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

(19) World Intellectual Property
Organization
International Bureau

(43) International Publication Date
28 June 2012 (28.06.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/087965 A3

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(54) Title: CELL CULTURE PLATFORM FOR SINGLE CELL SORTING AND ENHANCED REPROGRAMMING OF IPSCS
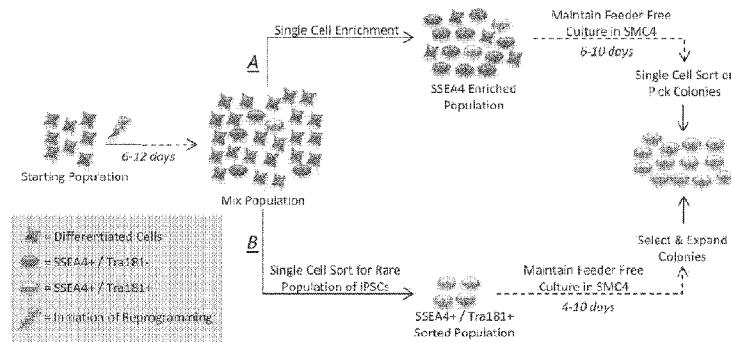
FIG. 9

(57) **Abstract**: The invention provides cell culture conditions for culturing stem cells, including feeder-free conditions for generating and culturing human induced pluripotent stem cells (iPSCs). More particularly, the invention provides a culture platform that allows long-term culture of pluripotent cells in a feeder-free environment; reprogramming of cells in a feeder-free environment; single-cell dissociation of pluripotent cells; cell sorting of pluripotent cells; maintenance of an undifferentiated status; improved efficiency of reprogramming; and generation of a naïve pluripotent cell.

CELL CULTURE PLATFORM FOR SINGLE CELL SORTING AND ENHANCED REPROGRAMMING OF IPSCS

STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is FATE_094_00WO_ST25.txt. The text file is 4 KB, was created on December 15, 2011, and is being submitted electronically via EFS-Web.

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 61/426,369, filed December 22, 2010, and U.S. Provisional Application No. 61/496,991, filed June 14, 2011, each of which is incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Technical Field

The present invention relates generally to cell culture conditions, media, and culture platforms for culturing stem cells, including feeder-free conditions for generating and culturing human induced pluripotent stem cells (iPSCs).

Description of Related Art

The application of pluripotent stem cell biology opens new doors for regenerative medicine. The derivation of human embryonic stem cells (hESC) by culturing pre-implantation blastocysts in cocktails of growth factors has led to many promising cell therapy approaches where the expanded self renewing population of cells can be differentiated to the therapy-relevant cell lineage *in vitro* or *in vivo*. In a further application of ESC biology and by using pre-implantation genetic analysis it has been possible to derive ESC lines from several genetic disease backgrounds, and thus, model these diseases in the tissue culture dish. There are, however, some limitations to ESC technology: the range of genetic backgrounds from which

ESC can be derived are both technically and politically limited, the genetic background of the ESCs are not always known and the use of ESC-derived cell therapy is essentially an allograft, running the same rejection risks as traditional tissue/organ transplants.

In a major advance, pluripotent cell populations were generated from adult, terminally differentiated cells: such derived cells are called induced pluripotent stem cells (iPSC). iPSC technology allows cells from any donor to be reprogrammed into a pluripotent, self renewing state and thus allow the expansion of a homogeneous population of cells from any genetic background. iPSCs overcome ethical considerations pertaining to ESCs and can be used to derive models of any genetic human disease for high throughput drug screening or hepatocytes and cardiomyocytes for xenobiotic drug toxicity screening. Further, iPSCs may ultimately result in cell therapies generated from the patient's own cells in an autologous transplantation that may prevent graft rejection. Expression and differentiation analysis has shown iPSCs to be very close to ESCs at the molecular level with variations between clonal iPSC cultures of similar magnitude to those seen when comparing multiple ESC lines.

iPSCs have generally been generated by ectopic expression of several key genes shown to be required for full reprogramming, namely combinations of: Oct4, Sox2, Klf4, c-Myc, Lin28 and Nanog. iPSCs were originally generated using integrating viral systems to express key transcription factors. Retroviral and lentiviral systems including polycistronic and inducible systems have now been successfully employed in iPSC generation. However, permanent genomic changes due to insertional mutagenesis and the potential for exogenous gene reactivation post iPSC differentiation may present potential problems for subsequent drug screening and therapeutic applications of cells generated by these methods. Indeed, significant differences between iPSC clones generated using the same viral systems have been reported, with a large percentage of clones forming tumors in rodents when transplanted as differentiated neurospheres. Research suggests that iPSCs generated using the same viral methods may behave differently once differentiated. Differences in ectopic gene integration site may result in different insertional mutagenesis and epigenetic regulation of transgene expression. For iPSC generation methods that include integrating systems, many clones may need to be derived and screened to identify those that are stable in both pluripotent and differentiated states. Thus, a

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method for the rapid derivation of clonal iPSCs from a given donor cell source would be beneficial. The use of non-integrating systems for iPSC generation such as adenoviral or episomal transient expression have also been demonstrated, albeit with lower efficiency. These systems may overcome safety and stability issues in iPSC generation, however there is a

5   potential for genomic integration when using any DNA-based reprogramming method and this would need to be assessed prior to their use in development of an iPSC-derived cell therapy.

Excisable viral systems and genome wide expression profiling show that iPSCs with integrated expression cassettes are less like ESCs than the same clones with the viral factors excised. Further, protein-only reprogramming has now been demonstrated in which the most

0   commonly used transcription factors were expressed in *E. coli* as fusion proteins with cell penetrating peptides. Multiple doses of the purified proteins were applied to murine fibroblasts resulting in iPSC generation. The efficiency of reprogramming using this protein-only system was very low. This may be due to the efficiency of the protein transduction, the specific activity of the protein and/or the stability of the proteins.

5   The process of differentiated cell reprogramming by the ectopic expression of pluripotency genes or their introduction via protein transduction or mRNA requires several months and the knowledge of a skilled stem-cell biologist. The identification of reprogrammed cells is initially by eye: screening for of ESC-like colony morphology. Such colonies must be picked by hand, are usually mechanically passaged and expanded. The introduction of the

20   pluripotency factors also produces transformed cell colonies as well as incompletely reprogrammed cells. A researcher may be able to identify the true iPSC colonies from the background of transformed cells, but this is not an efficient process. Further characterization and recognition as a true pluripotent population is then required and usually includes immunocytochemistry staining for markers of pluripotentcy, gene expression and epigenetic

25   analysis and the ability of the pluripotent population to differentiate to the three germ layers (ectoderm, mesoderm and endoderm). Once pluripotent cells are identified and selected, such cells are generally grown as colonies and require manual passaging by picking and mechanically dissociating cells prior to replating to maintain cells long-term.

Embryonic stem cells derived from various pre- and post-implantation stages display distinct states of pluripotency. For example, cells derived from the inner cell mass of a blastocyst are considered more "naïve" and have key properties that are quite different from the postimplantation derived cells that are considered more "primed" with higher propensity to randomly differentiate. Naïve cells appear to be in a more "grounded state" and do not require extrinsic signaling to maintain their undifferentiated status. On the other hand, primed cells require extrinsic signaling of key cytokines including TGFβ, Activin and bFGF and are quite dependent on the ERK/MAPK cellular pathway for maintaining their undifferentiated status.

Improvements to the iPSC generation process could dramatically lower the technical barriers, speed-up the process and enable subsequent scale-up and differentiation of cells for industrial applications of the technology such as drug screening and cell therapy. Methods for more efficient production of iPSCs without the use of exogenous material, and more efficient identification and selection of reprogrammed cells are required. Methods of generating iPSCs that promote the naïve state of human pluripotent stem cells would be greatly advantageous for future applications in regenerative medicine, such as disease correction, directed differentiation and manufacturing-scale expansion. Further, methods for more efficient production of iPSCs in defined culture conditions that enable single cell passage and scalability are required.

BRIEF SUMMARY OF THE INVENTION

One embodiment of the invention provides a method of culturing a pluripotent cell in a feeder-free environment comprising: culturing a pluripotent cell that is not a murine embryonic stem cell in a feeder-free environment in a culture medium comprising at least one agent that maintains pluripotency of the cell, wherein the agent is selected from the group consisting of: i) a TGFβ inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, and iv) a Rock inhibitor, while maintaining pluripotency of the cell during culturing.

In a particular embodiment the culture medium comprises a sufficient amount of the agent to allow for at least one cell division while maintaining pluripotency of the cell. In an additional embodiment, the culture medium comprises at least two agents that maintain

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pluripotency of the cell. In a particular embodiment, the culture medium comprises at least three agents or four agents that maintain pluripotency of the cell.

In another particular embodiment, the agent that maintains pluripotency of the cell comprises a Rock inhibitor. In a particular embodiment, the Rock inhibitor is thiazovivin or Y27632.

In one embodiment, the agent that maintains pluripotency of the cell comprises a TGFβ inhibitor, and in a particular embodiment the TGFβ inhibitor is A-83-01 or SB431542.

In a certain embodiment, the agent that maintains pluripotency of the cell comprises a GSK3 inhibitor, and in a particular embodiment the GSK3 inhibitor is CHIR99021 or BIO.

In one embodiment of the invention, the agent that maintains pluripotency of the cell comprises a MEK inhibitor. In a particular embodiment, the MEK inhibitor is PD98059 or PD032901.

In a particular embodiment, the culture medium comprises a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor. In a more particular embodiment of the invention, the TGFβ inhibitor is SB431542, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin.

In some embodiments of the invention, the pluripotency of the cell is maintained for at least five cell divisions. In other embodiments, pluripotency of the cell is maintained for at least ten cell divisions.

In some embodiments, the cells are cultured in the absence of growth factors and cytokines, optionally in the presence of soluble fibronectin. In a further embodiment, the cells are cultured in the absence of Matrigel™, and in yet another embodiment the culture medium is substantially free of bFGF.

In particular embodiments of the invention, the pluripotent cells are human embryonic stem cells or human induced pluripotent stem cells.

Another embodiment of the invention provides a method of culturing a pluripotent cell comprising culturing a pluripotent cell that is not a murine embryonic stem cell in the absence of growth factors and cytokines.

In certain embodiments, the method comprises culturing the pluripotent cell in a culture medium comprising at least one agent that maintains pluripotency of the cell to allow for at least one cell division while maintaining pluripotency of the cell, wherein the agent is selected from the group consisting of i) a TGFβ inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, and iv) a Rock inhibitor.

In one embodiment, the pluripotent cell is a human embryonic stem cell or a human induced pluripotent stem cell.

In further embodiments, the culture medium comprises at least two, at least three, or four agents selected from the group consisting of i) a TGFβ inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, and iv) a Rock inhibitor; in particular embodiments, the culture medium comprises a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor; in other particular embodiments the TGFβ inhibitor is SB431542, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin.

Yet another embodiment of the invention provides a method of obtaining dissociated human pluripotent cells comprising dissociating human pluripotent cells to obtain dissociated cells and contacting the dissociated cells with a culture medium comprising at least one agent that enhances viability of the dissociated cells, wherein the agent is selected from the group consisting of i) a TGFβ inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, and iv) a Rock inhibitor, whereby viability of the dissociated cells is enhanced. In particular embodiments, viability of the dissociated cells is enhanced by at least 10%, at least 50%, at least 100%, at least 200%, or at least 500%.

In other embodiments, the method of the invention further comprises culturing the dissociated cells in the culture medium for at least one, at least two, at least five, or at least ten passages while maintaining pluripotency of the dissociated cells.

In certain embodiments, the karyotype of the dissociated cells after culturing is substantially similar to the karyotype of the population of cells prior to dissociation.

In some embodiments, the method comprises dissociating in the presence of the agent. In yet other embodiments, the method comprises contacting the pluripotent cells with the

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agent prior to dissociating. In particular embodiments, contacting the dissociated cells with the culture medium comprises suspending the dissociated cells in the culture medium.

In other embodiments, the invention provides a method of increasing the potency of a cell in a feeder-free environment comprising contacting a cell in a feeder-free environment with a culture medium comprising at least one small molecule agent to obtain a cell having increased potency as compared to the cell prior to contacting with the culture medium, wherein the small molecule agent is selected from the group consisting of i) a TGFβ inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, and iv) a Rock inhibitor. In particular embodiments, the culture medium comprises a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor; in certain embodiments the TGFβ inhibitor is SB431542, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin.

In one embodiment, contacting comprises culturing the cell under conditions sufficient to increase the potency of the cell.

In some embodiments, the cell is selected from the group consisting of an embryonic stem cell; a pluripotent cell; a multipotent cell; a non-pluripotent cell; and a somatic cell.

In particular embodiments, the cell is not a murine embryonic stem cell. In other particular embodiments, the cell is a human cell, and in certain embodiments the cell is an induced pluripotent stem cell.

In some embodiments, the method further comprises contacting the cell with at least one pluripotency factor. In some embodiments, the pluripotency factor comprises a polynucleotide, polypeptide, or small molecule. In some embodiments, the pluripotency factor is an exogenous transcription factor. In particular embodiments, the exogenous transcription factor comprises an Oct4, Sox, Klf, Myc, Lin28, or Nanog polypeptide, or a polynucleotide encoding Oct4, Sox, Klf, Myc, Lin28, or Nanog. In other embodiments, the polypeptide comprises an amino acid sequence that allows transport across cell membranes. In other particular embodiments, the exogenous transcription factor comprises an Oct4, a Sox2, and a Klf4 polynucleotide.

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In yet other embodiments, the cell having increased potency is characterized by one or more of the following: expression of at least one pluripotent stem cell marker selected from the group consisting of Oct4, Nanog, KLF4, SSEA4 and TRA 1-81; pluripotent stem cell morphology; ability to contribute to germline transmission; teratoma formation, ability to differentiate or transdifferentiate into a lineage different from the starting lineage, and in vitro trilineage differentiation. In some embodiments, the cell having increased potency expresses at least a two-fold higher level of Oct4 as compared to the cell prior to contacting with the culture medium. In yet other embodiments, the cell having increased potency has Xist activity that is at least two fold lower compared to conventionally cultured iPSCs. In further embodiments, the cell having increased potency has a compact, domed colony morphology.

In some embodiments, the cell having increased potency replicates and maintains pluripotency in the absence of exogenous stimulation of the TGFβ, activin, and MEK signaling pathways, and optionally in the absence of exogenous stimulation of the bFGF pathway.

In other embodiments, the method further comprises culturing the cell having increased potency in a feeder-free environment in the presence of at least one of i) a TGFβ inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, or iv) a Rock inhibitor to allow for at least one cell division while maintaining the potency of the cell. In particular embodiments, cells are cultured in the presence of a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor; in certain embodiments the TGFβ inhibitor is SB431542, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin.

The invention also provides a cell having increased potency made by any of the above embodiments.

The invention further provides a method of improving the efficiency of reprogramming of a population of cells in a feeder-free environment comprising contacting a population of cells in a feeder-free environment with at least one small molecule agent selected from the group consisting of i) a TGFβ inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, and iv) a Rock inhibitor, under conditions sufficient to induce reprogramming, whereby the efficiency of reprogramming is improved by at least 10%, at least 50%, at least 100%, at least

300%, or at least 500% as compared to the efficiency of reprogramming without contacting the population of cells with the small molecule agent.

In particular embodiments, the cells are contacted with a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor; in certain embodiments the TGFβ inhibitor is SB431542, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin.

In some embodiments, the population of cells prior to reprogramming comprises non-pluripotent cells. In other embodiments, the efficiency of reprogramming is measured by time required for reprogramming or number of cells reprogrammed.

In yet other embodiments, the conditions sufficient to induce reprogramming comprise contacting the population of cells with at least one exogenous transcription factor selected from the group consisting of an Oct4, Sox, Klf, Myc, Lin28, or Nanog polypeptide, or a polynucleotide encoding Oct4, Sox, Klf, Myc, Lin28, or Nanog. In particular embodiments, the conditions comprise contacting the population of cells with an Oct4, Sox2, and Klf4 polypeptide or a polynucleotide encoding Oct4, Sox2, and Klf4.

Yet another embodiment of the invention provides a method of sorting a population of cells in a feeder-free environment to obtain a population of cells enriched for pluripotent cells comprising obtaining a suspension of dissociated cells comprising a mixed population of cells in a feeder-free environment and sorting the cells in the suspension to obtain cells expressing one or more markers of pluripotency, thereby obtaining an enriched population of cells enriched for pluripotent cells. In some embodiments, the suspension comprises at least one of i) a TGFβ inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, or iv) a Rock inhibitor. In further embodiments, the sorting is by magnetic beads or flow cytometry. In particular embodiments, the sorting is by magnetic beads. In other particular embodiments, the sorting is by flow cytometry.

In some embodiments, the method further comprises culturing the enriched population of cells in a culture medium comprising at least one of i) a TGFβ inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, or iv) a Rock inhibitor, optionally in combination with soluble fibronectin.

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In particular embodiments, the mixed population of cells in the suspension is contacted before sorting with at least one of i) a TGFβ inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, or iv) a Rock inhibitor. In certain embodiments, the mixed population of cells is contacted with a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor; in

5   other certain embodiments the TGFβ inhibitor is SB431542, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin.

In particular embodiments, the mixed population of cells comprises cells expressing one or more markers of pluripotency. In particular embodiments, the one or more markers of pluripotency comprises SSEA4, TRA160, TRA181, TRA1-85, TRA2-54, GCTM-2,

0   TG343, TG30, CD9, CD29, CD30, CD50, CD133/prominin, CD140a, CD56, CD73, CD105, CD31, CD34, OCT4, Nanog or Sox2. In specific embodiments, the marker of pluripotency is selected from the group consisting of SSEA4, TRA181, TRA160, and CD30

In particular embodiments, the method comprises contacting the mixed population of cells with one or more pluripotency factors to induce reprogramming. In some embodiments,

5   contacting comprises introducing one or more pluripotency factors into the cells in the mixed population of cells. In certain embodiments, the pluripotency factor comprises an Oct4, Sox, Klf, Myc, Lin28, or Nanog polypeptide, or a polynucleotide encoding Oct4, Sox, Klf, Myc, Lin28, or Nanog. In other particular embodiments, the pluripotency factor comprises an Oct4, a Sox2, and a Klf4 polypeptide, or polynucleotides encoding an Oct4, a Sox2, and a Klf4

20   polypeptide.

In certain particular embodiments, the pluripotent cells are induced pluripotent cells.

In some embodiments, the enriched population of cells is enriched by at least 20%, at least 50%, at least 100%, at least 200%, or at least 500% with respect to cells expressing

25   one or more markers of pluripotency.

In another embodiment the invention provides a method of obtaining induced pluripotent stem cells comprising: treating a population of cells to induce reprogramming; preparing a suspension of dissociated cells comprising the population of cells; sorting the cells in the suspension to obtain sorted cells expressing one or more markers of pluripotency; culturing

the sorted cells expressing one or more markers of pluripotency, wherein iPSCs are obtained. In particular embodiments, the sorted cells are cultured in the absence of cytokines and growth factors, optionally in a feeder-free environment, and optionally in the presence of soluble fibronectin.

In some embodiments, the population of cells is contacted with at least one of i) a TGFβ inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, or iv) a Rock inhibitor. In particular embodiments, the population of cells is contacted with a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor; in certain embodiments the TGFβ inhibitor is SB431542, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin.

In other embodiments, treating the population of cells to induce reprogramming comprises contacting the population of cells with one or more pluripotency factors. In particular embodiments, the pluripotency factor comprises an Oct4, Sox, Klf, Myc, Lin28, or Nanog polypeptide, or a polynucleotide encoding Oct4, Sox, Klf, Myc, Lin28, or Nanog. In certain embodiments, the pluripotency factor comprises an Oct4, a Sox2, and a Klf4 polypeptide, or polynucleotides encoding an Oct4, a Sox2, and a Klf4 polypeptide.

In other embodiments, treating the population of cells to induce reprogramming further comprises contacting the population of cells with at least one of i) a TGFβ inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, or iv) a Rock inhibitor. In particular embodiments, the cells are contacted with a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor; in certain embodiments the TGFβ inhibitor is SB431542, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin.

In other embodiments, the suspension comprises at least one of i) a TGFβ inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, or iv) a Rock inhibitor. In particular embodiments, the suspension comprises a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor; in certain embodiments the TGFβ inhibitor is SB431542, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin.

In some embodiments, sorting is by flow cytometry or magnetic beads. In particular embodiments, cells are sorted to obtain cells expressing at least one, two, three, four,

or more marker of pluripotency. In other particular embodiments, the one or more markers of pluripotency comprises SSEA4, TRA160, TRA181, TRA1-85, TRA2-54, GCTM-2, TG343, TG30, CD9, CD29, CD30, CD50, CD133/prominin, CD140a, CD56, CD73, CD105, CD31, CD34, OCT4, Nanog or Sox2. In other particular embodiments, the one or more markers of pluripotency is selected from the group consisting of SSEA4, TRA160, TRA181, and CD30. In some embodiments, the one or more markers of pluripotency are SSEA4, CD30, and TRA160 or TRA181. In another embodiment cells are sorted using specific markers to deplete non-reprogrammed cells from a reprogramming population.

In some embodiments, culturing comprises culturing the cells in a culture medium comprising at least one small molecule agent selected from the group consisting of i) a TGFβ inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, and iv) a Rock inhibitor. In particular embodiments, the culture medium comprises a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor; in certain embodiments the TGFβ inhibitor is SB431542, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin.

In particular embodiments, the cells are cultured in a feeder-free environment. In certain embodiments, the cells are treated, suspended, sorted, and cultured in a feeder-free environment.

In some embodiments, induced pluripotent stem cells are obtained in about 2 to 22 days. In particular embodiments, induced pluripotent stem cells are obtained in about 4 to about 18 days.

In other embodiments, induced pluripotent stem cells are obtained within about 4 to about 22 days after treating the population of cells to induce reprogramming. In certain embodiments, induced pluripotent stem cells are obtained within about 6 to about 18 days after treating the population of cells to induce reprogramming, and in other certain embodiments, induced pluripotent stem cells are obtained within about 10 to about 16 days after treating the population of cells to induce reprogramming.

The invention also provides in another embodiment an induced pluripotent stem cell obtained by any of the above methods.

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The invention further provides a method of depleting pluripotent cells from a population of cells comprising: obtaining a suspension of dissociated cells comprising a mixed population of cells having pluripotent cells, and sorting the cells in the suspension to remove cells expressing one or more markers of pluripotency, thereby depleting pluripotent cells from a population of cells.

In some embodiments, the mixed population of cells comprises multipotent cells or (adult) somatic cells.

In other embodiments, the mixed population of cells in the suspension is cultured prior to obtaining the suspension in a culture medium comprising at least one small molecule agent selected from the group consisting of i) a TGFβ inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, and iv) a Rock inhibitor. In particular embodiments, the culture medium comprises a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor; in certain embodiments the TGFβ inhibitor is SB431542, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin.

In further embodiments of the invention, the suspension comprises at least one small molecule agent selected from the group consisting of i) a TGFβ inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, and iv) a Rock inhibitor. In particular embodiments, the suspension comprises a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor; in certain embodiments the TGFβ inhibitor is SB431542, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin.

In some embodiments, sorting is by flow cytometry. In other embodiments sorting is by antibody-coated magnetic bead enrichment.

Some embodiments of the invention provide a method of obtaining a pluripotent cell having genomic stability comprising contacting a cell in a feeder-free environment with at least one small molecule agent selected from the group consisting of i) a TGFβ inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, and iv) a Rock inhibitor; in the absence of c-myc under conditions sufficient to obtain a pluripotent cell having genomic stability.

In some embodiments the cell is an embryonic stem cell, a pluripotent cell, a multipotent cell; a non-pluripotent cell; and a somatic cell. In some specific embodiments, the

cell comprises a non-pluripotent cell.

In certain embodiments, the conditions comprise contacting the cell with at least one pluripotency factor. In some embodiments, the pluripotency factor is an exogenous transcription factor selected from the group consisting of an Oct4, Sox, Klf, Myc, Lin28, or

5   Nanog polypeptide, or a polynucleotide encoding Oct4, Sox, Klf, Myc, Lin28, or Nanog. In particular embodiments, the pluripotency factor comprises an Oct4, a Sox2, and a Klf4 polynucleotide.

In some embodiments of the invention, the small molecule agent comprises a Rock inhibitor. In particular embodiments, the Rock inhibitor is thiazovivin or Y27632, and in

0   more particular embodiments the Rock inhibitor is thiazovivin.

In other embodiments of the invention, the small molecule agent comprises a TGFβ inhibitor. In some embodiments, the TGFβ inhibitor is A-83-01 or SB431542.

In some embodiments, the small molecule agent comprises a GSK3 inhibitor, and in particular embodiments the GSK3 inhibitor is CHIR99021 or BIO.

5   In other embodiments of the invention, the small molecule agent comprises a MEK inhibitor. In some particular embodiments, the MEK inhibitor is PD98059 or PD032901.

In some embodiments of the invention, the small molecule agent comprises a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor. In particular embodiments, the TGFβ inhibitor is SB431542, the GSK3 inhibitor is CHIR99021, the MEK

20   inhibitor is PD0325901, and the Rock inhibitor is thiazovivin.

The invention additionally comprises a method of culturing a pluripotent cell to maintain genomic stability of the cell comprising culturing a pluripotent cell in a feeder-free environment in a culture medium comprising at least one agent that maintains genomic stability of the pluripotent cell, wherein the agent is selected from the group consisting of: i) a TGFβ

25   inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, and iv) a Rock inhibitor, while maintaining genomic stability of the pluripotent cell during culturing.

In certain embodiments, the conditions comprise contacting the cell with at least one pluripotency factor. In some embodiments, the pluripotency factor is an exogenous transcription factor selected from the group consisting of an Oct4, Sox, Klf, Myc, Lin28, or

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Nanog polypeptide, or a polynucleotide encoding Oct4, Sox, Klf, Myc, Lin28, or Nanog. In particular embodiments, the pluripotency factor comprises an Oct4, a Sox2, and a Klf4 polynucleotide.

In some embodiments, the culture medium comprises at least two, at least three, or four agents.

In some embodiments of the invention, the agent comprises a Rock inhibitor. In particular embodiments, the Rock inhibitor is thiazovivin or Y27632, and in more particular embodiments the Rock inhibitor is thiazovivin.

In other embodiments of the invention, the agent comprises a TGFβ inhibitor. In some embodiments, the TGFβ inhibitor is A-83-01 or SB431542.

In some embodiments, the agent comprises a GSK3 inhibitor, and in particular embodiments the GSK3 inhibitor is CHIR99021 or BIO.

In other embodiments of the invention, the agent comprises a MEK inhibitor. In some particular embodiments, the MEK inhibitor is PD98059 or PD032901.

In some embodiments of the invention, the agent comprises a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor. In particular embodiments, the TGFβ inhibitor is SB431542, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin..

In some embodiments of the invention, the pluripotent cell is cultured in the culture medium for at least one, at least two, at least five, at least ten, or at least 15 passages while maintaining genomic stability of the pluripotent cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. The effects of various small molecule cocktails on single cell culture of human pluripotent stem cells on feeder free substratum. A. Different morphologies were evident when single cell dissociated human pluripotent stem cells were treated with various combinations of small molecules in a feeder free environment. Medium containing a combination of ROCKi/MEKi/TGFβi/GSKi delivered robust growth and viability of human pluripotent stem cells. **B.** While some small molecules supported maintenance of pluripotent

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stem cells, *i.e.*, MEKi/TGFβi/GSKi, and others facilitated single cell dissociation of pluripotent

stem cells, *i.e.*, Rocki, the unique combination of these small molecules, *i.e.*,

ROCKi/MEKi/TGFβi/GSKi, supported single cell dissociation while maintaining

undifferentiated status as indicated by highly proliferative colonies that were positive for

5   pluripotent marker Tra181. Tra181, red; DAPI, blue. **C.** The combination of

MEKi/TGFβi/GSKi/ROCKi supported viability and proliferation when hiPSCs were seeded at 1

x 10^5 cells on Matrigel and scored 4 days later for number of viable cells and percent viability as

measured by trypan blue. **D.** When combined with MEKi/TGFβi/GSKi, colonies cultured with

Thiazovivin appear to be more compact in morphology relative to culture supported by

0   MEKi/TGFβi/GSKi with Y27632. **E.** Flow-cytometry analysis for surface expression of SSEA4

and Tra181 of hiPSCs cultured in Y27632 or Thiazovivin in combination with

MEKi/TGFβi/GSKi shows that Thiazovivin better supports the maintenance of cells in an

undifferentiated state compared to the combination having Y27632.

Figure 2. Human pluripotent stem cell adaptation into feeder free and single

5   **cell culture. A.** iPSCs generated on feeder cells were readily adapted to feeder free and single

cell culture conditions with the utilization of SMC4 medium. Cell survival is perturbed and

limited cell attachment and growth is seen, however, upon single cell dissociation and culture in

conventional culture conditions. **B.** Once adapted to a feeder free environment, iPSCs were

routinely cultured as single cells on a feeder free surface when continuously maintained in SMC4

20   medium.

Figure 3. Long-term maintenance of single cell and feeder free human

pluripotent stem cell culture. A. Single cell dissociated human pluripotent stem cells

maintained in SMC4 medium in a feeder free culture for 10 passages expressed pluripotent

markers Nanog and Tra181 by immunoflourescence analysis. **B.** Figure 3B shows the percent

25   of 7 Aminoactinomysin D (7AAD) staining cells four hours after human pluripotent stem cells

were dissociated into single cells and placed into either conventional culture medium or SMC4

medium. Single cell dissociated human pluripotent stem cells displayed significantly increased

viability when cultured in SMC4 medium rather than conventional culture medium as seen by

7AAD staining and flow-cytometry analysis. **C.** Quantitative population analysis by flow

cytometry revealed that nearly all the cells dissociated into single cells and maintained in SMC4 medium expressed multiple markers of undifferentiated status. **D.** Global expression analysis revealed a high correlation between iPSCs passaged as single cells and maintained in a SMC4 medium free of feeder cells and human ESCs cultured on feeder cells and maintained as cell clumps during passaging, including H1 cells. **E.** iPSCs cultured in a feeder cell-free system using SMC4 medium and passaged as single cells, but not their original source fibroblast cells, expressed an array of pluripotent markers, similar to human ESCs, including H1 and HuES9. **F.** Exogenous expression of reprogramming factors was effectively shutdown in iPSCs maintained in a feeder-free environment, unlike exogenous factor positive controls (fibroblast cells infected with lentivirus expressing reprogramming factors post 4 days). Endogenous expression of the reprogramming factors in feeder free iPSCs was similar to ESCs, including H1 and HuES9. **G.** After 11 passages as single cells in feeder free environment, human pluripotent stem cells retained normal karyotype. **H.** 14 days post replacement of SMC4 medium with conventional differentiation medium, cells maintained in a feeder free environment displayed gene expression indicative of ectoderm, endoderm and mesoderm germ layers, in addition to extraembryonic markers, which are normally expressed by only naïve and totipotent stem cells. **I.** Immunoflourescence analysis: iPSCs cultured in a feeder cell-free system using SMC4 medium and passaged as single cells readily developed into all three somatic cell types upon transfer to conventional differentiation medium. Mesoderm, alpha smooth muscle actin (αSMA); Ectoderm, beta tubulin III (β TUB III); Endoderm, Sox17. **J.** Teratoma formation analysis demonstrating that human pluripotent stem cells cultured in SMC4 medium give rise to cell types representative of the three germ layers, and thus retain their pluripotent potential.

Figure 4. Enhanced single cell sorting and subsequent feeder-free culture of human pluripotent stem cells. Demonstration of sorting of human pluripotent stem cells based on pluripotent marker expression and subsequent maintenance of sorted cells in feeder free culture supplemented with an optimized SMC4 or SMC4+ fibronectin medium. **A.** Prior to sorting, the human pluripotent stem cells were dissociated into single cells and labeled with antibody markers to SSEA4 and Tra-181. Cells were sorted using FACs technology based on surface marker expression of pluripotent markers, SSEA4 and TRA181. Post sort cells were

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seeded initially in SMC4 + fibronectin medium which was exchanged for SMC4 medium 24-72 hrs later. One day post sorting, sorted cells were dividing and by day 5, large colonies consisting of hundreds of cells were present. **B.** Sorted cells retained their expression of pluripotency marker Tra160. Tra160, red; DAPI, blue. **C.** After 5 passages post sort, approximately 1 month in culture, quantitative flow cytometry analysis revealed that the majority of the sorted iPSCs retained their pluripotent status based on SSEA4 and Tra181 staining. **D.** Various densities derived from SSEA4+ and Tra181+ human pluripotent stem cells were plated, including clonal densities of 500 events per well of a 6-well plate, approximately 52 events per cm^2, on feeder-free surface and supplemented with SMC4 + fibronectin medium. After 7 days post sort, many alkaline phosphatase positive colonies appeared in each of the seeded wells. **E.** The derived alkaline phosphatase positive colonies were scored. Approximately 8-10% of the seeded events had produced colonies with 16,000 events per well producing a nearly confluent well. N.C., not counted due to confluency preventing accurate counts. **F.** SSEA4+ and Tra181+ human pluripotent stem cells were sorted directly into 96 well-plates at various densities including 1 event per well, approximately 3 events per cm^2. Wells were stained for alkaline phosphatase 8 days post sort. **G.** Each well was scored for alkaline phosphatase colonies on day 8.

Figure 5. Reprogramming kinetics in the absence and presence of SMC4 medium. A. Seven days after the initiation of reprogramming by the ectopic expression of Oct4, Sox2, Klf4 and cMyc, the cells were either maintained in conventional medium or switched to SMC4 medium. After 20 days of reprogramming, few if any iPSC-like colonies were formed in the conventional medium culture, while many iPSC-like colonies appeared in the cells switched to SMC4 medium. **B.** Many iPSC-like colonies in SMC4 medium expressed alkaline phosphatase while few colonies in the conventional culture expressed alkaline phosphatase.

Figure 6. Culture in SMC4 medium promotes naïve characteristics. A. Schematic illustration of the adaptation of human pluripotent stem cells to SMC4 medium and its comparison to conventionally maintained human pluripotent stem cells. **B.** Hierarchical clustering based on Affymetrix global gene expression and Pearson Coefficient between hiPSCs derived from IMR90 parental cells (conventionally derived hiPSC (Conv) and SMC4 medium-

adapted hiPSCs (SMC4 medium)), both cultured for 11 passages in respective media, demonstrating that both hiPSCs are similar to each other and different from their parental IMR90 cells. **C.** While Xist expression was modestly repressed in hiPSCs cultured in conventional format compared to their parental IMR90 cell line, it was significantly repressed in hiPSCs cultured in SMC4 medium and was similar to levels of female hESC, HUES9. **D.** X-chromosome located genes were more highly expressed in hiPSCs cultured in SMC4 medium as compared to expression in conventionally cultured hiPSCs. **E.** When transferred back onto feeder-cells, hiPSCs maintained in SMC4 medium exhibit a morphology that is more like mouse ESCs as seen by a more three-dimensional shape and less of a flat morphology as compared with hiPSCs cultured in conventional medium.

Figure 7. Generation and maintenance in SMC4 medium improves the undifferentiated state of human pluripotent stem cells. **A.** Schematic illustration of parallel generation of clones FTi91 and 93, generated in SMC4 medium and maintained in SMC4 medium in FF culture, and clone FTi99, generated in conventional medium and maintained on feeder cells. **B.** Hierarchical clustering based on Affymetrix global gene expression and Pearson Coefficient depicting a close relationship between the derived pluripotent lines and distant relationship from the starting cell line (FTC1). **C.** Hierarchical clustering based on all 4-fold differentially expressed genes amongst Hues9, H1, FTi99, FTi91 and FTi93 demonstrating a separation between pluripotent lines cultured in conventional system versus those cultured in SMC4 medium. **D.** Affymetrix gene expression of selected genes associated with naïve and lineage differentiation compared between the SMC4 medium group (FTi91 and 93) versus conventional culture group (H1, Hues9 and FTi99) showing an increase in expression of many genes associated with pluripotency and significant reduction in lineage-specific gene expression in the SMC4 medium set. **E.** Schematic illustration of various methods of deriving hiPSCs with naïve status. Bottom panel describes the advantages of naïve state over primed state.

Figure 8. non-iPSC colonies generated during reprogramming process. A. A typical cell population early in the reprogramming process: immune staining showed that the majority of fast growing cells formed colonies that were SSEA4 negative and inevitably non-pluripotent cells. These cells were transformed and highly proliferative, grew quickly, and

dominated the culture. Colony 1 shows a SSEA4 positive colony with the potential of becoming an iPSC colony while colony 2 shows a transformed population with a high proliferation rate but negative for pluripotentcy marker staining. **B.** Many non-iPSC colonies displayed expression of a single pluripotent marker, while true iPSC colonies displayed expression of multiple pluripotent markers. At a later stage of reprogramming, various colonies were formed with some colonies being double positive for both SSEA4 and Tra181, *i.e.*, colony 1, while other colonies were only positive for one of the markers or none, *e.g.*, colonies 4 and 5.

Figure 9. Selection of pluripotent cells such as iPSCs with the use of cell sorting. A schematic depiction of single cell culture systems and cell sorting methodologies used for the enrichment and selection of pluripotent cells from a mixture of pluripotent and non-pluripotent cells.

Figure 10. Identification of pluripotent markers. To survey for additional markers specific to SSEA4^{+}/Tra181^{+} cells, various surface markers were assessed. Detection of Tra160 expression was used as a positive control as it has been demonstrated to correlate well with Tra181 expression. CD200 and CD90 do not appear to be specific for pluripotent cells as they similarly stain both SSEA^{-}/Tra181^{-} and SSEA^{+}/Tra181^{+} populations, while little specificity for and the SSEA^{+}/Tra181^{+} population is seen with CD9. However, CD50 and CD30 appear to preferentially identify the SSEA4^{+}/Tra181^{+} population over the SSEA^{-}/Tra181^{-} population.

Figure 11. CD30 is specific to Nanog expressing clones. A. Representative flow profile of SSEA4/Tra181 expression of various hiPSC lines demonstrating that all derived clones are representative of the majority population expressing both SSEA4 and Tra181. **B.** Based on the gating seen in (A), CD30 and CD9 flow profile of each cell line was assessed. Only clones FTC8 clone 1 and FTi31 appear to express CD30 while all clones express CD9. **C.** Relative Nanog expression of various lines was also assessed. **D.** Summary table demonstrating that only CD30 expression correlates with Nanog expression.

Figure 12. Depletion of non-reprogrammed cells during the reprogramming process enriches for reprogrammed cells. During a reprogramming process, a mixed population of reprogramming and non-reprogramming cells existed, with only a minor population of cells representing cells reprogrammed to full pluripotency, as indicated by SSEA4

and Tra181 expression (indicated by black colored dotted lined arrow). However, when non-reprogrammed cells were removed or depleted from the mixed population, a significant enrichment of SSEA4 and Tra181 positive population was demonstrated. As indicated by the gray colored solid line arrows, the CD13 negative cell population represented a significantly

5   enriched population of SSEA4 and Tra181 positive cells over CD13 low and high population cells.

Figure 13. Enhanced cellular reprogramming by single cell sorting and enrichment of pluripotent stem cells from various sources including IMR90 and ASC. A. A population of cells early in the reprogramming process was enriched by single cell sorting of

0   SSEA4+ cells. The starting population of cells, containing reprogrammed and non-reprogrammed cells, was enzymatically dissociated into single cells, stained for unique markers of pluripotency and sorted based on surface marker expression by immunoconjugated magnetic beads to obtain a cell population positive for SSEA4. After this enrichment for SSEA4$^+$ cells, 10,000 SSEA4+ cells were seeded onto Matrigel™ coated dishes and cultured in either

5   conventional medium or SMC4 medium in a FF environment. AP staining was conducted 8 days post sorting. The smaller inserted panels in the right-hand corners of these are images of representative cell morphologies. No alkaline phosphatase positive colonies were detected in conventional culture while many alkaline phosphatase positive colonies were derived with SMC4 medium. **B.** The number of SSEA4$^+$/Tra181$^+$ colonies derived from cellular

20   reprogramming on Matrigel™ in the presence or absence of SMC4 medium were scored. **C.** iPSC clones derived from either IMR90 fibroblast cells or adipose stem cells in feeder free conditions and single cell culture using SMC4 medium expressed markers of pluripotency. **D.** Whole population flow cytometry analysis of iPSC clones derived using single cell sorting methods revealed that the majority of cells were positive for key pluripotent markers. **E.** iPSCs

25   generated using single cell sorting for SSEA4 and feeder-free culture, but not their original source fibroblast or adipose stem cells, expressed an array of pluripotent markers, similar to human ESCs, including H1 and HuES9. **F.** Exogenous expression of reprogramming factors was effectively silenced in the iPSCs generated using single cell sorting and feeder-free culture, unlike control fibroblast cells that were infected with lentivirus expressing reprogramming

factors post 3 days. **G.** iPSCs generated using single cell sorting based on SSEA4 readily developed into all three somatic cell types upon differentiation. Endoderm, FoxA2; Mesoderm, alpha smooth muscle actin (αSMA); Ectoderm, beta tubulin III (β TUB III).

Figure 14. Single cell sorting for the selection of a unique population of

5   **pluripotent stem cells. A.** Using FACS sorting, a unique and rare population of cells that were SSEA4$^+$/Tra181$^+$ early in the reprogramming process was selected and transferred to feeder free culture supplemented with SMC4 + fibronectin medium which was exchanged for SMC4 medium after 24-72 hours. After an additional 6 days of culture, SSEA4$^+$/Tra181$^+$ derived iPSC colonies appeared to be growing in feeder free culture. However, when SSEA4-/Tra181-

0   colonies were transferred and maintained in feeder free culture, no alkaline phosphatase expressing colonies were detected after 14 days of culture. **B.** Various SSEA4$^+$/Tra181$^+$ sorted cells developed into colonies while maintaining their SSEA4 and Tra181 expression.

Figure 15. High-throughput approach for the generation and primary

characterization of iPSCs under feeder-free conditions. Three-factor (Oct, Sox, Klf) induced

5   fibroblast cells were sorted in a two-step fashion to deliver an efficient 96-well plating platform using SMC4 medium (and using SMC4 + fibronectin medium during sorting). Wells containing individual colonies were marked and expanded into 4x96-well plates. While one set was designated as the master-plate and expanded, the other three plates were processed for characterization including flow-cytometry analysis for surface marker expression including

20   SSEA4 and Tra181, qRT PCR for expression of key markers including Nanog and transgene silencing and immunofluorescence for pluripotent markers including Oct4 and Nanog. Based on the characterization readouts, selected hiPSC clones were expanded for further analysis and banked. The data panels represent snapshots of the clones surveyed (marked by wells 31-40 of the 96-well plate) during the high-throughput platform hiPSC generation of FTC5. In the qPCR

25   panel, expression was normalized to Gapdh. Nanog expression is relative to H1 hESC while transgene expression is relative to day 4 post infection of FTC5 (Day 4 infection). Based on the characterization readouts, selected hiPSC clones were expanded for further analysis and banked. In the highlighted example, well 37 was identified as a candidate for expansion based on its

multi-parameter pluripotency profile and termed FTC5 clone 1. Immuno-fluorescence images were taken at 5x magnification.

Figure 16. High-throughput platform, clonal and FF derivation of 3-factor (polycistronic-OKS) hiPSCs in the presence of SMC4 medium. A. Clones FTi91 and FTi93 derived from the above strategy were stained for expression of pluripotent markers. **B.** qRT-PCR of endogenous expression of pluripotent markers for FTC1 (foreskin fibroblast), H1 and Hues9 (hESC lines), FTi 91 and 93 (FTC1 derived hiPSC clones) and Day 4 P.I. (Day 4 post infection). Expression was normalized to Gapdh and relative within each gene group. **C.** Oct4 promoter methylation status. Open circles represent unmethylated CpG islands while dark circles represent methylated CpG islands. **D.** EB formation and differentiation of clones FTi91 and 93 after 28 days of differentiation. Endoderm, FoxA2; Mesoderm, alpha smooth muscle actin (αSMA); Ectoderm, Tuj1. **E.** Histological sections of teratoma derived from hiPSC clone generated and maintained in FF culture and SMC4 medium. Black arrows point to areas of interest: Mesoderm, white adipose tissue; Ectoderm, neurons; Endoderm, glands.

Figure 17. Generation and maintenance of pluripotent stem cells in SMC4 medium maintain genomic integrity and can be enriched for pluripotent cells. A. Copy number variation as assessed by array comparative genomic hybridization. Bottom table is an interpretation summary of the data demonstrating minimal copy number variation between the SMC4 medium-cultured human pluripotent stem cells and their parental cell line. **B.** Cytogenic analysis of G-banded metaphase cells. Bottom table is a summary of the data depicting genomic stability after long-term feeder free culture in SMC4 medium.

Figure 18. Characterization of FTC5 and FTC7 derived clonal hiPSCs under FF and SMC4 culture. To determine reproducibility of the high-throughput platform, additional patient consented lines FTC5 and FTC7 were tested for hiPSC generation. **A.** Representative immunofluorecence staining of pluripotent markers Oct4, Tra181, Nanog and Tra160 expressed in individual FTC5 and FTC7 derived hiPSC clones induced with OKS and generated using multiplex platform. **B.** Representative lineage specific staining of FTC5 and FTC7 derived hiPSCs 28 days post induction of differentiation. Endoderm, FoxA2; Mesoderm, alpha smooth muscle actin (αSMA); Ectoderm, Tuj1. **C.** Cytogenetic analysis of G-banded

metaphase cells from FTC5 and FTC7 derived hiPSC clones after long-term FF and single cell culture in various passages.

Figure 19. Method of maintaining pluripotent culture using cell surface markers. A. A population of pluripotent stem cells was further enriched during culturing as demonstrated by Tra181 enrichment in feeder free and SMC4 platform. **B.** During culture of a pluripotent cell population, some proportion of cells within the population may begin to differentiate and lose their pluripotency. Using selection methods for pluripotency, *i.e.*, Tra181 enrichment, a homogeneous culture of undifferentiated cells was attained.

Figure 20. Separation of differentiated cells from a heterogeneous population of non-, semi- and fully-pluripotent stem cells. FACS sorting of a population of reprogrammed human fibroblast cells: when cells double negative for pluripotency markers SSEA4-/Tra181- (blue box; lower left box) were sorted, no alkaline phosphatase (AP) positive colonies were detected, indicating a loss in pluripotency and potential tumorigenecity after one week of culture, whereas the sorting of a double positive population SSEA4+/Tra181+ (orange box; upper right box) resulted in the selection and enrichment of the population for pluripotent cells, as evidenced by the formation of AP+ colonies.

Figure 21. Cytokine free culture of human pluripotent stem cells on feeder free culture. A. Human pluripotent stem cells maintained their growth and morphology when cultured on a gelatin coated surface and supplemented with SMC4 medium in the absence of any cytokines, including bFGF. **B.** Magnified image depicting individual cells within a human pluripotent stem cell colony. **C.** SSEA4 and Tra181 co-expression at passage 3 provided further evidence that the cytokine-free cultured human pluripotent stem cells maintained their undifferentiated status.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides a robust culture system for culturing stem cells, including feeder-free conditions for generating and culturing human induced pluripotent stem cells (iPSCs). Specifically, the invention provides a culture platform that allows long-term culture of pluripotent cells in a feeder-free environment; reprogramming of cells in a feeder-free

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environment; single-cell dissociation of pluripotent cells; cell sorting of pluripotent cells; improved efficiency of reprogramming; generation of a naïve pluripotent cell; and identification markers for the identification and selection of pluripotent cells. The media and culture methods of the invention support the viability and survival of single cell dissociated human stem cells, and maintain the undifferentiated status of stem cells to allow for cultivation and passaging of dissociated single cells without differentiation.

Definitions

As used herein, the terms "reprogramming" or "dedifferentiation" or "increasing cell potency" or "increasing developmental potency" refers to a method of increasing the potency of a cell or dedifferentiating the cell to a less differentiated state. For example, a cell that has an increased cell potency has more developmental plasticity (*i.e.,* can differentiate into more cell types) compared to the same cell in the non-reprogrammed state. In other words, a reprogrammed cell is one that is in a less differentiated state than the same cell in a non-reprogrammed state.

As used herein, the term "potency" refers to the sum of all developmental options accessible to the cell (*i.e.,* the developmental potency). One having ordinary skill in the art would recognize that cell potency is a continuum, ranging from the most plastic cell, a totipotent stem cell, which has the most developmental potency to the least plastic cell, a terminally differentiated cell, which has the least developmental potency. The continuum of cell potency includes, but is not limited to, totipotent cells, pluripotent cells, multipotent cells, oligopotent cells, unipotent cells, and terminally differentiated cells. In the strictest sense, stem cells are pluripotent; thus, being able to give rise to any mature cell type. However, multipotent, oligopotent or unipotent progenitor cells are sometimes referred to as lineage restricted stem cells (*e.g.,* mesenchymal stem cells, adipose tissue derived stem cells, *etc.*) and/or progenitor cells.

As used herein, the term "pluripotent" refers to the ability of a cell to form all lineages of the body or soma (*i.e.,* the embryo proper). For example, embryonic stem cells are a type of pluripotent stem cells that are able to form cells from each of the three germs layers, the

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ectoderm, the mesoderm, and the endoderm. Pluripotency is a continuum of developmental potencies ranging from the incompletely or partially pluripotent cell (*e.g.,* an epiblast stem cell or EpiSC), which is unable to give rise to a complete organism to the more primitive, more pluripotent cell, which is able to give rise to a complete organism (*e.g.,* an embryonic stem cell). The level of cell pluripotency can be determined by assessing pluripotency characteristics of the cells. Pluripotency characteristics include, but are not limited to: i) pluripotent stem cell morphology; ii) expression of pluripotent stem cell markers including, but not limited to SSEA1 (mouse only), SSEA3/4; TRA1-60/81; TRA1-85, TRA2-54, GCTM-2, TG343, TG30, CD9, CD29, CD133/prominin, CD140a, CD56, CD73, CD105, CD31, CD34, OCT4, Nanog and/or Sox2, and as described in the present invention, CD30 and CD50; iii) ability of pluripotent mouse stem cells to contribute to germline transmission in mouse chimeras; iv) ability of pluripotent stem cells to contribute to the embryo proper using tetraploid embryo complementation assays; v) teratoma formation of pluripotent stem cells; vi) formation of embryoid bodies: and vii) inactive X chromosome reactivation.

As used herein, the term "pluripotent stem cell morphology" refers to the classical morphological features of an embryonic stem cell. Normal embryonic stem cell morphology is characterized by being round and small in shape, with a high nucleus-to-cytoplasm ratio, the notable presence of nucleoli, and typical intercell spacing.

As used herein, the term "multipotent" refers to the ability of an adult stem cell to form multiple cell types of one lineage. For example, hematopoietic stem cells are capable of forming all cells of the blood cell lineage, *e.g.,* lymphoid and myeloid cells.

"Adhere" refers to cells attaching to a vessel, for example, a cell attaching to a sterile plastic (or coated plastic) cell culture dish or flask in the presence of an appropriate culture medium. Certain classes of cells are not sustained or do not grow in a culture unless they adhere to the cell culture vessel. Certain classes of cells ("non-adherent cells") are maintained and/or proliferate in culture without adhering.

"Culture" or "cell culture" refers to the maintenance, growth and/or differentiation of cells in an *in vitro* environment. "Cell culture media," "culture media"

(singular "medium" in each case), "supplement" and "media supplement" refer to nutritive compositions that cultivate cell cultures.

"Cultivate" refers to the sustaining, propagating (growing) and/or differentiating of cells outside of tissue or the body, for example in a sterile plastic (or coated plastic) cell culture dish or flask. "Cultivation" may utilize a culture medium as a source of nutrients, hormones and/or other factors helpful to propagate and/or sustain the cells.

As used herein, a "dissociated" cell refers to a cell that has been substantially separated or purified away from other cells or from a surface (*e.g.*, a culture plate surface). For example, cells can be dissociated from an animal or tissue by mechanical or enzymatic methods. Alternatively, cells that aggregate *in vitro* can be dissociated from each other, such as by dissociation into a suspension of clusters, single cells or a mixture of single cells and clusters, enzymatically or mechanically. In yet another alternative embodiment, adherent cells are dissociated from a culture plate or other surface. Dissociation thus can involve breaking cell interactions with extracellular matrix (ECM) and substrates (*e.g.*, culture surfaces), or breaking the ECM between cells.

As used herein, the terms "enrich" and "enriching" refer to increasing the amount of a specified component in a composition, such as a composition of cells, and "enriched", when used to describe a composition of cells such as a cell population, refers to a population of cells having an increased amount proportionally of a specified component as compared to the proportion of such component in the population of cells prior to being enriched. For example, a composition such as a population of cells may be enriched with respect to a target cell type (*i.e.*, cells having specified characteristics), thus having an increased proportion or percent of the target cell type as compared to the proportion of the target cells present in the population of cells before being enriched. A population of cells may be enriched for a target cell type by cell selection and sorting methods known in the art. In some embodiments of the invention, a population of cells is enriched by a sorting or selection process as described in the examples herein. In a particular embodiment of the invention, a method that enriches for a target cell population, enriches the cell population with respect to the target cell population by at least about 20%, meaning that the enriched cell population comprises proportionately about 20% more of

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the target cell type than in the population before the population was enriched. In one embodiment, a method that enriches for a target cell population enriches the cell population with respect to the target cell population proportionately by at least about 30+%, 40+%, 50+%, 60+%, 70+%, 80%, 85%, 90%, 95%, 97%, 98% or 99%, or at least about 98%, or in particular embodiments, about 99%.

In certain aspects of the invention, a population of cells is enriched with respect to the amount of pluripotent cells or cells exhibiting pluripotency characteristics. In particular embodiments of the invention, a population of cells undergoing reprogramming is enriched for target cells having characteristics of pluripotency, such as expression of pluripotency markers including, without limitation, SSEA4, TRA 1-60, TRA-1-81, CD30 or CD50. In another particular embodiment of the invention, a population of cells, such as a population of cells undergoing reprogramming, is depleted of nonpluripotent cells using surface markers specific to differentiating or nonpluripotent cells, which may include, for example, CD13, CD26, CD34, CD45, CD31, CD46, or CD7. The resulting cell population can thus be described as a population of cells enriched for pluripotent cells. In certain aspects of the invention, the cells in an enriched population of cells are enriched for target cells have a distinct gene or protein expression profile, for example, cell surface expression of at least two pluripotency markers such as SSEA4, TRA 1-60, TRA-1-81, CD30 and CD50. In some embodiments, the cell population is enriched for target cells expressing two or more pluripotency markers. In particular embodiments, the cell population is enriched for target cells expressing SSEA4 in combination with either Tra-181 or Tra-160. In more particular embodiments, the cell population is enriched for target cells expressing SSEA4, Tra181, and CD30. In one embodiment, at least about 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 70%, 75%, 80%, 90%, 95%, 97%, 98%, or 99% of the cells in an enriched population of cells are the target cell type, such as pluripotent cells.

Thus, in some embodiments the invention provides methods of enriching a population of cells for pluripotent cells by sorting the cell population based on cell surface expression of pluripotency markers, such as SSEA4, TRA 1-60, TRA-1-81, CD30 and CD50, and collecting the fraction of cells expressing such markers to obtain a population of cells that is enriched for pluripotent cells. In other embodiments of the invention, a population of cells is

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enriched for pluripotent cells by sorting the cell population based on cell surface expression of markers of differentiating or differentiated cells, such as CD13, CD26, CD34, CD45, CD31, CD46, and CD7, and depleting the cell population of such cells to obtain a population of cells that is enriched for pluripotent cells. In particular embodiments, the cell population is sorted based on the expression of CD13, and CD13+ cells are removed from the cell population to obtain a population of cells enriched for pluripotent cells.

As used herein, "feeder cells" or "feeders" are terms used to describe cells of one type that are co-cultured with cells of a second type to provide an environment in which the cells of the second type can grow, as the feeder cells provide growth factors and nutrients for the support of the second cell type. The feeder cells are optionally from a different species as the cells they are supporting. For example, certain types of human cells, including stem cells, can be supported by primary cultures of mouse embryonic fibroblasts, and immortalized mouse embryonic fibroblasts. The feeder cells may typically be inactivated when being co-cultured with other cells by irradiation or treatment with an anti-mitotic agent such as mitomycin c, to prevent them from outgrowing the cells they are supporting. Without limiting the foregoing, one specific feeder cell type may be a human feeder, such as a human skin fibroblast or a human embryonic stem cell. Another feeder cell type may be mouse embryonic fibroblasts (mEF).

As used herein, a "feeder-free" (FF) environment refers to an environment such as a cell culture or culture medium essentially free of feeder cells and which has not been pre-conditioned by the cultivation of feeder cells. "Pre-conditioned" medium refers to a medium harvested after feeder cells have been cultivated within the medium for a period of time, such as for at least one day. Pre-conditioned medium contains many mediator substances, such as growth factors and cytokines, that are secreted by the feeder cells cultivated in the medium.

In some embodiments of the invention, the feeder free environment is essentially free of human feeder cells, including without limitation human fibroblasts, keratinocytes, and embryonic stem cells, and in particular embodiments additionally is not pre-conditioned by feeder cells. In further embodiments of the invention, the feeder free environment is essentially free of animal feeder cells, and further, in particular embodiments is not pre-conditioned with feeder cells. In certain embodiments of the invention, the feeder free environment is essentially

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free of both human and animal feeder cells, and in other certain embodiments the feeder free environment is essentially free of both human and animal feeder cells and is not pre-conditioned with feeder cells.

Genomic stability refers to the ability of a cell to faithfully replicate DNA and maintain integrity of the DNA replication process. As used herein to describe cells of the invention, "genomically stable cells" and "cells having genomic stability" refer to cells that exhibit a frequency of mutations and chromosomal aberrations (such as translocations, aneuploidy, copy number variations and duplications) that is substantially similar to the frequency of mutations and chromosomal aberrations relative to normal somatic human cells.

"Ingredient" refers to any compound or other material, whether chemical or biological in origin that may be used in cell culture media to maintain and/or promote the growth and/or differentiation of cells. The terms "component" "nutrient" and "ingredient" may be used interchangeably. Conventional ingredients used for cell culture media may include but are not limited to amino acids, salts, metals, sugars, lipids, nucleic acids, hormones, vitamins, fatty acids, proteins and the like. Other ingredients that promote and/or maintain cultivation of cells *ex vivo* may be selected by those persons of ordinary skill in the art as required for a desired effect.

"Isolate" or "isolating" refers to separating and collecting a composition or material from its natural environment, such as the separating of individual cell or cell cultures from tissue or the body. In one aspect, a population or composition of cells is substantially free of cells and materials with which it can be associated in nature. "Isolated" or "purified" or "substantially pure", with respect to a target population of cells, refers to a population of cells that is at least about 50%, at least about 75%, at least about 85%, at least about 90%, and in particular embodiments, at least about 95% pure, with respect to the target cells making up a total cell population. Purity of a population or composition of cells can be assessed by appropriate methods that are well known in the art. For example, a substantially pure population of pluripotent cells refers to a population of cells that is at least about 50%, at least about 75%, at least about 85%, at least about 90%, and in particular embodiments at least about 95%, and in

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certain embodiments about 98% pure, with respect to pluripotent cells making up the total cell population. The term "essentially pure" is used interchangeably herein with "substantially pure".

"Passage" or "passaging" refers to the act of subdividing and plating cells into multiple cell culture surfaces or vessels when the cells have proliferated to a desired extent. In some embodiments "passage" or "passaging" refers to subdividing, diluting and plating the cells. As cells are passaged from the primary culture surface or vessel into a subsequent set of surfaces or vessels, the subsequent cultures may be referred to herein as "secondary culture" or "first passage," *etc.* Each act of subdividing and plating into a new culture vessel is considered one passage.

"Plating" refers to placing a cell or cells into a culture vessel such that the cells adhere to and spread on a cell culture vessel.

A "pluripotency factor" refers to an agent capable of increasing the developmental potency of a cell, either alone or in combination with other agents. Pluripotency factors include, without limitation, polynucleotides, polypeptides, and small molecules capable of increasing the developmental potency of a cell. Exemplary pluripotency factors include, for example, transcription factors and small molecule reprogramming agents. Transcription factors may refer to proteins (*i.e.,* polypeptides) as well as the polynucleotides encoding the proteins unless the usage herein indicates otherwise. Examplary transcription factors include, for example, Oct, Klf, Myc, and Sox polypeptides, as well as polynucleotides encoding these polypeptides. Examples of additional transcription factors are provided herein.

As used herein, the terms "polypeptide" and "protein" are used interchangeably, unless specified to the contrary, and according to conventional meaning, *i.e.,* as a sequence of amino acids. Polypeptides are not limited to a specific length, *e.g.,* they may comprise a full length protein sequence or a fragment of a full length protein, and may include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like, as well as other modifications known in the art, both naturally occurring and non-naturally occurring. Polypeptides used in the methods of the invention may be prepared using any of a variety of well known recombinant and/or synthetic techniques.

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The methods of the invention, in certain embodiments, employ active fragments of polypeptides described herein (*e.g.,* Sox-2, c-Myc, Oct3/4, Klf4, Lin28, Nanog, *etc.,* or a substrate, cofactor and/or downstream effector thereof), for example, comprising at least about 10, 15, 20, 25, 50, 75, 100, 200, 300, 400, 500, 1000, *etc.,* contiguous amino acids, or more, including all intermediate lengths, of a polypeptide described herein. In a particular embodiment, the fragment or combination of fragments employed retain the ability to modulate, induce and/or maintain pluripotency when used in the methods described herein.

In another aspect, the present invention employs variants of the polypeptide compositions described herein (*e.g.,* Sox-2, c-Myc, Oct3/4, Klf4, Lin28, Nanog, *etc.,* or a substrate, cofactor and/or downstream effector thereof). Polypeptide variants generally encompassed by the present invention will typically exhibit at least about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more identity (determined as described below), along its length, to a polypeptide sequences set forth herein. In a particular embodiment, the variant or combination of variants employed retain the ability to induce pluripotency as described herein.

In another aspect, the present invention employs polypeptide variants which exhibit at least about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more identity (determined as described below), along its length, to the corresponding region of a wild-type mammalian polypeptide used according to the present disclosure.

A polypeptide variant may differ from a naturally occurring polypeptide in one or more substitutions, deletions, additions and/or insertions. Such variants may be naturally occurring or may be synthetically generated, for example, by modifying one or more of the above polypeptide sequences used in the methods of the invention and evaluating their effects using any of a number of techniques well known in the art.

"Proliferate" refers to the property of one cell dividing into two essentially identical cells or a population of cells increasing in number (*e.g.,* to reproduce).

"Propagation" refers to growing (*e.g.,* reproducing via cell proliferation) cells outside of tissue or the body, for example, in a sterile container such as a plastic (or coated plastic) cell culture dish or flask.

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"Primary culture" refers to cells, tissue and/or culture where the isolated cells are placed in a first culture vessel with culture medium. The cells, tissue and/or culture may be sustained and/or may proliferate, however, as long as the cells, tissue and/or culture remain in the first vessel the cells, tissue and/or culture are referred to as the primary culture.

5   The terms "small molecule reprogramming agent" or "small molecule reprogramming compound" are used interchangeably herein and refer to small molecules that can increase developmental potency of a cell, either alone or in combination with other pluripotency factors. A "small molecule" refers to an agent that has a molecular weight of less than about 5 kD, less than about 4 kD, less than about 3 kD, less than about 2 kD, less than about

10   1 kD, or less than about .5kD. Small molecules can be nucleic acids, peptidomimetics, peptoids, carbohydrates, lipids or other organic or inorganic molecules. Libraries of chemical and/or biological mixtures, such as fungal, bacterial, or algal extracts, are known in the art and can be screened with any of the assays of the invention. In particular embodiments, the small molecule reprogramming agent used herein has a molecular weight of less than 10,000 daltons, for

15   example, less than 8000, 6000, 4000, 2000 daltons, e.g., between 50-1500, 500-1500, 200-2000, 500-5000 daltons.

As used herein, the terms "substantially free of" and "essentially free of" are used interchangeably, and when used to describe a composition, such as a cell population or culture media, refer to a composition that is free of a specified substance, such as, 95% free, 96% free,

20   97% free, 98% free, 99% free of the specified substance, or is undetectable as measured by conventional means. Similar meaning can be applied to the term "absence of," where referring to the absence of a particular substance or component of a composition.

Cells for use in the invention

A starting population of cells for use in the invention may be derived from

25   essentially any suitable source, and may be heterogeneous or homogeneous with respect to cell types or state of pluripotency. In one embodiment, the cells are mammalian cells, and in particular embodiments, the cells are isolated from a mammal selected from the group consisting of: a rodent, a cat, a dog, a pig, a goat, a sheep, a horse, a cow, or a primate. In a certain

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embodiment, the mammal is a human. In other certain embodiments, the cells to be used or treated according to the invention are adult cells, including essentially any accessible adult cell types.

The cells may be somatic, non-pluripotent, incompletely or partially pluripotent stem cells, multipotent cells, oligopotent cells, unipotent cells, terminally differentiated cells, or a mixed population of cells comprising any combination of the foregoing. Pluripotent cells used in the methods of the invention may be naturally-occurring stem cells, including embryonic stem cells, or can be induced pluripotent stem cells. A "mixed" population of cells is a population of cells of varying degrees of developmental potency. For example, a mixed population of cells may comprise cells undergoing reprogramming, so that the mixed population comprises pluripotent cells, partially pluripotent cells, and non-pluripotent cells, such as fully differentiated cells.

In one embodiment, the starting population of cells is selected from adult or neonatal stem/progenitor cells. In particular embodiments, the starting population of stem/progenitor cells is selected from the group consisting of: mesodermal stem/progenitor cells, endodermal stem/progenitor cells, and ectodermal stem/progenitor cells.

In another embodiment, the starting population of stem/progenitor cells is a mesodermal stem/progenitor cell. Illustrative examples of mesodermal stem/progenitor cells include, but are not limited to: mesodermal stem/progenitor cells, endothelial stem/progenitor cells, bone marrow stem/progenitor cells, umbilical cord stem/progenitor cells, adipose tissue derived stem/progenitor cells, hematopoietic stem/progenitor cells (HSCs), mesenchymal stem/progenitor cells, muscle stem/progenitor cells, kidney stem/progenitor cells, osteoblast stem/progenitor cells, chondrocyte stem/progenitor cells, and the like.

In other related embodiments, the starting population of stem/progenitor cells is an ectodermal stem/progenitor cell. Illustrative examples of ectodermal stem/progenitor cells include, but are not limited to neural stem/progenitor cells, retinal stem/progentior cells, skin stem/progenitor cells, and the like.

In other related embodiments, the starting population of stem/progenitor cells is an endodermal stem/progenitor cell. Illustrative examples of endodermal stem/progenitor cells

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include, but are not limited to liver stem/progenitor cells, pancreatic stem/progenitor cells, epithelial stem/progenitor cells, and the like.

In certain embodiments, the starting population of cells may be a heterogeneous or homogeneous population of cells selected from the group consisting of: pancreatic islet cells, CNS cells, PNS cells, cardiac muscle cells, skeletal muscle cells, smooth muscle cells, hematopoietic cells, bone cells, liver cells, an adipose cells, renal cells, lung cells, chondrocyte, skin cells, follicular cells, vascular cells, epithelial cells, immune cells, endothelial cells, and the like.

Inducing reprogramming and increasing potency of cells

Various strategies are being pursued to induce pluripotency, or increase potency, in cells (Takahashi, K., and Yamanaka, S., *Cell* 126, 663-676 (2006); Takahashi *et al.*, *Cell* 131, 861-872 (2007); Yu *et al.*, *Science* 318, 1917-1920 (2007); Zhou *et al.*, *Cell Stem Cell* 4, 381-384 (2009); Kim *et al.*, *Cell Stem Cell* 4, 472-476 (2009); Yamanaka *et al.*, 2009; Saha, K., Jaenisch, R., *Cell Stem Cell* 5, 584-595 (2009)), and improve the efficiency of reprogramming (Shi *et al.*, *Cell Stem Cell* 2, 525-528 (2008a); Shi *et al.*, *Cell Stem Cell* 3, 568-574 (2008b); Huangfu *et al.*, *Nat Biotechnol* 26, 795-797 (2008a); Huangfu *et al.*, *Nat Biotechnol* 26, 1269-1275 (2008b); Silva *et al.*, *Plos Bio* 6, e253. doi: 10.1371/journal. pbio. 0060253 (2008); Lyssiotis *et al.*, *PNAS* 106, 8912-8917 (2009); Ichida *et al.*, *Cell Stem Cell* 5, 491-503 (2009); Maherali, N., Hochedlinger, K., *Curr Biol* 19, 1718-1723 (2009b); Esteban *et al.*, *Cell Stem Cell* 6, 71-79 (2010); and Feng *et al.*, *Cell Stem Cell* 4, 301-312 (2009)).

Generally, techniques for reprogramming involve modulation of specific cellular pathways, either directly or indirectly, using polynucleotide-, polypeptide- and/or small molecule-based approaches. The developmental potency of a cell may be increased, for example, by contacting a cell with one or more pluripotency factors. "Contacting", as used herein, can involve culturing cells in the presence of a pluripotency factor (such as, for example, small molecules, proteins, peptides, *etc.*) or introducing pluripotency factors into the cell. Pluripotency factors can be introduced into cells by culturing the cells in the presence of the factor, including transcription factors such as proteins, under conditions that allow for

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introduction of the transcription factor into the cell. *See, e.g.*, Zhou H *et al.*, *Cell Stem Cell.* 2009 May 8;4(5):381-4; WO/2009/117439. Introduction into the cell may be facilitated for example, using transient methods, *e.g.*, protein transduction, microinjection, non-integrating gene delivery, mRNA transduction, *etc.*, or any other suitable technique. In some embodiments, the transcription factors are introduced into the cells by expression from a recombinant vector that has been introduced into the cell, or by incubating the cells in the presence of exogenous transcription factor polypeptides such that the polypeptides enter the cell.

In particular embodiments, the pluripotency factor is a transcription factor. Exemplary transcription factors that are associated with increasing, establishing, or maintaining the potency of a cell include, but are not limited to Oct-3/4, Cdx-2, Gbx2, Gsh1, HesX1, HoxA10, HoxA11, HoxB1, Irx2, Isl1, Meis1, Meox2, Nanog, Nkx2.2, Onecut, Otx1, Oxt2, Pax5, Pax6, Pdx1, Tcf1, Tcf2, Zfhx1b, Klf-4, Atbf1, Esrrb, Gcnf, Jarid2, Jmjd1a, Jmjd2c, Klf-3, Klf-5, Mel-18, Myst3, Nac1, REST, Rex-1, Rybp, Sall4, Sall1, Tif1, YY1, Zeb2, Zfp281, Zfp57, Zic3, Coup-Tf1, Coup-Tf2, Bmi1, Rnf2, Mta1, Pias1, Pias2, Pias3, Piasy, Sox2, Lef1, Sox15, Sox6, Tcf-7, Tcf7l1, c-Myc, L-Myc, N-Myc, Hand1, Mad1, Mad3, Mad4, Mxi1, Myf5, Neurog2, Ngn3, Olig2, Tcf3, Tcf4, Foxc1, Foxd3, BAF155, C/EBPβ, mafa, Eomes, Tbx-3; Rfx4, Stat3, Stella, and UTF-1. Exemplary transcription factors include Oct4, Sox2, Klf4, c-Myc, and Nanog.

Small molecule reprogramming agents are also pluripotency factors and may also be employed in the methods of the invention for inducing reprogramming and maintaining or increasing cell potency. In some embodiments of the invention, one or more small molecule reprogramming agents are used to induce pluripotency of a somatic cell, increase or maintain the potency of a cell, or improve the efficiency of reprogramming.

In some embodiments, small molecule reprogramming agents are employed in the methods of the invention to improve the efficiency of reprogramming. Improvements in efficiency of reprogramming can be measured by (1) a decrease in the time required for reprogramming and generation of pluripotent cells (*e.g.*, by shortening the time to generate pluripotent cells by at least a day compared to a similar or same process without the small molecule), or alternatively, or in combination, (2) an increase in the number of pluripotent cells

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generated by a particular process (*e.g.*, increasing the number of cells reprogrammed in a given time period by at least 10%, 30%, 50%, 100%, 200%, 500%, *etc.* compared to a similar or same process without the small molecule). In some embodiments, a 2-fold to 20-fold improvement in reprogramming efficiency is observed. In some embodiments, reprogramming efficiency is improved by more than 20 fold. In some embodiments, a more than 100 fold improvement in efficiency is observed over the method without the small molecule reprogramming agent (*e.g.*, a more than 100 fold increase in the number of pluripotent cells generated).

Several classes of small molecule reprogramming agents may be important to increasing, establishing, and/or maintaining the potency of a cell. Exemplary small molecule reprogramming agents include, but are not limited to: agents that inhibit H3K9 methylation or promote H3K9 demethylation; agents that inhibit H3K4 demethylation or promotes H3K4 methylation; agents that inhibit histone deacetylation or promote histone acetylation; L-type Ca channel agonists; activators of the cAMP pathway; DNA methyltransferase (DNMT) inhibitors; nuclear receptor ligands; GSK3 inhibitors; MEK inhibitors; TGFβ receptor/ALK5 inhibitors; HDAC inhibitors; Erk inhibitors; ROCK inhibitors; FGFR inhibitors; and PARP inhibitors. Exemplary small molecule reprogramming agents include GSK3 inhibitors; MEK inhibitors; TGFβ receptor/ALK5 inhibitors; HDAC inhibitors; Erk inhibitors; and ROCK inhibitors. Each of these classes of small molecule agents is described more fully below.

In some embodiments of the invention, small molecule reprogramming agents are used to replace one or more transcription factors in the methods of the invention to induce pluripotency, improve the efficiency of reprogramming, and/or increase or maintain the potency of a cell. For example, in some embodiments, a cell is contacted with one or more small molecule reprogramming agents, wherein the agents are included in an amount sufficient to improve the efficiency of reprogramming. In other embodiments, one or more small molecule reprogramming agents are used in addition to transcription factors in the methods of the invention. In one embodiment, a cell is contacted with at least one pluripotency transcription factor and at least one small molecule reprogramming agent under conditions to increase, establish, and/or maintain the potency of the cell or improve the efficiency of the reprogramming process.

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In another embodiment, a cell is contacted with at least one pluripotency transcription factor and at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or at least ten small molecule reprogramming agents under conditions and for a time sufficient to increase, establish, and/or maintain the potency of the cell or improve the efficiency of reprogramming. The state of potency or differentiation of cells can be assessed by monitoring the pluripotency characteristics described elsewhere herein.

In one embodiment, cells are contacted with a composition comprising one or more pluripotency factors and/or a combination of small molecule reprogramming agents, wherein the pluripotency factors and small molecules increase or induce the pluripotency of a cell. It is contemplated that the cells of the invention may be contacted *in vitro*, *ex vivo*, or *in vivo*.

Characterizing Pluripotent Cells

Following induction of reprogramming, reprogrammed cells can be selected based on relevant and detectable morphological, molecular and/or biochemical changes associated with pluripotency. Specific characteristics of cell pluripotency which may be monitored, separately or in combination, in assessing the potency of a cell include, but are not limited to, gene expression, methylation, and *in vivo* and *in vitro* characteristics such as: i) pluripotent stem cell morphology that is round and flat; ii) expression of pluripotent stem cell markers including SSEA1 (mouse pluripotent stem cells), SSEA3/4 (human pluripotent stem cells); TRA1-60/81; TRA1-85, TRA2-54, GCTM-2, TG343, TG30, CD9, CD29, CD133/prominin, CD140a, CD56, CD73, CD105, CD31, CD34, OCT4, Nanog and/or Sox2, and, as provided by the present invention, CD30 and CD50, and combinations of the foregoing; iii) ability of pluripotent stem cells to contribute to germline transmission in mouse chimeras; iv) ability of pluripotent stem cells to contribute to the embryo proper using tetraploid embryo complementation assays; v) teratoma formation of pluripotent stem cells; vi) formation of embryoid bodies and in vitro trilineage differentiation; and vii) inactive X chromosome reactivation. In certain embodiments, a subset of any of the above characteristics is used for monitoring cell potency. In one embodiment, pluripotent cells

are characterized by having a flat, round colony morphology, expression of SSEA4 and Oct4, and the ability to form chimeras and teratomas.

As discussed herein, pluripotency exists as a continuum and induced pluripotent stem cells appear to exist in both a "primed" state and a "naïve" state, with a cell in a naïve state possibly having greater differentiation potential. Induced pluripotent stem cells generated in conventional culture medium exist in a primed state and more closely resemble cells derived from a post-implantation blastocyst, while naïve iPSCs display pluripotency characteristics that more closely resemble mouse embryonic stem cells or cells derived from a pre-implantation blastocyst. The primed and naïve cell states can be defined by various differences, including differences in colony morphology, cellular response to inhibition or activation of key signaling pathways, gene expression signature, and ability to reactivate genes associated with extraembryonic cells. For example, conventional iPSCs, representing a primed pluripotent state, exhibit a colony morphology that is flat, while naïve iPSCs exhibit a compact domed colony morphology that is similar to mouse embryonic stem cells. Further, conventional iPSCs require extrinsic signaling of key cytokines, such as TGFβ, Activin, and bFGF and are dependent on ERK/MEK cellular signaling for maintenance of an undifferentiated state, and differentiate when these pathways are inhibited by contacting cells with, for example, a TGFβ or MEK inhibitor. In contrast, naïve cells do not require extrinsic signaling and maintain pluripotency even when treated with inhibitors of the TGFβ and MEK signaling pathways.

Additionally, gene expression analysis reveals significant differences between naïve and prime pluripotent cells. For example, naive iPSCs have significantly repressed Xist expression while conventional iPSCS show only modest repression of Xist expression; naïve cells show significant X chromosome reactivation and increased expression of genes located on the X chromosome over the expression seen in conventional iPSCs; and naïve cells express extraembryonic stem cell markers, including without limitation Gata6, CDX2, and CGB. In contrast, early markers of differentiation, such as lineage specific genes such as Foxa2, Sox17, and Brachyury, are more highly expressed in conventional iPSCs over naïve iPSCs. Additional markers useful for identifying cells in a naïve state of pluripotency include an increase in Klf4, Tbx3, Gbx2, Lin28, Soc3 or a decrease in Otx2, Sox17, Cer1, FoxA2, Zic1, Lhx2, Xist.

In particular embodiments of the invention, naïve cells show Xist expression that is decreased by at least two-fold, at least five-fold, or at least ten-fold as compared to conventional iPSCs. In some embodiments of the invention, cells in a naïve state of pluripotency have Xist expression 2-fold lower than conventional iPSCs and expression of at least five genes located on the X chromosome at levels three-fold higher than conventional iPSCs.

X chromosome reactivation can be shown by increased expression of at least five genes, at least 10 genes, or in particular embodiments, at least 100 genes located on the X chromosome at levels at least two-fold, three-fold, five-fold, or more over levels of such genes in conventional iPSCs.

In particular embodiments of the invention, the pluripotent cells of the invention retain characteristics of pluripotency for multiple cell passages, such as for example, at least 1, 3, 5, 7, 10, 15, 20 or more passages.

Culture media platforms for use in the methods of the invention

The culture media of the invention (*i.e.*, culture platforms) comprise a chemically defined stock basal media and various combinations of small molecules, including small molecule inhibitors, that allow:

long-term culture of pluripotent cells in a feeder-free environment;

reprogramming of cells in a feeder-free environment;

single-cell dissociation of pluripotent cells;

cell sorting of pluripotent cells;

maintenance of an undifferentiated status;

improved efficiency of reprogramming; and

generation of a naïve pluripotent cell.

The chemically defined stock basal media for use in the culture medium of the invention may be any defined basal media suitable for supporting the maintenance, growth, and/or differentiation of stem cells, such as conventional human embryonic stem cell media. Examples of defined basal media which may be used in accordance with the invention include, but are not limited to: Dulbecco's Modified Eagle Medium ("DMEM"), Basal Media Eagle

(BME), DMEM/F-12 (1:1 DMEM and F-12 vol:vol); Medium 199; F-12 (Ham) Nutrient

Mixture; F-10 (Ham) Nutrient Mixture; Minimal Essential Media (MEM), Williams' Media E;

and RPMI 1640, all of which are available from Gibco-BRL/Life Technologies, Inc.,

Gaithersburg, Md., among others. Several versions of many of these media are available, and

those that are particularly useful to construct the culture media of the invention include, but are

not limited to: DMEM 11966, DMEM 10314, MEM 11095, Williams' Media E 12251, Ham F12

11059, MEM-alpha 12561, and Medium-199 11151 (all available from Gibco-BRL/Life

Technologies (1995-1996 catalog)). The culture media may include, for example, one or more

of the following: amino acids, vitamins, organic salts, inorganic salts, trace elements, buffering

salts, sugars, ATP, and the like (suitable basal media ingredients are available from Sigma-

Aldrich of Saint Louis, Mo.).

Small molecules, and classes thereof, for use in the cell culture media of the

invention are described more fully below. In particular embodiments, the culture media of the

invention comprises one or more, two or more, or three or more of a TGFβ inhibitor, a GSK3

inhibitor, a MEK inhibitor, and a ROCK inhibitor.   In certain embodiments, the culture media of

the invention comprises a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a ROCK

inhibitor. Exemplary TGFβ inhibitors, GSK3 inhibitors, MEK inhibitors, and ROCK inhibitors

for use in the cell culture media and methods of the invention are described below.   The culture

media may additionally comprise a PARP inhibitor, such as Olaparib (AZD-2281).

GSK-3β Inhibitors

Inhibitors of GSK-3β include, but are not limited to, antibodies that bind GSK-3β,

dominant negative GSK-3β variants, and siRNA and antisense nucleic acids that target GSK-3β.

Other exemplary GSK-3β inhibitors include, but are not limited to, Kenpaullone, 1-

Azakenpaullone,CHIR99021, CHIR98014, AR-A014418, CT 99021, CT 20026, SB216763, AR-

A014418, lithium, SB 415286, TDZD-8, BIO, BIO-Acetoxime, (5-Methyl- lH-pyrazol-3-yl)-(2-

phenylquinazolin-4-yl)amine, Pyridocarbazole- cyclopenadienylruthenium complex, TDZD-8 4-

Benzyl-2-methyl-l,2,4- thiadiazolidine-3,5-dione, 2-Thio(3-iodobenzyl)-5-(l-pyridyl)-[l,3,4]-

oxadiazole, OTDZT, alpha-4-Dibromoacetophenone, AR-AO 144-18, 3- (l-(3-Hydroxypropyl)-

lH-pyrrolo[2,3-b]pyridin-3-yl]-4-pyrazin-2-yl-pyrrole-2,5-dione; TWSl 19 pyrrolopyrimidine

compound, L803 H-KEAPPAPPQSpP-NH2 or its myristoylated form; 2-Chloro-l- (4,5-dibromo-

thiophen-2-yl)-ethanone, SB216763, and SB415286.   Exemplary GSK3 inhibitors for use in the

cell culture media of the invention include CHIR99021, BIO, and Kenpaullone, while

CHIR99021 is preferred in particular embodiments.

ERK/MEK inhibitors

Exemplary inhibitors of the ERK/MEK pathway include, but are not limited to

antibodies to MEK or ERK, dominant negative MEK or ERK variants, and siRNA and antisense

nucleic acids that suppress expression of MEK and/or ERK.   Other exemplary ERK/MEK

inhibitors include, but are not limited to, PD0325901, PD98059, UO126, SL327, ARRY- 162,

PD184161, PD184352, sunitinib, sorafenib, Vandetanib, pazopanib, Axitinib, GSKl 120212,

ARRY-438162, RO5126766, XL518, AZD8330, RDEAl 19, AZD6244, FR180204 and PTK787.

Additional MEK/ERK inhibitors include those compounds disclosed in

International Published Patent Applications WO 99/01426, WO 02/06213, WO 03/077914, WO

05/051301 and WO2007/044084.

Further illustrative examples of MEK/ERK inhibitors include the following

compounds: -- 6-(4-Bromo-2-chloro-phenylamino)-7-fluoro-3-methyl-3H-benzoimidazol- e-5-

carboxylic acid (2,3-dihydroxy-propoxy)-amide; 6-(4-Bromo-2-chloro-phenylamino)-7-fluoro-3-

(tetrahydro-pyran-2-ylm- ethyl)-3H-benzoimidazole-5-carboxylic acid (2-hydroxy-ethoxy)-

amide, 1-[6-(4-Bromo-2-chloro-phenylamino)-7-fluoro-3-methyl-3H-benzoimida- zol-5-yl]-2-

hydroxy-ethanone, 6-(4-Bromo-2-chloro-phenylamino)-7-fluoro-3-methyl-3H-benzoimidazol- e-

5-carboxylic acid (2-hydroxy-1,1-dimethyl-ethoxy)-amide, 6-(4-Bromo-2-chloro-phenylamino)-

7-fluoro-3-(tetrahydro-furan-2-ylm- ethyl)-3H-benzoimidazole-5-carboxylic acid (2-hydroxy-

ethoxy)-amide, 6-(4-Bromo-2-fluoro-phenylamino)-7-fluoro-3-methyl-3H-benzoimidazol- e-5-

carboxylic acid (2-hydroxy-ethoxy)-amide, 6-(2,4-Dichloro-phenylamino)-7-fluoro-3-methyl-

3H-benzoimidazole-5-- carboxylic acid (2-hydroxy-ethoxy)-amide, 6-(4-Bromo-2-chloro-

phenylamino)-7-fluoro-3-methyl-3H-benzoimidazol- e-5-carboxylic acid (2-hydroxy-ethoxy)-

amide, referred to hereinafter as MEK inhibitor 1; 2-[(2-fluoro-4-iodophenyl)amino]-N-(2-

hydroxyethoxy)-1,5-dimethyl-6- -oxo-1,6-dihydropyridine-3-carboxamide; referred to hereinafter as MEK inhibitor 2; and 4-(4-bromo-2-fluorophenylamino)-N-(2-hydroxyethoxy)-1,5-dimethyl-6-- oxo-1,6-dihydropyridazine-3-carboxamide or a pharmaceutically acceptable salt thereof. In certain embodiments, the MEK/ERK inhibitor for use in the cell culture medium of the invention is PD98059.

TGFβ Receptor/ALK5 Inhibitors

Exemplary ALK5 inhibitors include antibodies to ALK5, dominant negative variants of ALK5, and antisense nucleic acids that suppress expression of ALK5. Other exemplary ALK5 inhibitors include, but are not limited to, SB431542, A-83-01, 2-(3-(6-Methylpyridin-2-yl)-lH- pyrazol-4-yl)-l ,5-naphthyridine, Wnt3a/BIO, BMP4, GW788388, SM16, IN-1130, GW6604, SB- 505124, and pyrimidine derivatives, see, *e.g.,* WO2008/006583, herein incorporated by reference.

Further, while "an ALK5 inhibitor" is not intended to encompass non-specific kinase inhibitors, an "ALK5 inhibitor" should be understood to encompass inhibitors that inhibit ALK4 and/or ALK7 in addition to ALK5, such as, for example, SB- 431542 (see, *e.g.,* Inman, *et al., J MoI. Phamacol.* 62(1): 65-74 (2002).

In view of the data herein showing the effect of inhibiting ALK5, it is believed that inhibition of the TGFβ/activin pathway will have similar effects. Thus, any inhibitor, *e.g.,* upstream or downstream of the TGFβ/activin pathway can be used in combination with, or instead of, ALK5 inhibitors as described in each paragraph herein. Exemplary TGFβ/activin pathway inhibitors include but are not limited to: TGFβ receptor inhibitors, inhibitors of SMAD 2/3 phosphorylation, inhibitors of the interaction of SMAD 2/3 and SMAD 4, and activators/agonists of SMAD 6 and SMAD 7. Furthermore, the categorizations described below are merely for organizational purposes and one of skill in the art would know that compounds can affect one or more points within a pathway, and thus compounds may function in more than one of the defined categories.

TGFβ receptor inhibitors can include antibodies to, dominant negative variants of and siRNA or antisense nucleic acids that target TGFβ receptors. Specific examples of inhibitors

include but are not limited to SU5416; 2-(5-benzo[l,3]dioxol-5-yl-2-tert-butyl-3H- imidazol-4-yl)-6-methylpyridine hydrochloride (SB-505124); lerdelimumb (CAT-152); metelimumab (CAT-192); GC-1008; IDl 1; AP-12009; AP-11014; LY550410; LY580276; LY364947; LY2109761 ; SB-505124; SB-431542; SD-208; SM16; NPC-30345; Ki26894; SB-203580; SD-093; Gleevec; 3,5,7,2',4'-pentahydroxyfiavone (Morin); activin-M108A; P144; soluble TBR2-Fc; and antisense transfected tumor cells that target TGFβ receptors. (*See, e.g.,* Wrzesinski, *et al., Clinical Cancer Research* 13(18):5262-5270 (2007); Kaminska, *et al., Acta Biochimica Polonica* 52(2):329-337 (2005); and Chang, *et al., Frontiers in Bioscience* 12:4393-4401 (2007).

Exemplary TGFβ receptor inhibitors for use in the cell culture media of the invention include SB431542, A-83-01, and RepSox. In particular embodiments, the TGFβ inhibitor is SB431542.

ROCK Inhibitors

ROCKs are serine/threonine kinases that serve as target proteins for Rho (of which three isoforms exist--RhoA, RhoB and RhoC). Exemplary ROCK inhibitors include, but are not limited to antibodies to ROCK, dominant negative ROCK variants, and siRNA and antisense nucleic acids that suppress expression of ROCK. Other exemplary ROCK inhibitors include, but are not limited to: thiazovivin, Y27632, Fasudil, AR122-86, Y27632 H-1152, Y-30141, Wf-536, HA-1077, hydroxyl-HA-1077, GSK269962A, SB-772077-B, N-(4-Pyridyl)-N'-(2,4,6-trichlorophenyl)urea, 3-(4-Pyridyl)-1H-indole, and (R)-(+)-trans-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide.

Exemplary ROCK inhibitors for use in the cell culture medium of the invention include thiazovivin, Y27632, pyrintegrin, and Blebbistatin. In certain embodiments, the ROCK inhibitor is thiazovivin.

FGFR Inhibitors

Exemplary FGFR inhibitors include, but are not limited to antibodies to FGFR, dominant negative FGFR variants, and siRNA and antisense nucleic acids that suppress expression of FGFR. Other exemplary FGFR inhibitors include, but are not limited to RO-

4396686, CHIR-258, PD 173074, PD 166866, ENK-834, ENK-835, SU5402, XL-999, SU6668, R04383596, and BIBF-1120.

PARP Inhibitors

PARP inhibitors inhibit Poly (ADP-ribose) polymerase ("PARP"). The PARP protein is a DNA repair enzyme which functions to regulate DNA repair pathways in cells. PARP is involved with base excision repair (BER) pathway, and PARP inhibition may promote genomic stability of cells during reprogramming or maintenance of pluripotent cells. Exemplary PARP inhibitors for use in the cell culture mediums of the invention include, without limitation, iniparib, veliparib, and olaparib (AZD – 2281).

The amount of the small molecules in the cell culture media of the invention can vary and may be optimized according to the specific culture conditions, including the specific molecules and combinations used, the type of cell being cultured in the media, and the specific application of use for the culture medium of the invention. In some embodiments, a small molecule is present in the media at a concentration sufficient to induce pluripotency, improve the efficiency of reprogramming, or increase or maintain the potency of a cell.

In particular embodiments, preferred concentrations and combinations of the small molecules in the cell culture media of the invention are shown in Table 1. In particular embodiments of the cell culture media of the invention, the cell culture medium is "SMC4" medium, as described in Table 1. SMC4 medium comprises conventional human ESC media and the specific pathway modulators and additives as shown in Table 1. The components of the medium may be present in the medium in amounts within the optimal range for such components shown in Table 1, and are present at the optimal concentrations shown in Table 1. Embodiments of SMC4 medium may optionally comprise any one or more of the alternative medium and pathway modulators and activators shown in Table 2, in concentrations within the optimal ranges shown in Table 2, and in certain embodiments, in concentrations within the optimal concentration shown in Table 2. In particular embodiments of the media, SMC4 medium comprises soluble fibronectin, and is referred to throughout as "SMC4 + fibronectin".

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In some embodiments, the culture medium of the invention further comprises one or more of an Oct polypeptide, a Klf polypeptide, a Myc polypeptide, and a Sox polypeptide. In some embodiments, the culture medium does not comprise cells. In some embodiments, the culture medium further comprises cells, e.g., non-pluripotent cells, partially pluripotent cells, pluripotent cells, or mixed cell populations containing cells of various states of potency.

Table 1. **Cell culture components and additives**. The table below lists examples of molecules, and the signaling pathways they affect, that can be used to enhance the viability and pluripotency of cells undergoing single cell passage and cell sorting and enrichment procedures, and to enhance the reprogramming process as well as maintain pluripotent stem cells in an undifferentiated state.

SMC4 MEDIUM	Optimal Concentration	Optimal Concentration Range
CONVENTIONAL/BASAL HUMAN ESC MEDIUM FORMULATION		
DMEM/F12 (or DMEM, or HIGH GLUCOSE DMEM, or KNOCK-OUT DMEM)	1 x	1 x
L-GLUTAMINE	2 mM	1 to 10 mM
NON-ESSENTIAL AMINO ACIDS	1 x	0.5 to 5 x
2-MERCAPTOETHANOL	100 μM	10 to 500 μM
bFGF	10ng /mL	1 to 100 ng/mL
SPECIFIC PATHWAY INHIBITORS AND ADDITIVES		
ERK/MEK/MAPK • PD0325901	0.4 μM	.01 to 100 μM
Wnt/βcatenin/GSK • CHIR99021	1 μM	.01 to 100 μM
Rho/ROCK/Myosin II • THIAZOVIVN	5 μM	.01 to 500 μM
TGFB/ALK • SB431542	2 μM	.01 to 100 μM
EXTRACELLULAR MATRIX		
MATRIGEL™	As per manufacturer's recommendations	
GELATIN	As per manufacturer's recommendations	

SMC4 + Fibronectin refers to SMC4 medium, as described above, with soluble fibronectin in a concentration of about 5 μg/mL. Fibronectin may be present in the SMC4 medium in a concentration range of about 05 to 500 μg/mL.

Table 2. Particular alternative cell culture components and additives to enhance cellular pluripotency and viability on single cell passaging, cell sorting and culture on feeder-free systems

EXAMPLES OF PARTICULAR ALTERNATIVE MEDIUM AND PATHWAY MODULATORS AND ADDITIVES		
	Optimal Concentration	Optimal Range
ERK/MEK/MAPK		
• PD98059	10 μM	.01 to 500 μM
Wnt/βcatenin/GSK		
• Bio	2 μM	.01 to 100 μM
• Kenpaullone	5 μM	.01 to 100 μM
• XAV939	1 μM	.01 to 100 μM
Rho/ROCK/Myosin II		
• Y27632	10 μM	.01 to 500 μM
• Blebbistatin	5 μM	.01 to 500 μM
• Pyrintegrin	1 μM	.01 to 100 μM
TGFB/ALK		
• A-83-01	1 μM	.01 to 100 μM
• RepSox	1 μM	.01 to 100 μM
• TGFβ	250 ng/mL	1 to 25,000 ng/mL
PI3K/AKT/PTEN		
• Insulin	10 μg/mL	1 to 100 μg/mL
• IGF	10 ng /mL	1 to 100 ng/mL
• PDK2 Agonist	1 μM	.01 to 100μM
• PDK1 Agonist PS48	5uM	.01 to 500 uM
FGF/FGFR		
• bFGF	10ng /mL	1 to 100 ng/mL
• PD173074	0.1 μM	.001 to 10 μM
• SU5401	2 μM	.01 to 100 μM
EGF/EGFR		
• EGF	10 ng /mL	1 to 100 ng/mL
• AG1478	1 μM	.01 to 100 μM
p53		
• Pifithrin-α	5 μM	.01 to 500 μM
• Pifithrin-μ	2 μM	.01 to 500 μM
Hedgehog		
• Cyclopamine	1 μM	.01 to 100 μM
Notch/Delta		
• DAPT	1 μM	.01 to 100 μM
Chromatin & Histone Modifier/Acetylation/Methylation		
• VALPROIC ACID	0.5 mM	0.05 to 5 mM
• 5 AZA	1 mM	.01 to 100 mM
• Butyrate	1 mM	.01 to 100 mM
• Parnate	2 μM	.01 to 200 μM
• BIX01294	1 μM	.01 to 200 μM
• RG108	1 μM	.01 to 200 μM
• Tranylcypromine hydrochloride	100 μM	.001 to 10 mM
• trichostatin A	100 nM	.01 to 1000 nM
Antioxidant		
• Ascorbic Acid	10 μg/mL	0.01 to 1000 μg/mL
• Reduced Oxygen	5 %	1 to 20 %
• Tocotrienols	25 μM	.1 to 1000 μM
JAK/STAT		
• LIF	1,000 U/mL	10 to 10,000 U/mL
• IL6	10 ng/mL	.1 to 1000 ng/mL
BMP		
• BMP	10 ng/mL	.01 to 1000 ng/mL

• Dorsomorphin • LDN-193189		1 μM 2 μM	.01 to 100 μM .01 to 500 μM
PARP • Olaparib (AZD2281)		1 μM	.01 to 100 μM

KLF
    • Forskolin
    • AICAR
		10 μM
		0.5 mM
			.01 to 100 μM
			.001 to 100 mM

Ca2+ channel activator • Bay K 8644		1 μM	.01 to 100 μM
KNOCKOUT SERUM REPLACEMENT		20 %v/v	2 to 80 %v/v
XENO-FREE KNOCKOUT SERUM REPLACEMENT		20 %v/v	2 to 80 %v/v
Transferrin		1 mg/mL	.01 to 100 mg/mL
Albumin		.5 mg/mL	.1 to 10 mg/mL
N2		1 x	.1x to 10 x
B27		1 x	.1x to 10 x

Cytokines and growth factors

In some embodiments of the invention, the cell culture media of the invention is substantially free of cytokines and/or growth factors, and optionally is a feeder-free environment.

5   In other embodiments, the cell culture media contains supplements such as serums, extracts, growth factors, hormones, cytokines and the like.

Various growth factors and their use in culture media are well known in the art and include, for example, ECM proteins, laminin 1, fibronectin, collagen IV isotypes, proteases, protease inhibitors, cell surface adhesion proteins, cell-signaling proteins, cadherins, chloride

10   intracellular channel 1, transmembrane receptor PTK7, insulin-like growth factor, Inhibin beta A, inducers of the TGFβ/Activin/nodal signaling pathway, and Activin A. Cytokines used in the culture media may include, for example, one or more of the following: growth factors such as epidermal growth factor (EGF), acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1), insulin-like

15   growth factor 2 (IGF-2), keratinocyte growth factor (KGF), nerve growth factor (NGF), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), vascular endothelial cell growth factor (VEGF) transferrin, various interleukins (such as IL-1 through IL-18), various colony-stimulating factors (such as granulocyte/macrophage colony-stimulating factor (GM-CSF)), various interferons (such as IFN-.gamma.) and other cytokines having effects upon stem

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cells such as stem cell factor (SCF) and erythropoietin (Epo). These cytokines may be obtained commercially, for example from R&D Systems, Minneapolis, Minn., and may be either natural or recombinant. In some embodiments, for culture of a wide variety of mammalian cells, the basal media will contain FGF at a concentration of about 0.01-100 ng/ml, about 0.2-20 ng/ml, and in particular embodiments about 0.5-10 ng/ml. Other cytokines, if used, may be added at concentrations that are determined empirically or as guided by the established cytokine art.

Additional components that may be included in the present media are insulin (especially as insulin Zn^{++}) and transferrin. These additional ingredients, available commercially (for example, from Sigma-Aldrich, St. Louis, Mo.), may be formulated into the present media at concentration ranges of about 0.1 to about 100 μg/ml or about 1 to about 10 μg/ml. Additionally, recombinant insulin or zinc based salt of insulin may be substituted for animal- or human-derived insulin. Other ingredients or substitutes may be added to the supplement compositions as are known to those persons of ordinary skill in the art.

Cytokines and like components of the supplements may instead (or in addition) be included in the basal media. Such components are typically included with the supplement compositions as the supplement compositions are conventionally stored at about -20°C rather than the about 4°C temperature regularly used for storing basal media. Cytokines and like components may fair better at temperatures closer to -20°C.

Substrates for Use in the methods of the invention

Any suitable vessel or cell culture container may be used as a support for cell cultures in the basal media and/or the cell culture supplements. No substrate coating on the support is necessary. Coating the surface of a culture vessel with adhesion-promoting substrata (for example, collagens, fibronectins, RGD-containing polypeptides, gelatins, and the like) however promotes attachment of the cells and thereby may enhance the effect of the cell culture media and supplements disclosed herein. Suitable substrates for culturing and passaging cells are known in the art and include, without limitation, gelatin, Laminin, Fibronectin, Collagen, Elastin, osteopontin, mixtures of naturally occurring cell line-produced matrices such as

Matrigel™ and synthetic or man-made surfaces such as Polyamine monolayers and carboxy-terminated monolayers .

Feeder Free Environments

While cells have typically been cultured on feeder cells or in a culture environment pre-conditioned with feeder cells and containing fetal bovine serum, such environments may be unsuitable for producing cells for clinical and therapeutic use. For example, cells cultivated in such xeno-contaminated environments are generally considered unsuitable for human cell transplantation because the exposure to animal components may present a serious risk of immune rejection and transmitting unidentified pathogens to the treated patients, and could potentially reactivate animal retroviruses. Culture systems using animal-free culture medium, such as the feeder free environment of the invention, facilitate the production of clinical-grade cell lines, particulary hESC and iPSC cell lines.

In some embodiments of the invention, the feeder free environment of the invention is essentially free of human feeder cells, including without limitation human fibroblasts, keratinocytes, and embryonic stem cells, and is not pre-conditioned by feeder cells. In further embodiments of the invention, the feeder free environment is essentially free of animal feeder cells, and further, in some embodiments is not pre-conditioned with feeder cells. In particular embodiments of the invention, the feeder free environment is essentially free of both human and animal feeder cells, and in more particular embodiments the feeder free environment is essentially free of both human and animal feeder cells and is not pre-conditioned with feeder cells.

The feeder free cell culture media of the invention are used in the methods of the invention, including culturing of pluripotent cells, reprogramming of cells, single-cell dissociation of pluripotent cells, cell sorting of pluripotent cells, generation of a naïve pluripotent cell, and maintenance of an undifferentiated status of cells. In particular methods of the invention, the feeder free environment is used in methods to induce pluripotency, improve the efficiency of reprogramming, and/or increase or maintain the potency of a cell. In certain

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embodiments, the feeder free environment is substantially free of cytokines and growth factors, including bFGF.

Dissociation

Dissociation of cells into single cells, such as into a single cell suspension, can be accomplished by enzymatic or mechanical means. Any enzymatic agent known in the art to allow dissociation of cells into single cells may be used in the methods of the invention. In one embodiment of the invention, the dissociation agent is selected from Trypsin/EDTA, TrypLE-Select, Collagenase IV and Dispase.

A chelator, such as EDTA, Accutase, or AccuMax, may also be used, alone or in combination with an enzymatic agent, in dissociating cells in accordance with the methods of the invention. The dissociation agent may be dissolved in calcium and magnesium free PBS to facilitate dissociation to single cells.

To enhance the survival of the cells during and after dissociation, a survival promoting substance can be added (*e.g.*, growth factor, inhibitors of cellular pathways involved in cell death and apoptosis, or conditioned media). In some embodiments, cells cultured in conventional medium are dissociated and the single cells are placed in a cell culture of the invention having one or more small molecule inhibitors, such as the SMC4 media or SMC4 + fibronectin. The dissociated single cells may optionally be placed in a feeder free environment. In other embodiments, cells are cultured in a feeder free environment before dissociating and placed in a cell culture of the invention having one or more small molecule inhibitors, such as the SMC4 media or SMC4 + fibronectin, which may optionally be a feeder free environment.

Enzymatic dissociation to single cells may be supported by mechanical force. Alternatively, the dissociation agent may be only a mechanical force, such as by using a mechanical tool, such as a pipette or a sharpened micro capillary to detach the cells.

General techniques in cell culture and media collection are outlined in Large Scale Mammalian Cell Culture (Hu *et al.*, *Curr. Opin. Biotechnol.* 8:148, 1997); Serum-free Media (K. Kitano, Biotechnology 17:73, 1991); Large Scale Mammalian Cell Culture (*Curr. Opin. Biotechnol.* 2:375, 1991); and Suspension Culture of Mammalian Cells (Birch *et al.*,

Bioprocess Technol. 19:251, 1990). Other reading of interest includes Understanding Media (M. McLuhan, Mentor N.Y., 1964) and The Medium is the Massage (M. McLuhan & Q. Fiore, Bantam N.Y., 1967).

For further elaboration of general techniques useful in the practice of this invention, the practitioner can refer to standard textbooks and reviews in cell biology, tissue culture, and embryology. Included are "Teratocarcinomas and embryonic stem cells: A practical approach" (E. J. Robertson, ed., IRL Press Ltd. 1987); "Guide to Techniques in Mouse Development" (P. M. Wasserman *et al.* eds., Academic Press 1993); "Embryonic Stem Cell Differentiation *in vitro*" (M. V. Wiles, Meth. Enzymol. 225:900, 1993); "Properties and uses of Embryonic Stem Cells: Prospects for Application to Human Biology and Gene Therapy" (P. D. Rathjen *et al.*, al., 1993). Differentiation of stem cells is reviewed in Robertson, *Meth. Cell Biol.* 75:173, 1997; and Pedersen, Reprod. Fertil. Dev. 10:31,1998.

Enrichment and Depletion Strategies

The invention also provides strategies for enriching a population of cells for pluripotent cells as a method of increasing the efficiency of generating iPSCs. The enrichment strategy provides a method of deriving clonal iPSC colonies in a relatively short time, improving the efficiency of iPSC generation. The enrichment methods of the invention comprise sorting a population of cells that have been induced to reprogram to identify and obtain cells expressing markers of pluripotency, thereby obtaining a population of cells enriched for pluripotent cells. The cells to be sorted may have been induced to reprogram and may comprise a mixed population of cells undergoing reprogramming, so that the population comprises pluripotent cells, partially pluripotent cells, and non-pluripotent cells, such as fully differentiated cells. In one embodiment, the population of cells to be sorted has been induced to reprogram and expresses markers of pluripotency. In some embodiments, the cells are cultured after reprogramming is induced for about 4 to 30 days, about 4 to 24 days, about 6 to 22 days, or about 8 to about 12 days. An additional enrichment methodology involves the depletion of cells expressing markers of differentiation or non-pluripotency to obtain an enriched population of pluripotent cells.

The enrichment strategy of the invention includes obtaining a single cell suspension of the population of cells to be sorted. In one embodiment of the invention, a single cell suspension is obtained by dissociating the cells in the population and resuspending the cells. The dissociated cells may be resuspended in any suitable solution or media for maintaining cells or performing cell sorting. In particular embodiments, the single cell suspension contains one or more of a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor. In certain embodiments, the single cell suspension comprises a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor, and in certain particular embodiments, the TGFβ inhibitor is SB431542, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin.

In the enrichment process of the invention, cells are sorted to obtain pluripotent cells, or cells are depleted of non-reprogrammed or non-pluripotent cells, thereby obtaining a population of cells enriched for pluripotent cells. In one embodiment, a single cell suspension is prepared, and then the single cells are prepared for sorting, such as by staining for markers of pluripotency using, *e.g.*, appropriate antibodies. Cells may be sorted by any suitable method of sorting cells, such as by magnetic bead or flow cytometry (FACS) sorting.

Cells may be sorted based on various markers of pluripotency, including expression of Oct, Sox, Nanog, SSEA3/4; TRA1-60/81; TRA1-85, TRA2-54, GCTM-2, TG343, TG30, CD9, CD29, CD133/prominin, CD140a, CD56, CD73, CD105, CD31, CD34, OCT4, KLF4, SSEA1 (Mouse), and as demonstrated in the present invention, CD30 and CD50. In various embodiments, cells are sorted based on at least two, at least three, or at least four markers of pluripotency. In certain embodiments, cells are sorted based on expression of SSEA4, and in certain particular embodiments based on expression of SSEA4 in combination with TRA181 or TRA160. In certain embodiments cells are sorted based on SSEA4, Tra181 or Tra160 and CD30. In certain embodiments, cells are initially depleted for non-reprogrammed cells using surface markers of differentiating cells, which may include but are not limited to, CD13, CD26, CD34, CD45, CD31, CD46, or CD7, and and then enriched for pluripotent markers such as SSEA4, Tra181 and CD30.

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After sorting to obtain cells positive for pluripotency markers, the desired cell fraction is a population of cells enriched for pluripotent cells. The population enriched for pluripotent cells may be placed in a cell culture system, such as conventional hESC media or the cell culture media of the invention. The cell culture system may be supplemented with feeder cells, or optionally be a feeder free environment. In some embodiments, the sorted cells expressing markers of pluripotency are placed in a feeder cell supplemented culture system and then transferred to a feeder free environment. The cell culture system may be supplemented with one or more of the specific pathway modulators and additives shown in Table 1. In one embodiment, the cell culture medium is a feeder free environment and comprises at least one of a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor; in particular embodiments, the cell culture media comprises a TGFβ inhibitor, a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor; and in certain embodiment, the TGFβ inhibitor is SB431542, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin. In other particular embodiments of the invention, the cell culture system is a feeder free environment comprising a Matrigel™ coated tissue plate, conventional hESC medium, and the specific pathway modulators shown in Table 1. In one embodiment, the cell culture system comprises the SMC4 medium described in Table 1, optionally combined with any of the alternative medium and pathway modulators shown in Table 2.

The enriched cell population may be cultured in the cell culture systems described herein to obtain iPSC colonies, typically appearing about 3 to about 25 days after sort; about 5-9 days post sort, or about 5-7 days post sort. iPSC colonies can be picked or sorted for clonal expansion. Using the enrichment strategy of the invention, the cell population is enriched 3-fold for pluripotent cells.

The invention also provides methods of depleting a population of cells of undesirable cells. In some embodiments, a population of cells, such as a population of cells undergoing reprogramming or a population of pluripotent cells, is depleted of differentiated cells. In the method of the invention, a population of pluripotent cells or cells induced to reprogram can be depleted of cells having cells surface markers of differentiated cells. The population of cells can be sorted based on surface markers of differentiating cells, such as CD13, CD26, CD34,

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CD45, CD31, CD46, or CD7, and cells expressing the markers of differentiating cells can be removed from the cell population to obtain a cell population enriched in pluripotent cells. CD13 is used as a surface marker of differentiating cells in particular embodiments of the invention.

In other embodiments, a population of cells induced to differentiate, such as a population of cells induced to differentiate into a desired lineage, is depleted of pluripotent cells to obtain a population of differentiating or differentiated cells. In some embodiments, the population of differentiated cells comprises a population of cells, such as ESCs or iPSCs, that has been induced to differentiate into a specific lineage. A population of cells may be depleted of pluripotent cells using the sorting techniques described above, such as sorting cells in the population according to magnetic beads or FACs based on markers of pluripotency. In some embodiments, a population of cells comprising differentiated cells is sorted by FACs using pluripotency markers, and a fraction is obtained that is depleted of cells expressing pluripotency markers. In other embodiments, a population of cells is sorted by FACs based on markers of differentiation, such as lineage-specific markers like CD13, CD26, CD34, CD45, CD31, CD46, or CD7, to obtain a fraction depleted of markers of pluripotency. CD13 is used as a surface marker of differentiating cells in particular embodiments of the invention.

The practice of the present invention will employ, unless indicated specifically to the contrary, conventional methods of chemistry, biochemistry, organic chemistry, molecular biology, microbiology, recombinant DNA techniques, genetics, immunology, cell biology, stem cell protocols, cell culture and transgenic biology that are within the skill of the art, many of which are described below for the purpose of illustration. Such techniques are explained fully in the literature. *See, e.g.,* Sambrook, *et al., Molecular Cloning: A Laboratory Manual* (3rd Edition, 2001); Sambrook, *et al., Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); Maniatis *et al., Molecular Cloning: A Laboratory Manual* (1982); Ausubel *et al., Current Protocols in Molecular Biology* (John Wiley and Sons, updated July 2008); *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology*, Greene Pub. Associates and Wiley-interscience; Glover, *DNA Cloning: A Practical Approach*, vol. I & II (IRL Press, Oxford, 1985); Anand, *Techniques for the Analysis of Complex Genomes*, (Academic Press, New York, 1992); Guthrie and Fink, *Guide to Yeast Genetics and Molecular Biology*

(Academic Press, New York, 1991); *Oligonucleotide Synthesis* (N. Gait, Ed., 1984); *Nucleic Acid Hybridization* (B. Hames & S. Higgins, Eds., 1985); *Transcription and Translation* (B. Hames & S. Higgins, Eds., 1984); *Animal Cell Culture* (R. Freshney, Ed., 1986); Perbal, *A Practical Guide to Molecular Cloning* (1984); Fire *et al.*, *RNA Interference Technology: From Basic Science to Drug Development* (Cambridge University Press, Cambridge, 2005); Schepers, *RNA Interference in Practice* (Wiley-VCH, 2005); Engelke, *RNA Interference (RNAi): The Nuts & Bolts of siRNA Technology* (DNA Press, 2003); Gott, *RNA Interference, Editing, and Modification: Methods and Protocols* (Methods in Molecular Biology; Human Press, Totowa, NJ, 2004); Sohail, *Gene Silencing by RNA Interference: Technology and Application* (CRC, 2004); Clarke and Sanseau, m*icroRNA: Biology, Function & Expression* (Nuts & Bolts series; DNA Press, 2006); *Immobilized Cells And Enzymes* (IRL Press, 1986); 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); Harlow and Lane, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1998); *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 andCC Blackwell, eds., 1986); Riott, *Essential Immunology*, 6th Edition, (Blackwell Scientific Publications, Oxford, 1988); *Embryonic Stem Cells: Methods and Protocols* (Methods in Molecular Biology) (Kurstad Turksen, Ed., 2002); *Embryonic Stem Cell Protocols: Volume I: Isolation and Characterization* (Methods in Molecular Biology) (Kurstad Turksen, Ed., 2006); *Embryonic Stem Cell Protocols: Volume II: Differentiation Models* (Methods in Molecular Biology) (Kurstad Turksen, Ed., 2006); *Human Embryonic Stem Cell Protocols* (Methods in Molecular Biology) (Kursad Turksen Ed., 2006); *Mesenchymal Stem Cells: Methods and Protocols* (Methods in Molecular Biology) (Darwin J. Prockop, Donald G. Phinney, and Bruce A. Bunnell Eds., 2008); *Hematopoietic Stem Cell Protocols* (Methods in Molecular Medicine) (Christopher A. Klug, and Craig T. Jordan Eds., 2001); *Hematopoietic Stem Cell Protocols* (Methods in Molecular Biology) (Kevin D. Bunting Ed., 2008) *Neural Stem Cells: Methods and Protocols* (Methods in Molecular Biology) (Leslie P. Weiner Ed., 2008); Hogan *et al.*, *Methods of Manipulating the Mouse Embyro* (2nd Edition, 1994); Nagy *et al.*, *Methods of Manipulating*

the Mouse Embryo (3rd Edition, 2002), and *The zebrafish book. A guide for the laboratory use of zebrafish* (Danio rerio), 4th Ed., (Univ. of Oregon Press, Eugene, Oreg., 2000).

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise.

Throughout this specification, unless the context requires otherwise, the words "comprise", "comprises" and "comprising" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of." Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications and publications to provide yet further embodiments.

These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the claims below, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

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EXAMPLES

EXAMPLE 1

EXPERIMENTAL METHODS

A.   Cell Culture of Pluripotent Stem Cells

5   Prior to feeder-free adaptation, human induced pluripotent stem cells (hiPSCs) were maintained on feeder cells (mitomycin C treated mouse embryonic fibroblast (MEF) cells (Millipore)), and cultured with conventional medium. As used in this application, "conventional medium" refers to basal human embryonic stem cell (hESC) medium containing DMEM/F12 (Mediatech), 10ng/mL bFGF (Invitrogen), 20% v/v knockout serum replacement (Invitrogen),

0   1% v/v non-essential amino acids (Mediatech), 2mM L-glutamine (Mediatech) and 100μM β-mercaptoethanol (Invitrogen). Conventional medium is also described in the first sections of Table 1. hiPSCs were passaged every 5-7 days by mechanically cutting and scrapping colonies into small pieces using a fine tip glass pipette (clump passaging), collected and dilute passaged 1:3-1:6 onto freshly seeded mitomycin C treated MEF cells with daily addition of hESC

5   medium. Cell cultures were maintained in a humidified incubator set at 37°C and 5% CO_2. Culturing cells in conventional medium with feeder cells and using clump passaging is referred to herein as "conventional culture".

For single cell dissociation, hiPSCs were washed once with phosphate buffered saline (PBS) (Mediatech) and treated with Accutase (Millipore) or TrypL (Invitrogen) for 3-5

20   min at 37°C followed by pipetting to break into single cells. The single cell suspension was then mixed in equal volume with conventional medium as described above, spun down at 300 g for 5 min and resuspended in SMC4 medium or SMC4 + fibronectin medium. In most cases, the single cell dissociated cells were maintained in SMC4 medium composed of conventional hESC medium supplemented with various small molecules and additives, including 0.4μM

25   PD0325901, 1μM CHIR99021, 5μM Thiazovivin and 2μM SB431542 (all Biovision). Small molecules were maintained at a stock concentration of 5-25μM in DMSO at -20°C prior to the addition to media. All working media were maintained in 4°C for up to 4 weeks. Of the ROCK inhibitors, culture with Thiazovivin was preferred over Y27632for maintaining cells in an undifferentiated state.

After resuspension in the appropriate medium, the cells were transferred to feeder-free tissue culture plates (BD Falcon) that were previously coated with Matrigel™ (1:25 dilution; BD Biosciences) for 1-2 hrs in 37°C. In this format, cells routinely received fresh medium every other day and were passaged when confluency had reached 66-75%, which normally occurred 4-5 days post passage. With each passage cells were re-dissociated into single cells and transferred to a new tissue culture plate coated with Matrigel™ (BD Biosciences) at a dilution passage of 1:5-1:10. For defined, growth-factor-free culture, cell suspensions were added to tissue culture plates previously coated with 1% Gelatin (Mediatech). Cells were maintained and passaged as described above, except that the SMC4 medium was substantially free of all cytokines and growth factors, including bFGF.

For the purpose of freezing, cells were dissociated into single cells, resuspended in SMC4 + fibronectin supplemented with 10% v/v DMSO (Mediatech) and placed into cryovials (Nalgene). Once capped, cryovials were placed inside a Mr. Frosty (Nalgene) and kept overnight at -80°C. The next day cryovials were transferred to liquid nitrogen for long-term storage. To thaw, frozen cryovials were placed in 37°C water bath for approximately 1-2 min, until most of the ice had melted. The thawed cell solution was then gently mixed with fresh conventional hESC medium and spun down at 300 g for 5 min. The cell solution was resuspended in SMC4 + fibronectin medium and transferred onto Matrigel™ (BD Biosciences) coated tissue culture plate. As with all other cell culture incubations, cells were maintained in a humidified incubator set at 37°C and 5% CO_2.

B. **Induction of Reprogramming**

To initiate the reprogramming process, ectopic expression of reprogramming factors (in variable combinations of human Oct4, Sox2, Klf4, c-Myc, Lin28, and Nanog) was achieved using lentiviral transduction or other methods such as protein only treatment. In most cases, the starting cells were plated at 10% confluency (*i.e.*, 1x10^5 cells per well of a 6-well plate) on a gelatin (Mediatech) coated surface. For the method of viral infection, freshly collected lentivirus was added to the starting cells at a dilution of 1:2, supplemented with 4µg/mL polybrene (Millipore), and spin-infected at 650 g at 32°C for 1.5 hrs. The culture was transferred to 37°C and 5% CO_2 for an additional 7 hrs. After the completion of the incubation,

the cells were washed three times with PBS and fed with fresh medium. With difficult to infect cells, such as IMR90 fibroblasts in feeder-free culture systems, this process was repeated one more time, 48 hrs post the initial infection. For non-genetic methods of inducing reprogramming such as the use of direct protein application to the cells, protein mixtures, or cocktails, consisting of reprogramming factors at 8μg/mL were added to cell solution and maintained for 24 hrs prior to medium change. This step was repeated for an additional two to four times. All starting cells were cultured in their own respective somatic cell medium until day 4 post initial protein addition, at which point the medium was switched to one part somatic cell medium and one part conventional hESC medium. Upon confluency (usually between days 4-6) the cells were trypsinized, mixed with equal part culture medium, spun down at 300 g for 5 min, resuspended in 1:1 somatic/conventional hESC medium and expanded 1:4-1:6 into a larger culture plate. For example, cells in two wells of a 6-well plate are usually expanded onto a 10 cm dish. The next day following the expansion, the medium is completely switched to conventional hESC medium. Once the expanded cells reach confluency (usually between days 8-12) they will be processed for enrichment (see Unique Population Enrichment). In all cases the medium was routinely changed every other day.

C.   Unique Population Enrichment

After the starting cells have been induced to reprogram with various strategies including individual lentivirus constructs or polycistronic vectors containing Oct4 and/or Klf4 and/or Sox2 and/or Myc and cultured for approximately 8-12 days (see above), cells are dissociated into single cells (see Cell Culture Of Pluripotent Stem Cells) and stained with various surface markers of pluripotency, markers of somatic cells and/or markers of incomplete reprogramming. Briefly, dissociated cells were resuspended in staining solution containing Hanks' Balanced Salt Solution (Invitrogen), 4% fetal bovine serum (Invitrogen) and 10mM Hepes (Invitrogen) and kept on ice. Per recommended manufacturers' dilution, conjugated primary antibodies were added to the cell solution and the solution was incubated on ice for 15 min. The cell solution was washed and resuspended in staining buffer and maintained on ice. At this point, various enrichment/depletion strategies were taken, including Fluorescent Activated

Cell Sorting (BD Biosciences, see below) and Magnetic Cell Sorting (Miltenyi Biotec, see below).

Flow cytometry sorting was performed on FACS Aria (BD, Biosciences). Primary antibodies used included SSEA4 (BD Biosciences), Tra-181 (Biosciences), Tra-161 (BD Biosciences), CD30 (BD Biosciences), and CD50 (BD Biosciences), as specified. The sorted cells were then spun down and resuspended in SMC4 + fibronectin medium and transferred to Matrigel™ coated tissue culture plates. When sorted into microwells, *i.e.*, 96 well plates, the plates were spun down for 2 min at 300 g. The SMC4 + fibronectin medium was replaced every other day for 3-4 days. After 3-4 days SMC4 + fibronectin medium was typically replaced with SMC4 medium for the remaining time in culture. Colony formation was typically seen 7-9 days post sort. Flow cytometry analysis was performed on Guava EasyCyte 8HT (Millipore).

MACS Microbeads (Miltenyi Biotec) separation was performed according to protocol. Briefly, cells were dissociated into single cells (See Cell Culture of Pluripotent Stem Cells) and stained with appropriate FITC-conjugated primary antibodies, including SSEA4 (BD Biosciences), Tra-1-81 (BD Biosciences), Tra-160 (BD Biosciences), CD30 (BD Biosciences), and CD50 (BD Biosciences), as specified. Cells were then magnetically labeled with Anti-FITC Microbeads (Miltenyi Biotec). The labeled cell suspension was then loaded onto a LS MACS Column (Miltenyi Biotec). The collected cells from either positively or negatively selected fractions were spun down at 300 g for 5 min and resuspended in SMC4 + fibronectin and transferred to Matrigel™ (BD Biosciences) coated tissue culture plates. The following day, fresh medium was added to the culture and subsequently replaced every other day. After 3-4 days, SMC4 + fibronectin medium was typically replaced with SMC4 media for the remaining time in culture. The colonies typically appeared 5-7 days post sort.

D.   Alkaline Phosphatase Staining

Cells were fixed in 4% v/v paraformaldehyde (Alfa Aesar) for 30 sec, washed three times with PBS and stained with Alkaline Phosphatase Staining Kit (Sigma-Aldrich). Briefly, 1 mL Sodium Nitrite Solution was added to 1 mL FRV-Alkaline Solution, mixed and incubated at 25°C for 2 min. The solution was then mixed with 45 mL of H_2O followed by the addition of 1mL Naphthol AS-BI Alkaline Solution. The alkaline-dye mixture was added to the

fixed cells and incubated at 25°C for 15 min followed by a PBS wash. The cells were then scored for the presence of alkaline phosphatase.

E.   Immunofluorescence Staining

Cells were fixed using 4% v/v paraformaldehyde (Alfa Aesar) for 15 min, washed

5   three times with PBS containing 0.2% v/v Tween (PBST) (Fisher Scientific) and permeablized using 0.15% v/v TritonX-100 (Sigma-Aldrich) in PBS for 1 hr at 25°C. After permeabilization, cells were blocked with 1% v/v BSA (Invitrogen) in PBST (PBSTB) (Fisher Scientific) for 30 min at 25°C. After gentle removal of PBSTB, cells were incubated with primary antibody in PBSTB overnight at 4°C. Primary antibodies used in this study include Nanog (Abcam), Tra-1-

0   60 (BD Biosciences), Tra-181 (BD Biosciences), SSEA4 (BD Biosciences), β-III Tubulin (R&D Systems), α-Smooth Muscle Actin (Sigma) and Sox17 (R&D Systems). After the overnight incubation, cells were washed three times with PBST and stained with secondary antibody (Alexa 488 or 555; Invitrogen) diluted 1:200 in PBSTB for 1 hr at 25°C. The cells were washed three times in PBST and stained with Hoechst dye (Invitrogen). Images of the stained cells were

5   captured using fluorescence microscopy and CCD camera.

F.   Induction of Differentiation and Teratoma Formation

Feeder-free iPSCs were differentiated as both monolayers and as embryoid bodies. For monolayer differentiation, iPSCs were allowed to reach near confluency prior to switching to differentiation medium as cells usually reduce their proliferation upon

20   differentiation. Briefly, upon confluency, SMC4 medium was switched to differentiation medium containing DMEM/F12 (Mediatech), 20% fetal bovine serum (Invitrogen), 1% non-essential amino acids (Mediatech), 2 mM L-glutamine (Mediatech) and 100 μM β-mercaptoethanol. Once the medium was switched, the iPSCs were allowed to differentiate for 14 days. Medium was changed every 2-3 days. For embryoid body ("EB") formation and

25   differentiation, hiPSCs were single cell dissociated with Accutase (Millipore) and resuspended in differentiation medium to a final concentration of 75,000 cells/mL and 5 uM Thiazovivan was added. Cells were seeded in 100μL/well to V-bottom 96-well non-tissue culture plate (Nunc) and centrifuged at 950g for 5 min. The following day compact "ball-like clumps" were transfer to ultra-low binding 6-well plate (Corning) using P1000 at approximately 30-40 EBs/well. After

7 days, transfer EBs were transferred at 1:1 to Matrigel coated 6-well plate. After 3 weeks in culture, cells were fixed and stained.

Teratoma grafting and analyses was conducted by Applied Stem Cells (Menlo Park, CA). Briefly, 1-2 million single cell dissociated hiPSCs were mixed in 100 uL SMC4 media supplemented medium and 100 uL Matrigel and introduced to the renal capsule and testis of Beige SCID mice. The developed teratomas were harvested, sectioned and analyzed for various differentiated cell types and structures.

G.   RT-qPCR and qPCR Analysis

RNA was isolated using the PicoPure RNA Isolation kit (MDS Analytical Technologies), and 0.5 μg RNA was used to generate first strand cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). Relative gene expression levels were determined using the TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and the FAM-labeled TaqMan probes listed below in Table 3.

Table 3: ABI Primers and Probes.

ABI Primers and Probes	
Symbol	**Assay ID**
Endogenous SOX2	Hs00602736_s1
Endogenous LIN28	Hs00702808_s1
Endogenous MYC	Hs00905030_m1
ZFP42 (REX1)	Hs00399279_m1
DPPA2	Hs00414521_g1
DPPA4	Hs00216968_m1
DNMT3B	Hs01003405_m1
GAPDH	Hs99999905_m1

Custom-Made Primers and Probes			
Gene	**Forward Primer**	**Reverse Primer**	**Probe**
Transgenic Oct4	CTGGTTGGAGGGAAGGTAATCTAG (SEQ ID NO:1)	TTTTGTAATCCAGAGGTTGATTGTTC (SEQ ID NO:2)	CCCCGACGCGTCT (SEQ ID NO:3)
Transgenic Klf4	GCCTTACACATGAAGAGGCATTT (SEQ ID NO:4)	TTTTGTAATCCAGAGGTTGATTGTTC (SEQ ID NO:2)	CCCCGACGCGTCT (SEQ ID NO:3)
Transgenic Myc	TCTTGTGCGTAACTCGAGTCTAGAG (SEQ ID NO:5)	TTTTGTAATCCAGAGGTTGATTGTTC (SEQ ID NO:2)	CCCCGACGCGTCT (SEQ ID NO:3)
Transgenic Lin28	CCGGAGGCACAGAATTGAC (SEQ ID NO:6)	TTTTGTAATCCAGAGGTTGATTGTTC (SEQ ID NO:2)	CCCCGACGCGTCT (SEQ ID NO:3)
Transgenic Sox2	CACTGCCCCTCTCACACATG (SEQ ID NO:7)	TTTTGTAATCCAGAGGTTGATTGTTC (SEQ ID NO:2)	CCCCGACGCGTCT (SEQ ID NO:3)
Transgenic Nanog	CATGCAACCTGAAGACGTGTAA (SEQ ID NO:8)	TTTTGTAATCCAGAGGTTGATTGTTC (SEQ ID NO:2)	CCCCGACGCGTCT (SEQ ID NO:3)
Endogenous Oct4	GGGTTTTTGGGATTAAGTTCTTCA (SEQ ID NO:9)	GCCCCCACCCTTTGTGTT (SEQ ID NO:10)	TCACTAAGGAAGGAATTG (SEQ ID NO:11)
Endogenous Klf4	AGCCTAAATGATGGTGCTTGGT (SEQ ID NO:12)	TTGAAAACTTTGGCTTCCTTGTT (SEQ ID NO:13)	AGTCTTGGTTCTAAAGGTACC (SEQ ID NO:14)
Endogenous Nanog	TGATGCCCATCCAGTCAATCT (SEQ ID NO:15)	CCTCGCTGATTAGGCTCCAA (SEQ ID NO:16)	ATGGAGGGTGGAGTATG (SEQ ID NO:17)

H.   Gene Expression Analysis

Total RNA was isolated from cells using a Pico Pure RNA Isolation Kit (Molecular Devices, Sunnyvale, CA). In brief, biotinylated aRNA was prepared using the standard protocol for MessageAmp II aRNA Amplification Kit (Applied Biosystems/Ambion,

Austin, TX) utilizing the optional Second Round Amplification and then transcribed into biotin labeled aRNA using MessageAmp II Biotin Enhanced Kit (Applied Biosystems/Ambion, Austin, TX) using the standard protocol. Biotin labeled aRNA was purified and fragmented according to Affymetrix recommendations. 20 µg of fragmented aRNA were used to hybridize to the Human Genome U133 Plus 2.0 chips (Affymetrix Inc. Santa Clara, CA) for 16 hrs at 45°C. The arrays were washed and stained in the Affymetrix Fluidics Station 450 and scanned using the Affymetrix GeneChip Scanner 3000 7G. The image data were analyzed using Affymetrix Expression Console software using default analysis settings. Arrays were normalized by log scale robust multi-array analysis (RMA) and visualized in Spotfire for Genomics 3.1 (Tibco Spotfire, Palo Alto, CA).

I.   Karyotype Analysis and Copy Number Variation Analysis

Cytogenetic analysis was performed on twenty G-banded metaphase cells by Cell Line Genetics located in Madison, WI.

High resolution comparative genomic hybridization (NimbleGen 12x135k) and subsequent copy number variation analysis was conducted by WiCell (Madison, WI).

EXAMPLE 2

CELL CULTURE CONDITIONS AND METHODS TO ENABLE FEEDER-FREE CULTURE ENVIRONMENTS AND ENZYMATIC SINGLE CELL DISSOCIATION AND PASSAGING OF PLURIPOTENT STEM CELLS

The present example relates to the culturing and dissociation of pluripotent cell populations. Such cell populations include but are not limited to, embryonic stem cells (ESC) and induced pluripotent cells such as those generated through somatic cell nuclear transfer (SCNT) or via the introduction of pluripotency factors – induced pluripotent stem cells (iPSC). Pluripotent stem cell culture conditions have traditionally included the use of feeder-cells that are rendered mitotically inactive via irradiation or mitomycin-C treatment but provide growth factors and nutrients required for the support of stem cell cultures. The culturing of stem cell populations without the use of feeder cells would be advantageous for research and industrial applications where homogeneous populations of the stem cells are required or where scaled,

industrial activities require xenogeneic-free, defined culture conditions for a stem cell product. In the present example several small molecule modulators of specific cell signaling pathways were tested to establish if individual or combinations ("cocktails") of small molecules could be used to enhance the culturing of pluripotent cells in feeder-free systems.

5   When pluripotent cell populations were cultured without the use of feeders but instead using more defined extracellular matrix such as Matrigel™ in conventional hESC cell culture media (such as the conventional/basal medium formulation described in the first section of Table 1) cell viability and pluripotency was not supported (Figure 1). However, the use of a small molecule inhibitor of Rock kinase improved the cell viability under these conditions.

0   Further, the use of small molecule inhibitors of the MAP Kinase, TGFβ and Wnt/β-catenin pathways maintained the pluripotent nature of cells in the absence of feeders, although changes in cell viability may be noticed. The combination of Rock kinase inhibitor, in combination with MEK, TGFβ and GSK3 inhibitors maintained both viability and pluripotency of pluripotent stem cells when cultured in feeder-free environments.

5   Pluripotent cells such as ESC or iPSCs typically grow as clumps. Traditionally these cells have been expanded and passaged by manually picking colonies with the morphology recognized by a researcher skilled in the art. Such procedures are described in Example 1 of this document (described as clump passaging). The picked colony is then mechanically broken up and the dissociated cells are replated. Rapid expansion of a pluripotent cell population would

20   benefit from the use of enzymatic, single-cell passaging. Enzymes such as trypsin and accutase are commonly used for the single cell dissociation of cells during passaging.

     In a specific demonstration, iPSC cells showed a significant drop in viability when enzymatically-passaged and seeded as single cells in feeder-free environments, as can be seen by 7AAD incorporation in Figure 3B. By using the culture platform of the present

25   invention comprising the media compositions such as those listed in Table 1), pluripotent cell populations were cultured in feeder-free culture and enzymatically dissociated for single cell passage, with vastly improved cell viability and maintenance of pluripotency. More specifically, by using combinations of conventional/basal hESC media formulation and modulators of cellular signaling pathways such as the MAP kinase pathway, TGFβ pathway, Wnt/β-catenin pathway

and Rho/Rock pathway, pluripotent stem cell viability and pluripotency were maintained during applications that require single-cell dissociation and feeder-free culture.

As shown in Figure 2, iPSCs generated on feeder-cell cultures and using clump passaging were single cell dissociated using enzymes such as accutase and plated as single cells in feeder-free systems such as on Matrigel™ with the use of SMC4 or SMC4 + fibronectin media compositions, as described in Table 1. Cell viability was maintained only when dissociated cells were placed in media containing combinations of the small molecules described in these media are used. The use of conventional medium, resulted in massive cell loss when pluripotent cells are enzymatically dissociated into single cells and plated in feeder-free environments. Once cells are adapted to feeder-free cultures and single cell passaging they were continually maintained in this way with the use of SMC4 medium. Full characterization of hiPSC cells adapted to feeder-free, single cell passaging conditions is shown in Figure 3. Pluripotent status of these cells was maintained after multiple passages: pluripotent markers were identified by immunofluorescence and gene expression. Further, about 99.8% of cells maintained SSEA4 and Tra1-81 positive staining as measured by flow cytometry. Gene expression profiles of these cells was similar to human ESC cultured on feeders. Transgene silencing in the hiPSC was maintained, karyotype was both normal and identical to the same clone grown in feeder-cell culture. Furthermore, the hiPSCs passaged and maintained in this way differentiated to the three germ layers as demonstrated by both in vitro differentiation and teratoma formation (Figures 3I and 3J).

EXAMPLE 3

METHODS AND CULTURE CONDITIONS TO ENABLE SINGLE CELL SORTING OF PLURIPOTENT CELLS WHILE MAINTAINING PLURIPOTENCY AND CELL VIABILITY

As described in Example 2, stem cell cultures of ESCs or iPSCs are routinely cultured on feeder cells and passaged by manual selection of cell colonies which are then mechanically dissociated prior to replating. The skilled researcher is able to recognize stem cell colonies having pluripotent, non-differentiated characteristics based on colony morphology and use this as a method of selecting pluripotent cells. Thus, pluripotent populations or populations with the desired characteristics can be picked and manually enriched from a population of cells

where some cells have less desirable characteristics, such as cells showing signs of differentiation in culture or clumps of dead cells. This process is laborious and dependent upon skilled researchers to pick the desired cell populations. The use of a cell-enrichment or sorting technology where cells are selected individually on the basis of a desired characteristic would

5   therefore be of great benefit to the field. Such an enrichment step using currently available techniques such as magnetic activated cell sorting (MACS) or Fluorescent Activated Cell Sorting (FACS) would require the enzymatic passaging of pluripotent cell populations into single cell format prior to enrichment and seeding back into culture. Further, the use of feeder-supported cultures would be less desirable for these techniques, necessitating the use of feeder-free culture

0   systems.

In particular embodiments, using media compositions of the invention as described in Table 1, and methods described in Examples 1 and 2, pluripotent cell populations were single cell dissociated, enriched using cell sorting processes such as Magnetic Activated Cell Sorting (MACS) or Fluorescent Activated cell sorting (FACS), and seeded on feeder-free

5   culture without loss of pluripotency or cellular viability.

In a specific example, as shown in Figure 4, a population of single cell dissociated pluripotent cells maintained in SMC4 medium (Table 1) was selected and sorted by FACS based on cells positive for the cell surface markers $SSEA4^+/Tra181^+$. These surface antigens are commonly used as markers for pluripotency. The sorted double-positive population was then

20   transferred to feeder-free culture (Matrigel™ ECM coating) and allowed to grow in either SMC4 medium (Table 1) or SMC4 + fibronectin medium for 2-4 days prior to switching back to SMC4 medium (Table 1). Cell division was seen within 24 hours of the sort and cells were near confluent by day 5 post sort. The sorted cells were single cell cultured for an additional 5 passages and shown to not only express markers of undifferentiated status but also represented a

25   substantially pure population of pluripotent cells as judged by double staining flow cytometry for $SSEA4^+/Tra181^+$ cells (Figure 4B and 4C).

In an example of enriching and sorting of pluripotent stem cells during culture, cultures containining both pluripotent and differentiated cells were enriched for cells positive for

Tra181, and then passaging of Tra181$^+$ cells was continued to maintain a pure population of pluripotent stem cells (Figure 17C).

The efficiency of single cell sorting of pluripotent cells was investigated. As can be seen in Figures 4 D and E various numbers of post-sort events, as measured by the FACS instrument, were plated in feeder-free culture systems using either SMC4 medium or SMC4 + fibronectin medium, and the numbers of alkaline phosphatase-positive cells were scored. Alkaline phosphatase is an early indicator of pluripotent colony formation. The quantified post-sort seeding efficiency, as shown by the number of alkaline positive colonies per number of cells seeded, was seen to be approximately 8-10% in this system (Figure 4E). In a further demonstration of the potential of single cell sorting in the maintenance of pluripotent cell populations, an iPSC culture was single cell dissociated, labeled with fluorescently-conjugated antibodies specific for the pluripotency markers SSEA4 and Tra181, applied to a FACS and selected based on these markers. The selected SSEA4$^+$/Tra181$^+$ cells were plated directly from the FACS instrument into a 96-well plate at events/well from 1 to 9. As few as 1 event per well produced clonal alkaline phosphatase-positive colonies (Figure 4F and 4G). Thus, such a single cell sorting system was used for the clonal selection of pluripotent cells based on preferred characteristics associated with certain cell surface markers.

EXAMPLE 4

METHODS AND CONDITIONS TO ENABLE EFFICIENT REPROGRAMMING OF CELLS TO A PLURIPOTENT STATE IN FEEDER-FREE CULTURE SYSTEMS

The use of iPSCs for industrial and/or clinical applications necessitates the generation, selection and maintenance of the cells in fully defined culture conditions, specifically xenogeneic-free conditions. Thus, cellular reprogramming in feeder-free culture conditions is highly desirable. However, while fibroblasts and keratinocytes are the most commonly used cell type for reprogramming due to access via skin biopsy or hair follicle, the efficiency of reprogramming for these cell types is extremely low, and an efficient method for reprogramming these cells in feeder-free cultures has yet to be demonstrated.

As described in Example 2, the use of conventional hESC stem cell media in the presence of inhibitors of cell signaling pathways – specifically the MAP kinase pathway, TGFβ pathway, Wnt/β-catenin pathway and Rho/Rock pathways enabled the growth and maintenance of pluripotent cultures in the absence of feeder-cells and expansion of these cultures using enzymatic, single-cell passaging. In the present example, iPSC cells were generated in culture systems devoid of feeder-cells. Specifically, human fibroblasts were infected with virus expressing the pluripotency factors Oct4, KLF4, Sox2 and C-myc. Reprogramming protocols were carried out as described in Example 1 with cells plated on Matrigel™™ rather than feeder cells.

A comparison was made of feeder-free cellular reprogramming using either conventional hESC stem cell medium or conventional hESC medium supplemented with the specific pathway modulators listed in the SMC4 medium (Table 1). As can be seen in Figure 5, 20 days after the induction of reprogramming using individual lentivirus expression Oct4, Klf4, Sox2 and Myc many iPSC-like colonies were seen in the feeder-free cultures supplemented with the SMC4 medium whereas few or no colonies were seen in the cultures maintained in conventional hESC medium alone. Subsequent characterization of the colonies generated in SMC4 medium showed many of them to be true iPSC colonies. The cells were determined to be pluripotent by expression of pluripotency markers Oct4, Nanog, Sox2, KLF4, SSEA4 and TRA 1-81 using immunofluorescence, flow cytometry and gene expression profiling (Figures 13C-E). Further, cells were shown to effectively differentiate to all three germ layers when cultured in differentiation medium. Thus, the efficient generation of iPSCs in a feeder-free culture system was demonstrated by the current invention. The efficiency of this approach in comparison to other methods of reprogramming is described in Example 8 and Table 4.

EXAMPLE 5

CELL CULTURE COMPOSITIONS USED IN THE GENERATION AND MAINTENANCE OF NAÏVE-STATE PLURIPOTENT CELLS

Recent studies have demonstrated that through epigenetic reprogramming, terminally differentiated cells have the ability to recourse into a progenitor-like state (Xie, H.,

Ye, M., Feng, R., and Graf, T. 2004), into a different differentiated-cell state (Szabo, Bhatia 2010) or even back into an embryonic-like state (Takahashi *et al.*, 2006), such as iPSCs. Although the generation of iPSCs has become more routine, only a very small percentage of the somatic cells in a given experiment reprogram into iPSCs. Several parameters attribute to this low efficiency including the proliferative state of the somatic cell, additional mutagenesis leading to gene activation or suppression, the format of gene delivery and environmental cues. It has also been reported that not all cells identified as iPSCs behave similarly to ESCs. For example, gene expression profiling has demonstrated that many iPSCs display significant differences in expression profiles to their ESC counterparts. In addition, studies of Xist activity and X-chromosome reactivation analysis show that while some ESCs are in a naïve state (*i.e.*, a grounded state of pluripotency) the majority, if not all derived iPSCs are in a primed state (*i.e.*, primed to differentiate). Combined, these differences may contribute to reduced pluripotency and low efficiency in the differentiation of iPSCs towards specific cell types, reducing the value of iPSCs in regenerative medicine.

By targeting key cellular pathways involved in the mechanisms behind naïve and primed states, the inventors have demonstrated the ability to transform pluripotent stem cells that exist in a primed state, including conventional iPSCs, into a naïve state. Using the media compositions listed in Table 1, it was possible to further reprogram such primed pluripotent cells and conventional iPSCs to a naïve state. More specifically, by reprogramming somatic cells or culturing iPSCs in a medium containing modulators of cellular signaling pathways, such as the MAP kinase pathway, TGFβ pathway, and/or Wnt/β-catenin pathways, the gene expression signature of the generated or cultured iPSCs become more ESC-like than conventional iPSCs (iPSCs generated and/or cultured in conventional culture medium and not contacted with the modulators of cellular signaling pathways). As demonstrated in hierarchical clustering, both conventional medium and SMC4 medium derived hiPSCs were similar to each other and different from their parental line IMR90 (Figure 6B). However, when iPSCs cultured in SMC4 medium were plated back onto feeder cells they more closely resembled mouse ESCs than conventionally cultured iPSCs, another demonstration of naïve status (Figure 6E). Furthermore, iPSCs cultured in SMC4 medium had significantly reduced Xist activity and enhanced

expression of genes of the X-chromosome (Figures 6C and 6D). Finally, iPSCs cultured in SMC4 medium not only differentiated into all three germ layers but also demonstrated the ability to reactivate genes associated with extraembryonic cells, an ability only reserved for cells in the naïve state (Figure 3H). Thus, culturing pluripotent stem cells, including iPSCs existing in a primed state, in SMC4 medium promoted naïve status and enhanced differentiation potential.

To determine the pluripotent state of hiPSC clones generated using SMC4 supplemented medium and the cell sorting platform (Figure 7), hiPSCs were derived from the same starting fibroblast line and 3-factor polycistronic vector expressing Oct4, Klf4 and Myc but maintained under conventional culture, including clump passage and feeder cells (FTi99). Affymetrix global gene expression analysis of the various clones as well as mRNA from hESCs was conducted (Figure 7B). All pluripotent lines were seen to have a different profile from the control fibroblast line (FTC1, Pearson score 0.886) and essentially similar expression profiles to each other (Pearson score 0.969). A heat map signature was generated to represent the 1,739 probes that were differentially expressed by 4-fold between the hESCs/FTi99 (hESCs or hiPSCs generated and maintained on feeder cells and conventional medium) and FTi91/FTi93 (hiPSCs generated and maintained on feeder-free and SMC4 medium) groups (Figure 7C). In depth analysis of the genes within the 1,739 differentially expressed transcripts identified an interesting trend: while several pluripotency genes commonly associated with naïve state were upregulated in the FTi91/FTi93 group, more significantly, many differentiated genes associated with the prime state were repressed within the this group (Figure 7D). Our overall analysis of the SMC4 medium generated hiPSCs suggests that culture conditions play a more influential role in deciding the undifferentiated state relative to the starting cell line or the derivation strategy. The data also suggest that clones derived in or adapted to SMC4 culture conditions demonstrate naïve characteristics with preferred qualities such as a highly undifferentiated state with reduced expression of early lineage markers (Figure 7E).

EXAMPLE 6

THE IDENTIFICATION OF ANTIBODY COCKTAIL TO IDENTIFY BONA FIDE HIPSCS

Pluripotent stem cell surface markers were surveyed. In addition to SSEA4 and Tra181 expression, expression of CD30 and CD50 was also identified and deemed to represent additional surface markers of pluripotency (Figure 10). Cells reprogrammed using polycistronic lentivirus expressing Oct4, Klf4 and Sox2 represent a variety of potency states, and are identified with few cells bearing the markers of true pluripotency, such as Nanog expression. To date, there is no reliable method of identifying truly pluripotent hiPSCs based on surface marker expression. For example, as seen in Figure 11, some clonal populations that were identified as positive for SSEA4, Tra181 and CD9 did not express Nanog, and would have been incorrectly identified as hiPSCs.

The current invention provides a combination of cell surface markers that identify populations of cells expressing Nanog, a marker of truly pluripotent cells. Specifically, cells positive for surface markers of CD30, SSEA4 and Tra181 identify cells expressing Nanog (Figure 11).

In a further example of added enrichment, a cell population undergoing reprogramming was sorted to identify cells positive for CD13 surface marker expression and these CD13+ cells were removed from the reprogramming cell population. The CD13+ population correlated to somatic cells and nonreprogrammed cells, and depleting the reprogramming population of cells of CD13+ cells enhanced enrichment of SSEA4/Tra181 positive cells (Figure 12). As demonstrated in Figure 12, when somatic cells were reprogammed using a polycistronic vector expressing Oct4, Klf4 and Sox2 for 21 days, various cell populations with diverse surface marker expression patterns were created, with only a minor subset of cells representing SSEA4 and Tra181 positive cells (dotted arrow, Figure 12). However, when the same population was first assessed based on CD13 expression (solid arrows, Figure 12), the population subset that was negative for CD13 (*i.e.*, depleted of CD13 cells) represented a population that had been significantly enriched for SSEA4 and Tra181 expressing cells (11.04%, Figure 12). On the contratry, the subset of cells that expressed high levels of CD13 represented few cells that expressed both SSEA4 and Tra181 (1.14%, Figure12).

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EXAMPLE 7

THE USE OF SINGLE CELL SORTING IN THE GENERATION OF INDUCED PLURIPOTENT STEM CELLS FROM DIFFERENTIATED CELLS

As can be seen in Figure 8A, a fibroblast cell culture early in the reprogramming

5   process, where reprogramming was induced using individual lentivirus expressing Oct4, Klf4,

Sox2 and myc, contained colonies of morphologically different cells. Some of these cells stained

positive for markers of pluripotency whereas others were merely transformed, fast growing cells.

At this stage in the reprogramming process it is not clear by cell morphology alone which cell

colonies will go on to form iPSCs. The faster growing, transformed but not pluripotent cells

0   quickly took over the culture. It would therefore be advantageous to have an enrichment step

that selected for iPSCs early in the reprogramming process. Further, and as shown in Figure 8,

some colonies expressed several pluripotency markers during the reprogramming process, and

were true iPSCs, whereas some colonies were not fully reprogrammed and only expressed some

markers of pluripotency. Colony 1 in Figure 8B was positive for both SSEA4 and Tra181,

5   whereas colonies 4 and 5 were only positive for one or the other marker. It would therefore be

more efficient and less technically challenging to select for pluripotent cells using cell surface

markers of pluripotency or several markers simultaneously rather than by colony morphology.

Using cell culture compositions described herein combined with cell enrichment

and/or sorting methodologies also provided herein, it was possible to derive iPSCs in greater

20   numbers and in shorter periods of time by selecting for individual cells that showed surface

markers of pluripotency during the reprogramming process. More specifically, and as shown

schematically in Figures 9A and B, two paths for the generation of iPSCs using single cell

sorting and enrichment based on markers of pluripotency during reprogramming were carried out

using the methods of the invention.

25   In path _A_, after the initiation of reprogramming, a mixed population of cells at

various states of potency was generated. The mixed population of cells contained differentiated

cells, partially-reprogrammed cells, reprogrammed cells, and cells undergoing reprogramming.

The cell population was enriched using methods such as magnetic bead sorting or flow-

cytometry sorting (see Example 1 for methodologies) for cells that expressed pluripotent markers

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such as SSEA4. Upon enrichment, the cells were maintained in SMC4 medium (Table 1) or, in particular embodiments, SMC4 + fibronectin medium for approximately 3 days followed by replacement with SMC4 medium, and after a culture period of approximately 6-10 days, iPSC colonies were identified based on live culture staining of markers such as SSEA4 and TRA181,

5   and were picked or sorted for clonal expansion (Figure 9).

Under the path _B_ schematic (Figure 9), early after the initiation of reprogramming, the mixed population of cells was sorted to obtain the rare population of cells positive for two or more markers of pluripotency. Such markers include but are not limited to SSEA4 and TRA181. The selected cells expressing a combination of pluripotency markers were

0   transferred to feeder cell supplemented culture systems or feeder-free culture systems, specifically those supplemented with cell culture media compositions described in Table 1. In this way iPSC colonies were generated with significantly reduced timelines and technical barriers compared with previously described methodologies.

In a specific demonstration of this technology, IMR90 fibroblast cells were

5   infected with lentivirus expressing Oct4 and Sox2 and Klf4 and c-Myc (OSKM). After several days of feeder-free culture, the reprogramming cells were switched from their somatic cell culture medium to feeder-free culture supplemented with SMC4 medium (Table 1). At 8 days post initiation of reprogramming, the infected cell population was seen by flow-cytometry analysis to contain a modest sub-population of cells that expressed the pluripotency marker

20   SSEA4 (Figure 13A). Using magnetic activated cell sorting as described in Example 1, the pluripotent population was enriched 3-fold for cells expressing SSEA4 (Figure 13A). After enrichment of cells for SSEA4 expressing cells, the sorted cells were transferred to feeder-free culture containing Matrigel™ and supplemented with either conventional hESC medium or SMC4 medium or in particular embodiments, SMC4 + fibronectin medium. Alkaline

25   phosphatase staining of the cultures shows that use of small molecule inhibitors of MEK, GSK3, Rock kinase and TGFβ supported the enrichment of pluripotent cells using single cell sorting whereas conventional basal medium did not (Figure 13A).

Quantification of three independent experiments clearly demonstrated pluripotent cell selection by single cell sorting only in the presence of the small molecule inhibitors (Figure

13B). Colonies from cells enriched in this way were cultured further in SMC4 medium and characterized as true iPSCs: the true iPSCs stained positive for pluripotency markers SSEA4 and Tra181 in immunofluorescence and flow cytometry (Figures 13C and D); showed similar gene expression profiles to human ESC (Figure 13E); showed significant silencing of exogenous

5   transgenes (Figure 13F) and were able to differentiate into all three germ layers (Figure 13G). Pluripotent cell colonies were rarely observed when the same protocol was used but with conventional culture conditions (consisting of conventional culture medium containing feeder cells and lacking SMC4). Thus the ability to robustly enrich a pluripotent cell population during cellular reprogramming using single cell sorting based on cell surface markers of pluripotency

0   was dependent on the use of the cell signaling pathway inhibitors and additives listed in Table 1. Additionally, improved post-sort seeding was observed when the additive fibronectin was used in the culture system for 3 days. This reprogramming process was also completed for other starting cell types including human adipose-derived stem cells.

In a further example of the technology, FACS was used for the enrichment of a

5   pluripotent population of cells from a mixture of non-reprogrammed, partially reprogrammed and fully reprogrammed cells. As with the previous examples, IMR90 fibroblast cells were infected with lentivirus expressing Oct4 and Sox2 andKlf4/c andMyc (OSKM). Several days following fibroblast infection the cell culture was switched to feeder-free culture in SMC4 medium. The infected cell population contained a modest population of cells positive for both

20   SSEA4 and Tra181 markers of pluripotentcy (Figure 14A). The cells that were positive for both pluripotency markers were selected and sorted away from cells that were either negative for both makers or positive for only one marker. Comparison of subsequent feeder free cultures with media supplemented as described in Table 1, in some embodiments containing fibronectin, showed that cultures from cells positive for both markers formed colonies that were subsequently

25   characterized as pluripotent iPSCs whereas cells gated by FACS to be negative for the markers of pluripotency did not produce alkaline phosphatase-positive colonies (Figure 14A,B). More specifically, inhibitors of the signaling molecules MEK, GSK, Rock and TGFβ as additives to the basal media formulation facilitated the use of FACS in the sorting selection and enrichment

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of pluripotent cells early in the reprogramming process. Additionally, improved post-sort

seeding was observed when the additive fibronectin was used in the culture system for 3 days.

The advantages of SMC4 medium and culture systems were next used to develop

a high-throughput method for generating feeder-free and clonally derived hiPSCs. A scheme

5   was devised to treat cells induced to reprogram with SMC4 medium and select for rare

individual cells that have faithfully reprogrammed as indicated by a combination of pluripotency

markers. Furthermore, we coupled the reprogramming process with a multiplex platform to

effectively select for the top tier clones based on selection assays of dual marker flow-cytometry,

qRTPCR and immunofluorescence (Figure 15).

0   In an optimized multiplex protocol, reprogramming was initiated using the 3-

factor (OKS) polycistronic virus, and an initial bulk FACS sort of the SSEA4$^+$/Tra181$^+$

population was completed on day 20 post infection followed by FACS resorting of

SSEA4$^+$/Tra181$^+$ cells into 96 well-plates on day 30 (Figures 15 and B). This strategy allowed

for the derivation of numerous clones and led to the generation of clones FTC1 clone 1 and 2 that

5   expressed pluripotent markers, exhibited attenuation of exogenous gene activity and de-

methylation of the Oct4 promoter (Figure 15A-C). FTC1 clone 1 and 2 were also shown to be

pluripotent by differentiating into the three somatic lineages in vitro and in vivo (Figure 15D, E).

The selected clones were also assessed for chromosomal integrity and demonstrated to have

minimal copy number variations from their parental line and normal karyotype even after 20

20   continuous single cell passages in FF culture, a marked improvement over other reprogramming

strategies (Figures17).

To determine the reproducibility of the platform, additional fibroblast lines, FTC5

and FTC7, were induced to reprogram using a polycistronic vector expressing Oct4, Klf4 and

Sox2, and applied to the high-throughput platform as described in Figure 15. As described in

25   Figure 18, the colonies derived from FTC5 and FTC7 fibroblast lines were shown to maintain

their undifferentiated state, retain their pluripotency and genomic stability. Thus, the

combination of 3 pluripotency factors, SMC4 culture medium and a multiplex characterization

platform significantly enhances the kinetics of feeder-free reprogramming while enabling the

identification, selection and expansion of clonally derived and genomically stable hiPSCs in a high-throughput manner.

Example 8

METHODS AND CULTURE COMPOSITIONS FOR
THE RAPID GENERATION OF MULTIPLE IPSC CLONES

The generation of human iPSCs by the ectopic expression of pluripotency genes such as Oct4, Sox2, Klf4, c-myc, Lin28 and Nanog is an inefficient and technically demanding process. Strategies involving lentiviral or retroviral integration of pluripotency factor transgenes into the host cell genome in combination with culture systems including feeder cell support have traditionally been the most efficient methods for iPSC generation. A literature review of historical studies using virus and feeder cell methodologies for human iPSC generation shows an efficiency of 0.001%-0.01% of infected cells becoming iPS cells, where potential pluripotent cells are seen at the 21-30 day period post infection and these are clonally derived by manual "clump" passaging between 30 and 45 days post infection (Table 4).

Other methods for introducing the pluripotency genes include episomal vector systems and transduction of modified protein. Such methods are regarded as important developments towards the ultimate clinical application of iPSC technology. However, these methodologies are of even lower efficiency than reprogramming using viral systems. Further, the efficiency of the reprogramming process is also reduced, or in some conditions is impossible, when feeder cell-free systems are used in combination with conventional stem cell media formulations, hindering the development of iPSCs for industrial and therapeutic use. Somatic cell reprogramming has been characterized as a stochastic process; the majority of cells will eventually reprogram over time. However a robust, technically easy, efficient and scalable method for producing multiple iPSC clones in a single reprogramming has yet to be described.

The present invention provides cell culture conditions and methodologies to derive clonal iPSC colonies in a relatively short time and with lower technical barriers than current methods. Specifically, and as can be seen in Figure 13B, the use of small molecule inhibitors of specific signaling pathways as additives in SMC4 medium allowed the generation

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of iPSC colonies in feeder free culture environments at an efficiency vastly greater than when SMC4 medium is not used. Inhibitors of MEK, GSK, Rock and TGFβ signaling pathways were used to allow efficient reprogramming in feeder-free environments.

As can be seen from Figure 13 and Table 4, in three independent reprogramming experiments either none or one colony was seen when conventional hESC medium was used in the reprogramming process in a feeder-free environment, whereas the small molecule culture additives (*i.e.*, SMC4 medium) enhanced the feeder-free reprogramming event to an efficiency of 0.035%, and resulted in colonies on days 14-21 post infection and the derivation of clonal iPSCs by days 28-35. Thus, the use of small molecule inhibitors increased the efficiency of reprogramming with respect to the time to reprogram and the percent of cells reprogrammed. The colonies that were generated using this approach were characterized as pluripotent using standard procedures such as immunofluorescence and gene expression profiling.

In a further demonstration of this technology, differentiated cells were infected with virus expressing individual pluripotency genes Oct4, Klf4, Sox2, and Myc, and were cultured in SMC4 medium and feeder-free culture environments (Table 1) for 8-12 days. At this time point, and as described in Example 6 and Figures 13 and 14, the cells were enriched using FACS or MACS single cell sorting to obtain the small population of pluripotent cells defined by positive staining for pluripotency markers such as SSEA4 and Tra181. Using this approach the timelines for iPSC production were greatly reduced: an efficiency of 0.22% in terms of the percent of cells reprogrammed was demonstrated, with colonies appearing in days immediately post sort (day 10-16). This efficiency of reprogramming allowed the derivation of clonal iPSCs by days 21-28 post infection.

In a further demonstration of the improved efficiency of reprogramming, a polycistronic vector system in which 3 (Oct4, Klf4, and Sox2) or 4 (Oct4, Klf4, Sox2, and myc) pluripotency factors were expressed from the same promoter element was used in combination with the optimized SMC4 medium, feeder-free culture and single cell sorting system using SSEA4 and TRA181, resulting in reprogramming efficiencies of 0.756% with colonies first seen at days 6-8 post infection. This method was so efficient that iPSC colonies were present just 4

days after infection. These techniques represent a significant improvement over traditional methods of iPSC generation.

Table 4: Kinetics of reprogramming in various strategies. N.I., not identified; *
Morphological appearance of iPSC-like colony; ** Calculated as number of SSEA4+/Tra181+
per seeded cells; *** Time required for an iPSC colony to be expanded and maintained as a
clonal line (based on Tra181/SSEA4 staining). Gray boxes represent data from literature search.
Individual Factors Virus; infection conducted by combining individual virus each expressing one
of the key transcription factors, 3 represents combination Oct4/Klf4/Sox2 and 4 represents
combination Oct4/Klf4/Sox2/cMyc.

Reprogramming strategy	Appearance of colonies (Days)*	Efficiency (%)**	Derivation of clonal population (Days)***
Individual 4 Factors Virus + Feeder Cells + Conventional Medium	21-30	0.001-0.01	30-45
Individual 4 Factors Virus + Feeder Cells + Conventional Medium + TGFβi + MEKi	14-21	0.01-0.1	28-42
Individual 4 Factors Virus - Feeder Cells + SMC4	14-21	0.035 ± 0.015	28-35
Individual 4 Factors Virus - Feeder Cells +Enrichment + Conventional Medium	N.I.	---	---
Individual 4 Factors Virus - Feeder Cells + Enrichment + SMC4	10-16	0.220 ± 0.120	21-28
Individual 3 Factors Virus - Feeder Cells + Enrichment + SMC4	18-24	0.032 ± 0.018	24-35
Polycistronic 4 Factors Virus - Feeder Cells + Enrichment + SMC4	6-8	0.756 ± 0.238	18-24
Polycistronic 3 Factors Virus - Feeder Cells + Enrichment + SMC4	10-14	0.276 ± 0.084	21-28

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EXAMPLE 9

METHODS OF DEPLETING A PLURIPOTENT CELL POPULATION FROM A DIFFERENTIATED CELL POPULATION USING SINGLE CELL SORTING AND ENRICHMENT

Drug screening and some clinical applications of stem cell biology require the generation of homogeneous populations of cells differentiated to a specific lineage from pluripotent cells such as ESCs or iPSCs. Contamination of a differentiated cell population with pluripotent cells could lead to misleading screening results or even tumor/teratoma formation *in vivo*. Methods of either enriching a population of cells for differentiated cells or depleting pluripotent cells from a cell population could include the sorting technologies described in Examples 3 and 6 herein. The use of small molecule additives in cell culture media to specifically prevent differentiation or partial differentiation of pluripotent cells during the single cell sorting process, as provided by the present invention, allows pluripotent cells to be negatively selected out of a population of fully differentiated cells. Inversely, the positive selection of differentiated cells from a cell population by cell sorting can be more effective under culture conditions where pluripotent cells remain fully positive for surface markers of pluripotency. As can be seen in Figure 20, a mixed population of fully differentiated fibroblasts and pluripotent cells was effectively separated using FACS when the cells were pre-cultured in culture environments described in Table 1. The small molecule culture additives can also be used during the sorting procedure, stabilizing the single cell suspension. It can be seen from Figure 20 that cells selected as negative for pluripotency markers were seen to be completely free of pluripotent cells on subsequent culturing and staining for alkaline phosphatase.

EXAMPLE 10

CYTOKINE AND GROWTH FACTOR FREE CULTURE OF PLURIPOTENT STEM CELLS ON FEEDER-FREE CULTURE

As discussed in Example 2, conventional human pluripotent culture systems include feeder cells and cytokines, such as bFGF, which serve as extrinsic stimuli for the maintenance of human pluripotent stem cells in an undifferentiated state. Feeder cells and the process for producing recombinant cytokines serve as a source of xenogeneic contaminants, however. In addition, the key factor(s) secreted from feeder cells and the exact cellular pathways stimulated by cytokines are yet to be identified. Thus, the conventional culture of human pluripotent stem cells represents an ill-defined system and may impede transition to clinical-grade manufacturing.

To address this issue, the present invention includes a further embodiment wherein the feeder-free and single cell passage system discussed in Example 2 was further modified by removing bFGF and other cytokines and growth factors from the SMC4 medium formulation. Further, in one embodiment of the invention, Matrigel™ was replaced with gelatin, since Matrigel™ represents an extracellular matrix that is animal derived and not fully characterized. These embodiments of the invention provided a fully defined and cytokine free culture system that allows for intrinsic self-renewal and maintenance of pluripotent stem cells, including iPSCs.

As demonstrated in Figures 21A and 21B, human pluripotent stem cells, such as iPSCs generated with a polycistronic vector system containing Oct4, Klf4, and Sox2 and maintained in a feeder free environment, were readily single cell passaged onto a gelatin coated culture surface with SMC4 medium devoid of additional cytokines or growth factors such as bFGF. After several passages in this fully defined system, iPSCs maintained their pluripotent status as demonstrated by co-expression of Tra181 and SSEA4 (Figures 21C). Further, we have demonstrated the generation of iPSCs in this fully defined and cytokine free system. Thus, in one embodiment the invention provides a fully defined culture system absent of growth factor and cytokines comprising a combination of SMC4 medium and gelatin.

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EXAMPLE 11

GENOMIC STABILITY IN THE GENERATION AND MAINTENANCE

OF PLURIPOTENT STEM CELLS

Studies suggest that the reprogramming process and subsequent culture of pluripotent stem cells may result in a higher propensity for genomic abnormalities. In addition, feeder-free culture has been shown to give rise to clonal outgrowth of karyotype abnormal cells. As demonstrated in Figures 17A, 17B and 18C, the invention provides a method of reprogramming to obtain cells having genomic stability as well as a method of maintaining reprogrammed cells having genomic stability. In the method of the invention, genomic stability was maintained during both the reprogramming process as well as during long-term feeder-free culture when cells were reprogrammed using our 3-factor polycistronic construct containing Oct4, Klf4 and Sox2 and cultured in SMC4 medium. As seen in Figure 17A, high resolution comparative genomic hybridization demonstrated that minimal copy number variations were detected when hiPSCs were generated and maintained in long-term feeder-free culture using SMC4 medium. In addition, genomic stability was maintained during routine long-term feeder-free and single cell culture as demonstrated in Figures 17B and 18C.

EXAMPLE 12

MAINTAINING A CELL POPULATION OF PLURIPOTENT CELLS DURING CULTURING USING CELL SURFACE MARKERS.

It is often useful to remove differentiated cells from pluripotent cell culture to maintain the pluripotency of a stem cell culture. To date, this process requires manual picking of differentiated cells away from the cell culture or collecting the undifferentiated cells from a substantially differentiated population. Both processes are labor intensive, require skilled training, and rely on selection of cells based on morphology, which may not always be indicative of the true pluripotency status of the cells in the culture (Figure 8).

In an improved process, the present invention provides the ability to efficiently and precisely select for undifferentiated cells during routine culture. As demonstrated in Figure 19A, a population of hiPSCs maintained in SMC4 medium and FF culture was readily enriched or sorted to maintain a cell culture of undifferentiated pluripotent cells. In another example, as described in Figure 19B, a population of mostly differentiated cells, which would normally be discarded, can be removed from the cell culture to achieve mostly undifferentiated pluripotent cells as described by Tra181 expression.

CLAIMS:

1.   A method of culturing a pluripotent cell in a feeder-free environment comprising: culturing a pluripotent cell that is not a murine embryonic stem cell in a feeder-free environment in a culture medium comprising the agents:

      i)   a TGFβ receptor inhibitor;

      ii)   a GSK3 inhibitor;

      iii)   a MEK inhibitor; and

      iv)   a Rock inhibitor,

      while maintaining pluripotency of the cell during culturing, and wherein the culture medium comprises a sufficient amount of the agents to allow for at least one cell division while maintaining pluripotency of the cell.

2.   The method of Claim 1, wherein:

      (A)   the pluripotency of the cell is maintained for at least five or for at least ten cell divisions; and/or

      the cell is cultured in the absence of growth factors and cytokines or in the absence of a gelatinous protein mixture from Englebreth-Holm-Swarm (EHS) mouse sarcoma cells; and/or

      the culture medium is substantially free of bFGF; and/or

      (B)   the TGFβ receptor inhibitor is an ALK5 inhibitor; and/or

      (C)   the Rock inhibitor is thiazovivin or Y27632;

      the TGFβ receptor inhibitor is A-83-01 or SB431542,

      the GSK3 inhibitor is CHIR99021 or BIO; and/or

      the MEK inhibitor is PD98059 or PD032901.

3.   A method of culturing a pluripotent cell comprising culturing a pluripotent cell that is not a murine embryonic stem cell in the absence of growth factors and cytokines, wherein the method comprises culturing the pluripotent cell in a culture medium comprising: i) a TGFβ receptor inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor; and iv) a Rock inhibitor, to allow for at least one cell division while maintaining pluripotency of the cell.

4.   A method of enhancing the viability of dissociated human pluripotent cells comprising:

a)   dissociating human pluripotent cells to obtain dissociated cells; and

b)   contacting the dissociated cells with a culture medium comprising the agents:

i)   a TGFβ receptor inhibitor;

ii)   a GSK3 inhibitor;

iii)   a MEK inhibitor; and

iv)   a Rock inhibitor;

whereby viability of the dissociated cells is enhanced as compared to viability of dissociated cells not contacted with the agents.

5.   The method of Claim 4:

(i)   wherein viability of the dissociated cells is enhanced by at least 10%, at least 50%, at least 100%, at least 200%, or at least 500% over viability of dissociated cells not contacted with the agent; and/or

(ii)   further comprising culturing the dissociated cells in the culture medium for at least one, at least two, at least five, or at least ten passages while maintaining pluripotency of the dissociated cells; and/or

(iii)   wherein the karyotype of the dissociated cells after culturing is substantially similar to the karyotype of the same population of cells prior to dissociation; and/or

(iv)   wherein the method comprises dissociating in the presence of the agents; or

(v)   further comprising contacting the pluripotent cells with the agents prior to dissociating; and/or

(vi)   wherein contacting the dissociated cells with the culture medium comprises suspending the dissociated cells in the culture medium.

6.   A method of increasing the pluripotency of a non-pluripotent or incompletely pluripotent cell in a feeder-free environment comprising:

contacting a non-pluripotent or incompletely pluripotent cell in a feeder-free environment with a culture medium comprising: i) a TGFβ receptor inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor; and iv) a Rock inhibitor, to obtain a cell having increased

potency as compared to the non-pluripotent or incompletely pluripotent cell prior to contacting with the culture medium.

7.   The method of Claim 6, wherein:

(i)   contacting comprises culturing the non-pluripotent or incompletely pluripotent cell under conditions sufficient to increase the pluripotency of the non-pluripotent or incompletely pluripotent cell, and/or

(ii)   the non-pluripotent or incompletely pluripotent cell is an embryonic stem cell; a pluripotent cell; a multipotent cell; a non-pluripotent cell; a somatic cell; a human cell; or an induced pluripotent stem cell; and/or

(iii)   the non-pluripotent or incompletely pluripotent cell having increased potency is characterized by one or more of the following:

a)   expression of at least one pluripotent stem cell marker selected from the group consisting of endogenous Oct4, Nanog, SSEA4, Sox2, Klf4, Tra181, and Lin28;

b)   pluripotent stem cell morphology;

c)   ability to contribute to germline transmission;

d)   teratoma formation;

e)   ability to differentiate or transdifferentiate into a lineage different from the starting lineage; and

f)   *in vitro* trilineage differentiation; and/or

(iv) the non-pluripotent or incompletely pluripotent cell having increased potency expresses at least a two-fold higher level of Oct4 as compared to the cell prior to contacting with the culture medium; and/or

(v)   the non-pluripotent or incompletely pluripotent cell having increased potency has Xist activity that is at least two fold lower compared to conventionally cultured iPSCs; and/or

(vi)   the non-pluripotent or incompletely pluripotent cell having increased potency has a compact domed colony morphology; and/or

(vii)   the non-pluripotent or incompletely pluripotent cell having increased potency replicates and maintains pluripotency in the absence of exogenous stimulation of the TGFβ

receptor, activin, and MEK signaling pathways, and optionally in the absence of exogenous stimulation of the bFGF pathway.

8.   The method of Claim 7, wherein the conditions sufficient to increase the pluripotency of the non-pluripotent or incompletely pluripotent cell comprise contacting the non-pluripotent or incompletely pluripotent cell with at least one pluripotency factor.

9.   The method of Claim 8, wherein the pluripotency factor comprises a polynucleotide, polypeptide, or small molecule.

10.   The method of Claim 8, wherein the pluripotency factor is an exogenous transcription factor.

11.   The method of Claim 10, wherein the exogenous transcription factor comprises an Oct4, Sox, Klf, Myc, Lin28, or Nanog polypeptide, or a polynucleotide encoding Oct4, Sox, Klf, Myc, Lin28, or Nanog.

12.   The method of Claim 9, wherein the polypeptide comprises an amino acid sequence that allows transport across cell membranes.

13.   The method of Claim 10, wherein the exogenous transcription factor comprises an Oct4, a Sox2, and a Klf4 polynucleotide.

14.   A method of improving the efficiency of reprogramming of a population of cells in a feeder-free environment comprising:

        culturing a population of cells in a feeder-free environment in the absence of growth factors and cytokines and contacting the population of cultured cells with the following agents:

        i)   a TGFβ receptor inhibitor;

        ii)   a GSK3 inhibitor;

        iii)   a MEK inhibitor; and

iv)   a Rock inhibitor

under conditions sufficient to induce reprogramming, whereby the efficiency of reprogramming is improved by at least 10%, at least 50%, at least 100%, at least 300%, or at least 500% as compared to the efficiency of reprogramming without contacting the population of cells with the small molecule agent; wherein optionally:

(a)   the population of cells prior to reprogramming comprises non-pluripotent cells; or

(b)   the efficiency of reprogramming is measured by time required for reprogramming or number of cells reprogrammed; or

(c)   the conditions comprise contacting the population of cells with at least one exogenous transcription factor selected from the group consisting of an Oct4, Sox, Klf, Myc, Lin28, or Nanog polypeptide, or a polynucleotide encoding Oct4, Sox, Klf, Myc, Lin28, or Nanog; or

(d)   the conditions comprise contacting the population of cells with an Oct4, Sox2, and Klf4 polypeptide or a polynucleotide encoding Oct4, Sox2, and Klf4.

15.   A method of sorting a population of cells in a feeder-free environment to obtain a population of cells enriched for pluripotent cells comprising:

a)   obtaining a suspension of dissociated cells comprising a mixed population of cells in a feeder-free environment;

b)   sorting the cells in the suspension to obtain cells expressing one or more markers of pluripotency, thereby obtaining an enriched population of cells enriched for pluripotent cells,

wherein the suspension comprises: i) a TGFβ receptor inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, and iv) a Rock inhibitor.

16.   The method of Claim 15:

(a)   wherein sorting is by magnetic beads or flow cytometry; and/or

(b)   further comprising culturing the enriched population of cells in a culture medium comprising: i) a TGFβ receptor inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, and iv) a Rock inhibitor, optionally in combination with soluble fibronectin; and/or

(c)   wherein the mixed population of cells in the suspension is contacted before sorting with i) a TGFβ receptor inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, and iv) a Rock inhibitor; and/or

(d)   wherein the mixed population of cells comprises cells expressing one or more markers of pluripotency,

wherein the one or more markers of pluripotency optionally comprises SSEA4, TRA160, TRA181, TRA1-85, TRA2-54, GCTM-2, TG343, TG30, CD9, CD29, CD30, CD50, CD133/ prominin, CD140a, CD56, CD73, CD105, CD31, CD34, OCT4, Nanog or Sox2; and/or

(e)   wherein the method comprises contacting the mixed population of cells with one or more pluripotency factors to induce reprogramming,

wherein contacting optionally comprises introducing one or more pluripotency factors into the cells in the mixed population of cells, and/or

(f)   wherein the pluripotent cells are induced pluripotent cells; and/or

(g)   wherein the enriched population of cells is enriched by at least 20%, at least 50%, at least 100%, at least 200%, or at least 500% with respect to cells expressing one or more markers of pluripotency.

17.   The method of Claim 16, wherein the pluripotency factor comprises an Oct4, Sox, Klf, Myc, Lin28, or Nanog polypeptide, or a polynucleotide encoding Oct4, Sox, Klf, Myc, Lin28, or Nanog.

18.   The method of Claim 16, wherein the pluripotency factor comprises an Oct4, a Sox2, and a Klf4 polypeptide, or polynucleotides encoding an Oct4, a Sox2, and a Klf4 polypeptide.

19.   A method of obtaining induced pluripotent stem cells (iPSCs) comprising:

a)   treating a population of cells to induce reprogramming comprising contacting the population of cells with: i) a TGFβ receptor inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, and iv) a Rock inhibitor;

b)   preparing a suspension of dissociated cells comprising the population of cells;

c)   sorting the cells in the suspension to obtain sorted cells expressing one or more markers of pluripotency to obtain iPSCs;

d)   culturing the iPSCs expressing one or more markers of pluripotency in a feeder free environment in a culture medium comprising: i) a TGFβ receptor inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor; and iv) a Rock inhibitor, to allow for at least one cell division while maintaining pluripotency of the iPSCs.

20.   The method of Claim 19:

(a)   wherein treating the population of cells to induce reprogramming comprises contacting the population of cells with one or more pluripotency factors,

wherein treating the population of cells to induce reprogramming further comprises contacting the population of cells i) a TGFβ receptor inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, and iv) a Rock inhibitor; and/or

(b)   wherein the suspension comprises: i) a TGFβ receptor inhibitor; ii) a GSK3 inhibitor; iii) a MEK inhibitor, and iv) a Rock inhibitor; and/or

(c)   wherein sorting comprises sorting the cells using flow cytometry or magnetic beads; and/or

(d)   wherein cells are sorted to obtain cells expressing two, three, four, or more markers of pluripotency; and/or

(e)   wherein the one or more markers of pluripotency comprises SSEA4, TRA160, TRA181, TRA1-85, TRA2-54, GCTM-2, TG343, TG30, CD9, CD29, CD30, CD50, CD133/prominin, CD140a, CD56, CD73, CD105, CD31, CD34, OCT4, Nanog or Sox2; and/or

(f)   wherein the one or more markers of pluripotency are SSEA4, CD30, and TRA160 or TRA181; and/or

(g)   wherein culturing comprises culturing the cells in a culture medium comprising:

    i)   a TGFβ receptor inhibitor;

    ii)   a GSK3 inhibitor;

    iii)   a MEK inhibitor; and

iv)   a Rock inhibitor,

and/or

(h)   wherein the cells are treated, suspended, sorted, and cultured in a feeder-free environment; and/or

(i)   wherein induced pluripotent stem cells are obtained in about 2 to 22 days or in about 4 to about 18 days; or

(j)   whereby induced pluripotent stem cells are obtained within about 4 to about 22 days, within about 6 to about 18 days, or within about 10 to about 16 days after treating the population of cells to induce reprogramming.

21.   The method of Claim 20, wherein the pluripotency factor comprises an Oct4, Sox, Klf, Myc, Lin28, or Nanog polypeptide, or a polynucleotide encoding Oct4, Sox, Klf, Myc, Lin28, or Nanog; or an Oct4, a Sox2, and a Klf4 polypeptide, or polynucleotides encoding an Oct4, a Sox2, and a Klf4 polypeptide.

22.   The method of Claim 20, wherein culturing the cells in a culture medium comprising culturing the cells in a feeder-free environment;

23.   A method of depleting pluripotent cells from a population of cells comprising:

a)   obtaining a suspension of dissociated cells comprising a mixed population of cells having pluripotent cells;

b)   sorting the cells in the suspension to remove cells expressing one or more markers of pluripotency, thereby depleting pluripotent cells from a population of cells, wherein the suspension comprises:

i)   a TGFβ receptor inhibitor;

ii)   a GSK3 inhibitor;

iii)   a MEK inhibitor; and

iv)   a Rock inhibitor;

and wherein optionally

(I)   the mixed population of cells comprises multipotent cells or somatic cells;

    (II) the mixed population of cells in the suspension is cultured prior to obtaining the suspension in a culture medium comprising:

       i) a TGFβ receptor inhibitor;

       ii) a GSK3 inhibitor;

       iii) a MEK inhibitor; and

       iv) a Rock inhibitor;

    (III) sorting comprises sorting the cells using flow cytometry or magnetic beads.

24. A method of obtaining a pluripotent cell having genomic stability comprising: contacting a cell in a feeder-free environment with:

  i) a TGFβ receptor inhibitor;

  ii) a GSK3 inhibitor;

  iii) a MEK inhibitor; and

  iv) a Rock inhibitor;

  in the absence of c-myc under conditions sufficient to obtain a pluripotent cell having genomic stability.

25. The method of Claim 24, wherein:

  (a) the cell is selected from the group consisting of:

    an embryonic stem cell;

    a pluripotent cell;

    a multipotent cell;

    a non-pluripotent cell; and

    a somatic cell; and/or

  (b) the contacted cell comprises a non-pluripotent cell; and/or

  (c) the conditions comprise contacting the cell with at least one pluripotency factor, and/or

  (d) the Rock inhibitor is thiazovivin or Y27632; and/or

  (e) the TGFβ receptor inhibitor is an ALK5 inhibitor; and/or

(f)   the TGFβ receptor inhibitor is A-83-01 or SB431542; and/or

(g)   the GSK3 inhibitor is CHIR99021 or BIO; and/or

(h)   the MEK inhibitor is PD98059 or PD032901; and/or

(i)   the TGFβ inhibitor is SB431542, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin.

26.   The method of Claim 25, wherein the pluripotency factor is an exogenous transcription factor.

27.   The method of Claim 26, wherein the exogenous transcription factor comprises an Oct4, Sox, Klf, Myc, Lin28, or Nanog polypeptide, or a polynucleotide encoding Oct4, Sox, Klf, Myc, Lin28, or Nanog.

28.   The method of Claim 25, wherein the pluripotency factor comprises an Oct4, a Sox2, and a Klf4 polynucleotide.

29.   A method of culturing a pluripotent cell to maintain genomic stability of the cell comprising:

culturing a pluripotent cell in a feeder-free environment in a culture medium comprising:

i)   a TGFβ receptor inhibitor;

ii)   a GSK3 inhibitor;

iii)   a MEK inhibitor; and

iv)   a Rock inhibitor, while maintaining genomic stability of the pluripotent cell during culturing.

30.   The method of Claim 29, wherein:

(a)   the Rock inhibitor is thiazovivin or Y27632;

(b)   the Rock inhibitor is thiazovivin;

(c)   the TGFβ receptor inhibitor is an ALK5 inhibitor;

(d)   the TGFβ receptor inhibitor is A-83-01 or SB431542;

(e)   the GSK3 inhibitor is CHIR99021 or BIO;

(f)   the MEK inhibitor is PD98059 or PD032901;

(g)   the TGFβ receptor inhibitor is SB431542, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin; and/or

(h)   the pluripotent cell is cultured in the culture medium for at least one, at least two, at least five, at least ten, or at least 15 passages while maintaining genomic stability of the pluripotent cell.

31.   The method of any of Claims 1, 3, 23 or 29 wherein the pluripotent cell is a human embryonic stem cell or a human induced pluripotent stem cell.

32.   A method according to any one of Claims 1 to 31 substantially as herein described with reference to the Figures and/or Examples.
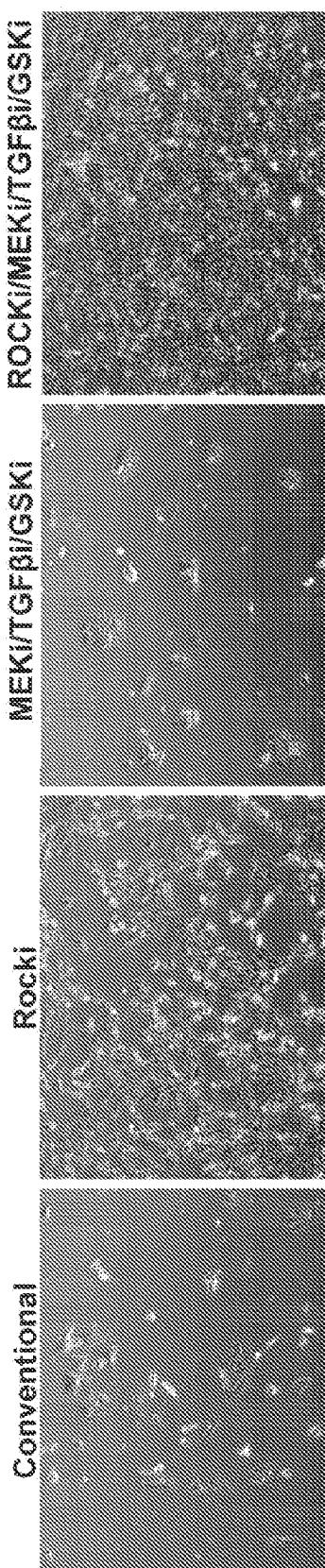
FIG. 1A
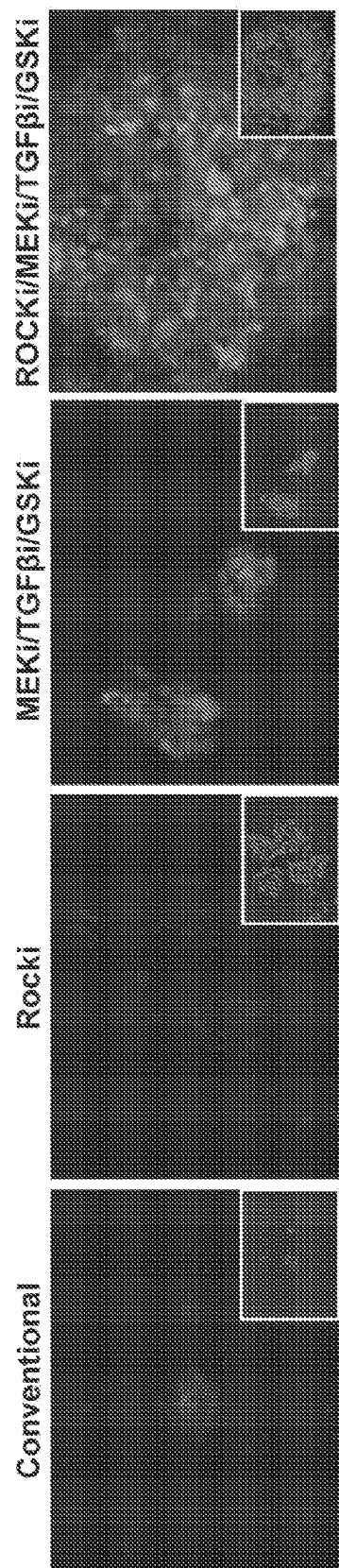
FIG. 1B
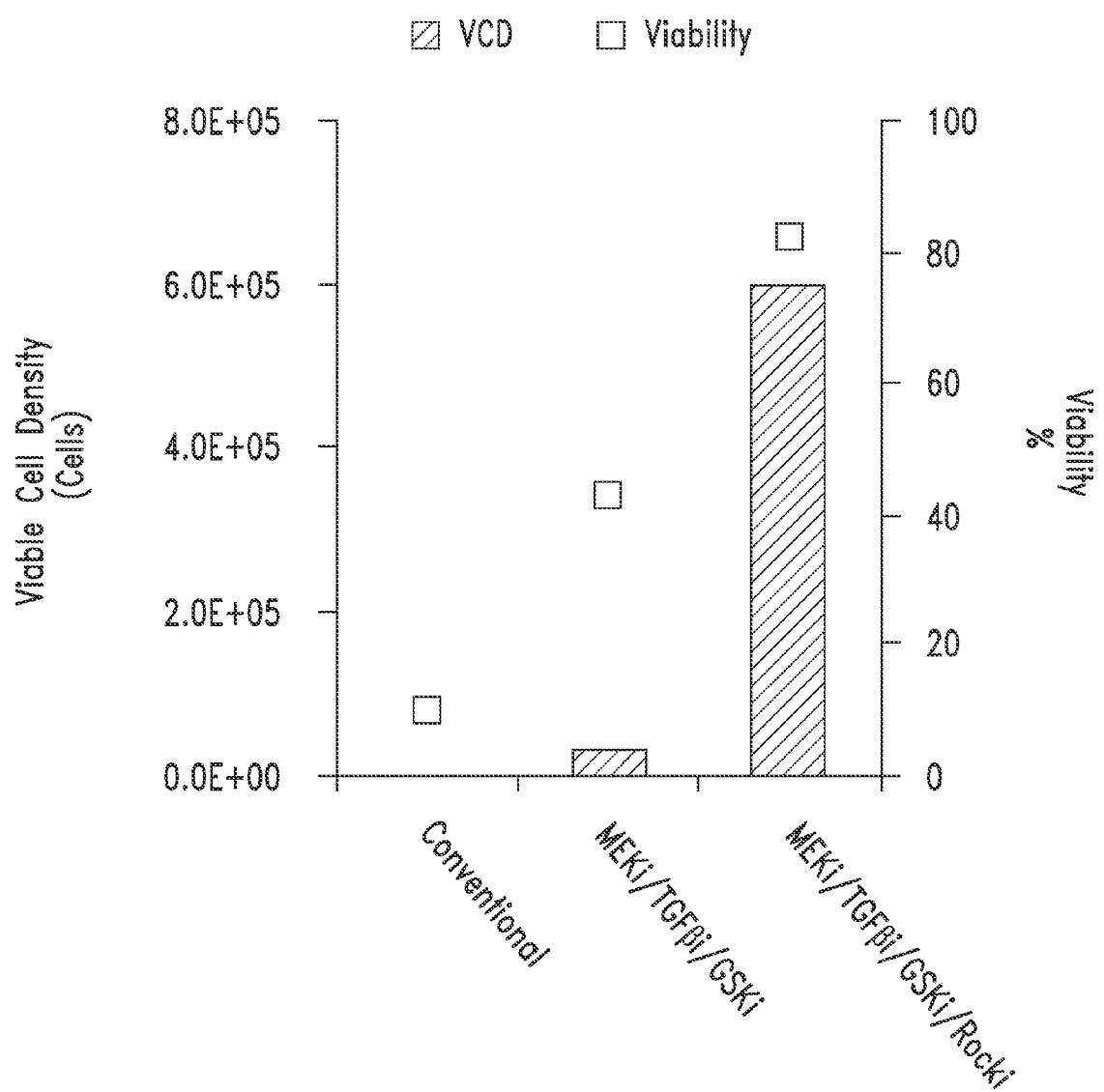
FIG. 1C
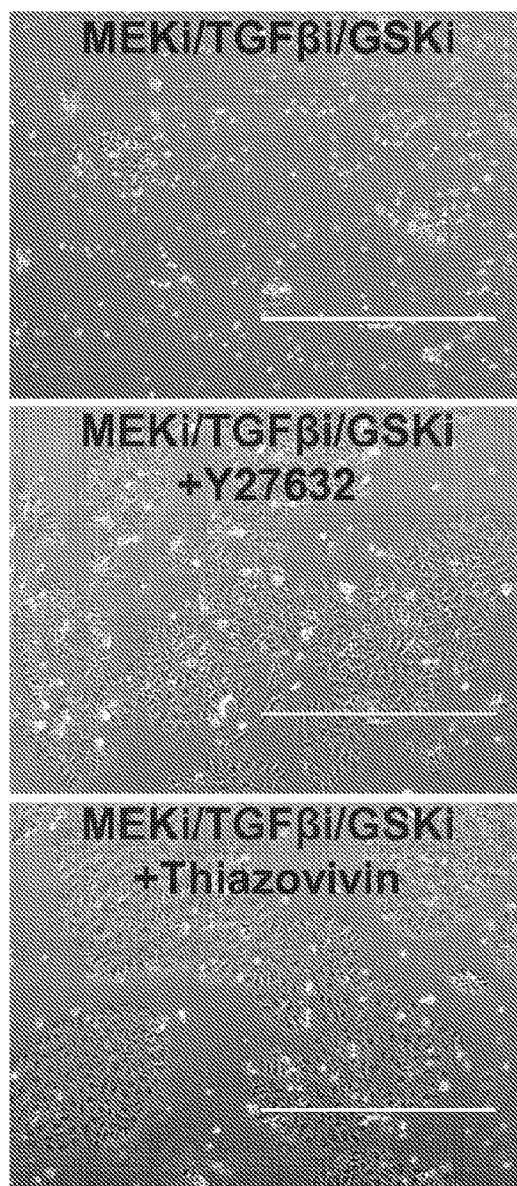
FIG. 1D

MEKi/TGFβi/GSKi +Y27632
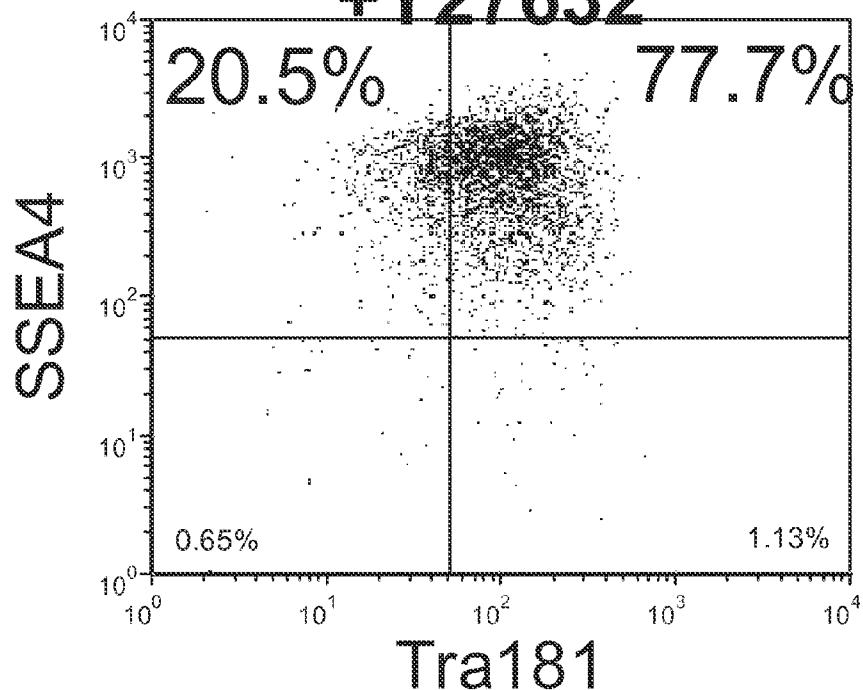
MEKi/TGFβi/GSKi +Thiazovivin

FIG. 1E

FIG. 2A
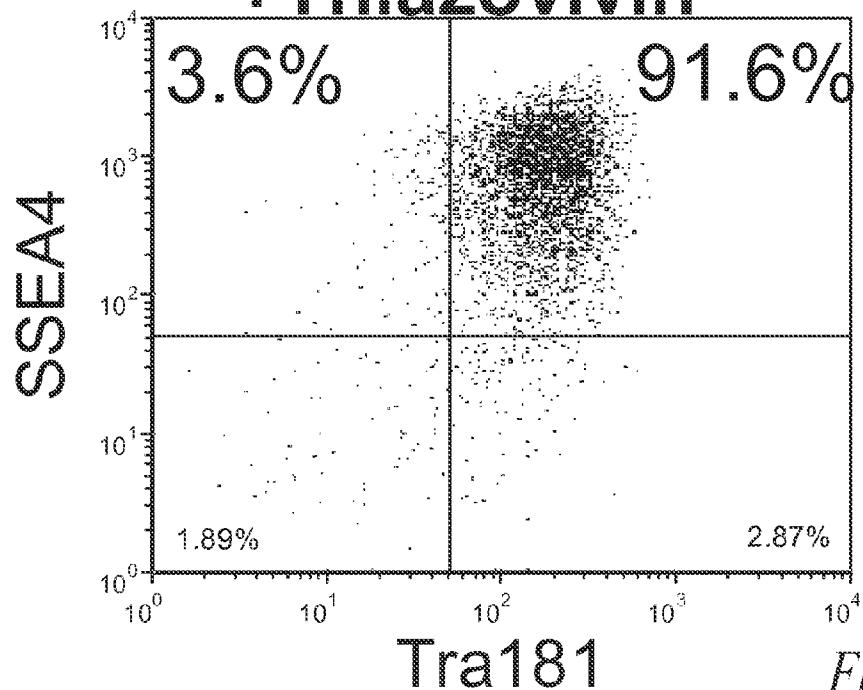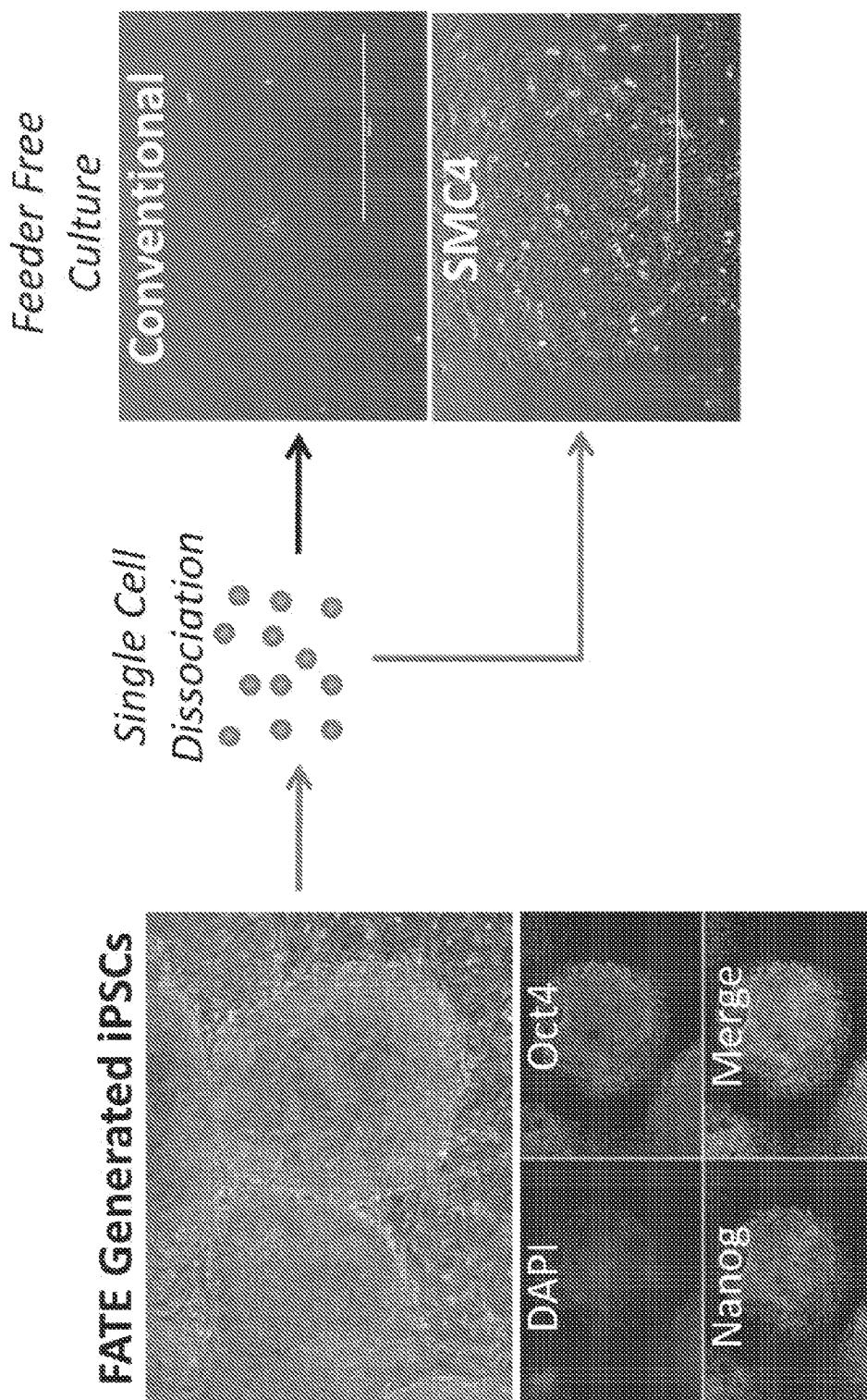
Routine Single Cell Culture on Feeder-Free Culture
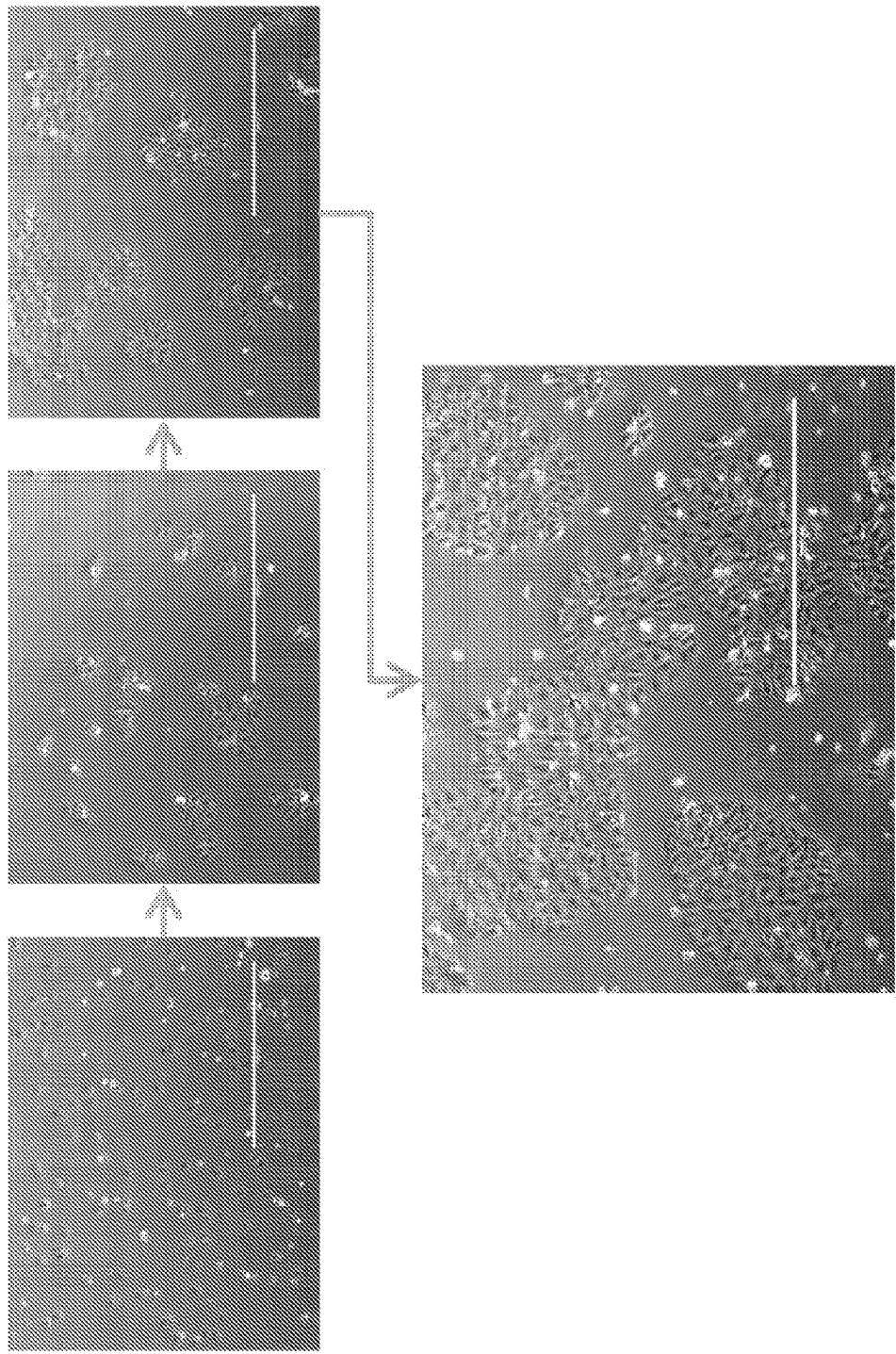
FIG. 2B
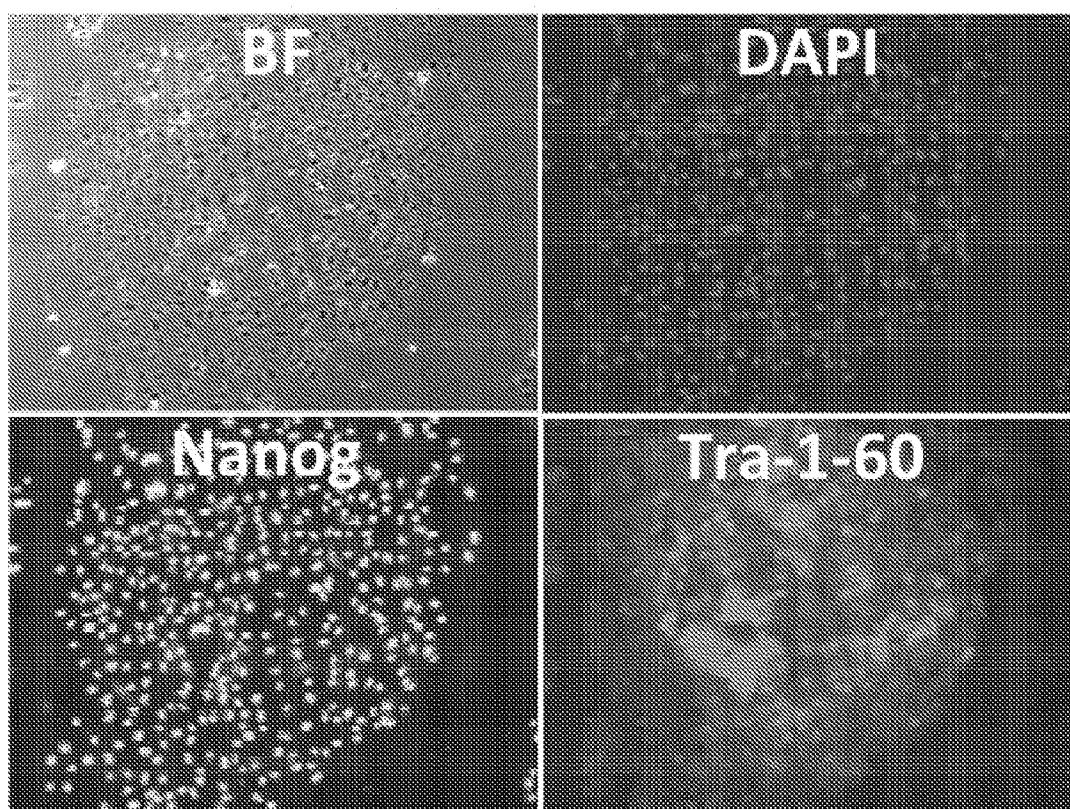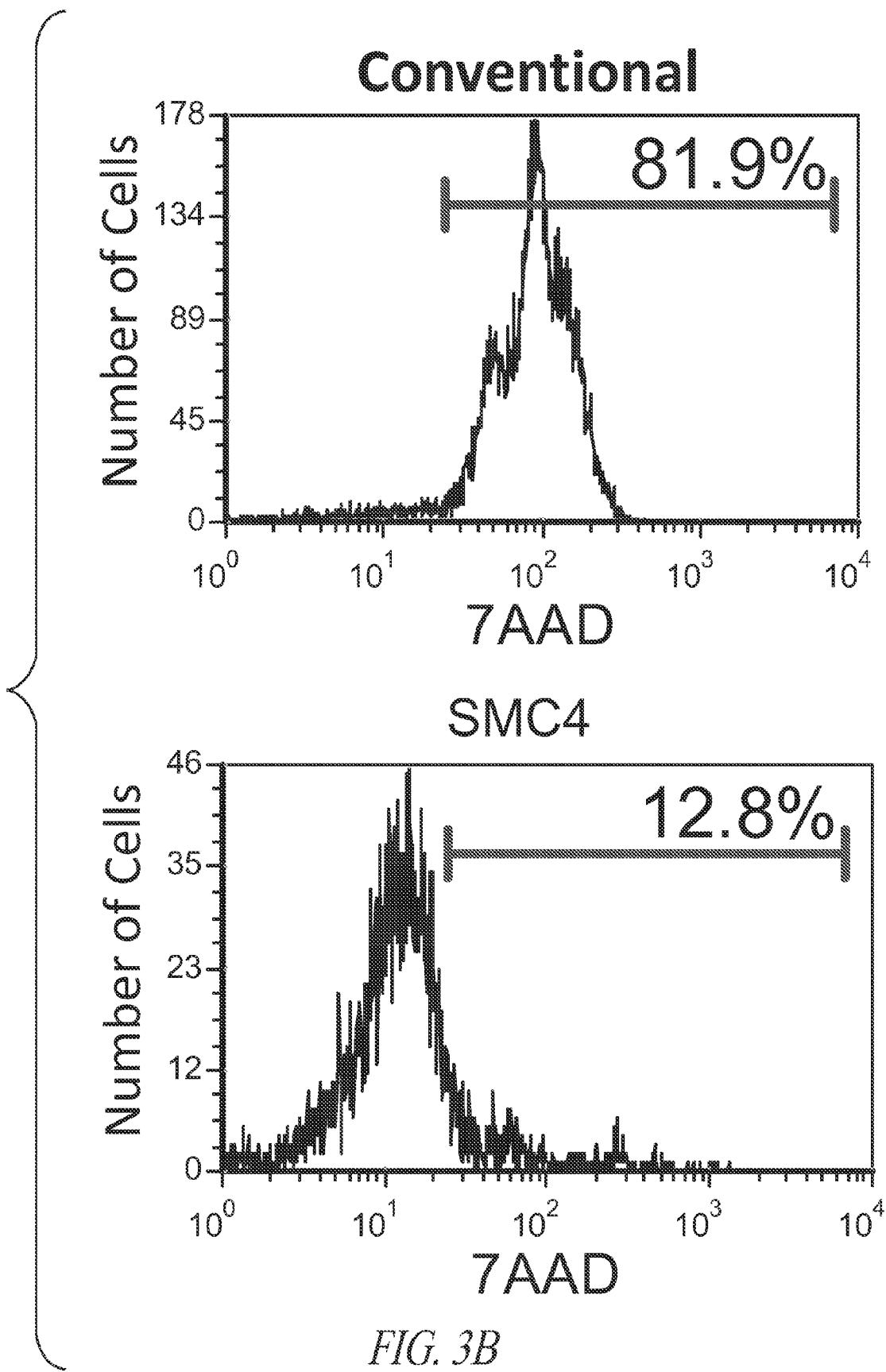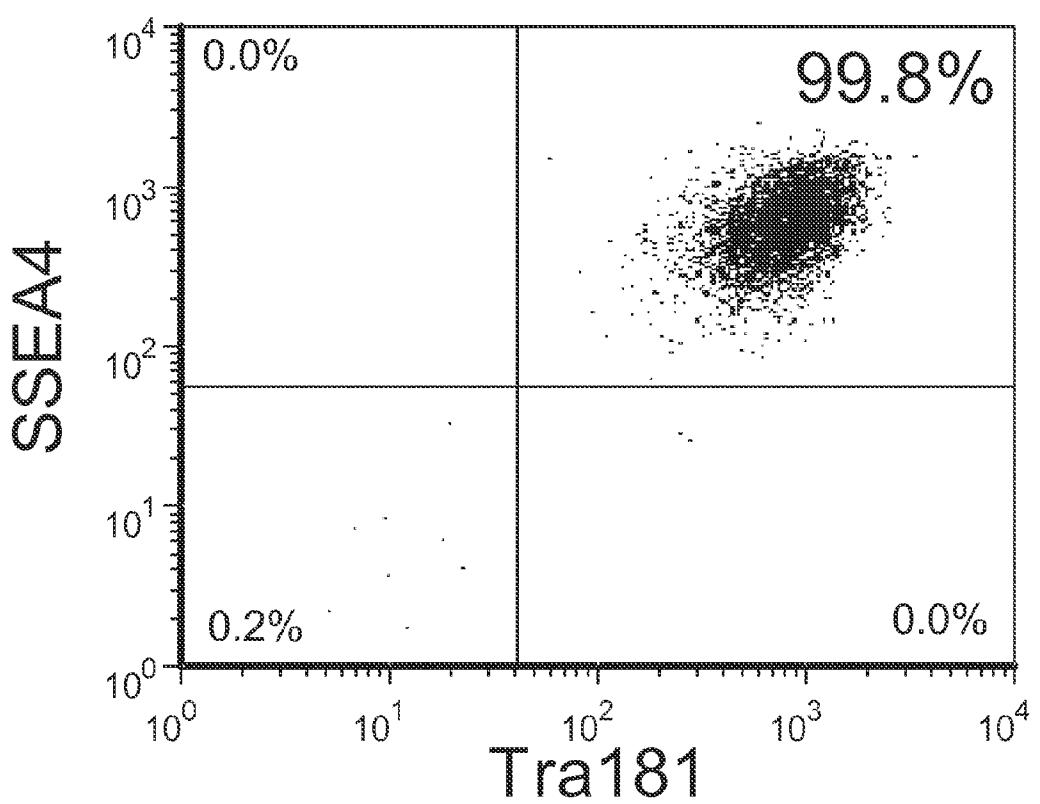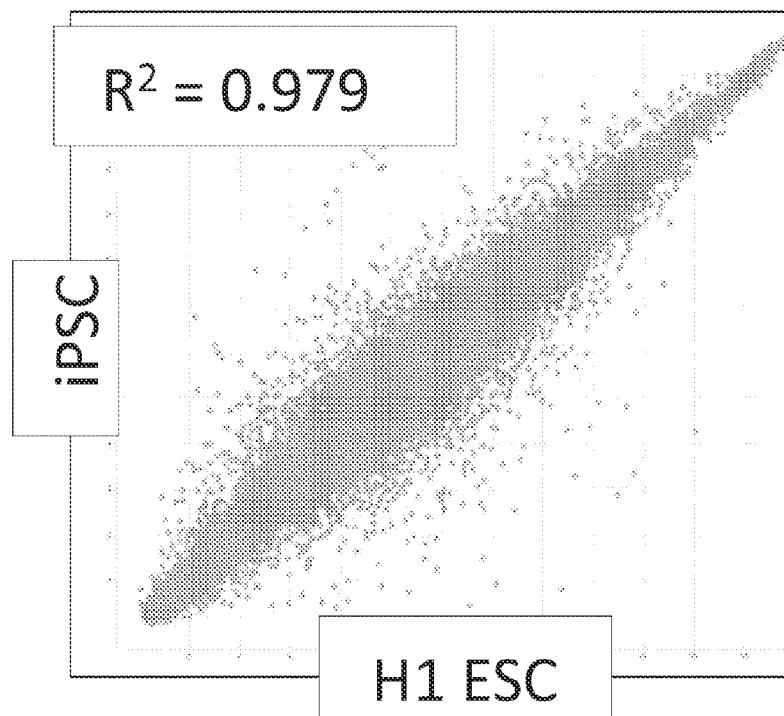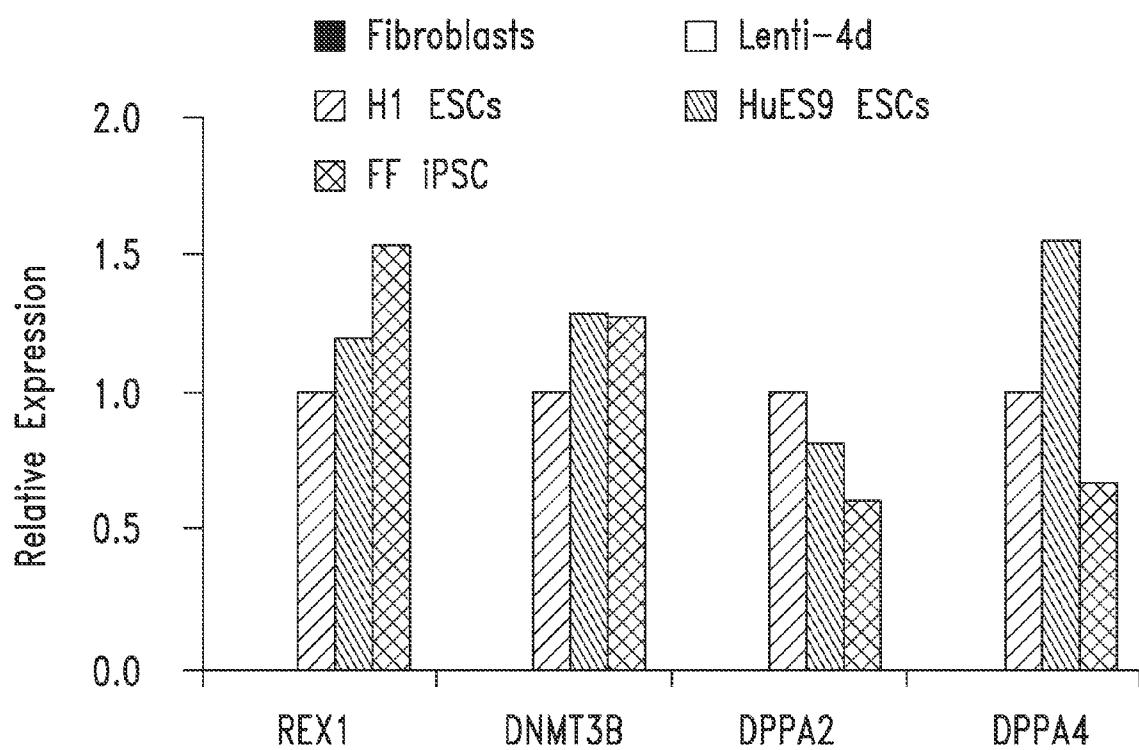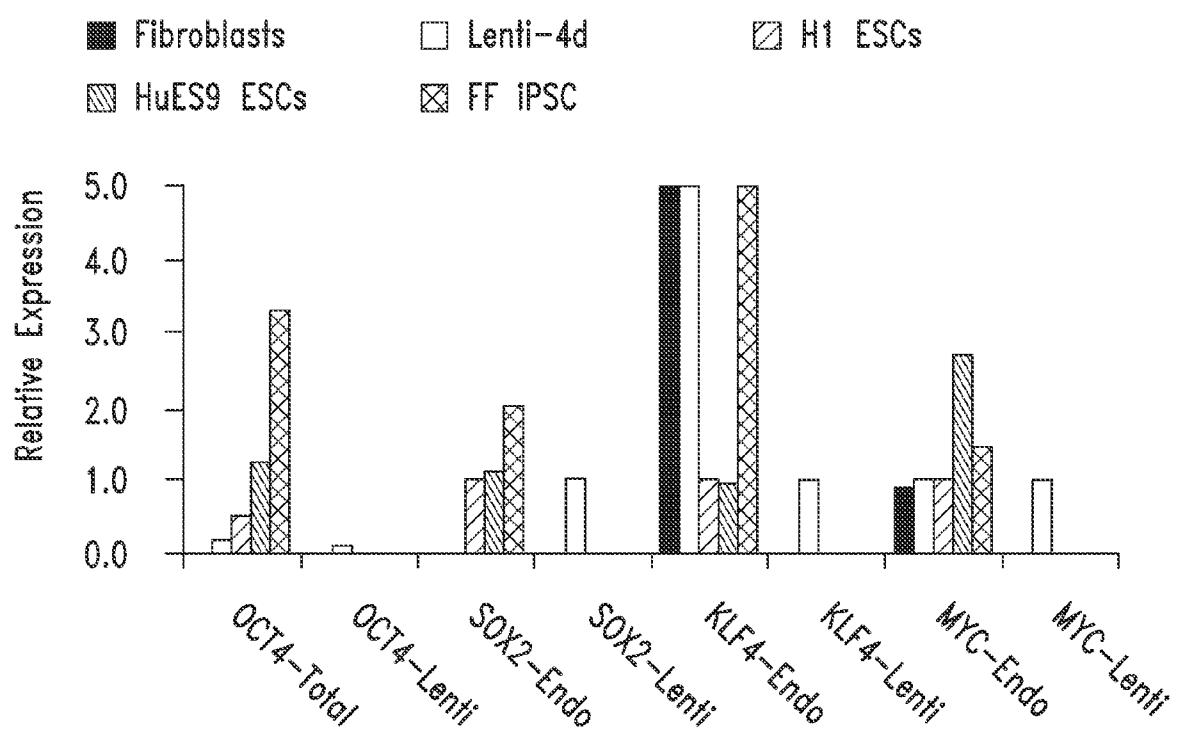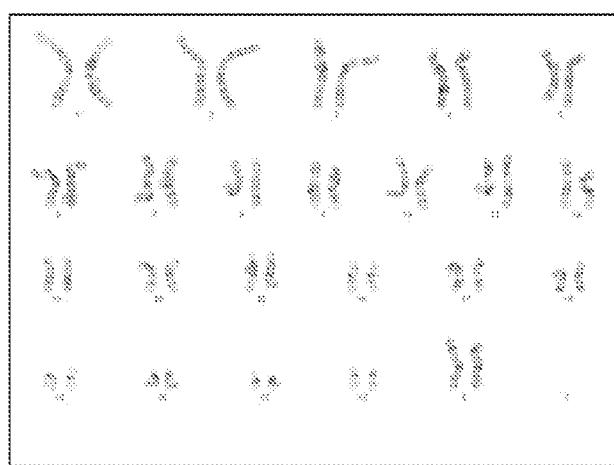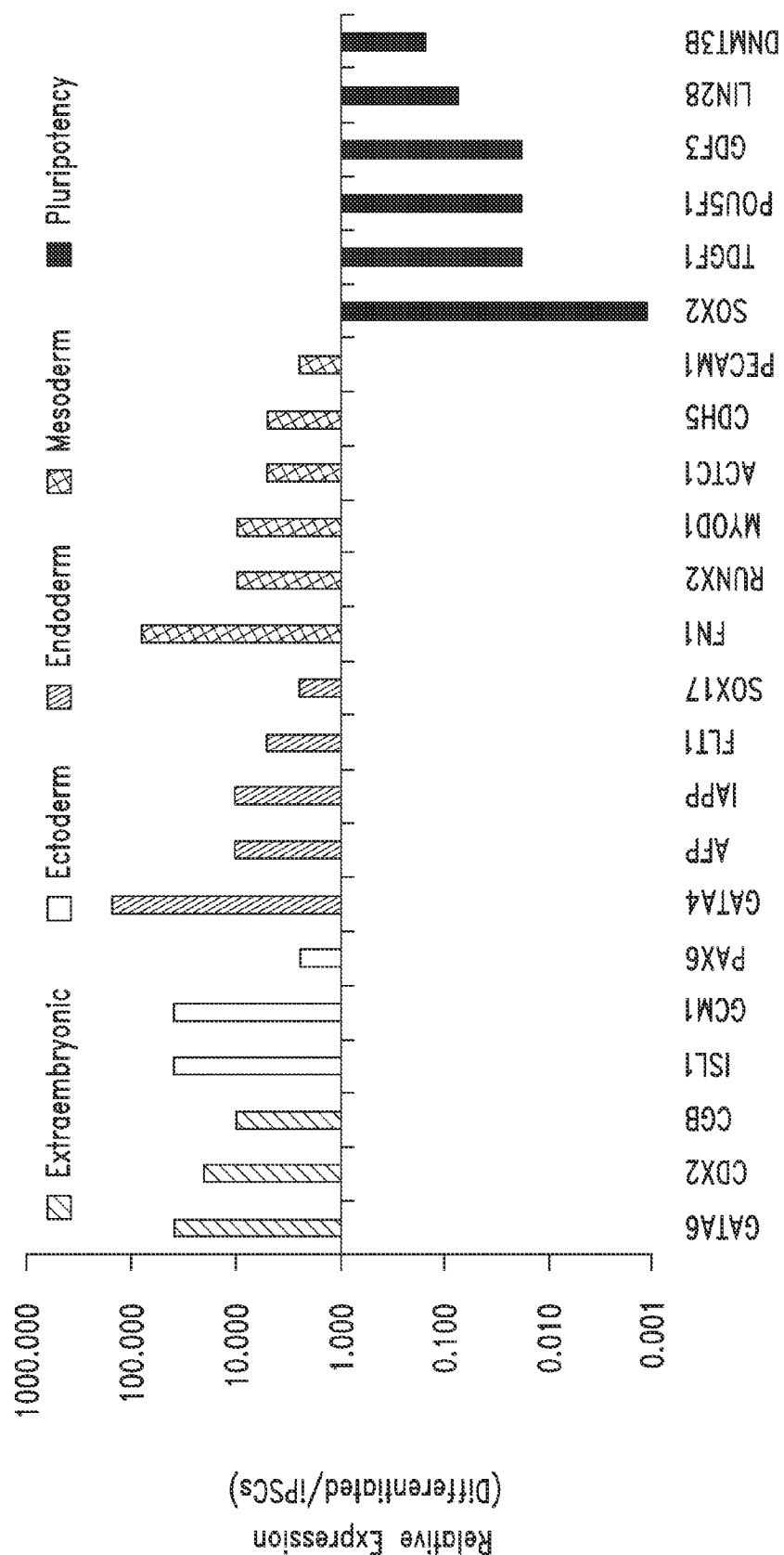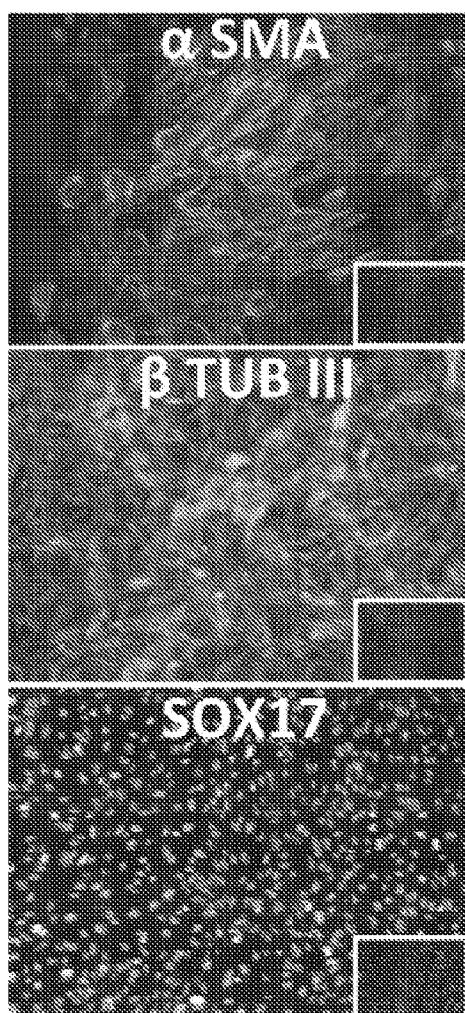
FIG. 3A

FIG. 3B

FIG. 3C

$$R^2 = 0.979$$

iPSC

H1 ESC

FIG. 3D

Legend:
- ■ Fibroblasts
- ☐ Lenti-4d
- ▨ H1 ESCs
- ▧ HuES9 ESCs
- ⊠ FF iPSC

FIG. 3E

FIG. 3F

FIG. 3G

FIG. 3H

FIG. 3I

FIG. 3J

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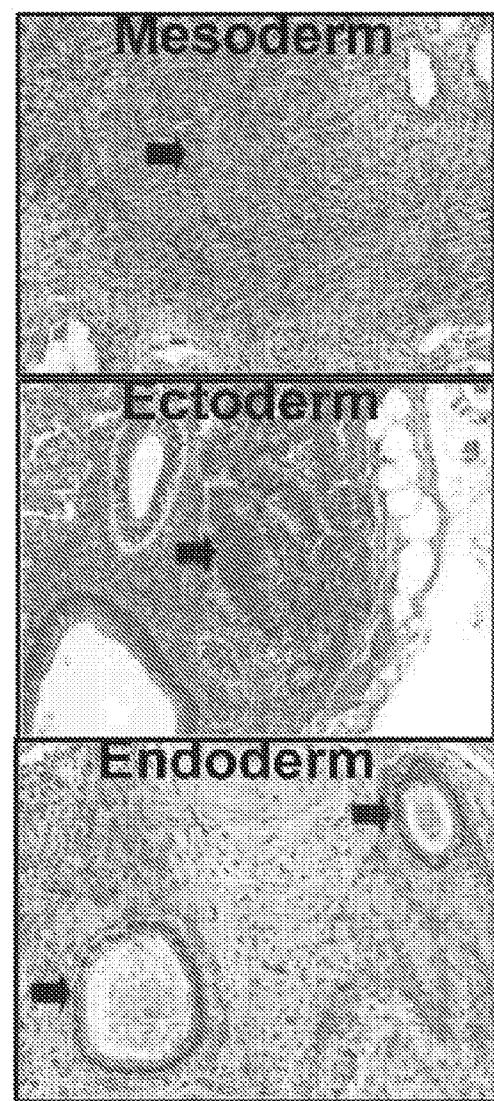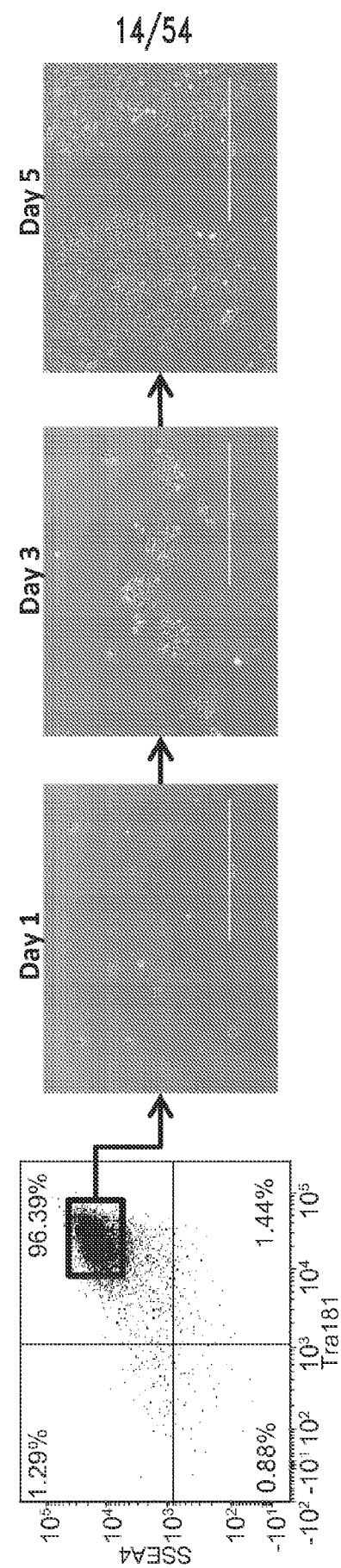
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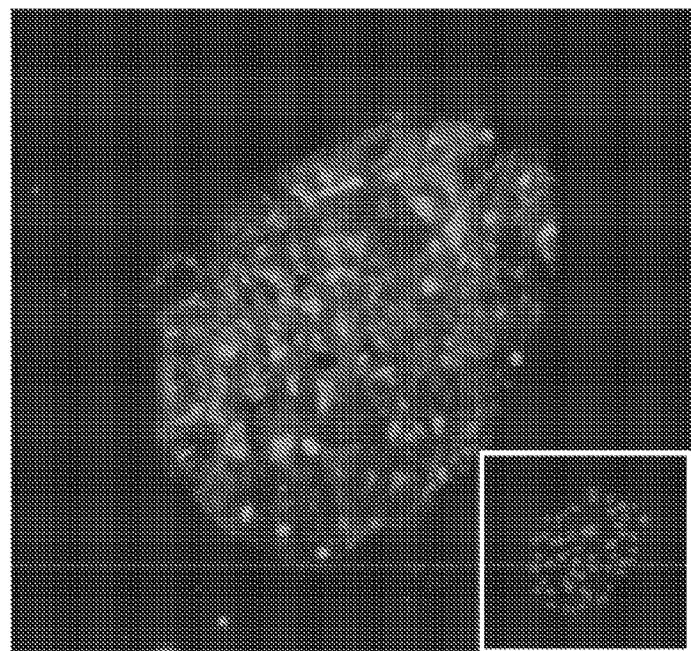
FIG. 4B
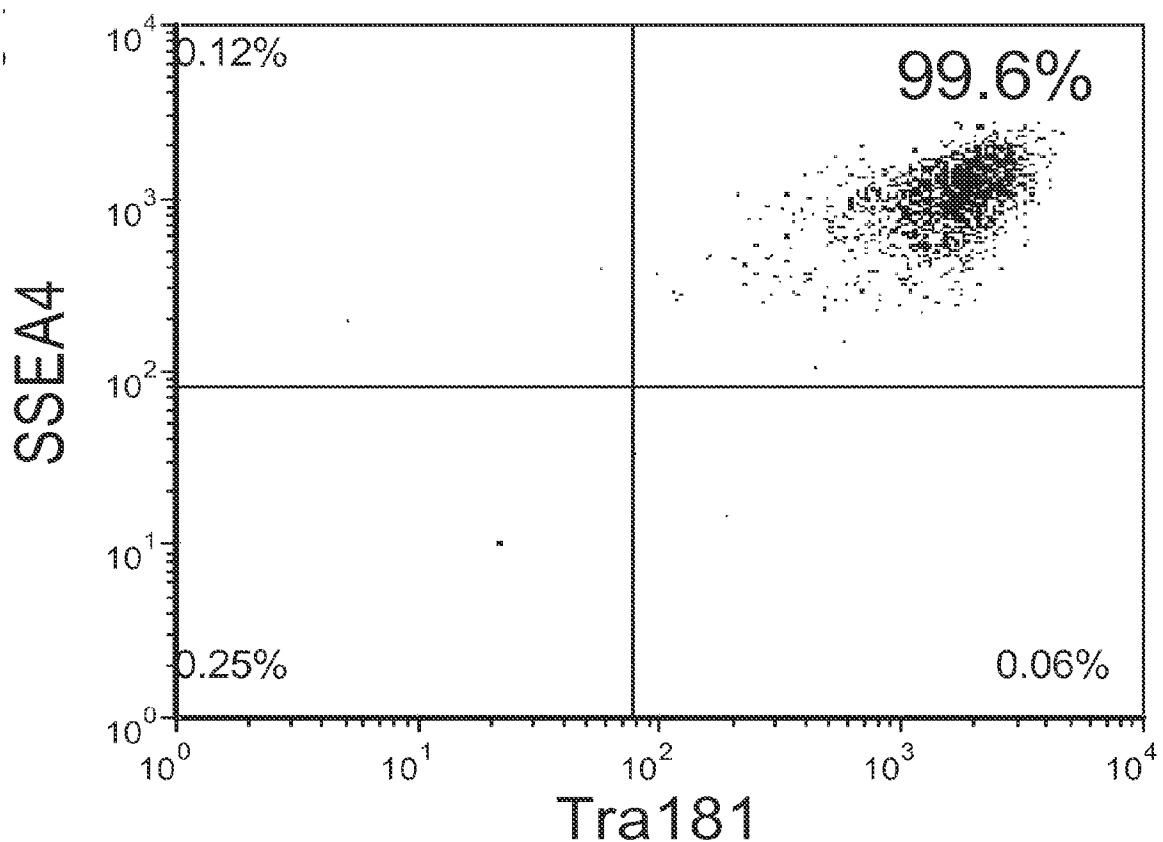
FIG. 4C
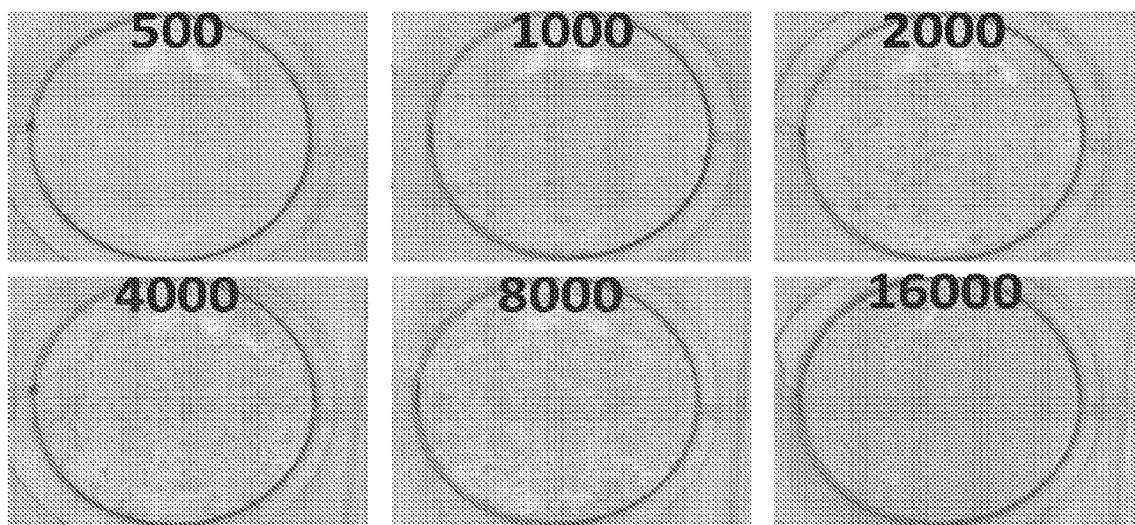
FIG. 4D
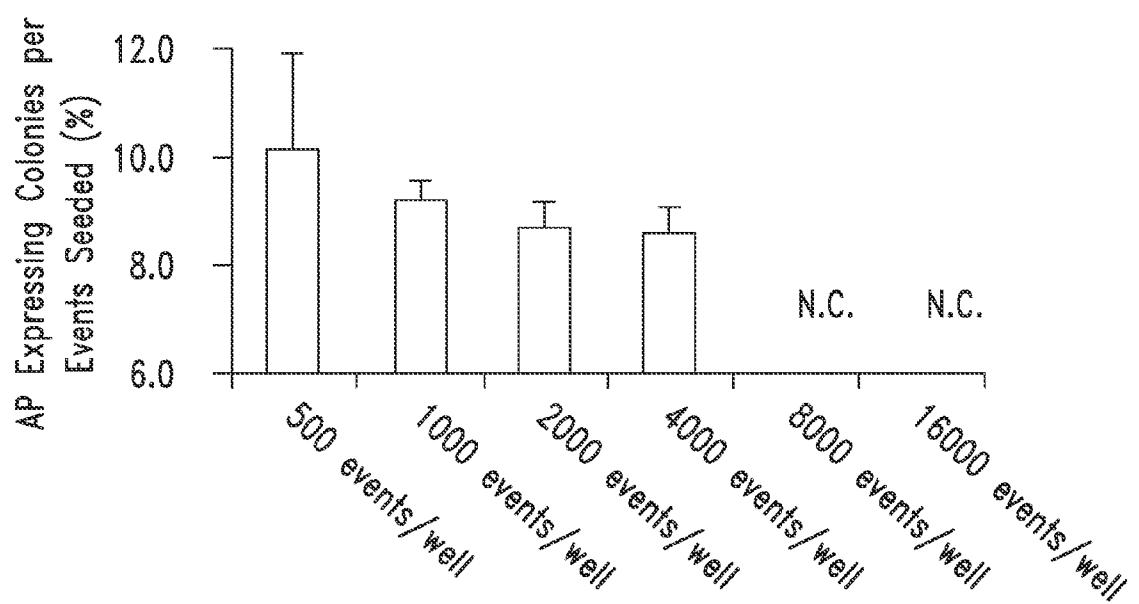
FIG. 4E
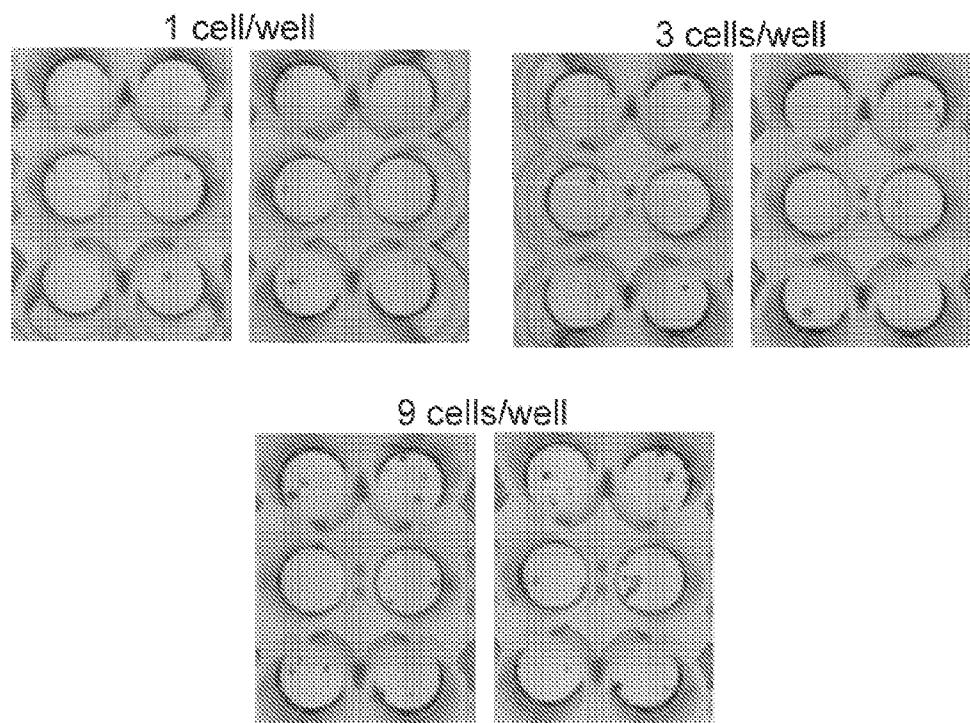
FIG. 4F
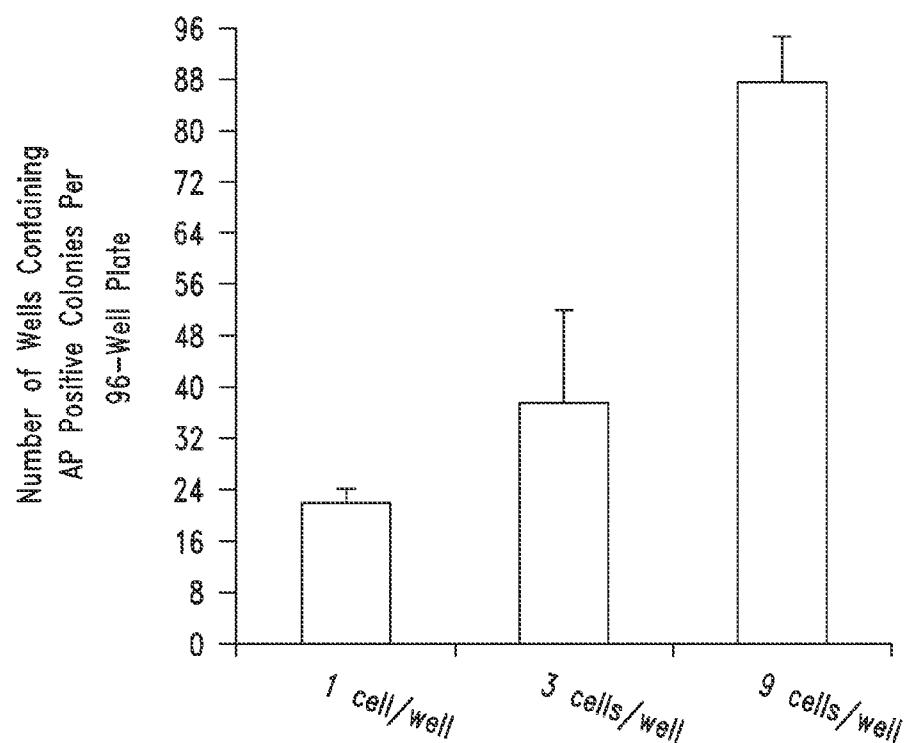
FIG. 4G
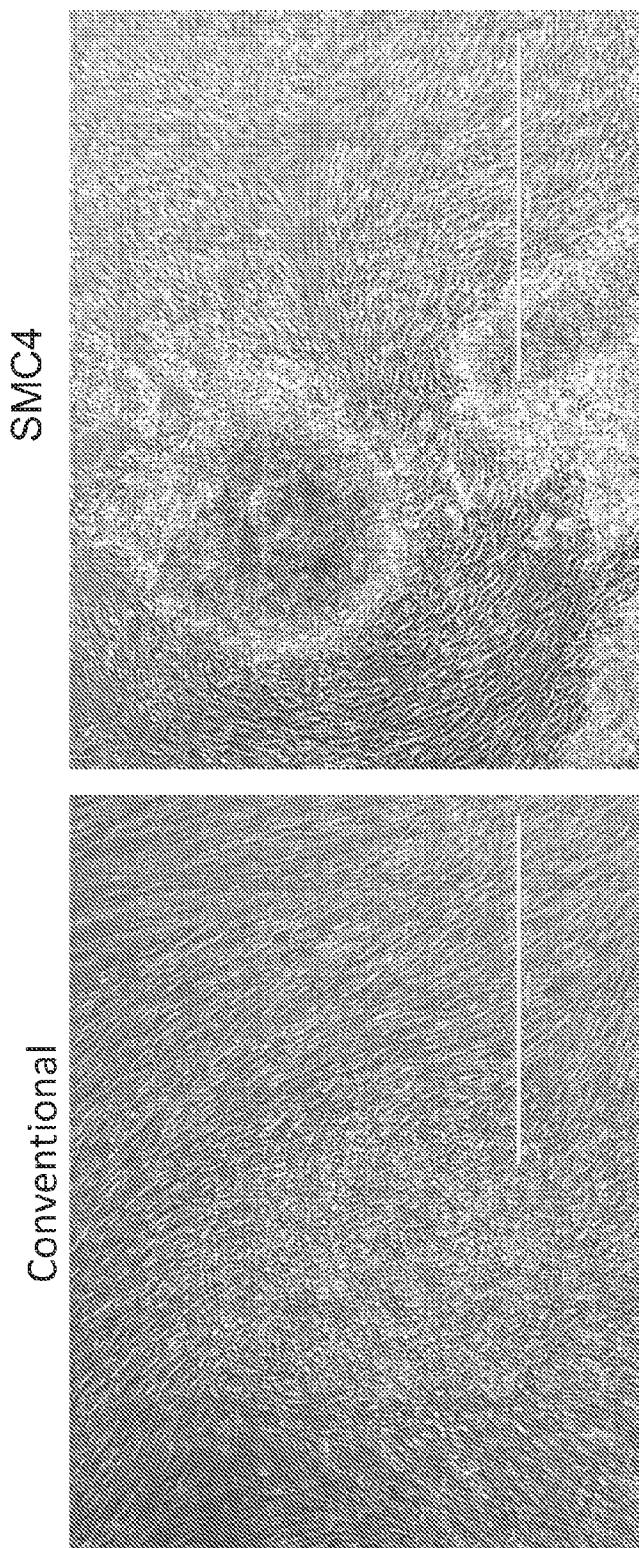
Conventional   SMC4

FIG. 5A
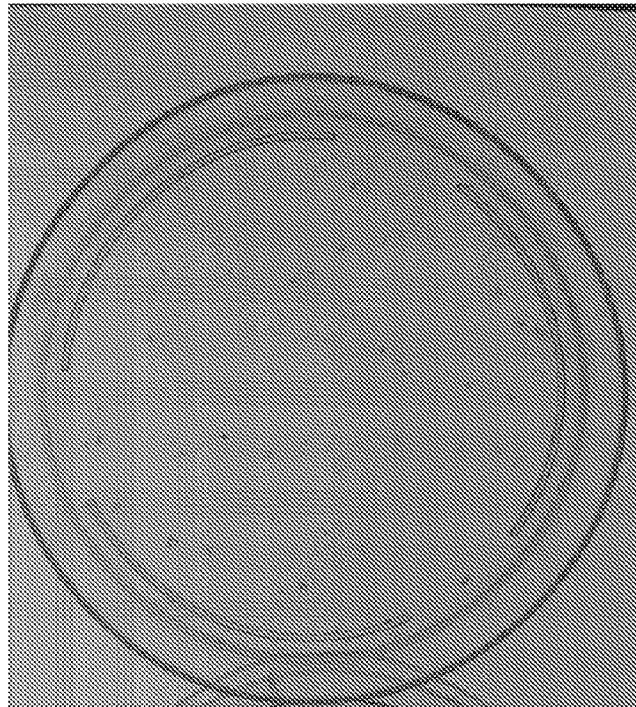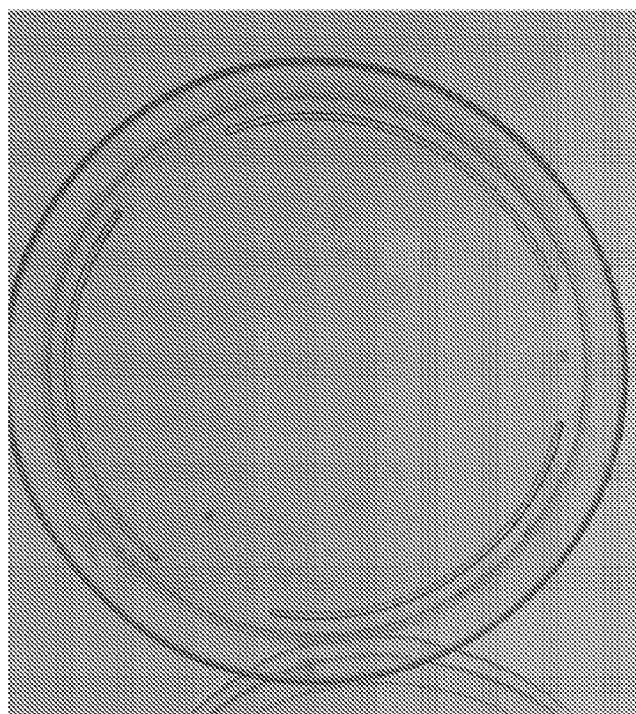
FIG. 5B
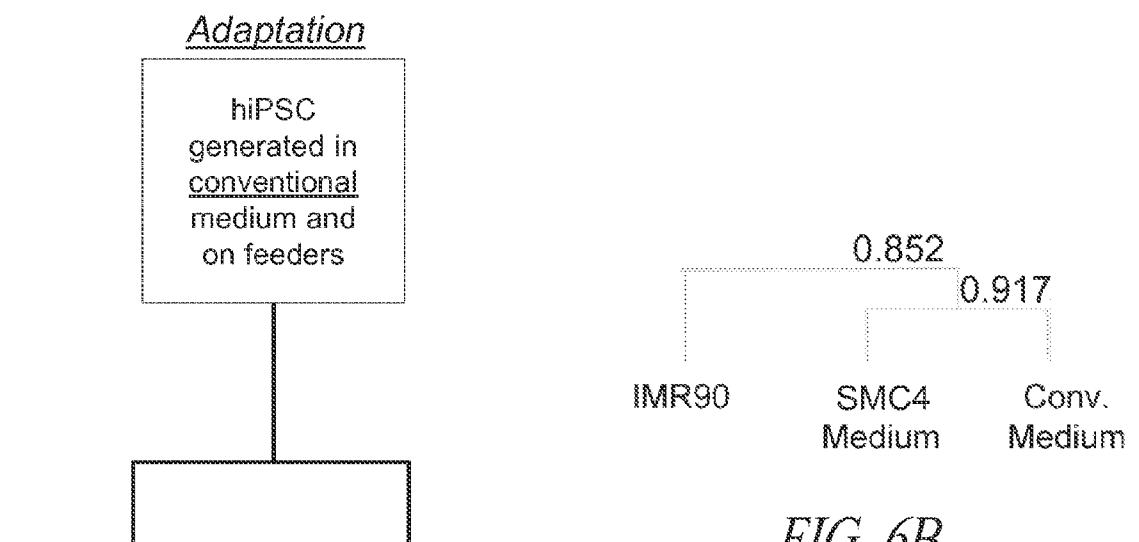
FIG. 6A
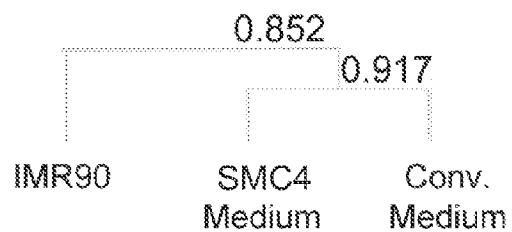
FIG. 6B
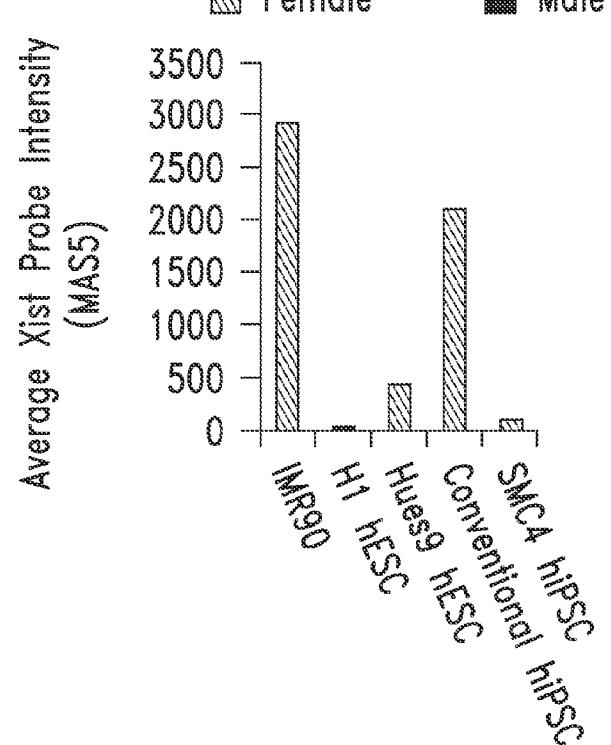
FIG. 6C
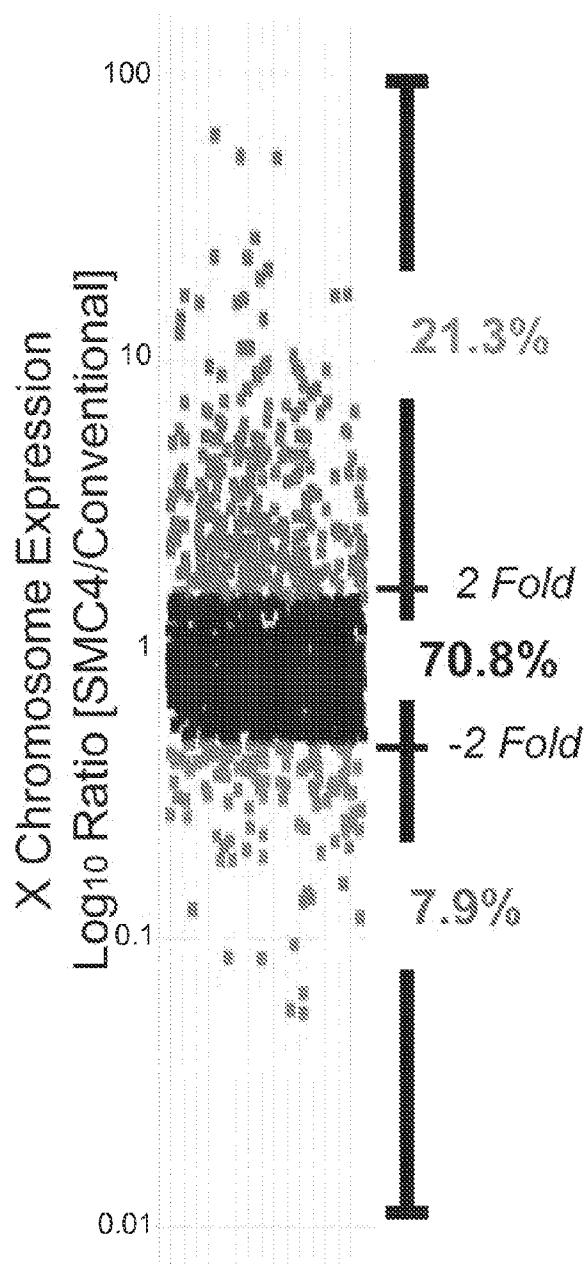
FIG. 6D
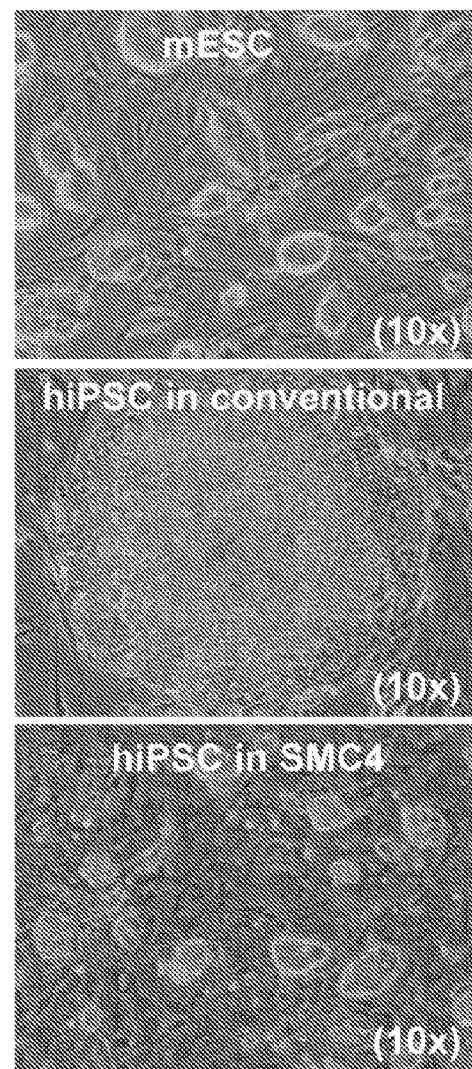
FIG. 6E
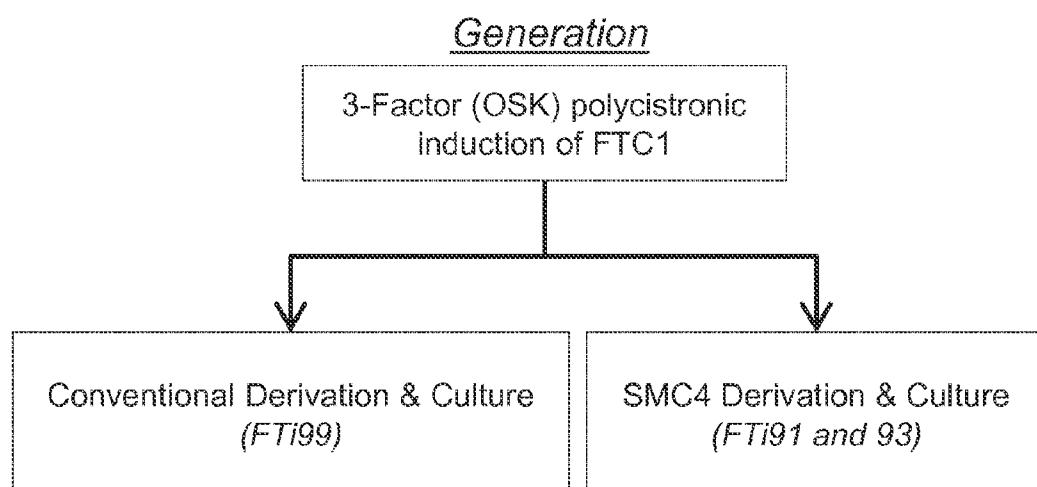
FIG. 7A
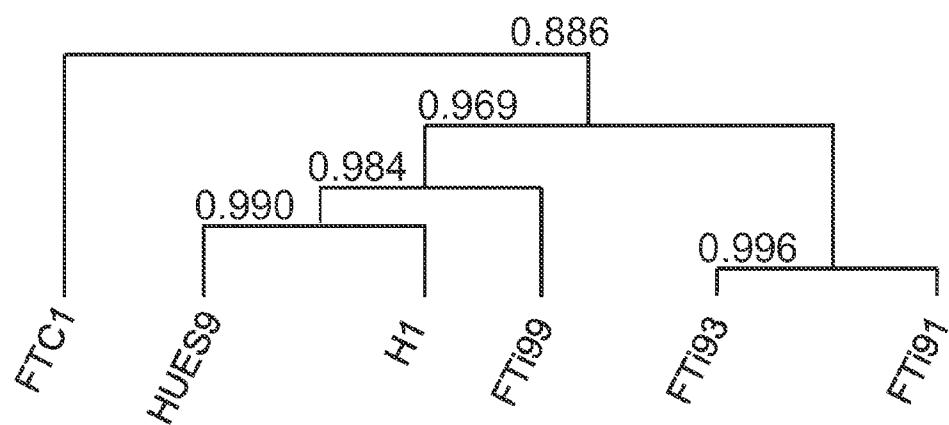
FIG. 7B
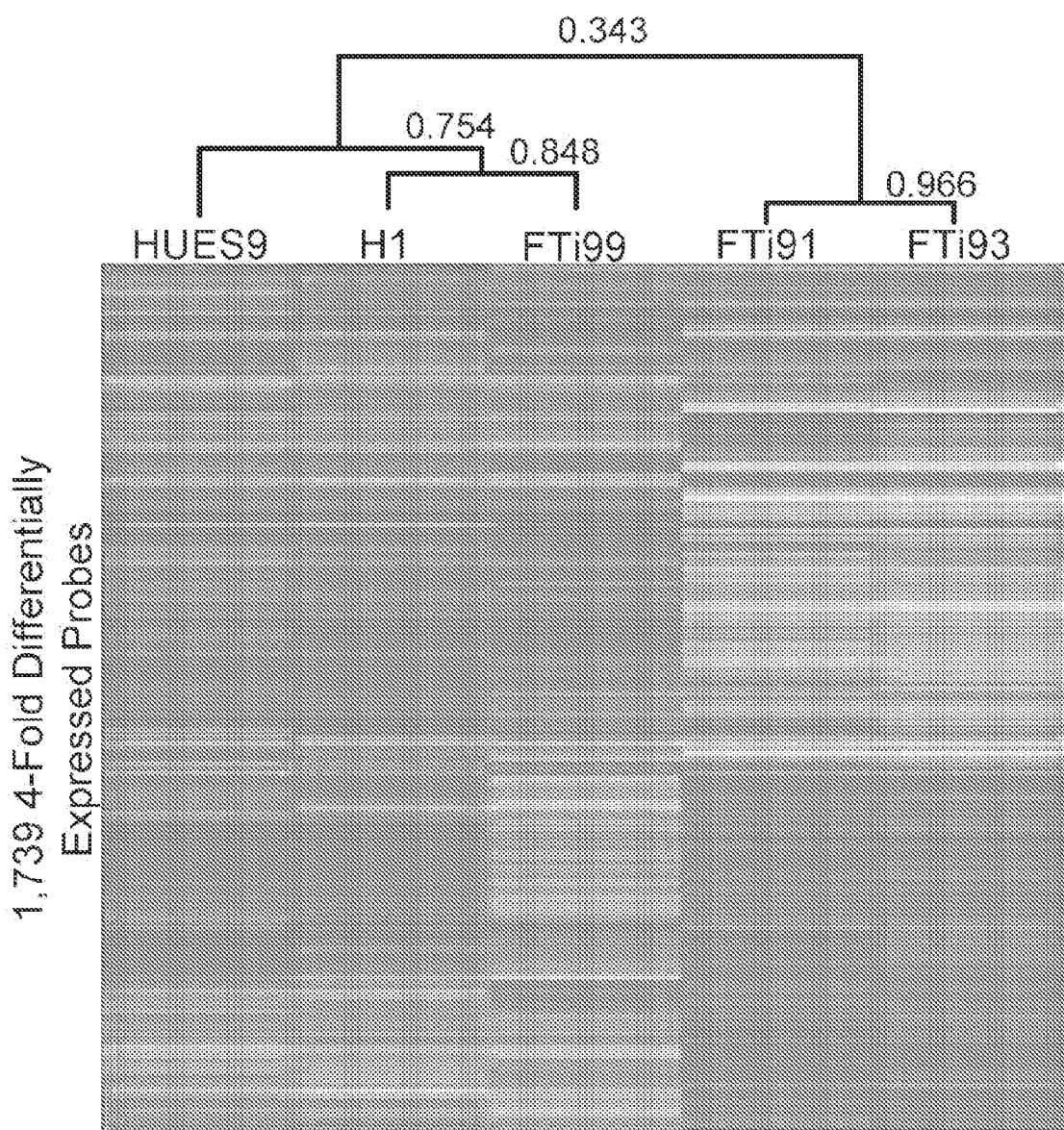
FIG. 7C
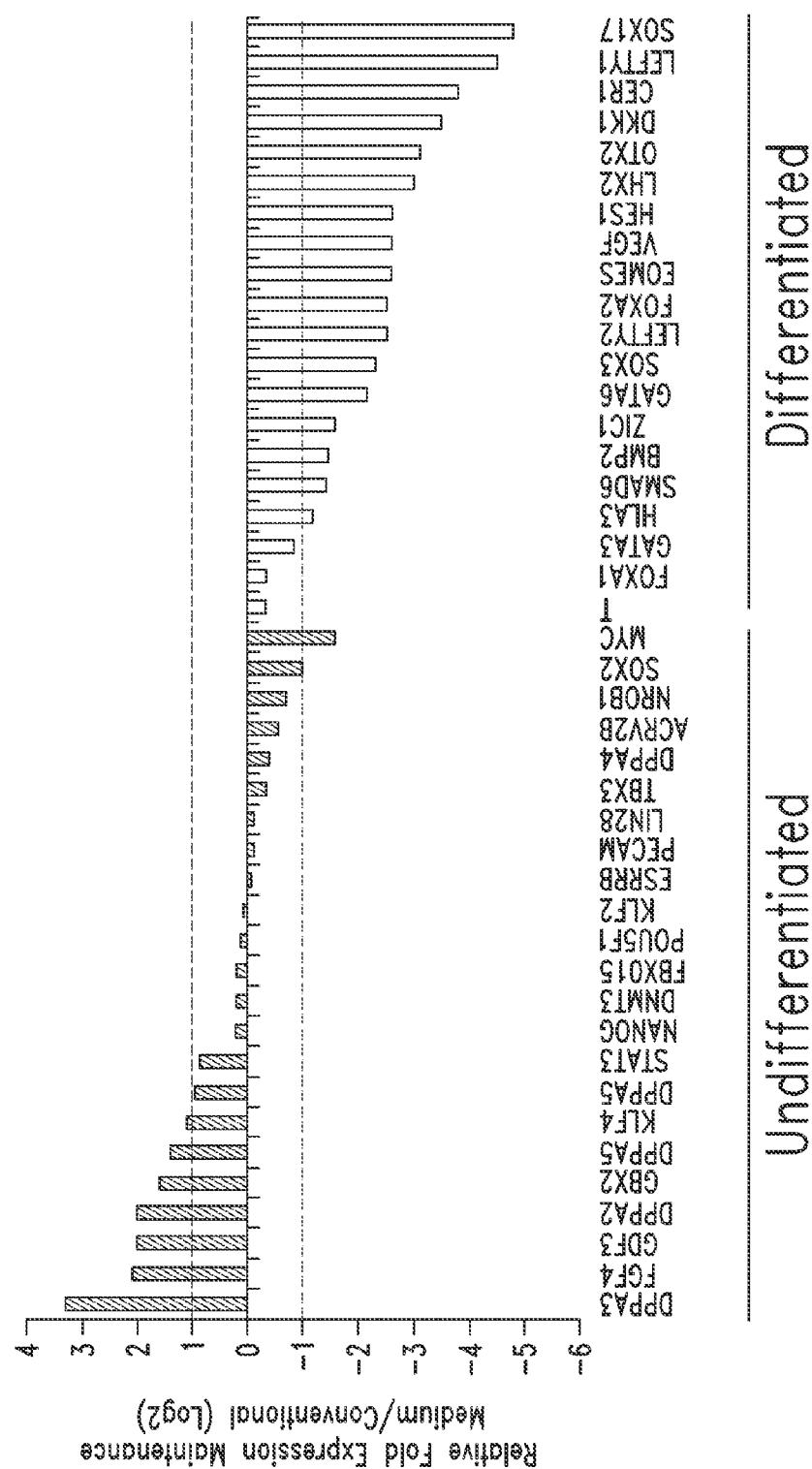
FIG. 7D
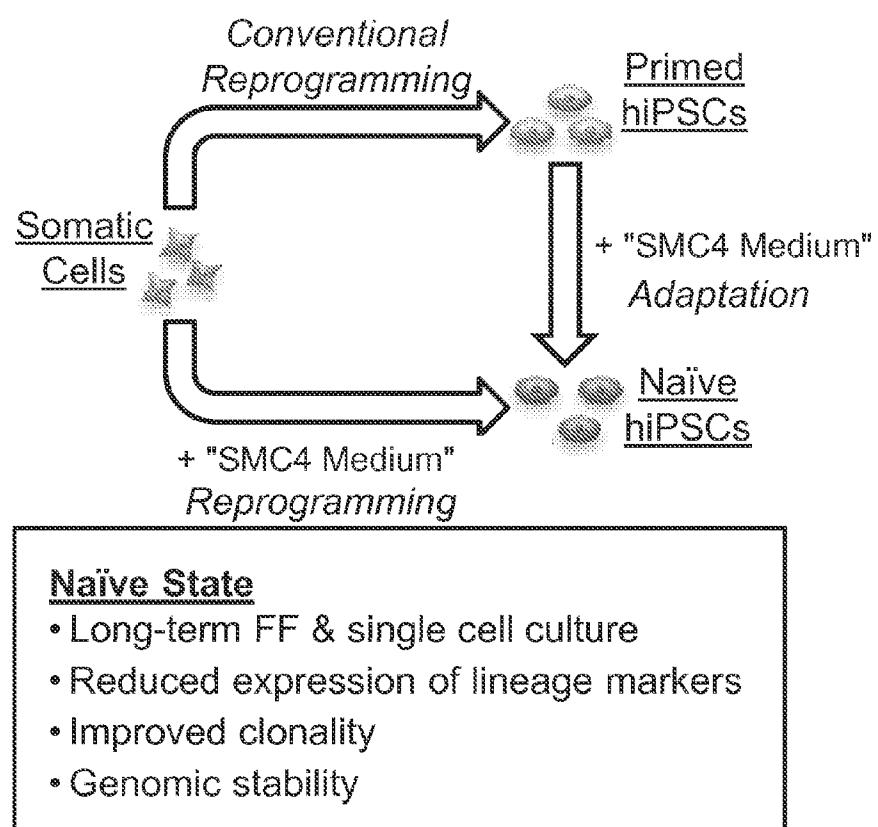
FIG. 7E
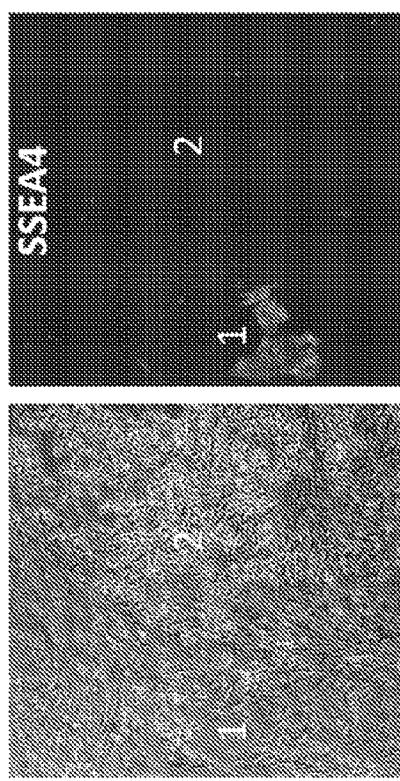
FIG. 8A
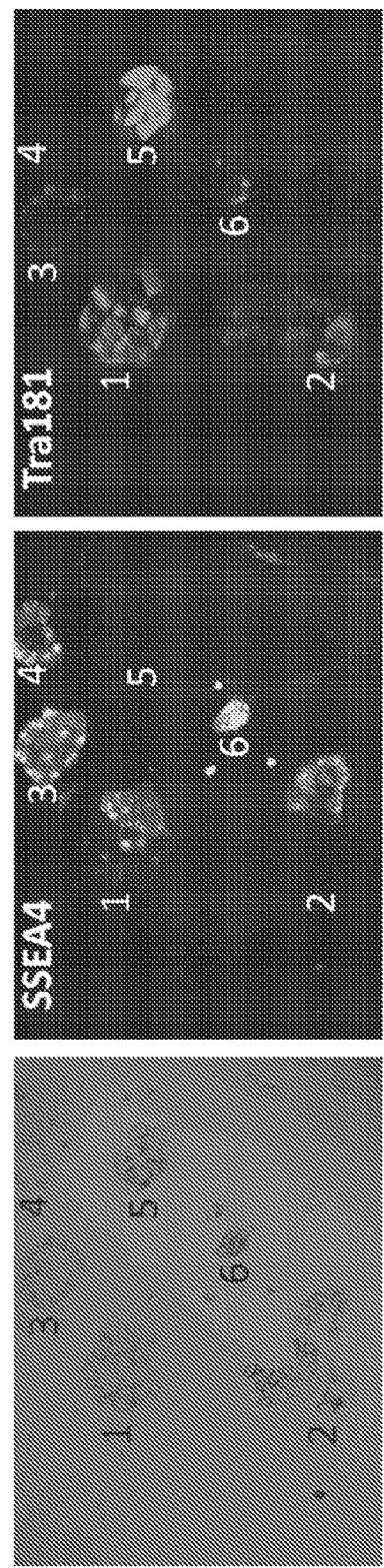
FIG. 8B
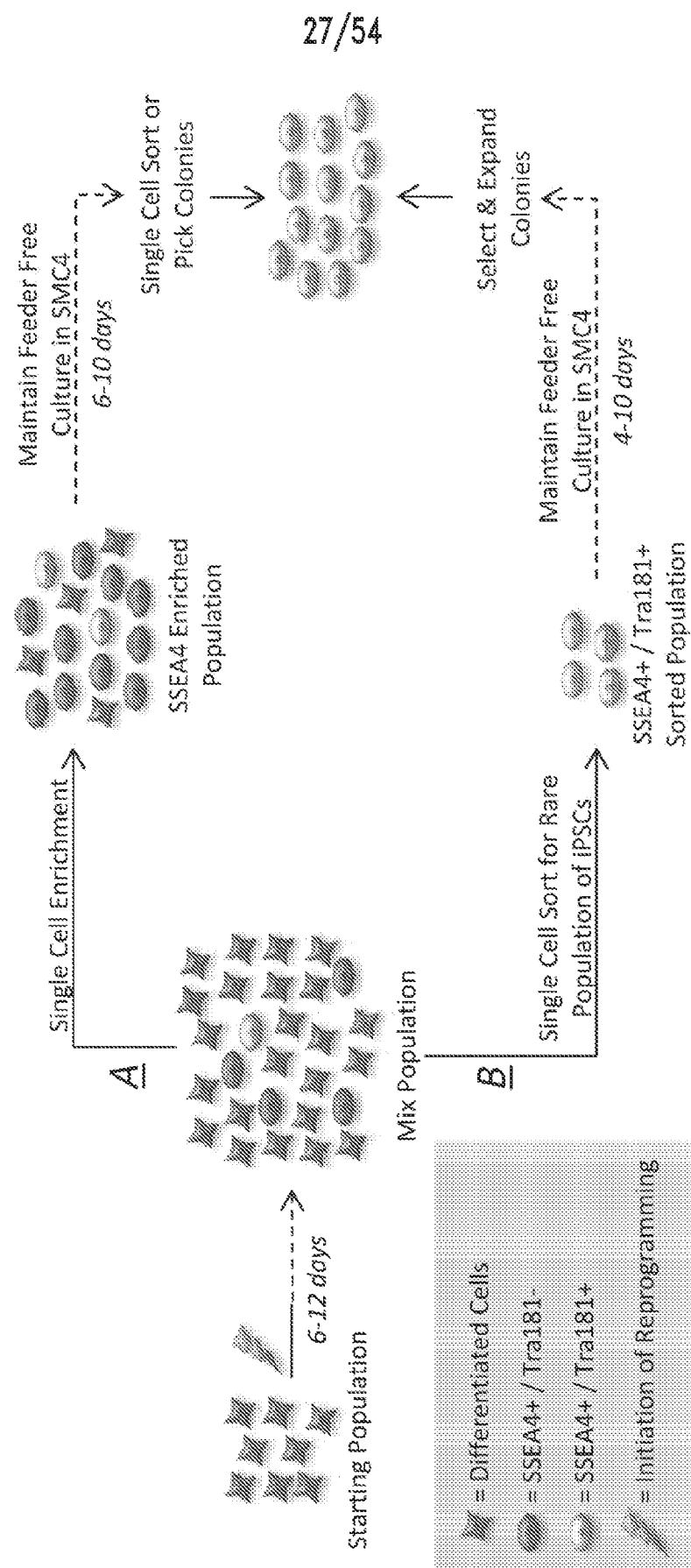
FIG. 9
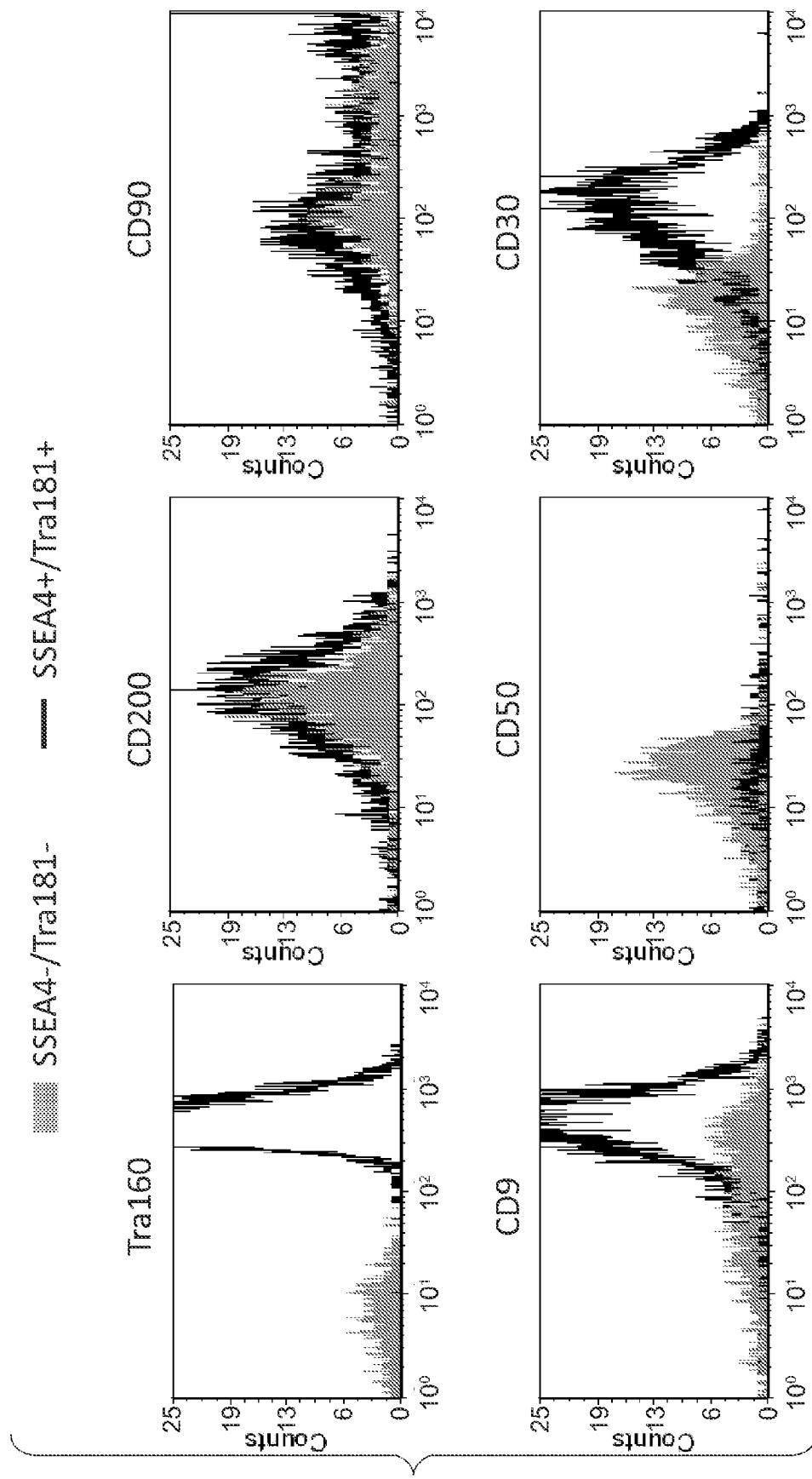
FIG. 10
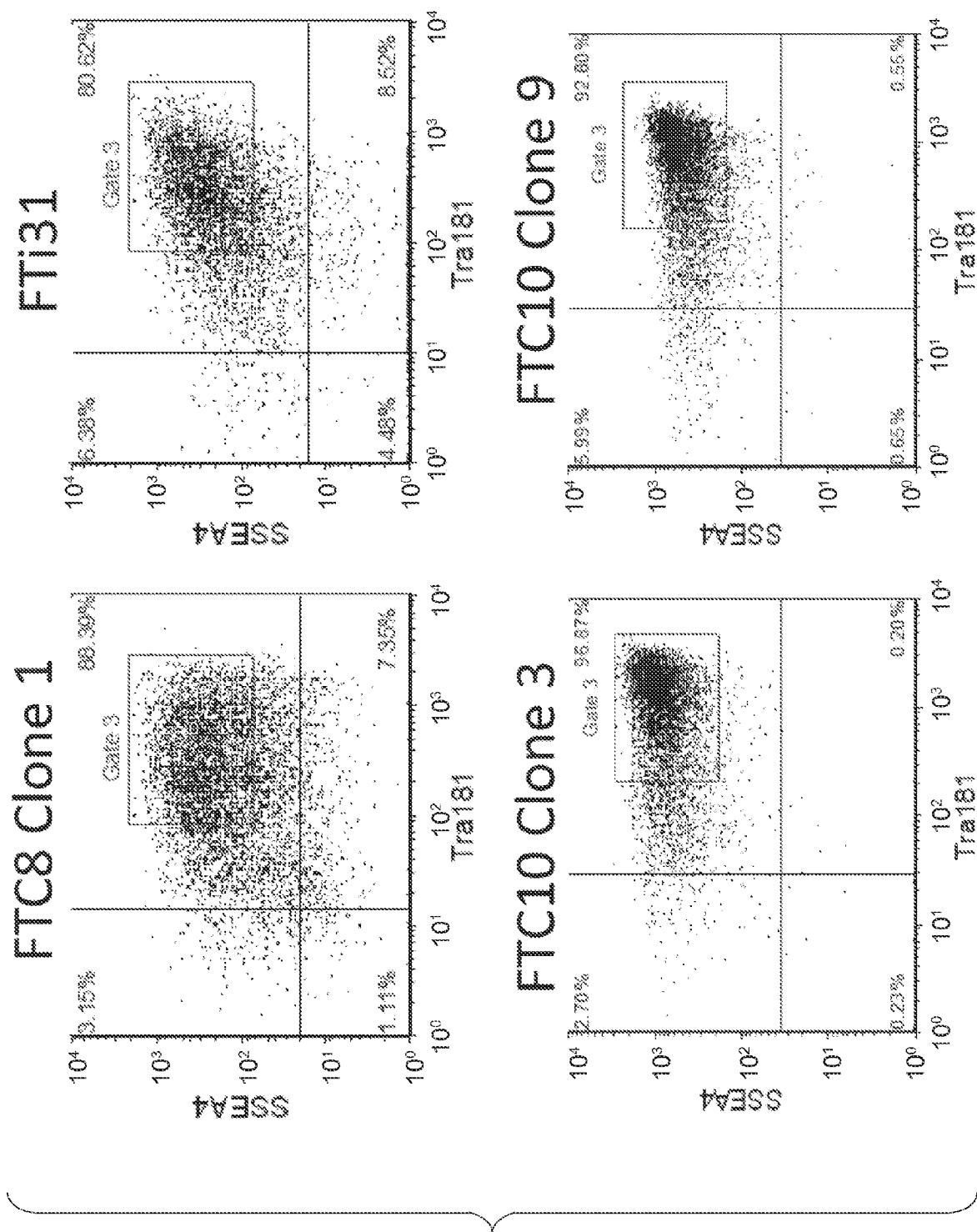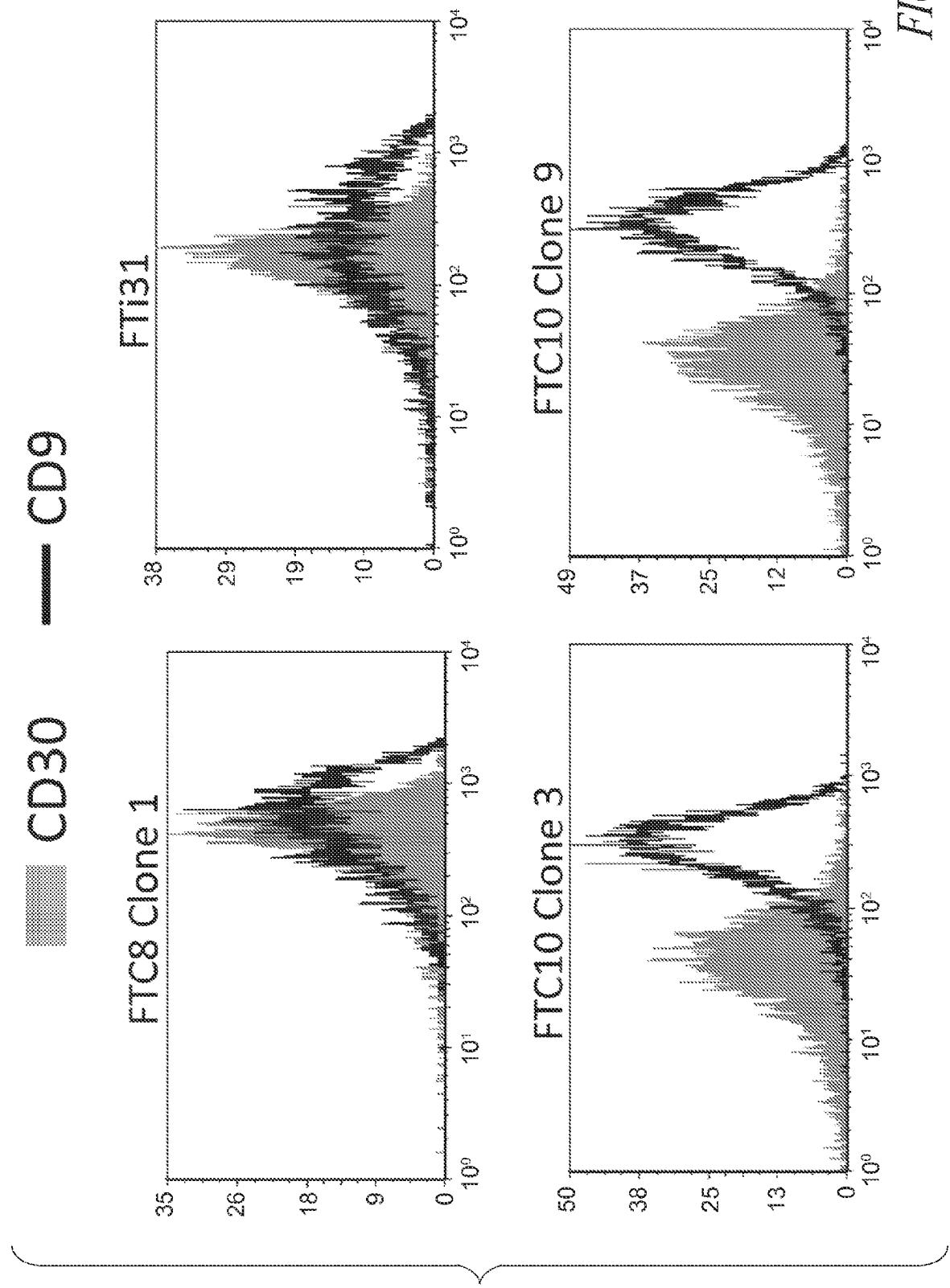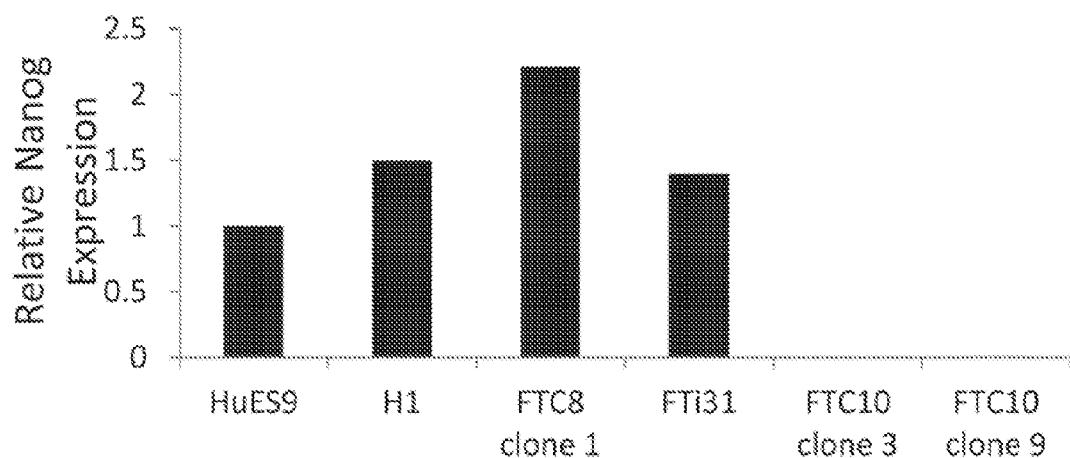
FIG. 11A

FIG. 11B

FIG. 11C

Clone	CD30 expression	Nanog Expression
FTC8 clone 1	+	+
FTi31	+	+
FTC10 clone 3	-	-
FTC10 clone 9	-	-

FIG. 11D
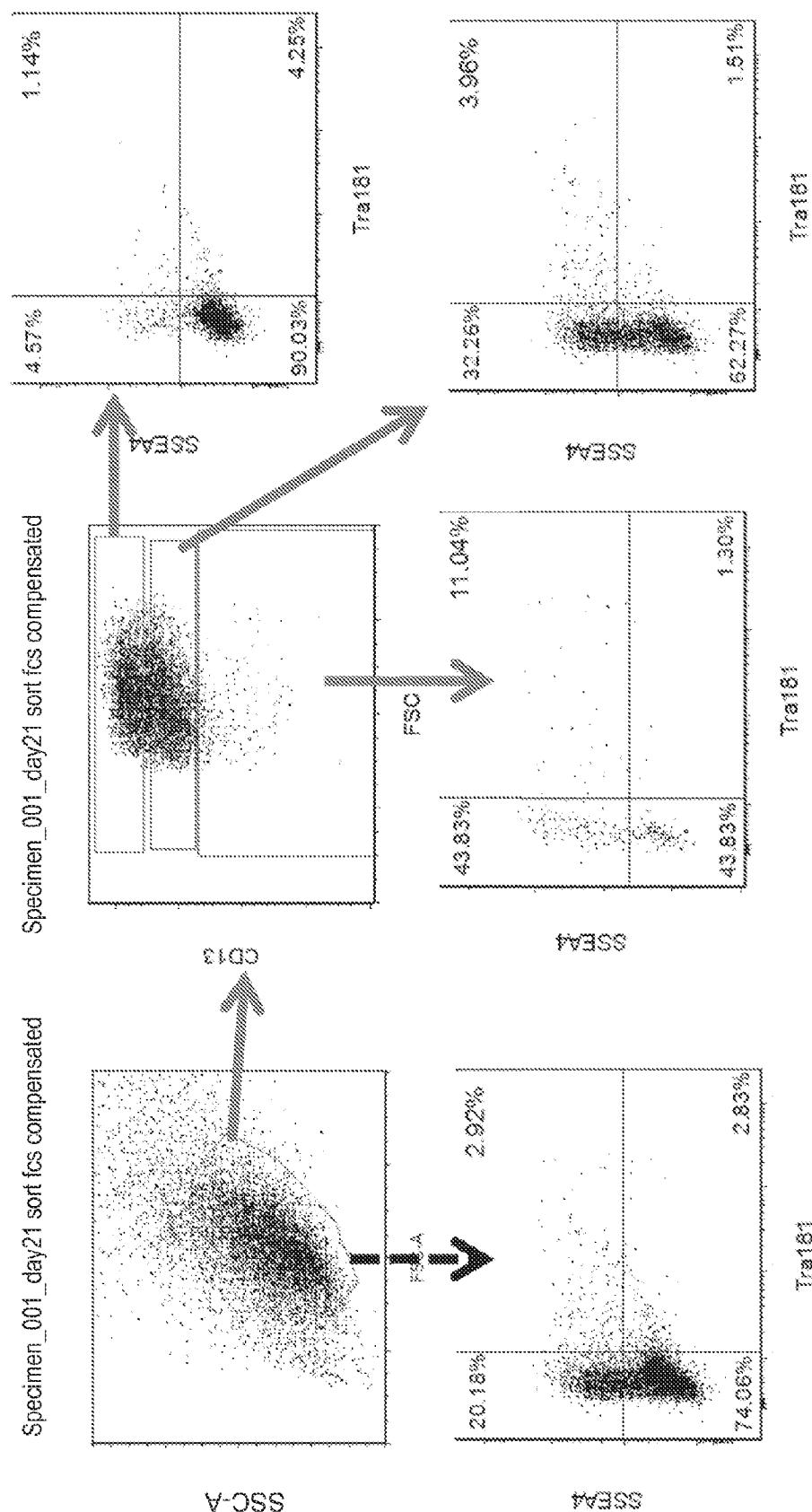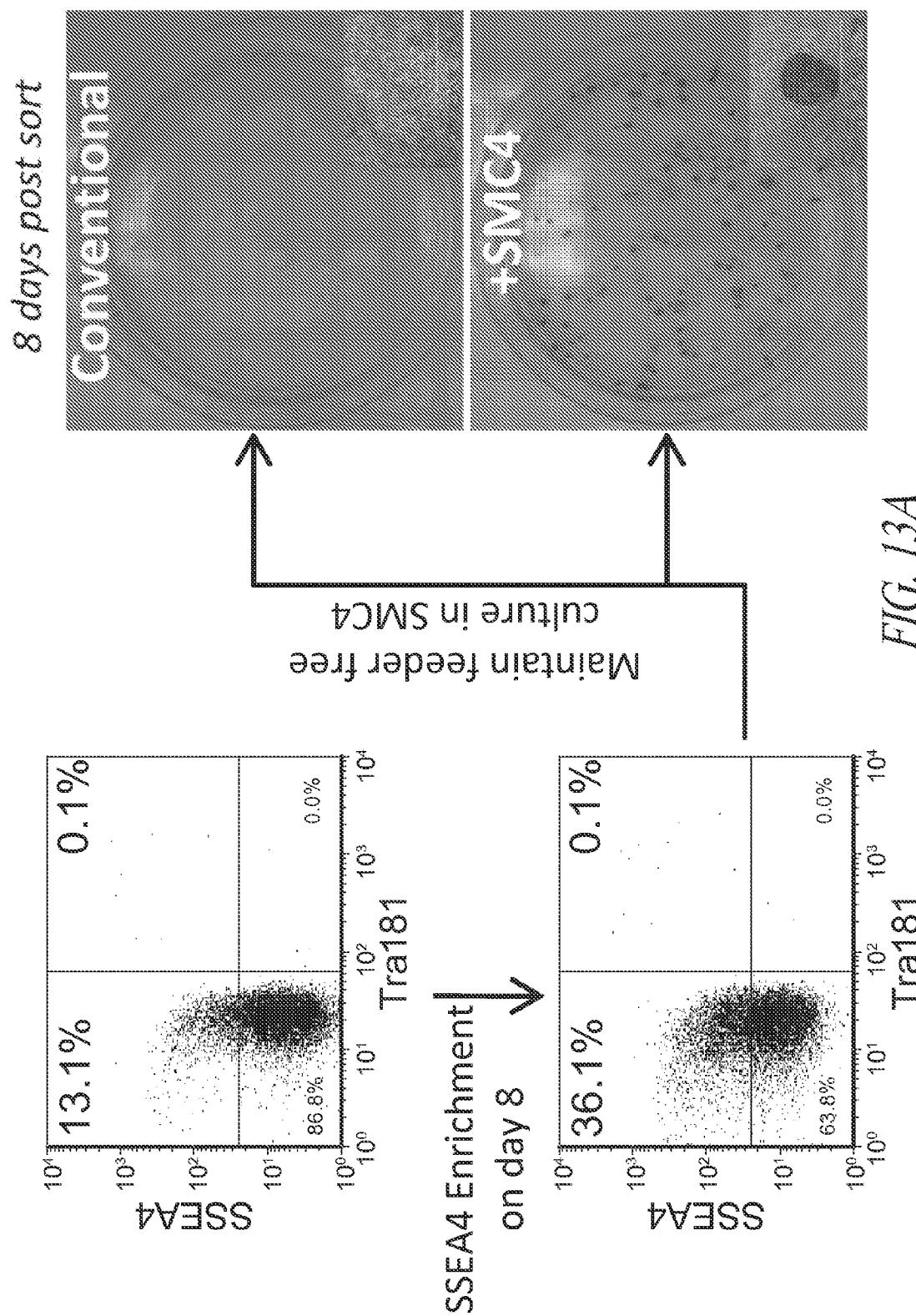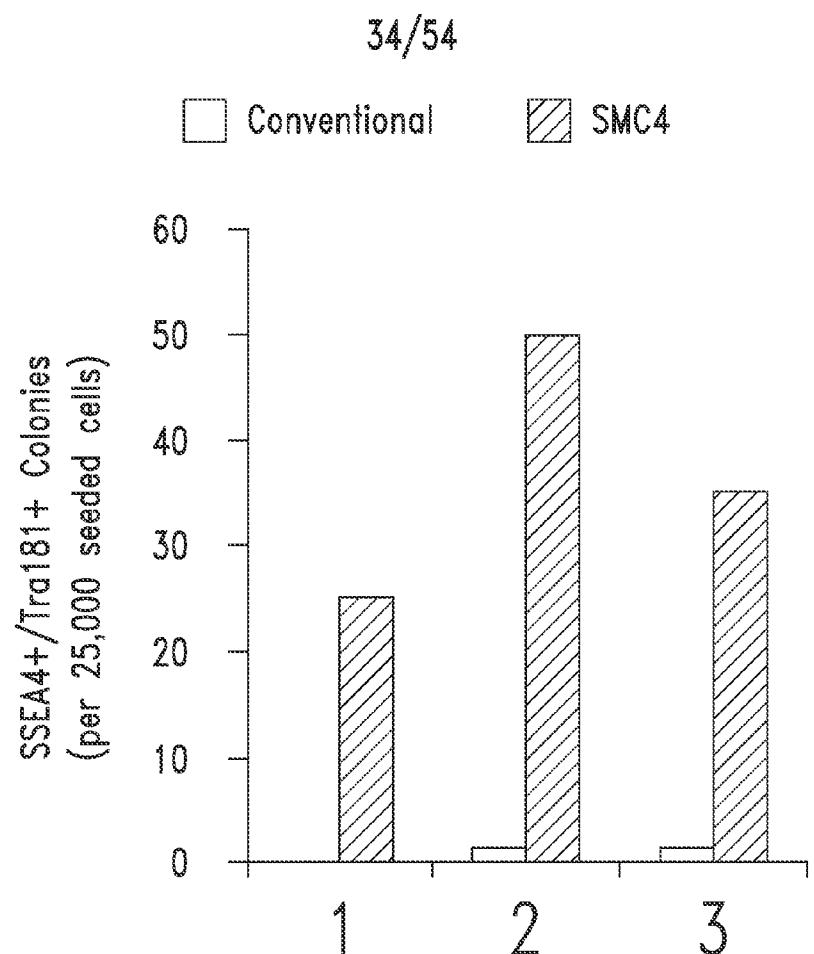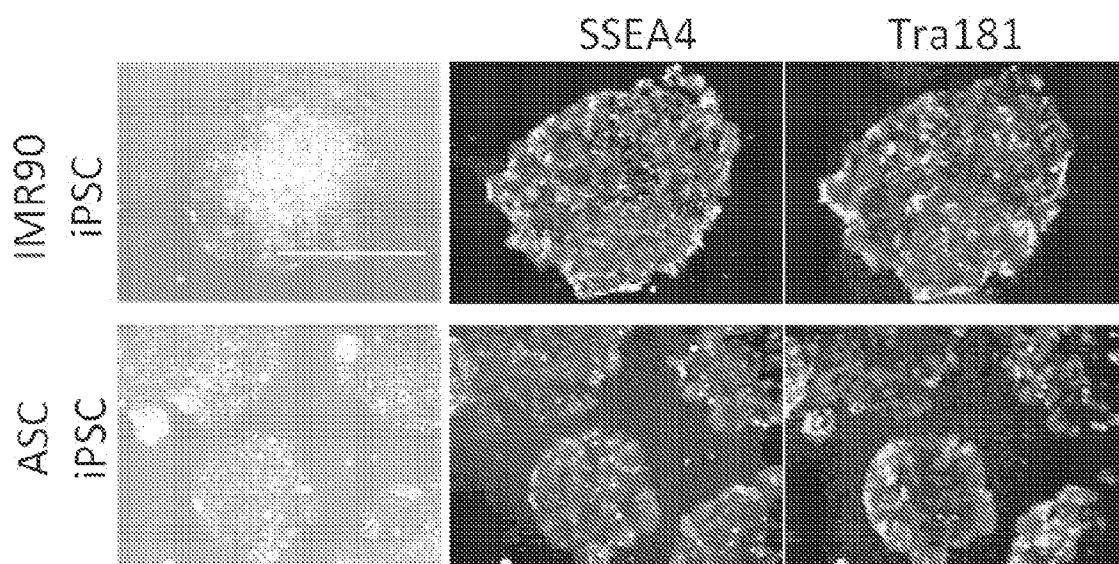
FIG. 12

FIG. 13A

FIG. 13B

FIG. 13C
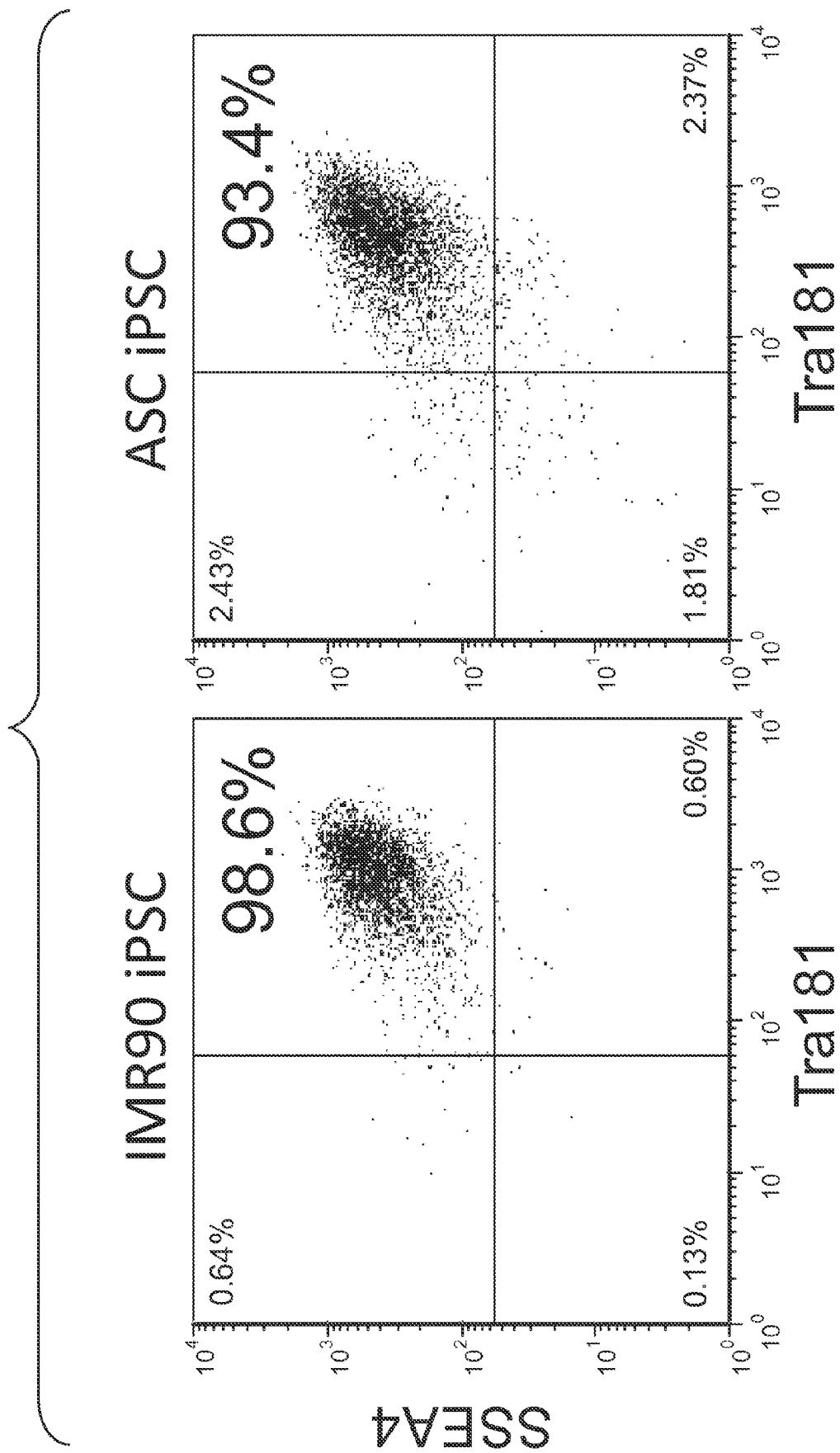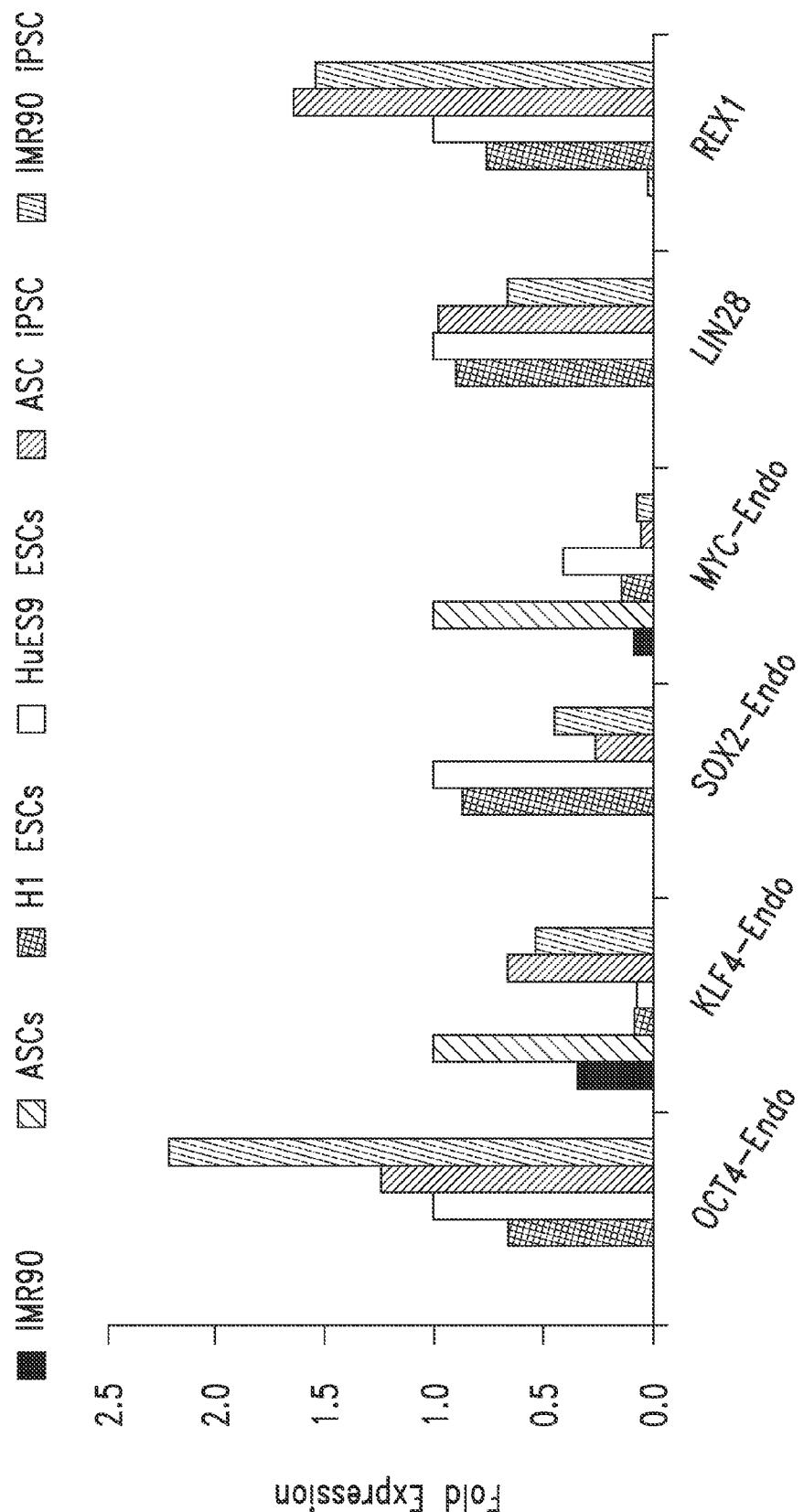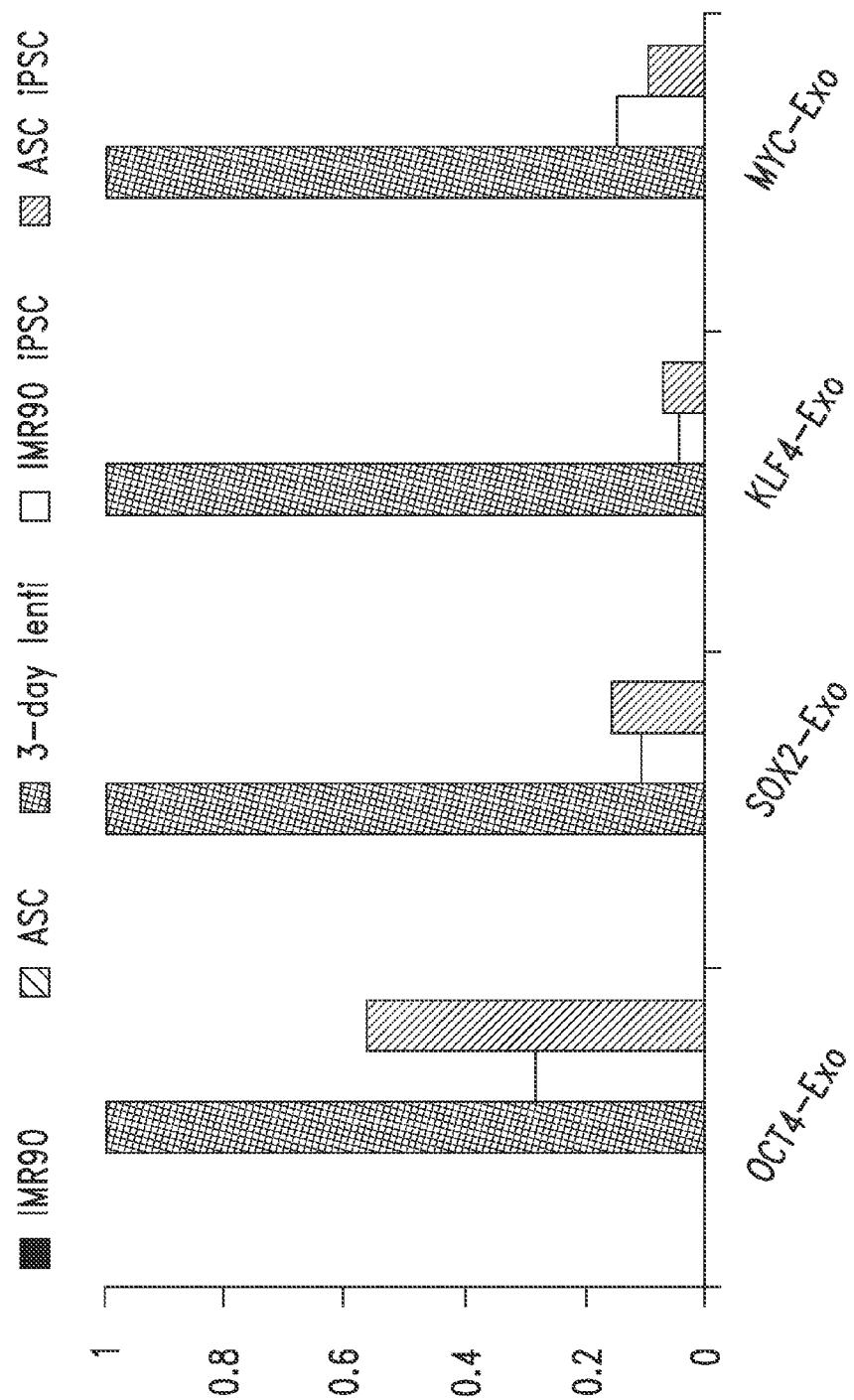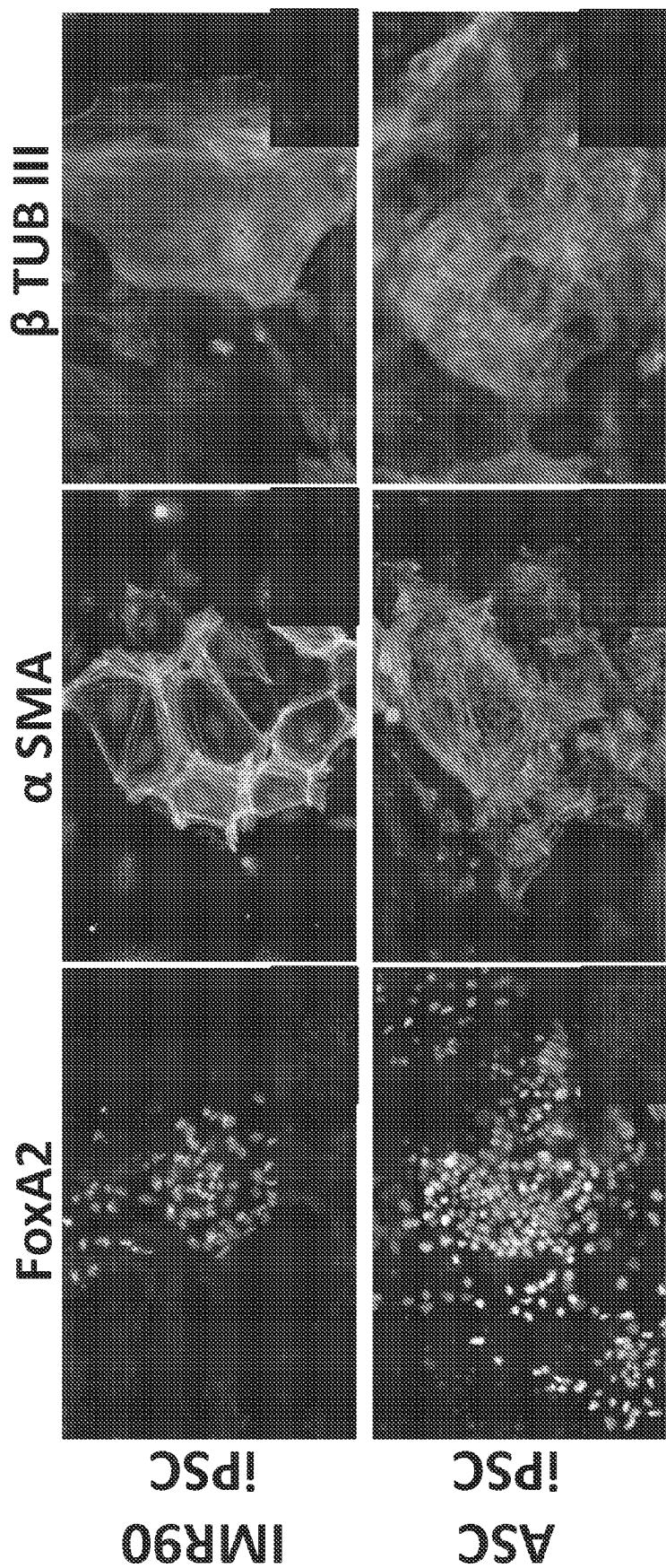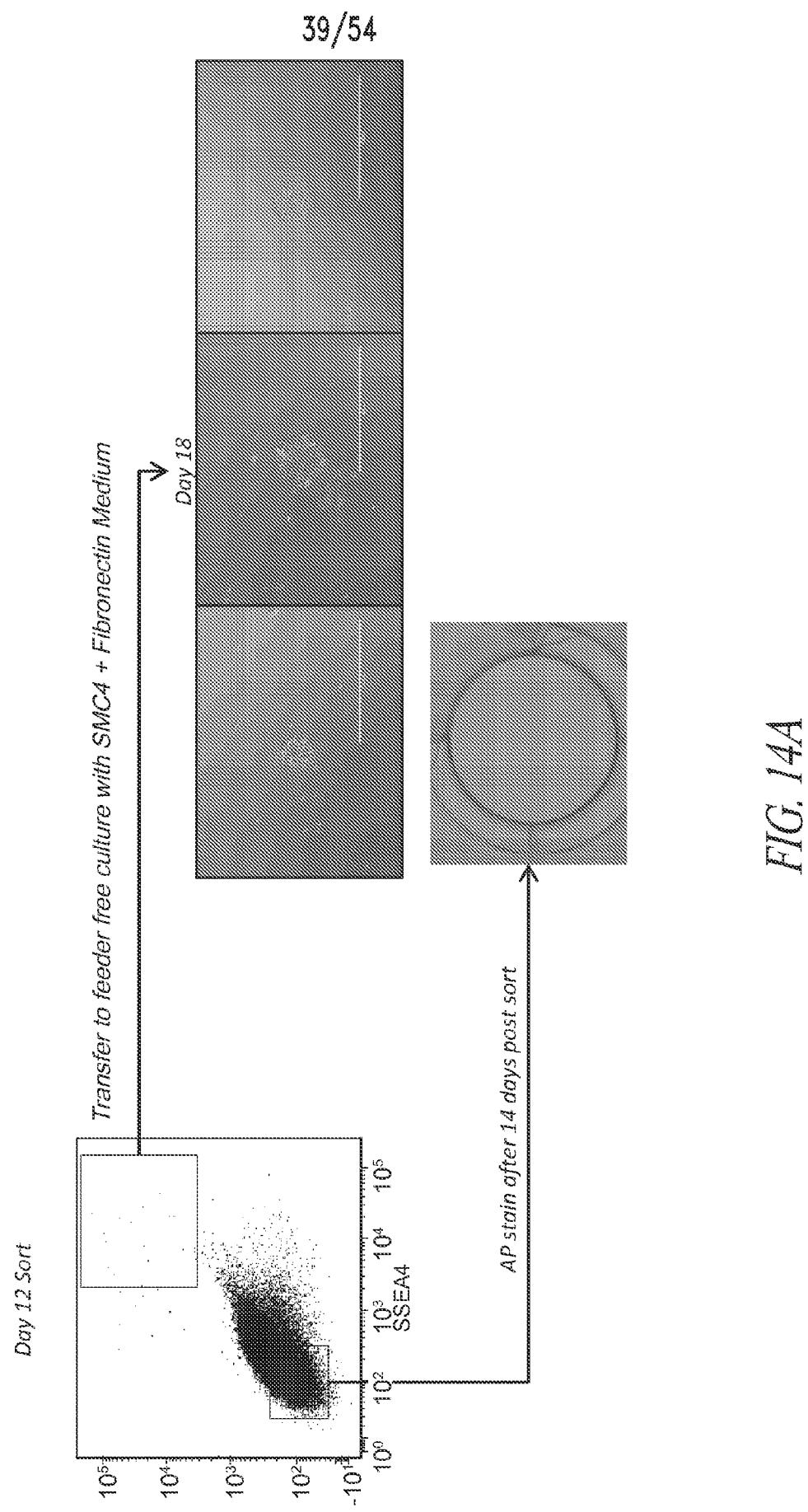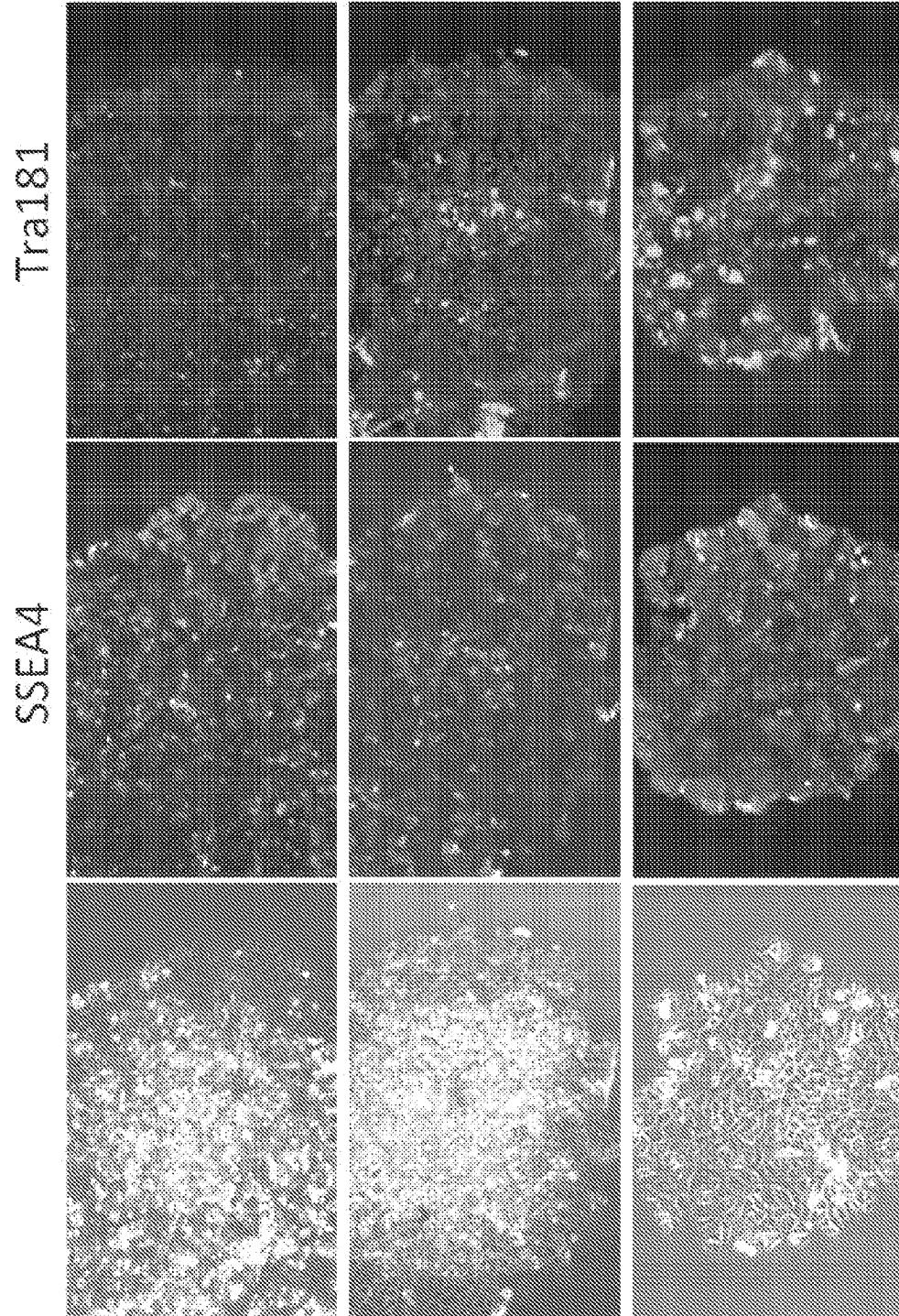
FIG. 13D

FIG. 13E

FIG. 13F

FIG. 13G

39/54

FIG. 14A

FIG. 14B
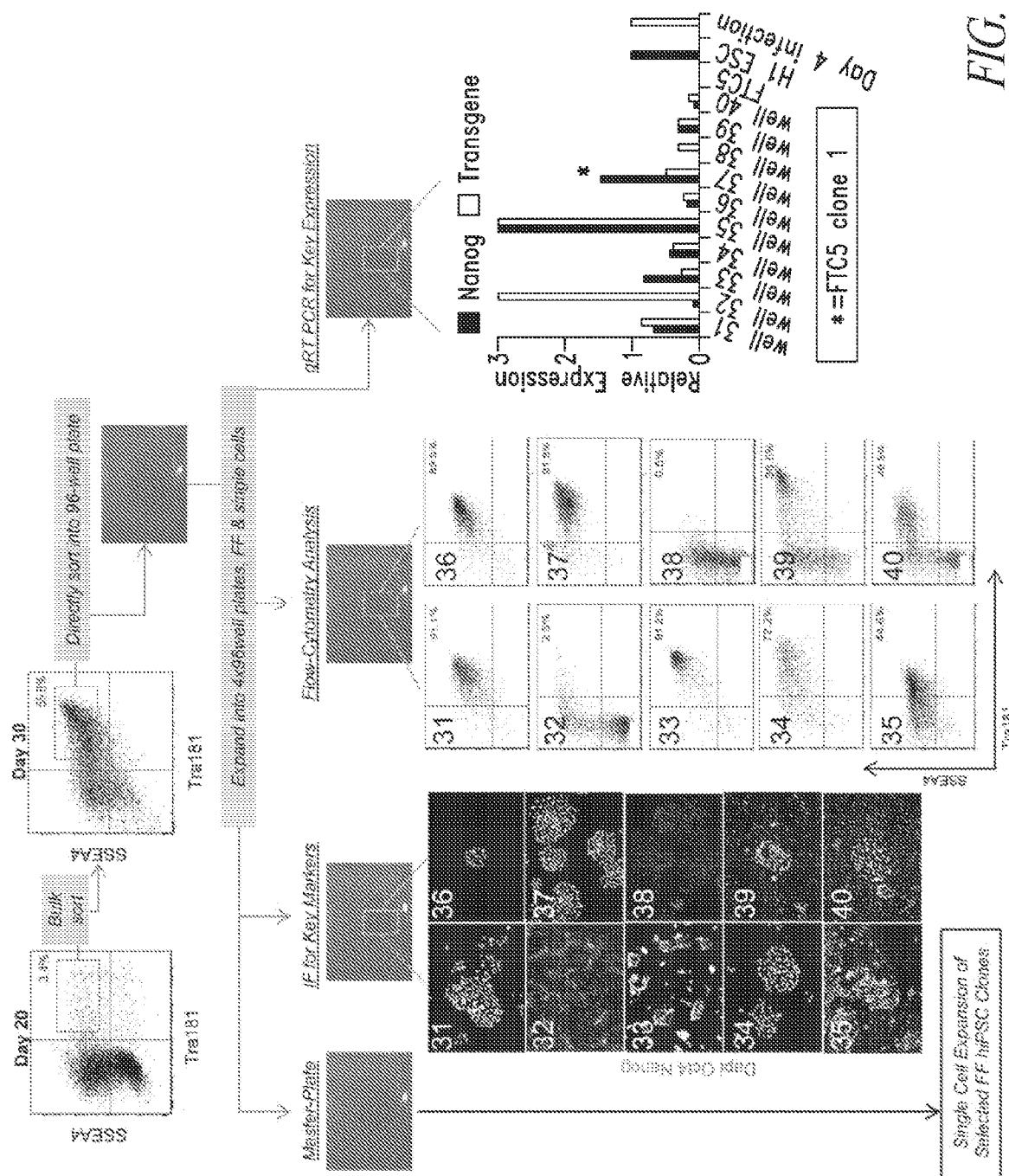
FIG. 15
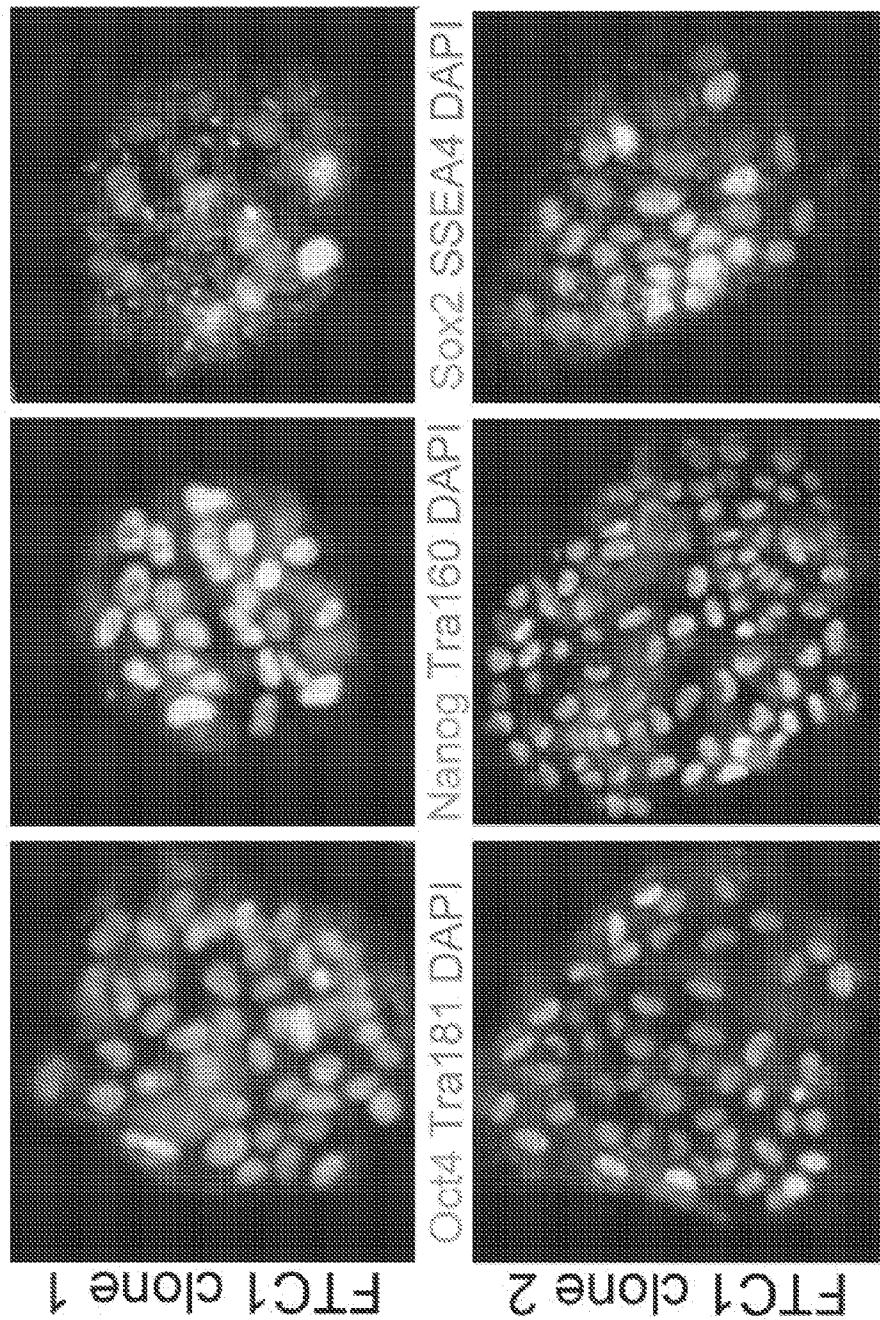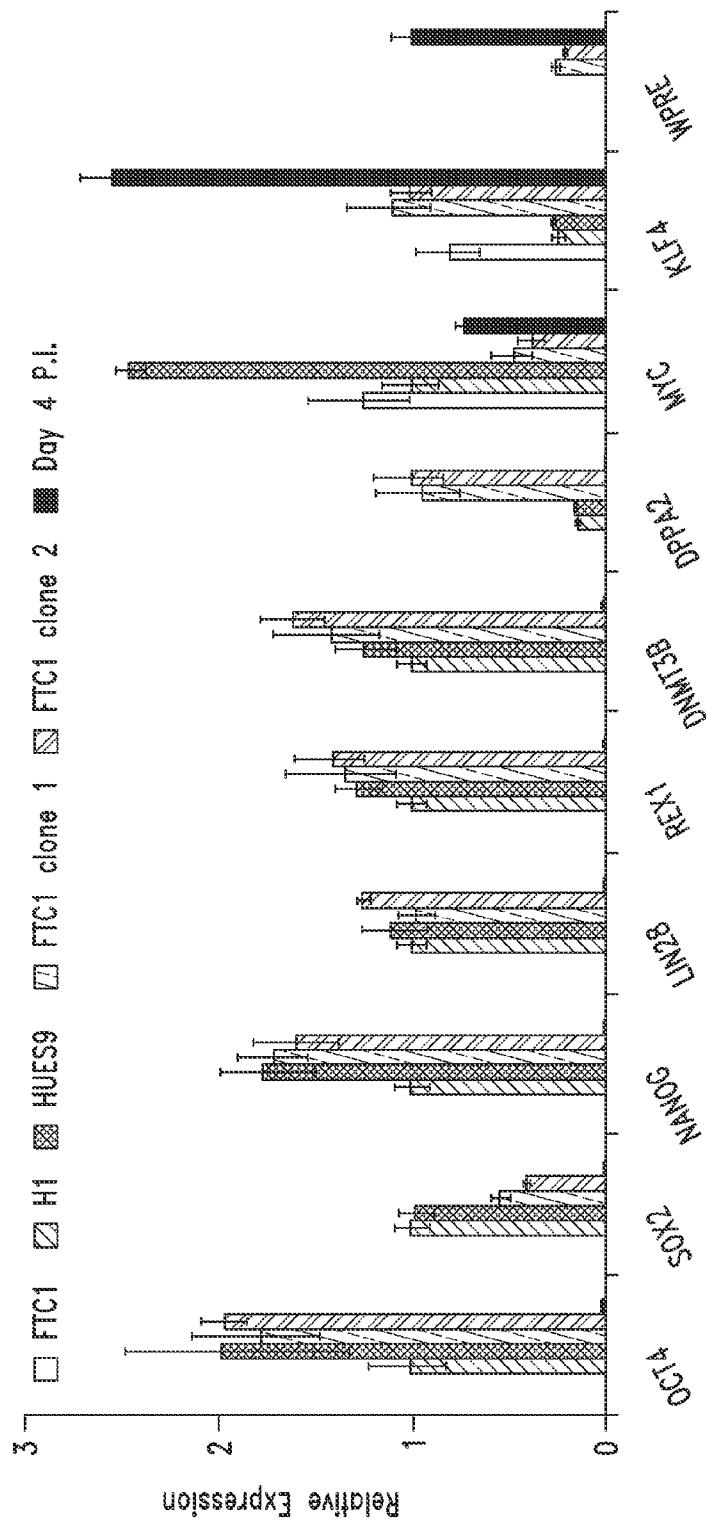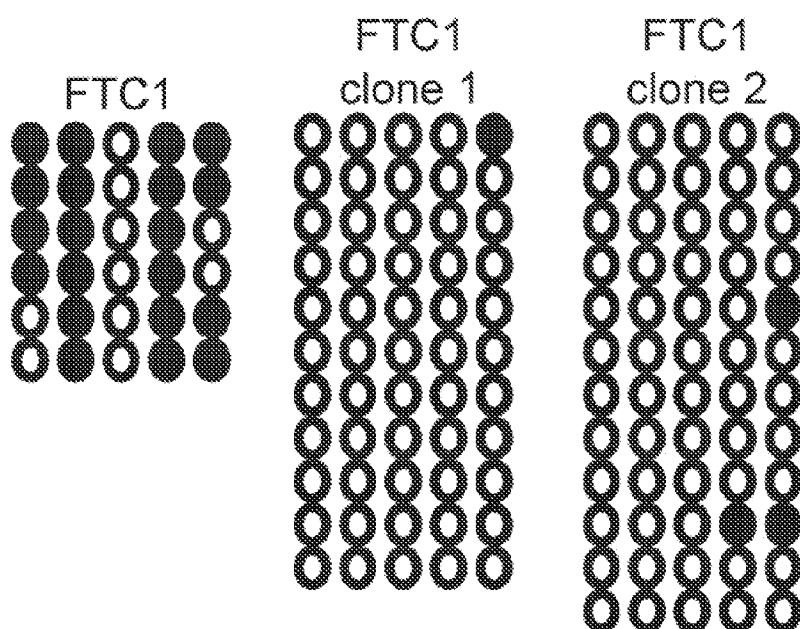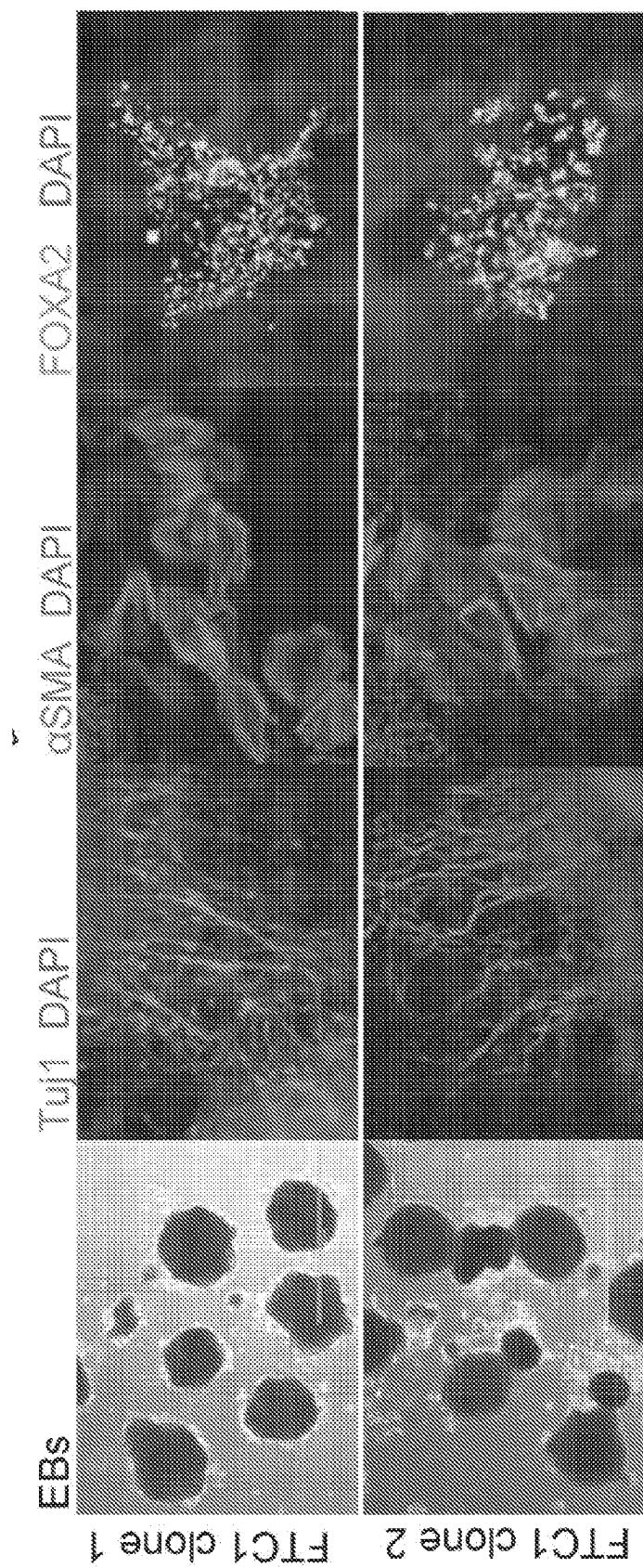
FIG. 16A
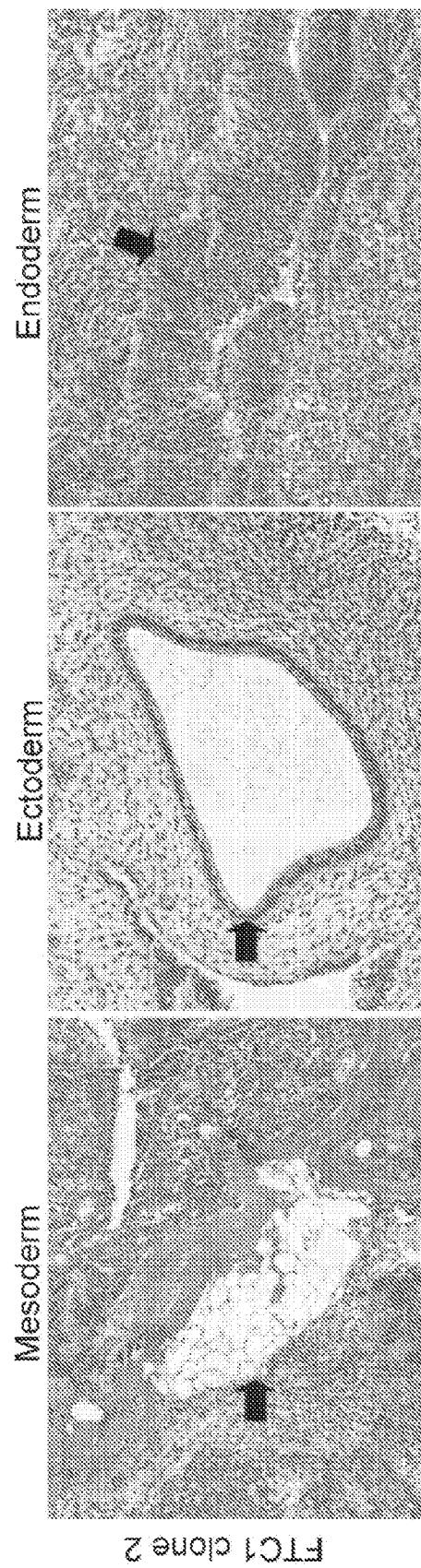
FIG. 16B

FIG. 16C

FIG. 16D

FIG. 16E
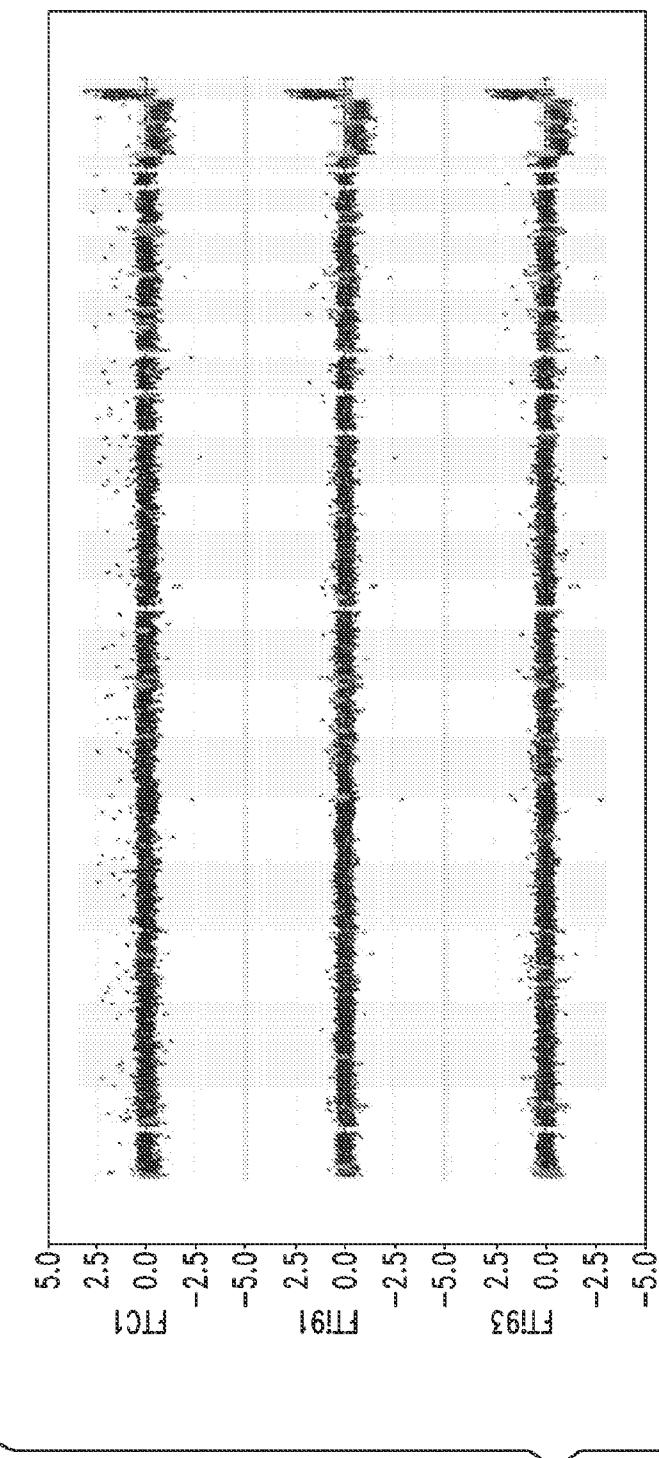
| Cytogenetics Report	Array	Total CNVs	Test Sample CNVs	Conclusion
FTC1 p4	NimbleGen 12x135K	31	14	Consistent With A Balanced Karyotype
FTi91 p10	NimbleGen 12x135K	35	12	Consistent With A Balanced Karyotype
FTi93 p9	NimbleGen 12x135K	41	13	Consistent With A Balanced Karyotype

FIG. 17A
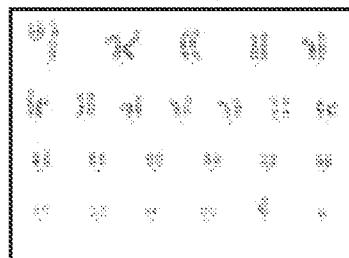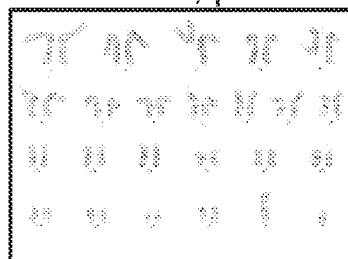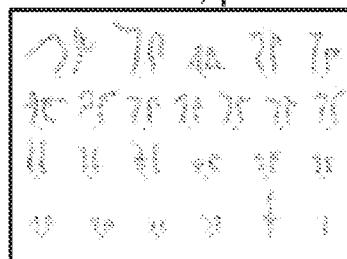
FIG. 17B
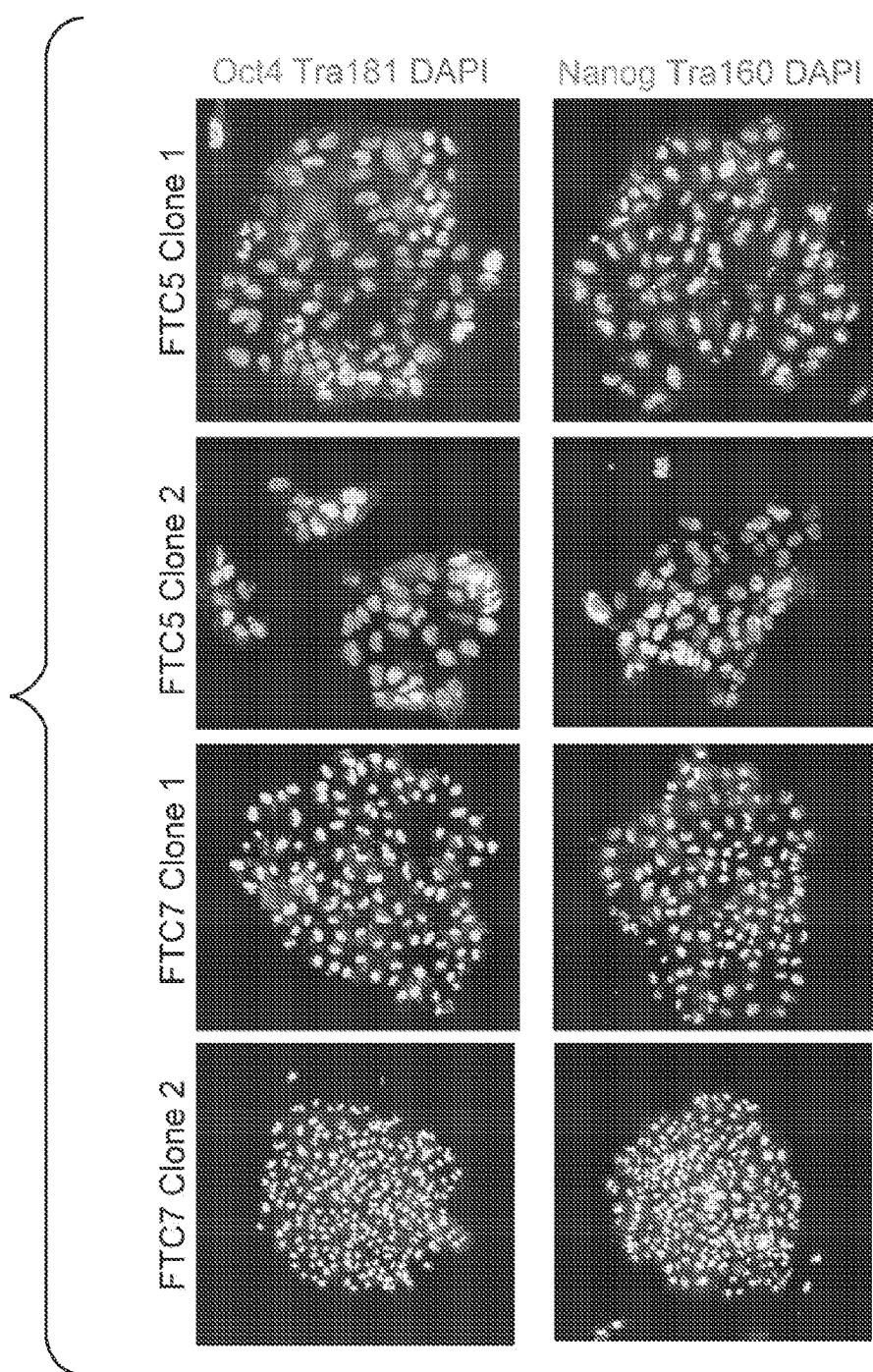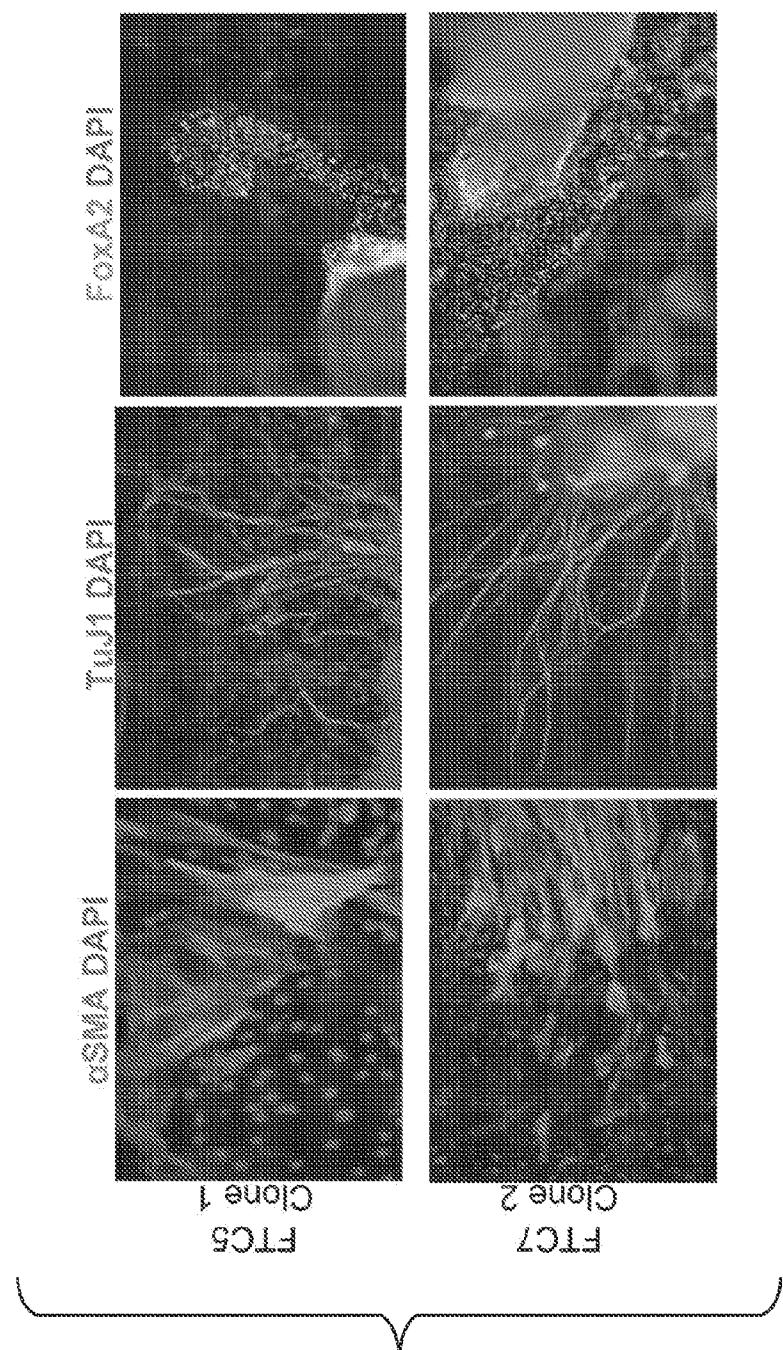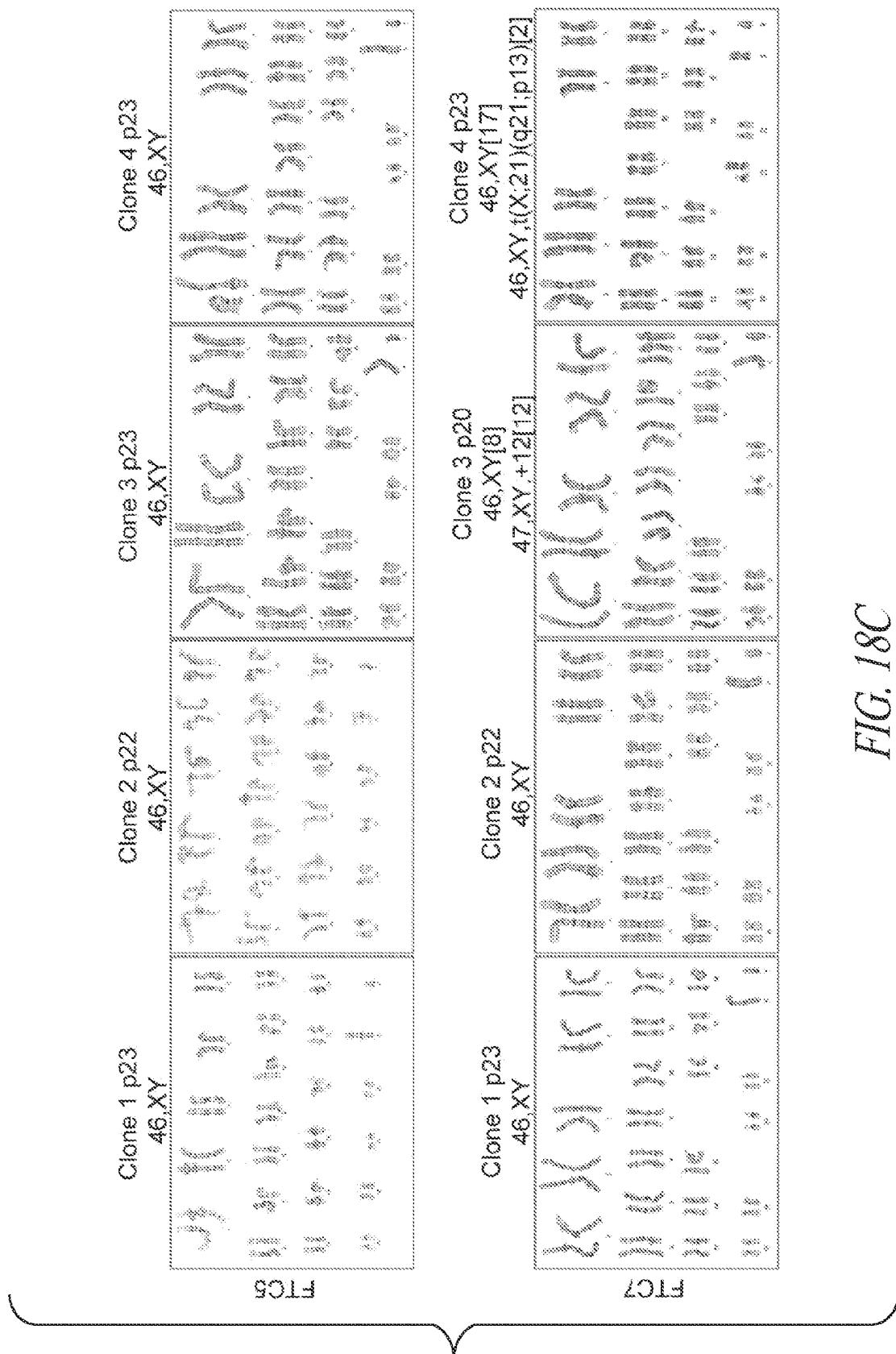
FIG. 18A

FIG. 18B

FIG. 18C
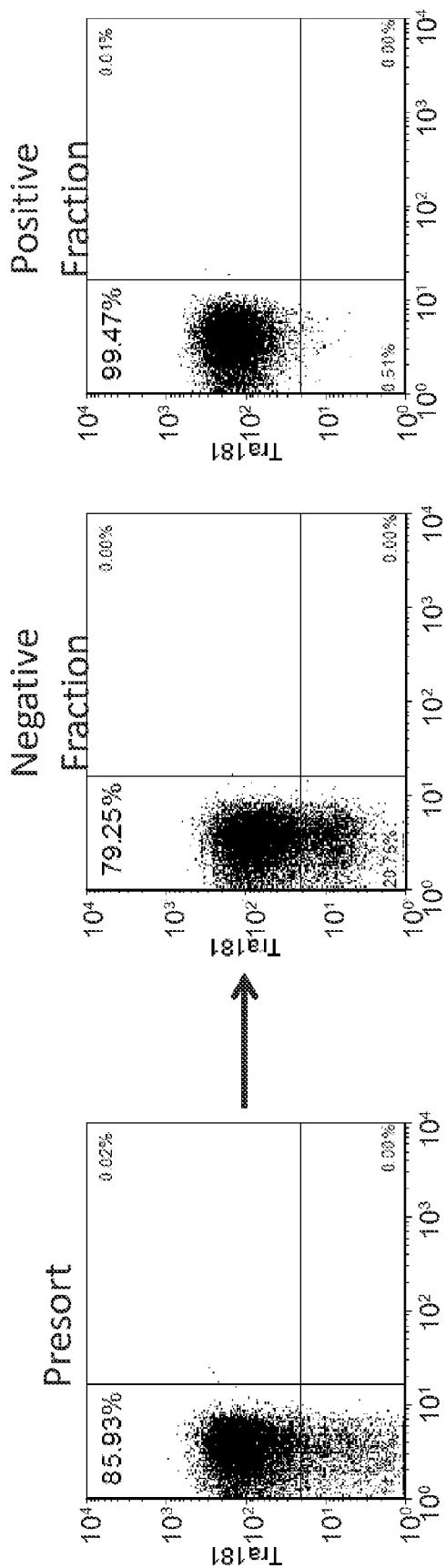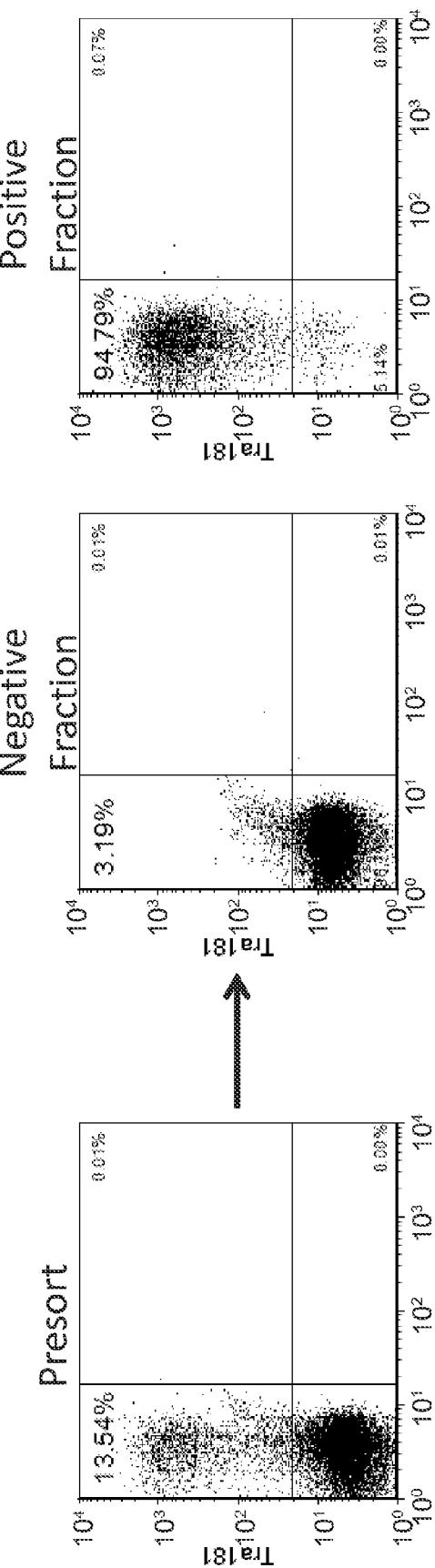
FIG. 19A

FIG. 19B
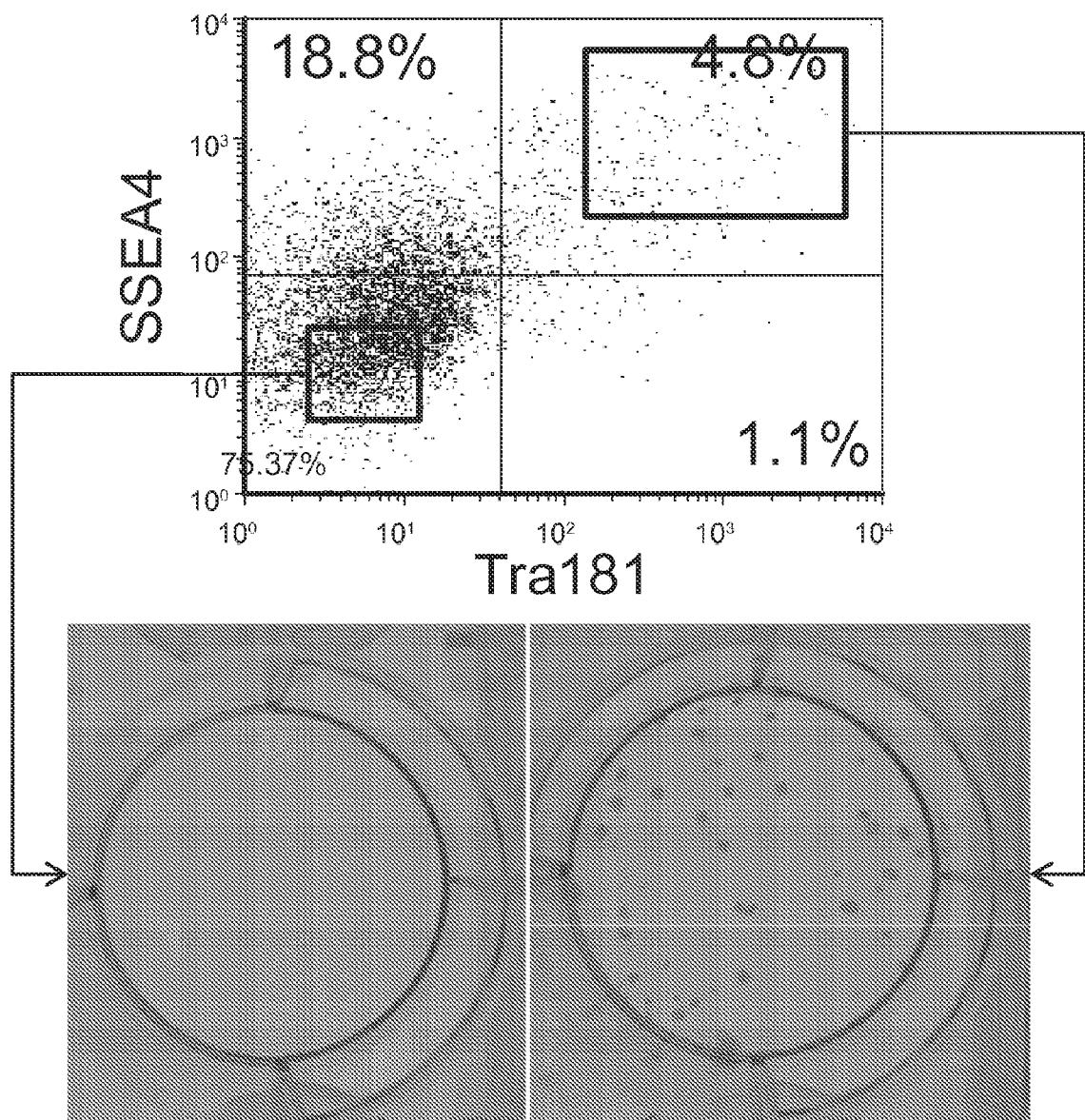
FIG. 20
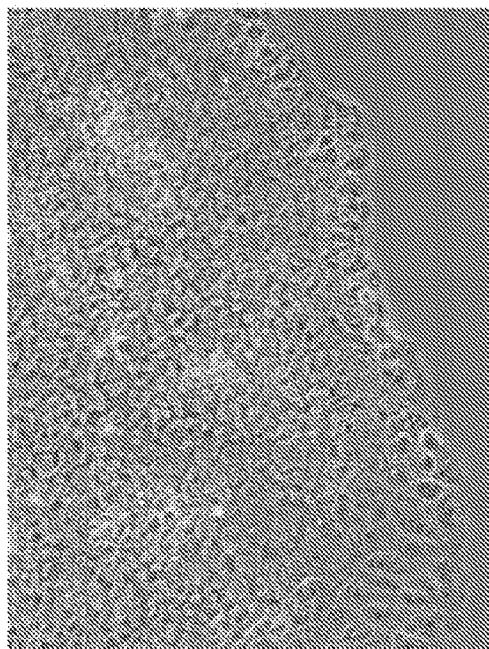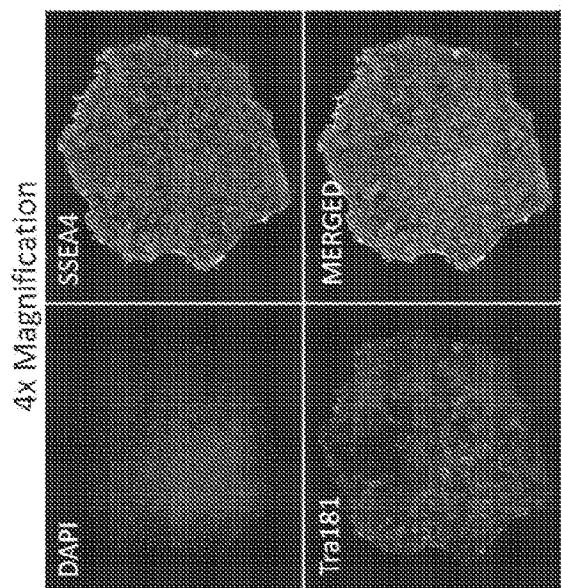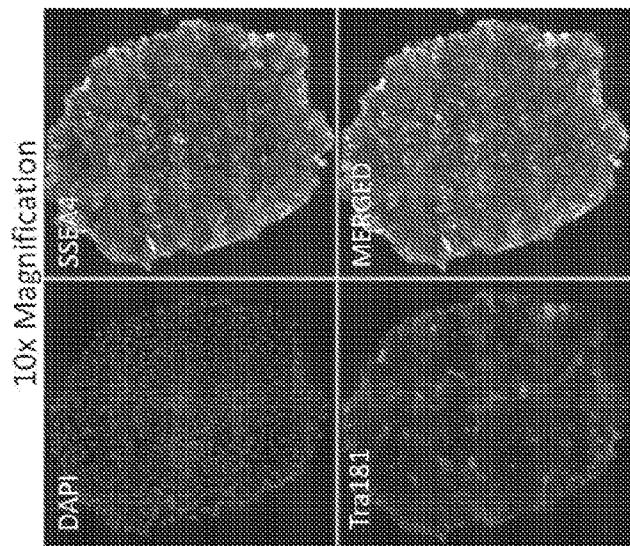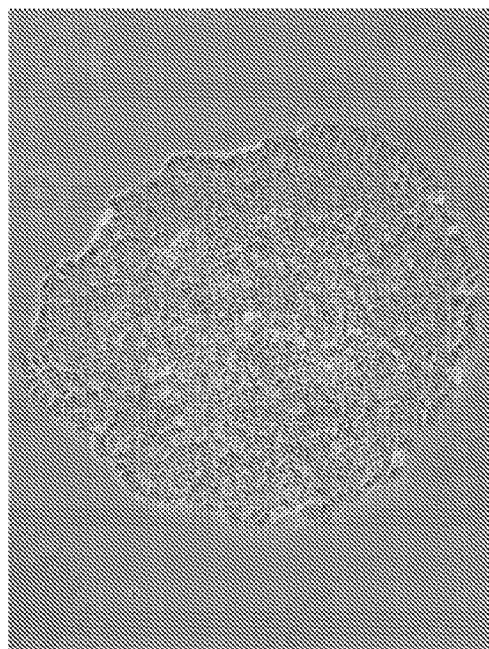
FIG. 21B

FIG. 21A

FIG. 21C

<110> Fate Therapeutics
       Valamehr, Bahram
       Abujarour, Ramzey
       Flynn, Peter

<120> CELL CULTURE PLATFORM FOR SINGLE CELL SORTING AND ENHANCED
       REPROGRAMMING OF IPSCS

<130> FATE-094/02WO 310854-2437

<150> US 61/496,991
<151> 2011-06-14

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<151> 2010-12-22

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