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(71) Applicant: **STEMGENT, INC.** [US/US]; One Kendall Square, Cambridge, MA 02139 (US).

(72) Inventors; and
(71) Applicants : **MAHON, Kerry** [US/US]; 122 Hamilton Street, Unit 1, Cambridge, MA (US). **HAMILTON, Jonathon, Bradley** [US/US]; 19 Aspen Road, Littleton, MA 01460 (US). **LUO, Chenmei** [US/US]; 22 Redland Street, Shrewsbury, MA 01545 (US).

(74) Agent: **SUNSTEIN, Bruce, D.**; Sunstein Kann Murphy & Timbers LLP, 125 Summer Street, Boston, MA 02110 (US).

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[Continued on next page]

(54) **Title**: METHODS OF PREPARING PLURIPOTENT STEM CELLS

(57) **Abstract**: The invention relates to pluripotent stems cells and their methods of use. The invention also relates to methods of producing pluripotent stem cells.

FIG.1A

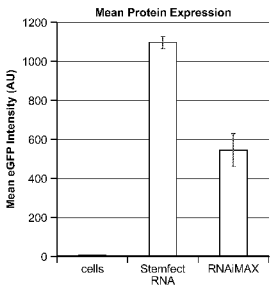


FIG.1B

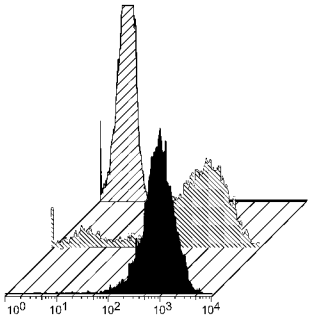
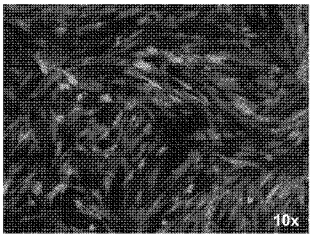


FIG.1C



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METHODS OF PREPARING PLURIPOTENT STEM CELLS

CROSS REFERENCE

This application claims priority to U.S. Provisional Application No. 61/659,240 filed June 13, 2013, herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

The invention relates to methods of preparing pluripotent stem cells and their method of use.

BACKGROUND OF THE INVENTION

The widespread adoption of induced pluripotent stem (iPS) cell technology for regenerative medicine and drug screening applications has been limited by the inability to efficiently derive human iPS cell lines that are free from both genomic perturbation and viral contaminants.

iPS cells were first described by Yamanaka in 2006 (Cell 2006 126(4):663-676) and were immediately recognized for their potential to revolutionize the field of personalized medicine. Yamanaka describe the results of experiments, first performed in mice and then in human cells, wherein the addition of four transcription factors (reprogramming factors), Oct4, Sox2, Klf4 and c-Myc, to a fibroblast led to the de-differentiation of the somatic fibroblast cell to a cell in a pluripotent state.

Early methods of generating iPS cells focused on the use of retroviruses for delivering the reprogramming factors. Such viruses require significant safety precautions when handling, and their mode of action requires integration of the virus into the host cell genome to express the encoded transcription factor. DNA-based methods of generating iPS cells have also been developed and, although these methods are safer than retrovirus based methods, with regards to handling, these methods carry a risk of homologous recombination with the host cell genome. Both viral and DNA based methods of generating iPS cells therefore cannot be used to produce clinical grade cells. Further, iPS cells

produced by viral and DNA based methods must be extensively screened prior to use to ensure that any genomic modifications that may have occurred do not affect the function of the cells.

Recent developments using messenger RNA (mRNA) to generate induced pluripotent stem (iPS) cells have led to improved methods of producing iPS cells. However, certain cell lines remain refractory to reprogramming with mRNA. Further, methods of generating iPS cells generate only a small number of iPS colonies per culture. mRNA based methods for producing iPS cells require multiple transfections (for example, a culture must be transfected every day for 16-18 days) and often requires growth on a feeder layer of cells.

There is a long felt need for a method of preparing pluripotent stem cells that can be rapidly prepared, for example from primary patient cells, do not require a step of screening for genetic modifications, and are safe to use for clinical applications. The novel methods of preparing pluripotent stems cells of the invention wherein a combination of mRNA and miRNA is introduced into the cells, as well as the cells themselves, satisfy this long felt need.

BRIEF SUMMARY OF THE INVENTION

The present invention is directed to a novel method of generating iPS cells wherein cells are combined with a combination of mRNA and miRNA. The method of the invention offers numerous surprising and unexpected advantages as compared to methods of producing iPS cells known in the art, including virus based methods, DNA based methods and mRNA based methods that do not utilize miRNA.

The claimed method of producing iPS cells by the addition of microRNA (miRNA) in combination with mRNA improves upon any known methods of producing iPS cells because it provides for: 1) faster kinetics for the reprogramming process as compared to any known methods; and 2) higher productivity as compared to any known methods.

A decrease in the amount of time required to produce iPS cells is clearly an

advantage. The novel claimed method of producing iPS cells of the invention also offers the advantage of requiring significantly fewer transfections, as compared to any known method of producing iPS cells.

The novel method of the invention also provides for production of an increased number of iPS cell colonies from typical patient lines as compared to other methods. Further, the claimed method of producing iPS cells of the invention enables the generation of iPS cells from cells that have not yielded any colonies when subjected to any known method of producing iPS cells.

Unlike virus based methods of producing iPS cells the novel claimed method is safe and provides an efficient method for producing iPS cells suitable for clinical use. Differentiated progeny cells derived from iPS cells of the invention are also suitable for clinical use. The claimed method can be performed without the need for any significant safety precautions. Unlike iPS cells prepared by methods that require viral vectors, iPS cells produced by the novel method of the invention offer the advantage of being free from viral contaminants and therefore are suitable for clinical applications. iPS cells and differentiated progeny cells produced from the iPS cells, such as those produced by the claimed methods, are advantageous over cells produced by other methods because they can be used for the development of personalized treatments and for regenerative medicine applications.

The claimed methods provide for a method of producing iPS cells wherein there is no risk of the occurrence of homologous recombination with the host cell genome. The claimed method produces iPS cells that have no genomic integrations and therefore require no pre-screening to determine genomic modifications as do iPS cells prepared by other methods known in the art.

Unlike art-accepted methods of producing iPS cells, the claimed method eliminates inherent variability associated with feeder based reprogramming methods by pairing a defined, xeno-free cell culture medium (that is, the medium contains no non-human components) with pluripotent cell culture attachment substrates. In addition, according to the claimed methods, a reduced number of

transfections are ultimately required to establish iPS cell colonies. Further, a reduced amount of mRNA is needed per daily transfection.

Unlike iPS cells produced by methods currently known in the art, the claimed method offers the advantage of producing iPS cells that can be banked and used for experiments 4-5 weeks or more following production.

In addition, unlike other methods, the claimed method does not require post-colony isolation screening for genomic integrations or viral contaminants.

The methods of preparing pluripotent stem cells of the current invention clearly provide at least the following advantages over other methods known in the art: the use of mRNA and miRNA allows for fine control of stoichiometry and expression levels; the use of mRNA and miRNA allows for temporal control of stoichiometry and expression levels; because there is no integration of either mRNA or miRNA the method of the invention is suitable for the production of clinically relevant cells as compared to methods known in the art which use virus and therefore create a safety concern for clinical use; the timeline for colony formation, identification and isolation can be under 14 days according to the methods described herein, as compared to prior art methods that may require 40 days or more for production of pluripotent stem cells; the methods described herein do not require reseeding the cells although reseeding can be done, in contrast to methods known in the art that require reseeding after viral transduction; and the method is performed in the absence of a feeder layer as compared to methods known in the art that require the use of a feeder layer.

The effect on cell reprogramming is to enhance reprogramming. The methods of the present invention include inducing pluripotency in a cell, such that the cell becomes capable of dividing and differentiating into any cell type other than embryonic cells. Cellular reprogramming also induces de-differentiation of a cell. Altering cell reprogramming can enhance the level of pluripotency or de-differentiation that has been induced by an agent other than the combination of mRNA and microRNA. The pluripotent or multipotent cells, also called stem cells, have the ability to divide (self-replicate or self-renew) or differentiate into multiple

different phenotypic lineages for indefinite periods. The cells of the present invention, under specific conditions, or in the presence of optimal regulatory signals, can become pluripotent and differentiate themselves into many different cell types that make up the organism.

The pluripotent or multipotent cells of the present invention possess the ability to differentiate into cells that have characteristic attributes and specialized functions, such as hair follicle cells, blood cells, heart cells, eye cells, skin cells, pancreatic cells, or nerve cells. In particular, pluripotent cells of the invention can differentiate into multiple cell types including but not limited to: cells derived from the endoderm, mesoderm or ectoderm, including but not limited to cardiac cells, neural cells (for example, astrocytes and oligodendrocytes), hepatic cells (for example, pancreatic islet cells), osteogentic, muscle cells, epithelial cells, chondrocytes, adipocytes, dendritic cells and, haematopoietic and retinal pigment epithelial (RPE) cells.

iPS cells are promising tools for the treatment of neurodegenerative disorders. For example, somatic cells from a patient with a disorder can be transformed into iPS cells using the methods of the invention and further differentiated to the desired neural subtype. Such cells can then be used in the development of disease models for the discovery of new compounds or other agents capable of treating the disease and/or for treating compounds used for therapy. In certain cases, the differentiated cells can be used for cell therapy to replace damaged tissue.

Examples of differentiation methods to the neural subtypes motor neuron and dopaminergic neuron and their application to the development of new therapies are found in the following references: Saporta et al. “Induced pluripotent stem cells in the study of neurological diseases” Stem Cell Research & Therapy 2011, 2:37; Lopez-Gonzalez, R. and Velasco, I. “Therapeutic Potential of Motor Neurons Differentiated from Embryonic Stem Cells and Induced Pluripotent Stem Cells” Arch Med Res 2012, 43:1, 1-10; Cooper et al. “Differentiation of human ES and Parkinson’s disease iPS cells into ventral midbrain dopaminergic neurons

requires a high activity form of SHH, FGF8a and specific regionalization by retinoic acid" Molecular and Cellular Neuroscience 45 (2010) 258–266; Dimos et al., "Induced Pluripotent Stem Cells Generated from Patients with ALS Can Be Differentiated into Motor Neurons" Science 2008 321, 1218; Hu et al., "Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency" Proc. Nat. Acad. Sci. USA 2010, 107, 9, 4335; Mohamad O, Drury-Stewart D, Song M, Faulkner B, Chen D, et al. (2013) Vector-Free and Transgene-Free Human iPS Cells Differentiate into Functional Neurons and Enhance Functional Recovery after Ischemic Stroke in Mice. PLoS ONE 8(5): e64160. doi:10.1371/journal.pone.0064160; Osadaka et al., "Control of neural differentiation from pluripotent stem cells" Inflammation and Regeneration 2008 Vol.28 No.3 166; Marchetto et al., "Induced pluripotent stem cells (iPSCs) and neurological disease modeling: progress and promises" Human Molecular Genetics, 2011, Vol. 20, Review Issue 2 R109–R115.

Methods of differentiating stem cells are well known in the art and include, for example, contacting pluripotent stem cells with appropriate growth factors and/or cytokines.

Cell reprogramming can further include partial de-differentiation to a closely related cell or cell type and/or trans-differentiation, wherein a cell of the present invention converts from one differentiated cell type into another differentiated cell type.

Moreover, to enhance the efficiency to establish induced pluripotent stem (iPS) cells, the following cytokines and/or small molecules, in addition to the above-mentioned miRNAs, may further be introduced into somatic cells to be reprogrammed: i.e., basic fibroblast growth factor (bFGF), stem cell factor (SCF), etc. for the cytokines; and histone deacetylase inhibitors such as valpronic acid, DNA methylase inhibitors such as 5'-azacytidine, histone methyltransferase (G9a) inhibitors such as BIX01294 (BIX), etc. for the small molecules (D. Huangfu et al., Nat. Biotechnol., 26, pp. 795-797, 2008; S. Kubicek et al., Mol. Cell, 25, pp. 473-481, 2007; Y. Shi et al., Cell Stem Cell, 3, 568-574, 2008, Yan Shi et al., Cell Stem Cell, 2, pp. 525-528, 2008. In addition, p53 inhibitors such as

shRNA or siRNA for p53 and/or UTF1 may be introduced into somatic cells (Yang Zhao et al., Cell Stem Cell, 3, pp 475-479, 2008). Also, activation of the Wnt signal (Marson A. et al., Cell Stem Cell, 3, pp 132-135, 2008) or inhibition of signaling by mitogen-activated protein kinase or glycogen synthase kinase-3 (Silva J. et al., PloS Biology, 6, pp 2237-2247 2008) can serve as a means for increasing the efficiency of generating iPS cells.

The invention provides for a method of producing a pluripotent stem cell comprising: introducing at least one mRNA into a target cell; introducing at least one miRNA into a target cell; and culturing the target cell to produce a pluripotent stem cell.

The step of introducing the at least one mRNA into the cell and or the step of introducing the at least one miRNA into the target cell can be repeated at least once.

Prior to step (a), at least one miRNA can be introduced into the target cell.

Steps (a) and (b) can be sequential.

Steps (a) and (b) can occur simultaneously.

The stem cell can be produced in less than 2 weeks from the initiation of step (a), for example, 13 days, 12 days, 11 days, 10 days, 9 days, 8 days, 7 days, 6 days, 5 days or less from the initiation of step (a). Alternatively, a stem cell of the invention is produced in more than 2 weeks, for example 2-10 weeks, 2-5 weeks and 2-3 weeks from the initiation of step (a).

The stem cell that is produced can express at least one of a surface marker selected from the group consisting of: SSEA3, SSEA4, Tra-1-81, Tra-1-60, Rex1, Oct4, Nanog and Sox2.

The stem cells can divide *in vitro* for greater than one year; and/or divide in vitro for more than 30 passages; and/or stain positive by alkaline phosphatase or Hoechst Stain, and/or form a teratoma.

The stem cell can form an embryoid body and express one or more endoderm markers selected from the group consisting of: AFP, FOXA2 and GATA4, and/or one or more mesoderm markers selected from the group consisting of: CD34, CDH2 (N-cadherin), COL2A1, GATA2, HAND1, PECAM1, RUNX1, RUNX2; and/or one or more ectoderm markers selected from the group consisting of: ALDH1A1, COL1A1, NCAM1, PAX6 andTUBB3 (Tuj1).

At least 1 stem cell is produced, for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 500, 1000 or more.

One or both of the at least one miRNA and the at least one mRNA can comprise at least one modified nucleotide as defined herein.

Neither of the at least one miRNA and the at least one mRNA are provided in a DNA vector or a viral vector.

One or both of the at least one miRNA and the at least one mRNA can comprise a modified nucleotide, for example 5-methylcytosine or pseudouracil, or any modified nucleotide as defined herein.

The at least one mRNA is not integrated into the genome of the stem cell.

The mRNA and miRNA introduced into the target cells in steps (a) and (b) are not present in the stem cell.

The culturing can be performed in the absence of a feeder layer.

The method can be performed at $\leq$5% O_2.

The method can be performed at 5%-21% O_2, for example, 6, 7, 8, 9, 10, 15, 20 and 21%, for example, at 21% O_2

The target cell includes but is not limited to fibroblasts, peripheral blood derived cell types (specifically late - endothelial progenitor cell (L-EPCs)), cord blood derived cell types (CD34+), epithelial cells and keratinocyte.

The at least one mRNA encodes a reprogramming factor.

The at least one mRNA can encode at least one of OCT4, SOX2, KLF4, c-MYC and LIN28.

The at least one miRNA can comprise at least one miRNA that is 80% or more identical to an miRNA selected from the group consisting of hsa-miR-302a, hsa-miR-302b, hsa-miR-302c, hsa-miR302d, hsa-miR367, hsa-miR-200c, hsa-miR-369-3p and hsa-miR-369-5p.

The at least one miRNA can also comprise a combination of hsa-miR-302a, hsa-miR-302b, hsa-miR-302c, hsa-miR302d and hsa-miR367.

The at least one miRNA can also comprise a combination of hsa-miR-302a, hsa-miR-302b, hsa-miR-302c, hsa-miR302d, hsa-miR-200C, hsa-miR-369-3p and hsa-miR-369-5p.

The at least one miRNA can also comprise a combination of hsa-miR-302a, hsa-miR-302b, hsa-miR-302c, hsa-miR302d, hsa-miR-200c, hsa-miR-369-3p and hsa-miR-369-5p.

The at least one miRNA can comprise the combination of: hsa-miR-302a, hsa-miR-302b, hsa-miR-302c, hsa-miR-302d and hsa-miR-367; or hsa-miR-302a, hsa-miR-302b, hsa-miR-302c, hsa-miR-302d, hsa-miR-200C, hsa-miR-369-3p, hsa-miR-369-5p; or the combination of hsa-miR-302a, hsa-miR-302b, hsa-miR-hsa-miR-302c, hsa-miR-302d, hsa-miR-200C, hsa-miR-369-3p, hsa-miR-369-5p

The target cell can be a mammalian cell, including but not limited to a human cell.

The invention also provides for a method of inducing pluripotency in a target cell comprising: introducing at least one mRNA into the target cell; introducing at least one miRNA into the target cell; and culturing the target cell to produce a pluripotent cell.

The invention also provides for an isolated pluripotent stem cell comprising at least one mRNA encoding a reprogramming factor in combination with at least one miRNA produced according to any one of the methods described herein.

The invention also provides for a formulation comprising the isolated pluripotent stem cell as defined herein and produced by any one of the methods described herein, or a differentiated cell derived from an isolated pluripotent stem cell as defined herein, for example, in combination with a pharmaceutical carrier.

The formulation can further comprise a compound that suppresses an immune response.

As used herein, compound includes any one of a protein, an antibody, a nucleic acid, for example, siRNA, miRNA, antisense RNA, mRNA and/or a small molecule.

The invention also provides for a kit for producing a pluripotent stem cell or a differentiated progeny cell comprising at least one mRNA and at least one miRNA.

The kit can further comprise culture media and/or a transfection reagent.

The kit can further comprise a compound that suppresses an immune response.

The invention also provides for a method of treating a subject with any of the diseases described herein comprising administering to the subject the isolated pluripotent stem cell of the invention and produced by any of the methods described herein.

The invention also provides for a method of treating a subject with any of the diseases described herein, comprising administering to the subject a progeny cell produced by differentiation of the isolated pluripotent stem cell obtained by the methods of the invention.

The invention also provides for a method of identifying a compound for treatment of a disease comprising contacting a cell produced by differentiation of a stem cell produced by the methods of the invention with a compound of interest.

The invention also provides for a method of determining the activity of a compound for treating a disease comprising contacting a cell produced by differentiation of a stem cell produced by the methods of the invention with a compound known to treat a disease.

The invention also provides a method of determining the toxicity of a compound for treating a disease comprising contacting a cell produced by differentiation of a stem cell produced by the methods of the invention with a compound known to treat a disease.

According to the methods of the invention, the cell produced by differentiation of a stem cell is selected from the group consisting of: fibroblast, peripheral blood derived cells including but not limited to endothelial progenitor cell (L-EPCs)), cord blood derived cell types (CD34+), epithelial cells, and keratinocytes .

The invention also provides for the use of a cell produced by differentiation of a stem cell produced by the methods of the invention for the manufacture of a medicament for treating a subject with a disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 presents the results of transfections of fibroblasts with eGFP mRNA (A: fluorescence intensity as determined by flow cytometry; B: representative histograms; C: fluorescent imaging of cells transfected with eGFP mRNA).

Figure 2 presents A: the timeline for production of iPS cells from primary patient fibroblasts; and B: the morphology progression during iPS cell production.

Figure 3 presents the effect of target cell number and mRNA dose on iPS cell generation.

Figure 4 presents a graph demonstrating the number of mRNA transfections

required to generate Tra-1-81(+) iPS cell colonies.

Figure 5 presents results demonstrating that iPS cell colonies can be formed when cells are treated with miRNA in the presence of low levels of mRNA.

Figure 6 demonstrates the continued expansion and maintenance of pluripotency of clonal mRNA iPS cells lines under feeder free conditions.

Figure 7 presents morphological progression of an iPS colony from cells refractory to other methods of reprogramming.

Figure 8 presents the nucleotide sequence (A) and amino acid sequence (B) of OCT4.

Figure 9 presents the nucleotide sequence (A) and amino acid sequence (B) of SOX2.

Figure 10 presents the amino acid sequence of NANOG.

Figure 11 presents the nucleotide sequence (A) and amino acid sequence (B) of LIN28.

Figure 12 presents the nucleotide sequence (A) and amino acid (B) sequence of KLF4

Figure 13 presents the nucleotide sequence (A) and amino acid sequence (B) of cMYC.

Figure 14 presents the nucleotide sequence of GFP.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, "pluripotent" as it refers to a "pluripotent stem cell" means a cell with the developmental potential, under different conditions, to differentiate to cell types characteristic of all three germ cell layers, i.e., endoderm (e.g., gut tissue), mesoderm (including blood, muscle, and vessels), and ectoderm (such as skin and nerve). Pluripotent cell as used herein, includes a cell that can form a

teratoma which includes tissues or cells of all three embryonic germ layers, or that resemble normal derivatives of all three embryonic germ layers (i.e., ectoderm, mesoderm, and endoderm) are formed. A pluripotent cell of the invention also means a cell that can form an embryoid body (EB) and express markers for all three germ layers including but not limited to the following: endoderm markers-AFP, FOXA2, GATA4; mesoderm markers-CD34, CDH2 (N-cadherin), COL2A1, GATA2, HAND1, PECAM1, RUNX1, RUNX2; and Ectoderm markers-ALDH1A1, COL1A1, NCAM1, PAX6, TUBB3 (Tuj1).

A pluripotent cell of the invention also means a human cell that expresses at least one of the following markers: SSEA3, SSEA4, Tra-1-81, Tra-1-60, Rex1, Oct4, Nanog, Sox2 as detected using methods known in the art. A pluripotent stem cell of the invention includes a cell that stains positive with alkaline phosphatase or Hoechst Stain.

A pluripotent cell has a lower developmental potential than a totipotent cell. The ability of a cell to differentiate to all three germ layers can be determined using, for example, a nude mouse teratoma formation assay. In some embodiments, pluripotency can also be evidenced by the expression of embryonic stem (ES) cell markers. Pluripotency of a cell or population of cells generated using the compositions and methods described herein is also determined by the developmental potential to differentiate into cells of each of the three germ layers.

In some embodiments, a pluripotent cell is termed an "undifferentiated cell." Accordingly, the terms "pluripotency" or a "pluripotent state" as used herein refer to the developmental potential of a cell that provides the ability of the cell to differentiate into all three embryonic germ layers (endoderm, mesoderm and ectoderm). Those of skill in the art are aware of the embryonic germ layer or lineage that gives rise to a given cell type. A cell in a pluripotent state typically has the potential to divide *in vitro* for a long period of time, e.g., greater than one year or more than 30 passages.

As used herein, the term "induced pluripotent stem cells (iPS cells)" refers to cells having similar properties to those of ES cells. In particular, an "iPS" cell as used herein, includes an undifferentiated cell which is reprogrammed from somatic cells and have pluripotency and proliferation potency. However, this term is not to be construed as limiting in any sense, and should be construed to have its broadest meaning. As used herein, the term "pluripotent stem cell", as it refers to the cell produced by the claimed methods is synonymous with the term "iPS". iPS cells of the invention are generated from a variety of cell types including but not limited to fibroblasts, peripheral blood derived cell types (specifically late - endothelial progenitor cell (L-EPCs)), cord blood derived cell types (CD34+), epithelial cells and keratinocytes.

The invention also provides for colonies of iPS cells produced, for example, by providing a non-pluripotent cell (somatic), culturing this cell in a media, culturing this cell on a surface, culturing this cell with a feeder cell (for example, NuFF or MEF- mouse embryonic fibroblast) introducing mRNA, introducing miRNA, introducing mRNA and miRNA, optionally splitting the cell culture, identifying stem cell colonies using surface markers or morphology, isolating the colony, and subculturing the isolated colony. A pluripotent stem cell colony will exhibit some or all of the characteristics described above for pluripotent stem cells.

As used herein, the term "somatic cell" also 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 cell forming the body of an organism, as opposed to a germline cell. 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--apart from the sperm and ova, the cells from which they are made (gametocytes) and undifferentiated, pluripotent, embryonic 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*. Unless otherwise indicated, the compositions and methods for reprogramming a somatic cell described herein can be performed both *in vivo* and *in vitro* (where *in vivo* is practiced when a somatic cell is present within a subject, and where *in vitro* is practiced using an isolated somatic cell maintained in culture).

As used herein, the term "reprogramming factor," refers to factor that can alter the developmental potential of a cell, such as a protein, an RNA, or a small molecule, the expression of which contributes to the reprogramming of a cell, e.g. a somatic cell, to a less differentiated or undifferentiated state, e.g. to a cell of a pluripotent state or partially pluripotent state. A reprogramming factor can be, for example, transcription factors that can reprogram cells to a pluripotent state, such as, but not limited to, SOX2, OCT3/4, KLF4, NANOG, LIN-28, c-MYC, Glis1, Sal4, Esrbb1 and the like, including but not limited to, any gene, protein, RNA or small molecule, that can substitute for one or more of these transcription factors in a method of reprogramming cells *in vitro*.

The term "cell reprogramming" refers to altering the natural state of the cell such that the cell becomes pluripotent and is capable of dividing and differentiating into any cell type other than embryonic cells. Cellular reprogramming can include inducing pluripotency in or de-differentiation of the cell. Altering cell reprogramming can also refer to enhancing the level of pluripotency or de-differentiation that has been induced by an agent other than a microRNA. Pluripotent or multipotent cells, also called stem cells, have the ability to divide (self-replicate or self-renew) or differentiate into multiple different phenotypic lineages for indefinite periods; in some cases throughout the life of the organism. A stem cell population is a population that possesses at least one stem cell. When pluripotent stem cells are derived from a non-pluripotent cell, such as for

example a somatic cell, they are termed induced pluripotent stem cells (iPS or iPSCs). Cell reprogramming can further include partial de-differentiation to a closely related cell or cell type. Cell reprogramming can also include trans-differentiation. Trans-differentiation is defined as the conversion of one differentiated cell type into another, such as for example conversion of exocrine cells into beta-islet-like cells. (See, e.g., Blelloch, et al., Short cut to cell replacement, Nature, 455:604-605 (2008).)

The term "progenitor cell" is used herein to refer to cells that have greater developmental potential, i.e., a cellular phenotype that is more primitive (e.g., is at an earlier step along a developmental pathway or progression) relative to a cell which it can give rise to by differentiation. Often, progenitor cells have significant or very high proliferative potential. Progenitor cells can give rise to multiple distinct cells having lower developmental potential, i.e., differentiated cell types, or to a single differentiated cell type, depending on the developmental pathway and on the environment in which the cells develop and differentiate.

As used herein, the term “nucleic acid” refers to deoxyribonucleotides, ribonucleotides, or modified nucleotides, and polymers thereof in single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

As used herein, "nucleotide" is used as recognized in the art to include those with natural bases (standard), and modified bases well known in the art. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other; see,

e.g., Usman and McSwiggen, supra; Eckstein, et al., International PCT Publication No. WO 92/07065; Usman et al, International PCT Publication No. WO 93/15187; Uhlman & Peyman, supra, all are hereby incorporated by reference herein). Some of the non-limiting examples of base modifications that can be introduced into nucleic acid molecules include, hypoxanthine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2,4,6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (e.g., 5-methylcytidine), 5-alkyluridines (e.g., ribothymidine), 5-halouridine (e.g., 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (e.g. 6-methyluridine and pseudouridine), propyne, and others (Burgin, et al., Biochemistry 35:14090, 1996; Uhlman & Peyman, supra). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents.

As used herein, the term "deoxyribonucleotide" encompasses natural and synthetic, unmodified and modified deoxyribonucleotides. Modifications include changes to the sugar moiety, to the base moiety and/or to the linkages between deoxyribonucleotide in the oligonucleotide.

By "RNA" is meant a molecule comprising at least one ribonucleotide residue. By "ribonucleotide" is meant a nucleotide with a hydroxyl group at the 2' position of a β-D-ribofuranose moiety. The term RNA includes double-stranded RNA, single-stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Nucleotides in the RNA molecules of the instant invention can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA.

As used herein, “modified nucleotide” refers to a nucleotide that has one or more modifications to the nucleoside, the nucleobase, pentose ring, or phosphate

group. For example, modified nucleotides exclude ribonucleotides containing adenosine monophosphate, guanosine monophosphate, uridine monophosphate, and cytidine monophosphate and deoxyribonucleotides containing deoxyadenosine monophosphate, deoxyguanosine monophosphate, deoxythymidine monophosphate, and deoxycytidine monophosphate. Modifications include those naturally occurring that result from modification by enzymes that modify nucleotides, such as methyltransferases. Modified nucleotides also include synthetic or non-naturally occurring nucleotides. Synthetic or non-naturally occurring modifications in nucleotides include those with 2' modifications, e.g., 2'-O-methyl, 2'-methoxyethoxy, 2'-fluoro, 2'-allyl, 2'-O-[2-(methylamino)-2-oxoethyl], 4'-thio, 4'-CH_2-O-2'-bridge, 4'-$(CH_2)_2$-O-2'-bridge, 2'-LNA, and 2'-O-(N-methylcarbamate) or those comprising base analogs. In connection with 2'-modified nucleotides as described for the present disclosure, by "amino" is meant 2'-NH_2 or 2'-O-NH_2, which can be modified or unmodified. Such modified groups are described, *e.g.*, in Eckstein *et al.*, U.S. Pat. No. 5,672,695 and Matulic-Adamic *et al.*, U.S. Pat. No. 6,248,878.

As used herein, "microRNA" or "miRNA" refers to a nucleic acid that forms a single-stranded RNA, which single-stranded RNA has the ability to alter the expression (reduce or inhibit expression; modulate expression; directly or indirectly enhance expression) of a gene or target gene when the miRNA is expressed in the same cell as the gene or target gene. In one embodiment, a miRNA refers to a nucleic acid that has substantial or complete identity to a target gene and forms a single-stranded miRNA. In some embodiments miRNA may be in the form of pre-miRNA, wherein the pre-miRNA is double-stranded RNA. The sequence of the miRNA can correspond to the full length target gene, or a subsequence thereof. Typically, the miRNA is at least about 15-50 nucleotides in length (e.g., each sequence of the single-stranded miRNA is 15-50 nucleotides in length, and the double stranded pre-miRNA is about 15-50 base pairs in length). In some embodiments the miRNA is 20-30 base nucleotides. In some embodiments the miRNA is 20-25 nucleotides in length. In some

embodiments the miRNA is 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

The invention also provides for pluripotent stem cells that are produced by introducing into the cells a combination of mRNA and miRNA mimics. As used herein, the term, “miRNA mimic” means synthetic miRNA that has enhanced stability due to modified nucleotides or structural modifications (e.g. bulges or loops). As used herein, the term “miRNA mimic” also means small, chemically modified double-stranded RNAs that mimic endogenous miRNAs and enable miRNA functional analysis by up-regulation of miRNA activity. They are typically hairpins, for example, formed by single stranded miRNA that forms a double stranded portion that is a hairpin loop.

The term "contacting" or "contact" as used herein in connection with contacting a cell with one or more mRNAs or miRNAs as described herein, includes subjecting a cell to a culture medium which comprises one or more mRNAs or miRNAs at least one time, or a plurality of times, or to a method whereby such mRNAs and/or miRNAs are forced to contact a cell at least one time, or a plurality of times, i.e., a transfection system. Preferably, the mRNA and miRNA, when introduced into a cell, are not present in a DNA or viral vector. mRNA and miRNA of the invention that are not in a DNA or viral vector can be introduced or transfected into a cell according to methods known in the art, for example, electroporation and lipofection.

As used herein, the term "transfection reagent" refers to any agent that induces uptake of a synthetic, mRNA or miRNA into a host cell. Also encompassed are agents that enhance uptake e.g., by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 25-fold, at least 500-fold, at least 100-fold, at least 1000-fold, or more, compared to an mRNA or miRNA administered in the absence of such a reagent. In one embodiment, a cationic or non-cationic lipid molecule useful for preparing a composition or for co-administration with an mRNA or miRNA is used

as a transfection reagent. In other embodiments, the mRNA or miRNA comprises a chemical linkage to attach e.g., a ligand, a peptide group, a lipophilic group, a targeting moiety etc. In other embodiments, the transfection reagent comprises a charged lipid, an emulsion, a liposome, a cationic or non-cationic lipid, an anionic lipid, or a penetration enhancer as known in the art or described herein.

As used herein, the term "repeated transfections" refers to repeated transfection of the same cell culture with an mRNA or miRNA of the invention, a plurality of times (e.g., more than once or at least twice). In some embodiments, the cell culture is transfected at least twice, at least 3 times, at least 4 times, at least 5 times, at least 6 times, at least 7 times, at least 8 times, at least 9 times, at least 10 times, at least 11 times, at least 12 times, at least 13 times, at least 14 times, at least 15 times, at least 16 times, at least 17 times at least 18 times, at least 19 times, at least 20 times, at least 25 times, at least 30 times, at least 35 times, at least 40 times, at least 45 times, at least 50 times or more. The transfections can be repeated until a desired phenotype of the cell is achieved.

The time between each repeated transfection is referred to herein as the "frequency of transfection." In some embodiments, the frequency of transfection occurs every 6 h, every 12 h, every 24 h, every 36 h, every 48 h, every 60 h, every 72 h, every 96 h, every 108 h, every 5 days, every 7 days, every 10 days, every 14 days, every 3 weeks, or more during a given time period in any method of producing a pluripotent stem cell or any method of inducing pluripotency in a cell according to the invention. The frequency can also vary, such that the interval between each dose is different (e.g., first interval 36 h, second interval 48 h, third interval 72 h etc). It should be understood depending upon the schedule and duration of repeated transfections, it will often be necessary to split or passage cells or change or replace the media during the transfection regimen to prevent overgrowth and replace nutrients. For the purposes of the methods described herein, transfections of a culture resulting from passaging an earlier transfected culture is considered "repeated transfection," "repeated contacting" or "contacting a plurality of times," unless specifically indicated otherwise.

The term "introducing" when used in the context of "introducing" an miRNA or mRNA into a cell refers to any of the well-known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, biolistics, liposomes, microinjection, plasma vectors, viral vectors and any of the other well-known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into a host cell (see, e.g., Sambrook et al., supra). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one miRNA into the host cell.

A variety of different types of cells can be utilized for the methods of the present invention. Cells that may express an mRNA and/or miRNAs of the invention can include, e.g., fibroblast cells, peripheral blood derived cells including but not limited to endothelial progenitor cell (L-EPCs)), cord blood derived cell types (CD34+), epithelial cells, and keratinocytes .

The cells can be any of the cells typically utilized in generating cells that harbor recombinant nucleic acid constructs. Cells useful according to the methods of the invention include, but are not limited mouse embryonic fibroblasts (MEFs). The cells can be mammalian cells, for example, human, rodent or primate. Cell types utilized for the methods of the present invention can also include cells from tissue samples including but not limited to blood, bone, brain, kidney, muscle, spinal cord, nerve, endocrine system, uterine, ear, foreskin, liver, intestine, bladder or skin, for example, as derived from a subject diagnosed with a particular disease or in need of pluripotent stem cells. The cells can include neural cells, lymphocytes, epidermal cells, islet cells, intestinal cells or fibroblasts. The cells of the present invention can be autologous or heterologous cells. The cells useful for the methods of the present invention can include animal cells. In some embodiments the cells are mammalian. In some embodiments the cell are from rodents or primates. In some embodiments the cells are mouse cells. In some embodiments are pig cells.

The types of target or somatic cells to be used for the formation of pluripotent stem cells of the invention or reprogrammed by the method of the present invention are not specifically limited, and any somatic cells can be used. For example, various somatic cells such as (1) tissue stem cells, e.g., neural stem cells, hematopoietic stem cells, mesenchymal stem cells, and dentis stem cells; (2) tissue precursor cells; and (3) differentiated cells, e.g., lymphocytes, epidermal cells, endothelial cells, muscle cells, fibroblast cells, pilary cells, skin cells, liver cells, gastric mucosa cells, intestine cells, spleen cells, pancreatic cells (including pancreatic exocrine cells), brain cells, lung cells, and renal cells can be reprogrammed. Blood cells including platelets, erytrocytes, leukocytes (neutrophils, eosinophils, basophils, lymphocytes, monocytes) and thrombocytes can be used to produce pluripotent stem cells according to the methods of the invention. For use of induced pluripotent stem cells or progeny cells differentiated from iPS cells in therapies against diseases, it is desirable to use somatic cells isolated from the patient. For example, somatic cells involved in a disease and somatic cells associated with a therapy for a disease can also be used.

As used herein "culture" means maintain for an appropriate amount of time under controlled conditions in a controlled and defined medium.

As used herein, "culture medium" means a medium optimized for mRNA based cellular reprogramming of human cells or a medium suitable for expanding and maintaining iPS cell lines. In one embodiment, a "culture medium" according to the invention is xeno-free. Culture medium useful according to the invention includes any medium known in the art to provide for production of pluripotent stem cells. Culture medium useful according to the invention also includes any medium known in the art to support maintenance of pluripotent stem cells. Culture medium according to the invention includes but is not limited to Pluriton™ Reprogramming Medium (Stemgent) for production of iPS cells, and Nutristem™ XF/FF Culture Medium (Stemgent) for maintenance of iPS cells.

By "subject" is meant an organism, which is a donor or recipient of explanted somatic cells or the pluripotent cells themselves. "Subject" also refers to an organism to which the pluripotent cells or differentiated progeny of the pluripotent cells of the invention can be administered. A subject can be a mammal or mammalian cells, including a human or human cells.

“Subject,” as used herein, is preferably, but not necessarily limited to, a human subject. The subject is male or female, and may be of any race or ethnicity. Subject as used herein may also include an animal, particularly a mammal such as a canine, feline, bovine, caprine, equine, ovine, porcine, rodent (*e.g.*, a rat and mouse), a lagomorph, a primate (including non-human primate), *etc.*, that may be treated in accordance with the methods of the present invention or screened for veterinary medicine or pharmaceutical drug development purposes. A subject according to some embodiments of the present invention includes a patient, human or otherwise, in need of therapeutic treatment for a disease according to the invention.

As used herein, “control subject” means a subject that has not been diagnosed with a disease according to the invention. A “control subject” also means a subject that is not at risk of developing a disease, as defined herein.

The phrase "pharmaceutically acceptable carrier" refers to a carrier for the administration of a therapeutic agent. Exemplary carriers include saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof.

Various methodologies of the instant invention include steps that involves comparing a value, level, feature, characteristic, property, etc. to a "suitable control", referred to interchangeably herein as an "appropriate control". A "suitable control" or "appropriate control" is any control or standard familiar to one of ordinary skill in the art useful for comparison purposes. In one embodiment, a "suitable control" or "appropriate control" is a value, level, feature, characteristic, property, etc. determined prior to performing a method of producing a pluripotent stem cell or a method of inducing pluripotency, as described herein. For example, a transcription rate, mRNA level, translation rate, protein level,

biological activity, cellular characteristic or property, genotype, phenotype, etc. can be determined prior to introducing an mRNA and miRNA of the invention into a cell or organism. In another embodiment, a "suitable control" or "appropriate control" is a value, level, feature, characteristic, property, etc. determined in a cell or organism, *e.g.*, a control or normal cell or somatic cell or organism, exhibiting, for example, normal traits. In yet another embodiment, a "suitable control" or "appropriate control" is a predefined value, level, feature, characteristic, property, etc.

The term "*in vitro*" has its art recognized meaning, *e.g.*, involving purified reagents or extracts, *e.g.*, cell extracts. The term "*in vivo*" also has its art recognized meaning, *e.g.*, involving living cells, *e.g.*, immortalized cells, primary cells, cell lines, and/or cells in an organism.

"Treatment", or "treating" as used herein, is defined as the application or administration of a pluripotent stem cell or a differentiated cell derived therefrom of the invention to a patient who has a disorder with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect the disease or disorder, or symptoms of the disease or disorder. The term "treatment" or "treating" is also used herein in the context of administering agents prophylactically. The term "effective dose" or "effective dosage" is defined as an amount sufficient to achieve or at least partially achieve the desired effect. The term "therapeutically effective dose" is defined as an amount sufficient to cure or at least partially arrest the disease and its complications in a patient already suffering from the disease. The term "patient" includes human and other mammalian subjects that receive either prophylactic or therapeutic treatment.

As used herein, the term "biological sample" includes tissue; cultured cells, e.g., primary cultures, explants, and transformed cells; cellular extracts, e.g., from cultured cells, tissue, embryos, cytoplasmic extracts, nuclear extracts; blood, etc.

The term "autologous" when used herein designates host derived and transplanted re-inserted, re-administered or returned to the host from which the nucleic acid, protein, cell or tissue was derived.

A given miRNA sequence includes both the human and murine homologues or orthologs having structural and functional similarity to the referenced miRNA. The term, homolog applies to the relationship between genes separated by the event of speculation (ortholog) or to the relationship between genes separated by the event of genetic duplication (paralog). Orthologous miRNAs are miRNAs in different species that are similar to each other because they originated from a common ancestor. Homologous sequences are similar sequences which share a common ancestral DNA sequence or which would have been expected to share such given their high degree of sequence identity. Accordingly, in some embodiments, the ortholog or homologue is any sequence which differs from the sequence of the referenced miRNA by at most one, two or three nucleic acid residues.

An inhibitor of a miRNA can be an antisense nucleic acid or siRNA which is complementary to or shares substantial identity with the miRNA and can block the function of the miRNA.

As used herein, the term "substantial identity" refers to a sequence that hybridizes to a reference sequence under stringent conditions, or to a sequence that has a specified percent identity over a specified region of a reference sequence.

As used herein, the phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Exemplary stringent hybridization conditions can be as following: 50% formamide, 5X SSC, and 1% SDS, incubating at 42°C, or, 5X SSC, 1% SDS, incubating at 65°C, with wash in 0.2X SSC, and 0.1% SDS at 65°C.

Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Additional guidelines for determining hybridization parameters are provided in numerous references, e.g., and Current Protocols in Molecular Biology, ed. Ausubel, et al.

The terms "substantially identical" or "substantial identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (i.e., at least about 60%, preferably 65%, 70%, 75%, preferably 80%, 85%, 90%, or 95% identity over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition, when the context indicates, also refers analogously to the complement of a sequence. Preferably, the substantial identity exists over a region that is at least about 6-7 amino acids or 25 nucleotides in length, or more preferably over a region that is 50-100 amino acids or nucleotides in length, or the entire length.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared.

A preferred example of algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., Nuc. Acids Res. 25:3389-3402 (1977) and Altschul et al., J. Mol. Biol. 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention.

The term “administering,” as used herein, refers to any mode of transferring, delivering, introducing, or transporting an iPS cell or a differentiated progeny of

the iPS of the invention to a subject. Such modes include, but are not limited to, oral, topical, intravenous, intraperitoneal, intramuscular, intradermal, intranasal, and subcutaneous administration. Preferably, administration is (1) intravenous, for example, wherein the iPS cells are contained in an IV bag or (2) via a medical device, for example, a stent, valve, balloon or a catheter, wherein the medical device is in combination with, or coated with, an iPS cell or iPS cell population of the invention.

In one embodiment, administration can be via an implantable or non-implantable drug delivery device in combination with an iPS cell or iPS cell population of the invention or via an implantable or non-implantable time release delivery device which may comprise a delivery device associated with the iPS cells of the invention.

By "ameliorate" is meant decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease.

By "delivering" is meant delivery of a therapeutic iPS cell or differentiated cell derived therefrom of the invention to a subject in need of treatment. For example, a therapeutic cell that has been differentiated from an iPS of the invention may be delivered to a vein, artery, capillary, heart, or tissue of a subject, as well as to a specific population, or sub-population, of cells. Delivery of a therapeutic cell of the invention may be assessed by adding tracking agents, such as gold, gadolinium, and/or the like, to the exosomes to allow identification of the tissues that take up the cells with MRI.

By "effective amount" or "therapeutically effective amount" is meant the amount of iPS cells or a population of iPS cells or differentiated cells derived from an iPS cell required to ameliorate the symptoms of a disease. By "effective amount" or "therapeutically effective amount" is also meant the amount of iPS cells or a population of iPS cells or differentiated cells derived therefrom, required to induce a therapeutic or prophylactic effect for use in therapy to treat a disease according to the invention. The effective amount of active compound(s), for example, cells of the invention, used to practice the present invention for

therapeutic treatment of a disease varies depending upon the manner of administration, the age, body weight, and general health of the subject. Ultimately, the attending physician or veterinarian will decide the appropriate amount and dosage regimen. Such amount is referred to as an "effective" amount.

"Disease," "disorder," and "condition" are commonly recognized in the art and designate the presence of signs and/or symptoms in an individual or patient that are generally recognized as abnormal and/or undesirable. Diseases or conditions may be diagnosed and categorized based on pathological changes.

As used herein, the terms "treat," "treated," "treating," "treatment," and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition, or symptoms associated therewith be completely eliminated.

A subject is said to be treated for a disease, if following administration of the cells of the invention, one or more symptoms of the disease are decreased or eliminated.

The cells of the invention, including differentiated progeny derived from iPS cells of the invention, are useful for treatment of a disease. In particular, any disease wherein cell therapy is appropriate can be treated using the iPS or differentiated progeny derived therefrom of the invention. Diseases where cell therapy is known in the art to be an appropriate method of therapy include but are not limited to: automimmune disease, diseases wherein treatment involves regeneration of neural cells/tissue, diseases wherein treatment involves regeneration of cardiac cells/tissues, Parkinson's Disease and Alzheimer's Disease. Cells differentiated from the iPS cells of the invention including myocardial cells, insulin producing cells or nerve cells can be safely utilized in stem cell transplantation therapies for treatment of various disease such as heart failure, insulin dependent diabetes mellitus, Parkinson's disease and spinal cord injury. iPS cells or differentiated cells derived therefrom can be used for

autologous cells therapy, wherein the therapy is specific/personalized for a particular subject, for example to prevent an immune response, or non-autologous.

As used herein, the term “disease” includes any one or more of the following autoimmune diseases or disorders: diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, myasthenia gravis, systemic lupus erythematosis, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjögren’s Syndrome, including keratoconjunctivitis sicca secondary to Sjögren’s Syndrome, alopecia areata, allergic responses due to arthropod bite reactions, Crohn’s disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener’s granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Graves ophthalmopathy, sarcoidosis, primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis.

In another embodiment, disease refers to any one of Wilson's disease, spinocerebellar ataxia, prion disease, Parkinson's disease, Huntington's disease, amytrophic lateral sclerosis, amyloidosis, Alzheimer's disease, Alexander's disease, alcoholic liver disease, cystic fibrosis, Pick's Disease, spinal muscular dystrophy or Lewy body dementia.

“Disease” also includes any one of rheumatoid spondylitis; post ischemic perfusion injury; inflammatory bowel disease; chronic inflammatory pulmonary disease, eczema, asthma, ischemia/reperfusion injury, acute respiratory distress syndrome, infectious arthritis, progressive chronic arthritis, deforming arthritis, traumatic arthritis, gouty arthritis, Reiter's syndrome, acute synovitis and

spondylitis, glomerulonephritis, hemolytic anemia, aplastic anemia, neutropenia, host versus graft disease, allograft rejection, chronic thyroiditis, Graves' disease, primary binary cirrhosis, contact dermatitis, skin sunburns, chronic renal insufficiency, Guillain-Barre syndrome, uveitis, otitis media, periodontal disease, pulmonary interstitial fibrosis, bronchitis, rhinitis, sinusitis, pneumoconiosis, pulmonary insufficiency syndrome, pulmonary emphysema, pulmonary fibrosis, silicosis, or chronic inflammatory pulmonary disease.

"Disease" also refers to any one of cancer, tumor growth, cancer of the colon, breast, bone, brain and others (e.g., osteosarcoma, neuroblastoma, colon adenocarcinoma), chronic myelogenous leukemia (CML), acute myeloid leukemia (AML), acute promyelocytic leukemia (APL), cardiac cancer (e.g., sarcoma, myxoma, rhabdomyoma, fibroma, lipoma and teratoma); lung cancer (e.g., bronchogenic carcinoma, alveolar carcinoma, bronchial adenoma, sarcoma, lymphoma, chondromatous hamartoma, mesothelioma); various gastrointestinal cancer (e.g., cancers of esophagus, stomach, pancreas, small bowel, and large bowel); genitourinary tract cancer (e.g., kidney, bladder and urethra, prostate, testis; liver cancer (e.g., hepatoma, cholangiocarcinoma, hepatoblastoma, angiosarcoma, hepatocellular adenoma, hemangioma); bone cancer (e.g., osteogenic sarcoma, fibrosarcoma, malignant fibrous histiocytoma, chondrosarcoma, Ewing's sarcoma, malignant lymphoma, multiple myeloma, malignant giant cell tumor chordoma, osteochronfroma, benign chondroma, chondroblastoma, chondromyxofibroma, osteoid osteoma and giant cell tumors); cancers of the nervous system (e.g., of the skull, meninges, brain, and spinal cord); gynecological cancers (e.g., uterus, cervix, ovaries, vulva, vagina); hematologic cancer (e.g., cancers relating to blood, Hodgkin's disease, non-Hodgkin's lymphoma); skin cancer (e.g., malignant melanoma, basal cell carcinoma, squamous cell carcinoma, Karposi's sarcoma, moles dysplastic nevi, lipoma, angioma, dermatofibroma, keloids, psoriasis); and cancers of the adrenal glands (e.g., neuroblastoma).

As used herein, “diagnosing” or “identifying a patient or subject having” refers to a process of determining if an individual is afflicted with a disease or ailment, for example a disease as defined herein.

Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

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 invention, yet open to the inclusion of unspecified elements, whether essential or not.

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 of the invention.

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.

Reprogramming Factors

The term "factor" according to the invention when used in conjunction with the expression “reprogramming factor” thereof by RNA includes proteins and peptides as well as derivatives and variants thereof. For example, the term "reprogramming factor" includes but is not limited to: OCT4, SOX2, NANOG, LIN28, KLF4, c-MYC, L-Myc, Glis-1, Sal4, Esrbb1, LRH-1, RAR-gamma and any factor known in the art to have the ability to reprogram a cell as defined herein. The invention contemplates the use of any of the reprogramming factors described herein, either alone or in any combination.

The invention also contemplates the use of any of the following reprogramming factors: members of the Oct family, Klf family, Sox family, Myc family, Lin family, and Nanog family including, but are not limited to: Oct3/4 (also referred to as Oct3, Oct4 or POU5F1) for Oct family; Sox1, Sox2, Sox3, Sox4, Sox11 and

Sox15 for Sox family; c-Myc, N-Myc and L-Myc for Myc family; Lin28 and Lin28b for Lin family; and Nanog for Nanog family.

The factors can be of any animal species; e.g., mammals and rodents.

OCT4 is a transcription factor of the eukaryotic POU transcription factors and an indicator of pluripotency of embryonic stem cells. It is a maternally expressed Octomer binding protein. It has been observed to be present in oocytes, the inner cell mass of blastocytes and also in the primordial germ cell. The gene POU5F1 encodes the OCT4 protein. Synonyms to the gene name include OCT3, OCT4, OTF3 and MGC22487. The presence of OCT4 at specific concentrations is necessary for embryonic stem cells to remain undifferentiated.

Preferably, "OCT4 protein" or simply "OCT4" relates to human OCT4 and preferably comprises an amino acid sequence encoded by the nucleic acid according to Figure 8A, preferably the amino acid sequence according to Figure 8B. One skilled in the art would understand that the cDNA sequence of OCT4 as described above would be equivalent to OCT4 mRNA, and can be used for the generation of RNA capable of expressing OCT4.

Sox2 is a member of the Sox (SRY-related HMG box) gene family that encode transcription factors with a single HMG DNA-binding domain. SOX2 has been found to control neural progenitor cells by inhibiting their ability to differentiate. The repression of the factor results in delamination from the ventricular zone, which is followed by an exit from the cell cycle. These cells also begin to lose their progenitor character through the loss of progenitor and early neuronal differentiation markers.

Preferably, "SOX2 protein" or simply "SOX2" relates to human SOX2 and preferably comprises an amino acid sequence encoded by the nucleic acid according to Figure 9A, preferably the amino acid sequence according to Figure 9B. One skilled in the art would understand that the cDNA sequence of SOX2 as described above would be equivalent to SOX2 mRNA, and can be used for the generation of RNA capable of expressing SOX2.

NANOG is a NK-2 type homeodomain gene, and has been proposed to play a key role in maintaining stem cell pluripotency presumably by regulating the expression of genes critical to embryonic stem cell renewal and differentiation. NANOG behaves as a transcription activator with two unusually strong activation domains embedded in its C terminus. Reduction of NANOG expression induces differentiation of embryonic stem cells.

Preferably, "NANOG protein" or simply "NANOG" relates to human NANOG and preferably comprises an amino acid sequence encoded by the amino acid sequence according to Figure 10. One skilled in the art would understand that the cDNA sequence of NANOG as described above would be equivalent to NANOG mRNA, and can be used for the generation of RNA capable of expressing NANOG.

LIN28 is a conserved cytoplasmic protein with an unusual pairing of RNA-binding motifs: a cold shock domain and a pair of retroviral-type CCHC zinc fingers. In mammals, it is abundant in diverse types of undifferentiated cells. In pluripotent mammalian cells, LIN28 is observed in RNase-sensitive complexes with Poly(A)-Binding Protein, and in polysomal fractions of sucrose gradients, suggesting it is associated with translating mRNAs.

Preferably, "LIN28 protein" or simply "LIN28" relates to human LIN28 and preferably comprises an amino acid sequence encoded by the nucleic acid according to Figure 11A, preferably the amino acid sequence according to Figure 11B. One skilled in the art would understand that the cDNA sequence of LIN28 as described above would be equivalent to LIN28 mRNA, and can be used for the generation of RNA capable of expressing LIN28.

Krueppel-like factor (KLF4) is a zinc-finger transcription factor, which is strongly expressed in postmitotic epithelial cells of different tissues, e.g. the colon, the stomach and the skin. KLF4 is essential for the terminal differentiation of these cells and involved in the cell cycle regulation.

Preferably, "KLF4 protein" or simply "KLF4" relates to human KLF4 and preferably comprises an amino acid sequence encoded by the nucleic acid

according to Figure 12A, preferably the amino acid sequence according to Figure 12B. One skilled in the art would understand that the cDNA sequence of KLF4 as described above would be equivalent to KLF4 mRNA, and can be used for the generation of RNA capable of expressing KLF4.

MYC (cMYC) is a protooncogene, which is overexpressed in a wide range of human cancers. When it is specifically-mutated, or overexpressed, it increases cell proliferation and functions as an oncogene. MYC gene encodes for a transcription factor that regulates expression of 15% of all genes through binding on Enhancer Box sequences (E-boxes) and recruiting histone acetyltransferases (HATs). MYC belongs to MYC family of transcription factors, which also includes N-MYC and L-MYC genes. MYC-family transcription factors contain the bHLH/LZ (basic Helix-Loop-Helix Leucine Zipper) domain

Preferably, "cMYC protein" or simply "cMYC" relates to human cMYC and preferably comprises an amino acid sequence encoded by the nucleic acid according to Figure 13A, preferably the amino acid sequence according to Figure 13B. One skilled in the art would understand that the cDNA sequence of cMYC as described above would be equivalent to cMYC mRNA, and can be used for the generation of RNA capable of expressing cMYC.

A reference herein to specific factors such as OCT4, SOX2, NANOG, LIN28, KLF4 or c-MYC or to specific sequences thereof is to be understood so as to also include all variants of these specific factors or the specific sequences thereof as described herein. In particular, it is to be understood so as to also include all splice variants, posttranslationally modified variants, conformations, isoforms and species homologs of these specific factors/sequences which are naturally expressed by cells.

A reprogramming factor or nuclear reprogramming factor useful according to the invention includes any of the reprogramming factors recited herein. A reprogramming factor useful according to the invention also includes a factor identified by the method of screening for reprogramming factors described in International Publication No. WO2005/80598 A1, incorporated by reference

herein in its entirety. Those skilled in the art are able to screen a reprogramming factor for use in the method of the present invention by referring to the above publication. In addition, the reprogramming factor can also be confirmed by using a method in which appropriate modification or alteration has been made in the above screening method.

Examples of the combination reprogramming factors are disclosed in International Publication No, WO2007/069666 A1 and its family member U.S. patent application Ser. No. 12/213,035 and U.S. patent application Ser. No. 12/289,873, filed Nov. 6, 2008, entitled "Nuclear Reprogramming Factor and Induced Pluripotent Stem Cells" which are incorporated by reference herein in their entireties. Those skilled in the art are able to appropriately select a reprogramming factor that can be preferably used for the method of the present invention by referring to the above publication. In addition, other examples of the combinations of reprogramming factors are disclosed, for example, in Yu et al., Science 318:1917-20, 2007, incorporated by reference herein in its entirety. Accordingly, those skilled in the art are able to understand that any variety and combination of reprogramming factors can be used for the methods of the present invention, which combination is not disclosed in International Publication No. WO2007/069666 A1 or Yu et al., Science 318:1917-20, 2007. Reprogramming factors useful for the invention are identified by using the screening method of reprogramming factor described in International Publication No. WO2005/80598 A1.

The amino acid and nucleotide sequences of nuclear reprogramming factors usable alone or in combination in the present application, for example Oct3/4 Nanog, Lin28, Lin28b, ECAT1, ECAT2, ECAT3, ECAT5, ECAT7, ECAT8, ECAT9, ECAT10, ECAT15-1, ECAT15-2, Fthl17, Sal14, Rex1, Utf1, Tcl1, Stella, β-catenin, Stat3, Grb2, c-Myc, N-Myc, L-Myc, Sox1, Sox2, Sox3, Sox4, Sox11, Myb12, Klf1, Klf2, Klf4, and Klf5; and FoxD3, ZNF206, and Otx2 (which are also described in International Publication No. WO2008/118820), are available from

GenBank (NCBI, USA). Accession numbers thereof regarding human, mouse or rat are described below:

Oct3/4 (human NM_203289 or NM_002701, mouse NM_013633, rat NM_001009178), Nanog (human NM_024865, mouse NM_028016, rat NM_001100781), Lin28 (human NM_024674, mouse NM_145833, rat NM_001109269), Lin28b (human NM_001004317, mouse NM_001031772), ECAT1 (human AB211062, mouse AB211060), ECAT2 (human NM_001025290, mouse NM_025274), ECAT3 (human NM_152676, mouse NM_015798), ECAT5 (human NM_181532, mouse NM_181548), ECAT7 (human NM_013369, mouse NM_019448), ECAT8 (human AB211063, mouse AB211061), ECAT9 (human NM_020634, mouse NM_008108), ECAT10 (human NM_006942NM_009235, mouse NM_009235), ECAT15-1 (human NM_018189, mouse NM_028610), ECAT15-2 (human NM_138815NM_028615, mouse NM_028615), Fthl17 (human NM_031894, mouse NM_031261), Sal14 (human NM_020436, mouse NM _175303), Rex1 (human NM_174900, mouse NM_609556), Utf1 (human NM_003577, mouse NM_009482), Tcl1 (human NM_021966, mouse NM_ 009337), Stella (human NM_199286, mouse NM_139218), β-catenin (human NM_001904, mouse NM_007614), Stat3 (human NM_139276, mouse NM_213659), Grb2 (human NM_002086, mouse NM_008163), FoxD3 (human NM_012183, mouse NM_010425), ZNF206 (human NM_032805, mouse NM_001033425), Myb12 (human NM_002466, mouse NM_008652), Otx2 (human NM_172337, mouse NM_144841), c-Myc (human NM _002467, mouse NM_010849), N-Myc (human NM_005378, mouse NM_008709), L-Myc (human NM_005376, mouse NM_008506), Sox1 (human NM_005986 NM_005986, NM_009233, mouse NM_005986, NM_009233), Sox2 (human NM_003106, mouse NM_011443), Sox3 (human NM_005634, mouse NM_009237), Sox4 (human NM_003107, mouse NM_009238), Sox11 (human NM_003108, mouse NM_009234), Myb12 (human NM_002466, mouse NM_008652), Klf1 (human NM_006563, mouse NM_010635), Klf2 (human NM_016270, mouse NM_008452), Klf4 (human NM_004235, mouse NM_010637), and Klf5 (human NM_001730, mouse NM-009769).

The reprogramming factors of the invention may further be combined with one or more gene product(s) of gene(s) selected from: Fbx15, Nanog, ERas, ECAT15-2, Tcl1, and β-catenin. Further, these factors may also be combined with one or more gene product(s) of gene(s) selected from: ECAT1, Esg1, Dnmt3L, ECAT8, Gdf3, Sox15, ECAT15-1, Fthl17, Sal14, Rex1, UTF1, Stella, Stat3, and Grb2, for example. However, gene products that can be included with the reprogramming factors of the present invention are not limited to the gene products of genes specifically described above. The nuclear reprogramming factors of the present invention can include other gene products which can function as a reprogramming factor, as well as one or more factors involving differentiation, development, or proliferation, and factors having other physiological activities. It should be understood that the aforementioned aspect may also be included within the scope of the present invention.

According to the present invention, the term "peptide" comprises oligo- and polypeptides and refers to substances comprising two or more, preferably 3 or more, preferably 4 or more, preferably 6 or more, preferably 8 or more, preferably 10 or more, preferably 13 or more, preferably 16 or more, preferably 21 or more and up to preferably 8, 10, 20, 30, 40 or 50, in particular 100 amino acids joined covalently by peptide bonds. The term "protein" refers to large peptides, preferably to peptides with more than 100 amino acid residues, but in general the terms "peptides" and "proteins" are synonyms and are used interchangeably herein.

Proteins and peptides described according to the invention may be isolated from biological samples such as tissue or cell homogenates and may also be expressed recombinantly in a multiplicity of pro- or eukaryotic expression systems.

For the purposes of the present invention, "variants" of a protein or peptide or of an amino acid sequence comprise amino acid insertion variants, amino acid deletion variants and/or amino acid substitution variants.

Amino acid insertion variants comprise amino- and/or carboxy-terminal fusions and also insertions of single or two or more amino acids in a particular amino acid sequence. In the case of amino acid sequence variants having an insertion, one or more amino acid residues are inserted into a particular site in an amino acid sequence, although random insertion with appropriate screening of the resulting product is also possible.

"Conservative substitutions" may be made, for instance, on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. Preferably the degree of similarity, preferably identity between a specific amino acid sequence described herein and an amino acid sequence which is a variant of said specific amino acid sequence will be at least 70%, preferably at least 80%, preferably at least 85%, even more preferably at least 90% or most preferably at least 95%, 96%, 97%, 98% or 99%. The degree of similarity or identity is given preferably for a region of at least about 20, at least about 40, at least about 60, at least about 80, at least about 100, at least about 120, at least about 140, at least about 160, at least about 200 or 250 amino acids. In preferred embodiments, the degree of similarity or identity is given for the entire length of the reference amino acid sequence.

According to the invention, a variant of a protein or peptide preferably has a functional property of the protein or peptide from which it has been derived. Such functional properties are described above for OCT4, SOX2, NANOG, LIN28, KLF4 and c-MYC, respectively. Preferably, a variant of a protein or peptide has the same property in reprogramming an animal differentiated cell as the protein or peptide from which it has been derived. Preferably, the variant induces or enhances reprogramming of an animal differentiated cell.

miRNAs

The invention provides for methods of producing a pluripotent stem cell wherein one or more miRNA(s) is introduced into a target cell in combination with mRNA.

One microRNA cluster, designated the miR-290 cluster, constitutes over 70% of the entire miRNA population in mouse ES cells (Marson, A. et al. Connecting

microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells Cell 134:521-533 (2008)). Expression of the miR-290 cluster is rapidly down-regulated upon ES cell differentiation (See, e.g., Houbaviy, H. B., Murray, M. F. & Sharp, P. A. Embryonic stem cell-specific MicroRNAs Dev Cell 5:351-358 (2003)). A subset of the miR-290 cluster, called the embryonic stem cell cycle (ESCC) regulating miRNAs, enhances the unique stem cell cycle and includes miR-291-3p, miR-294, and miR-295, as well as the human homologues hsa-mir-302a, hsa-miR-302b, hsa-miR-302c, hsa-miR-302d, hsa-miR-371-5p, hsa-miR-372, hsa-miR-373. (See, e.g., Wang, Y. et al. Embryonic stem cell-specific microRNAs regulate the G1-S transition and promote rapid proliferation Nat Genet 40:1478-1483 (2008)). This subset includes miR-291-3p, miR-294, and miR-295 and their homologues.

Removal of genes required for maturation of all miRNAs has shown that miRNAs play essential roles in the proliferation and differentiation of Embryonic Stem Cells (ESCs)(Wang, Y. et al., Nat Genet 39:380-5 (2007); Kanellopoulou, C. et al. Genes Dev 19:489-501 (2005); Murchison, E. P. et al., Proc Natl Acad Sci USA 102:12135-40 (2005)). For example, the loss of the RNA binding protein DGCR8, which is required for the production of all canonical miRNAs, results in a cell cycle defect and an inability to silence the self-renewal program of ESCs when they are placed in differentiation-inducing conditions (Wang, Y. et al., Nat Genet 39:380-5 (2007). The introduction of individual members of a family of miRNAs, the ESCC miRNAs, into Dgcr8-/-ESCs can rescue the cell cycle defect (Wang, Y. et al., Nat Genet, 40:1478-1483 (2008)).

According to the methods of the invention, miRNA comprises one or more miRNA(s) included in the RNA sequences specified by the registration names of the miRBase database or the accession numbers shown in the tables below or the sequences or combination of sequences shown in the tables below or any possible combination of the sequences shown below. In the registration names, the symbols "hsa" and "mmu" represent Homo sapiens and Mus musculus, respectively.

The invention provides for an miRNA that is 18-25 nucleotides, for example, 20-25 nucleotides, 21-23 nucleotides and 19-23 nucleotides. Such miRNAs can be induced from precursor RNAs including pri-miRNAs (i.e., transcription products from genomic DNAs) and pre-miRNAs (i.e., processed products from pri-miRNAs).

The present invention provides methods comprising the use of miRNA that provide for a higher reprogramming efficiency in the presence of the miRNA than in the absence thereof, for preparation of induced pluripotent stem cells. For example, the presence of an added miRNA supports the production of an induced pluripotent stem cell as compared to in the absence of the miRNA. Further, when reprogramming is performed on the same number of somatic cells in the presence of a reprogramming factor containing the same components in the same concentrations with and without addition of miRNA, increased efficiency can be observed wherein a greater number of induced pluripotent stem cells are generated in the sample which comprises miRNA as compared to the sample that does not comprise miRNA.

Regarding miRNA useful according to the invention, its classification and in vivo functions are described in Jikken Igaku (Experimental Medicine), 24, pp. 814-819, 2006; microRNA Jikken Purotokoru (microRNA Experimental Protocol), pp. 20-35, 2008, YODOSHA CO., LTD. At present, a database storing data relating to about 1,000 miRNA sequences is available (for example, miRBase, Griffiths-Jones et al. Nucleic Acids Research 36:D154-D158, 2008 (published online Nov. 8, 2007), see also http://microma.sanger.ac.uk/sequences/index.shtml [online]), and it is possible for those skilled in the art to obtain any miRNA data therefrom, and to readily extract an miRNA that is expressed in embryonic stem cells at a higher level than in somatic cells. In addition, it is also possible to readily identify miRNA expressed in embryonic stem cells at a higher level than in somatic cells by confirming the difference in miRNA expression between embryonic stem cells and somatic cells by methods including but not limited to miRNA microarray and real-time PCR analyses.

It is preferable to use miRNA derived from the same animal species as the target animal whose somatic cells are to be reprogrammed. miRNA useful according to the invention includes wild type miRNA as well as miRNAs in which one to several nucleotides (for example 1 to 6 nucleotides, preferably 1 to 4 nucleotides, more preferably 1 to 3 nucleotides, yet more preferably 1 or 2 nucleotides, and most preferably 1 nucleotide) are substituted, inserted, and/or deleted, and which are capable of exerting equivalent functions to those of the wild type miRNA in vivo. For example, the miRNA of the present invention includes miRNAs in which one to several nucleotides are substituted, inserted, and/or deleted, and which increase the efficiency of iPS cell production. The miRNA of the present invention also includes miRNAs in which one to several nucleotides are substituted, inserted, and/or deleted, and which improve the efficiency of nuclear reprogramming. The miRNA of the present invention also includes miRNAs in which one to several nucleotides are substituted, inserted, and/or deleted, and which regulate DNA methylation. The present invention also includes such miRNAs wherein the DNA methylation is down-regulated. The present invention also includes such miRNAs wherein the DNA methylation is de novo DNA methylation.

According to the methods of the present invention, miRNAs that have been confirmed to improve the reprogramming efficiency in the above manner can be used either alone or in combinations of two or more types. In addition, a plurality of miRNAs forming a cluster may also be used. For example, hsa-miR-302-367 which is available as a miRNA cluster, or individual miRNAs from the hsa-miR-302-367 cluster, and the like may be used. Among these RNA sequences, some RNA sequences may include a plurality of miRNAs within one sequence. Use of such an RNA sequence may achieve efficient production of iPS cells. Further, an RNA sequence including a plurality of miRNAs within one sequence and one or more other RNA sequence(s) including one or more miRNA(s) can also be used in combination. In the invention, preferably, the miRNAs are one or two or more miRNAs contained in one or two or more RNAs selected from RNAs represented in the tables presented below.

An miRNA is non-coding RNA which is not translated into a protein. miRNA is first transcribed as pri-miRNA from a corresponding gene. A pri-miRNA generates pre-miRNA having a characteristic hairpin structure of about 60 to about 120 nucleotides or more, for example about 70 nucleotides, and this pre-miRNA is further processed into mature miRNA, which is mediated by Dicer. In the present invention, not only mature miRNA but also precursor RNA thereof (i.e., pri-miRNA or pre-miRNA), or a vector comprising DNA encoding the miRNA or precursor RNA, can be used as long as the effect of the present invention is not impaired. In addition, miRNA for use in the present invention may be either natural type or non-natural type. Thus, any small RNA or RNA precursor may be used as long as the effect of the present invention is not impaired.

The production method of miRNA for use in the present invention is not specifically limited, although the production can be achieved, for example, by a chemical synthetic method or a method using genetic recombination technique. When the production is carried out by a method using genetic recombination technique, miRNA for use in the present invention can, for example, be produced through a transcription reaction with use of a DNA template and a RNA polymerase obtained by means of gene recombination. Examples of usable RNA polymerase include a T7 RNA polymerase, a T3 RNA polymerase, and a SP6 RNA polymerase.

Alternatively, a recombinant vector capable of expressing miRNA can be produced by insertion of miRNA-encoding DNA or precursor RNA (pri-miRNA or pre-miRNA)-encoding DNA into an appropriate vector under the regulation of expression control sequences (promoter and enhancer sequences and the like). The type of vector used herein is not specifically limited, although DNA vectors are preferred. Examples thereof can include plasmid vectors. In addition, as to the above plasmids, mammalian expression plasmids well known to those skilled in the art can be employed.

The invention also provides for methods of producing pluripotent stem cells using miRNA mimics, as defined herein, in combination with mRNA. The invention

also provides for pluripotent stem cells comprising at least one miRNA mimic and at least one mRNA.

An miRNA useful for the methods of the invention includes any miRNA known to be involved in pluripotency of a cell or the mesenchymal to epithelial transition. miRNA useful according to the invention include but are not limited to the following:

Table 1. Sequences of miRNA used to enhance cellular reprogramming

miRNA	Sequence
hsa-mir-302a	CCACCACUUAAACGUGGAUGUACUUGCUUUGAAACUAAAGAAG UAAGUGCUUCCAUGUUUUGGUGAUGG or UAAGUGCUUCCAUGUUUUGGUGA
hsa-mir-302b	GCUCCCUUCAACUUUAACAUGGAAGUGCUUUCUGUGACUUUA AAAGUAAGUGCUUCCAUGUUUUAGUAGGAGU or UAAGUGCUUCCAUGUUUUAGUAG
hsa-mir-302c	CCUUUGCUUUAACAUGGGGGUACCUGCUGUGUGAAACAAAAG UAAGUGCUUCCAUGUUUCAGUGGAGG or UAAGUGCUUCCAUGUUUCAGUGG
hsa-mir-302d	CCUCUACUUUAACAUGGAGGCACUUGCUGUGACAUGACAAAA AUAAGUGCUUCCAUGUUUGAGUGUGG or UAAGUGCUUCCAUGUUUGAGUGU
hsa-mir-367	CCAUUACUGUUGCUAAUAUGCAACUCUGUUGAAUAUAAAUUG GAAUUGCACUUUAGCAAUGGUGAUGG or AAUUGCACUUUAGCAAUGGUGA
hsa-mir-200c	CCCUCGUCUUACCCAGCAGUGUUUGGGUGCGGUUGGGAGUC UCUAAUACUGCCGGGUAAUGAUGGAGG
hsa-mir-369-3p	AAUAAUACAUGGUUGAUCUUU
hsa-mir-369-5p	AGAUCGACCGUGUUAUAUUCGC

In certain embodiments, combinations of miRNAs are introduced into a somatic cell to facilitate production of a pluripotent stem cell (see for example Table 2).

Table 2.
miRNA cocktails used in combination with mRNA reprogramming

Cluster A	Cluster B
302a, 302b, 302c, 302d, 367	302a, 302b, 302c, 302d, 200c, 369-3p, 369-5p

Table 3. Sequences of miRNA used to enhance cellular reprogramming

miRNA	miR Base Accession Number

mmu-miR-150	MI0000172
mmu-miR-182	MI0000224
mmu-miR-126	MI0000153
4 mmu-miR-290-295 cluster	
mmu-miR-290	MI0000388
(mmu-miR-290-5p/290-3p)	
mmu-miR-291a	MI0000389
(mmu-miR-291a-5p/291a-3p)	
mmu-miR-292	MI0000390
(mmu-miR-292-5p/292-3p)	
mmu-miR-294	MI0000392
(mmu-miR-294/294*)	
mmu-miR-295	MI0000393
(mmu-miR-295/295*)	
mmu-miR-17-92 cluster	
mmu-miR-323	MI0000592
mmu-miR-130b	MI0000408
mmu-miR-7a-1	MI0000728
14 mmu-miR-7a-2	MI0000729
mmu-miR-205	MI0000248
mmu-miR-200a	MI0000554
17 mmu-miR-200c	MI0000694
mmu-miR-mix	

*indicates star form of miRNA.

Table 4. Sequences of miRNA used to enhance cellular reprogramming

miRNA	miR Base Accession Number
hsa-miR-371	MI0000779
(hsa-miR-371-5p/371-3p)	
hsa-miR-372	MI0000780
hsa-miR-373	MI0000781
(hsa-miR-373/373*)	
hsa-miR-371-373 cluster	
hsa-miR-93	MI0000095
(hsa-miR-93/93*)	
hsa-miR-302a	MI0000738
(hsa-miR-302a/302a*)	
hsa-miR-302b	MI0000772
(hsa-miR-302b/302b*)	
hsa-miR-302c	MI0000773
(hsa-miR-302c/302c*)	

hsa-miR-302d	MI0000774
(hsa-miR-302d/302d*)	
hsa-miR-367	MI0000775
(hsa-miR-367/367*)	
hsa-miR-302-367 cluster	
hsa-miR-520a	MI0003149
(hsa-miR-520a-5p/520a-3p)	
hsa-miR-520b	MI0003155
hsa-miR-520c	MI0003158
(hsa-miR-520c-5p/520c-3p)	
hsa-miR-520d	MI0003164
(hsa-miR-520d-5p/520d-3p) hsa-miR-520e	MI0003143
mmu-miR-290-295 cluster	
mmu-miR-290	MI0000388
(mmu-miR-290-5p/290-3p)	
mmu-miR-291a	MI0000389
(mmu-miR-291a-5p/291a-3p)	
mmu-miR-292	MI0000390
(mmu-miR-292-5p/292-3p)	
mmu-miR-293	MI0000391
(mmu-miR-293/293*)	
mmu-miR-294	MI0000392
(mmu-miR-294/294*)	
mmu-miR-295	MI0000393
(mmu-miR-295/295*)	

TABLE 5

microRNA mimic	**sequence**
mmu-miR-302b	UAAGUGCUUCCAUGUUUUAGUAG
mmu-miR-302	UAAGUGCUUCCAUGUUUUGGUGA
mmu-miR-495	AAACAAACAUGGUGCACUUCUU
mmu-miR-26a	UUCAAGUAAUCCAGGAUAGGCU
mmu-miR-19a*	UAGUUUUGCAUAGUUGCACUAC
mmu-miR-302d	UAAGUGCUUCCAUGUUUGAGUGU
mmu-miR-10b	UACCCUGUAGAACCGAAUUUGUG
mmu-miR-294	AAAGUGCUUCCCUUUUGUGUGU
mmu-miR-302c	AAGUGCUUCCAUGUUUCAGUGG
mmu-miR-183*	GUGAAUUACCGAAGGGCCAUAA
mmu-miR-200a	UAACACUGUCUGGUAACGAUGU
mmu-miR34c*	AAUCACUAACCACACAGCCAGG
mmu-miR-293	AGUGCCGCAGAGUUUGUAGUGU
mmu-miR-181b	AACAUUCAUUGCUGUCGGUGGGU
mmu-miR-151	CUAGACUGAGGCUCCUUGAGG

mmu-miR-680	GGGCAUCUGCUGACAUGGGGG
mmu-miR-295	AAAGUGCUACUACUUUUGAGUCU
mmu-miR-880	UACUCCAUCCUCUCUGAGUAGA
mmu-miR-93	CAAAGUGCUGUUCGUGCAGGUAG
mmu-miR-455-5p	UAUGUGCCUUUGGACUACAUCG
mmu-miR-144	UACAGUAUAGAUGAUGUACU
mmu-miR-467d	UAAGUGCGCGCAUGUAUAUGCG
mmu-miR-484	UCAGGCUCAGUCCCCUCCCGAU
mmu-miR-205	UCCUUCAUUCCACCGGAGUCUG
mmu-miR-582-5p	UACAGUUGUUCAACCAGUUACU
mmu-miR-290-3p	AAAGUGCCGCCUAGUUUUAAGCCC or AAAGUGCCGCCUAGUUUUAAGCC
mmu-miR-138*	CGGCUACUUCACAACACCAGGG
mmu-miR-181d	AACAUUCAUUGUUGUCGGUGGGU
mmu-miR-324-3p	CCACUGCCCCAGGUGCUGCU
mmu-miR-877*	UGUCCUCUUCUCCCUCCUCCCA
mmu-miR-23a	AUCACAUUGCCAGGGAUUUCC
mmu-miR-379	UGGUAGACUAUGGAACGUAGG
mmu-miR-673	CUCACAGCUCUGGUCCUUGGAG
mmu-miR-876-5p	UGGAUUUCUCUGUGAAUCACUA
mmu-miR-291-3p	AAAGUGCUUCCACUUUGUGUGC
mmu-miR-30d	UGUAAACAUCCCCGACUGGAAG
mmu-miR-421	AUCAACAGACAUUAAUUGGGCGC
mmu-miR-879*	GCUUAUGGCUUCAAGCUUUCGG
mmu-miR-542-3p	UGUGACAGAUUGAUAACUGAAA
mmu-miR-124*	CGUGUUCACAGCGGACCUUGAU
mmu-miR-363	AAUUGCACGGUAUCCAUCUGUA
mmu-miR-871	UAUUCAGAUUAGUGCCAGUCAUG
mmu-miR-19a	UGUGCAAAUCUAUGCAAAACUGA
mmu-miR-16*	CCAGUAUUGACUGUGCUGCUGA
mmu-miR-873	GCAGGAACUUGUGAGUCUCCU
mmu-miR-199b	CCCAGUGUUUAGACUACCUGUUC
mmu-miR-106a	CAAAGUGCUAACAGUGCAGGUAG
mmu-miR-181b	AACAUUCAUUGCUGUCGGUGGGU
mmu-miR-200a*	CAUCUUACCGGACAGUGCUGGA
mmu-miR-431*	CAGGUCGUCUUGCAGGGCUUCU
mmu-miR-689	CGUCCCCGCUCGGCGGGGUCC
mmu-miR-721	CAGUGCAAUUAAAAGGGGGAA

TABLE 6

microRNA mimic	**sequence**
mmu-miR-744*	CUGUUGCCACUAACCUCAACCU
mmu-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU
mmu-miR-669c	AUAGUUGUGUGUGGAUGUGUGU

mmu-let-7c	UGAGGUAGUAGGUUGUAUGGUU
mmu-miR-466h	UGUGUGCAUGUGCUUGUGUGUA
mmu-miR-654-3p	UAUGUCUGCUGACCAUCACCUU
mmu-miR-470*	AACCAGUACCUUUCUGAGAAGA
mmu-miR-24	UGGCUCAGUUCAGCAGGAACAG
mmu-miR-182	UUUGGCAAUGGUAGAACUCACACCG
mmu-miR-335	UCAAGAGCAAUAACGAAAAAUGU
mmu-miR-181c	AACAUUCAACCUGUCGGUGAGU
mmu-miR-330	GCAAAGCACAGGGCCUGCAGAGA
mmu-miR-134	UGUGACUGGUUGACCAGAGGGG
mmu-miR-675-3p	CUGUAUGCCCUAACCGCUCAGU
mmu-miR-218	UUGUGCUUGAUCUAACCAUGU
mmu-let-7f	UGAGGUAGUAGGUUGUAUGGUU
mmu-miR-491	AGUGGGGAACCCUUCCAUGAGG
mmu-miR-466g	AUACAGACACAUGCACACACA
mmu-miR-465c-3p	GAUCAGGGCCUUUCUAAGUAGA
mmu-miR-202	AGAGGUAUAGCGCAUGGGAAGA
mmu-miR-681	CAGCCUCGCUGGCAGGCAGCU
mmu-miR-877	GUAGAGGAGAUGGCGCAGGG
mmu-miR-875-5p	UAUACCUCAGUUUUAUCAGGUG
mmu-miR-712	CUCCUUCACCCGGGCGGUACC
mmu-miR-297	AUGUAUGUGUGCAUGUGCAUGU
mmu-let-7d	AGAGGUAGUAGGUUGCAUAGUU
mmu-miR-142-3p	UGUAGUGUUUCCUACUUUAUGGA
mmu-miR-328	CUGGCCCUCUCUGCCCUUCCGU
mmu-miR-485-5p	AGAGGCUGGCCGUGAUGAAUUC
mmu-miR-122a	UGGAGUGUGACAAUGGUGUUUG
mmu-miR-877*	UGUCCUCUUCUCCCUCCUCCCA
mmu-miR-135a	UAUGGCUUUUUAUUCCUAUGUGA
mmu-miR-674-3p	CACAGCUCCCAUCUCAGAACAA
mmu-miR-497	CAGCAGCACACUGUGGUUUGUA
mmu-miR-7b	UGGAAGACUUGUGAUUUUGUUGU
mmu-miR-30b*	CUGGGAUGUGGAUGUUUACGUC
mmu-miR-34b	AGGCAGUGUAAUUAGCUGAUUGU
mmu-miR-466e-5p	GAUGUGUGUGUACAUGUACAUA
mmu-miR-193b	AACUGGCCCACAAAGUCCCGCU
mmu-miR-883a-5p	UGCUGAGAGAAGUAGCAGUUAC
mmu-let-7i*	CUGCGCAAGCUACUGCCUUGCU
mmu-miR-342	UCUCACACAGAAAUCGCACCCGU
mmu-miR-140*	UACCACAGGGUAGAACCACGG
mmu-miR-24-2*	GUGCCUACUGAGCUGAAACAGU
mmu-miR-195	UAGCAGCACAGAAAUAUUGGC
mmu-miR-297a	AUGUAUGUGUGCAUGUGCAUGU
mmu-miR-344	UGAUCUAGCCAAAGCCUGACUGU
mmu-miR-18	UAAGGUGCAUCUAGUGCAGAUAG

mmu-miR-93*	ACUGCUGAGCUAGCACUUCCCG
mmu-miR-297	AUGUAUGUGUGCAUGUGCAUGU
mmu-miR-16	UAGCAGCACGUAAAUAUUGGCG
mmu-miR-380-5p	AUGGUUGACCAUAGAACAUGCG
mmu-miR-672	UGAGGUUGGUGUACUGUGUGUGA
mmu-miR-431	UGUCUUGCAGGCCGUCAUGCA
mmu-miR-715	CUCCGUGCACACCCCCGCGUG
mmu-miR-669a	AGUUGUGUGUGCAUGUUCAUGU
mmu-miR-103	AGCAGCAUUGUACAGGGCUAUGA
mmu-miR-124*	CGUGUUCACAGCGGACCUUGAU
mmu-miR-15b	UAGCAGCACAUCAUGGUUUACA
mmu-miR-450b*	AUUGGGAACAUUUUGCAUGCAU
mmu-miR-882	AGGAGAGAGUUAGCGCAUUAGU
mmu-miR-686	AUUGCUUCCCAGACGGUGAAGA
mmu-miR-222	AGCUACAUCUGGCUACUGGGU
mmu-miR-684	AGUUUUCCCUUCAAGUCAA
mmu-miR-450b	UUUUGCAGUAUGUUCCUGAAUA
mmu-miR-582-3p	CCUGUUGAACAACUGAACCCAA
mmu-miR-135b	UAUGGCUUUUCAUUCCUAUGUGA
mmu-miR-493	UGAAGGUCCUACUGUGUGCCAGG
mmu-miR-546	AUGGUGGCACGGAGUC
mmu-miR-708	AAGGAGCUUACAAUCUAGCUGGG
mmu-miR-433-3p	AUCAUGAUGGGCUCCUCGGUGU
mmu-miR-494	UGAAACAUACACGGGAAACCUC
mmu-miR-203	GUGAAAUGUUUAGGACCACUAG
mmu-miR-9	UCUUUGGUUAUCUAGCUGUAUGA
mmu-miR-574-5p	UGAGUGUGUGUGUGUGAGUGUGU
mmu-miR-376c	AACAUAGAGGAAAUUUCACGU
mmu-miR-433-5p	UACGGUGAGCCUGUCAUUAUUC
mmu-miR-181a-2*	ACCGACCGUUGACUGUACCUUG
mmu-miR-218-2*	CAUGGUUCUGUCAAGCACCGCG
mmu-miR-196a	UAGGUAGUUUCAUGUUGUUGGG
mmu-miR-542-5p	CUCGGGGAUCAUCAUGUCACGA
mmu-miR-7	UGGAAGACUAGUGAUUUUGUUGU
mmu-miR-743b-5p	UGUUCAGACUGGUGUCCAUCA
mmu-miR-377	AUCACACAAAGGCAACUUUUGU
mmu-miR-683	CCUGCUGUAAGCUGUGUCCUC
mmu-miR-675-5p	UGGUGCGGAAAGGGCCCACAGU
mmu-miR-598	UACGUCAUCGUCGUCAUCGUUA
mmu-miR15b*	CGAAUCAUUAUUUGCUGCUCUA
mmu-miR-9	UCUUUGGUUAUCUAGCUGUAUGA
mmu-miR-450a-3p	AUUGGGGAUGCUUUGCAUUCAU
mmu-miR-449b	AGGCAGUGUUGUUAGCUGGC
mmu-miR-707	CAGUCAUGCCGCUUGCCUACG
mmu-miR-335-3p	UUUUUCAUUAUUGCUCCUGACC

mmu-miR-147	GUGUGCGGAAAUGCUUCUGCUA
mmu-miR-466c-5p	GAUGUGUGUGUGCAUGUACAUA
mmu-miR-16	UAGCAGCACGUAAAUAUUGGCG
mmu-miR-127	UCGGAUCCGUCUGAGCUUGGCU
mmu-miR- 673-3p	UCCGGGGCUGAGUUCUGUGCACC
mmu-miR- 466b-5p	GAUGUGUGUGUACAUGUACAUG
mmu-miR- 27a*	AGGGCUUAGCUGCUUGUGAGCA
mmu-miR-1	UGGAAUGUAAAGAAGUAUGUAU
mmu-miR-201	UACUCAGUAAGGCAUUGUUCUU
mmu-miR-376b	AUCAUAGAGGAACAUCCACUU
mmu-miR-187	UCGUGUCUUGUGUUGCAGCCGG
mmu-miR-299	UGGUUUACCGUCCCACAUACAU
mmu-miR-299	UAUGUGGGACGGUAAACCGCUU
mmu-miR-574-3p	CACGCUCAUGCACACACCCACA
mmu-miR- 193*	UGGGUCUUUGCGGGCAAGAUGA
mmu-miR-679	GGACUGUGAGGUGACUCUUGGU
mmu-miR- 540-5p	CAAGGGUCACCCUCUGACUCUGU
mmu-miR- 466a-5p	UAUGUGUGUGUACAUGUACAUA
mmu-miR-470	UUCUUGGACUGGCACUGGUGAGU
mmu-miR-1224	GUGAGGACUGGGGAGGUGGAG
mmu-miR-191	CAACGGAAUCCCAAAAGCAGCUG
hsa-miR-199b-5p	CCCAGUGUUUAGACUAUCUGUUC
hsa-let-7a	UGAGGUAGUAGGUUGUAUAGUU
hsa-let-7b	UGAGGUAGUAGGUUGUGUGGUU
hsa-let-7c	UGAGGUAGUAGGUUGUAUGGUU
hsa-let-7d	AGAGGUAGUAGGUUGCAUAGUU
hsa-let-7e	UGAGGUAGGAGGUUGUAUAGUU
hsa-let-7f	UGAGGUAGUAGAUUGUAUAGUU
hsa-let-7g	UGAGGUAGUAGUUUGUACAGUU
hsa-let-7i	UGAGGUAGUAGUUUGUGCUGUU
hsa-miR-100	AACCCGUAGAUCCGAACUUGUG
hsa-miR-100	AACCCGUAGAUCCGAACUUGUG
hsa-miR-122	UGGAGUGUGACAAUGGUGUUUG
hsa-miR-127-3p	UCGGAUCCGUCUGAGCUUGGCU
hsa-miR-128	UCACAGUGAACCGGUCUCUUU
hsa-miR-129-5p	CUUUUUGCGGUCUGGGCUUGC
hsa-miR-134	UGUGACUGGUUGACCAGAGGGG
hsa-miR-140-5p	CAGUGGUUUUACCCUAUGGUAG
hsa-miR-145	GUCCAGUUUUCCCAGGAAUCCCU
hsa-miR-149	UCUGGCUCCGUGUCUUCACUCCC
hsa-miR-18a	UAAGGUGCAUCUAGUGCAGAUAG
hsa-miR-18b	UAAGGUGCAUCUAGUGCAGUUAG or UAAGGUGCAUCUAGUGCUGUUA
hsa-miR-193a-3p	AACUGGCCUACAAAGUCCCAGU
hsa-miR-199a-5p	CCCAGUGUUCAGACUACCUGUUC

hsa-miR-216a	UAAUCUCAGCUGGCAACUGUGA
hsa-miR-216b	AAAUCUCUGCAGGCAAAUGUGA
hsa-miR-218	UUGUGCUUGAUCUAACCAUGU
hsa-miR-26a	UUCAAGUAAUCCAGGAUAGGCU
hsa-miR-31	AGGCAAGAUGCUGGCAUAGCU
hsa-miR-345	GCUGACUCCUAGUCCAGGGCUC
hsa-miR-34c-5p	AGGCAGUGUAGUUAGCUGAUUGC
hsa-miR-362-5p	AAUCCUUGGAACCUAGGUGUGAGU
hsa-miR-378	ACUGGACUUGGAGUCAGAAGG
hsa-miR-384	AUUCCUAGAAAUUGUUCAUA
hsa-miR-409-3p	GAAUGUUGCUCGGUGAACCCCU
hsa-miR-450a	UUUUGCGAUGUGUUCCUAAUAU
hsa-miR-450b-5p	UUUUGCAAUAUGUUCCUGAAUA
hsa-miR-452	AACUGUUUGCAGAGGAAACUGA
hsa-miR-98	UGAGGUAGUAAGUUGUAUUGUU
hsa-miR-99a	AACCCGUAGAUCCGAUCUUGUG
hsa-miR-99b	CACCCGUAGAACCGACCUUGCG
mmu-let-7a	UGAGGUAGUAGGUUGUAUAGUU
mmu-let-7b	UGAGGUAGUAGGUUGUGUGGUU
mmu-let-7c	UGAGGUAGUAGGUUGUAUGGUU
mmu-let-7d	AGAGGUAGUAGGUUGCAUAGUU
mmu-let-7e	UGAGGUAGGAGGUUGUAUAGUU
mmu-let-7f	UGAGGUAGUAGAUUGUAUAGUU
mmu-let-7g	UGAGGUAGUAGUUUGUACAGUU
mmu-let-7i	UGAGGUAGUAGUUUGUGCUGUU
mmu-miR-100	AACCCGUAGAUCCGAACUUGUG
mmu-miR-100	AACCCGUAGAUCCGAACUUGUG
mmu-miR-122	UGGAGUGUGACAAUGGUGUUUG
mmu-miR-127	UCGGAUCCGUCUGAGCUUGGCU
mmu-miR-128	UCACAGUGAACCGGUCUCUUU
mmu-miR-129-5p	CUUUUUGCGGUCUGGGCUUGC
mmu-miR-134	UGUGACUGGUUGACCAGAGGGG
mmu-miR-140	CAGUGGUUUUACCCUAUGGUAG
mmu-miR-145	GUCCAGUUUUCCCAGGAAUCCCU
mmu-miR-149	UCUGGCUCCGUGUCUUCACUCCC
mmu-miR-18a	UAAGGUGCAUCUAGUGCAGAUAG
mmu-miR-18b	UAAGGUGCAUCUAGUGCUGUUAG
mmu-miR-193	AACUGGCCUACAAAGUCCCAGU
mmu-miR-199a-5p	CCCAGUGUUCAGACUACCUGUUC
mmu-miR-199b	ACAGUAGUCUGCACAUUGGUUA
mmu-miR-216a	UAAUCUCAGCUGGCAACUGUGA
mmu-miR-216b	AAAUCUCUGCAGGCAAAUGUGA
mmu-miR-218	UUGUGCUUGAUCUAACCAUGU
mmu-miR-26a	UUCAAGUAAUCCAGGAUAGGCU
mmu-miR-31	AGGCAAGAUGCUGGCAUAGCUG

mmu-miR-345	GCUGACCCCUAGUCCAGUGCUU
mmu-miR-34c	AGGCAGUGUAGUUAGCUGAUUGC
mmu-miR-362-5p	AAUCCUUGGAACCUAGGUGUGAAU
mmu-miR-378 (old mmu-miR-422b)	ACUGGACUUGGAGUCAGAAGG
mmu-miR-384-3p	AUUCCUAGAAAUUGUUCACAAU
mmu-miR-409-3p	GAAUGUUGCUCGGUGAACCCCU
mmu-miR-450a-5p	UUUUGCGAUGUGUUCCUAAUAU
mmu-miR-450b-5p	UUUUGCAGUAUGUUCCUGAAUA
mmu-miR-452	UGUUUGCAGAGGAAACUGAGAC
mmu-miR-464	UACCAAGUUUAUUCUGUGAGAUA
mmu-miR-465a-5p	UAUUUAGAAUGGCACUGAUGUGA
mmu-miR-465b-5p	UAUUUAGAAUGGUGCUGAUCUG
mmu-miR-465c-5p	UAUUUAGAAUGGCGCUGAUCUG
mmu-miR-468	UAUGACUGAUGUGCGUGUGUCUG
mmu-miR-98	UGAGGUAGUAAGUUGUAUUGUU
mmu-miR-99a	AACCCGUAGAUCCGAUCUUGUG
mmu-miR-99b	CACCCGUAGAACCGACCUUGCG
old mmu-miR-422b	CUGGACUUGGAGUCAGAAGGC
miR-106b	UAAAGUGCUGACAGUGCAGAU
miR-20b	CAAAGUGCUCAUAGUGCAGGUAG
miR-17	CAAAGUGCUUACAGUGCAGGUAG
miR-291a	CAUCAAAGUGGAGGCCCUCUCU
miR-291b-5p	GAUCAAAGUGGAGGCCCUCUCC
miR-25	CAUUGCACUUGUCUCGGUCUGA
miR-32	UAUUGCACAUUACUAAGUUGCA
miR-92a-1	UAUUGCACUUGUCCCGGCCUG
miR-92a-2	UAUUGCACUCGUCCCGGCCUCC
miR-92b	UAUUGCACUCGUCCCGGCCUCC
miR-367	AAUUGCACUUUAGCAAUGGUGA
miR-19b	UGUGCAAAUCCAUGCAAAACUGA
miR-290-5p	ACUCAAACUAUGGGGGCACUUU
miR-292	ACUCAAACUGGGGGCUCUUUUG
miR-200c	UAAUACUGCCGGGUAAUGAUGGA
miR-20a	UAAAGUGCUUAUAGUGCAGGUAG
miR-291b-3p	AAAGUGCAUCCAUUUUGUUUGU

Moreover, to enhance the efficiency of establishing induced pluripotent stem (iPS) cells, the following cytokines and/or small molecules, may further be introduced into somatic cells, in addition to miRNA and mRNA of the invention, to be reprogrammed: i.e., basic fibroblast growth factor (bFGF), stem cell factor (SCF), etc. (cytokines); and histone deacetylase inhibitors such as valpronic acid, DNA methylase inhibitors such as 5'-azacytidine, histone methyltransferase

(G9a) inhibitors such as BIX01294 (BIX), (small molecules) (D. Huangfu et al., Nat. Biotechnol., 26, pp. 795-797, 2008; S. Kubicek et al., Mol. Cell, 25, pp. 473-481, 2007; Y. Shi et al., Cell Stem Cell, 3, 568-574, 2008, Yan Shi et al., Cell Stem Cell, 2, pp. 525-528, 2008. In addition, p53 inhibitors such as shRNA or siRNA for p53 and/or UTF1 may be introduced into somatic cells (Yang Zhao et al., Cell Stem Cell, 3, pp 475-479, 2008). Also, activation of the Wnt signal (Marson A. et al., Cell Stem Cell, 3, pp 132-135, 2008) or inhibition of signaling by mitogen-activated protein kinase or glycogen synthase kinase-3 (Silva J. et al., PloS Biology, 6, pp 2237-2247 2008) can serve as a means for increasing the efficiency of generating iPS cells.

Identification of iPS Cells

The invention provides for methods of determining if a cell is a pluripotent stem cell. These methods include but are not limited to teratoma assays; antibody staining for Oct4, NANOG, Rex-1, SSEA3, SSEA4, SSEA1 (mouse only), Tra-1-60, Tra-1-80; morphological observations; RT-PCR for pluripotency factors; methylation pattern comparisons to hES cells (bisulfate sequencing); spontaneous differentiation to all three germ layers (analyzed by RT-PCR or Ab staining); and pluritest analysis.

A cell can also be determined to be a pluripotent stem cell by analysis of the presence or absence of various markers specific to undifferentiated cells, for example, by RT-PCR. For example, some pluripotent cell markers include: Oct3/4; Nanog; alkaline phosphatase (AP); ABCG2; stage specific embryonic antigen-1 (SSEA-1); SSEA-3; SSEA-4; Tra-1-60; TRA-1-81; Tra-2-49/6E; ERas/ECAT5, E-cadherin; βIII-tubulin; .alpha.-smooth muscle actin (.alpha.-SMA); fibroblast growth factor 4 (Fgf4), Cripto, Dax1; zinc finger protein 296 (Zfp296); N-acetyltransferase-1 (Nat1); (ES cell associated transcript 1 (ECAT1); ESG1/DPPA5/ECAT2; ECAT3; ECAT6; ECAT7; ECAT8; ECAT9; ECAT10; ECAT15-1; ECAT15-2; Fthl17; Sal14; undifferentiated embryonic cell transcription factor (Utf1); Rex1; p53; G3PDH; telomerase, including TERT; silent X chromosome genes; Dnmt3a; Dnmt3b; TRIM28; F-box containing protein 15

(Fbx15); Nanog/ECAT4; Oct3/4; Sox2; Klf4; c-Myc; Esrrb; TDGF1; GABRB3; Zfp42, FoxD3; GDF3; CYP25A1; developmental pluripotency-associated 2 (DPPA2); and T-cell lymphoma breakpoint 1 (Tcl1); DPPA3/Stella; DPPA4. Other markers can include Dnmt3L; Sox15; Stat3; Grb2; SV40 Large T Antigen; HPV16 E6; HPV16 E7, β-catenin, and Bmi1. Such cells can also be characterized by the down-regulation of markers characteristic of the differentiated cell from which the iPS cell is induced. For example, iPS cells derived from fibroblasts may be characterized by down-regulation of the fibroblast cell marker Thy1 and/or up-regulation of SSEA-3 and 4. It is understood that the present invention is not limited to those markers listed herein, and encompasses markers such as cell surface markers, antigens, and other gene products including ESTs, RNA (including microRNAs and antisense RNA), DNA (including genes and cDNAs), and portions thereof.

iPS cells may be further identified by semipermanent cell proliferation, pluripotency, or cell morphology (Takahashi, K. et al., Cell 131:861-872 (2007)). Briefly, regarding semipermanent proliferation, the ability of cells to expand exponentially is tested by culturing the cells over about 4-6 months. In the case of human iPS cells, because the population doubling time is known to be about 46.9.±.12.4 hr, 47.8.±.6.6 hr, or 43.2±11.5 hr for example, this value can be indicative of the ability of proliferation. Alternatively, high telomerase activity may be detected by the telomeric repeat amplification protocol (TRAP) because iPS cells normally have high telomerase activity.

Pluripotency can be confirmed by forming teratoma and identifying tissues or cells of three embryonic germ layers (i.e., ectoderm, mesoderm, and endoderm). Specifically, cells are injected intradermally in a nude mouse (where the cells are induced from murine somatic cells) or in the spermary of a SCID mouse (where the cells are induced from human somatic cells), followed by confirming the formation of a tumor then confirming that the tumor tissues are composed of tissues including neural rosettes (ectoderm), cartilage (mesoderm), cardiac

myocyte (mesoderm), gut-like epithelium (endoderm), adipose (mesoderm), and the like.

Because human or mouse iPS cell colonies are known to have a morphology similar to that of human or mouse ES cell colonies, the morphology of iPS cells can be used as an indicator of pluripotency. In general, human iPS cells form flat colonies, while mouse iPS cells tend to form swollen colonies.

Kits of Pharmaceutical Systems

The present invention provides for kits for producing a pluripotent stem cell or pharmaceutical compositions comprising iPS cells of the invention. Kits according to this aspect of the invention comprise a carrier means, in combination with an mRNA and miRNA of the invention. In one embodiment, a kit of the invention further comprises one or more of a culture medium suitable for producing pluripotent cells of the invention, a medium suitable for growth and maintenance of pluripotent cell colonies of the invention, and a transfection reagent. The carrier means may comprise any one of a box, carton, tube or the like, having in close confinement therein one or more container means, such as vials, tubes, ampules, bottles and the like.

If desired, the kit is provided together with instructions for using the kit to produce pluripotent stem cells. The instructions will generally include information about how to produce pluripotent stem cells.

Formulations comprising pluripotent stem cells or differentiated cells derived from pluripotent stem cells of the invention may be provided in combination with carrier means and may include instructions that generally include information about the use of the cells for treating a subject having a disease. In other embodiments, the instructions include at least one of the following: description of the therapeutic agent (iPS cells or cells derived therefrom); warnings; indications; counter-indications; animal study data; clinical study data; and/or references. The instructions may be printed directly on the container (when present), as a

label applied to the container, or as a separate sheet, pamphlet, card, or folder supplied in or with the container. In certain embodiments, kits of the invention may also include B18R protein or any other component known in the art to suppress an immune response.

Animal Models

The iPS cells of the invention are also applicable to animals, and may also be used to facilitate biomedical research of disease in a variety of animal model systems.

Uses

The methods of the invention provide for production of pluripotent stem cells that can be used for clinical applications including disease treatment and prevention. In particular, the iPS cells of the invention, or differentiated progeny cells can be used for applications in the field of regenerative medicine. The cells of the invention also provide for methods of designing personalized treatments for subjects in need thereof.

The iPS cells of the invention and their differentiated progeny can also be used to identify compounds with a particular function, for example, treatment or prevention of disease, determine the activity of a compound of interest and or determine the toxicity of a compound of interest. Further, the present invention provides a stem cell therapy comprising transplanting somatic cells into a patient, wherein the somatic cells are obtained by inducing differentiation from induced pluripotent stem cells that are obtained according to the methods of the invention by using somatic cells isolated and collected from a patient.

In addition, the present invention provides a method for evaluation of physiological effect or toxicity of a compound, a drug, or a toxic agent, with use of various cells obtained by inducing differentiation from induced pluripotent stem cells that are obtained by the methods of the invention.

The application of induced pluripotent stem cells produced by the method of the present invention is not specifically limited, and these cells can be used for every

examination/study to be performed with use of ES cells, and for any disease therapy which utilizes ES cells. For example, induced pluripotent stem cells obtained by the method of the present invention can be induced to produce desired differentiated cells or precursor cells (such as nerve cells, myocardial cells, blood cells and insulin-producing cells) or by treatment with retinoic acid, a growth factor such as EGF, glucocorticoid, activin A/BMP4 (bone morphogenetic protein 4), or VEGF (vascular endotherial growth factor), so that appropriate tissues can be formed. Stem cell therapies through autologous cell transplantation can be achieved by returning these differentiated cells or tissue obtained in the above manner, into the patient. However, the application of the induced pluripotent stem cells (iPS cells) of the present invention is not to be limited to the abovementioned specific aspects. The iPS cells have a capacity of germline transmission *in vivo*. Thus, when the iPS cells are introduced into the blastocyst from a non-human mammal, and then transplanted into the uterus of a surrogate mother of the same animal, a chimeric animal to which part of the genotypes of the iPS cell has been transmitted (WO 2007/069666) is produced. The iPS cells can also be used for modification of a gene, introduction (or knock-in) of a gene, or knock-out of a gene, thereby enabling clarification of the function of a gene, to create a non-human animal model with disease, or to produce a substance such as protein.

Methods of Treatment

The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disease or disorder treatable *via* administration of the pluripotent stem cells of the invention or differentiated progenitor cells.

In one aspect, the invention provides a method for preventing in a subject, a disease or disorder as described above by administering to the subject an iPS or differentiated progenitor cell of the invention. Subjects at risk for the disease can be identified by, for example, any or a combination of diagnostic or prognostic

assays as described herein. Administration of a prophylactic agent can occur prior to the detection of, *e.g.*, cancer in a subject, or the manifestation of symptoms characteristic of the disease or disorder, such that the disease or disorder is prevented or, alternatively, delayed in its progression.

Another aspect of the invention pertains to methods of treating subjects therapeutically, *i.e.*, altering the onset of symptoms of the disease or disorder. These methods can be performed *in vivo* (*e.g.*, by administering the pluripotent stem cells or differentiated progeny of the invention to a subject).

Therapeutic agents (e.g. pluripotent cells of the invention) can be tested in an appropriate animal model. For example, a pluripotent stem cell or differentiated progeny cell, as described herein can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with the cell. Alternatively, an agent (*e.g.*, a pluripotent stem cell of the invention) can be used in an animal model to determine the mechanism of action of such an agent.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA, genetics, immunology, cell biology, cell culture and transgenic biology, which are within the skill of the art. See, e.g., Maniatis et al., 1982, Molecular Cloning (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Sambrook et al., 1989, Molecular Cloning, 2nd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Sambrook and Russell, 2001, Molecular Cloning, 3rd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Ausubel et al., 1992), Current Protocols in Molecular Biology (John Wiley & Sons, including periodic updates); Glover, 1985, DNA Cloning (IRL Press, Oxford); Anand, 1992; Guthrie and Fink, 1991; Harlow and Lane, 1988, Antibodies, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Jakoby and Pastan, 1979; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide

To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Riott, Essential Immunology, 6th Edition, Blackwell Scientific Publications, Oxford, 1988; Hogan et al., Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986); Westerfield, M., The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio), (4th Ed., Univ. of Oregon Press, Eugene, 2000).

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. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

EXAMPLES

The present invention is described by reference to the following Examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE 1

Transfection of eGFP mRNA using the Stemfect™ RNA Transfection Kit

Figure 1 demonstrates the results of experiments wherein fibroblasts are transfected with eGFP mRNA using the Stemfect™ RNA Transfection Kit. BJ fibroblast cells (fibroblasts derived from human foreskin that are not mature) were seeded in a 24-well format and transfected with 250 ng of eGFP mRNA. The cells were cultured at 37°C and 5% CO_2 and analyzed by flow cytometry at 18-24 hours post-transfection. Figure 1A is a graph demonstrating the mean fluorescence intensity as determined by flow cytometry. Stemfect™ RNA Transfection Kit yielded 2-3 fold higher average protein expression than that observed using RNAiMAX™. Figure B presents representative histograms comparing the transfection efficiency of Stemfect™ RNA Transfection Kit (purple) to RNAiMAXTM (green) alongside an untransfected cells control (red). Stemfect™ Transfection Kit led to >98% transfection efficiency of eGFP mRNA without any significant toxicity, while enabling a tighter distribution of protein expression.

Figure 1C presents the results of an experiment wherein 75,000 BJ fibroblasts were seeded in 24-well format and transfected with 250 ng of eGFP mRNA using the Stemfect™ RNA Transfection Kit. Fluorescent image captured 18-24 hours post-transfection.

EXAMPLE 2

Derivation of integration-free iPS cells from primary patient fibroblasts in a feeder-free environment.

iPS cells of the invention are generated from primary patient fibroblasts in a feeder-free environment.

The Experimental timeline for production of iPS cells from primary patient fibroblasts in a feeder free environment is presented in Figure 2A.

Experimental Timeline:

On day 1, 50,000 human fibroblasts were seeded in a single well of a 6-well plate, pre-coated with Matrigel™ and cultured overnight at 37°C, 5% CO_2, and

21% O_2. During days 0-12 target fibroblasts were transfected in medium previously conditioned with NuFFs (Human Newborn Foreskin Fibroblasts). The cells were transfected with miRNA and mRNA cocktail of the invention (for example, Cluster A or Cluster B) as follows:

Day 0-pluripotency miRNA cocktail;

Days 1-3-1.5 µg of mRNA cocktail (OSKML- Oct4, Sox2, Klf4, Myc and Lin28);

Day 4 □both mRNA and miRNA cocktails (sequentially added);

Days 5-12-1.5 µg of mRNA cocktail.

Figure 2B presents the morphology Progression. 50,000 diseased patient dermal fibroblasts were seeded in one well of a 6-well plate and were then transfected as outlined above. Images were captured at defined time-points (purple dots in Figure 2A). Day 2: The fibroblasts display typical compact morphologies in response to repeated transfection with mRNA. Day 5: Cells have initiated mesenchymal to epithelial transition and begin to assemble into small, loose clusters. Day 8: The rate of proliferation of cells within the clusters has increased as the edges of the colonies are emerging. Day 10: The cluster of cells has expanded, and the edges of a burgeoning colony are more well defined. Day 12: TRA-1-81(+) iPS cell colonies with defined edges and tight cell clustering are present in the primary culture. TRA-1-81 is a surface marker for pluripotency.

Figure 3 presents the effect of target cell number and mRNA dose on iPS cell generation. Human fibroblasts were seeded at either 25,000 or 50,000 cells per well on a MatrigelTM coated 6-well plate and allowed to adhere overnight. The cells were transfected daily with either 1.0 or 1.5 µg mRNA (encoding at least one of Oct4, Sox2, Klf4, Myc and Lin28) in NuFF conditioned Medium containing 300 ng/ml B18R protein for 10 days. Cultures were incubated at 37°C, 5% CO_2, and 5% O_2. Wells were assessed for the number of TRA-1-81 positive colonies at Day 11.

Transfection of mRNA elicits an immune response from the cells that ultimately leads to apoptosis and death in the cell culture. This response is abrogated by

using modified nucleotide or by using the B18R protein to block the immune response (see Angel and Yannik PLOS ONE, 2010)

EXAMPLE 3

Number of Transfections Required for Generating iPS cell colonies

The number of transfections required for generating iPS cell colonies when transfecting with an mRNA cocktail only was determined. Two patient derived human dermal fibroblast cultures were each seeded at 50,000 cells in one well of a Matrigel™ coated 6-well plate and cultured overnight at 37°C, 5% CO_2, and 21% O_2. Cells were transfected daily with 1.5 µg of mRNA reprogramming cocktail in Pluriton™ Reprogramming Medium for the indicated number of days (see Figure 4) and incubated overnight at 37°C, 5% CO_2, and 21% O_2. After completing the transfections, the media was changed daily until Day 12. Each well was then individually stained with Stemgent StainAlive™ (Stemgent) TRA-1-81 Antibody for iPS cell colony identification to assess reprogramming productivity at Day 12. Colonies emerged in wells receiving as few as 6 transfections. Maximal iPS cell colony productivity was observed when primary patient fibroblasts samples received 8 to 12 mRNA transfections

EXAMPLE 4

iPS Cell Colony Formation in a Scaled-Down Format and Atmospheric Oxygen Tension

As demonstrated in Figure 5, addition of miRNA supports iPS cell colony derivation in a scaled-down, 12-well format and atmospheric oxygen tension. Two patient- derived dermal fibroblast cultures were seeded at 25,000 cells per well of a Matrigel™ coated 12-well plate and cultured overnight at 37°C and 5% CO_2. The cultures were then transfected with either 0.5 µg or 0.75 µg of mRNA cocktail Oct4, Sox2, Klf4, c-Myc, Lin28 and nGFP at a molar ration of 3:1:1:1:1:1 and miRNA cocktail, for example, Cluster A or Cluster B, under indicated O_2

tensions according to the timeline outlined in Figure 2A. Wells at 5% O_2 were counted on Day 13, while wells at 21% O_2 were counted on Day 16 due to delayed emergence of iPS cell colonies. Each well was individually assayed with Stemgent StainAlive™ TRA- 1-81 Antibody and counted to assess reprogramming productivity. Inclusion of miRNA cocktail allows iPS cell colony generation in a 12-well culture format in both reduced (5%) and atmospheric (21%) oxygen tensions.

EXAMPLE 5

Continued expansion and maintenance of pluripotency of clonal mRNA iPS cell lines under feeder-free conditions

Figure 6 demonstrates the continued expansion and maintenance of pluripotency of clonal mRNA iPS cell lines under feeder-free conditions. A primary mRNA iPS cell colony derived in Pluriton™ Reprogramming Medium on Matrigel™ was manually isolated at Day 13 and continued to express surface markers for pluripotency (TRA-1-81), as it was subsequently passaged in NutriStem™ XF/FF Culture Medium on Matrigel™, resulting in an integration-free, virus-free iPS cell line that has never been in contact with a feeder layer.

EXAMPLE 6

The Use of miRNA to Enable Reprogramming in Refractory Lines

Using miRNA mimics in conjunction with mRNA, iPS cell colonies were generated from cell lines that are refractory to methods involving mRNA alone or miRNA alone. These target cells were primary patient fibroblasts seeded onto a feeder layer at 5,000 cells/ well. Typically, reprogramming experiments require >100,000 cells/ well in a 6-well format. According to the novel claimed methods, such high numbers of target cells are not required. In one embodiment, the novel methods provides for production of pluripotent stem cells from 1,000-10,000 cells/ well in a 6-well format.

As indicated in the timeline presented below, cells were treated with miRNA (cluster A or B) at day 0 and prior to any mRNA transfection. They were then cotransfected with miRNA and mRNA, wherein the mRNA encodes at least one of Oct4, Sox2, Klf4, Myc and Lin28 at day 3. Every day through day 16, cells were treated with mRNA alone. In parallel, control cells were transfected with only mRNA for 16 straight days and compared to the miRNA + mRNA transfected cells grown under identical conditions. All cells were trypsinized and replated at specified cell densities on NuFFs at day 7. Cells treated with either miRNA cluster A (hsa-mir-302a, hsa-mir-302b, hsa-mir-302c, hsa-mir-302d and hsa-mir-367) or miRNA cluster B (hsa-mir-302a, hsa-mir-302b, hsa-mir-302c, hsa-mir-302d, hsa-mir-200c, hsa-mir-369-3p and hsa-mir-369-5p), in addition to mRNA encoding at least one of Oct4, Sox2, Klf4, Myc and Lin28, produced 1-2 colonies that stained positive for Tra-1-81, a pluripotent stem cell marker, while cells treated with mRNA alone did not yield any iPS cell colonies.

Feeder: Nuff 300k/well

Target cells: primary patient fibroblasts- 5k/well, LN0005 x 3 well, LN0013x 3 wells,

Media: LN-Media for first 5 days then Pluriton 2532

Protocol: mRNA alone or mRNA plus 2x miRNA transfection using RNAiMAX reagent, cells split at day 7, each well re-plated with 50k and 20k cells on Nuffs

mRNA required: 1.2ug/well (encoding at least one of Oct4, Sox2, Klf4, Myc and Lin28), R1 for LN-cells

LN0005,LN-medium, mRNA	LN0005, LN-medium mRNA+ miRNA cluster A	LN0005, LN-medium mRNA+ miRNA cluster B
LN0013, LN-medium, mRNA	LN0013, LN-medium, mRNA + miRNA cluster A	LN0013, LN-medium, mRNA + miRNA cluster B

At day -2 NuFF were seeded onto 6-well plate at 300,000 cells/ well. At day -1 LNH primary patient fibroblasts were seeded onto the feeder layer at 5,000 cells/

well. At day 0 2 wells were transfected with mRNA and 4 wells were transfected with miRNA (2 wells with cluster A and 2 wells with cluster B). At day 3 the 2 wells were transfected with mRNA and the 4 wells were co-transfected with mRNA + miRNA (2 wells with cluster A and 2 wells with cluster B). On the remaining days all cells were transfected with mRNA through d18. At day 5 cells in the wells treated with mRNA + miRNA show more morphology changes than the wells with mRNA alone. At day 7 the cells are split and counted. The cell count per well is below:

LN005 mRNA control = 5.35×10^5

LN005 mRNA + miRNA cluster A = 8.1×10^5

LN005 mRNA + miRNA cluster B = 8.1×10^5

LN013 mRNA control = 6.6×10^5

LN013 mRNA + miRNA cluster A = 1.12×10^6

LN005 mRNA + miRNA cluster B = 1.0×10^6

The cells were re-plated at 100k/well and 50k/well for each condition

At day 14 iPS colonies appeared in the LN0013-mRNA + miRNA-B 100K/well sample. At days 20- d22 LiveStain Tra-1-81 was performed. 4 positive iPS , two iPS derived from LN0013 co-transfection with cluster A and B and two iPS derived from LN0005 co-transfection with cluster A were detected and one iPS from each condition were expanded.

Results are presented in Figure 7.

These data demonstrate that LN cell lines (primary patient fibroblasts) are difficult to be reprogrammed using mRNA alone (3 x transfections were done). These data also demonstrate that miRNA, in combination with mRNA enhances cell proliferation and iPS reprogramming of patient primary fibroblast cells designated LN cells

EXAMPLE 7

Comparison of miRNA to siRNA on Enhancement of iPS cell Generation

These data present experiments wherein the ability of miRNA and siRNA to enhance iPS cell generation are compared.

Inhibition of p53 has been shown to increase cellular proliferation. The generation of iPS cells treated with mRNA encoding at least one of Oct4, Sox2, Klf4, Myc and Lin28 supplemented with p53 was compared to the generation of iPS cells treated with mRNA and miRNA cluster A or cluster B (see details presented below). As in prior experiments, the target primary patient fibroblasts were seeded and grown on a NuFF layer for the duration of the experiment. The effect of splitting the culture on the output number of iPS cell colonies was also determined.

While previous attempts to reprogram certain primary patient fibroblasts with mRNA alone were unsuccessful, addition of miRNA cluster A or cluster B increased the efficiency of mRNA reprogramming. In the no-split protocol starting with 5,000 cells/well 57 colonies under either cluster A or cluster B conditions were produced. This efficiency of over 1% is higher than any efficiency typically observed in other reprogramming systems. In contrast, addition of siRNA targeting p53 had a minimal effect, yielding only 1 colony when starting with the same number of cells.

mRNA, siRNA and miRNA Transfection on primary patient cells

Feeder: Nuff 300k/well, 3002M lot# 868

Target cells: For No split- primary patient fibroblasts: 5k cells/well x 3 wells (in one plate)

For split- primary patient fibroblasts: 10k cells/well x 3 wells (in other plate)

Media: Pluriton 2532 with supplement Lot# 2567 B18R lot#1633

Protocol: Split 10k wells after 6-7 transfections, re-plate each well (condition) at 50 and100k cells/well (6 wells from 3 of 10k wells)

mRNA required: 1.2ug/well encoding at least one of Oct4, Sox2, Klf4, Myc and Lin28, R4

mRNA transfection was performed every day for 4 h except day 0; miRNA and siRNA were added at day 0.

miRNA for 4 wells/6-well plate was introduced into the cells by transfecting the cells for 4 h at day 0 and day 3 (day 3-miRNA was cotransfected with mRNA)

miRNA- cluster A: miRNA 302A, 302B, 302C, 302D, 367

miRNA- cluster B: miRNA 302A, 302B, 302C, 302D, 200C, 369-3p, 369-5p.

1 vial of miRNA powder(1.0OD) + 250 ul RNase free TE =20uM stock was used.

Equal amount (ul) of each miRNA stock was mixed into the cocktail and aliquoted.

For transfections 3.5ul of the cluster cocktail was adder per well/6-well plate/ in 2ml media

(20000nM x 3.5ul = $\underline{X}$ ul x 2000 ul, X = 35nM miRNA cluster A and cluster B, final concentration of miRNA in the well is 35nM)

A tube: 7ul miRNA A or B + 117ul Opti-M

B tube: 10.25ul RNAiMAX + 117ul Opti-M

A and B were mixed and maintained at room temperature for 15 min. 120ul of complex was added into each well

siRNA for 2 wells/6-well plate at day 0 and day 4 (day 4-cotransfected with mRNA):

p53 siRNA stock 20pmol/ul

A tube: 1.5ul siRNA + 250ul Opti-M

B tube: 5ul RNAiMAX + 250ul Opti-M

A and B were mixed and maintained at room temperature for 15min. 250ul of complex was added into each well

6-well plate format:

UTC 5K + miRNA cluster A	UTC 5K + miRNA cluster B	UTC 5K + p53 siRNA
UTC 10K + miRNA cluster A	UTC 10K + miRNA cluster B	UTC 10K + p53 siRNA

At day -2: NuFF seeded onto 6-well plate at 300,000 cells/ well

At day -1: primary patient fibroblasts seeded onto the feeder layer at 5,000 or 10,000 cells/ well

At day 0: miRNA or siRNA transfections were performed. No mRNA transfections were performed at day 0.

At day 3:

2 wells were transfected with mRNA;

4 wells were transfected with mRNA + miRNA (2 wells with cluster A and 2 wells with cluster B);

At day 4:

The 4 wells were transfected with mRNA transfection (miRNA wells)

The 2 wells were transfected with mRNA + siRNA

Other days:

mRNA transfection was performed daily through d17

At days 4-5, early morphology changes were beginning to occur in the cells in the wells transfected with miRNA. Cells at a higher cell density were observed in cells treated with siRNA, although these cells exhibited fewer morphology changes.

Day 6: the culture was split and cells were plated (primary patient fibroblasts) at 10k and the cells were counted. The cell count/well was as below

mRNA + miRNA A = 9.1×10^5

mRNA + miRNA B = 8.77×10^5

mRNA + siRNA A = 8.3×10^5

Cells were re-plated at 60k, 40k and 20k/well for each condition

Day 12:

Certain small iPS appear in the 5k-miRNA A and B wells

Many “loose” clusters were observed in the split wells-miRNA-A and B

Day 17: LiveStain Tra1-81 in no split wells and colony count

UTC 5K + miRNA cluster A 57	UTC 5K + miRNA cluster B 57	UTC 5K + p53 siRNA 1

Day 18: LiveStain Tra1-81 in the split wells and colony count

UTC 60K +miRNA cluster A 44	UTC 40K +miRNA cluster A 40	UTC 20K +miRNA cluster A 14
UTC 60K +miRNA cluster B 68	UTC 40K +miRNA cluster B 58	UTC 20K +miRNA cluster B 21
UTC 60K + P53 siRNA 4	UTC 40K + P53 siRNA 7	UTC 20K + P53 siRNA 3

These data demonstrate that UTC cells are not reprogrammed by the addition of mRNA alone. These data also demonstrate that UTC cells treated with miRNA are efficiently reprogrammed as compared with UTC cells treated with either mRNA alone or with mRNA and siRNA in combination.

All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications can be made to the invention disclosed herein without departing from the scope and spirit of the invention.

The invention illustratively described herein suitably can be practiced in the absence of any element or elements, limitation or limitations that are not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments, optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the description and the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or

exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

Embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description.

The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

References

1. Warren, L., Manos, P.D., Ahfeldt, T., Loh, Y.H., Li, H., Lau, F., Ebina, W., Mandal, P.K., Smith, Z.D., Meissner, A., Daley, D.Q., Brack, A.S., Collins, J.J., Cowan, C., Schlaeger, T.M., Rossi, D.J. (2010) Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell, 7:618-30.
2. Angel, M., Yanik, M.F. (2010) Innate Immune Suppression Enables Frequent Transfection with RNA Encoding Reprogramming Proteins. PLoS One 5:e11756.
3. Yakubov, E., Rechavi, G., Rozenblatt, S., Givol, D. (2010) Reprogramming of Human Fibroblasts to Pluripotent Stem Cells using mRNA of Four Transcription Factors. Biochem Biophys Res Commun. 394:189.
4. Anokye-Danso, F., Snitow, M. and Morrisey, E.E. (2012) How microRNAs Facilitate Reprogramming to Pluripotency J. Cell Science 125, 1-9.
5. Subramanyam, D. Lamouille S., Judson, R.L., Liu, J.Y., Bucay, N., Derynck, R., Blelloch, R. (2011) Multiple Targets of miR-302 and miR-372 Promote Reprogramming of Human Fibroblasts to Induced Pluripotent Stem Cells Nature Biotechnology May; 29(5): 443-8.
6. U.S. 2013/0102768

CLAIMS

We claim:

1. A method of producing a pluripotent stem cell comprising:

    a. introducing at least one mRNA into a target cell;

    b. introducing at least one miRNA into a target cell; and

    c. culturing the target cell to produce a pluripotent stem cell.

2. The method of claim 1, wherein the step of introducing the at least one mRNA into the cell and or the step of introducing the at least one miRNA into the target cell is repeated at least once.

3. The method of any one of the preceding claims, wherein prior to step (a), at least one miRNA is introduced into the target cell.

4. The method of any one of the preceding claims, wherein steps (a) and (b) are sequential.

5. The method of any one of the preceding claims, wherein steps (a) and (b) occur simultaneously.

6. The method of any one of the preceding claims, wherein the stem cell is produced in less than 2 weeks from the initiation of step (a).

7. The method of any one of the preceding claims, wherein the stem cell is produced in greater than 2 weeks from the initation of step (a).

8. The method of any one of the preceding claims, wherein the stem cell is produced in 2-3 weeks from the initiation of step (a).

9. The method of any one of the preceding claims, wherein the stem cell expresses at least one of a surface marker selected from the group

consisting of: SSEA3, SSEA4, Tra-1-81, Tra-1-60, Rex1, Oct4, Nanog and Sox2.

10. The method of any one of the preceding claims, wherein the stem cells can divide *in vitro* for greater than one year; and/or divide in vitro for more than 30 passages; and/or stain positive by alkaline phosphatase or Hoechst Stain, and/or form a teratoma.

11. The method of any one of the preceding claims, wherein the stem cell can form an embryoid body and express one or more endoderm markers selected from the group consisting of: AFP, FOXA2 and GATA4, and/or one or more mesoderm markers selected from the group consisting of: CD34, CDH2 (N-cadherin), COL2A1, GATA2, HAND1, PECAM1, RUNX1, RUNX2; and/or one or more ectoderm markers selected from the group consisting of: ALDH1A1, COL1A1, NCAM1, PAX6 andTUBB3 (Tuj1).

12. The method of any one of the preceding claims, wherein, at least one stem cell is produced.

13. The method of any one of the preceding claims, wherein one or both of the at least one miRNA and the at least one mRNA comprise a modified nucleotide.

14. The method of any one of the preceding claims, wherein the at least one mRNA is not integrated into the genome of the stem cell.

15. The method of any one of the preceding claims, wherein the mRNA and miRNA introduced into the target cells in steps (a) and (b) are not present in the stem cell.

16. The method of any one of the preceding claims, wherein the culturing is performed in the absence of a feeder layer.

17. The method of any one of the preceding claims, wherein the method is performed at $\leq$5% O_2.

18. The method of any one of the preceding claims, wherein the method is performed at 5%-21% O_2.

19. The method of any one of the preceding claims, wherein the method is performed at 21% O_2

20. The method of any one of the preceding claims, wherein the target cell is selected from the group consisting of: fibroblast, peripheral blood derived cells including but not limited to endothelial progenitor cell (L-EPCs)), cord blood derived cell types (CD34+), epithelial cells, and keratinocytes.

21. The method of any one of the preceding claims, wherein the at least one mRNA encodes a reprogramming factor.

22. The method of any one of the preceding claims, wherein the at least one mRNA encodes at least one of OCT4, SOX2, KLF4, c-MYC, LIN28, Nanog, Glis1, Sal4 and Esrbb1.

23. The method of any one of the preceding claims, wherein the at least one miRNA comprises at least one miRNA that is 80% or more identical to an miRNA selected from the group consisting of hsa-miR-302a, hsa-miR-302b, hsa-miR-302c, hsa-miR302d, hsa-miR-367, hsa-miR-200c, hsa-miR-369-3p and hsa-miR-369-5p.

24. The method of any one of the preceding claims, wherein the at least one miRNA comprises a combination of hsa-miR-302a, hsa-miR-302b, hsa-miR-302c, hsa-miR302d and hsa-miR367.

25. The method of any one of the preceding claims, wherein the at least one miRNA comprises a combination of hsa-miR-302a, hsa-miR-302b, hsa-miR-302c, hsa-miR302d, hsa-miR-200c, hsa-miR-369-3p and hsa-miR-369-5p.

26. The method of any one of the preceding claims, wherein the at least one miRNA comprises the combination of: hsa-miR-302a, hsa-miR-302b, hsa-miR-302c, hsa-miR-302d and hsa-miR-367; or hsa-miR-302a, hsa-miR-hsa-miR-302b, hsa-miR-302c, hsa-miR-302d, hsa-miR-200C, hsa-miR-369-3p, hsa-miR-369-5p; or the combination of hsa-miR-302a, hsa-miR-302b, hsa-miR-302c, hsa-miR-302d, hsa-miR-200C, hsa-miR-369-3p, hsa-miR-369-5p

27. The method of any one of the preceding claims, wherein the cell is a human cell.

28. A method of inducing pluripotency in a target cell comprising:

a. introducing at least one mRNA into the target cell;

b. introducing at least one miRNA into the target cell; and

c. culturing the target cell to produce a pluripotent cell.

29. A isolated pluripotent stem cell comprising at least one mRNA encoding a reprogramming factor in combination with at least one miRNA produced according to the method of any one of the preceding claims.

30. The isolated pluripotent stem cell produced according to the method of any one of the preceding claims, wherein the at least one mRNA is not integrated into the genome of the cell.

31. The isolated pluripotent stem cell produced according to the method of any one of the preceding claims, wherein the mRNA and miRNA introduced into the target cells in steps (a) and (b) are not present in the stem cell.

32. The isolated pluripotent stem cell of any one of claims 29-31, wherein the at least one miRNA comprises at least one miRNA that is 80% or more identical to an miRNA selected from the group consisting of hsa-miR-

302a, hsa-miR-302b, hsa-miR-302c, hsa-miR302d, hsa-miR367, hsa-miR-200c, hsa-miR-369-3p and hsa-miR-369-5p.

33. The isolated pluripotent stem cell of any one of claims 29-31, wherein the at least one mRNA encodes at least one of OCT4, SOX2, KLF4, c-MYC and LIN28.

34. A formulation comprising the isolated pluripotent stem cell of any one of claims 29-33.

35. The formulation of claim 34, further comprising a compound that suppresses an immune response.

36. A kit for producing a pluripotent stem cell comprising at least one mRNA and at least one miRNA.

37. The kit of claim 36, further comprising culture media and a transfection reagent.

38. The kit of claim 36, further comprising a compound that suppresses an immune response.

39. A method of treating a subject with a disease comprising administering to the subject a cell produced by differentiation of the isolated pluripotent stem cell of any one of claims 29-33.

40. A method of treating a subject with a disease comprising administering to the subject a cell produced by differentiation of the isolated pluripotent stem cell produced by the method of any one of claims 1-28.

41. A method of identifying a compound for treatment of a disease comprising contacting a cell produced by differentiation of a stem cell produced by the method of any one of claims 1-28 with a compound of interest.

42. A method of determining the activity of a compound for treating a disease comprising contacting a cell produced by differentiation of a stem cell produced by the method of any one of claim 1-28 with a compound known to treat a disease.

43. A method of determining the toxicity of a compound for treating a disease comprising contacting a cell produced by differentiation of a stem cell produced by the method of any one of claims 1-28 with a compound known to treat a disease.

44. The method of any of claims 40-43, wherein the cell produced by differentiation of a stem cell produced by any one of claims 1-28, is selected from the group consisting of: fibroblast, peripheral blood derived cells including but not limited to endothelial progenitor cell (L-EPCs)), cord blood derived cell types (CD34+), epithelial cells, and keratinocytes .

45. The use of a cell produced by differentiation of a stem cell produced by the method of any one of claims 1-28 for the manufacture of a medicament for treating a subject with a disease.

FIG.1A

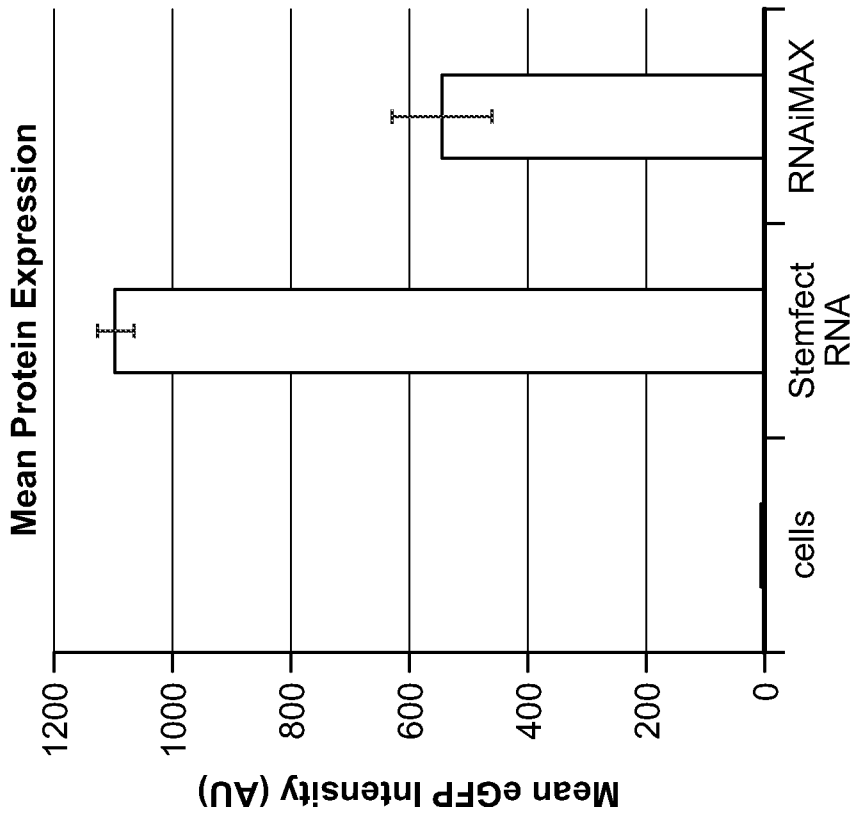

Mean Protein Expression
Mean eGFP Intensity (AU)
0
200
400
600
800
1000
1200
cells
Stemfect RNA
RNAiMAX

FIG.1B

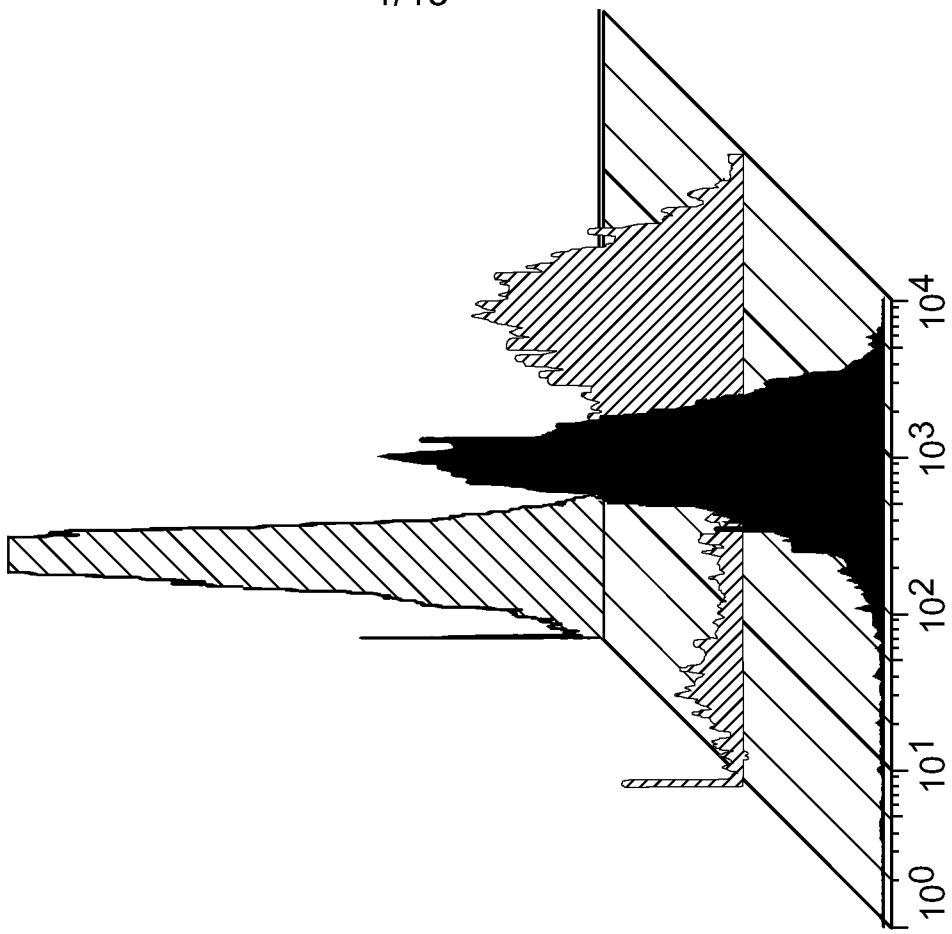

10^0
10^1
10^2
10^3
10^4

FIG.1C

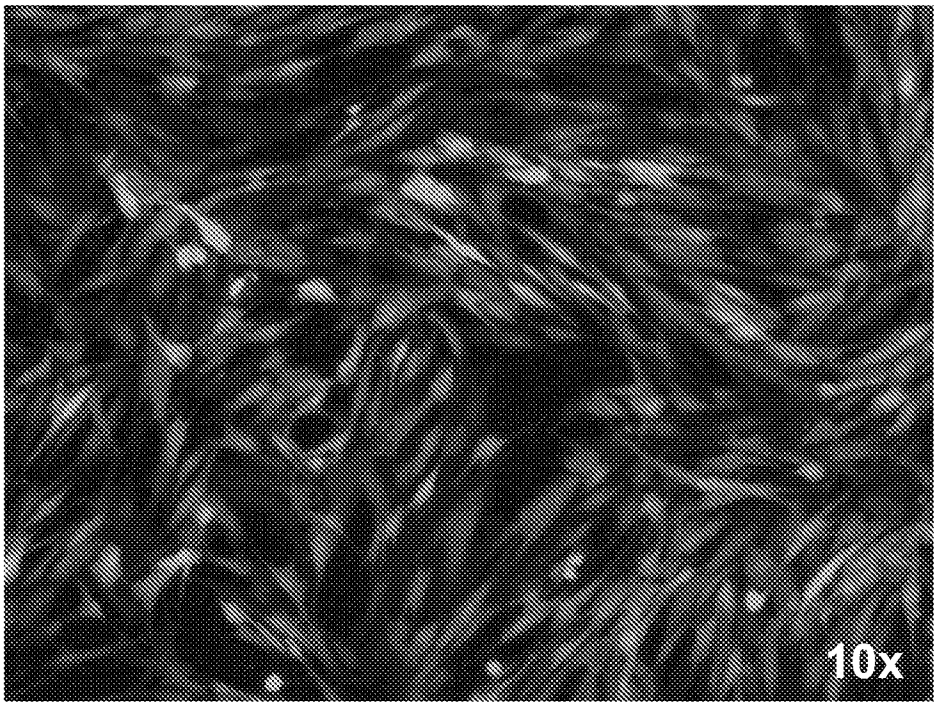


FIG. 2A

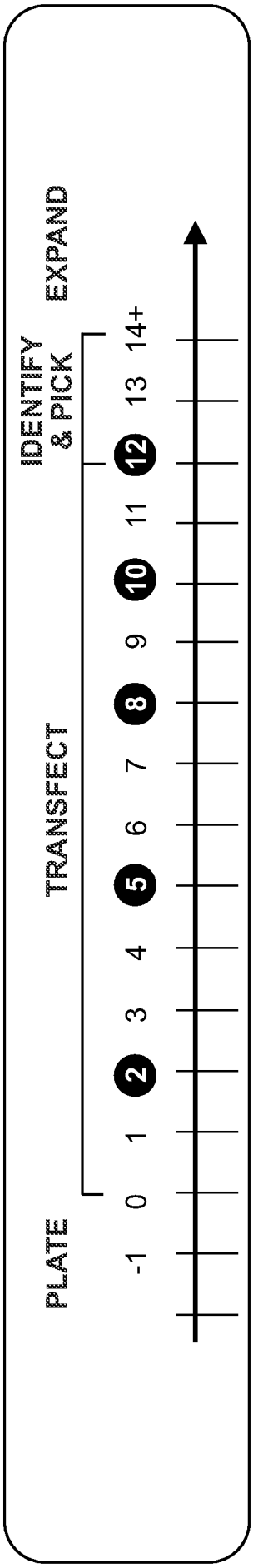

PLATE
TRANSFECT
IDENTIFY & PICK
EXPAND
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14+

FIG. 2B

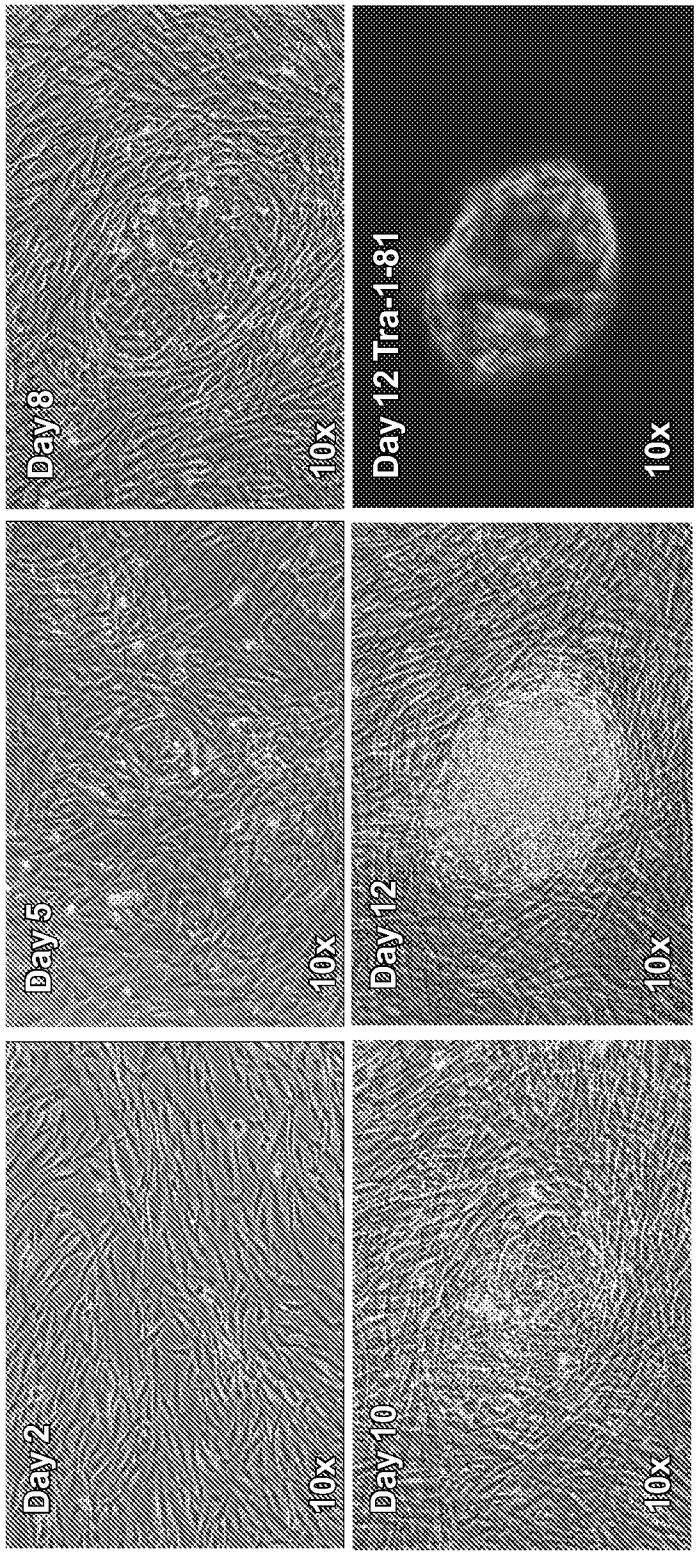

Day 2
10x
Day 5
10x
Day 8
10x
Day 10
10x
Day 12
10x
Day 12 Tra-1-81
10x

FIG. 3

BJ Cell Density	mRNA Dose per Well	# of mRNA Cocktail Transfections	TRA-1-81 (+) Colonies Day 11
25K	1µg	10	>1000
25K	1.5µg	10	850
50K	1µg	10	600
50K	1.5µg	10	>1000

FIG. 4

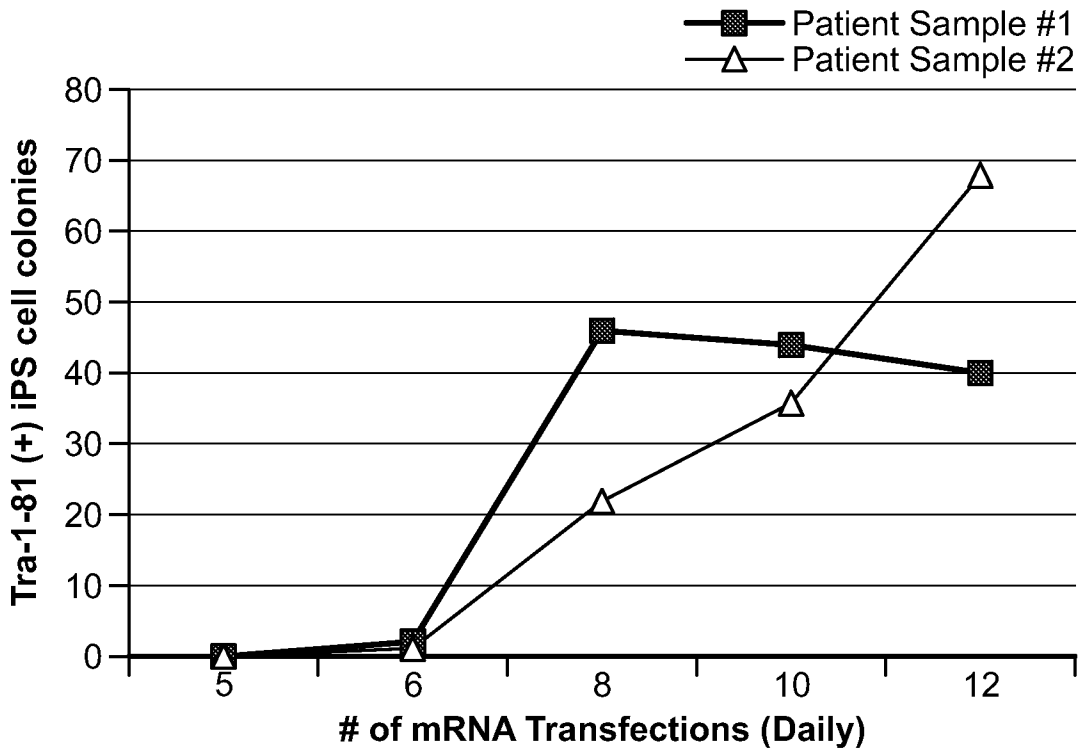


FIG. 5

	O_2 Tension	0.5 µg mRNA Tra-1-81 (+) Count	0.75 µg mRNA Tra-1-81 (+) Count
Patient sample #1	5%	113	170
	20%	3	5
Patient sample #2	5%	68	39
	20%	11	21

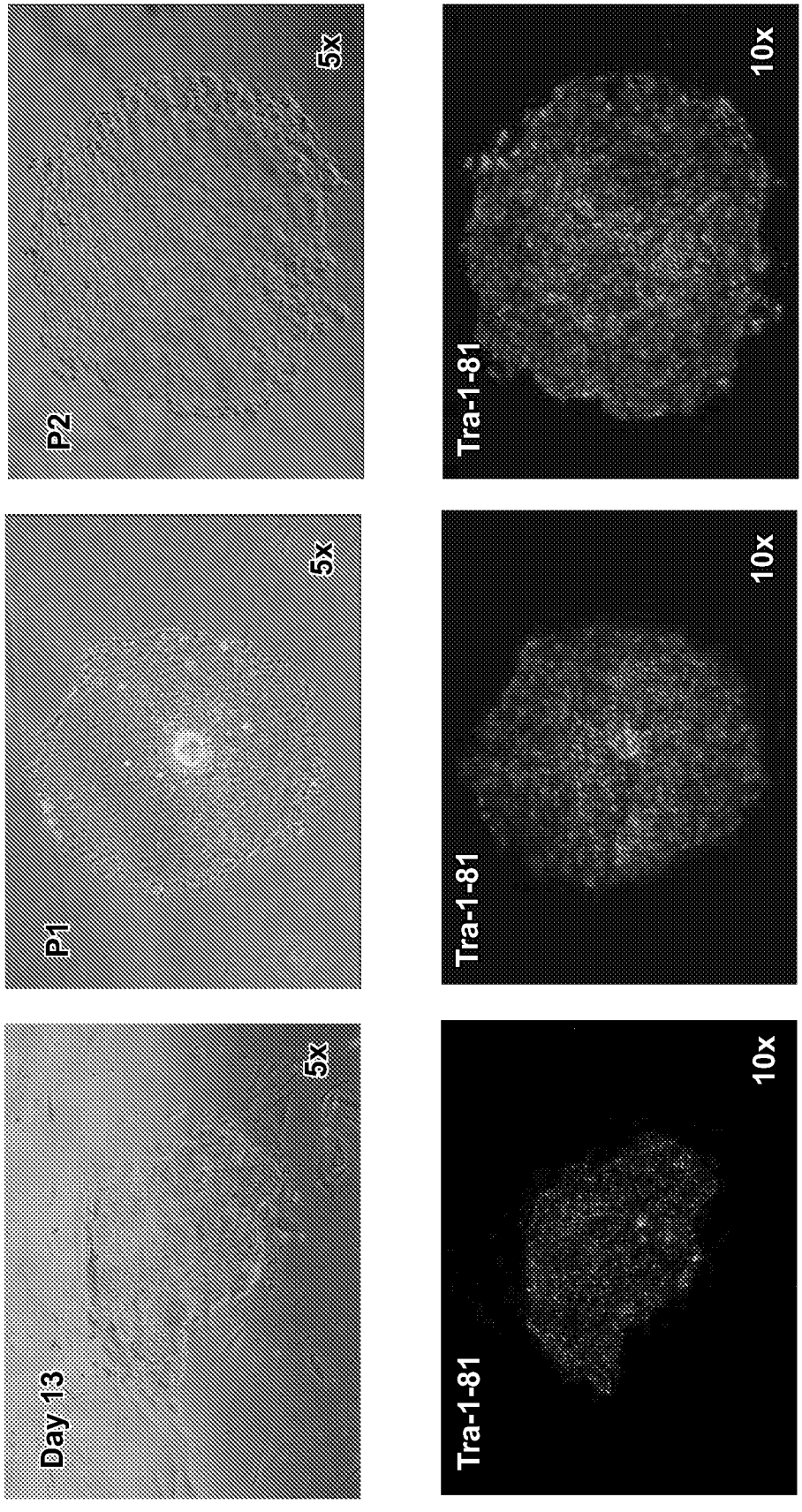


FIG. 6

FIG. 7

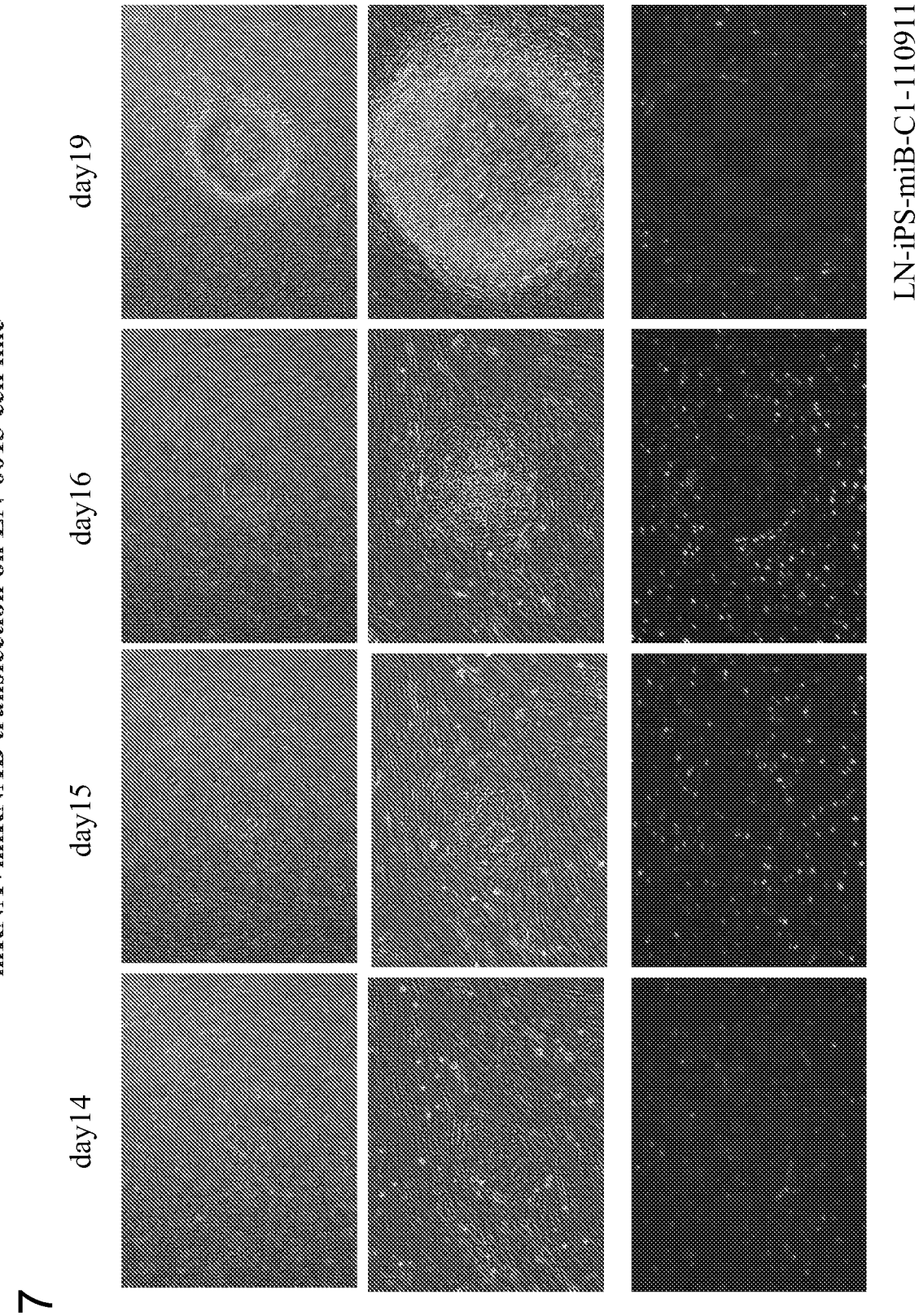

mRNA+miRNAB transfection on LN-0013 cell line
day14
day15
day16
day19
4x
10x
GFP
LN-iPS-miB-C1-110911

FIG. 8A

OCT4

```
   1 gcgtcgacac tagttctaga ccctcattgg accctcgtac agaagctaat acgactcact
  61 atagggaaat aagagagaaa agaagagtaa gaagaaatat aagagccacc atggcgggac
 121 acctggcttc ggatttcgcc ttctcgcccc ctccaggtgg tggaggtgat gggccagggg
 181 ggccggagcc gggctgggtt gatcctcgga cctggctaag cttccaaggc cctcctggag
 241 ggccaggaat cgggccgggg gttgggccag gctctgaggt gtgggggatt cccccatgcc
 301 ccccgccgta tgagttctgt ggggggatgg cgtactgtgg gccccaggtt ggagtggggc
 361 tagtgcccca aggcggcttg gagacctctc agcctgaggg cgaagcagga gtcggggtgg
 421 agagcaactc cgatggggcc tccccggagc cctgcaccgt cacccctggt gccgtgaagc
 481 tggagaagga gaagctggag caaaacccgg aggagtccca ggacatcaaa gctctgcaga
 541 aagaactcga gcaatttgcc aagctcctga agcagaagag gatcaccctg ggatatacac
 601 aggccgatgt ggggctcacc ctgggggttc tatttgggaa ggtattcagc caaacgacca
 661 tctgccgctt tgaggctctg cagcttagct tcaagaacat gtgtaagctg cggcccttgc
 721 tgcagaagtg ggtggaggaa gctgacaaca atgaaaatct tcaggagata tgcaaagcag
 781 aaaccctcgt gcaggcccga aagagaaagc gaaccagtat cgagaaccga gtgagaggca
 841 acctggagaa tttgttcctg cagtgcccga aacccacact gcagcagatc agccacatcg
 901 cccagcagct tgggctcgag aaggatgtgg tccgagtgtg gttctgtaac cggcgccaga
 961 agggcaagcg atcaagcagc gactatgcac aacgagagga ttttgaggct gctgggtctc
1021 ctttctcagg gggaccagtg tcctttcctc tggccccagg gccccatttt ggtaccccag
1081 gctatgggag ccctcacttc actgcactgt actcctcggt ccctttccct gaggggggaag
1141 cctttccccc tgtctccgtc accactctgg gctctcccat gcattcaaac tgagctgcct
1201 tctgcggggc ttgccttctg gccatgccct tcttctctcc cttgcacctg tacctcttgg
1261 tctttgaata aagcctgagt aggaagtgag ggtctagaac tagtgtcgac gc
```

FIG. 8B

```
  1 maghlasdfa fspppggggd gpggpepgwv dprtwlsfqg ppggpgigpg vgpgsevwgi
 61 ppcpppyefc ggmaycgpqv gvglvpqggl etsqpegeag vgvesnsdga spepctvtpg
121 avklekekle qnpeesqdik alqkeleqfa kllkqkritl gytqadvglt lgvlfgkvfs
181 qtticrfeal qlsfknmckl rpllqkwvee adnnenlqei ckaetlvqar krkrtsienr
241 vrgnlenlfl qcpkptlqqi shiaqqlgle kdvvrvwfcn rrqkgkrsss dyaqredfea
301 agspfsggpv sfplapgphf gtpgygsphf talyssvpfp egeafppvsv ttlgspmhsn
```

FIG. 9A

SOX2

```
   1 gcgtcgacac tagttctaga ccctcattgg accctcgtac agaagctaat acgactcact
  61 atagggaaat aagagagaaa agaagagtaa gaagaaatat aagagccacc atgtacaaca
 121 tgatggagac ggagctgaag ccgccgggcc cgcagcaaac ttcgggggc ggcggcggca
 181 actccaccgc ggcggcggcc ggcggcaacc agaaaaacag cccggaccgc gtcaagcggc
 241 ccatgaatgc cttcatggtg tggtcccgcg ggcagcggcg caagatggcc caggagaacc
 301 ccaagatgca caactcggag atcagcaagc gcctgggcgc cgagtggaaa cttttgtcgg
 361 agacggagaa gcggccgttc atcgacgagg ctaagcggct gcgagcgctg cacatgaagg
 421 agcacccgga ttataaatac cggccccggc ggaaaaccaa gacgctcatg aagaaggata
 481 agtacacgct gcccggcggg ctgctggccc ccggcggcaa tagcatggcg agcggggtcg
 541 gggtgggcgc cggcctgggc gcgggcgtga accagcgcat ggacagttac gcgcacatga
 601 acggctggag caacggcagc tacagcatga tgcaggacca gctgggctac ccgcagcacc
 661 cgggcctcaa tgcgcacggc gcagcgcaga tgcagcccat gcaccgctac gacgtgagcg
 721 ccctgcagta caactccatg accagctcgc agacctacat gaacggctcg cccacctaca
 781 gcatgtccta ctcgcagcag ggcacccctg gcatggctct tggctccatg ggttcggtgg
 841 tcaagtccga ggccagctcc agccccctg tggttacctc ttcctcccac tccagggcgc
 901 cctgccaggc cggggacctc cgggacatga tcagcatgta tctccccggc gccgaggtgc
 961 cggaacccgc cgcccccagc agacttcaca tgtcccagca ctaccagagc ggcccggtgc
1021 ccggcacggc cattaacggc acactgcccc tctcacacat gtgagctgcc ttctgcgggg
1081 cttgccttct ggccatgccc ttcttctctc ccttgcacct gtacctcttg gtctttgaat
1141 aaagcctgag taggaagtga gggtctagaa ctagtgtcga cgc
```

FIG. 9B

```
  1 mynmmetelk ppgpqqtsgg gggnstaaaa ggnqknspdr vkrpmnafmv wsrgqrrkma
 61 qenpkmhnse iskrlgaewk llsetekrpf ideakrlral hmkehpdyky rprrktktlm
121 kkdkytlpgg llapggnsma sgvgvgaglg agvnqrmdsy ahmngwsngs ysmmqdqlgy
181 pqhpglnahg aaqmqpmhry dvsalqynsm tssqtymngs ptysmsysqq gtpgmalgsm
241 gsvvkseass sppvvtsssh srapcqagdl rdmismylpg aevpepaaps rlhmsqhyqs
301 gpvpgtaing tlplshm
```

FIG. 10

NANOG

```
1   msvdpacpqs lpcfeasdck esspmpvicg peenypslqm ssaemphtet vsplpssmdl
61  liqdspdsst spkgkqptsa eksvakkedk vpvkkqktrt vfsstqlcvl ndrfqrqkyl
121 slqqmqelsn ilnlsykqvk twfqnqrmks krwqknnwpk nsngvtqkas aptypslyss
181 yhqgclvnpt gnlpmwsnqt wnnstwsnqt qniqswsnhs wntqtwctqs wnnqawnspf
241 yncgeeslqs cmqfqpnspa sdleaaleaa geglnviqqt tryfstpqtm dlflnysmnm
301 qpedv
```

FIG. 11A

LIN28

```
  1 gcgtcgacac tagttctaga ccctcattgg accctcgtac agaagctaat acgactcact
 61 atagggaaat aagagagaaa agaagagtaa gaagaaatat aagagccacc atgggctccg
121 tgtccaacca gcagtttgca ggtggctgcg ccaaggcggc agaagaggcg cccgaggagg
181 cgccggagga cgcggcccgg gcggcggacg agcctcagct gctgcacggt gcgggcatct
241 gtaagtggtt caacgtgcgc atggggttcg gcttcctgtc catgaccgcc cgcgccgggg
301 tcgcgctcga ccccccagtg gatgtctttg tgcaccagag taagctgcac atggaagggt
361 tccggagctt gaaggagggt gaggcagtgg agttcacctt taagaagtca gccaagggtc
421 tggaatccat ccgtgtcacc ggacctggtg gagtattctg tattgggagt gagaggcggc
481 caaaaggaaa gagcatgcag aagcgcagat caaaaggaga caggtgctac aactgtggag
541 gtctagatca tcatgccaag gaatgcaagc tgccacccca gcccaagaag tgccacttct
601 gccagagcat cagccatatg gtagcctcat gtccgctgaa ggcccagcag ggccctagtg
661 cacagggaaa gccaacctac tttcgagagg aagaagaaga aatccacagc cctaccctgc
721 tcccggaggc acagaattga gctgccttct gcggggcttg ccttctggcc atgcccttct
781 tctctccctt gcacctgtac ctcttggtct ttgaataaag cctgagtagg aagtgagggt
841 ctagaactag tgtcgacgc
```

FIG. 11B

```
1   mgsvsnqqfa ggcakaaeea peeapedaar aadepqllhg agickwfnvr mgfgflsmta
61  ragvaldppv dvfvhqsklh megfrslkeg eaveftfkks akglesirvt gpggvfcigs
121 errpkgksmq krrskgdrcy ncggldhhak ecklppqpkk chfcqsishm vascplkaqq
181 gpsaqgkpty freeeeeihs ptllpeaqn
```

FIG. 12A

KLF4

```
   1 gcgtcgacac tagttctaga ccctcattgg accctcgtac agaagctaat acgactcact
  61 atagggaaat aagagagaaa agaagagtaa gaagaaatat aagagccacc atggctgtca
 121 gcgacgcgct gctcccatct ttctccacgt tcgcgtctgg cccggcggga agggagaaga
 181 cactgcgtca agcaggtgcc ccgaataacc gctggcggga ggagctctcc cacatgaagc
 241 gacttccccc agtgcttccc ggccgcccct atgacctggc ggcggcgacc gtggccacag
 301 acctggagag cggcggagcc ggtgcggctt gcggcggtag caacctggcg cccctacctc
 361 ggagagagac cgaggagttc aacgatctcc tggacctgga ctttattctc tccaattcgc
 421 tgacccatcc tccggagtca gtggccgcca ccgtgtcctc gtcagcgtca gcctcctctt
 481 cgtcgtcgcc gtcgagcagc ggccctgcca gcgcgccctc cacctgcagc ttcacctatc
 541 cgatccgggc cgggaacgac ccgggcgtgg cgccgggcgg cacgggcgga ggcctcctct
 601 atggcaggga gtccgctccc cctccgacgg ctcccttcaa cctggcggac atcaacgacg
 661 tgagcccctc gggcggcttc gtggccgagc tcctgcggcc agaattggac ccggtgtaca
 721 ttccgccgca gcagccgcag ccgccaggtg gcgggctgat gggcaagttc gtgctgaagg
 781 cgtcgctgag cgcccctggc agcgagtacg gcagcccgtc ggtcatcagc gtcagcaaag
 841 gcagccctga cggcagccac ccggtggtgg tggcgcccta caacggcggg ccgccgcgca
 901 cgtgccccaa gatcaagcag gaggcggtct cttcgtgcac ccacttgggc gctggacccc
 961 ctctcagcaa tggccaccgg ccggctgcac acgacttccc cctggggcgg cagctcccca
1021 gcaggactac cccgaccctg ggtcttgagg aagtgctgag cagcagggac tgtcaccctg
1081 ccctgccgct tcctcccggc ttccatcccc acccggggcc caattaccca tccttcctgc
1141 ccgatcagat gcagccgcaa gtcccgccgc tccattacca agagctcatg ccacccggtt
1201 cctgcatgcc agaggagccc aagccaaaga ggggaagacg atcgtggccc cggaaaagga
1261 ccgccaccca cacttgtgat tacgcgggct gcggcaaaac ctacacaaag agttcccatc
1321 tcaaggcaca cctgcgaacc cacacaggtg agaaacctta ccactgtgac tgggacggct
1381 gtggatggaa attcgcccgc tcagatgaac tgaccaggca ctaccgtaaa cacacggggc
1441 accgcccgtt ccagtgccaa aaatgcgacc gagcatttc caggtcggac cacctcgcct
1501 tacacatgaa gaggcatttt taagctgcct tctgcggggc ttgccttctg gccatgccct
1561 tcttctctcc cttgcacctg tacctcttgg tctttgaata aagcctgagt aggaagtgag
1621 ggtctagaac tagtgtcgac gc
```

FIG. 12B

```
1   mavsdallps fstfasgpag rektlrqaga pnnrwreels hmkrlppvlp grpydlaaat
61  vatdlesgga gaacggsnla plprreteef ndlldldfil snslthppes vaatvsssas
121 asssspsss gpasapstcs ftypiragnd pgvapggtgg gllygresap pptapfnlad
181 indvspsggf vaellrpeld pvyippqqpq ppggglmgkf vlkaslsapg seygspsvis
241 vskgspdgsh pvvvapyngg pprtcpkikq eavsscthlg agpplsnghr paahdfplgr
301 qlpsrttptl gleevlssrd chpalplppg fhphpgpnyp sflpdqmqpq vpplhyqgqs
361 rgfvaragep cvcwphfgth gmmltppssp lelmppgscm peepkpkrgr rswprkrtat
421 htcdyagcgk tytksshlka hlrthtgekp yhcdwdgcgw kfarsdeltr hyrkhtghrp
481 fqcqkcdraf srsdhlalhm krhf
```

FIG. 13A-1

c-Mye

```
   1 gacccccgag ctgtgctgct cgcggccgcc accgccgggc cccggccgtc cctggctccc
  61 ctcctgcctc gagaagggca gggcttctca gaggcttggc gggaaaaaga acggagggag
 121 ggatcgcgct gagtataaaa gccggttttc ggggctttat ctaactcgct gtagtaattc
 181 cagcgagagg cagagggagc gagcgggcgg ccggctaggg tggaagagcc gggcgagcag
 241 agctgcgctg cgggcgtcct gggaagggag atccggagcg aataggggggc ttcgcctctg
 301 gcccagccct cccgctgatc ccccagccag cggtccgcaa cccttgccgc atccacgaaa
 361 ctttgcccat agcagcgggc gggcactttg cactggaact tacaacaccc gagcaaggac
 421 gcgactctcc cgacgcgggg aggctattct gcccatttgg ggacacttcc ccgccgctgc
 481 caggacccgc ttctctgaaa ggctctcctt gcagctgctt agacgctgga ttttttcgg
 541 gtagtggaaa accagcagcc tcccgcgacg atgcccctca acgttagctt caccaacagg
 601 aactatgacc tcgactacga ctcggtgcag ccgtatttct actgcgacga ggaggagaac
 661 ttctaccagc agcagcagca gagcgagctg cagcccccgg cgcccagcga ggatatctgg
 721 aagaaattcg agctgctgcc caccccgccc ctgtccccta gccgccgctc cgggctctgc
 781 tcgccctcct acgttgcggt cacacccttc tcccttcggg gagacaacga cggcggtggc
 841 gggagcttct ccacggccga ccagctggag atggtgaccg agctgctggg aggagacatg
 901 gtgaaccaga gtttcatctg cgacccggac gacgagacct tcatcaaaaa catcatcatc
 961 caggactgta tgtggagcgg cttctcggcc gccgccaagc tcgtctcaga gaagctggcc
1021 tcctaccagg ctgcgcgcaa agacagcggc agcccgaacc ccgcccgcgg ccacagcgtc
1081 tgctccacct ccagcttgta cctgcaggat ctgagcgccg ccgcctcaga gtgcatcgac
1141 ccctcggtgg tcttccccta ccctctcaac gacagcagct cgcccaagtc ctgcgcctcg
1201 caagactcca gcgccttctc tccgtcctcg gattctctgc tctcctcgac ggagtcctcc
1261 ccgcagggca gccccgagcc cctggtgctc catgaggaga caccgcccac caccagcagc
1321 gactctgagg aggaacaaga agatgaggaa gaaatcgatg ttgtttctgt ggaaaagagg
```

FIG. 13A-2

1381 caggctcctg gcaaaaggtc agagtctgga tcaccttctg ctggaggcca cagcaaacct
1441 cctcacagcc cactggtcct caagaggtgc cacgtctcca cacatcagca caactacgca
1501 gcgcctccct ccactcggaa ggactatcct gctgccaaga gggtcaagtt ggacagtgtc
1561 agagtcctga gacagatcag caacaaccga aaatgcacca gccccaggtc ctcggacacc
1621 gaggagaatg tcaagaggcg aacacacaac gtcttggagc gccagaggag gaacgagcta
1681 aaacggagct tttttgccct gcgtgaccag atcccggagt tggaaaacaa tgaaaaggcc
1741 cccaaggtag ttatccttaa aaaagccaca gcatacatcc tgtccgtcca agcagaggag
1801 caaaagctca tttctgaaga ggacttgttg cggaaacgac gagaacagtt gaaacacaaa
1861 cttgaacagc tacggaactc ttgtgcgtaa ggaaaagtaa ggaaaacgat tccttctaac
1921 agaaatgtcc tgagcaatca cctatgaact tgtttcaaat gcatgatcaa atgcaacctc
1981 acaaccttgg ctgagtcttg agactgaaag atttagccat aatgtaaact gcctcaaatt
2041 ggactttggg cataaaagaa ctttttatg cttaccatct ttttttttc tttaacagat
2101 ttgtatttaa gaattgtttt taaaaaattt taagatttac acaatgtttc tctgtaaata
2161 ttgccattaa atgtaaataa ctttaataaa acgtttatag cagttacaca gaatttcaat
2221 cctagtatat agtacctagt attataggta ctataaaccc taatttttt tatttaagta
2281 cattttgctt tttaaagttg atttttttct attgttttta gaaaaaataa aataactggc
2341 aaatatatca ttgagccaaa tcttaaaaaa aaaaaaaaa

FIG. 13B

```
   1 MDFFRVVENQ QPPATMPLNV SFTNRNYDLD YDSVQPYFYC DEEENFYQQQ QQSELQPPAP
  61 SEDIWKKFEL LPTPPLSPSR RSGLCSPSYV AVTPFSLRGD NDGGGGSFST ADQLEMVTEL
 121 LGGDMVNQSF ICDPDDETFI KNIIIQDCMW SGFSAAAKLV SEKLASYQAA RKDSGSPNPA
 181 RGHSVCSTSS LYLQDLSAAA SECIDPSVVF PYPLNDSSSP KSCASQDSSA FSPSSDSLLS
 241 STESSPQGSP EPLVLHEETP PTTSSDSEEE QEDEEEIDVV SVEKRQAPOK RSESGSPSAG
 301 GHSKPPHSPL VLKRCHVSTH QHNYAAPPST RKDYPAAKRV KLDSVRVLRQ ISNNRKCTSP
 361 RSSDTEENVK RRTHNVLERQ RRNELKRSFF ALR1JQ1PELE NNEKAPKVVI LKKATAYILS
 421 VQAEEQKLIS EEDLLRKRRE QLKHKLEQLR NSCA
```

FIG. 14

GFP

```
1    gcgtcgacac tagttctaga ccctcattgg accctcgtac agaagctaat acgactcact
61   atagggaaat aagagagaaa agaagagtaa gaagaaatat aagagccacc atggtgagca
121  agggcgagga gctgttcacc ggggtggtgc ccatcctggt cgagctggac ggcgacgtaa
181  acggccacaa gttcagcgtg tccggcgagg gcgagggcga tgccacctac ggcaagctga
241  ccctgaagtt catctgcacc accggcaagc tgcccgtgcc ctggcccacc ctcgtgacca
301  ccctgaccta cggcgtgcag tgcttcagcc gctaccccga ccacatgaag cagcacgact
361  tcttcaagtc cgccatgccc gaaggctacg tccaggagcg caccatcttc ttcaaggacg
421  acggcaacta caagacccgc gccgaggtga agttcgaggg cgacaccctg gtgaaccgca
481  tcgagctgaa gggcatcgac ttcaaggagg acggcaacat cctggggcac aagctggagt
541  acaactacaa cagccacaac gtctatatca tggccgacaa gcagaagaac ggcatcaagg
601  tgaacttcaa gatccgccac aacatcgagg acggcagcgt gcagctcgcc gaccactacc
661  agcagaacac ccccatcggc gacggccccg tgctgctgcc cgacaaccac tacctgagca
721  cccagtccgc cctgagcaaa gaccccaacg agaagcgcga tcacatggtc ctgctggagt
781  tcgtgaccgc cgccgggatc actctcggca tggacgagct gtacaagaga tctcgagctg
841  atccaaaaaa gaagagaaag gtagatccaa aaaagaagag aaaggtagat ccaaaaaaga
901  agagaaaggt aggatccacc ggatctagat aagctgcctt ctgcggggct tgccttctgg
961  ccatgcccct cttctctccc ttgcacctgt acctcttggt ctttgaataa agcctgagta
1021 ggaagtgagg gtctagaact agtgtcgacg c
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/45686

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07H 21/02, C12N 15/09, C12N 15/113, C12N 15/63, C12N 5/00, C12N 5/0789 (2013.01)
USPC - 435/377, 435/455
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C07H 21/02, C12N 15/09, C12N 15/113, C12N 15/63, C12N 5/00, C12N 5/0789, C12N 5/10 (2013.01)
USPC - 435/377, 435/455

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/375, 435/325, 536/24.5
(keyword limited; terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Scholar, ProQuest Dialog
Search terms: iPS, induced pluripotent stem cell, miRNA, microRNA, mRNA, introduce, transfect, kit, immune, suppress

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2012/0134966 A1 (BLELLOCH et al.) 31 May 2012 (31.05.2012) para [0018], [0026], [0052]-]0054], [0077]	1-3, 28 --- 36-38
Y	US 2011/0143397 A1 (KARIKO et al.) 16 June 2011 (16.06.2011) abstract, para [0029], [0043], [0045]	36-38
A	US 2011/0189137 A1 (RANA) 4 August 2011 (04.08.2011) Entire document	1-3, 28, 36-38

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 October 2013 (28.10.2013)	08 NOV 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/45686

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-27, 29-35, and 39-45
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.