered to a subject. A “therapeutically effective amount” is an amount of pluripotent stem cells described herein, and/or their at least partially differentiated progeny, sufficient to produce a measurable improvement in a symptom or marker of the condition being treated. Actual dosage levels of cells in a therapeutic composition can be varied so as to administer an amount of the cells that is effective to achieve the desired therapeutic response for a particular subject. The selected dosage level will depend upon a variety of factors including, but not limited to, the activity of the therapeutic composition, formulation, the route of administration, combination with other drugs or treatments, severity of the condition being treated, the physical condition of the subject, prior medical history of the subject being treated and the experience and judgment of the clinician or practitioner administering the therapy. Generally, the dose and administration scheduled should be sufficient to result in slowing, and preferably inhibiting progression of the condition and also preferably causing a decrease in one or more symptoms or markers of the condition. Determination and adjustment of a therapeutically effective dose, as well as evaluation of when and how to make such adjustments, are known to those of ordinary skill in the art of medicine.

[00108] The dosage of pluripotent stem cells described herein, and/or their at least partially differentiated progeny administered according to the methods described herein can be determined by a physician and adjusted, as necessary, to suit observed effects of the treatment. With respect to duration and frequency of treatment, it is typical for skilled clinicians to monitor subjects in order to determine when the treatment is providing therapeutic benefit, and to determine whether to administer another dose of cells, increase or decrease dosage, discontinue treatment, resume
treatment, or make other alteration to the treatment regimen. Where cells administered are expected to engraft and survive for medium to long term, repeat dosages can be necessary. However, administration can be repeated as necessary and as tolerated by the subject. The dosage should not be so large as to cause substantial adverse side effects. The dosage can also be adjusted by the individual physician in the event of any complication. Typically, however, the dosage can range from 100 to 1 x 10^9 pluripotent stem cells as described herein, and/or their at least partially differentiated progeny for an adult human, e.g. 100 to 10,000 cells, 1,000 to 100,000 cells, 10,000 to 1,000,000 cells, or 1,000,000 to 1 x 10^9 cells. Effective doses can be extrapolated from dose-response curves derived from, for example, animal model test bioassays or systems.

[00109] Therapeutic compositions comprising pluripotent stem cells described herein, and/or their at least partially differentiated progeny prepared as described herein are optionally tested in one or more appropriate in vitro and/or in vivo animal models of disease, such as a SCID mouse model, to confirm efficacy, evaluate in vivo growth of the transplanted cells, and to estimate dosages, according to methods well known in the art. In particular, dosages can be initially determined by activity, stability or other suitable measures of treatment vs. non-treatment (e.g., comparison of treated vs. untreated animal models), in a relevant assay. In determining the effective amount of pluripotent stem cells described herein, and/or their at least partially differentiated progeny, the physician evaluates, among other criteria, the growth and volume of the transplanted cells and progression of the condition being treated. The dosage can vary with the dosage form employed and the route of administration utilized.

[00110] With respect to the therapeutic methods described herein, it is not intended that the administration of pluripotent stem cells described herein, and/or their at least partially differentiated progeny be limited to a particular mode of administration, dosage, or frequency of dosing. All modes of administration are contemplated, including intramuscular, intravenous, intraperitoneal, intravascular, intraarticular, intralesional, subcutaneous, or any other route sufficient to provide a dose adequate to treat the condition being treated.

[00111] Efficacy of treatment can be assessed, for example by measuring a marker, indicator, symptom or incidence of, the condition being treated as described herein or any other measurable parameter appropriate, e.g. number of pluripotent cell progeny. It is well within the ability of one skilled in the art to monitor efficacy of treatment or prevention by measuring any one of such parameters, or any combination of parameters.
Effective treatment is evident when there is a statistically significant improvement in one or more markers, indicators, or symptoms of the condition being treated, or by a failure to worsen or to develop symptoms where they would otherwise be anticipated. As an example, a favorable change of at least about 10% in a measurable parameter of a condition, and preferably at least about 20%, about 30%, about 40%, about 50% or more can be indicative of effective treatment. Efficacy for pluripotent cells generated according to the methods described herein and/or the at least partially differentiated progeny of the pluripotent cell can also be judged using an experimental animal model known in the art for a condition described herein. When using an experimental animal model, efficacy of treatment is evidenced when a statistically significant change in a marker is observed, e.g. the number of hematopoietic cells present in a mouse following bone marrow ablation and treatment with pluripotent cells as described herein.

In some aspects, the technology described herein relates to a system for generating a pluripotent cell from a somatic cell, comprising removing a portion of the cytoplasm and/or mitochondria from the somatic cell.

A system for generating a pluripotent cell from a somatic cell, according to the methods described herein, can comprise a container in which the somatic cells are subjected to stress. The container can be suitable for culture of somatic and/or pluripotent cells, as for example, when cells are cultured for days or longer under low oxygen conditions in order to reduce the amount of cytoplasm and/or mitochondria according to the methods described herein. Alternatively, the container can be suitable for stressing the cells, but not for culturing the cells, as for example, when somatic cells are triturated in a device having a narrow aperture for a limited period, e.g. less than 1 hour. A container can be, for example, a vessel, a tube, a microfluidics device, a pipette, a bioreactor, or a cell culture dish. A container can be maintained in an environment that provides conditions suitable for the culture of somatic and/or pluripotent cells (e.g. contained within an incubator) or in an environment that provides conditions which will cause environmental stress on the somatic cell (e.g. contained within an incubator providing a low oxygen content environment). A container can be configured to provide 1 or more of the environmental stresses described above herein, e.g. 1 stress, 2 stresses, 3 stresses, or more. Containers suitable for manipulation and/or culturing somatic and/or pluripotent cells are well known to one of ordinary skill in the art and are available commercially (e.g. Cat No CLS430597
In some embodiments, the container is a microfluidics device. In some embodiments, the container is a cell culture dish, flask, or plate.

In some embodiments, the system can further comprise a means for selecting pluripotent cells, e.g. the system can comprise a FACS system which can select cells expressing a pluripotency marker (e.g. Oct4-GFP) or select by size as described above herein. Methods and devices for selection of cells are well known to one of ordinary skill in the art and are available commercially, e.g. BD FACSARIA SORPTM coupled with BD LSRIITM and BD FACSDIVATM Software (Cat No. 643629) produced by BD Biosciences; Franklin Lakes, NJ.

In some embodiments, somatic cells which are not present in a tissue are provided to the system. In some embodiments, tissues are provided to the system and the system further comprises a means of isolating one or more types of somatic cells. By way of non-limiting example, the system can comprise a tissue homogenizer. Tissue homogenizers and methods of using them are known in the art and are commercially available (e.g. FASTH21TM, Cat No. 21-82041 Omni International; Kennesaw, GA). Alternatively, the system can comprise a centrifuge to process blood or fluid samples.

In some embodiments, the system can be automated. Methods of automating cell isolation, cell culture, and selection devices are known in the art and are commercially available. For example, the FASTH21TM Tissue Homogenizer (Cat No. 21-82041 Omni International; Kennesaw, GA) and the BD FACSARIA SORPTM.

In some embodiments, the system can be sterile, e.g. it can be operated in a sterile environment or the system can be operated as a closed, sterile system.

The description of embodiments of the disclosure is not intended to be exhaustive or to limit the disclosure to the precise form disclosed. While specific embodiments of, and examples for, the disclosure are described herein for illustrative purposes, various equivalent modifications are possible within the scope of the disclosure, as those skilled in the relevant art will recognize. For example, while method steps or functions are presented in a given order, alternative embodiments may perform functions in a different order, or functions may be performed substantially concurrently. The teachings of the disclosure provided herein can be applied to other procedures or methods as appropriate. The various embodiments described herein can be combined to provide further embodiments. Aspects of the disclosure can be modified, if necessary, to employ the compositions, functions and concepts of the above references and
application to provide yet further embodiments of the disclosure. These and other changes can be made to the disclosure in light of the detailed description.

Specific elements of any of the foregoing embodiments can be combined or substituted for elements in other embodiments. Furthermore, while advantages associated with certain embodiments of the disclosure have been described in the context of these embodiments, other embodiments may also exhibit such advantages, and not all embodiments need necessarily exhibit such advantages to fall within the scope of the disclosure.

All patents and other publications identified are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the present invention. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

This invention is further illustrated by the following examples which should not be construed as limiting.

**EXAMPLES**

**EXAMPLE 1**

All organisms possess a primitive survival instinct. When plants are subjected to significant external stresses they activate a mechanism to survive that causes dedifferentiation of somatic cells and enables regeneration of the injured area or the entire organism. While such mechanisms appear to be essential for lower organisms to survive extreme environmental changes, they have yet to be documented in mammals.

The inventors hypothesized that physical stress may cause mature mammalian somatic cells to revert to a stem cell state, similar to that seen in plants and lower organisms. To examine this hypothesis, mature cells procured from seven adult somatic tissues were studied. To first focus on which physical stresses might be most effective in altering mature cells to revert to a less mature state, CD45 positive lymphocytes harvested from Oct4-GFP mice were studied. Cells from these mice provide a readout of reversion to a stem cell phenotype when the stem cell specific
Oct4 promoter is activated. The mature, fully differentiated somatic cells were exposed to several significant external stimuli.

[00127] For example, CD45 positive lymphocytes were exposed to low pH solution to provide a strong chemical stress. Within 3 days of exposure, GFP expressing cells were observed, and within 5 days, spherical colonies composed of GFP expressing cells were observed. Cells generated in this manner are referred to in this Example as Stress Altered Stem Cells (SASCs or SACs). SACs can also be referred to as rejuvenated stem cells (RSCs) or animal callus cells (ACCs). SACs expressed several markers normally associated with embryonic stem cells. SACs exhibited a differentiation potency equivalent to ES cells, contributed to the generation of chimera mice and were capable of generating whole fetuses when injected into 4N blastocysts. Cells generated in this manner initially showed low mitochondrial activity and other conditions normally associated with the induction of cell based injury defense mechanisms. They then exhibited demethylation of the Oct4 and Nanog gene promoters. The reprogramming of stress altered cells appeared to be induced via mesenchymal-epithelial transition. The findings are consistent with descriptions of cells contained in the plant callus, in response to injury (external stimuli). A plant callus is formed from a stress induced conversion of somatic cells to pluripotent plant stem cells, capable of forming clonal bodies. Such a spherical colony, generated from mature fully differentiated somatic mammalian cells in response to significant external stimuli, is referred to herein as an Animal Callus, and to the stress altered cells contained in such a colony or callus, as "Animal Callus Cells" (ACCs) or SACs.

[00128] Thus, significant physical and chemical stresses caused normal mature adult cells to be reprogrammed to pluripotent stem cells capable of embryogenesis. While not wishing to be bound by theory, the mechanism of reprogramming appears to include the induction of a cellular survival and repair process normally seen in response to injury. It is demonstrated herein that mammalian somatic cells possess a survival mechanism very similar to that of plants, to revert to reprogrammed state in response to significant stressful external stimuli.

[00129] Various types of somatic cells have reportedly been reprogrammed to a pluripotent stem cell state through induction or forced expression of specific genes\(^1\)\(^-\)\(^5\). It is also believed that damage to cells as a result of exposure to irritants, such as burns, chemical injury, trauma and radiation, may alter normal somatic cells to become cancer cells.

[00130] Introduction
All organisms appear to have a common instinct to survive injury related to stressful stimuli by adapting themselves to the environment and regenerating their bodies. In plants, ontogenesis is observed not only in zygotes but also in fully differentiated somatic cells and immature pollen. In vertebrates, newts are capable of regenerating several anatomical structures and organs, including their limbs\(^1\). Of particular note is that the remarkable regenerative capacity demonstrated by both plants and newts is induced by external stimuli, which cause cellular dedifferentiation of previously fully differentiated somatic cells. While billions of years have passed from the earliest form of life, and different organisms have evolved in unique ways, this survival instinct may be inherited from a common ancestor to modern-era organisms. Although terminally differentiated mammalian cells are normally believed to be incapable of reversing the differentiation process, mammals may retain a previously unappreciated program to escape death in response to drastic environmental changes.

The plant callus, a mass of proliferating cells formed in response to external stimuli, such as wounding, which can be stimulated in culture by the plant hormones\(^2\). The callus contains reprogrammed somatic cells, referred to as callus cells, each of which is capable of clonally regenerating the entire body. Callus cells are not inherent in plants, but are generated from somatic cells in response to external stimuli. Although recent studies demonstrated that mammalian somatic cells can be reprogrammed by exogenous processes, such as gene induction\(^3\)\(^-\)\(^7\), reprogramming of mammalian somatic cells in response to external physical and or chemical stimuli, in a manner that parallels plants, has not been reported. Interestingly, it is believed that extreme external stimuli, such as exposure to irritants, including burns, chemical injury, trauma and radiation, may alter normal somatic cells to become cancer cells. Such experiences seem to indicate that external stimuli will result in mammalian cellular change.

In this study, it was hypothesized that mammalian cells retain a mechanism to survive exposure to significant external stress, in the same manner as plants. This report presents evidence that application of significant physical and chemical stimuli can cause reprogramming of mature, fully differentiated mammalian somatic cells, procured from various tissues, and that such stress altered cells are capable of forming an animal callus containing "animal callus cells", which can regenerate the clonal body.

Results

**Significant physical and chemical stimuli applied to mature somatic cells.** Since
the embryonic transcription factor Oct4 is thought to be crucial in regulation of the pluripotent status of cells, the initial strategy was to identify which external stimuli most efficiently altered mature cells to become reprogrammed to express Oct4. CD45 positive hematopoietic lineage cells were first studied in order to avoid contamination with undifferentiated cells. CD45 positive cells harvested from spleens procured from Oct4-GFP (GOF) mice, were exposed to various significant physical and chemical stimuli. The exposures included: osmotic pressure treatment, treatment with significant mechanical trituration, exposure to low pH, application of cell membrane damage using streptolysin O (SLO), exposure to under nutrition and exposure to hypoxia and high Ca²⁺ concentration. Next, GFP expressing cells were identified, sorted and collected using FACS. Gene expression of Oct4 was confirmed by R-T PCR. Exposure to each of the applied stimuli resulted in reprogramming of the mature cells to express GFP to some degree (Figure 5A). Exposure of the mature cells to the chemical stress of low pH and the physical stress of significant mechanical trituration appeared to be the most effective treatments in altering mature cells to express Oct4. To determine the optimal pH for inducing conversion to Oct4 expressing cells, CD45 positive cells were exposed to solutions of varying acidity, from pH 4.0 to pH 6.8. At 3 days after exposure to an acidic solution, GFP expression of cells was analyzed using FACS. An acid solution with a pH 5.4-5.6 most efficiently altered cells to express GFP (Figure 5B). Consequently, exposure to low pH was focused upon as the stress treatment of choice for the remainder of the study.

[00136] The optimum culture conditions for maintaining stress altered Oct4 expressing cells were then determined. Several previously described culture media, including: ES establishment culture medium, 3⁰ and ACTH¹⁰, ES culture condition, ES-LIF¹¹, embryonic neural stem cell culture condition, B27-LIF¹², and EpiSCs culture condition¹³, were studied. Cells were plated into each medium, and GFP expressed colonies were counted (Figure 5C). The medium B27-LIF appeared to be the most effective in generating GFP expressing spherical colonies. Therefore B27-LIF medium was utilized for culture of the treated cells.

[00137] Stress treated CD45 positive cells were cultured in B27-LIF medium, and within 5 days, GFP expressing spherical colonies were observed while no GFP expressing colonies were observed in the untreated control (Figure 1A). Spherical colonies grew to approximately 70 μm in diameter over the first 7 days, and spherical colonies could be maintained for another 7 days in that culture condition. The configuration of the colonies was slightly baroque, appearing more similar
in shape to the callus seen in botany, rather than spheres. A cell colony generated by stress treatment was therefore referred to as an Animal Callus (AC). Cultured cells were dissociated and population analysis was then performed using FACS. The analysis revealed that the application of certain significant stimuli resulted in the generation of stress altered cells, now referred to as Animal Callus Cells (ACCs), that did not previously exist in the CD45 positive cell populations (Figure 1B). The phenotypic change of CD45 positive cells as a result stress treatment was observed at the single cell level. While CD45 positive cells did not express GFP, ACCs expressed GFP associated with a diminished expression of CD45 (Figure 1C). Examination of single cells revealed that the cell size of treated cells appeared smaller than untreated cells. Therefore, cell size of ACCs population was analyzed by FACS. The cell size of ACCs was quite small, with 80% of cells being less than 8 μm in diameter (Figure 1D).

[00138] To examine chronological phenotypic change associated with CD45 diminution and Oct4 expression, stress treated CD45 positive cells were analyzed at day 1, day 3 and day 7. At day 1, most of cells still expressed CD45, but not Oct4. At day 3, marker expression transitioned to reveal CD45 negative cells or CD45 negative/Oct4positive (dim) cells. At day 7, CD45 expression disappeared, and Oct4 expressing cells were observed (Figure 1E). Notably, during the first 7 days of culture, the number of PI positive cells (dead cells) gradually increased (Data not shown), which suggested that the stress treatment and the culture condition gradually changed the character of cells and selected for successfully altered cells, which expressed Oct4.

[00139] Characterization of ACCs. To confirm the reprogramming of somatic cells as a result of exposure to external stimuli, early embryogenesis marker gene expression of ACCs was investigated. As a positive control of early embryogenesis, ES cells were utilized in following experiments. Marker expression and DNA methylation was characterized as follows: Immunofluorescence staining at day 7, showed that spherical colonies containing ACCs, uniformly expressed pluripotent cell markers, E-cadherin antigen, Nanog, SSEA-1, PCAM-1, and AP, and were positive for Oct4-GFP (Figure 2A). Gene expression analysis showed that ACCs and ES cells, but not primary CD45 positive cells, expressed comparable levels of Oct4, Nanog, Sox2, Ect1, Esg1, Dax1, Fgf5, Klf4 and Rex1 genes (Figure 2B). Gene expression of ES specific genes in ACCs reached a peak at day 7 (Figure 2B). Bisulfite sequencing was performed to determine the methylation status of Oct4 and Nanog gene promoters in ACCs. Native lymphocytes and cultured lymphocyte control samples displayed extensive methylation at both
promoters, whereas ACCs showed widespread demethylation of these regions similar to that seen in ES cells (Figure 2C). Thus, it is demonstrated that mammalian somatic cells were reprogrammed by external stress.

[00140] To confirm that the Oct4 gene expression resulted from stress treatment of mature cells not only in GOF mice but also in wild type mice, CD45 positive lymphocytes were harvested from spleens procured from ICR mice. The lymphocytes were then exposed to the stress treatment and chronologically analyzed until day 7 using FACS. A SSEA-1 positive/E-cadherin positive cell population was seen in the stress treated group, while SSEA-1/E-cadherin expression was not observed in the non-stress treated control group (Figure 6A). Those double positive cells expressed Oct4 gene expression, which was confirmed by R-T PCR (Figure 6B). These results demonstrated that as a result of the stress treatment, ACCs, Oct4 positive and pluripotent marker expressing cells, were generated from CD45 positive cells irrespective of mouse strain.

[00141] These results imply that the mature fully differentiated adult somatic cells reverted to “stemness” as a result of the stress treatment.

[00142] To assess the stemness of ACCs, their self-renewal potency and their differentiation potency were examined. To study their self-renewal potency, ACCs colonies derived from previously mature CD45 positive lymphocytes were dissociated into single cells, and plated into 96 well plates, with one cell per well in an effort to generate clonally derived populations. Ten days after plating, spherical colonies were seen in 4 of the 96 wells. The dividing time of ACCs varied from well to well. Some divided in 12-16h and others divided in 30-34h. ACCs were passaged at least 5 times, with continued expression of Oct4 observed. Consequently, ACCs demonstrated a potential for self-renewal, and the potential to differentiate into cells from all three germ layers in vitro.

[00143] ACs derived from mature GOF lymphocytes were again dissociated into single cells, sorted to contain only a population of cells that expressed GFP and then cultured in differentiation media. At 14-21 days after plating, cells expressed the ectoderm marker, βIII-tubulin and GFAP, the mesoderm marker, α-smooth muscle actin, and the endoderm marker, α-fetoprotein and Cytokeratin 7 (Figure 9A). Thus, ACCs differentiated into cells representative of the three germ layers in vitro.

[00144] Stress alteration of mature somatic cells procured from various adult tissues. To examine whether ACCs could be generated not only mature lymphocytes but also other types of
somatic cells, brain, skin, muscle, fat, bone marrow, lung and liver were harvested from Oct4-GFP (GOF) mice. Cells were isolated from the tissue samples, dissociated into single cells, and treated with different physical and or chemical stress conditions. The efficiency of the process to alter the cells differed as a function of both the source of cells and the stress condition(s) to which the cells were exposed (Figure 7A). The ability of stress to alter mature cells to express Oct4, differed depending on the derivation of cells, but stress was able to alter cells to express Oct4 to some degree in mature cells derived from all three germ layers (Figure 7A). ACC colonies derived from any mature tissue expressed pluripotent markers, E-cadherin, Nanog, PCAM-1 and AP (Figure 7B), and ES specific marker genes (Figure 7C). Significant physical and chemical stresses altered mature somatic cells to revert to a stem cell state, despite of the source of tissues and derivation of the germ layers.

**Cellular modification in the initial phase of ACCs generation.** These results demonstrate that strong physical and chemical stimuli result in reprogramming of somatic cells. Stress treated lymphocytes were observed to form an AC within 5 days. It was hypothesized that drastic change of molecular events occurred as a result of the stress exposure. Studies were therefore focused on the initial phase of the reprogramming, which was the during the first 7 days after the exposure to the stimuli.

Because ACCs survived after the significant stress exposure, it was speculated that survival mechanisms normally turned on to repair cellular damage were induced during the ACCs generation. First the expression of a number of candidate genes involved in cellular response to stress and DNA repair was compared in in native CD45 positive cells and stress-treated CD45 positive cells at day 1, day 3 and day 7. Cellular response gene expression was already observed at day 1, and those genes were up-regulated over 7 days when the mixtures of ACC generating cells and other cells were analyzed (Figure 8). Because the up-regulation of cellular response genes was correlated with ACCs generation, ACCs at day 3 and day 7 were sorted, and gene expression was analyzed. With the exception of Hif3a, all candidate genes were up-regulated to various degrees during the ACCs generation (Figure 3A). Four heat shock genes and one DNA repair gene were found to be up-regulated during the ACCs generation. Furthermore, seven of the up-regulated genes are known to be directly involved in the regulation of the cellular redox state. These results suggested that the self-repair or self-defense potency was induced during the ACCs generation.

**Since ACCs exhibited the up-regulation of cellular redox associated genes, the**
mitochondrial function of ACCs was next examined. Mitochondria are organelles responsible for production of the vast majority of ATP via the redox reaction using oxygen within eukaryotic cells. GFP expression of ACC spherical colonies gradually diminished from peripheral located cells after 7 days when colonies were cultured without passage. ACCs contained at day 10 contained GFP expressing central cells and non-GFP differentiated peripheral cells (Figure 3B). Mitochondrial morphology was evaluated in ACCs and differentiated cells by staining with a mitochondrial-specific dye, MitoTracker Red. ACC mitochondria were observed as peri-nuclear clusters that appear punctate and globular (Figure 3B) while differentiated cell contained many mitochondria which were filamentous and wide-spread in cytoplasm (Figure 3B). ATP production of ACCs was less than that in native CD45 positive cells (Figure 3C). Also, reactive oxygen species (ROS) production of ACCs was less than in native CD45 positive cells (Figure 3D). Finally the key factors involved in mtDNA replication were assessed; which are mitochondrial transcription factor A (Tfam), the mitochondrial-specific DNA polymerase gamma (Polg) and its accessory unit (Polg2). The gene expression of Tfam, Polg, and Polg2 in ACCs was lower than those in differentiated cells (Figure 3E). Consequently, ACCs contained small numbers of mitochondria and ACCs' mitochondrial activity was lower than differentiated cells. These results implied that ACCs acquired a metabolic system distinct from differentiated cells to survive after the severe stress response.

[00148] Developmental potential of ACCs. Finally, it was assessed whether ACCs possessed a developmental potential similar to that of plant callus cells. As an initial test for developmental potency, ACCs implanted subcutaneously in immunodeficient (SCID) mice were studied. Six weeks after transplantation, ACCs generated tissues representing all three germ layers (Figure 9B).

[00149] ACCs differentiated into cells representative of all three germ layers in vivo and in vitro. Therefore, the chimera contribution potency of ACCs was assessed. ACCs for use in chimera generation studies were prepared using CD45 positive cells derived from F1 GFP (C57BL/6GFP×DBA/2 or 129/SvGFP×C57BL/6GFP) or GOF. Because gene expression analysis had revealed that at day 7, ACCs expressed the highest level of pluripotent marker genes, day 7 ACCs were utilized for the chimera mouse generation study. Initially, conventional methods for chimera generation were utilized. ACs were dissociated into single cells via treatment with trypsin. The ACCs were then injected into blastocysts (Figure 4A). Using this approach, the chimera
contribution of dissociated ACCs was quite low (Table 1). Therefore ACCs without prior trypsin treatment, which often causes cellular damage, were injected into blastocysts. ACs were cut into small clusters using a micro-knife under the microscopy. Small clusters of ACs were then injected into blastocysts (Figure 4A). Using this approach, the chimera contribution of ACCs dramatically increased (Figure 4B). Chimera mice generated with ACCs grew up healthy (Figure 4C, Figure 9C) and germ line transmission has been observed. The chimera contribution rate of each tissue was analyzed by FACS. The results showed that ACCs derived from lymphocytes contributed to all tissue (Figure 4D).

[00150] As demonstrated above, ACCs can be generated from various cells derived from all three germ layers (Figure 7A-7C). In order to examine whether ACCs derived from various tissues had different differentiation tendencies, ACCs were generated from various tissues derived from F1GFP mice, and injected into ICR blastocysts. Then, using FACS, the contribution ratio of each tissue in the generated chimera mouse was analyzed. It was found that ACCs derived from any tissue contributed to chimeric mouse generation (Figure 9D). In addition, the contribution ratio to skin, brain, muscle, fat, liver and lung was analyzed in chimera mice generated using ACCs derived from various tissues. ACCs derived from any tissue contributed to generate tissues representative of all three germ layers, and no differentiation tendency was observed (Figure 9D).

[00151] The generation of mice by tetraploid complementation, which involves injection of pluripotent cells in 4N host blastocysts, represents the most rigorous test for developmental potency because the resulting embryos are derived only from injected donor cells. ACCs were generated from lymphocytes derived from DBAxB6GFP F1 mice or 129/SvGFPxB6GFP F1. ACCs resulted in the generation of (mid) late-gastration ‘all ACC embryos’ after injection into 4N blastocysts (Figures 4E and 9E). Genotyping analysis demonstrated that ‘all ACC embryos’ had specific genes of strain which was utilized to generate ACCs. Thus, ACCs possessed the potential to generate a clonal body just like plant callus cells.

[00152] Discussion

[00153] Mammalian somatic cells exhibit the ability for animal callus (AC) formation as a result of exposure to significant external stimuli, in a fashion very similar to plants. The cells contained in these calli (animal callus cells, ACCs) have the ability to generate chimeric mice and to generate new embryos fully consisting of only cells generated from ACCs. The results described herein demonstrate that mammalian somatic cells regain the ability to differentiate into...
any of the three germ layers by external stimuli. This implies that somatic cells have a greater plasticity than previously believed. Furthermore, this study demonstrates the potential of somatic cell reprogramming without gene induction or the introduction of foreign proteins, and offers new insight into the potential of adult stem cells; representing a significant milestone in the elucidation of stem cell biology.

[00154] **Materials and Methods**

[00155] _Tissue harvesting and Cell culture._ For mature lymphocytes isolation, spleens derived from GOF mice or ICR mice were minced by scissors and mechanically-dissociated with pasteure pipettes. Dissociated spleens were strain through a cell strainer (BD Biosciences, San Jose). Collected cells were re-suspended in DMEM medium and added the same volume of lympholyte (CEDARLANE®, Ontario, Canada), then centrifuged at 1000g for 15min. Lymphocytes layer was taken out and attained with CD45 antibody (ab25603, abcam, Cambridge, MA). CD45 positive cells were sorted by FACS Aria (BD Biosciences). Then, CD45 positive cells were treated with stress treatment (pH5.5 solution for 15min) and plated into B27 medium supplemented with 1000U LIF (Sigma) and 10 ng/ml FGF 2 (Sigma).

[00156] _Exposure to external stimuli - stress treatment._ To give a mechanical stress to mature cells, pasteure pipette were heated and then stretched to create lumens approximately 50 microns in diameters, and then broken. Mature somatic cells were then triturated through these pipettes for 20 min, and cultured for 7 days. To provide a hypoxic stimulus to mature cells, cells were cultured in a 5% oxygen incubator for 3 weeks. An under nutrition stimulus was provided to mature cells, by culturing the cells in a basic culture medium for 3 weeks. To expose the mature cells to a physiological stress, they were treated with low pH (pH5.5) solution, and cultured for 7 days. Also, cells were given more serious damage. To create pores in mature cell membranes, cells were treated with SLO (Streptolysine O).

[00157] SLO-treated cells were incubated in HBSS containing 10 µg/mL SLO at 37°C for 50 min and then cultured in culture medium without SLO for 7 days. Cells exposed to under-nutrition stress were cultured in basal medium for 2 to 3 weeks. Cells exposed to “ATP” stress were incubated in HBSS containing 2.4 mM ATP at 37°C for 15 min and then cultured in culture medium for 7 days. Cells exposed to “Ca” stress were cultured in culture medium containing 2 mM CaCl₂ for 2 weeks.

[00158] _Bisulfite sequence._ For cells procured from GOF mice were dissociated into single
cells. GFP positive cells collected using by FACS Aria. Genome DNA was extracted from ACCs and studied. Bisulfite treatment of DNA was done using the CpGenome DNA Modification Kit (Chemicon, Temecula, CA, http://www.chemicon.com) following the manufacturer's instructions. The resulting modified DNA was amplified by nested polymerase chain reaction PCR using two forward (F) primers and one reverse (R) primer: Oct4 (F1, GTTGTGTTTGGTTTTGGATAT (SEQ ID NO: 1); F2, ATGGGTGAAATTTGTTTATTTA (SEQ ID NO: 2); R, CCACCCCTCTAACCTAAACCTCTAAC (SEQ ID NO: 3)). And Nanog (F1, GAGGATGTGTTTTAAGTTTTTTT (SEQ ID NO:4); F2, AATGTTTATGGTGGATTTGTAGGT (SEQ ID NO: 5); R, CCCACACTCATATCAATATAAACC (SEQ ID NO:6)). PCR was done using TaKaRa Ex Taq Hot Start Version (RR030A). DNA sequencing was performed using M13 primer with the assistance of GRAS (The Genome Resource and Analysis Unit).

**[00159] Immunohistochemistry.** Cultured cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100/PBS prior blocking with 1% BSA solution (Life Technology, Tokyo, Japan). Secondary antibodies were goat anti-mouse or -rabbit coupled to Alexa-488 or -594 (Invitrogen). Cell nuclei were visualized with DAPI (Sigma). Slides were mounted with SlowFade Gold antifade reagent (Invitrogen).

**[00160] Fluorescence-Activated Cell Sorting and Flow Cytometry.** Cells were prepared according to standard protocols and suspended in 0.1% BSA/PBS on ice prior to FACS. PI (BD Biosciences) was used to exclude dead cells. Cells were sorted on a BD FACS Aria SORP and analyzed on a BD LSRII with BD FACSDiva Software (BD Biosciences).

**[00161] RNA Preparation and RT-PCR Analysis.** RNA was isolated with the RNeasy Micro kit (QIAGEN). Reverse transcription was performed with the SupeSACript III First Strand Synthesis kit (Invitrogen). SYBR Green Mix I (Roche Diagnostics) was used for amplification, and samples were run on a Lightcycler-II Instrument (Roche Diagnostics).

**[00162] Animal Studies.** For tumorigenicity studies, cells suspended in 100 ml PBS were injected subcutaneously in the flanks of age-matched immunodeficient SCID mice. Mice were sacrificed and necropsied after 6 weeks.

**[00163] ATP and ROS Assay.** Intercellular ATP level was measured by the ATP Bioluminescence Assay Kit HS II (Roche) according to supplier's protocol. The luminescence
intensity was measured by using a Gelomax 96 Microplate Luminometer (Promega, Madison, WI) and the luminescence readings were normalized by cell count. For measurement of ROS levels, cells were incubated in a medium contain 2 μM dihydroethidium (Molecular Probes) at 37°C in dark for 15 minutes. Cells were then washed with PBS and suspended in PBS containing 0.5% BSA. The fluorescence intensity of 30000 cells was recorded with the help of a BD Biosciences LSR II (BD Bioscience, Spark, MD).

Chimera mice generation and analyses. Production of Diploid and Tetraploid Chimeras. Diploid embryos were obtained from ICR strain females mated with ICR males and tetraploid embryos were obtained from BDF1 strain females mated with BDF1 males. Tetraploid embryos were produced by the electofusion of 2-cell embryos\textsuperscript{17}. In this study, because trypsin treatment caused low chimerism, ACCs spherical colonies were cut into small pieces using a micro-knife under the microscopy, then small clusters of ACCs were injected into day 4.5 blastocyst by large pipette. Next day, the chimeric blastocysts were transferred into day 2.5 pseudopregnant females.

References


Table 1: Generation of chimera mice from ACCs

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<tr>
<th>Mouse strain</th>
<th>Cell preparation for injection</th>
<th>Culture period of SACs</th>
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* All fetuses were collected at 13.5 dpc to 15.5 dpc and the contribution rate of ACCs into each organ was examined by FACS

** The contribution of SACs into each chimera was scored as high (>50% of the coat color of GFP expression)
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Table 3 – Percent of cells demonstrating pluripotent phenotype after 1 week of stress treatment. Treatments are shown in the first column and the tissue of origin of the somatic cells is shown in the second row. Numbers are percentages.

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What is claimed herein is:

1. A method to generate a pluripotent cell, comprising subjecting a somatic cell to a stress, and selecting cells exhibiting pluripotency, wherein the somatic cell is not present as part of a tissue.

2. The method of claim 1, wherein the stress comprises removing at least about 40% of the cytoplasm from the somatic cell.

3. The method of claim 1, wherein the stress comprises removing at least about 40% of the mitochondria from the somatic cell.

4. The method of any of claims 1-3, wherein the somatic cell is an isolated somatic cell.

5. The method of any of claims 1-4, wherein the somatic cell is present in a heterogeneous population of somatic cells.

6. The method of any of claims 1-5, wherein the somatic cell is present in a homogenous population of somatic cells.

7. The method of any of claims 1-6, wherein selecting the cells exhibiting pluripotency comprises selecting cells expressing Oct4 or Nanog, or Oct4 and Nanog expression.

8. The method of any of claims 1-7, wherein selecting cells exhibiting pluripotency comprises selecting cells which are not adherent.

9. The method of any of claims 1-8, wherein at least about 50% of the cytoplasm is removed from the somatic cell.

10. The method of any of claims 1-9, wherein at least about 60% of the cytoplasm is removed from the somatic cell.

11. The method of claim 10, wherein between 60-80% of the cytoplasm is removed from the somatic cell.

12. The method of claim 10, wherein at least about 80% of the cytoplasm is removed from the somatic cell.

13. The method of claim 12, wherein at least about 90% of the cytoplasm is removed from the somatic cell.

14. The method of any of claims 1-13, wherein the stress comprises exposure of the somatic cell to at least one environmental stimulus selected from: trauma, mechanical stimuli,
chemical exposure, ultrasonic stimulation, oxygen-deprivation, radiation, and exposure to extreme temperatures.

15. The method of claim 14, wherein the exposure to extreme temperatures comprises exposing the somatic cell to temperatures below 35°C or above 42°C.

16. The method of claim 14, wherein the exposure to extreme temperatures comprises exposing the somatic cell to temperatures at, or below freezing or exposure of the somatic cell to temperatures at least about 85°C.

17. The method of claim 14, wherein the mechanical stimulus comprises passing the somatic cell through at least one device with a smaller aperture than the size of the somatic cell.

18. The method of claim 14, wherein the mechanical stimulus comprises passing the somatic cell through several devices having progressively smaller apertures.

19. The method of any of claims 1-18, wherein the removal of a portion of the cytoplasm removes at least about 50% of the mitochondria from the cytoplasm.

20. The method of any of claims 1-19, wherein the removal of cytoplasm or mitochondria removes about 50%-90% of the mitochondria from the cytoplasm.

21. The method of any of claims 1-20, wherein the removal of cytoplasm or mitochondria removes more than 90% of the mitochondria from the cytoplasm.

22. The method of any of claims 1-21, further comprising culturing the pluripotent cell to allow propagation of the stem cell.

23. The method of any of claims 1-22, wherein the pluripotent stem cell expresses one or more pluripotent stem cell markers selected from the group consisting of: Oct4 and Nanog.

24. The method of any of claims 1-23, wherein the somatic cell is a mammalian somatic cell.

25. The method of any of claims 1-24, wherein the somatic cell is a human somatic cell.

26. The method of any of claims 1-25, wherein the somatic cell is an adult somatic cell or a neonatal somatic cell.

27. The method of any of claims 1-26, further comprising maintaining the pluripotent cell in vitro.

28. An assay comprising;
   contacting a pluripotent stem cell produced by the method according to any of claims 1 to 27 with a candidate agent.
29. The assay of claim 28, for use to identify agents which affect one or more of the viability, differentiation, proliferation of the pluripotent stem cell.

30. Use of a pluripotent stem cell produced by the method according to any one of claims 1 to 27 in a method of cell therapy for a subject.

31. A method of autologous cell therapy in a subject in need of cell therapy, comprising
   a. generating a pluripotent stem cell from a somatic cell according to any one of claims 1 to 27, wherein the somatic cell is obtained from the subject, and
   b. administering a composition comprising the pluripotent stem cell or a differentiated progeny thereof to the subject.

32. The method of claim 31, further comprising differentiating the pluripotent stem cell along a pre-defined cell lineage prior to administering the composition to the subject.

33. A composition comprising a pluripotent stem cell, wherein the pluripotent stem cell is generated from a somatic cell by the methods according any of claims 1 to 27.
Figures 1a-1e (cont)
Figures 1a-1e (cont)
Figures 2a-2c (cont)
Figures 4a-4e
Figures 4a-4e (cont)
Figures 5A-5C
11 / 20
Figures 5A-5C (cont)
Figures 6A-6B
Figures 7A-7C
Figures 9A-9E
Figures 9A-9E (cont)
Figure 10
GFP expression is plotted on the x-axis with Side Scatter plotted on the y-axis.

Figure 11
Provisional Application for Patent Cover Sheet
This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c)

Inventor(s)

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All Inventors Must Be Listed – Additional Inventor Information blocks may be generated within this form by selecting the Add button.

Title of Invention
GENERATING PLURIPOTENT CELLS DE NOVO

Attorney Docket Number (if applicable) 043214-071760-P

Correspondence Address

Direct all correspondence to (select one):

- The address corresponding to Customer Number
- Firm or Individual Name
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

- [ ] No.
- [ ] Yes, the name of the U.S. Government agency and the Government contract number are:
Entity Status
Applicant claims small entity status under 37 CFR 1.27

☐ Yes, applicant qualifies for small entity status under 37 CFR 1.27
☐ No

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Signature
Please see 37 CFR 1.4(d) for the form of the signature.

Signature: /Mark J. Fitzgerald/
First Name: Mark, Last Name: Fitzgerald
Date (YYYY-MM-DD): 2012-04-24
Registration Number (If appropriate): 45928

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<td><strong>First Named Inventor/Applicant Name:</strong></td>
<td>Charles A. Vacanti</td>
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<td><strong>Customer Number:</strong></td>
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<td><strong>Filer:</strong></td>
<td>Mark Fitzgerald/Nicole Aguirre</td>
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The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

- Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)
- Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)
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