



(51) International Patent Classification:

C12N 5/02 (2006.01) *C12N 5/074* (2010.01)
C12N 5/071 (2010.01) *C12Q 1/24* (2006.01)
C12N 5/0735 (2010.01) *G01N 33/53* (2006.01)

(21) International Application Number:

PCT/IB2017/050613

(22) International Filing Date:

3 February 2017 (03.02.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/290,737 3 February 2016 (03.02.2016) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))

Published:

— with international search report (Art. 21(3))

(54) Title: METHODS AND COMPOSITIONS FOR CULTURING STEM CELLS

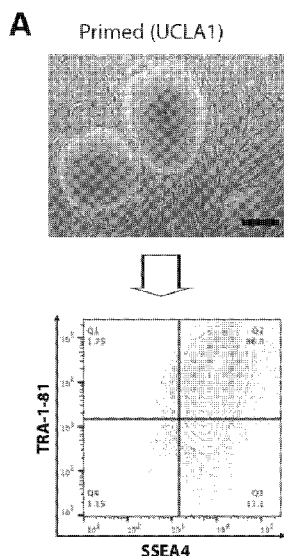


FIG. 1A

(57) Abstract: The current disclosure relates to methods for isolating and culturing naïve embryonic stem cells that phenotypically resemble pre-implantation-derived stem cells. Also disclosed are therapeutic methods and compositions comprising such cells.

METHODS AND COMPOSITIONS FOR CULTURING STEM CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of U.S. Provisional Patent Application No. 62/290,737 filed February 3, 2016, which is hereby incorporated by reference in its entirety.

STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made with Government support under HD079546, awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

[0003] The present invention relates generally to the field of cell culture and development. More particularly, it concerns the culturing and identification of stem cell populations.

2. Description of Related Art

[0004] Human embryonic stem cells (hESCs) are *in vitro* pluripotent cell types with the capacity for unlimited self-renewal and differentiation, making them critical models for understanding mechanisms required for human embryo development and differentiation. Although hESCs are derived from pre-implantation human blastocysts, they are morphologically and transcriptionally similar to murine epiblast stem cells (EpiSCs), which are derived from post-implantation mouse embryos. As such, hESCs and EpiSCs are said to exhibit a “primed pluripotent state” while, mouse ESCs derived from the pre-implantation blastocyst exhibit a “naïve pluripotent state” corresponding to an earlier stage of development (Nichols and Smith, 2009).

[0005] hESCs have many applications in basic science research and in therapeutic methods. However, it is not clear how to recapitulate or identify a naïve hESC in culture, given the predominance of the primed state in standard culturing protocols. The naïve state may not only represent a more undifferentiated cell, but may also represent a cell that has the potential to differentiate in a more appropriate and biologically relevant way than a primed cell. Therefore, there is a need in the art for methods for identifying and isolating naïve human ESCs.

SUMMARY OF THE INVENTION

[0006] SSEA-4 is known as a marker of human embryonic stem cells and as a marker of induced pluripotent stem cells. Surprisingly, it was found that a more undifferentiated and

perhaps more appropriately programmed cell can be detected on the basis of SSEA-4 antigen expression. SSEA-4-negative cells represent a stem cell population that has many research and therapeutic applications, and methods of the disclosure relate to novel methods for isolating and identifying such cells as well as therapeutic methods using such cells.

5 **[0007]** Aspects of the disclosure relate to a method for separating naïve human embryonic stem cells (hESCs) or naïve human induced pluripotent cells (hiPSCs) from non-naïve hESCs or non-naïve hiPSCs *in vitro*, the method comprising: contacting the cells *in vitro* with an agent that binds to Stage Specific Embryonic Antigen 4 (SSEA4) antigen on the cells; and separating SSEA4-negative unbound cells from the SSEA4-positive agent-bound cells; 10 wherein the fraction of unbound cells represents the naïve hESCs or naïve hiPSCs and the fraction of bound cells represents the non-naïve hESCs or non-naïve hiPSCs.

[0008] A further method aspect relates to a method for selectively labeling non-naïve hESCs or hiPSCs in a composition comprising naïve and non-naïve hESCs or iPSCs, the method comprising: contacting the cells *in vitro* with a labeled agent that binds to SSEA4 15 antigen on the cells; wherein the binding of the labeled agent to the non-naïve hESCs or iPSCs selectively labels the non-naïve hESCs or hiPSCs. In some embodiments, there is a method comprising: selectively labeling non-naïve hESCs or hiPSCs by contacting the cells *in vitro* with a labeled agent that binds to SSEA-4; separating labeled cells from unlabeled cells, wherein the unlabeled cells are naïve hESCs or hiPSCs; culturing the 20 unlabeled naïve hESCs or hiPSCs. In embodiments, the naïve hESCs or hiPSCs are undifferentiated or have not been contacted with a differentiation medium.

[0009] Further method aspects relate to a method for culturing or for maintaining a population of naïve hESCs or naïve hiPSCs comprising contacting the cells with an agent that binds to Stage Specific Embryonic Antigen 4 (SSEA4) antigen on the cells; and separating 25 SSEA4-negative unbound cells from the SSEA4-positive agent-bound cells; and culturing the unbound cells in cell media.

[0010] In some embodiments, the methods of the disclosure further comprises culturing the fraction of unbound cells. The cells may be cultured for at least, at most, or exactly 1, 2, 3, 4, 5, 6, 7, 10, 20, 30, 40, 50 or more passages or any range derivable therein. In some 30 embodiments, the cells are cultured in media comprising one or more inhibitors. In some embodiments the inhibitors are inhibitors of MAPK/ERK kinase, ROCK, Src, LCK, B-raf kinase, and/or GSK-3 β . Such inhibitors are commercially available. For example, Stemolecule™ PD0325901 is an inhibitor of MAPK/ERK kinase or MEK), Stemolecule™ Y27632 is an inhibitor of Rho-associated kinase (ROCK), Stemolecule™ WH-4-023 is an

inhibitor of Src and LCK, Stemolecule™ SB590885 is an inhibitor of B-RAF kinase, and Stemolecule™ IM-12 and Stemolecule™ CHIR99021 are inhibitors of glycogen synthase kinase 3 β (GSK-3 β). In some embodiments, the media further comprises one or more of fibroblast growth factor (FGF), Activin A, and/or leukemia inhibitor factor (LIF). In some
5 embodiments, the media comprises a compound or composition described herein.

[0011] In some embodiments, the agent is labeled with a detectable label. Detectable labels are known in the art and described herein. In some embodiments, the agent is an antibody. In further embodiments, the method further comprises contacting the cells with a second agent that binds to the SSEA4-binding agent. In some embodiments, the the SSEA-4
10 binding agent and/or second agent are linked to a solid support and/or detectable label.

[0012] In some embodiments, the agent-bound cells and unbound cells are separated by fluorescent-activated cell sorting (FACS), chromatography, solid-support assays, magnetic activated cell sorting (MACS), and panning. Other suitable methods for separating cell populations are also described herein.

15 [0013] In some embodiments the non-naïve cell of the disclosure is a primed cell.

[0014] In some embodiments, the method further comprises culturing or passaging the naïve hESCs or hiPSCs (i.e. SSEA-4 negative cells). In some embodiments, the method further comprises expanding the naïve hESCs or naïve hiPSCs. In some embodiments, the the method further comprises freezing the naïve hESCs or naïve hiPSCs. In some
20 embodiments, the method further comprises thawing the frozen cells.

[0015] Further embodiments relate to a naïve hESC or hiPSC isolated, identified, or cultured according to a method of the disclosure. Yet further aspects relate to compositions comprising cells isolated, identified, or cultured according to methods of the disclosure.

[0016] A further aspect relates to a therapeutic method comprising administering the cell
25 isolated, identified, or cultured cell (i.e. a naïve human pluripotent cell) according to methods of the disclosure, or a progeny of such cell.

[0017] In some embodiments of the methods and compositions described herein, the SSEA-4-negative cell is a naïve pluripotent cell. In some embodiments, the cell is a progeny of the naïve pluripotent cell and may be SSEA-4 negative or SSEA-4 positive. In some
30 embodiments, the progeny is SSEA-4 positive. In some embodiments, the SSEA-4 negative cell is a naïve human pluripotent cell.

[0018] It is also contemplated that the cell may be a mouse cell. Therefore, in some embodiments, the cell is a naïve mouse ESC, iPC, pluripotent cell.

[0019] A further method aspect relates for a method for evaluating a cell culture medium for culturing hESCs or hiPSCs, the method comprising: culturing the hESCs or hiPSCs in the medium; contacting the hESCs or hiPSCs with a detectable agent that binds to SSEA4 antigen on cells; detecting the agent binding to the antigen; and evaluating the medium on the basis of the detected agent. In some embodiments of the methods of the disclosure, a medium with a percentage of SSEA-4 negative cells of less than 25% is determined to be a naïve hESC or hiPSC maintenance medium. In some embodiments, a medium with a percentage of SSEA-4 negative cells of less than 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1% (or any derivable range therein) is determined to be a naïve hESC or hiPSC maintenance medium.

[0020] Aspects of the disclosure relate to a cell medium evaluated according to any methods of the disclosure.

[0021] In some embodiments of the methods of the disclosure, the method further comprises quantifying the SSEA4-negative unbound cells, SSEA4-positive bound cells, or both. Quantification methods are known in the art, and quantification may be done based on the detected label, such as a quantification of fluorescence, chemiluminescence, or enzyme activity.

[0022] A further aspect of the disclosure relates to a method for identifying culturing compositions that revert a primed hESC or hiPSC to a naïve hESC or hiPSC comprising: contacting the primed hESC or hiPSC cultured *in vitro* with a candidate culturing composition; contacting the cell with a detectable agent that binds to SSEA4 antigen on cells; detecting the presence or absence of the agent binding to the antigen; and identifying the culturing composition as a composition that reverts a primed hESC or hiPSC to a naïve hESC or hiPSC when the absence or reduction of agent-antigen binding is detected. In some embodiments, the method further comprises determining SSEA-4 status prior to contact with the candidate composition. In some embodiments, the reduction in SSEA-4-expressing cells is at least, at most, or exactly 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, or 99 % (or any derivable range therein).

[0023] In some embodiments, the candidate culturing composition comprises one or more of the inhibitors of MAPK/ERK kinase, ROCK, Src, LCK, B-raf kinase, and GSK-3 β . In some embodiments, the culturing composition comprises or further comprises one or more of FGF, Activin A, and LIF.

[0024] Further aspects relate to a culturing composition that reverses a primed hESC or hiPSC to a naïve hESC or hiPSC, wherein the composition is identified according to a method of the disclosure.

[0025] Further method aspects relate to a method for distinguishing a naïve hESC or a primed hESC, the method comprising: contacting the cell with a detectable agent that binds to Stage Specific Embryonic Antigen 4 (SSEA4)-positive cells; and detecting a naïve hESC as a cell not bound with the detectable agent; and/or detecting a primed hESC as a cell bound with the detectable agent.

[0026] Further aspects relate to A therapeutic method comprising contacting a naïve human pluripotent cell *in vitro* with a detectable agent that binds to SSEA4 antigen on cells; administering the naïve human pluripotent cell to the patient; wherein the binding of the agent to the SSEA4 antigen is not detectable. In some embodiments, the method comprises contacting a naïve human pluripotent cell and wherein the naïve human pluripotent cell is in a blastocyst. In some embodiments, the therapeutic method is *in vitro* fertilization (IVF).

[0027] Further aspects relate to a composition comprising an isolated naïve hESC or hiPSC and cell culture media, wherein the cell is SSEA4-negative. In some embodiments, the cell is a naïve hiPSC. In some embodiments, the cell is cryopreserved. In some embodiments, the cell is in cell culture media comprising one or more components described herein.

[0028] Further method aspects relate to methods for making a naïve iPSC cell comprising: isolating an adult stem cell; inducing pluripotency in the cell by methods known in the art or described herein; and screening the cell for SSEA-4 negativity, wherein a negative SSEA-4 cell represents a naïve iPSC.

[0029] Other culturing conditions can be appropriately defined. For example, the culturing temperature can be about 20 to 40°C, for example, at least, at most, or about 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40°C (or any range derivable therein), but particularly not limited to them. The CO₂ concentration can be about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10% (or any range derivable therein), such as about 2% to 10%, for example, about 2 to 5%, or any range derivable therein. The oxygen tension can be at least or about 1, 5, 8, 10, 20%, or any range derivable therein.

[0030] In some embodiments of the therapeutic method, the methods further comprise differentiating the cell into a hematopoietic stem or progenitor cell or an immune cell such as a T cell.

[0031] In certain embodiments, any of the cell population, may comprise at least, about, or at most, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 1×10^3 , 2×10^3 , 3×10^3 , 4×10^3 , 5×10^3 , 6×10^3 , 7×10^3 , 8×10^3 , 9×10^3 , 1×10^4 , 2×10^4 , 3×10^4 , 4×10^3 , 5×10^4 , 6×10^4 , 7×10^4 , 8×10^4 , 9×10^4 , 1×10^5 , 2×10^5 , 3×10^5 , 4×10^5 , 5×10^5 , 6×10^5 , 7×10^5 , 8×10^5 , 9×10^5 , 1×10^6 , or 2×10^6 cells (or any range derivable therein).

[0032] The subject or patient of the therapeutic methods may be any animal, in particular a mouse, non-human primate, or human. In some embodiments, the subject is a woman that has experienced infertility or has been diagnosed with infertility. In some embodiments, the subject is a woman that has not been successful with prior infertility treatments.

[0033] As used herein the specification, “a” or “an” may mean one or more. As used herein in the claim(s), when used in conjunction with the word “comprising”, the words “a” or “an” may mean one or more than one.

[0034] The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.” As used herein “another” may mean at least a second or more.

[0035] Throughout this application, the term “about” is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

[0036] Any embodiments described herein may be used interchangeably in the methods and compositions described herein. Furthermore, it is specifically contemplated that any method step, embodiment, or component may be specifically excluded from the methods and compositions described herein.

[0037] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0038] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better

understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0039] FIG. 1A-F: 5iLAF SSEA4 negative subpopulation recapitulates naïve expression pattern. **A)** Upper: brightfield image of primed UCLA1 hESCs. Lower: Flow cytometry plot of primed UCLA1 hESCs stained for SSEA4 and TRA-1-81. **B)** Upper: UCLA1 hESCs reverted in 5iLAF. A mixture of round and flat colonies are observed. Lower: Flow cytometry plot of 5iLAF cultured UCLA1 hESCs stained for SSEA4 and TRA-1-81. Scale bar indicates 200µm. **C)** 5iLAF cells were sorted into SSEA4⁺ and SSEA4⁻ populations. Upon re-plating, the SSEA4⁺ cells formed flat colonies and the SSEA4⁻ cells formed round colonies (n=2 biological replicates). Scale bar= 100µm. **D)** hESC line called UCLA20n, derived from a 5-day human blastocyst in 5iLAF. Left: Brightfield image. Scale bar indicates 200µm. Right: Flow cytometry plot of TRA-1-85+ (human) UCLA20n hESCs stained for SSEA4 and TRA-1-81. **E)** Expression of genes identified by others as associating with the naïve and primed states in mice. Expression level is determined by RNA-seq. For 5iLAF SSEA4 negative (neg) and primed hESCs, n=4. For 5iLAF SSEA4 positive (pos), n=2. Other data comes from published RNA-seq or microarray datasets. Methodology, cell type, and citation are indicated. **F)** A set of “pre-implantation epiblast” and “primed” specific genes were defined based on published data. Expression of these genes is shown for various methodologies, relative to primed controls from the same dataset. UCLA20n was normalized to a primed UCLA1 library generated and sequenced at the same time.

[0040] FIG. 2A-H: Naïve hESCs fail to recapitulate naïve-specific methylation pattern. **A)** Average genome wide-CG methylation level in primed and 5iLAF UCLA1 hESCs, shown in comparison with published datasets. For 5iLAF SSEA4 negative (neg) and primed hESCs, n=3. For 5iLAF SSEA4 positive (pos), n=2. **B)** DNA methylation is shown for a region of chromosome 10. Each bar indicates a single CG, and the height of the bar indicates the percentage of CG methylation. Where multiple CGs are too close to be visually rendered separately, an average value is shown. **C)** Correlation plots relative to human oocyte using 100kb genome bins. **D)** DNA methylation over stable primary imprints. The average methylation level of each imprint in a given sample is represented as one point in the box and whisker point. **E)** DNA methylation over the paternally imprinted *H19* locus. Each bar indicates a single CG, and the height of the bar indicates the fraction of CG methylation. Where multiple CGs are too close to be visually rendered separately, an average value is shown. **F)** Total DNA methylation for three competing approaches for culturing naïve cells. Because the Gafni 2013 data was generated by RRBS, only CGs that had coverage in the

Gafni 2013 dataset are included in this analysis to make the data comparable. **G)** Expression (RPKM) of DNA methyltransferases, DNMT cofactors, and Tet-family oxidases as measured by RNA-seq (n=4). **H)** RNA and protein levels of DNA methyltransferases in 5iLAF SSEA4 neg UCLA1 hESCs relative to primed. RNA level is determined from RNA-seq data (n=4), protein level from quantitative westerns (UHRF1, n=6 Western blots; DNMT1, DNMT3A, DNMT3B n=2; DNMT3L n=1).

[0041] FIG. 3A-K: Properties of 5iLAF SSEA4 negative and SSEA4 positive cells generated by reversion of primed hESCs. **A-C)** Immunofluorescence for SSEA4 and OCT4. Note that all colonies are OCT4 positive. **A)** A colony of SSEA4 positive primed UCLA1 hESCs. **B)** A colony of 5iLAF SSEA4 positive UCLA1 hESCs. **C)** A colony of 5iLAF SSEA4 negative UCLA1 hESCs. **D-F)** Immunofluorescence for TRA-1-81 and NANOG. Note that all populations are NANOG positive. **D)** A colony of TRA-1-81 positive UCLA1 hESCs. **E)** A colony of 5iLAF TRA-1-81 positive UCLA1 hESCs. **F)** A colony of 5iLAF TRA-1-81 negative UCLA1 hESCs. **G)** Flow cytometry of control primed and re-plated 5iLAF SSEA4 negative UCLA1 hESCs grown for one passage. Fluorescence of unstained cells is indicated in red and percentage showing positive staining is indicated. After re-plating, the vast majority of sorted SSEA4 negative cells remain SSEA4 negative. **H-K)** Similar to UCLA1, SSEA4 positive 5iLAF cells from UCLA4 (**H)** and UCLA5 (**J)** yield flat colonies upon re-plating while the SSEA4 positive 5iLAF cells yield round colonies. **I,K)** Unlike UCLA1, most cells in the SSEA4 negative subpopulation have high TRA-1-81 expression (compare to Figure 1B).

[0042] FIG. 4A-H: **A)** DNA methylation over transiently imprinted CG islands. The average methylation level of each imprint in a given sample is represented as one point in the box and whisker point. **B)** Reads mapped over an annotated SNP in the maternally imprinted SNRPB locus. Reads over each base are plotted, and the SNP sequence is indicated by color. Only one allele is expressed in the parent primed UCLA1 line, but both alleles are expressed in 5iLAF cells. **C)** Global DNA methylation in naïve and primed cells before and after sixteen days of culture in EpiLC-like conditions to restore the primed state. **D)** DNA methylation over imprints in naïve and primed cells before and after sixteen days of culture in EpiLC-like conditions to restore the primed state. Each imprint is represented as a single point in the box plot. Note the modest increase in methylation at imprints as the naïve cells are converted to primed conditions, whereas the global increase in methylation is much greater. **E)** Hypomethylation of young LINE elements including L1 human specific (L1HS) and its descendent L1PA2 in 5iLAF SSEA4 negative UCLA1 hESCs, as shown by metaplot.

Note the dramatic loss of methylation in the vicinity of the element promoter. **F)** DNA methylation over imprints for three alternate approaches for culturing naïve cells. Because the Gafni 2013 data was generated by RRBS, only CGs that had coverage in the Gafni 2013 dataset are included in this analysis to make the data comparable. Only sixteen stable imprints had sufficient coverage for robust analysis. **G)** Karyotyping results from reverted UCLA1 lines and new lines derived from blastocyst in 5iLAF. **H)** Comparative Genomic Hybridization (CGH) data is shown over two chromosomes for the UCLA20n line cultured in 5iLAF. Most chromosomes showed normal karyotype (e.g chromosome 6, left), but several showed regions of elevated DNA content consistent with aneuploidy (e.g. chromosome 12, right).

[0043] FIG. 5 depicts the SSEA-4 antigen.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

I. Definitions

[0044] The terms “separating,” “isolating,” and “purifying” refer to a segregation of one component from one or more other components, such as a separation of one cell type from another cell type. In certain methods described herein, the separation is from one component defined in the method from another component defined in the method (eg. separation of naïve and primed cells). The separation, isolation, or purification may be substantial in that less than 10% of the other component remains. The separation may also be such that less than 10, 9, 8, 7, 6, 5, 4, 3, 2, 1 % of the isolated, separated, or purified composition comprises the other component defined in the claims.

[0045] The term “antigen” refers to a molecule that binds to Ag-specific receptors, such as an antibody. Antigens are usually peptides, polysaccharides or lipids.

[0046] The term “xeno-free (XF)” or “animal component-free (ACF)” or “animal free,” when used in relation to a medium, an extracellular matrix, or a culture condition, refers to a medium, an extracellular matrix, or a culture condition which is essentially free from heterogeneous animal-derived components. For culturing human cells, any proteins of a non-human animal, such as mouse, would be xeno components.

[0047] The term “defined,” when used in relation to a medium, an extracellular matrix, or a culture condition, refers to a medium, an extracellular matrix, or a culture condition in which the nature and amounts of approximately all the components are known.

[0048] A “chemically defined medium” refers to a medium in which the chemical nature of approximately all the ingredients and their amounts are known. These media are also called synthetic media.

[0049] A "conditioned medium" is prepared by culturing a first population of cells in a medium, and then harvesting the medium. The conditioned medium (along with anything secreted into the medium by the cells) may then be used to support the growth of a second population of cells. Where a particular ingredient or factor is described as having been added to the medium, what is meant is that the factor (or a cell or particle engineered to secrete the factor) has been mixed into the medium by deliberate manipulation.

[0050] By contrast, "fresh medium" is a medium that has not been purposely conditioned by culturing with a different cell type before being used with the cell type it is ultimately designed to support. Otherwise, no limitations are intended as to its manner of preparation, storage, or use. It is added fresh (by exchange or infusion) into the ultimate culture, where it may be consumed or otherwise processed by the cell types that are present.

[0051] By "cell culture" is meant cells or tissues that are maintained, cultured or grown in an artificial, *in vitro* or *ex vivo* environment.

[0052] As used herein, reference to stem cell "attachment" means binding of the stem cell to the support surface such that the stem cell is not eluted by conventional washing or handling procedures. By stem cell "survival," particularly in regards to an undifferentiated stem cell, is meant sustained viability.

[0053] The terms "proliferation" and "propagation" are used interchangeably herein to denote an increase in the number of cells. The term "expanded" is also intended to mean that the resultant cell population is derived from *ex vivo* culture of stem cells, where the outgoing (cultured) number of cells exceeds the ingoing (non-cultured) number of cells. The term "expanded" is not to be construed or limited by any mechanism or theory of cellular origin and may comprise cells that originate *de novo* in culture.

[0054] Cells are “substantially free” of certain reagents or elements, such as serum, signaling inhibitors, animal components or feeder cells, exogenous genetic elements or vector elements, as used herein, when they have less than 10% of the element(s), and are “essentially free” of certain reagents or elements when they have less than 1% of the element(s). However, even more desirable are cell populations wherein less than 0.5% or less than 0.1% of the total cell population comprise exogenous genetic elements or vector elements.

[0055] A culture, matrix or medium are “essentially free” of certain reagents or elements, such as serum, signaling inhibitors, animal components or feeder cells, when the culture, matrix or medium respectively have a level of these reagents lower than a detectable level using conventional detection methods known to a person of ordinary skill in the art or these agents have not been extrinsically added to the culture, matrix or medium. The serum-free medium may be essentially free of serum.

[0056] A "gene," "polynucleotide," "coding region," "sequence," "segment," "fragment," or "transgene" which "encodes" a particular protein, is a nucleic acid molecule which is transcribed and optionally also translated into a gene product, *e.g.*, a polypeptide, *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The coding region may be present in either a cDNA, genomic DNA, or RNA form. When present in a DNA form, the nucleic acid molecule may be single-stranded (*i.e.*, the sense strand) or double-stranded. The boundaries of a coding region are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A gene can include, but is not limited to, cDNA from prokaryotic or eukaryotic mRNA, genomic DNA sequences from prokaryotic or eukaryotic DNA, and synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the gene sequence.

[0057] The term "cell" is herein used in its broadest sense in the art and refers to a living body which is a structural unit of tissue of a multicellular organism, is surrounded by a membrane structure which isolates it from the outside, has the capability of self-replicating, and has genetic information and a mechanism for expressing it. Cells used herein may be naturally-occurring cells or artificially modified cells (*e.g.*, fusion cells, genetically modified cells, *etc.*).

[0058] As used herein, the term "stem cell" refers to a cell capable of self-replication and pluripotency or multipotency. Typically, stem cells can regenerate an injured tissue. Stem cells herein may be, but are not limited to, embryonic stem (ES) cells, induced pluripotent stem cells or tissue stem cells (also called tissue-specific stem cell, or somatic stem cell).

[0059] “Embryonic stem (ES) cells” are pluripotent stem cells derived from early embryos. An ES cell was first established in 1981, which has also been applied to production of knockout mice since 1989. In 1998, a human ES cell was established, which is currently becoming available for regenerative medicine.

[0060] The term “naïve hESC” or “naïve iPC” refers to cells that are phenotypically, morphologically, and/or transcriptionally most similar to endogenous human pre-implantation state. In some embodiments, the naïve cells are distinct from primed cells based

on their comparative hypomethylated state, for example. In some embodiments, naïve cells exhibit a DNA methylation pattern of the pre-implantation blastocyst.

[0061] The term “primed hESC” or “primed iPC” refers to cells that are phenotypically, morphologically, and/or transcriptionally most similar to endogenous human post-implantation state. In some embodiments, the primed cells are distinct from naïve cells based on their comparative methylation state, for example. In some embodiments, primed cells exhibit a DNA methylation pattern of the post-implantation blastocyst.

[0062] Unlike ES cells, tissue stem cells have a limited differentiation potential. Tissue stem cells are present at particular locations in tissues and have an undifferentiated intracellular structure. Therefore, the pluripotency of tissue stem cells is typically low. Tissue stem cells have a higher nucleus/cytoplasm ratio and have few intracellular organelles. Most tissue stem cells have low pluripotency, a long cell cycle, and proliferative ability beyond the life of the individual. Tissue stem cells are separated into categories, based on the sites from which the cells are derived, such as the dermal system, the digestive system, the bone marrow system, the nervous system, and the like. Tissue stem cells in the dermal system include epidermal stem cells, hair follicle stem cells, and the like. Tissue stem cells in the digestive system include pancreatic (common) stem cells, liver stem cells, and the like. Tissue stem cells in the bone marrow system include hematopoietic stem cells, mesenchymal stem cells, and the like. Tissue stem cells in the nervous system include neural stem cells, retinal stem cells, and the like.

[0063] “Induced pluripotent stem cells,” commonly abbreviated as iPS cells or iPSCs, refer to a type of pluripotent stem cell artificially prepared from a non-pluripotent cell, typically an adult somatic cell, or terminally differentiated cell, such as fibroblast, a hematopoietic cell, a myocyte, a neuron, an epidermal cell, or the like, by introducing certain factors, referred to as reprogramming factors. hiPSCs or hiPS cells refers to human induced pluripotent stem cells. In this case, the stem cell is derived from a human cell.

[0064] “Pluripotency” refers to a stem cell that has the potential to differentiate into all cells constituting one or more tissues or organs, or particularly, any of the three germ layers: endoderm (interior stomach lining, gastrointestinal tract, the lungs), mesoderm (muscle, bone, blood, urogenital), or ectoderm (epidermal tissues and nervous system). “Pluripotent stem cells” used herein refer to cells that can differentiate into cells derived from any of the three germ layers, for example, direct descendants of totipotent cells or induced pluripotent cells.

[0065] By “operably linked” with reference to nucleic acid molecules is meant that two or more nucleic acid molecules (e.g., a nucleic acid molecule to be transcribed, a promoter, and

an enhancer element) are connected in such a way as to permit transcription of the nucleic acid molecule. "Operably linked" with reference to peptide and/or polypeptide molecules is meant that two or more peptide and/or polypeptide molecules are connected in such a way as to yield a single polypeptide chain, i.e., a fusion polypeptide, having at least one property of each peptide and/or polypeptide component of the fusion. The fusion polypeptide is particularly chimeric, i.e., composed of heterologous molecules.

[0066] The term "binds to" or when one protein or molecule is referred to as binding another, such binding is specific, in that the protein, agent, or molecule has a specific binding activity for the stated molecule. The term "selective" or "selectively" refers to an action that is discriminating. For example, "selectively labeling non-naïve hESCs" refers to a labeling action that discriminates between non-naïve and naïve hESCs and labels non-naïve hESCs (eg. primed cells).

[0067] The phrase "maintaining a population" with respect to a specific cell type refers to the culturing of cells for a period of time, wherein the cells are maintained in a specific state. The maintenance may be done by periodically removing any cells not of the specified cell type or by culturing the specified cell type in conditions that do not allow the cells to differentiate or drift into another cell type.

II. Human Embryonic Stem Cells

[0068] Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of a blastocyst. ES cells can be isolated by removing the outer trophectoderm layer of a developing embryo, then culturing the inner mass cells on a feeder layer of non-growing cells. Under appropriate conditions, colonies of proliferating, undifferentiated ES cells are produced. The colonies can be removed, dissociated into individual cells, then replated on a fresh feeder layer. The replated cells can continue to proliferate, producing new colonies of undifferentiated ES cells. The new colonies can then be removed, dissociated, replated again and allowed to grow. This process of "subculturing" or "passaging" undifferentiated ES cells can be repeated a number of times to produce cell lines containing undifferentiated ES cells (U.S. Patent Nos. 5,843,780; 6,200,806; 7,029,913). A "primary cell culture" is a culture of cells directly obtained from a tissue such as the inner cell mass of a blastocyst. A "subculture" is any culture derived from the primary cell culture.

[0069] Methods for obtaining mouse ES cells are well known. Human ES cells can be obtained from blastocysts using previously described methods (Thomson *et al. Proc. Natl. Acad. Scie. USA*, 92:7844-7848, 1995; Thomson and Marshall, *Curr. Top. Dev. Biol.*, 38:133-

165, 1998). In one method, day-5 human blastocysts are exposed to rabbit anti-human spleen cell antiserum, then exposed to a 1:5 dilution of Guinea pig complement to lyse trophoctoderm cells. After removing the lysed trophoctoderm cells from the intact inner cell mass, the inner cell mass is cultured on a feeder layer of gamma-inactivated mouse embryonic fibroblasts and in the presence of fetal bovine serum. After 9 to 15 days, clumps of cells derived from the inner cell mass can be chemically (*i.e.* exposed to trypsin) or mechanically dissociated and replated in fresh medium containing fetal bovine serum and a feeder layer of mouse embryonic fibroblasts. Upon further proliferation, colonies having undifferentiated morphology are selected by micropipette, mechanically dissociated into clumps, and replated (see U.S. Patent No. 6,833,269). ES-like morphology is characterized as compact colonies with apparently high nucleus to cytoplasm ratio and prominent nucleoli. Resulting ES cells can be routinely passaged by brief trypsinization or by selection of individual colonies by micropipette. In some methods, human ES cells can be grown without serum by culturing the ES cells on a feeder layer of fibroblasts in the presence of basic fibroblast growth factor (Amit *et al.*, *Dev. Bio.*, 227:271-278, 2000). In other methods, human ES cells can be grown without a feeder cell layer by culturing the cells on a protein matrix such as MatrigelTM or laminin in the presence of “conditioned” medium containing basic fibroblast growth factor (Xu *et al.*, *Nat. Biotechnol.*, 19:971-974, 2001). The medium is previously conditioned by coculturing with fibroblasts.

[0070] Another source of ES cells are established ES cell lines. Various human ES cell lines are known and conditions for their growth and propagation have been defined.

[0071] The source of ES cells can be a blastocyst, cells derived from culturing the inner cell mass of a blastocyst, or cells obtained from cultures of established cell lines. Thus, as used herein, the term “ES cells” can refer to inner cell mass cells of a blastocyst, ES cells obtained from cultures of inner mass cells, and ES cells obtained from cultures of ES cell lines.

[0072] Induced pluripotent stem (iPS) cells are cells which have the characteristics of ES cells but are obtained by the reprogramming of differentiated somatic cells. Induced pluripotent stem cells have been obtained by various methods. In one method, adult human dermal fibroblasts are transfected with transcription factors Oct4, Sox2, and Klf4 using retroviral transduction (Takahashi *et al.*, *Cell*, 126(4):663-76, 2007; Takahashi *et al.*, *Cell*, 131:861-872, 2007.). In some embodiments, the cells are also transfected with c-myc. The transfected cells are plated on SNL feeder cells (a mouse cell fibroblast cell line that produces LIF) in medium supplemented with basic fibroblast growth factor (bFGF). After

approximately 25 days, colonies resembling human ES cell colonies appear in culture. The ES cell-like colonies are picked and expanded on feeder cells in the presence of bFGF.

[0073] Based on cell characteristics, cells of the ES cell-like colonies are induced pluripotent stem cells. The induced pluripotent stem cells are morphologically similar to human ES cells, and express various human ES cell markers. Also, when growing under conditions that are known to result in differentiation of human ES cells, the induced pluripotent stem cells differentiate accordingly. For example, the induced pluripotent stem cells can differentiate into cells having neuronal structures and neuronal markers.

[0074] In another method, human fetal or newborn fibroblasts are transfected with four genes, Oct4, Sox2, Nanog and Lin28 using lentivirus transduction (Yu *et al.*, *Science*, 318:1917-1920, 2007). At 12-20 days post infection, colonies with human ES cell morphology become visible. The colonies are picked and expanded. The induced pluripotent stem cells making up the colonies are morphologically similar to human ES cells, express various human ES cell markers, and form teratomas having neural tissue, cartilage and gut epithelium after injection into mice.

[0075] Methods of preparing induced pluripotent stem cells from mouse are also known (Takahashi and Yamanaka, *Cell.*, 126(4):663-676, 2006). Induction of iPS cells typically require the expression of or exposure to at least one member from Sox family and at least one member from Oct family. Sox and Oct are thought to be central to the transcriptional regulatory hierarchy that specifies ES cell identity. For example, Sox may be Sox-1, Sox-2, Sox-3, Sox-15, or Sox-18; Oct may be Oct-4. Additional factors may increase the reprogramming efficiency, like Nanog, Lin28, Klf4, or c-Myc; specific sets of reprogramming factors may be a set comprising Sox-2, Oct-4, Nanog and, optionally, Lin-28; or comprising Sox-2, Oct4, Klf and, optionally, c-Myc.

[0076] IPS cells, like ES cells, have characteristic antigens that can be identified or confirmed by immunohistochemistry or flow cytometry. Pluripotency of embryonic stem cells can be confirmed by injecting approximately $0.5-10 \times 10^6$ cells into the rear leg muscles of 8-12 week old male SCID mice. Teratomas develop that demonstrate at least one cell type of each of the three germ layers.

III. SSEA-4 and Agents that bind thereto

[0077] Stage-specific embryonic antigen-4 (SSEA-4) is a glycolipid carbohydrate antigen expressed on the surface of human embryonic stem cells (hESC) and induced pluripotent stem (iPS) cells. Surprisingly, it has been found that naïve hESCs that resemble pre-

implantation stem cells do not express SSEA-4. These SSEA-4 negative cells represent a more undifferentiated cell that may be more applicable to therapeutic and research applications. For example, the SSEA-4 negative cells, as opposed to the primed SSEA-4 positive cells, may be able to effectively and appropriately differentiate into all the required cell types that are necessary for proper development, while a primed SSEA-4 positive cell may represent a cell with a more limited differentiation potential or a cell that is not capable of appropriately differentiating into a particular cell type, which may be problematic for research applications and for therapeutic applications. Therefore, methods for identifying the naïve stem cell have valuable applications.

[0078] The structure of SSEA-4 is known in the art, and antibodies directed to SSEA-4 are commercially available. The structure of SSEA-4 is shown in FIG. 5. Antibodies that bind to SSEA-4 are commercially available and include, for example, anti-SSEA4 antibody [MC813] (ab16287) from Abcam®, anti-human SSEA-4 Antibody, Clone MC-813-70 from Stemcell Technologies™, SSEA-4 Antibody (MC-813-70) from ThermoFisher Scientific™, for example. Since the structure of SSEA-4 is known, and SSEA-4 binding agents are known in the art, one could easily envision other binding agents that are within the scope of the current disclosure.

IV. Cell Culture Compositions and Methods

[0079] In accordance with the methods of the disclosure, the stem cells are cultured in an environment that includes a nutrient medium or culture medium that is capable of supporting the attachment, survival and/or proliferation of a stem cell *in vitro* or *ex vivo*. A person skilled in the art of culturing stem cells would be familiar with techniques for culturing these cells, for instance, as evidenced in U.S. Pat. No. 6,875,607, the entire contents of which are incorporated herein by reference.

[0080] The terms "nutrient medium", "cell culture medium," "culture medium" and "medium formulation" and the like refer to a nutritive solution for culturing or growing cells, preferably stem cells (whether differentiated or undifferentiated) that contain nutrients that are capable of supporting the survival and/or proliferation of the cells, and/or their attachment a support surface. The nutrient medium may contain any of the following in an appropriate combination: isotonic saline, buffer, amino acids, serum or serum replacement, and other exogenously added factors.

[0081] The culture media may be one that denotes conditions that result in a measurable amount of cell attachment, survival and/or proliferation. Effective conditions can be readily

determined and/or optimized by a skilled worker using conventional methods. Among the factors to be varied include, for example, the seeding density, the vessel, the culture medium, the temperature, the O₂/CO₂ concentrations, and the like.

[0082] A variety of culture medium capable of supporting the attachment, survival and/or proliferation of stem cells may be used in conjunction with the methods of the disclosure. Commercially available culture medium, such as DMEM, F12, aMEM, Hepatostim.TM., RPMI, or combinations thereof, may be used, either in the presence or absence of serum. In some embodiments, the serum is human serum. In some embodiments, the serum is non-human. Suitable sera include calf serum, fetal calf serum, horse serum, or the like. The skilled addressee would also know that a serum supplement could be used in place of serum in the culture media.

[0083] The stem cell may be brought into contact with the support surface by any suitable means. For example, a stem cell in a culture medium may be poured, pipetted or dispensed into a culture vessel comprising the support surface, or a medical device or scaffold comprising the support surface may be submerged in culture medium in which the stem cell is suspended.

[0084] The medium in certain aspects can be prepared using a medium used for culturing animal cells as its basal medium, such as any of AIM V, X-VIVO-15, NeuroBasal, EGM2, TeSR, BME, BGJb, CMRL 1066, Glasgow MEM, Improved MEM Zinc Option, IMDM, Medium 199, Eagle MEM, αMEM, DMEM, Ham, RPMI-1640, and Fischer's media, as well as any combinations thereof, but the medium may not be particularly limited thereto as far as it can be used for culturing animal cells. Particularly, the medium may be xeno-free or chemically defined.

[0085] The medium can be a serum-containing or serum-free medium, or xeno-free medium. From the aspect of preventing contamination with heterogeneous animal-derived components, serum can be derived from the same animal as that of the stem cell(s). The serum-free medium refers to medium with no unprocessed or unpurified serum and accordingly, can include medium with purified blood-derived components or animal tissue-derived components (such as growth factors).

[0086] The medium may contain or may not contain any alternatives to serum. The alternatives to serum can include materials which appropriately contain albumin (such as lipid-rich albumin, bovine albumin, albumin substitutes such as recombinant albumin or a humanized albumin, plant starch, dextrans and protein hydrolysates), transferrin (or other iron transporters), fatty acids, insulin, collagen precursors, trace elements, 2-mercaptoethanol, 3'-

thioglycerol, or equivalents thereto. The alternatives to serum can be prepared by the method disclosed in International Publication No. 98/30679, for example (incorporated herein in its entirety). Alternatively, any commercially available materials can be used for more convenience. The commercially available materials include knockout Serum Replacement (KSR), Chemically-defined Lipid concentrated (Gibco), and Glutamax (Gibco).

[0087] In further embodiments, the medium may be a serum-free medium that is suitable for neural cell development. For example, the medium may comprise B-27[®] supplement, xeno-free B-27[®] supplement (available at world wide web at <http://www.thermofisher.com/us/en/home/technical-resources/media-formulation.250.html>), NS21 supplement (Chen et al., J Neurosci Methods, 2008 Jun 30; 171(2): 239–247, incorporated herein in its entirety), GS21TM supplement (available at world wide web at amsbio.com/B-27.aspx), or a combination thereof at a concentration effective for producing T cells from the 3D cell aggregate.

[0088] In certain embodiments, the medium may comprise one, two, three, four, five, six, seven, eight, nine, ten, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more of the following B-27 supplement ingredients: Vitamins such as biotin; DL Alpha Tocopherol Acetate; DL Alpha-Tocopherol; Vitamin A (acetate); proteins such as BSA (bovine serum albumin) or human albumin, fatty acid free Fraction V; Catalase; Human Recombinant Insulin; Human Transferrin; Superoxide Dismutase; Other Components such as Corticosterone; D-Galactose; Ethanolamine HCl; Glutathione (reduced); L-Carnitine HCl; Linoleic Acid; Linolenic Acid; Progesterone; Putrescine 2HCl; Sodium Selenite; and T3 (triiodo-L-thyronine).

[0089] In further embodiments, the medium may comprise externally added ascorbic acid. The medium can also contain externally added fatty acids or lipids, amino acids (such as non-essential amino acids), vitamin(s), growth factors, cytokines, antioxidant substances, 2-mercaptoethanol, pyruvic acid, buffering agents, and inorganic salts.

[0090] One or more of the medium components may be added at a concentration of at least, at most, or about 0.1, 0.5, 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 180, 200, 250 ng/L, ng/ml, µg/ml, mg/ml, or any range derivable therein.

[0091] The medium used may be supplemented with at least one externally added cytokine at a concentration from about 0.1 ng/mL to about 500 ng/mL, more particularly 1 ng/mL to 100 ng/mL, or at least, at most, or about 0.1, 0.5, 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 180, 200, 250 ng/L, ng/ml, µg/ml, mg/ml, or any range derivable therein. Suitable cytokines, include but are not limited to,

FLT3 ligand (FLT3L), interleukin 7 (IL-7), stem cell factor (SCF), thrombopoietin (TPO), IL-2, IL-4, IL-6, IL-15, IL-21, TNF-alpha, TGF-beta, interferon-gamma, interferon-lambda, TSLP, thymopentin, pleiotrophin, midkine. Particularly, the culture medium may include at least one of FLT3L and IL-7. More particularly, the culture may include both FLT3L and IL-

5 7.

V. Cell Culture Conditions

[0092] The culture of human stem cells shares many of the same protocols as standard mammalian cell culture. However, the successful culture and maintenance of human stem cells in an undifferentiated state requires additional considerations to ensure that cells maintain their key characteristics of self-renewal and pluripotency.

[0093] Unlike many cell types, human stem cells may be grown in aggregates, or colonies. Standard culture of human stem cells involves exposure to media enriched with growth factors found in fetal bovine serum (FBS) or defined serum replacements. In addition, standard hPSC culture systems utilize support cells such as an inactivated mouse embryonic fibroblast (MEF) feeder layer to support growth and prevent differentiation. These cells provide necessary intercellular interactions, extracellular scaffolding and factors creating a robust and stable hPSC culture environment.

[0094] There are several basic techniques needed for the culturing of mammalian cells, including thawing frozen stocks, plating cells in culture vessels, changing media, passaging and cryopreservation. Passaging refers to the removal of cells from their current culture vessel and transferring them to one or more new culture vessels. Passaging is necessary to reduce the harmful effects of overcrowding and for expansion of the culture.

[0095] Cell culture conditions may be provided for the culture of cells according to methods described herein. In certain aspects, starting cells of a selected population may comprise at least, at most, or exactly 10^4 , 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} , 10^{12} , 10^{13} cells or any range derivable therein. The starting cell population may have a seeding density of at least, at most, or exactly 10, 10^1 , 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 cells/ml, or any range derivable therein.

[0096] A culture vessel used for culturing methods and cells of the disclosure can include, but is particularly not limited to: flask, flask for tissue culture, dish, petri dish, dish for tissue culture, multi dish, micro plate, micro-well plate, multi plate, multi-well plate, micro slide, chamber slide, tube, tray, CellSTACK® Chambers, culture bag, and roller bottle, as long as it is capable of culturing the stem cells therein. The stem cells may be cultured in a volume of

at least, at most, or exactly 0.2, 0.5, 1, 2, 5, 10, 20, 30, 40, 50 ml, 100 ml, 150 ml, 200 ml, 250 ml, 300 ml, 350 ml, 400 ml, 450 ml, 500 ml, 550 ml, 600 ml, 800 ml, 1000 ml, 1500 ml, or any range derivable therein, depending on the needs of the culture. In a certain embodiment, the culture vessel may be a bioreactor, which may refer to any device or system that supports a biologically active environment. The bioreactor may have a volume of at least, at most, or exactly 2, 4, 5, 6, 8, 10, 15, 20, 25, 50, 75, 100, 150, 200, 500 liters, 1, 2, 4, 6, 8, 10, 15 cubic meters, or any range derivable therein.

[0097] The culture vessel can be cellular adhesive or non-adhesive and selected depending on the purpose. The cellular adhesive culture vessel can be coated with any of substrates for cell adhesion such as extracellular matrix (ECM) to improve the adhesiveness of the vessel surface to the cells. The substrate for cell adhesion can be any material intended to attach stem cells or feeder cells (if used). The substrate for cell adhesion includes collagen, gelatin, poly-L-lysine, poly-D-lysine, laminin, and fibronectin and mixtures thereof for example MatrigelTM, and lysed cell membrane preparations.

[0098] In some embodiments the invention provides methods for cryo-preserving (i.e. freezing) the cells described herein. In other embodiments the invention provides compositions comprising cryopreserved cells, wherein the cell is one or more cells described herein.

[0099] In certain embodiments the disclosure provides a composition comprising a cryopreserved naïve hESC or naïve hiPSC. The composition may comprise at least 1, 10, 100, 1000, 10000, 100000, 1000000, or 10000000 cells. The cryopreserved cell may include a suitable media containing one or more cryoprotectants, such as DMSO or FBS to facilitate freezing the cells.

[0100] In other embodiments the disclosure provides a composition comprising a cryo-preserved in vitro differentiated progeny of a cell described herein.

[0101] The cryopreserved compositions may be used in research and therapeutic applications. For example a subject in need of cell therapy may be treated with the cryopreserved composition described herein.

VI. Labels

[0102] As used herein, the term "label" intends a directly or indirectly detectable compound or composition that is conjugated directly or indirectly to the agent to be detected, e.g., agent that binds to SSEA4. The label may be detectable by itself (e.g. radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical

alteration of a substrate compound or composition which is detectable. The labels can be suitable for small scale detection or more suitable for high-throughput screening. As such, suitable labels include, but are not limited to radioisotopes, fluorochromes, chemiluminescent compounds, dyes, and proteins, including enzymes. The label may be simply detected or it may be quantified. A response that is simply detected generally comprises a response whose existence merely is confirmed, whereas a response that is quantified generally comprises a response having a quantifiable (e.g., numerically reportable) value such as an intensity, polarization, and/or other property. In luminescence or fluorescence assays, the detectable response may be generated directly using a luminophore or fluorophore associated with an assay component actually involved in binding, or indirectly using a luminophore or fluorophore associated with another (e.g., reporter or indicator) component.

[0103] Examples of luminescent labels that produce signals include, but are not limited to bioluminescence and chemiluminescence. Detectable luminescence response generally comprises a change in, or an occurrence of, a luminescence signal. Suitable methods and luminophores for luminescently labeling assay components are known in the art and described for example in Haugland, Richard P. (1996) Handbook of Fluorescent Probes and Research Chemicals (6.sup.th ed.). Examples of luminescent probes include, but are not limited to, aequorin and luciferases.

[0104] Examples of suitable fluorescent labels include, but are not limited to, fluorescein, rhodamine, tetramethylrhodamine, eosin, erythrosin, coumarin, methyl-coumarins, pyrene, Malacite green, stilbene, Lucifer Yellow, Cascade Blue.TM., and Texas Red. Other suitable optical dyes are described in the Haugland, Richard P. (1996) Handbook of Fluorescent Probes and Research Chemicals (6.sup.th ed.).

[0105] In another aspect, the fluorescent label is functionalized to facilitate covalent attachment to a cellular component present in or on the surface of the cell or tissue such as a cell surface marker. Suitable functional groups, including, but not are limited to, isothiocyanate groups, amino groups, haloacetyl groups, maleimides, succinimidyl esters, and sulfonyl halides, all of which may be used to attach the fluorescent label to a second molecule. The choice of the functional group of the fluorescent label will depend on the site of attachment to either a linker, the agent, the marker, or the second labeling agent.

[0106] Attachment of the fluorescent label may be either directly to the cellular component or compound or alternatively, can be via a linker. Suitable binding pairs for use in indirectly linking the fluorescent label to the intermediate include, but are not limited to,

antigens/antibodies, e.g., rhodamine/anti-rhodamine, biotin/avidin and biotin/streptavidin.

Examples of suitable labels are also described in the table below:

Table 1 Fluorescent Protein Properties

Protein (Acronym)	Excitation Maximum (nm)	Emission Maximum (nm)	Molar Extinction Coefficient	Quantum Yield	<i>in vivo</i> Structure	Relative Brightness (% of EGFP)
GFP (wt)	395/475	509	21,000	0.77	Monomer*	48
Green Fluorescent Proteins						
EGFP	484	507	56,000	0.60	Monomer*	100
AcGFP	480	505	50,000	0.55	Monomer*	82
TurboGFP	482	502	70,000	0.53	Monomer*	110
Emerald	487	509	57,500	0.68	Monomer*	116
Azami Green	492	505	55,000	0.74	Monomer	121
ZsGreen	493	505	43,000	0.91	Tetramer	117
Blue Fluorescent Proteins						
EBFP	383	445	29,000	0.31	Monomer*	27
Sapphire	399	511	29,000	0.64	Monomer*	55
T-Sapphire	399	511	44,000	0.60	Monomer*	79
Cyan Fluorescent Proteins						
ECFP	439	476	32,500	0.40	Monomer*	39
mCFP	433	475	32,500	0.40	Monomer	39
Cerulean	433	475	43,000	0.62	Monomer*	79
CyPet	435	477	35,000	0.51	Monomer*	53
AmCyan1	458	489	44,000	0.24	Tetramer	31
Midori-Ishi Cyan	472	495	27,300	0.90	Dimer	73
mTFP1 (Teal)	462	492	64,000	0.85	Monomer	162
Yellow Fluorescent Proteins						
EYFP	514	527	83,400	0.61	Monomer*	151
Topaz	514	527	94,500	0.60	Monomer*	169
Venus	515	528	92,200	0.57	Monomer*	156
mCitrine	516	529	77,000	0.76	Monomer	174
YPet	517	530	104,000	0.77	Monomer*	238
PhiYFP	525	537	124,000	0.39	Monomer*	144
ZsYellow1	529	539	20,200	0.42	Tetramer	25
mBanana	540	553	6,000	0.7	Monomer	13

Orange and Red Fluorescent Proteins						
Kusabira Orange	548	559	51,600	0.60	Monomer	92
mOrange	548	562	71,000	0.69	Monomer	146
dTomato	554	581	69,000	0.69	Dimer	142
dTomato-Tandem	554	581	138,000	0.69	Monomer	283
DsRed	558	583	75,000	0.79	Tetramer	176
DsRed2	563	582	43,800	0.55	Tetramer	72
DsRed-Express (T1)	555	584	38,000	0.51	Tetramer	58
DsRed-Monomer	556	586	35,000	0.10	Monomer	10
mTangerine	568	585	38,000	0.30	Monomer	34
mStrawberry	574	596	90,000	0.29	Monomer	78
AsRed2	576	592	56,200	0.05	Tetramer	8
mRFP1	584	607	50,000	0.25	Monomer	37
JRed	584	610	44,000	0.20	Dimer	26
mCherry	587	610	72,000	0.22	Monomer	47
HcRed1	588	618	20,000	0.015	Dimer	1
mRaspberry	598	625	86,000	0.15	Monomer	38
HcRed-Tandem	590	637	160,000	0.04	Monomer	19
mPlum	590	649	41,000	0.10	Monomer	12
AQ143	595	655	90,000	0.04	Tetramer	11
* Weak Dimer						

VII. Kits

[0107] This disclosure also includes kits comprising the products disclosed herein such as the cell culture medium, cells, and/or SSEA-4 binding agents. Further, the current disclosure provides kits for isolating and/or purifying stem cells, and kits for culturing and/or identifying the stem cells.

[0108] The kits according the present invention can comprise as their constituents, for example, SSEA-4 binding agents, culture media or solutions and such for culturing cells.

VIII. Methods and Therapeutic Applications

[0109] The methods and compositions described herein are useful for purification, isolation, and identification of naïve stem cells. In certain embodiments, the methods include detection or isolation of naïve SSEA-4-negative stem cells on the basis of their SSEA-4-negative status. Such methods may include methods known in the art such as fluorescent activated cell sorting (FACS), immunostaining, immunohistochemistry, histological methods, precipitation, chromatography, solid-support assays, magnetic activated cell sorting (MACS), and panning.

[0110] The methods and compositions described herein are useful for therapeutic applications, such as stem cell therapy. In certain embodiments, human embryonic stem cell therapy comprises first identifying and isolating SSEA-4 negative cells. Treatment aspects relate to treating a patient with a stem cell, wherein the stem cell is a SSEA-4-negative cell. The method may further comprise differentiation of the SSEA-4 negative naïve stem cell. The differentiation may be done *in vitro* or *in vivo*. Therefore, treatment aspects relate to the administration of the SSEA-4 negative stem cell or its progeny. Treatment aspects relate to treating a patient with a stem cell, wherein the stem cell is a SSEA-4-negative cell. In some embodiments, the therapy is for the treatment of conditions of the blood and immune system. In some embodiments, the therapy is for the treatment of leukemias and lymphomas, including: acute myelogeneous leukemia (AML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), chronic myelogeneous leukemia (CML), juvenile myelomonocytic leukemia, Hodgkin lymphoma, and non-Hodgkin lymphoma (NHL); for bone marrow diseases and other diseases when bone marrow fails to work, including: severe aplastic anemia, fanconi anemia, paroxysmal nocturnal hemoglobinuria (PNH), pure red cell aplasia, and amegakaryocytosis/congenital thrombocytopenia; for inherited immune system disorders, including: severe combined immunodeficiency (SCID, all types) and Wiskott-Aldrich syndrome (WAS); for hemoglobinopathies (diseases with poorly functioning red blood cells), including: beta thalassemia major and sickle cell disease (SCD); for inherited metabolic disorders, including: Krabbe disease (GLD), Hurler syndrome (MPS-IH), adrenoleukodystrophy (ALD), metachromatic leukodystrophy (MLD); and for treating myelodysplastic syndromes and myeloproliferative disorders and multiple myeloma and other plasma cell disorders. Further embodiments relate to methods for in vitro fertilization, wherein the in vitro fertilized embryo is first screened for SSEA-4 expression and wherein

the individual is implanted with a embryo (cleavage or blastocyst) that has been first screened and determined to be SSEA-4-negative.

IX. Examples

[0111] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1 – Naïve human pluripotent cells feature a methylation landscape devoid of blastocyst or germline memory

[0112] Human embryonic stem cells (hESCs) typically exhibit “primed” pluripotency, analogous to stem cells derived from the mouse post-implantation epiblast. This has led to a search for growth conditions that support self-renewal of hESCs akin to hypomethylated naïve epiblast cells in human pre-implantation embryos. In the current study, Applicants discovered that reverting primed hESCs to a hypomethylated naïve state or deriving a new hESC line under naïve conditions results in the establishment of Stage Specific Embryonic Antigen 4 (SSEA4) negative hESC lines with a transcriptional program resembling the human pre-implantation epiblast. In contrast, Applicants discovered that the methylome of naïve hESCs *in vitro* is distinct from the human epiblast *in vivo* with loss of DNA methylation at primary imprints and young transposable elements and a lost “memory” of the methylation state of the human oocyte. This failure to recover the naïve epiblast methylation landscape appears to be a consistent feature of self-renewing hypomethylated naïve hESCs *in vitro*.

[0113] Human embryonic stem cells (hESCs) are *in vitro* pluripotent cell types with the capacity for unlimited self-renewal and differentiation, making them critical models for understanding mechanisms required for human embryo development and differentiation. Although hESCs are derived from pre-implantation human blastocysts, they are morphologically and transcriptionally similar to murine epiblast stem cells (EpiSCs), which are derived from post-implantation mouse embryos. As such, hESCs and EpiSCs are said to

exhibit a “primed pluripotent state” while, mouse ESCs derived from the pre-implantation blastocyst exhibit a “naïve pluripotent state” corresponding to an earlier stage of development (Nichols and Smith, 2009).

[0114] A number of culture conditions have recently been developed that promote maintenance and self-renewal of naïve human pluripotent stem cells (Chan et al., 2013; Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014; Ware et al., 2014). Each protocol generates cell types with slightly different molecular characteristics, which may reflect metastable states in the spectrum of naïve to primed pluripotency. Recent meta-analysis of sequencing data indicates that two of these protocols generate cells with a close transcriptional resemblance to the human pre-implantation epiblast (Huang et al., 2014). In the first protocol, hESCs are transfected with *KLF2* and *NANOG* and cultured in media with titrated two inhibitors plus leukemia inhibitory factor and Gö6983 (t2iL+Gö) (Takashima et al., 2014). In the second protocol, primed cells can be reverted by transferring to a media containing a cocktail of five inhibitors plus LIF, Activin and/or Fibroblast Growth Factor 2 (5iLA/F)(Theunissen et al., 2014). Using t2iL+Gö reversion of the H9 primed hESC line, it was shown that DNA methylation is globally reduced to the average level measured in human pre-implantation epiblasts (Takashima et al., 2014), with additional locus-specific erosion in the 5' region of the LINE1 human specific (LIHS) retrotransposons (Gkoutela et al., 2015). The DNA methylation profile of cells cultured in 5iLAF has never been evaluated.

[0115] Applicants reverted primed hESCs to the naïve state using 5iLAF to evaluate gene expression and DNA methylation and also derived a new hESC line called UCLA20n entirely under 5iLAF naïve culture conditions. It was discovered that reversion and maintenance in 5iLAF creates a heterogeneous mixture of cells in which the Stage Specific Embryonic Antigen 4 (SSEA4) negative cells are enriched in the transcriptional program of the pre-implantation epiblast. In contrast UCLA20n hESCs are almost entirely devoid of SSEA4 surface expression. Regardless of their origin (reversion or derivation) it was found that hESCs in 5iLAF as well as t2iL+Gö have a methylome compellingly distinct from that of the human pre-implantation epiblast, with globally erased oocyte memory and loss of imprints. Thus, self-renewing naïve hypomethylated hESCs appear unable to recapitulate or retain the methylation pattern of the pre-implantation blastocyst.

A. Reversion of primed UCLA1 hESCs in 5iLAF results in heterogeneous colonies

[0116] In order to revert UCLA1 hESCs (Diaz Perez et al., 2012) to the naïve state Applicants used 5iLAF (Theunissen et al., 2014) and first performed n=4 independent
5 reversions of the hESC line UCLA1. Upon reversion Applicants observed a mixture of small, round colonies similar to naïve mESCs as well as flat, cobblestone-like colonies (Figure 1A,B). Applicants evaluated one reversion using two classic human pluripotency surface markers called SSEA4 and TRA-1-81. Unlike primed UCLA1 hESCs, which are double positive for SSEA4 and TRA-1-81, the 5iLAF reverted hESCs have a large fraction of double
10 negative cells (Figure 1A,B). Immunofluorescence staining showed that the SSEA4 and TRA-1-81 negative cells were still positive for OCT4 and NANOG (Figures 3A-F).

[0117] Next, Applicants sorted the 5iLAF-cultured cells into SSEA4 positive and negative populations using fluorescence activated cell sorting (FACS) and re-plated the sorted cells onto MEFs in 5iLAF media (Figure 1C). Applicants discovered that SSEA4 positive cells
15 yielded mostly flat colonies, whereas SSEA4 negative cells yielded mostly round colonies. One passage after sorting, the SSEA4 negative population remained SSEA4 negative, indicating that this is a relatively stable state (Figure 3G). Applicants then reverted two additional lines called UCLA4 and UCLA5 (Diaz Perez et al., 2012) and found that small, round colony morphology was always enriched in the SSEA4 negative fraction whereas the
20 SSEA4 positive cells yielded mostly flat, cobblestone colonies (Figure 3H-K).

[0118] In order to determine whether the heterogeneity in SSEA4 expression was also observed when deriving hESC lines completely under naïve 5iLAF conditions, Applicants derived n=2 new hESC lines, which Applicants have called UCLA19n and UCLA20n after thawing n=7 day 5 vitrified human blastocysts. Colonies were uniformly round and flow
25 cytometry revealed that UCLA19n was 85% SSEA4 negative (results not shown), whereas UCLA20n was almost completely SSEA4 negative (Figure 1D). In contrast, TRA-1-81 was expressed on a significant portion of SSEA4 negative cells in UCLA20n as well as reverted UCLA4 and UCLA5 hESC lines (Figure 1D, Figure 3I, Figure 3K). Therefore, loss of TRA-1-81 is not a consistent marker of naïve morphology, whereas absence of SSEA4 is a highly
30 correlated feature of naïve round colony morphology. In summary, reversion of primed hESCs in 5iLAF generates a heterogeneous mixture of colonies, with SSEA4 negative hESCs correlating with small round colony morphology similar to naïve hESCs derived from the human pre-implantation blastocyst.

B. 5iLAF SSEA4 negative cells display expression patterns consistent with pre-implantation epiblasts.

[0119] On the basis of morphology, Applicants speculated that 5iLAF SSEA4 negative hESCs resemble the cells of the human pre-implantation epiblast. To address this, Applicants performed RNA-sequencing of 5iLAF-cultured SSEA4 positive or SSEA4 negative fractions of UCLA1, and compared them to SSEA4 positive primed UCLA1 hESCs at equivalent passages.

[0120] Applicants also performed RNA-Seq of UCLA20n at passage 20 after derivation. Applicants did not analyze UCLA19n as it was found to be 70% polyploid by passage 14. Consistent with the expression patterns of genes associated with naïve pluripotency in mice, the 5iLAF SSEA4 negative cells and UCLA20n had elevated levels of *NANOG* as well as a dramatic up-regulation of *KRUPPLE LIKE FACTOR (KLF)* family transcription factors and reduced expression primed state master regulators such as *ZINC FINGER OF THE CEREBELLUM (ZIC)* family transcription factors and *OTX2* (Buecker et al., 2014; Tang et al., 2011; Yang et al., 2014) (Figure 1E). To further confirm the similarity of 5iLAF SSEA4 negative and UCLA20n hESCs to the human pre-implantation blastocyst Applicants used the previously published single cell expression data from late pre-implantation epiblast and primed hESCs. (Yan et al., 2013). Applicants defined a set of “pre-implantation epiblast specific” and “primed-specific” genes, which showed >4 fold difference in expression between these two cell types. Using these genes as a reference Applicants found that the 5iLAF SSEA4 negative hESCs and UCLA20n had global up-regulation of naïve epiblast-specific genes, and down-regulation of primed-specific genes (Figure 1F).

[0121] In contrast, the SSEA4 positive hESCs sorted from 5iLAF cultures had an intermediate correlation between primed and naïve, suggesting that SSEA4 positive cells that stably self-renew in 5iLAF are partially reverted to the naïve state (Figure 1E,F). Comparing to published datasets, Applicants found that the SSEA4 negative population in UCLA1 and the new hESC line UCLA20n is analogous to the original 5iLAF hESC lines created by reverting WIBR2 (Theunissen et al., 2014) and to t2iL+Gö-cultured hESCs created by reverting H9 (Takashima et al., 2014). In contrast, other published naïve methods showed a less pronounced shift toward the naïve state and failure to repress primed markers (Chan et al., 2013; Gafni et al., 2013; Ware et al., 2014). Interestingly, lines generated by these methods are also reported to be SSEA4 positive. Given these results, Applicants focused the methylation analysis on the 5iLAF and t2iL+Gö conditions.

C. 5iLAF SSEA4 negative cells do not recapitulate the oocyte memory of naïve epiblast cells in vivo.

[0122] To determine the methylation pattern of hESCs in 5iLAF Applicants performed whole genome bisulfite sequencing (WGBS) on 2-4 independent sorts of SSEA4 negative or
 5 SSEA4 positive reverted UCLA1 cells, SSEA4 negative UCLA20n cells and primed UCLA1 cells that had been in culture a similar length of time to the reverted lines. Applicants discovered that similar to the levels observed in t2iL+Gö (Gkoutela et al., 2015; Takashima et al., 2014) 5iLAF cultured SSEA4 negative hESCs and UCLA20n had an average CG methylation level that resembled the human blastocyst (Figure 2A) (Okoe et al., 2014).

10 [0123] In mammals the methylation pattern of the blastocyst is shaped by events during gametogenesis and early embryogenesis. The male pronucleus is selectively demethylated in early embryonic development, with only a few regions such as paternally methylated imprinted loci protected from DNA demethylation (Okoe et al., 2014; Smith et al., 2014; Smith et al., 2012). Thus in humans the methylation pattern of the blastocyst strongly
 15 resembles Metaphase II human oocytes (Figure 2B, C). In contrast, the methylation landscapes of SSEA4 negative 5iLAF-cultured hESCs, UCLA20n and t2iL+Gö-cultured cells are only weakly correlated with the human blastocyst and human oocyte (Figure 2B, C). Naïve cells, even if cultured by different methodologies or derived directly from the human blastocyst, converge toward a methylation pattern that is different from the pre-implantation
 20 human blastocyst (Figure 2B,C). A striking example of this is trend is observed at 332 CpG islands identified previously as “transient maternal imprints”: sites that are highly methylated in oocytes and the maternal chromosomes of blastocyst that lose methylation upon implantation (Smith et al., 2014). Applicants discovered that reversion does not regenerate methylation at these sites, nor is methylation retained at these transient maternal imprints in
 25 the UCLA20n hESC line (Figure 4A).

[0124] An additional, striking deviation from oocyte and blastocyst methylation patterns in 5iLAF and t2iL+Go cultured cells occurred at stable imprints. These are regions where DNA methylation is established exclusively during germ-cell development. These methylated sites are protected from DNA demethylation during pre-implantation embryo development,
 30 remaining differentially methylated in somatic cells through the life of the organism and promoting a parent-of-origin specific expression pattern in the neighboring genes. Applicants examined DNA methylation at 29 maternally methylated stable primary imprints and 2 paternally methylated stable primary imprints (Okoe et al., 2014) (Figure 2D). There is roughly 50% methylation in somatic tissue and slightly below 50% methylation in blastocysts

as expected. In the primed UCLA1 hESCs used in this study, the median methylation of these imprinted sites was close to 50%, though some imprints were hyper or hypomethylated, similar to what has been observed previously for other hESC lines (Rugg-Gunn et al., 2007). Strikingly, the 5iLAF SSEA4 negative hESCs and UCLA20n had near complete loss of methylation from all thirty-one primary stable imprints evaluated in this study, with loss over many imprints found in t2iL+Gö (Figure 2D,E). Taking advantage of single nucleotide polymorphisms (SNPs) present in the UCLA1 hESC line, Applicants observed a shift in allelic expression upon reversion in 5iLAF from monoallelic to biallelic expression of several imprinted genes including *H19* and *SNRPN*. (Figure 4B). In order to determine whether methylation could be restored at imprinted genes by reverting the naïve hESCs back to a primed state, Applicants cultured 5iLAF SSEA4 negative and primed UCLA1 cells in primed epiblast like cell (EpiLC) media (Hayashi et al., 2011) for sixteen days. During this time, Applicants discovered that 5iLAF SSEA4 negative cells showed a global shift toward expression of primed-specific genes and gained DNA methylation genome-wide (Figure 3C). However, increased methylation over imprinted regions was very modest, and biallelic expression was still observed (Figure 3D). Thus, when lost, imprinting is not re-established upon culturing in primed conditions, a similar scenario to the rescue of global DNA methylation but not imprint methylation in *Dnmt1* knockout ESCs by the re-expression of *Dnmt1* (Holm et al., 2005). Furthermore, consistent with data observed in hESCs cultured in t2iL+Gö (Gkoutela et al., 2015), young LINE elements also show dramatic promoter hypomethylation in 5iLAF (Figure 3E).

[0125] Given the problem with maintenance of imprint methylation in naïve cells, Applicants considered the possibility that the 5iLAF SSEA4 positive cells may represent a useful intermediate. However, Applicants discovered that the SSEA4 positive cells showed intermediate levels of global and imprint methylation loss (Figure 2C, 2D), with biallelic expression of *SNRPN* and *H19* (Table S2). Applicants also compared the methylation loss to naïve human stem cell (NHSM) media (Gafni et al., 2013), which shows the smallest transcriptional shift towards naïve pluripotency (Figure 1E,F). In order to directly compare the methylome data to Gafni et al., 2013, Applicants modified all whole-genome data sets to simulate the Reduced Representation Bisulfite Sequencing approach used by Gafni to measure DNA methylation. Applicants discovered that imprint methylation was unperturbed in NHSM (Figure 4F), but very little global change in methylation was observed either (Figure 2F).

[0126] Consistent with an initial report of karyotypic instability in 5iLAF culture (Theunissen et al., 2014), Applicants discovered that twenty-four passages after reversion the 5iLAF UCLA1 hESCs developed widespread karyotypic abnormalities, which was not observed in the first thirteen passages following reversion (Figure 4G). Similarly, UCLA20n had evidence of trisomies at chromosomes 3, 7, 12 and 20 by passage 14 and as discussed above, UCLA19n was 70% polyploid at passage 15 (Figure 4H). Therefore, karyotypic instability may also be a frequent consequence of naïve hESC culture.

D. Altered DNA methyltransferase expression in 5iLAF SSEA4 negative hESCs.

[0127] To determine the cause of the failure to maintain DNA methylation, Applicants analyzed changes in RNA and protein levels of DNA methylation and demethylation machinery. Applicants found that the RNA and protein levels of the *de novo* DNA methyltransferase DNMT3B dropped sharply in the 5iLAF SSEA4 negative cells, while DNMT3A was unchanged and DNMT3L increased dramatically relative to primed hESCs. *UHRF1* RNA levels were slightly elevated in naïve hESCs. However, at the protein level, Applicants observe a 65% loss of UHRF1, and both *DNMT1* RNA and protein levels were reduced by 50% in the naïve state. Furthermore, expression of the 5mC oxidases TET1 and TET2 increased substantially in the naïve state. (Figure 2G, 2H).

E. Discussion

[0128] In the current study Applicants have shown that naïve hESCs have a transcriptional program enriched in human pre-implantation-specific genes but with a global DNA methylation landscape that is distinct from the normal state of the human pre-implantation blastocyst. The negative effect of the loss of “transient imprints”, and the failure to recapitulate the oocyte-like methylation pattern is unclear. However, the loss of stable primary imprints is potentially serious in human pluripotent stem cell research. Correct imprinting is necessary for organism survival, and a number of rare human medical disorders have been linked to aberrant imprinting (Butler, 2009). Of note, murine embryonic germ cell lines are transcriptionally similar to murine ESCs but have widespread loss of imprints and contribute poorly to chimeras (Leitch et al., 2013; Oliveros-Etter et al., 2015; Tada et al., 1998), demonstrating the importance of imprints in correct differentiation of pluripotent cells in vivo. Accompanying the loss of imprints, Applicants observed extensive karyotypic

abnormalities in cells after prolonged culture in 5iLAF. Loss of DNA methylation has been linked to karyotypic instability (Haaf, 1995).

[0129] Applicants note that methylation at the imprinted loci is clearly depressed relative to surrounding regions. This may reflect the observation that many imprinted loci are promoters or regulatory elements that are active in the blastocyst (Rugg-Gunn et al., 2007). Thus if methylation is partially eroded at the imprint, the relevant transcription factors bind and cause further demethylation (as is generally the case at these genetic elements). In other words, methylation may be a very weak barrier to locus activation in 5iLAF. Similar dynamics may be at work at LHS elements.

[0130] Although Applicants observed a reduction in DNMT3B protein in the naive cells, Applicants propose that this has only modest effects on creating the 5iLAF methylome given that DNMT3A^{-/-} DNMT3B^{-/-} DKO primed hESCs maintain primary imprints and show only modest DNA demethylation even after extended culture (Liao et al., 2015). Applicants therefore propose that a combination of impaired maintenance methylation and increased TET activity could explain the majority of the 5iLAF hypomethylation phenotype. In a cell type with impaired maintenance and some continuous *de novo* methylation (imparted by DNMT3A and the remaining DNMT3B), DNA methylation levels will reach a steady state, but memory of previous methylation will be lost with DNA replication.

* * *

[0131] All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims. All references cited in this application are specifically incorporated by reference for all purposes.

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CLAIMS

1. A method for separating naïve human embryonic stem cells (hESCs) or naïve hiPSCs from non-naïve hESCs or non-naïve hiPSCs *in vitro*, the method comprising:
contacting the cells *in vitro* with an agent that binds to Stage Specific Embryonic Antigen 4 (SSEA4) antigen on the cells; and
separating SSEA4-negative unbound cells from the SSEA4-positive agent-bound cells;
wherein the fraction of unbound cells represents the naïve hESCs or naïve hiPSCs and the fraction of bound cells represents the non-naïve hESCs or non-naïve hiPSCs.
2. The method of claim 1, wherein the method further comprises culturing the fraction of unbound cells.
3. The method of claim 2, wherein the cells are cultured in media comprising one or more of the inhibitors of MAPK/ERK kinase, ROCK, Src, LCK, B-raf kinase, and GSK-3 β .
4. The method of claim 3, wherein the media further comprises one or more of FGF, Activin A, and LIF.
5. The method of any one of claims 1-4, wherein the agent is labeled with a detectable label.
6. The method of any one of claims 1-5, wherein the agent is an antibody.
7. The method of any one of claims 1-6, wherein the method further comprises contacting the cells with a second agent that binds to the SSEA4-binding agent.
8. The method of any one of claims 1-7, wherein the SSEA-4 binding agent and/or second agent are linked to a solid support.
9. The method of any one of claims 1-7, wherein the agent-bound cells and unbound cells are separated by fluorescent-activated cell sorting (FACS), chromatography, solid-support assays, magnetic activated cell sorting (MACS), and panning.
10. The method of any one of claims 1-9, wherein the non-naïve cell is a primed cell.
11. The method of any one of claims 1-10, wherein the method further comprises expanding the naïve hESCs or naïve hiPSCs.
12. The method of any one of claims 1-11, wherein the method further comprises freezing the naïve hESCs or naïve hiPSCs.
13. The method of claim 12, wherein the method further comprises thawing the frozen cells.
14. A naïve hESC or hiPSC isolated according to the method of any one of claims 1-13.

15. A composition comprising the cell of claim 14.
16. A therapeutic method comprising administering the cell of claim 14 or composition of claim 15 to a human.
17. A method for selectively labeling non-naïve hESCs or hiPSCs in a composition comprising naïve and non-naïve hESCs or iPSCs, the method comprising: contacting the cells *in vitro* with a labeled agent that binds to Stage Specific Embryonic Antigen 4 (SSEA4) antigen on the cells; wherein the binding of the labeled agent to the non-naïve hESCs or iPSCs selectively labels the non-naïve hESCs or hiPSCs.
18. The method of claim 17, wherein the agent is labeled with a detectable label.
19. The method of claim 17 or 18, wherein the agent is an antibody.
20. The method of any one of claims 17-19, wherein the method further comprises contacting the cells with a second agent that binds to the SSEA4-binding agent.
21. The method of any one of claims 17-20, wherein the SSEA-4 binding agent and/or second agent are linked to a solid support.
22. The of any one of claims 17-21, wherein the method is for therapeutic treatment of a patient and wherein the method further comprises administering an unlabeled cell to the patient.
23. A method for culturing or for maintaining a population of naïve hESCs or naïve hiPSCs comprising
 - contacting the cells with an agent that binds to Stage Specific Embryonic Antigen 4 (SSEA4) antigen on the cells;
 - separating SSEA4-negative unbound cells from the SSEA4-positive agent-bound cells; and
 - culturing the unbound cells in cell media.
24. The method of claim 23, wherein the cells are cultured in media comprising one or more of the inhibitors of MAPK/ERK kinase, ROCK, Src, LCK, B-raf kinase, and GSK-3 β .
25. The method of claim 24, wherein the media further comprises one or more of FGF, Activin A, and LIF.
26. The method of any one of claims 23-25, wherein the agent is labeled with a detectable label.
27. The method of any one of claims 23-26, wherein the agent is an antibody.
28. The method of any one of claims 23-27, wherein the method further comprises contacting the cells with a second agent that binds to the SSEA4-binding agent.

29. The method of any one of claims 23-28, wherein the SSEA-4 binding agent and/or second agent are linked to a solid support.
30. The method of any one of claims 23-29, wherein the agent-bound cells and unbound cells are separated by fluorescent-activated cell sorting (FACS), chromatography, solid-support assays, magnetic activated cell sorting (MACS), and panning.
31. The method of any one of claims 23-30, wherein the non-naïve cell is a primed cell.
32. The method of any one of claims 23-31, wherein the method further comprises expanding the naïve hESCs or naïve hiPSCs.
33. The method of any one of claims 23-32, wherein the method further comprises freezing the naïve hESCs or naïve hiPSCs.
34. The method of claim 33, wherein the method further comprises thawing the frozen cells.
35. A naïve hESC or hiPSC cultured according to the method of any one of claims 23-34.
36. A composition comprising the cell of claim 35.
37. A therapeutic method comprising administering the cell of claim 35 or composition of claim 36 to a human.
38. A method for evaluating a cell culture medium for culturing hESCs or hiPSCs, the method comprising:
 - culturing the hESCs or hiPSCs in the medium;
 - contacting the hESCs or hiPSCs with a detectable agent that binds to SSEA4 antigen on cells;
 - detecting the agent binding to the antigen;
 - evaluating the medium on the basis of the detected agent.
39. The method of claim 38, wherein the agent is labeled with a detectable label.
40. The method of any one of claims 38-39, wherein the agent is an antibody.
41. The method of any one of claims 38-40, wherein the method further comprises contacting the cells with a second agent that binds to the SSEA4-binding agent.
42. The method of any one of claims 38-41, wherein the SSEA-4 binding agent and/or second agent are linked to a solid support.
43. The method of any one of claims 38-42, wherein the method further comprises quantifying the SSEA4-negative unbound cells, SSEA4-positive bound cells, or both.
44. The method of claim 43, wherein a medium with a percentage of SSEA-4 negative cells of less than 25% is determined to be a naïve hESC or hiPSC maintenance medium.
45. A cell medium evaluated according to any one of claims 38-44.

46. A method for identifying culturing compositions that revert a primed hESC or hiPSC to a naïve hESC or hiPSC comprising:
- contacting the primed hESC or hiPSC cultured *in vitro* with a candidate culturing composition;
 - contacting the cell with a detectable agent that binds to SSEA4 antigen on cells;
 - detecting the presence or absence of the agent binding to the antigen;
 - identifying the culturing composition as a composition that reverts a primed hESC or hiPSC to a naïve hESC or hiPSC when the absence of agent-antigen binding is detected.
47. The method of claim 46, wherein the candidate culturing composition comprises one or more of the inhibitors of MAPK/ERK kinase, ROCK, Src, LCK, B-raf kinase, and GSK-3 β .
48. The method of claims 46 or 47, wherein the culturing composition comprises or further comprises one or more of FGF, Activin A, and LIF.
49. A culturing composition that revers a primed hESC or hiPSC to a naïve hESC or hiPSC, wherein the composition is identified according to the method of any one of claims 46-48.
50. A method for distinguishing a naïve hESC or a primed hESC, the method comprising:
- contacting the cell with a detectable agent that binds to Stage Specific Embryonic Antigen 4 (SSEA4)-positive cells; and
 - detecting a naïve hESC as a cell not bound with the detectable agent; and/or
 - detecting a primed hESC as a cell bound with the detectable agent.
51. A therapeutic method comprising contacting a naïve human pluripotent cell *in vitro* with a detectable agent that binds to SSEA4 antigen on cells; administering the naïve human pluripotent cell to the patient; wherein the binding of the agent to the SSEA4 antigen is not detectable.
52. The method of claim 51, wherein the method comprises contacting a naïve human pluripotent cell and wherein the naïve human pluripotent cell is in a blastocyst.
53. The method of claim 51, wherein the therapeutic method is *in vitro* fertilization (IVF).
54. A composition comprising an isolated naïve hESC or hiPSC and cell culture media, wherein the cell is SSEA4-negative.
55. The composition of claim 54, wherein the cell is a naïve hiPSC.
56. The composition of claim 54 or 55, wherein the cell culture media comprises one or more inhibitors of MAPK/ERK kinase, ROCK, Src, LCK, B-raf kinase, and GSK-3 β .

57. The composition of any one of claims 54-56, wherein the cell culture media further comprises one or more of FGF, Activin A, and LIF.

A

Primed (UCLA1)

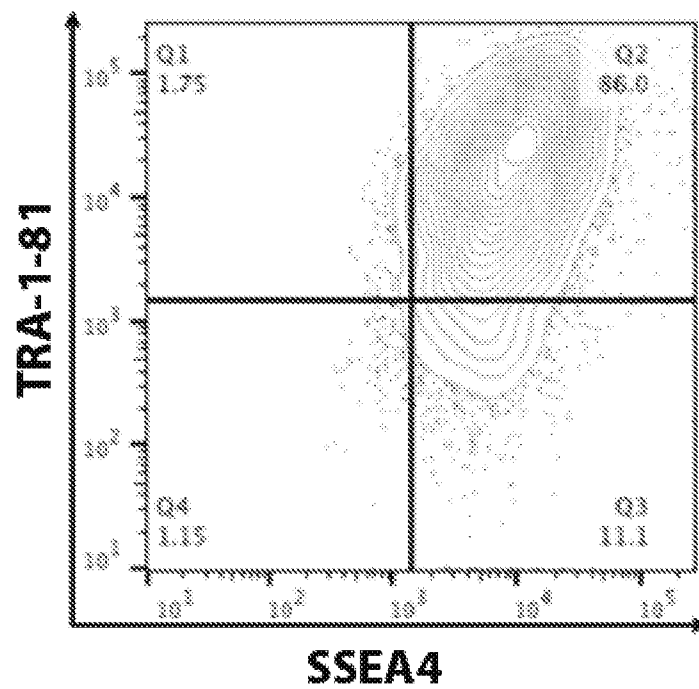
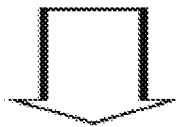
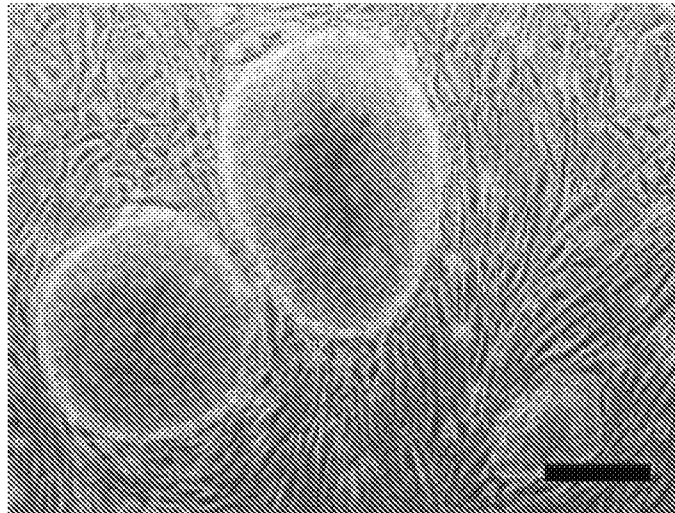


FIG. 1A

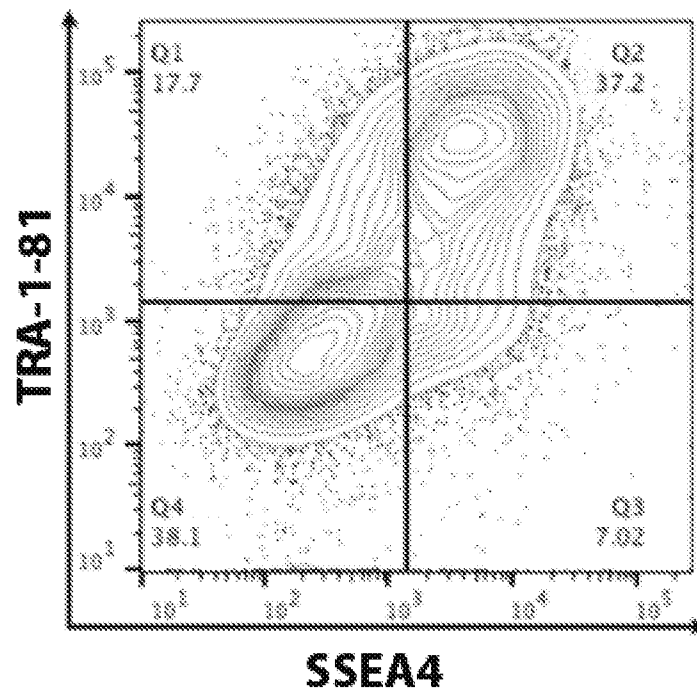
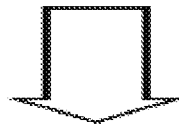
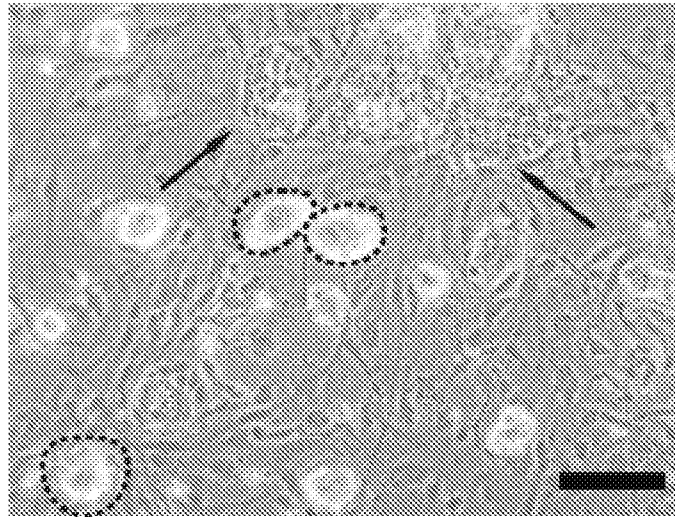
B 5iLAF (UCLA1)

FIG. 1B

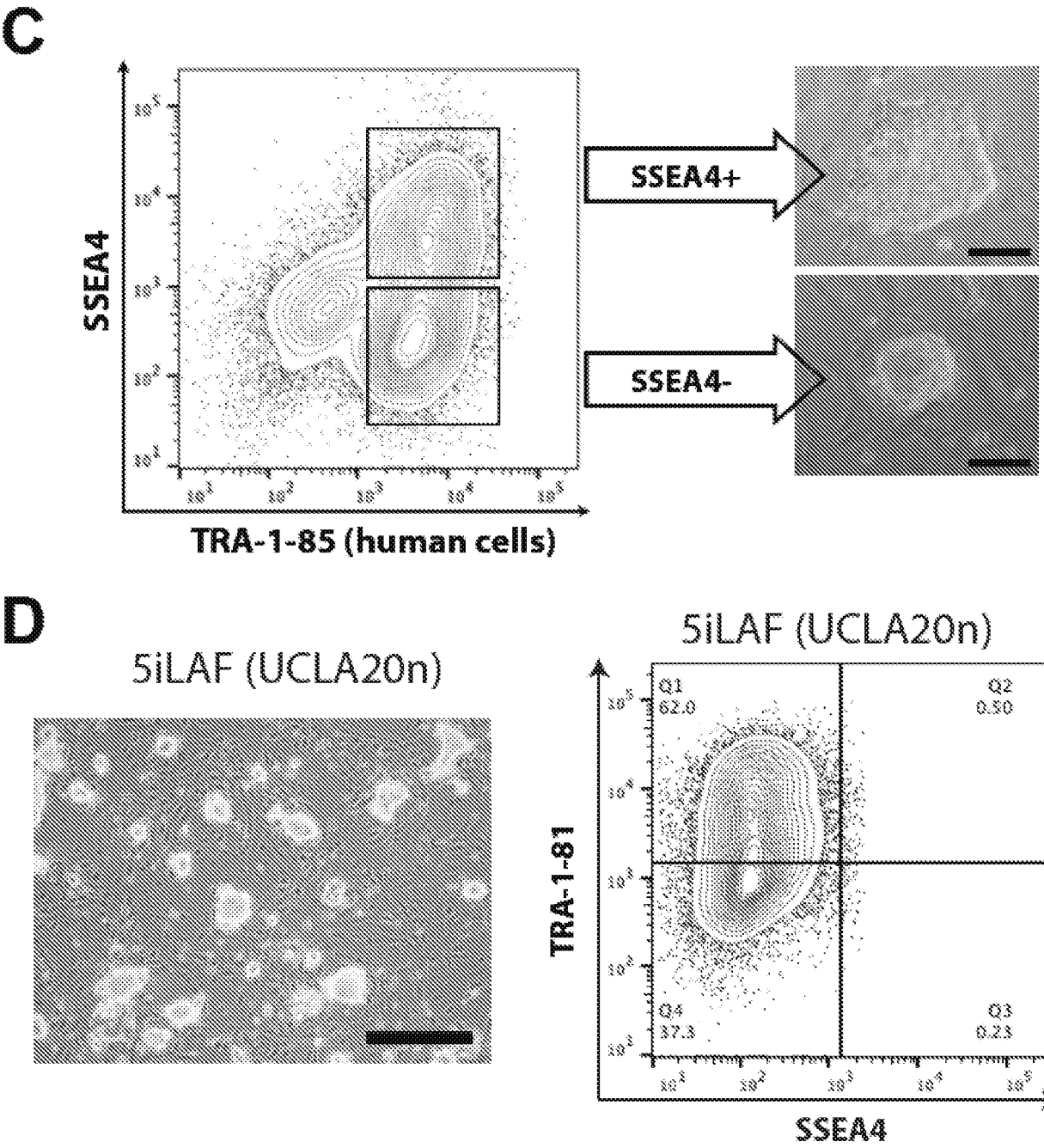


FIG. 1C-D

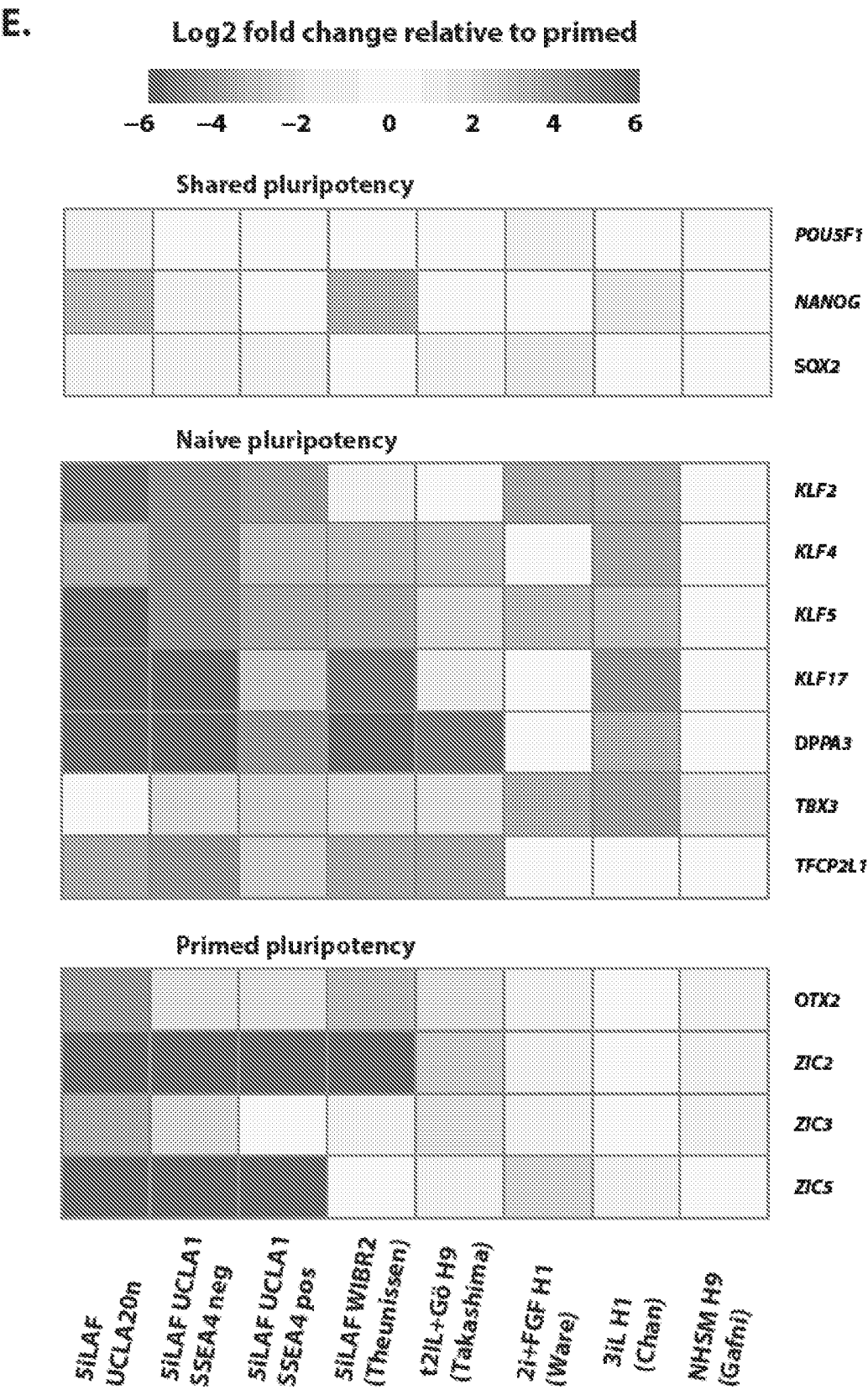


FIG. 1E

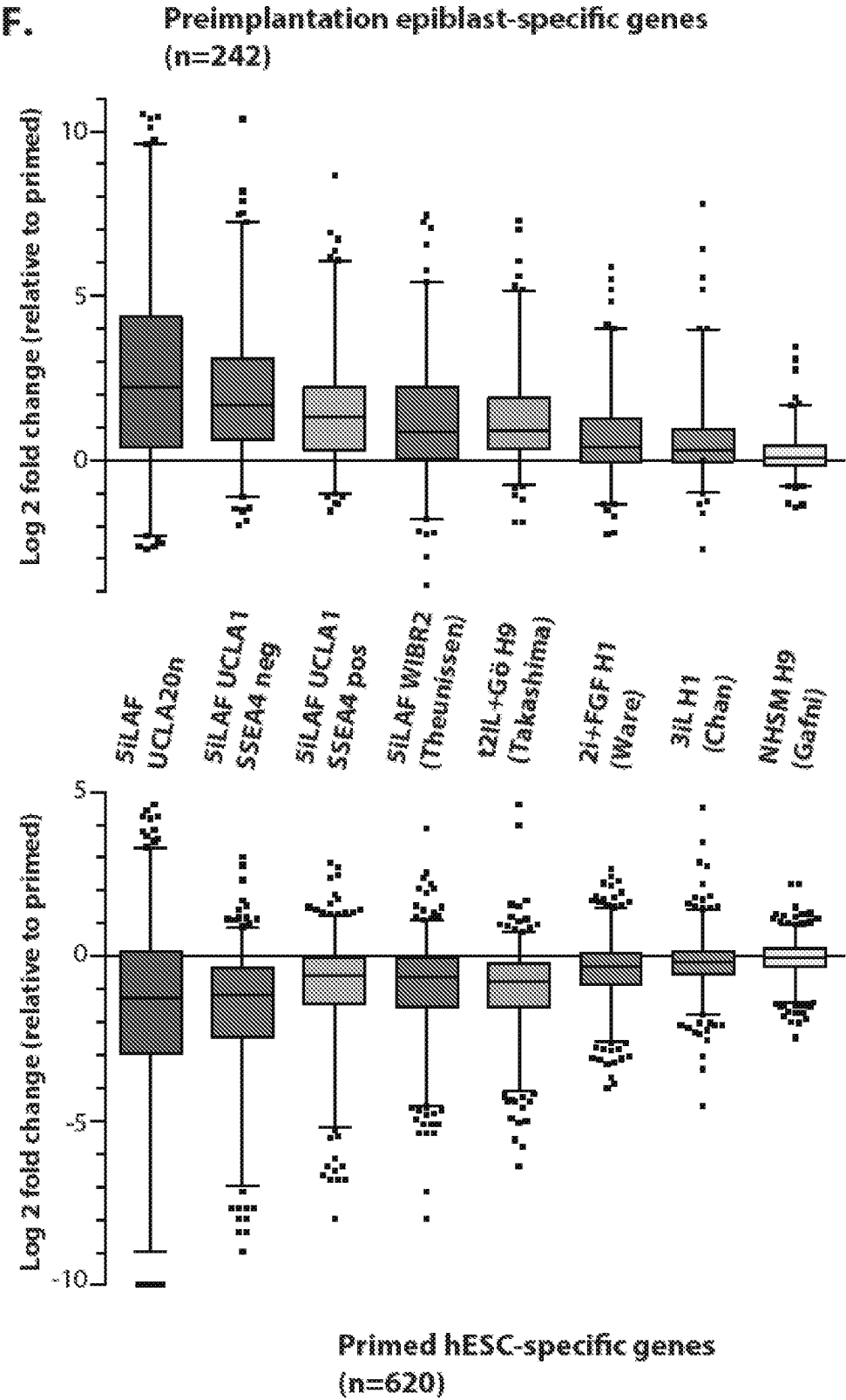


FIG. 1F

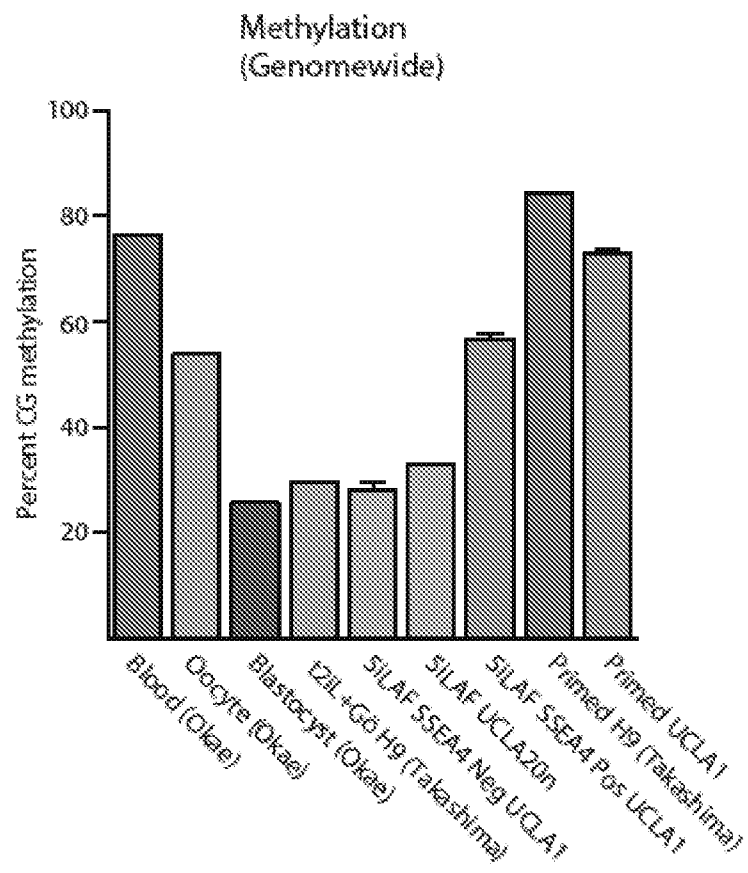


FIG. 2A

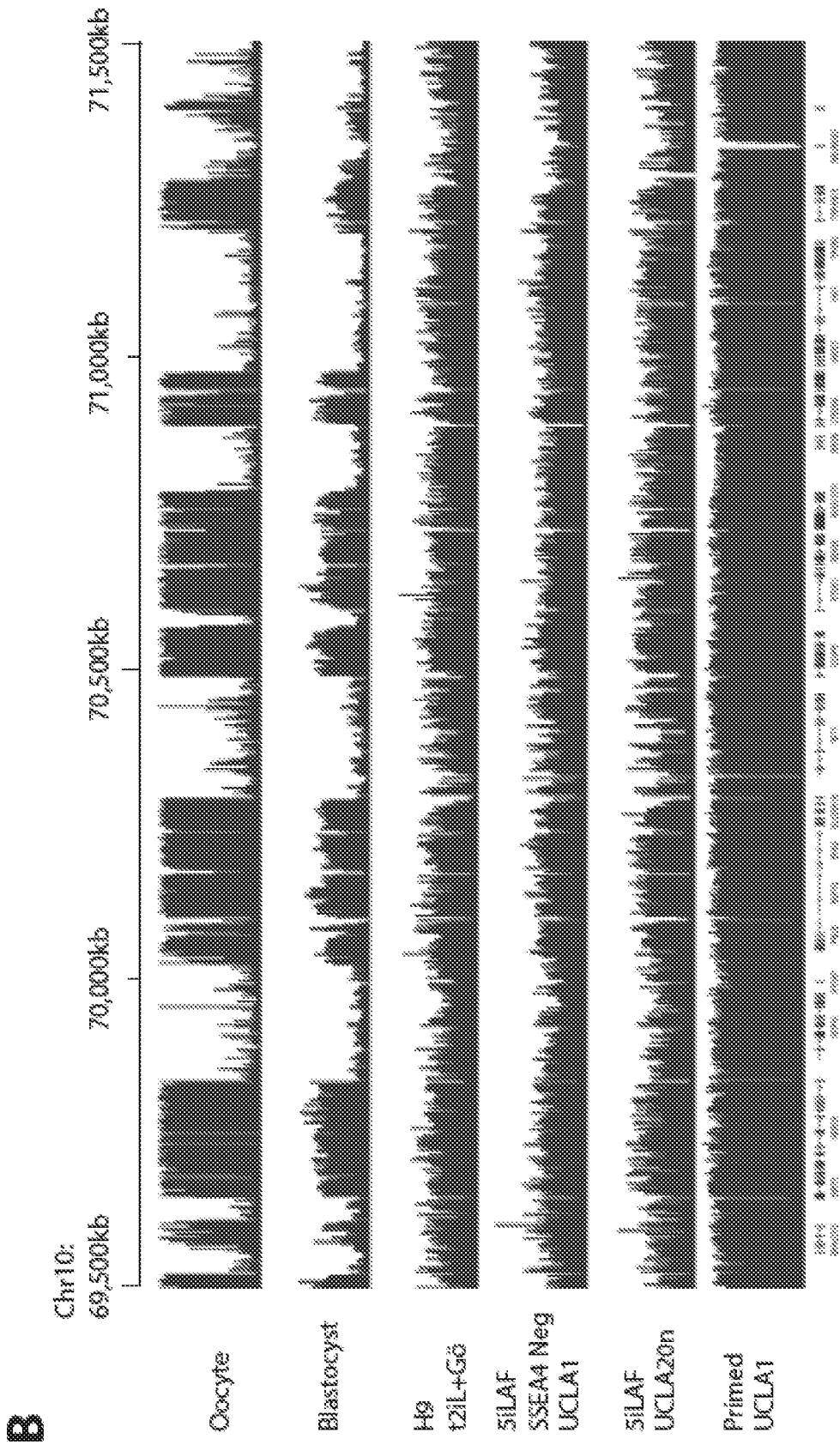


FIG. 2B

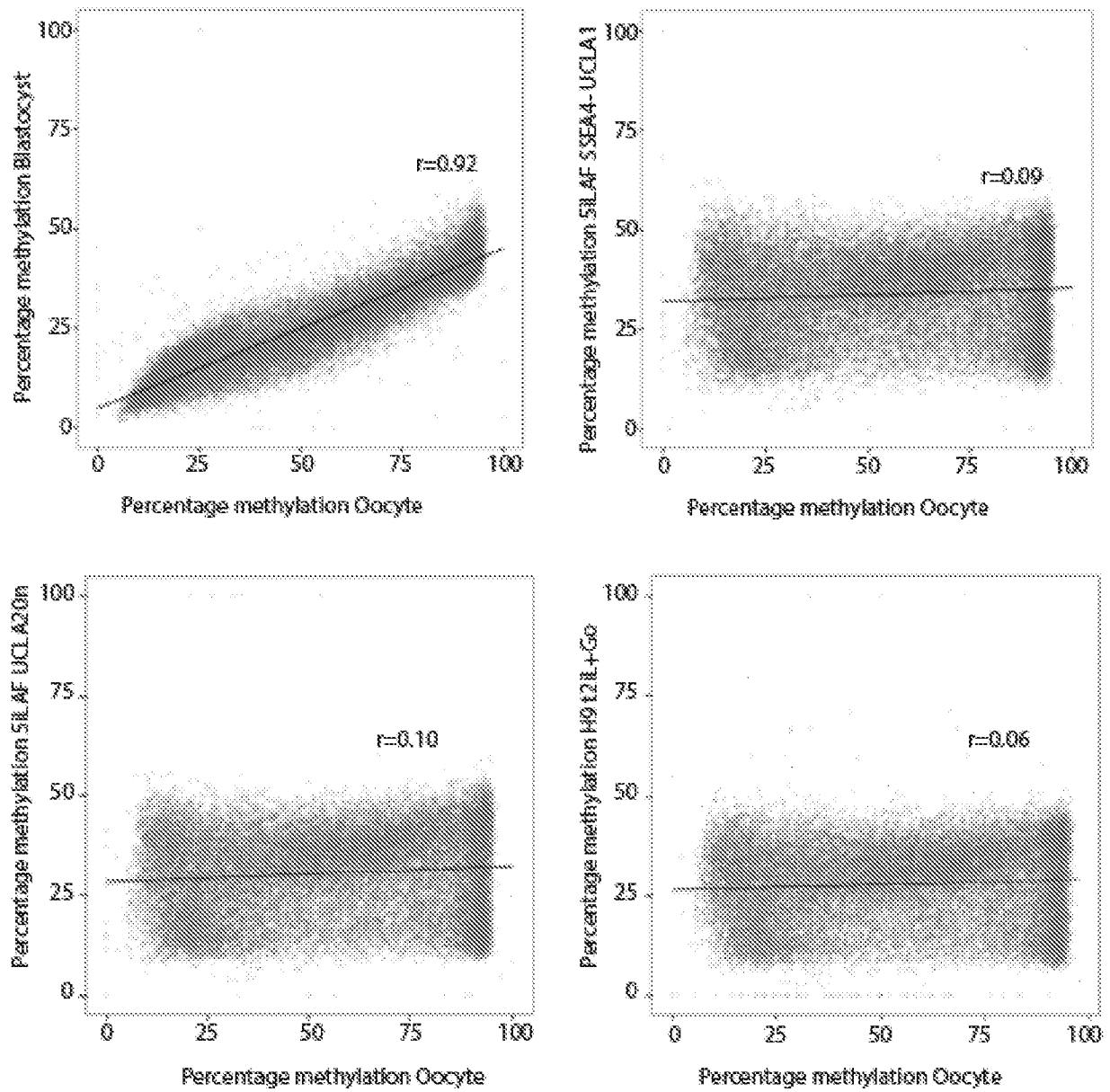


FIG. 2C

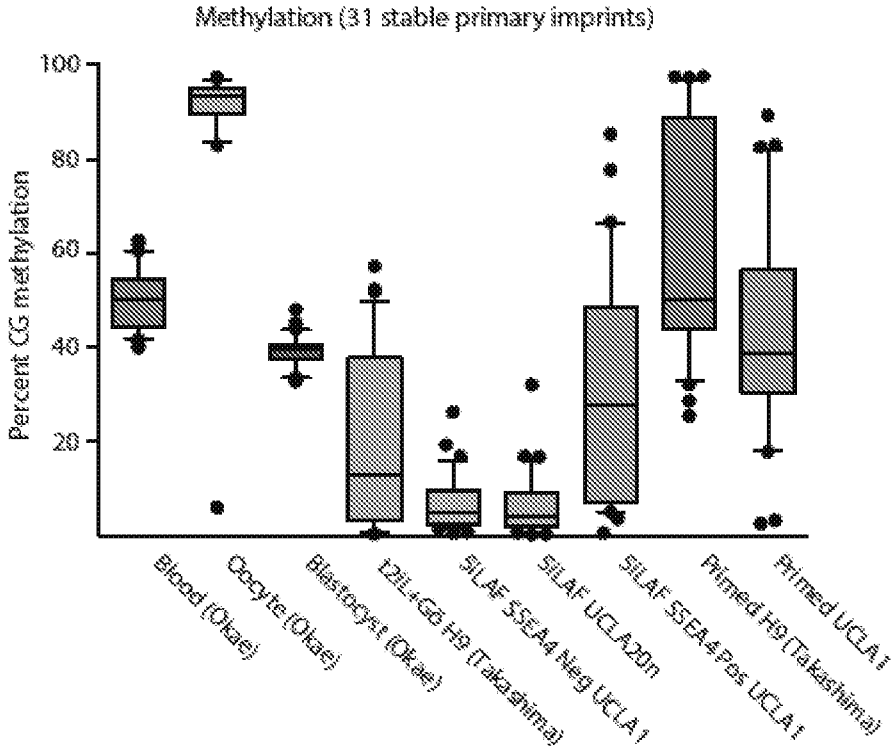


FIG. 2D

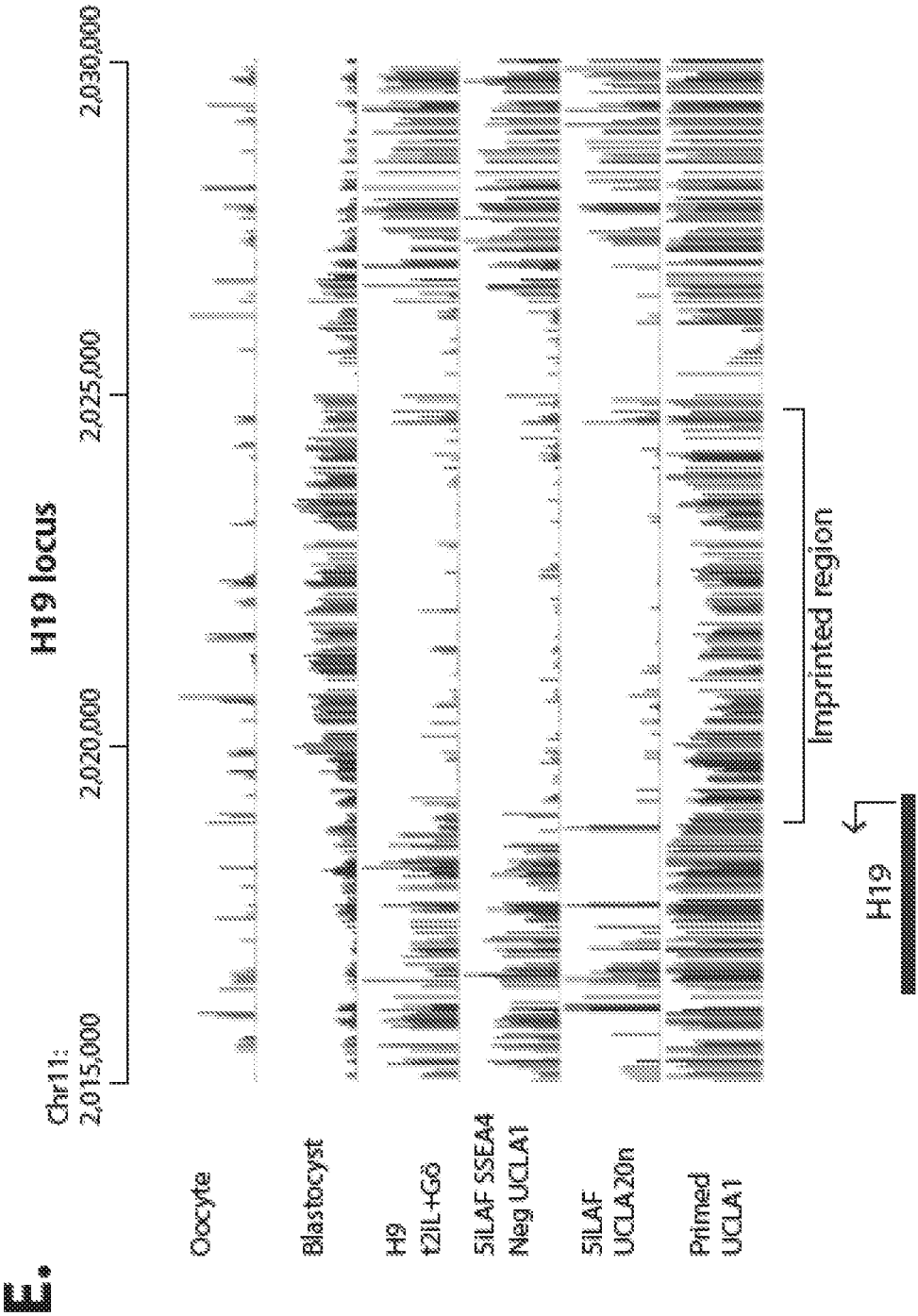


FIG 2E

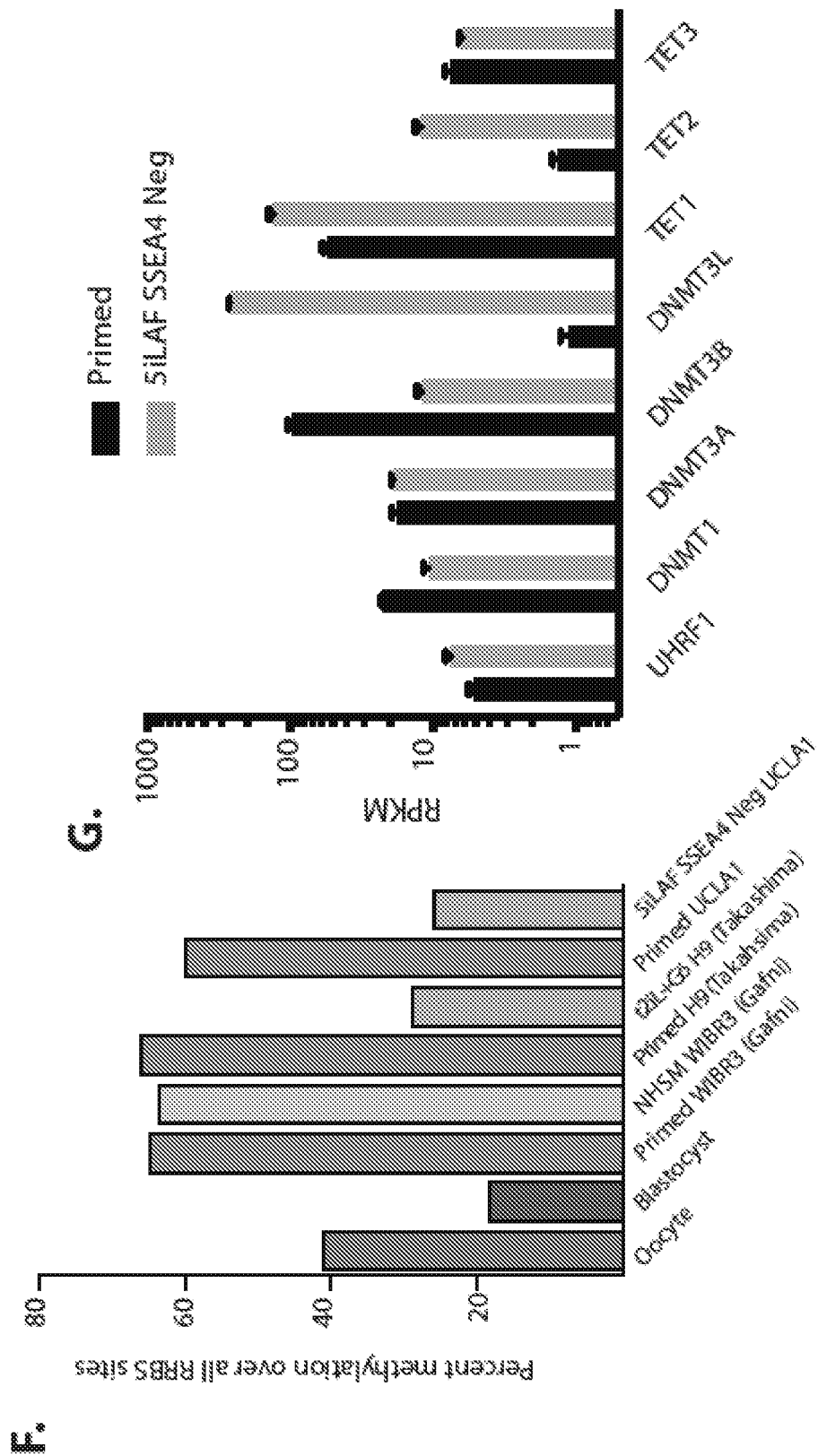


FIG. 2F-G

12 / 24

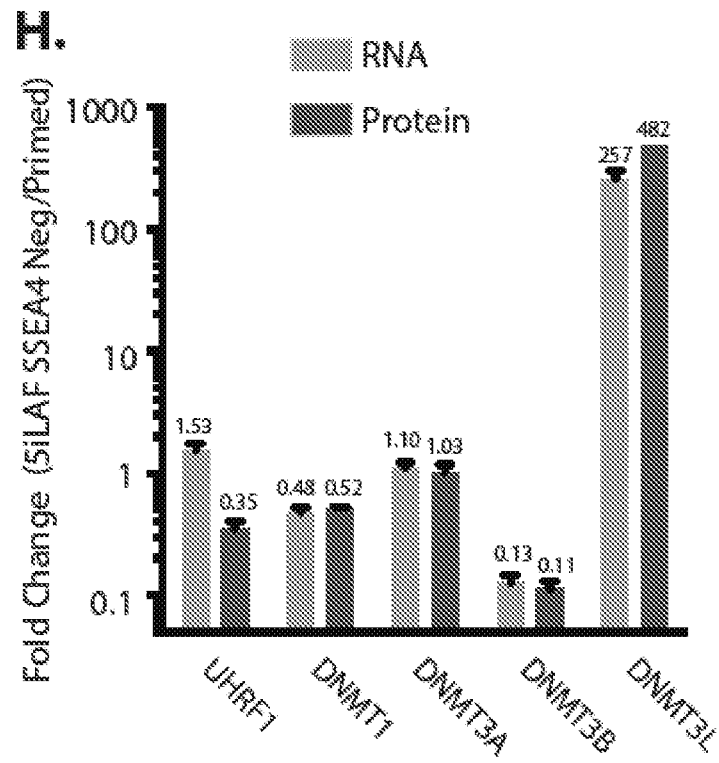


FIG. 2H

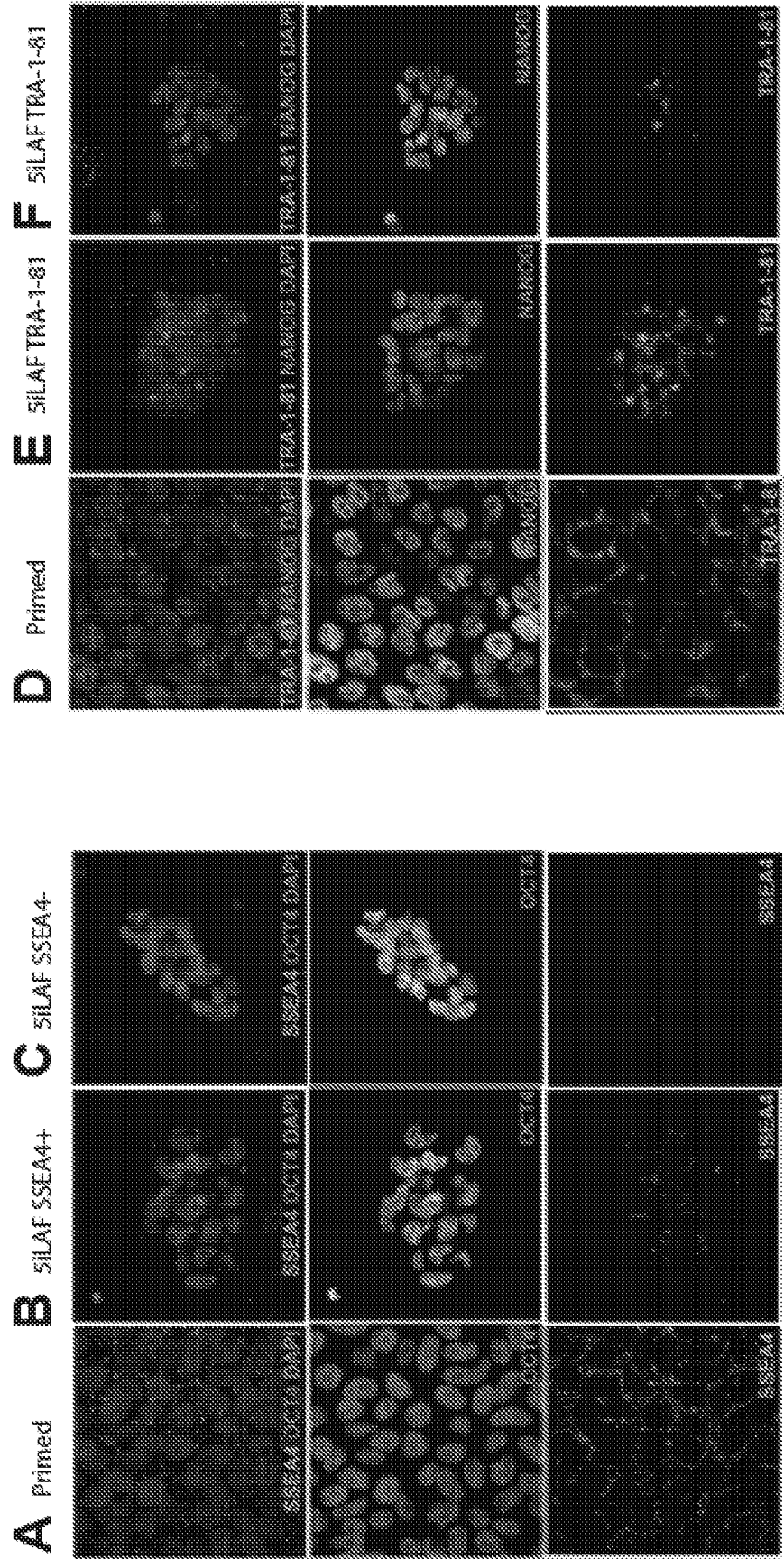


FIG. 3A-3F

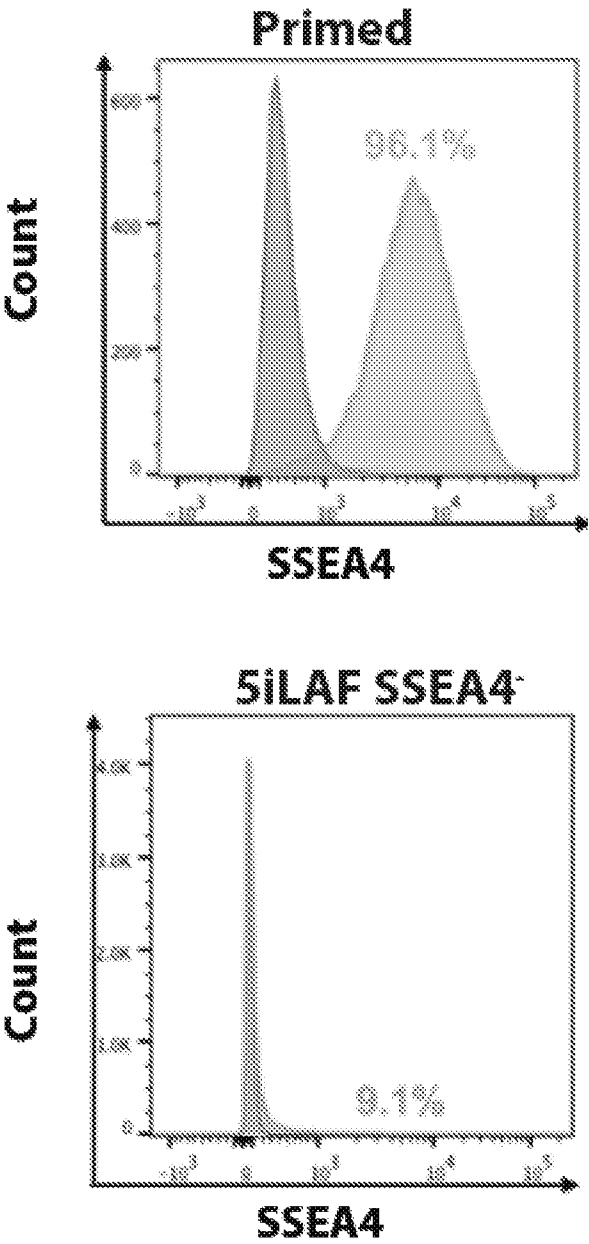


FIG. 3G

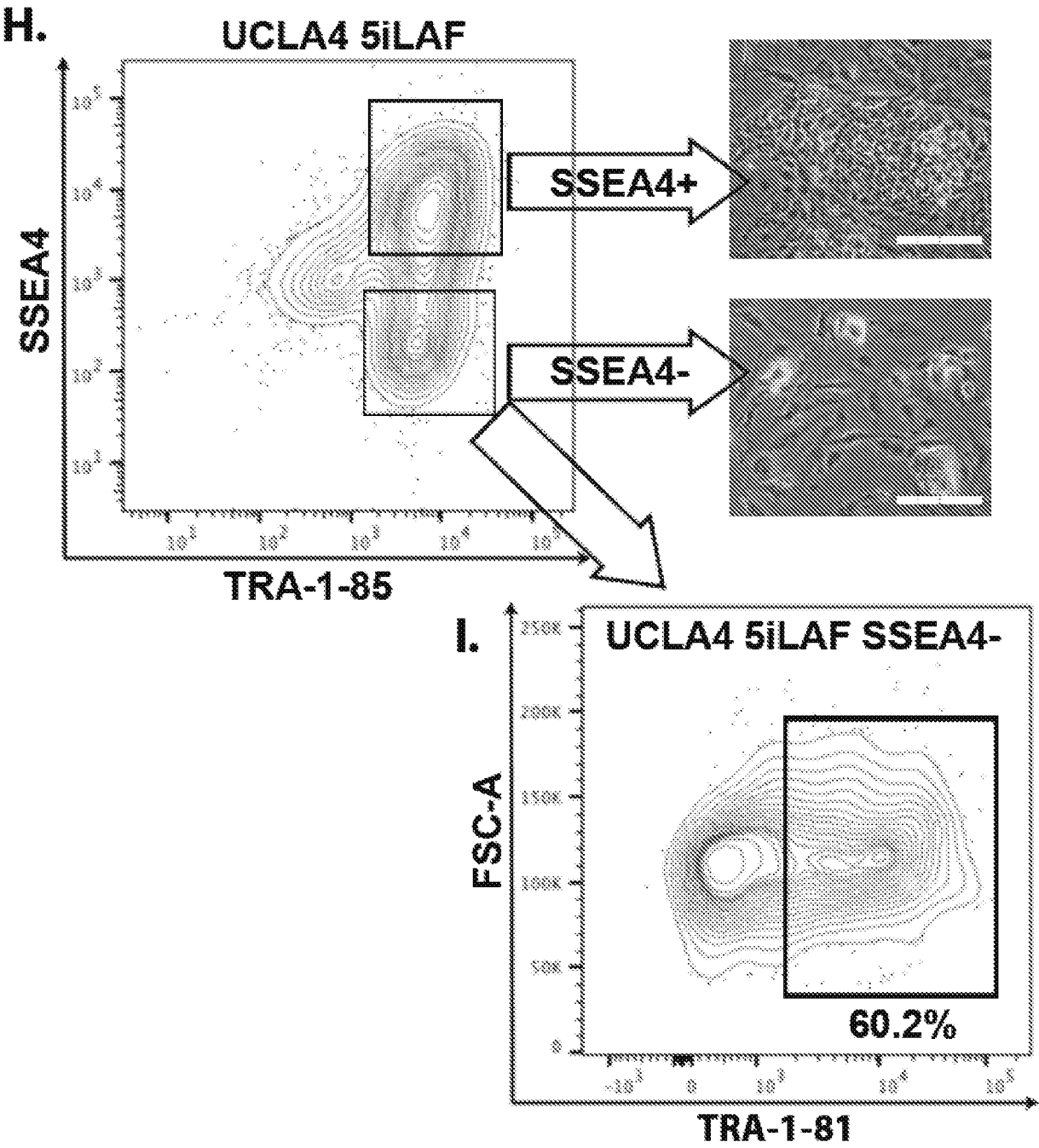


FIG. 3H-I

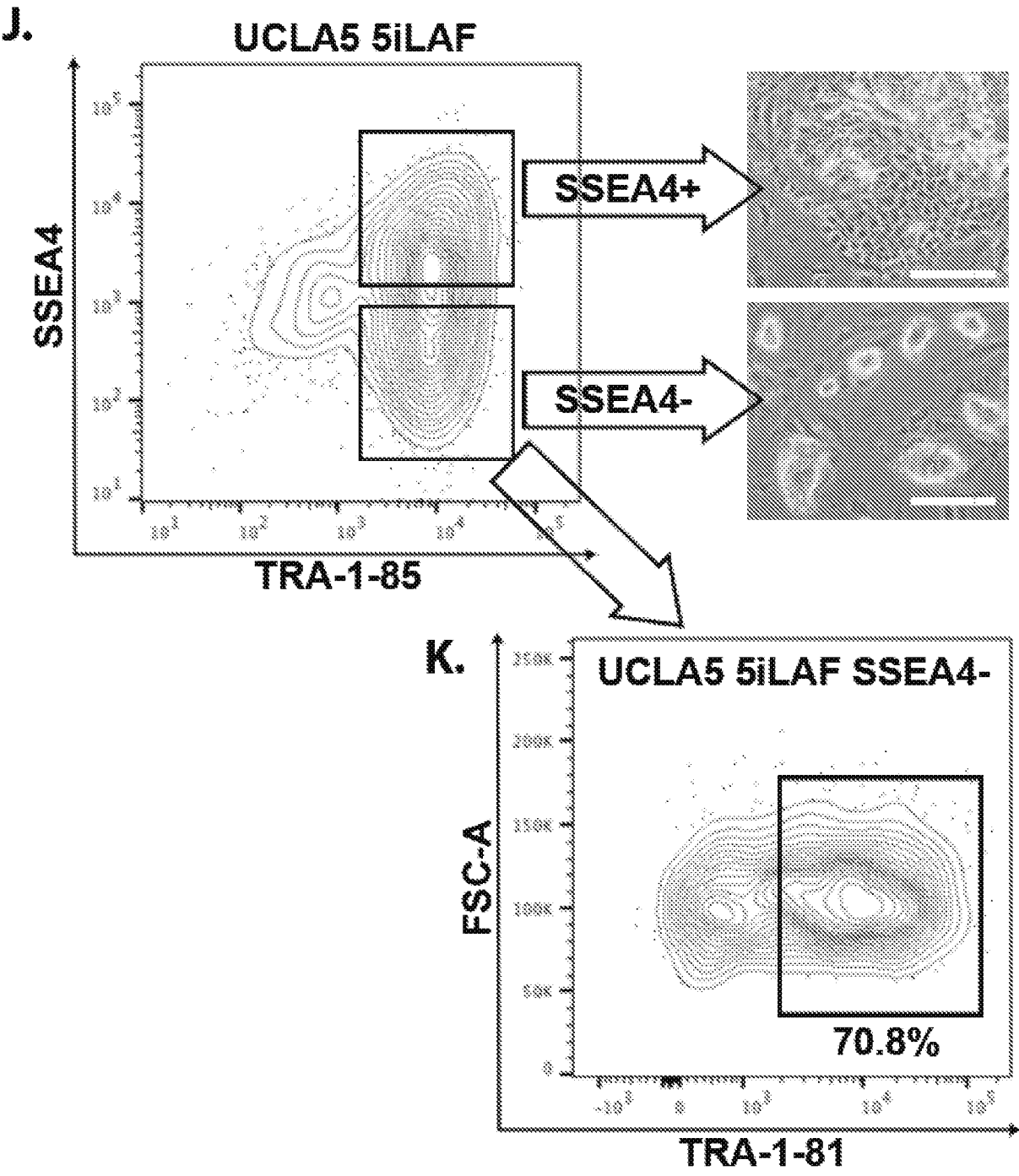


FIG. 3J-K

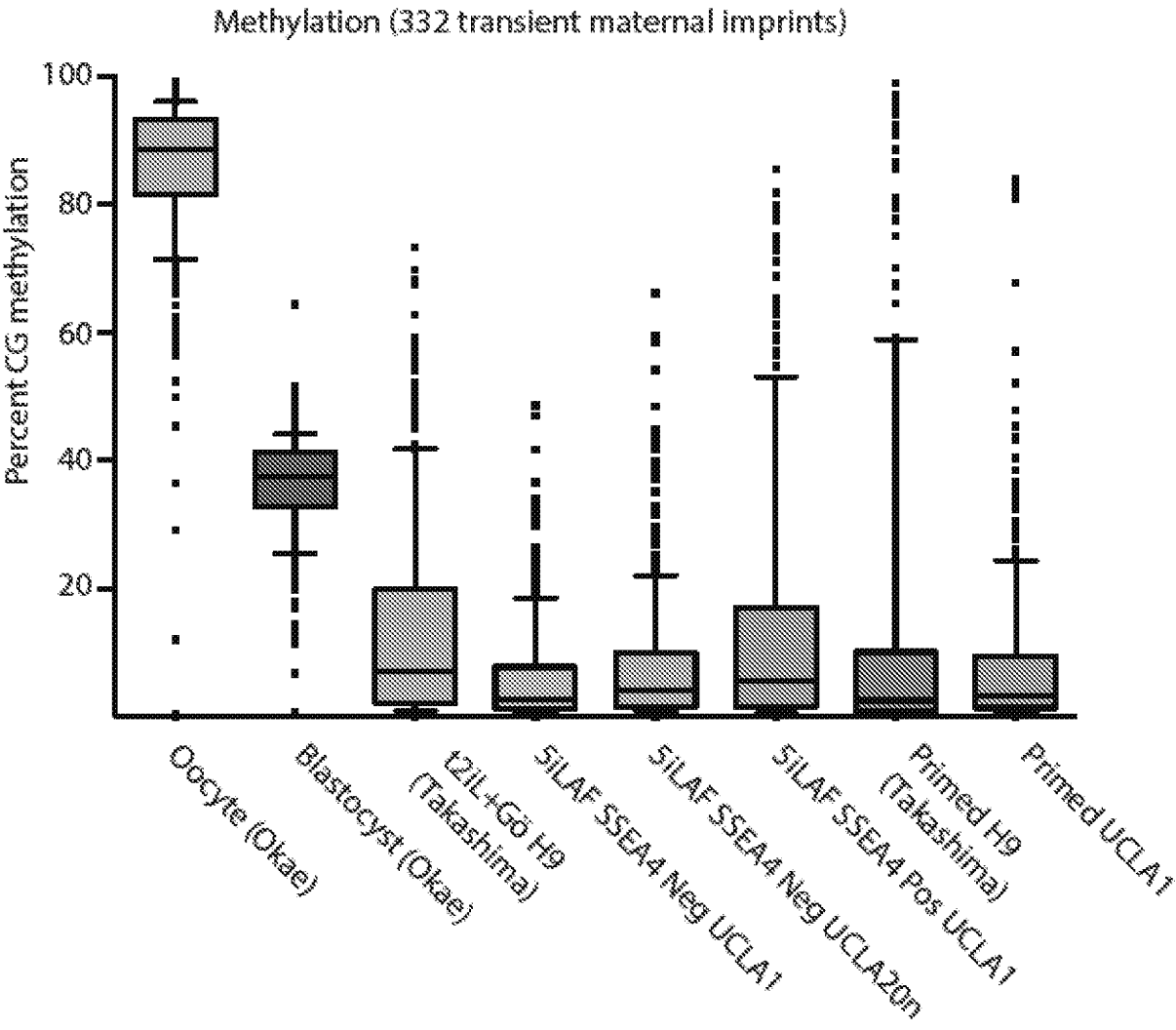


FIG. 4A

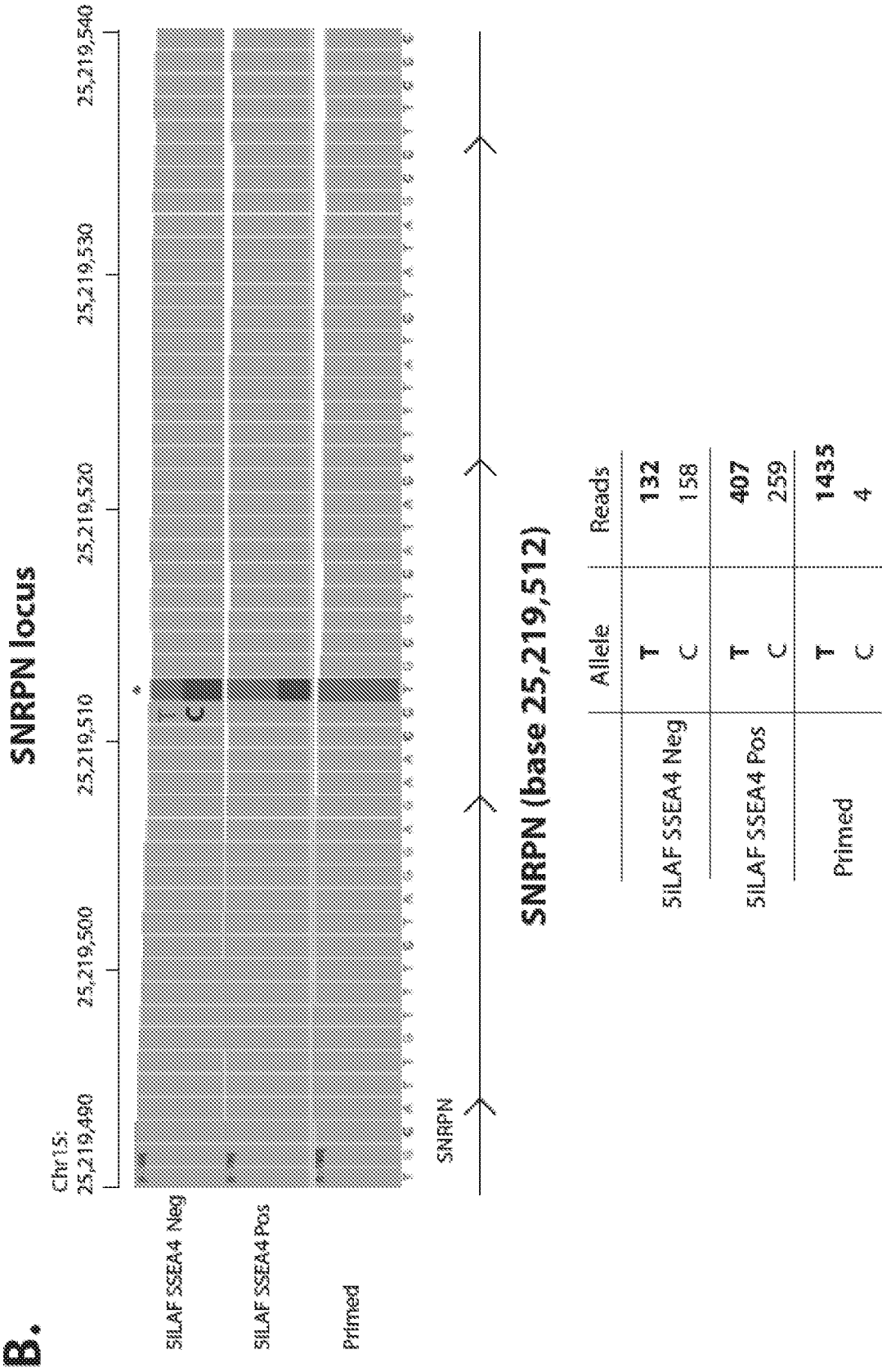


FIG. 4B

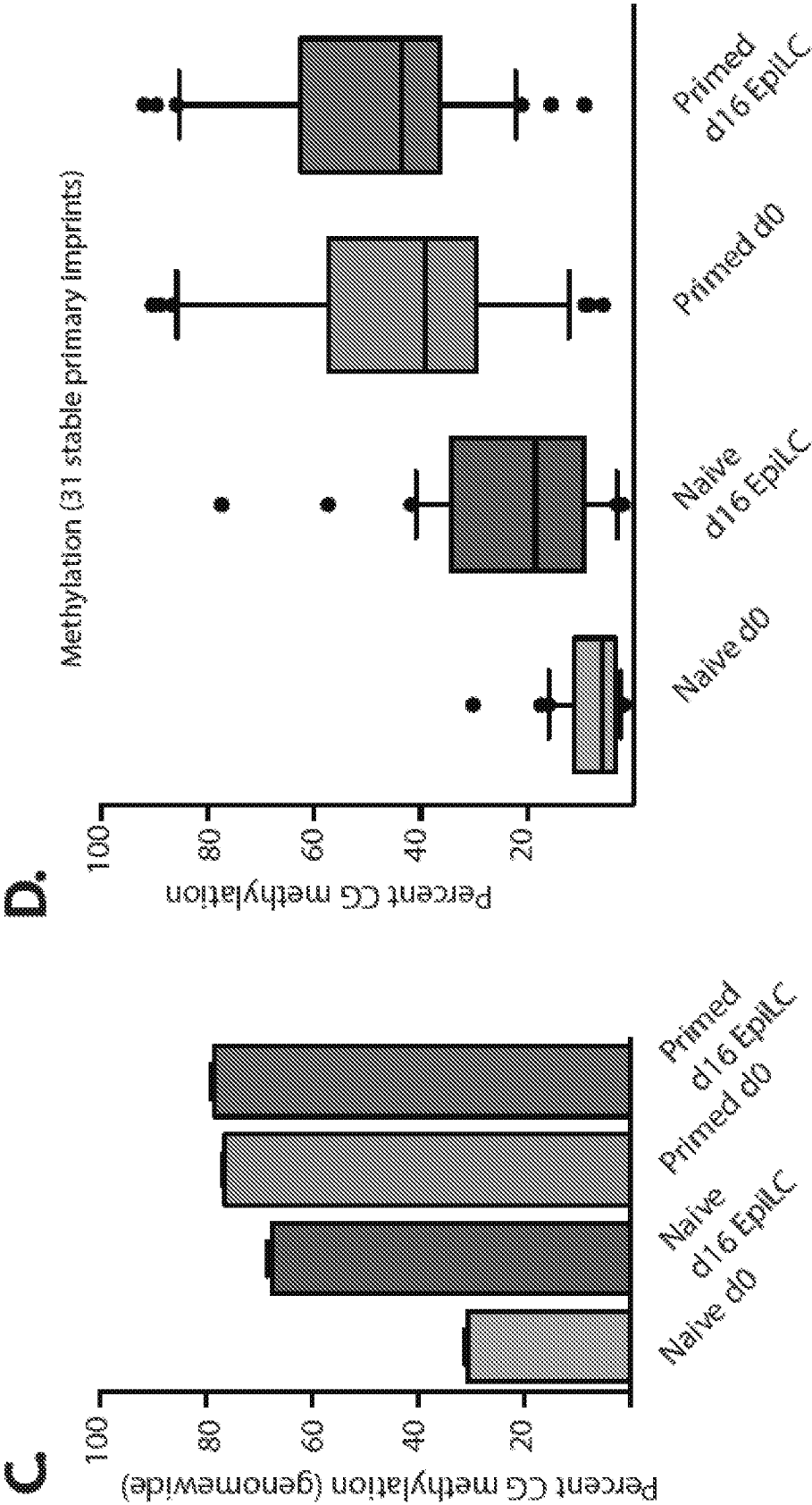


FIG. 4C-D

E.

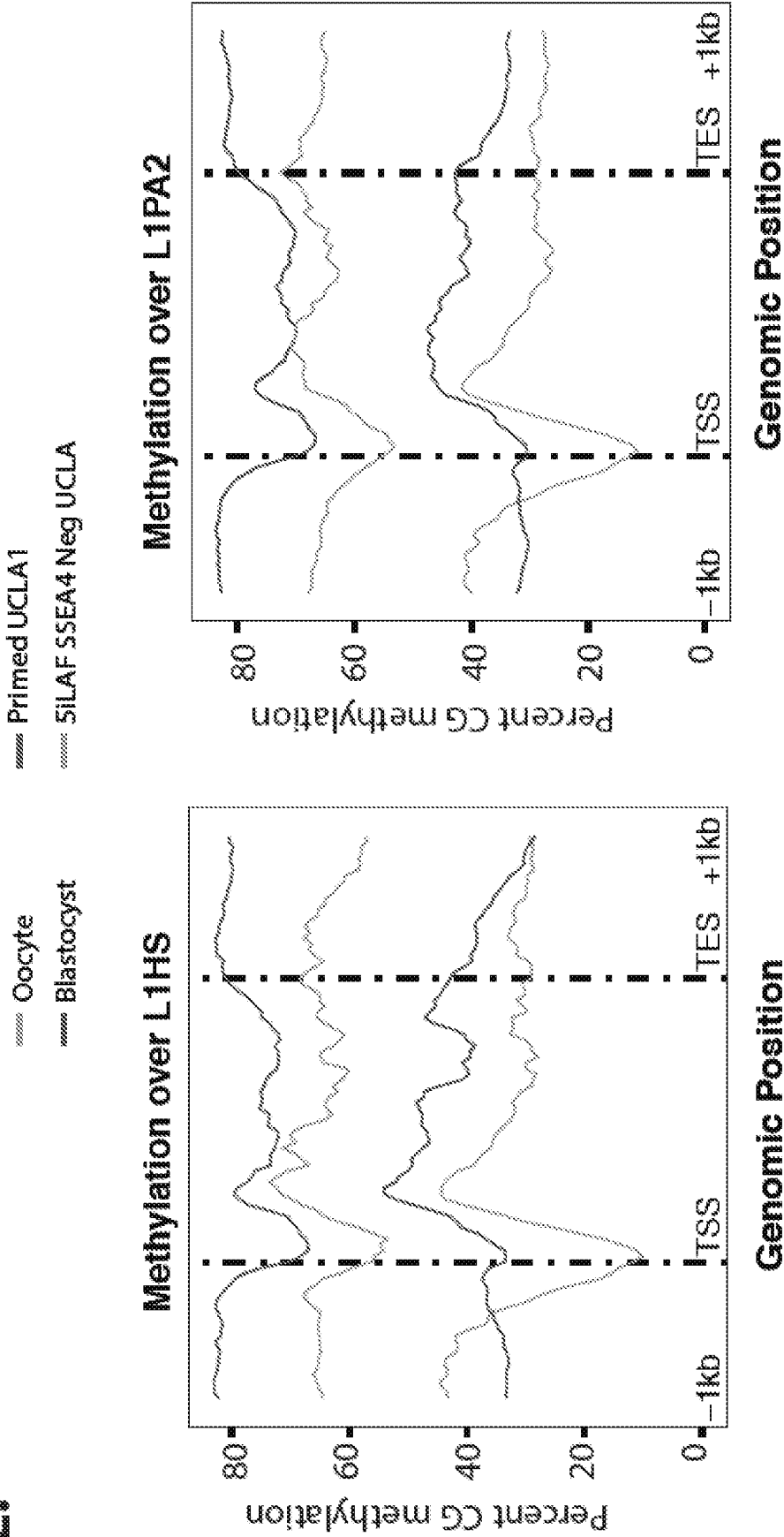


FIG. 4E

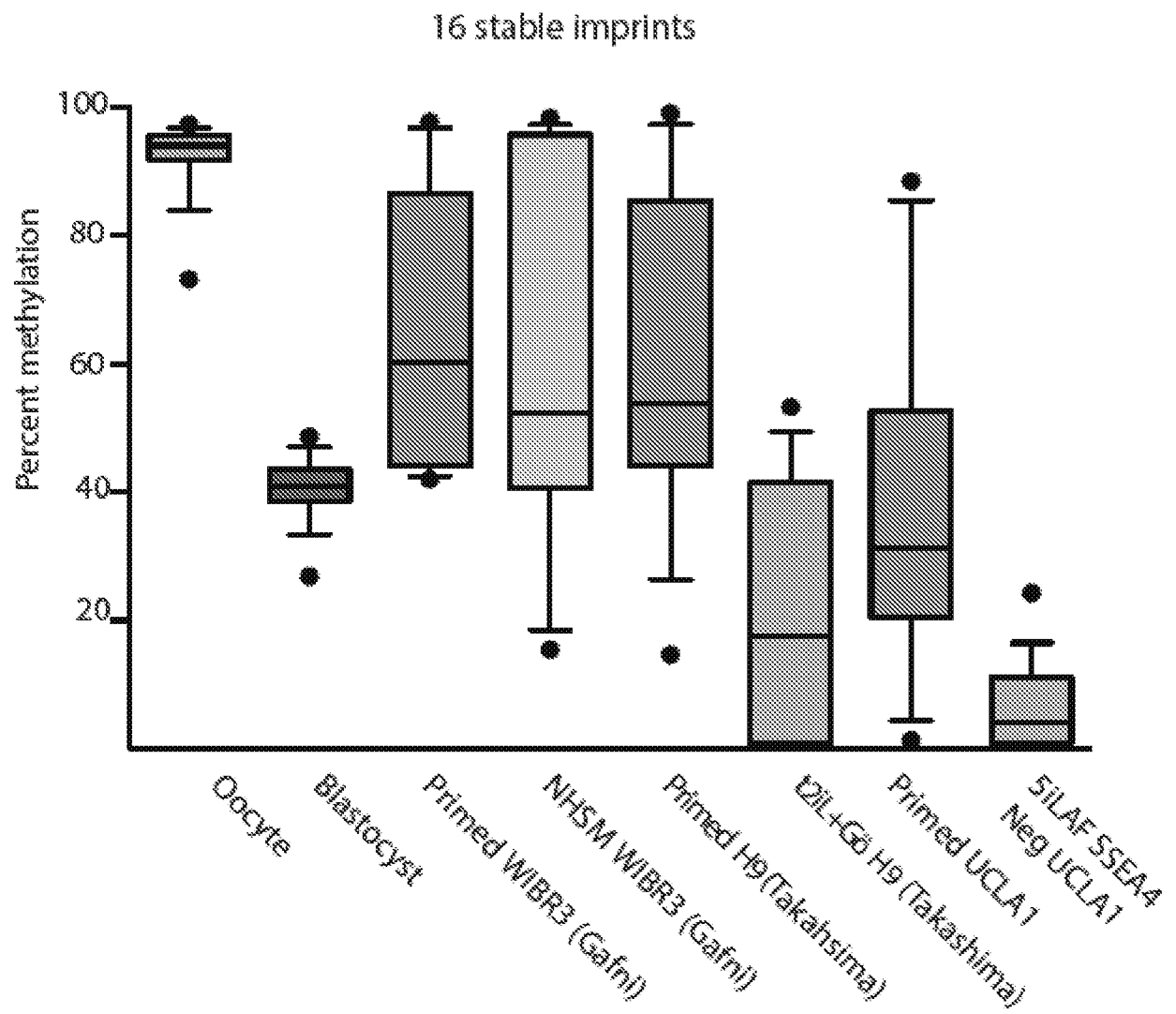
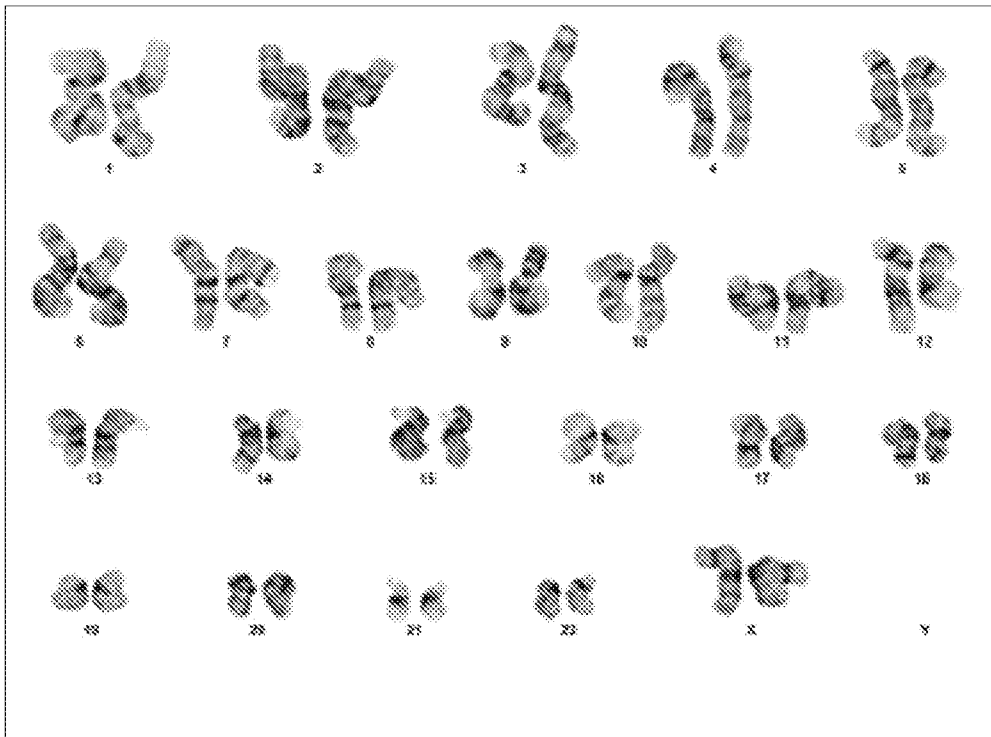


FIG. 4F

5iLAF Passage 13 (18/20 cells karyotypically normal)



5iLAF Passage 24 (2/20 cells karyotypically normal)

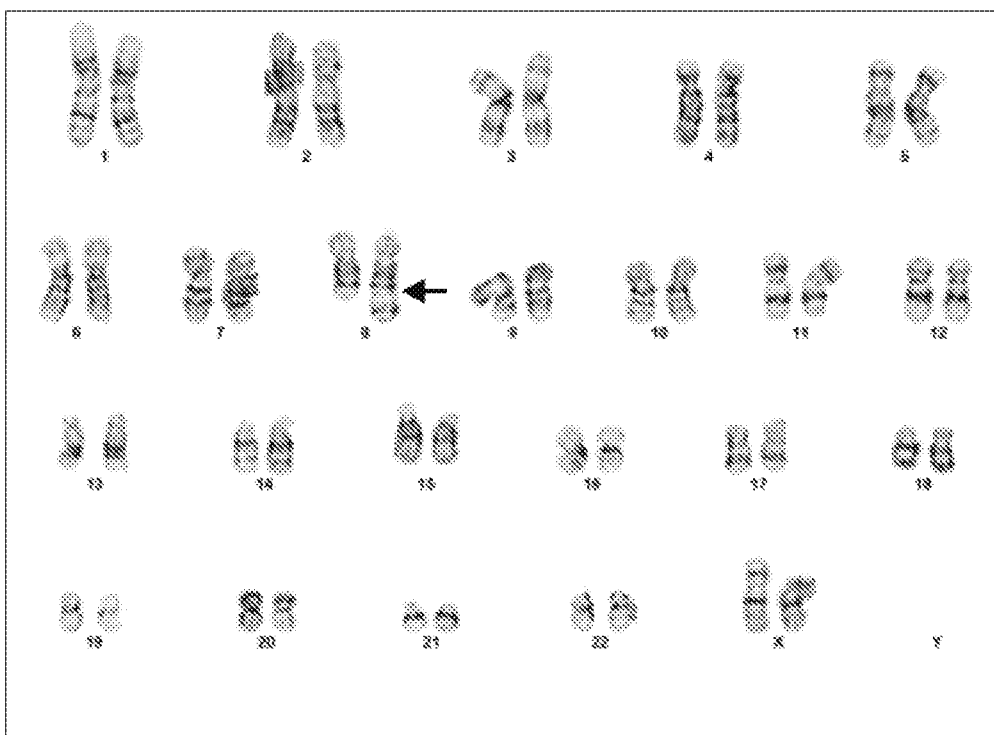


FIG. 4G

Passage 14 UCLA20n

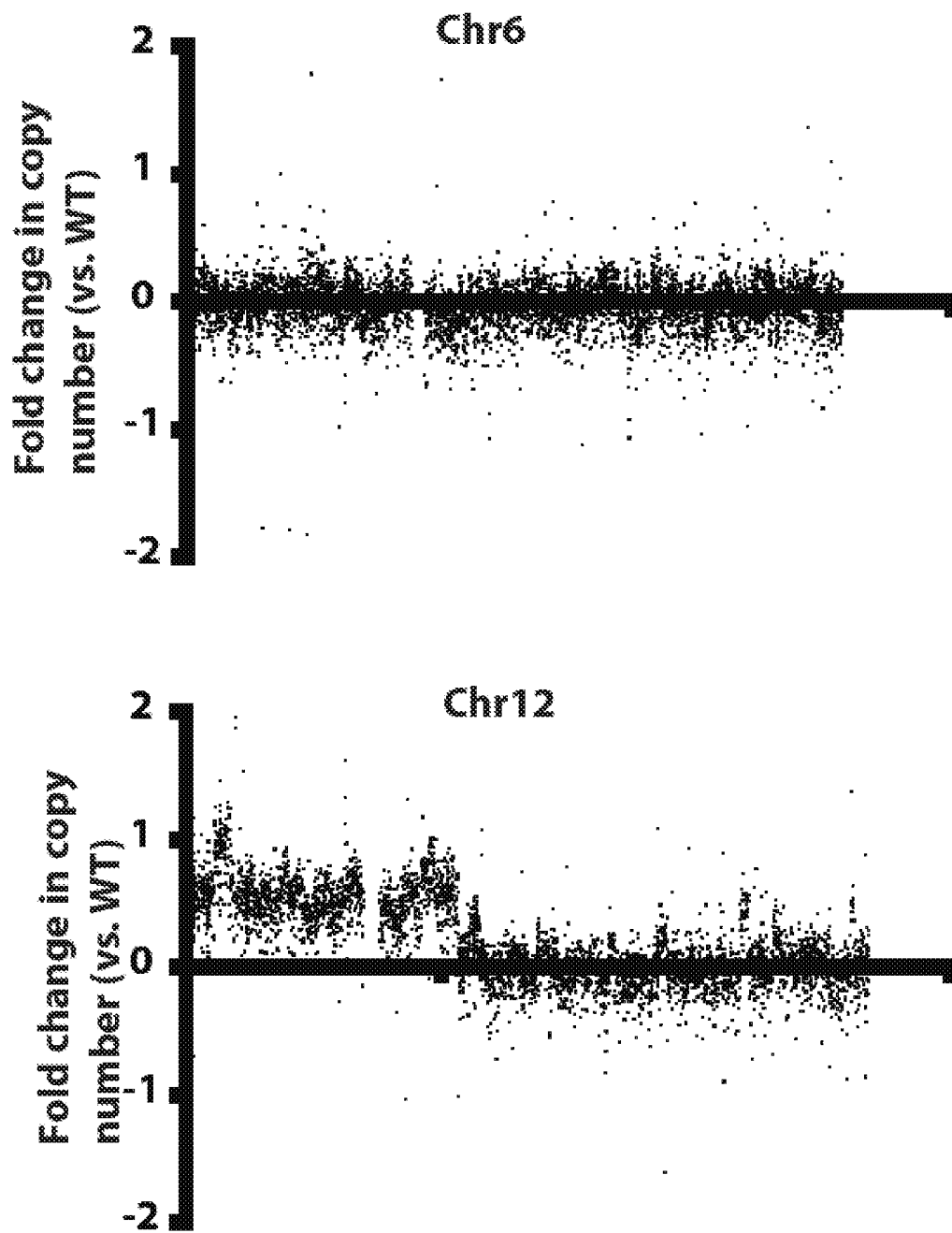


FIG. 4H

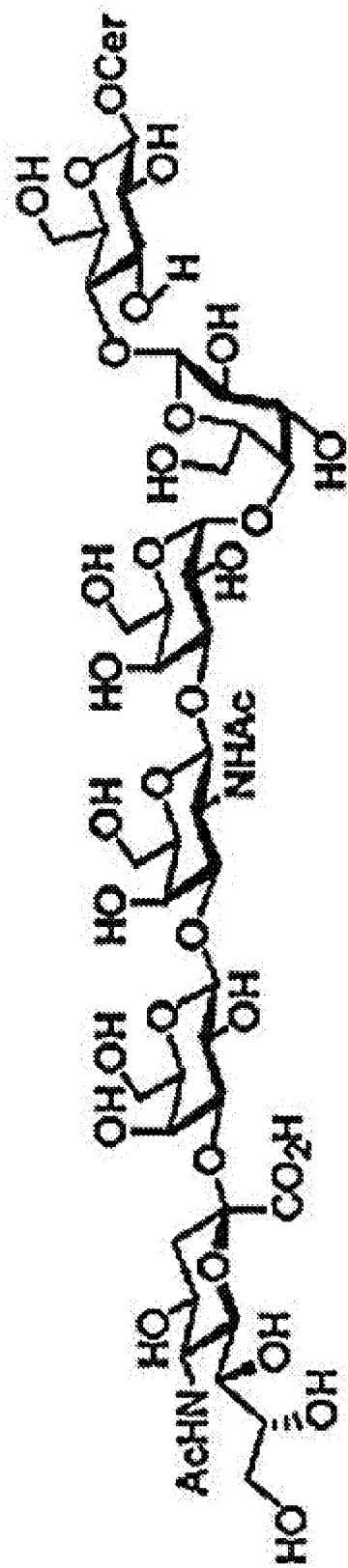


FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 17/50613

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 5/02, C12N 5/071, C12N 5/0735, C12N 5/074, C12Q 1/24, G01N 33/53 (2017.01)

CPC - C12N 2502/03, C12N 2502/02, C12N 5/0606, C12N 5/060, G01N 33/5073, G01N 33/53, C12N 2502/45

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

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

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2014/0220681 A1 (VALAMEHR et al.) 7 August 2014 (07.08.2014) para [0011], [0012], [0084], [0087], [0089], [0153], [0154], [0183], [0218], [0237], [0242], [0243], [0255]	1-5, 17-19, 23-26, 38-40, 46-48, 50-56
Y	WO 2014/072960 A2 (NATIONAL CENTRE FOR BIOLOGICAL SCIENCES) 15 May 2014 (15.05.2014) p 6, ln 24-30; p 6, ln 31-32 to p 7, ln 1-18; p 25, ln 10-30	1-5, 17-19, 23-26, 38-40, 46-48, 50-56

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


* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

22 April 2017

Date of mailing of the international search report

18 MAY 2017

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

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Lee W. Young

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 17/50613

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

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

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

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

3. ☒ Claims Nos.: 6-16, 20-22, 27-37, 41-45, 49, and 57
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

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

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.