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DESCRIPTION

Description

FIELD OF INVENTION

[0001] The invention relates to cells or a cell population that express markers characteristic of intestinal midgut endoderm, as set out in the appended set of claims.

BACKGROUND

[0002] Advances in the knowledge of incretin hormone mechanism of action coupled with advancements in the understanding of intestinal differentiation, both at the stem cell and endocrine cell stages, have led to interest in developing sources of incretin hormone producing cells, appropriate for engraftment. One approach is the generation of functional enteroendocrine L- or K-cells from pluripotent stem cells, such as human embryonic stem cells ("hESC") or induced pluripotent stem cells ("iPS").

[0003] The production/secretion of glucagon-like peptide 1 (GLP-1) from intestinal L-cells or glucose-dependent insulinotropic polypeptide (GIP) from intestinal K-cells has beneficial effects for the treatment of diabetes mellitus. Incretin hormones have systemic effects beneficial for the treatment of diabetes mellitus (Type 1 and Type 2) (Unger, J., Curr Diab Rep., 2013; 13(5):663-668). Benefits may include augmentation of many aspects of beta (β) cell function and number, suppression of glucagon secretion, increases in the insulin sensitivity of peripheral metabolic tissues, reduction of hepatic gluconeogenesis, and reduction of appetite. Two classes of incretin-based therapeutic agents have been identified for the treatment of diabetes mellitus (GLP-1 receptor agonists and dipeptidyl peptidase 4 (DPP-4) inhibitors). However, there is currently no incretin-based cell therapy option that would encompass an endogenous and cellular barometer for improved and efficient GLP1-based diabetes treatment. Furthermore, current incretin-based therapies are not regulated by circulating blood glucose levels and thus provide non-physiologically regulated GLP production.

[0004] In vertebrate embryonic development, a pluripotent cell gives rise to a group of cells comprising three germ layers (ectoderm, mesoderm, and endoderm) in a process known as gastrulation. The mesenchyme tissue is derived from the mesoderm, and is marked by the genes heart and neural crest derivatives expressed 1 (HAND 1), and forkhead box F 1 (FOXF 1), among others. Tissues such as, thyroid, thymus, pancreas, gut and liver, will develop from the endoderm, via an intermediate stage. The intermediate stage in this process is the

formation of the definitive endoderm. By the end of gastrulation, the endoderm is partitioned into anterior-posterior domains that can be recognized by the expression of a panel of factors that uniquely mark anterior foregut, posterior foregut, midgut, and hindgut regions of the endoderm.

[0005] The level of expression of specific transcription factors ("TFs") may be used to designate the identity of a tissue, as described in Grapin-Botten et al., Trends Genet, 2000; 16(3): 124-130. FOXA2 marks the entire endoderm along the anterior-posterior axis. During transformation of the definitive endoderm into a primitive gut tube, the gut tube becomes regionalized into broad domains that can be observed at the molecular level by restricted gene expression patterns. The anterior foregut is marked broadly by the high expression of SOX2, and encompasses organ domains such as the thyroid, lung, and esophagus. The midgut (includes the duodenum, ileum, jejunum) and hindgut (includes the colon) are marked by high expression of caudal type homeobox 2 (CDX2). The SOX2-CDX2 boundary occurs within the posterior foregut, within which additional TFs mark specific organ domains. The regionalized pancreas domain within the posterior foregut shows a very high expression of PDX1 and very low expression of CDX2 and SOX2. PTF1A is highly expressed in pancreatic tissue. Low PDX1 expression, together with high CDX2 expression marks the duodenum domain. The intestinal endoderm is patterned by specific homeobox (HOX) genes. For example, HOXC5 is preferentially expressed in midgut endoderm cells. In addition, the expression of HOXA13 and HOXD13 are restricted to hindgut endoderm cells. The ALB gene, or albumin 1 protein, marks the earliest liver progenitors in the posterior foregut endoderm (Zaret et al., Curr Top Dev Biol, 2016; 117:647-669).

[0006] Strides have been made in improving protocols to generate intestinal endoderm cells from human pluripotent stem cells. For example, the following publications (Spence et al., Nature, 2011; 470(7332):105-109; Watson et al., Nature Medicine, 2014; 20(11):1310-1314; and Kauffman et al., Front Pharmacol, 2013; 4(79):1-18) outline differentiation protocols using either fibroblast growth factor (FGF)-4, Wingless-type MMTV integration site family, member 3A (WNT3A), Chiron 99021, or retinoic acid (RA) and FGF7 starting at the definitive endoderm stage, that generate mid-/hindgut spheroids, containing not only a $CDX2^+/FOXA2^+$ endodermal population, but also a significant mesenchymal $CDX2^+$ cell population. The process of differentiating enteroendocrine cells from these hESC-derived mid-/hindgut spheroids is very inefficient, requiring a long time period, and is directed non-discriminately towards the generation of all intestinal cell types of the intervillus and villus regions. A need still exists for technology to generate intestinal midgut endoderm cells, without substantial contaminating mesenchyme, so as to be able to produce with high efficiency intestinal enteroendocrine cells for cell therapeutics.

SUMMARY OF THE INVENTION

[0007] The present invention relates to the embodiments as characterized in the claims and

described in the following. Accordingly, the invention relates to a population of human cells in culture medium, wherein greater than 50% of the cells express markers characteristic of intestinal midgut endoderm, wherein the cells expressing markers characteristic of intestinal midgut endoderm express CDX2, FOXA2, SOX9, PDX1, KLF5 and HOXC5, and do not express a transcription factor selected from SOX2, ALB, PTF1A, HOXA13 and LGR5. Further described are cells, cell populations and methods of generating the cells by differentiating human pluripotent stem cells. In particular, described are methods of directed differentiation of human pluripotent stem cells, to generate intestinal midgut endoderm cells, more particularly an endodermal monolayer of intestinal midgut endoderm cells.

[0008] Further disclosed is a method of producing a population of intestinal midgut endoderm cells comprising culturing human pluripotent stem cells in culture media. The method may comprise inducing differentiation of human pluripotent stem cells to intestinal midgut endoderm cells. A population of intestinal midgut endoderm cells may be produced. Furthermore, a population of substantially intestinal midgut endoderm cells may be produced. The intestinal midgut endoderm cells may form and may be stable as a monolayer in culture. Greater than 50% of the differentiated cells express markers characteristic of intestinal midgut endoderm, preferably greater than 60% of the differentiated cells express markers characteristic of intestinal midgut endoderm, more preferably greater than 70%, greater than 80%, and greater than 90% express markers characteristic of intestinal midgut endoderm. Differentiated cells expressing markers characteristic of intestinal midgut endoderm may be intestinal midgut endoderm cells. The intestinal midgut endoderm cells may express CDX2 and FOXA2. The intestinal midgut endoderm cells may express transcription factors selected from SOX9, PDX1, KLF5 and HOXC5. The intestinal midgut endoderm cells may not express transcription factors selected from SOX2, ALB, PTF1A, HOXA13 and LGR5.

[0009] Human pluripotent stem cells may be differentiated to intestinal midgut endoderm cells by steps including: a) culturing the human pluripotent stem cells in a first culture media containing GDF-8 and a GSK3β inhibitor, such as MCX compound, to induce differentiation into definitive endoderm cells; b) culturing the definitive endoderm cells in a second culture media containing ascorbic acid and FGF7 to induce differentiation into primitive gut tube cells; and c) culturing the primitive gut tube cells in a third culture media containing retinoic acid and BMP2 or BMP4 in acidic conditions to induce differentiation into intestinal midgut endoderm cells. In particular, acidic conditions may be culture with BLAR medium. The pH of the acidic culture can range from 6.8 to 7.2. The intestinal midgut endoderm cells may form a monolayer in culture. The monolayer of intestinal midgut endoderm cells may be maintained in culture.

[0010] Further disclosed for reference only is a method of treating a patient suffering from or at risk of developing diabetes comprising differentiating human pluripotent stem cells to intestinal midgut endoderm cells, and administering the differentiated intestinal midgut endoderm cells in a patient with diabetes. Diabetes may be Type 1 or Type 2. As disclosed for reference only, administering the cells may be via implantation, injection or otherwise administration directly or indirectly to the site of treatment. As also only disclosed for reference, the intestinal midgut endoderm cells may be implanted in the body, such as in subcutaneous

space, omentum, liver, kidney, etc. Further disclosed for reference only is the encapsulated delivery of the cells including encapsulation of macro- or micro-encapsulation devices.

[0011] Further disclosed is a method of producing intestinal midgut endoderm cells comprising inducing differentiation of definitive endoderm cells in culture to primitive gut tube cells. The definitive endoderm cells may be cultured in culture media containing ascorbic acid and FGF7. The primitive gut tube cells may be cultured in culture media containing retinoic acid and BMP2 or BMP4. The primitive gut tube cells are differentiated to intestinal midgut endoderm cells. Primitive gut tube cells may be differentiated to intestinal midgut endoderm cells in acidic conditions (acidic culture media). Acidic conditions may be culture with BLAR medium. The pH of the acidic culture can range from 6.8 to 7.2. The intestinal midgut endoderm cells may form and maintain a monolayer in culture.

[0012] In the disclosure discussed above, human pluripotent stem cells are human embryonic stem cells or induced pluripotent stem cells. In the disclosure above, the intestinal midgut endoderm cells express CDX2 and FOXA2. The intestinal midgut endoderm cells express transcription factors selected from SOX9, PDX1, KLF5 and HOXC5. The intestinal midgut endoderm cells may not express transcription factors selected from SOX2, ALB, PTF1A, HOXA13 and LGR5. The intestinal midgut endoderm cells may express CDX2, FOXA2, SOX9, PDX1, KLF5 and HOXC5. The intestinal midgut endoderm cells may not express SOX2, ALB, PTF1A, HOXA13 and LGR5. Greater than 50% of the differentiated cells express markers characteristic of intestinal midgut endoderm, preferably greater than 60% of the differentiated cells express markers characteristic of intestinal midgut endoderm, more preferably greater than 70%, greater than 80%, and greater than 90% express markers characteristic of intestinal midgut endoderm. Differentiated cells expressing markers characteristic of intestinal midgut endoderm may be intestinal midgut endoderm cells. The intestinal midgut endoderm cells may express CDX2 and FOXA2. The intestinal midgut endoderm cells may express transcription factors selected from SOX9, PDX1, KLF5 and HOXC5. The intestinal midgut endoderm cells may not express transcription factors selected from SOX2, ALB, PTF1A, HOXA13 and LGR5. It is possible that intestinal midgut endoderm cells do not express HAND1.

[0013] In the disclosure discussed above, the population of intestinal midgut endoderm cells may be substantially intestinal midgut endoderm cells. The population of intestinal midgut endoderm cells may comprise greater than 70% intestinal midgut endoderm cells, preferably greater than 80%, greater than 90%, and greater than 95% of intestinal midgut endoderm cells. The population of intestinal midgut endoderm cells may comprise less than 20% mesenchymal cells, preferably less than 15%, more preferably less than 10%, less than 5%, less than 2%, less than 1%, less than 0.5%. The intestinal midgut endoderm cells may lack expression of HAND1.

[0014] In the disclosure described above, differentiation may be induced *in vitro*. As disclosed for reference only, intestinal midgut endoderm cells may differentiate further *in vivo*. As disclosed for reference only, the intestinal midgut endoderm cells may further differentiate into enteroendocrine cells *in vivo*. The enteroendocrine cells express or secrete incretin hormones.

The incretin hormones may be GLP1 and GIP.

[0015] Intestinal midgut endoderm cells may serve as starting material for the identification of small molecules that promote at high efficiency the *in vitro* differentiation of intestinal midgut endoderm cells into, first, enteroendocrine precursors, and ultimately, incretin expressing or secreting enteroendocrine cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016]

FIGS. 1A - 1D depict a differentiation method for intestinal midgut endoderm cells. FIG. 1A is a summary of the differentiation method, including medium components, growth factors and small molecules added to each stage, and key stage-specific markers of the differentiating intestinal midgut endoderm cells (FOXA2, forkhead box A2; CDX2, caudal type homeobox 2; KLF5, Kruppel-like factor 5; SOX9, SRY (sex determining region Y)-box 9; PDX1, pancreatic and duodenal homeobox 1; LO, low expression and protein presence). Compared to the neutral pH noted at S2D2 (7.35±0.04), cells were exposed to slightly acidic conditions in BLAR medium (used interchangeably with "BLAR acidic medium"), during stage 3 (pH; S3D1, 6.98±0.05; S3D2, 7.02±0.04; S3D5, 7.18±0.03) (FIG. 1B), and as a result of lower sodium bicarbonate levels in BLAR medium. FIG. 1C depicts representative phase-contrast images of a S3D5 monolayer *(left),* and human epithelial colon adenocarcinoma cell line ("Caco-2") *(right)*, which was used as a benchmark for characterization of differentiation. A uniform morphology at S3D5 was consistently observed. Characterization of cell number using the Nucleocounter® NC-100 (Chemometec, Alleroed, Denmark, Catalog No. 900-004) shows that one hESC differentiated into 4.56±2.60 S3D5 intestinal midgut endoderm cells (FIG. 1D).

FIGS. 2A - 2D demonstrate the differentiation method, utilizing Bone morphogenic protein-4 (BMP4), generates intestinal midgut endoderm cells in monolayer, comprising both CDX2 and FOXA2 on transcript and protein levels. FIG. 2A *(bottom)* shows that 90.0±5.85 percent of S3D5 cells were co-present for both CDX2 and FOXA2 protein, similar to percentage seen in Caco-2 cells (86.0±6.67). Conversely, definitive endoderm (DE - S1D3) cells were devoid of CDX2 and FOXA2 co-presence (2.3±1.2). Gene expression analysis shows that CDX2 was induced (FIG. 2B), and FOXA2 maintained (FIG. 2C) during Stage 3. FIG. 2D shows that the induction of CDX2 protein levels and CDX2/FOXA2 protein co-presence after the establishment of the FOXA2-positive primitive gut endoderm stage, S2D2 (FIG. 2D-i), progressively increased through S3D2 (FIG. 2D-ii), and at S3D5 (FIG. 2D-iii) reached similar levels as seen in Caco-2 cells (FIG. 2D-iv). CDX2 protein is depicted on the bottom row, and FOXA2 protein is depicted on the top row. Each image was taken using the same parameters to allow for quantitative analysis. Protein expression was assessed by FACS; gene expression was assessed by qPCR.

FIGS. 3A - 3Q demonstrate the induction by S3D5 of transcript and protein levels of additional transcription factors (TF) that constitute robust intestinal midgut endoderm induction; proper

intestinal midgut endoderm was achieved. In addition to CDX2 and FOXA2 co-expression, S3D5 cells also exhibited co-expression of SOX9, PDX1, KLF5, HOXC5 (homeobox C5), but not SOX2 (SRY-box 2), ALB (albumin), PTF1A (pancreas specific transcription factor, 1a), and LGR5 (leucine rich repeat containing G protein coupled receptor 5). The protein presence of all TFs is depicted in separate single channel images. FIG. 3A *(bottom)* demonstrates that 98.7±0.25 percent of cells were co-present for both CDX2 and SOX9 at S3D5. Strong induction of SOX9 gene expression to levels seen in Caco-2 cells (FIG. 3B), and protein presence as assessed by immunofluorescence (IF)-analysis were observed (FIG. 3C). 69.4±14.2 percent of cells were co-positive for both CDX2 and PDX1 (FIG. 3D - *bottom)*. PDX1 gene expression was induced at low levels, when compared to pancreas-biased S4D3 cells (See, e.g., US2014/0242693) (FIG. 3E), and low to absent protein levels was reflected in the IF-analysis (FIG. 3F). Anterior endoderm TF SOX2 was not observed in S3D5 cells, as 1.45±0.15 of S3D5 cells exhibited SOX2 and CDX2 co-presence (FIG. 3G - *bottom;* FIG. 3I), and gene expression was below levels seen in hESC and Caco-2 cells (FIG. 3H). The gene expression of KLF5, essential for proper development of intestinal mid-/hindgut endoderm, was upregulated at S3D5 (FIG. 3J). Protein co-presence of KLF5 within CDX2-positive cells at S3D5 was observed (FIG. 3K). ALB gene expression (FIG. 3L), and protein presence (FIG. 3M) was not observed in S3D5 cells. The gene expression of pancreas lineage allocating TF, PTF1A, was not induced in S3D5 cells, unlike pancreas-biased S4D3 cells (FIG. 3N). The homeobox gene, HOXC5, present in the embryonic midgut endoderm was induced in S3D5 cells (FIG. 3O). FIG. 3P demonstrates that LGR5, a marker of embryonic intestinal endoderm beginning at mid-gestation in the mouse, was not induced in S3D5 cells. FIG. 3Q demonstrates that HOXA13, a marker of the intestinal hindgut endoderm, was not induced in S3D5 cells (FIG. 3P). Gene expression was assessed by qPCR.

FIGS. 4A - 4B characterize the proliferative profile of differentiating S3D5 cells. FIG. 4A depicts Caco-2 cells, where most of CDX2-protein positive cells were in active cell cycle (as indicated by the co-expression with KI67 protein) *(left),* and the proliferative index of the H1-hESC-derived cells (disclosed for reference only) during Stage 3 that decreased over time (S3D2 - *middle;* S3D5 - *right)*. CDX2 *(top* row) and KI67 *(bottom* row) protein levels are depicted as single channel images. The percentage KI67-protein positive cells of total S3D5 cells (total cells are >90% CDX2-positive), assessed by FACS, was 16.8±3.12, in contrast with percentage seen at S1D3 (97.3±1.3), and in Caco-2 cells (99.2±0.2) (FIG. 4B).

FIGS. 5A - 5C demonstrate use of BMP2 as an alternative to BMP4, during Stage 3 to achieve a monolayer of intestinal midgut endoderm cells with CDX2 and FOXA2 protein co-presence. FIG. 5A summarizes the differentiation method, including the medium components, growth factors and small molecules that were added to each stage, and stage-specific markers of the differentiating intestinal midgut endoderm cells (FOXA2, CDX2, KLF5, SOX9, and PDX1^{LO}). Compared to the neutral pH noted at S2D2 (7.35±0.04), cells were exposed to slightly acidic conditions in BLAR medium, during the entirety of stage 3 (pH; S3D1, 6.92; S3D2, 7.01; S3D5, 7.22) (FIG. 5B), and as a result of lower sodium bicarbonate levels in BLAR medium. FIG. 5C depicts representative phase-contrast images of a S3D5 monolayer *(left),* and Caco-2 cells *(right)*; a uniform morphology at S3D5 was observed.

FIGS. 6A - 6U demonstrate generation of proper intestinal midgut endoderm cells in monolayer, each comprising of CDX2, FOXA2, KLF5, SOX9, PDX1^{LO} and HOXC5 on transcript and protein levels. For IF-images, all TF protein levels are depicted as single channel images. FIG. 6A (bottom) shows that 94 percent of S3D5 cells were co-present for both CDX2 and FOXA2 protein, similar to or greater than the percentage seen in Caco-2 cells (86.0±6.67). Gene expression analysis shows CDX2 induced (FIG. 6B), and FOXA2 maintained (FIG. 6C) during Stage 3. FIG. 6D shows CDX2 protein levels and complete CDX2/FOXA2 protein co-presence induced at S3D5, reaching similar levels as seen in Caco-2 cells (FIG. 2D-iv). FIG. 6E (bottom) demonstrates that 99.8 percent of cells were co-present for both CDX2 and SOX9 at S3D5. Strong induction of SOX9 gene expression to levels seen in Caco-2 cells (FIG. 6F), and protein presence as assessed by IF-analysis were observed (FIG. 6G). 45.5 percent of cells were co-positive for both CDX2 and PDX1 (FIG. 6H - bottom). PDX1 gene expression was induced at low levels compared to pancreas-biased S4D3 cells (FIG. 6I); low to absent protein levels was reflected in the IF-analysis (FIG. 6J). Anterior endoderm TF SOX2 was not observed in S3D5 cells, as 0.8 percent of S3D5 cells exhibited SOX2 and CDX2 co-presence (FIG. 6K - bottom; FIG. 6M), and gene expression was below levels seen in hESC and Caco-2 cells (FIG. 6L). Gene expression of KLF5, essential for proper development of intestinal mid-/hindgut endoderm, was upregulated at S3D5 (FIG. 6N). Protein co-presence of KLF5 within CDX2-positive cells was observed at S3D5 (FIG. 6O). ALB gene expression (FIG. 6P), and protein presence (FIG. 6Q) was not observed in S3D5 cells. Gene expression of pancreas lineage allocating TF, PTF1A, was not induced in S3D5 cells, unlike pancreas-biased S4D3 cells (FIG. 6R). The homeobox gene HOXC5, present in the embryonic intestinal midgut endoderm, was induced in S3D5 cells (FIG. 6S). Figure 6T demonstrates that LGR5, a marker of embryonic intestinal endoderm beginning at mid-gestation, was not induced in S3D5 cells. FIG. 6U demonstrates that HOXA13, a marker of the intestinal hindgut endoderm, was not induced in S3D5 cells (FIG. 6U).

FIG. 7 characterizes the proliferative profile of differentiating S3D5 cells. Compared to Caco-2 cells, where most of CDX2-protein positive cells were in active cell cycle (as indicated by the co-expression with KI67 protein) (left), the proliferative index of the H1-hESC-derived cells (disclosed for reference only) during Stage 3 was lower (S3D5 - right). CDX2 (top row) and KI67 (bottom row) protein levels are depicted as single channel images. The percentage KI67-protein positive cells of total S3D5 cells (total cells are >90% CDX2-positive), assessed by FACS, was 14.1 percent, in contrast with percentage seen at S1D3 (97.3±1.3), and in Caco-2 cells (99.2±0.2).

FIGS. 8A-8F demonstrate the induction of a heterogeneous population of CDX2$^+$ cells. FIG. 8A is a summary of the differentiation methods, including medium components, growth factors and small molecules added to each stage, and key stage-specific markers of the differentiating intestinal mid-/hindgut endoderm cells (HAND1). FIG. 8B shows phase-contrast images of H1-hESC cells (disclosed for reference only) (top row, left), post-Stage 1 cells conditioned two days with 500 ng/ml FGF4 and 3µM Chiron99021 (top row, middle), post-Stage 1 cells conditioned two days with 500 ng/ml FGF4 and 500 ng/ml WNT3A (top row, right), a S3D5 monolayer conditioned by RA/BMP4 (bottom row, left), and a S3D5 monolayer conditioned by

RA/BMP2 (*bottom row, right*). The induction of gene expression after two days of conditioning is shown for CDX2 at low levels (FIG. 8C), is maintained for the endoderm marker FOXA2 (FIG. 8D), and is induced for the mesoderm/mesenchyme marker HANOI (FIG. 8F). KLF5 was not induced (FIG. 8E).

DETAILED DESCRIPTION

[0017] It is to be understood that this invention is not limited to particular methods, reagents, compounds, compositions or biological systems, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

[0018] Disclosed herein is the generation of intestinal midgut endoderm cells. The cells were generated using a specific culturing sequence. Accordingly, disclosed is an *in vitro* cell culture for differentiating cells derived from pluripotent stem cells into cells expressing markers characteristic of the intestinal midgut endoderm cell lineage, such as expression of CDX2 and FOXA2. Further disclosed is a method for obtaining and maintaining such cells in a monolayer via an *in vitro* cell culture. The invention is also based on the discovery that the inclusion of retinoic acid and BMP4 or BMP2 or analogues thereof, act to induce CDX2 and maintain FOXA2 protein expression in differentiating cells to facilitate differentiation towards intestinal midgut endoderm cells. CDX2 is not expressed at the protein level at definitive endoderm (Stage 1) or primitive gut tube (Stage 2). Accordingly, further disclosed are methods of differentiating pluripotent stem cells to generate intestinal midgut endoderm cells that express CDX2 and FOXA2.

Definitions

[0019] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred materials and methods are described herein. The following terminology will be used.

[0020] Stem cells are undifferentiated cells defined by the ability of a single cell both to self-renew, and to differentiate to produce progeny cells, including self-renewing progenitors, non-renewing progenitors, and terminally differentiated cells. Stem cells are also characterized by their ability to differentiate *in vitro* into functional cells of various cell lineages from multiple germ layers (endoderm, mesoderm and ectoderm), as well as to give rise to tissues of multiple germ layers following transplantation, and to contribute substantially to most, if not all, tissues following injection into blastocysts.

[0021] Stem cells are classified according to their developmental potential as: (1) totipotent; (2) pluripotent; (3) multipotent; (4) oligopotent; and (5) unipotent. Totipotent cells are able to give rise to all embryonic and extraembryonic cell types. Pluripotent cells are able to give rise to all embryonic cell types. Multipotent cells include those able to give rise to a subset of cell lineages, but all within a particular tissue, organ, or physiological system (for example, hematopoietic stem cells (HSC) can produce progeny that include HSC (self-renewal), blood cell-restricted oligopotent progenitors, and all cell types and elements (e.g., platelets) that are normal components of the blood). Cells that are oligopotent can give rise to a more restricted subset of cell lineages than multipotent stem cells; and cells that are unipotent are able to give rise to a single cell lineage (e.g., spermatogenic stem cells).

[0022] Stem cells are also categorized on the basis of the source from which they may be obtained. An adult stem cell is generally a multipotent undifferentiated cell found in tissue comprising multiple differentiated cell types. The adult stem cell can renew itself. Under normal circumstances, it can also differentiate to yield the specialized cell types of the tissue from which it originated, and possibly other tissue types. Induced pluripotent stem cells (iPS cells) are adult cells that are converted into pluripotent stem cells. (Takahashi et al., Cell, 2006; 126(4):663-676; Takahashi et al., Cell, 2007; 131:1-12). An embryonic stem cell is a pluripotent cell from the inner cell mass of a blastocyst-stage embryo. A fetal stem cell is one that originates from fetal tissues or membranes.

[0023] Embryonic tissue is typically defined as tissue originating from the embryo (which in humans refers to the period from fertilization to about six weeks of development). Fetal tissue refers to tissue originating from the fetus, which in humans refers to the period from about six weeks of development to parturition. Extraembryonic tissue is tissue associated with, but not originating from, the embryo or fetus. Extraembryonic tissues include extraembryonic membranes (chorion, amnion, yolk sac and allantois), umbilical cord and placenta (which itself forms from the chorion and the maternal decidua basalis).

[0024] Differentiation is the process by which an unspecialized ("uncommitted") or less specialized cell acquires the features of a specialized cell, such as an intestinal cell or pancreatic cell, for example. A differentiated cell is one that has taken on a more specialized ("committed") position within the lineage of a cell. The term committed, when applied to the process of differentiation, refers to a cell that has proceeded in the differentiation pathway to a point where, under normal circumstances, it will continue to differentiate into a specific cell type or subset of cell types, and cannot, under normal circumstances, differentiate into a different cell type or revert to a less differentiated cell type. De-differentiation refers to the process by which a cell reverts to a less specialized (or committed) position within the lineage of a cell. As used herein, the lineage of a cell defines the heredity of the cell, i.e. which cells it came from and what cells it can give rise to. The lineage of a cell places the cell within a hereditary scheme of development and differentiation.

[0025] In a broad sense, a progenitor cell is a cell that has the capacity to create progeny that

are more differentiated than itself, and yet retains the capacity to replenish the pool of progenitors. By that definition, stem cells themselves are also progenitor cells, as are the more immediate precursors to terminally differentiated cells. In a narrower sense, a progenitor cell is often defined as a cell that is intermediate in the differentiation pathway, i.e., it arises from a stem cell and is intermediate in the production of a mature cell type or subset of cell types. This type of progenitor cell is generally not able to self-renew. Accordingly, if this type of cell is referred to herein, it will be referred to as a non-renewing progenitor cell or as an intermediate progenitor or precursor cell.

[0026] "Markers", as used herein, are nucleic acid or polypeptide molecules that are differentially expressed in a cell of interest. In this context, differential expression means an increased level for a positive marker and a decreased level for a negative marker as compared to an undifferentiated cell. The detectable level of the marker nucleic acid or polypeptide is sufficiently higher or lower in the cells of interest compared to other cells, such that the cell of interest can be identified and distinguished from other cells using any of a variety of methods known in the art.

[0027] As used herein, a cell is "positive for" a specific marker or "positive" when the specific marker is sufficiently detected in the cell. Similarly, the cell is "negative for" a specific marker, or "negative" when the specific marker is not sufficiently detected in the cell. In particular, positive by fluorescence-activated flow cytometry (FACS) is usually greater than 2%, whereas the negative threshold by FACS is usually less than 1%.

[0028] As used herein, positive by real-time PCR (RT-PCR) had less than 28 cycles (Cts), and using Taqman$^®$ Low Density Array (TLDA) had less than 33 Cts; whereas negative by Open Array$^®$ is more than 28.5 cycles and negative by TLDA is more than 33.5 Cts.

[0029] To differentiate pluripotent stem cells into functional intestinal midgut endoderm cells in static *in vitro* cell culture, the differentiation process is often viewed as progressing through consecutive stages. Here, the differentiation process to intestinal midgut endoderm occurs through three stages In this step-wise progression, "Stage 1" refers to the first step in the differentiation process, the differentiation of pluripotent stem cells into cells expressing markers characteristic of definitive endoderm cells (hereinafter referred to alternatively as "Stage 1 cells"). "Stage 2" refers to the second step, the differentiation of cells expressing markers characteristic of definitive endoderm cells into cells expressing markers characteristic of primitive gut tube cells (hereinafter referred to alternatively as "Stage 2 cells"). "Stage 3" refers to the third step, the differentiation of cells expressing markers characteristic of gut tube cells into cells expressing markers characteristic of intestinal midgut endoderm cells (hereinafter referred to alternatively as "Stage 3 cells").

[0030] However, it should be noted that not all cells in a particular population progress through these stages at the same rate. Consequently, it is not uncommon in *in vitro* cell cultures to detect the presence of cells that have progressed less, or more, down the differentiation pathway than the majority of cells present in the population, particularly at the later

differentiation stages. For purposes of illustration, characteristics of the various cell types associated with the above-identified stages are described herein.

[0031] "Definitive endoderm cells," as used herein, refers to cells which bear the characteristics of cells arising from the epiblast during gastrulation and which form the gastrointestinal tract and its derivatives. Definitive endoderm cells express at least one of the following markers: FOXA2 (also known as hepatocyte nuclear factor 3-β ("HNF3β")), GATA4, SOX17, CXCR4, Brachyury, Cerberus, OTX2, goosecoid, C-Kit, CD99, and MIXL1. Markers characteristic of the definitive endoderm cells include CXCR4, FOXA2 and SOX17. Thus, definitive endoderm cells may be characterized by their expression of CXCR4, FOXA2 and SOX17. In addition, depending on the length of time cells are allowed to remain in Stage 1, an increase in HNF4α may be observed.

[0032] "Primitive gut tube cells," as used herein, refers to endoderm cells derived from definitive endoderm that can give rise to all endodermal organs, such as lungs, liver, pancreas, stomach, and intestine. Primitive gut tube cells may be characterized by their substantially increased expression of HNF4α over that expressed by definitive endoderm cells.

[0033] "Foregut endoderm cells," as used herein, refers to endoderm cells that give rise to the esophagus, lungs, stomach, liver, pancreas, gall bladder, and the most anterior portion of the duodenum. Foregut endoderm cells may be characterized by their expression of SOX2, PDX1, ALB, SOX17 and FOXA2, among others.

[0034] "Intestinal midgut endoderm cell," as used herein, refers to endoderm cells that give rise to small intestine. Intestinal midgut endoderm cells may be characterized by their expression of CDX2, FOXA2, and low expression of PDX1 (PDX1^{LO}). The expression of certain HOX genes can distinguish between midgut and hindgut endoderm. For example, HOXC5 is preferentially expressed in midgut endoderm cells.

[0035] "Hindgut endodermal cell" as used herein, refers to endoderm cells that give rise to large intestine. Hindgut endoderm cells may be characterized by their expression of CDX2, FOXA2, HOXA13 and HOXD13.

[0036] "Mesenchyme cell," as used herein, refers to mesoderm cells that give rise to connective tissues, such as bones, cartilage, lymphatic, and circulatory systems. Expression of HAND1 and FOXF1 define mesenchyme cells.

[0037] The term "patient" or "subject" or "host" refers to animals, including mammals, preferably humans, who are treated with compositions or pharmaceutical compositions, or in accordance with the methods described herein for reference.

[0038] The term "effective amount" or equivalents thereof refers to an amount of an agent or compound including but not limited to a growth factor, which is sufficient to promote and differentiate human pluripotent stem cells to a differentiated cell population, for example, to a

definitive endoderm, foregut endoderm, intestinal midgut endoderm, hindgut endoderm, pancreatice endoderm and the like.

[0039] As disclosed for reference, the terms "administering" and "administration" are used interchangeably herein and mean the cells may be implanted, injected, transplanted or otherwise administered directly or indirectly to the site of treatment. When cells are administered in semisolid or solid devices, implantation is a suitable means of delivery, particularly surgical implantation into a precise location in the body, such as into subcutaneous space, omentum, liver, kidney (kidney capsule). As disclosed for reference only, liquid or fluid pharmaceutical compositions may be administered to a more general location.

[0040] As used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a cell" includes a combination of two or more cells, and the like.

[0041] As used herein, the term "about" when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of between ± 20% and ± 0.1%, preferably ± 20% or ± 10%, more preferably ± 5%, even more preferably ± 1%, and still more preferably ± 0.5%, ± 0.1%. 0.05% or 0.01% of the specified value, as such variations are appropriate to perform the disclosed methods.

[0042] The following abbreviations may appear throughout the specification and claims:

ABCG2 - ATP-Binding Cassette, Sub-Family G, Member 2;

ALB - albumin;

BMP - bone morphogenic protein;

CDX2 - caudal type homeobox 2;

CXCR4 - C-X-C chemokine receptor type 4;

FAF-BSA - Fatty-acid Free Bovine Serum Albumin

FGF - fibroblast growth factor;

FOXA2 - Forkhead Box A2;

GATA4 - GATA binding protein 4;

GDF - growth differentiation factor;

GIP - glucose-dependent insulinotropic polypeptide;

GLP-1 - glucagon-like peptide 1;

GSK3B - Glycogen synthase kinase 3 beta;

HANOI - heart and neural crest derivatives expressed 1;

HOX - homeobox;

hTERT - human telomerase reverse transcriptase;

KLF - Kruppel-like factor;

LGR5 - leucine rich repeat containing G protein coupled receptor 5;

MIXL1 - Mix Paired-Like Homeobox-1;

OCT4 - octamer-binding transcription factor 4;

OTX2 - orthodenticle homeobox 2;

PDX1 - pancreatic and duodenal homeobox 1;

PTF1A - pancreas specific transcription factor, 1a;

SOX - sex determining region Y (SRY)-box;

TRA1-60 - T cell receptor alpha-1-60;

UTF1 - Undifferentiated embryonic cell transcription factor 1;

WNT3A - wingless-type MMTV integration site family, member 3A; and

ZFP42 - zinc finger protein 42.

[0043] Pluripotent stem cells have the potential to differentiate into cells of all three germinal layers: endoderm, mesoderm, and ectoderm tissues. Exemplary types of pluripotent stem cells that are disclosed for reference only, include established lines of pluripotent cells, including pre-embryonic tissue (such as, for example, a blastocyst), embryonic tissue, or fetal tissue taken any time during gestation, typically but not necessarily, before approximately 10 to 12 weeks gestation. Non-limiting examples that are disclosed for reference only are established lines of human embryonic stem cells or human embryonic germ cells, such as, for example the human embryonic stem cell lines H1, H7, and H9 (WiCell Research Institute, Madison, WI, USA). As disclosed for reference, cells taken from a pluripotent stem cell population already cultured in the absence of feeder cells are also suitable. iPS, or reprogrammed pluripotent cells, derived from adult somatic cells using forced expression of a number of pluripotent related transcription factors, such as OCT4, NANOG, SOX2, KLF4, and ZFP42 (Annu Rev Genomics Hum Genet 2011, 12:165-185; *see also* iPS, Cell, 126(4): 663-676) may be used. As disclosed for reference only, the human embryonic stem cells may also be prepared as described by Thomson et al. (U.S. Patent No. 5,843,780; Science, 1998, 282:1145-1147; Curr Top Dev Biol 1998, 38:133-165; Proc Natl Acad Sci U.S.A. 1995, 92:7844-7848). Mutant human embryonic stem cell lines, such as, BG01v (BresaGen, Athens, Ga.) are disclosed for

reference only. Cells derived from adult human somatic cells, such as, cells disclosed in Takahashi et al., Cell 131: 1-12 (2007) may be used. As disclosed herein, pluripotent stem cells may be derived according to the methods described in: Li et al. (Cell Stem Cell 4: 16-19, 2009); Maherali et al. (Cell Stem Cell 1: 55-70, 2007); Stadtfeld et al. (Cell Stem Cell 2: 230-240); Nakagawa et al. (Nature Biotechnol 26: 101-106, 2008); Takahashi et al. (Cell 131: 861-872, 2007); and U.S. Patent App. Pub. No. 2011/0104805. The pluripotent stem cells may be of non-embryonic origins.

[0044] Pluripotent stem cells differentiate through various stages each of which may be characterized by the presence or absence of particular markers. Differentiation of the cells into these stages is achieved by the specific culturing conditions, including the presence or lack of certain factors added to the culture media. In general, this differentiation may involve differentiation of pluripotent stem cells into definitive endoderm cells, referred to herein as Stage 1. These definitive endoderm cells may then be further differentiated into primitive gut tube cells, referred to herein as Stage 2. Primitive gut tube cells in turn may then be differentiated into intestinal midgut endoderm cells, referred to herein as Stage 3.

Differentiation of Pluripotent Stem Cells into Cells Expressing Markers

Characteristic of Intestinal Midgut Endoderm Cells

[0045] Characteristics of pluripotent stem cells are well known to those skilled in the art, and additional characteristics of pluripotent stem cells continue to be identified. Pluripotent stem cell markers include, for example, the expression of one or more of the following: ABCG2; Cripto; FOXD3; CONNEXIN43; CONNEXIN45; OCT4; SOX2; NANOG; hTERT; UTF1; ZFP42; SSEA-3; SSEA-4; TRA-1-60; and TRA-1-81.

[0046] Exemplary pluripotent stem cells, disclosed for reference only, include the human embryonic stem cell line H1 (NIH code: WA01), the human embryonic stem cell line H9 (NIH code: WA09), the human embryonic stem cell line H7 (NIH code: WA07), and the human embryonic stem cell line SA002 (Cellartis, Sweden). Suitable are cells that express at least one of the following markers characteristic of pluripotent cells: ABCG2, Cripto, CD9, FOXD3, CONNEXIN43, CONNEXIN45, OCT4, SOX2, NANOG, hTERT, UTF1, ZFP42, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81.

[0047] Also suitable in the context of the present disclosure is a cell that expresses at least one of the markers characteristic of the definitive endoderm lineage. A cell expressing markers characteristic of the definitive endoderm lineage may be a primitive streak precursor cell. Furthermore, a cell expressing markers characteristic of the definitive endoderm lineage may be a mesendoderm cell. Furthermore, a cell expressing markers characteristic of the definitive endoderm lineage may be a definitive endoderm cell.

[0048] Also suitable in the context of the present disclosure is a cell that expresses at least one of the markers characteristic of the intestinal midgut endoderm lineage. A cell expressing markers characteristic of the intestinal endoderm lineage may be an intestinal midgut endoderm cell in which the cell expresses FOXA2 and CDX2. It is possible that the cell does not express SOX2, ALB, PTF1A, HOXA13 or LGR5. A cell expressing markers characteristic of the intestinal endoderm lineage may be an intestinal midgut endoderm cell in which the cell expresses each of FOXA2, CDX2, SOX9, PDX1, KLF5 and HOXC5. A cell expressing markers characteristic of the intestinal endoderm lineage may be an intestinal midgut endoderm cell in which the cell does not express any of SOX2, ALB, PTF1A, HOXA13 and LGR5.

[0049] Further disclosed herein is a staged, directed differentiation of pluripotent stem cells toward intestinal midgut endoderm cells using cell culture conditions and media. To arrive at a cell expressing markers characteristic of intestinal midgut endoderm cell, a protocol starting with pluripotent stem cells, such as embryonic stem cells and induced pluripotent cells, may be employed. This protocol includes the following stages.

Stage 1:	Pluripotent stem cells, such as embryonic stem cells obtained for cell culture lines, are treated with appropriate factors to induce differentiation into cells expressing markers characteristic of definitive endoderm cells.
Stage 2:	Cells resulting from Stage 1 are treated with appropriate factors to induce further differentiation into cells expressing markers characteristic of primitive gut tube cells.
Stage 3:	Cells resulting from Stage 2 are treated with appropriate factors to induce further differentiation into cells expressing markers characteristic of intestinal midgut endoderm cells.

[0050] Methods for assessing expression of protein and nucleic acid markers in cultured or isolated cells are standard in the art. These methods include RT-PCR, Northern blots, *in situ* hybridization (*see, e.g.,* Current Protocols in Molecular Biology (Ausubel et al., eds. 2001 supplement)), and immunoassays (such as immunohistochemical analysis of sectioned material), Western blotting, and for markers that are accessible in intact cells, FACS (*see, e.g.,* Harlow and Lane, Using Antibodies: A Laboratory Manual, New York: Cold Spring Harbor Laboratory Press (1998)). The efficiency of differentiation may be determined by exposing a treated cell population to an agent (such as an antibody) that specifically recognizes a protein marker expressed by cells expressing markers characteristic of the cell type of interest.

1. Differentiation of pluripotent stem cells into cells expressing markers characteristic of definitive endoderm cells

[0051] Pluripotent stem cells may be differentiated into cells expressing markers characteristic of definitive endoderm cells by any suitable method known in the art, or by any method disclosed herein. Pluripotent stem cells may be treated with a medium, such as MCDB-131

medium (Life Technologies, Carlsbad, California) supplemented with factors including GDF8 and a GSK3β inhibitor (such as the cyclic aniline-pyridinotriazine compounds disclosed in U.S. Patent App. Pub. No. 2010/0015711) to induce differentiation into cells expressing markers characteristic of definitive endoderm cells. There is a broad range of GSK3β inhibitors, such as staurosporine, and the preferred GSKβ inhibitor (14-Prop-2-en-1-yl-3,5,7,14,17,23,27-heptaazatetracyclo [19.3.1.1~2,6~.1~8,12~]heptacosa-1(25),2(27),3,5,8(26),9,11,21,23-nonaen-16-one, referred to herein as "MCX Compound". Treatment may involve contacting pluripotent stem cells with a medium supplemented with about 10 ng/ml to 1000 ng/ml, preferably 50 ng/ml to about 150 ng/ml, alternatively about 75 ng/ml to about 125 ng/ml, alternatively about 100 ng/ml of GDF8. The treatment may also involve contacting the cells with about 0.1 to 10 μM, preferably about 0.1 to 5 μM, alternatively about 0.5 to about 2.5 μM, preferable about 1.5 μM or about 1.0 μM of MCX compound. Other components of the medium may include: Sodium bicarbonate at about 2.7g/1000ml to 3.6g/1000ml, preferably 2.7g/1000ml; FAF-BSA at about 0.1% to 2.0%, preferably about 0.5%; GlutaMAX™ (Life Technologies Corporation, Carlsbad, California) at 1:100 dilution ("1X concentration"); and D-Glucose at a concentration range of about 2 mM to 20 mM, preferably 4.5mM to obtain a concentration of 10mM D-Glucose.

[0052] The pluripotent cells may be cultured for approximately two to five days, preferably about three to four days, to facilitate their differentiation into definitive endoderm cells. The pluripotent cells may be cultured in the presence of an effective amount of TGFβ signaling molecule and/or GSK3β inhibitor, for example, an effective amount of GDF8 and MCX compound for one day, followed by culturing in the presence of GDF8 and a lower concentration of MCX compound for one day, followed by culturing in the presence of GDF8 for one day in the absence of the MCX compound. In particular, the cells may be cultured in the presence of GDF8 and about 1.5 μM of MCX compound for one day, followed by culturing in the presence of GDF8 and about 0.1 μM MCX compound for one day, followed by culturing in the presence of GDF8 for one day in the absence of the MCX compound. Furthermore, the cells may be cultured in the presence of GDF8 and about 1.5 μM of MCX compound for one day, followed by culturing in the presence of GDF8 and about 0.1 μM MCX compound for one day.

[0053] Generation of cells expressing markers characteristic of definitive endoderm cells may be determined by testing for the presence of the markers before and after following a particular protocol. Pluripotent stem cells typically do not express such markers. Thus, differentiation of pluripotent cells can be detected when the cells begin to express markers characteristic of definitive endoderm cells, such as CXCR4, FOXA2 and SOX17. Cells expressing markers characteristic of definitive endoderm cells may be definitive endoderm cells.

2. Differentiation of cells expressing markers characteristic of definitive endoderm cells into cells expressing markers characteristic of primitive gut tube cells

[0054] The cells expressing markers characteristic of definitive endoderm cells may be further differentiated into cells expressing markers characteristic of primitive gut tube cells. The formation of cells expressing markers characteristic of primitive gut tube cells includes culturing cells expressing markers characteristic of definitive endoderm cells with a medium, such as MCDB-131, containing FGF7 to differentiate these cells. For example, the culture medium may include from about 10 ng/ml to 100 ng/ml, preferably about 25 ng/ml to about 75 ng/ml, alternatively from about 30 ng/ml to about 60 ng/ml, alternatively about 50 ng/ml of FGF7. The cells may be cultured under these conditions for about two to three days, preferably about two days.

[0055] Furthermore, differentiation into cells expressing markers characteristic of primitive gut tube cells may include culturing cells expressing markers characteristic of definitive endoderm cells with FGF7 and ascorbic acid (vitamin C). The culture medium, such as MCDB-131, may include from about 0.1 mM to about 1.0 mM ascorbic acid, preferably about 0.1 mM to about 1.0 mM, alternatively from about 0.2 mM to about 0.4 mM, alternatively about 0.25 mM of ascorbic acid. The culture medium may also include from about 10 ng/ml to 100 ng/ml, preferably about 10 ng/ml to about 50 ng/ml, alternatively from about 15 ng/ml to about 30 ng/ml, alternatively about 50 ng/ml or about 25 ng/ml of FGF7. For example, the culture medium may include about 0.25 mM ascorbic acid and about 50 ng/ml FGF7. Other components of the medium may include: sodium bicarbonate at about 2.7g/1000ml to 3.6g/1000ml, preferably 2.7g/1000ml; FAF-BSA at about 0.1% to 2.0%, preferably about 0.5%; GlutaMAX™ at 1:100 dilution ("1X concentration"); and D-Glucose at a concentration range of about 2 mM to 20 mM, preferably 4.5mM to obtain a concentration of 10mM D-Glucose. Cells expressing markers characteristic of definitive endoderm cells may be treated for 2 days with FGF7 and ascorbic acid. Differentiation of definitive endoderm cells can be detected when the cells begin to express markers characteristic of primitive gut tube cells, such as expression of FOXA2 and increased expression of HNF4α. Cells expressing markers characteristic of primitive gut tube cells may be primitive gut tube cells.

3. Differentiation of cells expressing markers characteristic of primitive gut tube cells into cells expressing markers characteristic of intestinal midgut endoderm cells

[0056] Cells expressing markers characteristic of primitive gut tube cells may be further differentiated into cells expressing markers characteristic of intestinal midgut endoderm cells. Primitive gut tube cells may be further differentiated into intestinal midgut endoderm cells by culturing the primitive gut tube cells in a culture medium, such as BLAR medium (Life Technologies, Corporation, Carlsbad, California), supplemented with retinoic acid and a BMP4 or BMP2. Preferably, the medium may be supplemented with from about 0.5 µM to about 5 µM of retinoic acid, preferably about 1 µM, and from about 10 ng/ml to about 100 ng/ml BMP4 or BMP2, preferably about 50 ng/ml of BMP4 or BMP2. Other supplements to the medium may include: FAF-BSA at about 0.1% to 2.0%, preferably about 0.5%; GlutaMAX™; and D-Glucose at a concentration range of about 2 mM to 20 mM, preferably 4.5mM to obtain a concentration

of 10mM D-Glucose. Cells expressing markers characteristic of primitive gut cells may be treated for 3 to 5 days, preferably for 5 days with BMP4 or BMP2 and retinoic acid. The pH of the culture can range from 6.8 to 7.2 during the 5-day Stage 3 conditioning period (compared to normal pH at S2D2 being 7.3 or more).

[0057] Further disclosed is a method of producing a population of intestinal midgut endoderm cells by culturing human pluripotent stem cells in selected culture media for generating intestinal midgut endoderm cells. The method may induce differentiation of human pluripotent stem cell to intestinal midgut endoderm cells in a staged process. A population of intestinal midgut endoderm cells may be produced. Furthermore, a population of substantially intestinal midgut endoderm cells may be produced. The intestinal midgut endoderm cells may form and maintain a monolayer on planar culture. The intestinal midgut endoderm cells may be stable as a monolayer in culture. Cells stable as a monolayer, or remain stable as a monolayer, herein refers to a monolayer of cells that do not form spheroids in culture.

[0058] Greater than 50% of the differentiated cells express markers characteristic of intestinal midgut endoderm. Greater than 60%, greater than 70%, greater than 80%, greater than 90% or greater than 95% of the differentiated cells may express markers characteristic of intestinal midgut endoderm. Differentiated cells express markers characteristic of intestinal midgut endoderm may be intestinal midgut endoderm cells. The intestinal midgut endoderm cells may express CDX2 and FOXA2 as determined by FACS analysis and qPCR. Intestinal midgut endoderm cells may express transcription factors selected from SOX9, PDX1, KLF5 and HOXC5 as determined by IF analysis and qPCR. The intestinal midgut endoderm cells may not express transcription factors selected from SOX2, ALB, PTF1A as determined by IF analysis and qPCR, and HOXA13 and LGR5 as determined by qPCR.

[0059] Further disclosed herein is a method of producing intestinal midgut endoderm cells comprising inducing differentiation of definitive endoderm cells in culture to primitive gut tube cells. The definitive endoderm cells may be cultured in culture medium containing ascorbic acid and FGF7. The primitive gut tube cells may be cultured in culture medium containing retinoic acid and BMP2 or BMP4. The primitive gut tube cells are differentiated to intestinal midgut endoderm cells. Primitive gut tube cells may be differentiated to intestinal midgut endoderm cells in acidic conditions (acidic culture media). Acidic conditions may be culture in BLAR media. The pH of the acidic culture can range from 6.8 to 7.2 during the 5-day differentiation conditioning period from primitive gut tube cells to intestinal midgut endoderm cells (compared to normal pH at S2D2 being 7.3 or more). The intestinal midgut endoderm cells may form and maintain a monolayer in culture.

[0060] Herein, human pluripotent stem cells are human hESC or iPS cells. Herein, the intestinal midgut endoderm cells express CDX2 and FOXA2 as determined by FACS analysis and qPCR. The intestinal midgut endoderm cells express transcription factors selected from SOX9, PDX1, KLF5 and HOXC5 as determined by IF analysis and qPCR. The intestinal midgut endoderm cells may not express transcription factors selected from SOX2, ALB, PTF1A as determined by IF analysis and qPCR, and HOXA13 and LGR5 as determined by qPCR. The

intestinal midgut endoderm cells may express CDX2, FOXA2, SOX9, PDX1, KLF5 and HOXC5 by IF analysis and qPCR. The intestinal midgut endoderm cells do not express SOX2, ALB and PTF1A as determined by IF analysis and qPCR, and HOXA13 and LGR5 as determined by qPCR.

[0061] As a result of the differentiation protocol described above and herein, using specific culture components and culture conditions, in particular acidic culture condition, such as culture in BLAR medium, a culture of cells expressing markers for intestinal midgut endoderm cells is generated; the cells lack expression of HAND 1 as determined by qPCR, a marker of mesoderm/mesenchymal lineage. Varying the differentiation protocol to induce pluripotent stem cells to midgut/hindgut endoderm lineage, such as inducing stem cells at definitive endoderm stage 1 rather than primitive gut tube cell stage 2, results in a mixed population of endoderm-mesenchyme CDX2$^+$ mid-/hindgut cells that express HAND1 as determined by qPCR.

[0062] The population of intestinal midgut endoderm cells may be substantially intestinal midgut endoderm cells. The population of intestinal midgut endoderm cells may comprise greater than 70% intestinal midgut endoderm cells, preferably greater than 80%, greater than 90%, and greater than 95% of intestinal midgut endoderm cells. The population of intestinal midgut endoderm cells may comprise less than 20% mesenchymal cells, preferably less than 15%, more preferably less than 10%, less than 5%, less than 2%, less than 1%, less than 0.5%. Intestinal midgut endoderm cells may lack expression of HANOl.

Use of Differentiated Intestinal Midgut Endoderm Cells

[0063] The differentiated intestinal midgut endoderm cells may be used for treating a patient suffering from or at risk of developing diabetes alone or in combination with differentiated or mature endocrine cells, for example, enteroendocrine cells. As disclosed for reference only, differentiated intestinal midgut endoderm cells, or mixtures thereof, may be administered to a patient having diabetes, for example Type 1 or Type 2 diabetes. Intestinal midgut endoderm cells may differentiate and mature to enteroendocrine cells. Intestinal midgut endoderm cells may differentiate and mature to enteroendocrine cells, and the enteroendocrine cells may express or secrete incretin type hormones. Incretin hormones may include GLP1 and GIP. As disclosed for reference only, administration of the cells may be via implantation or injection in the body, in particular implantation into subcutaneous space, omentum, liver, kidney, etc.

[0064] In the context of the above disclosure, differentiation of intestinal midgut endoderm cells may be induced *in vitro*. As disclosed herein, intestinal midgut endoderm cells may further differentiate and mature *in vivo*. As disclosed herein for reference, the intestinal midgut endoderm cells may further differentiate into enteroendocrine cells *in vivo* or in a mixture with enteroendocrine cells *in vivo*. Such enteroendocrine cells express or secrete incretin hormones. The enteroendocrine cell-secreted incretin hormones may include GLP1 and GIP.

[0065] Intestinal midgut endoderm cells may serve as starting material for the identification of small molecules that promote at high efficiency the *in vitro* differentiation of intestinal midgut endoderm cell type into, first, enteroendocrine precursors, and then to incretin expressing or secreting enteroendocrine cells.

[0066] As disclosed for reference only, cells and cell populations and mixtures such as those described herein may be micro- or macro-encapsulated and subsequently transplanted into a mammalian host. As disclosed for reference only, encapsulated cells or cells alone may be transplanted (administered) subcutaneously or anywhere else in the body whereby the cells may be vascularized and differentiate and mature *in vivo.*

EXAMPLES

[0067] The invention can be further understood in view of the following non-limiting examples.

Reference Example 1

Method of producing an intestinal midgut endoderm cell population with CDX2 and FOXA2 co-presence/co-expression

[0068] The following example describes a directed-based method to generate intestinal midgut endoderm cells from human embryonic stem cell ("hESC"). "Intestinal midgut endoderm" refers to a corresponding *in vivo* or *in situ* cell type which is CDX2-positive and FOXA2-positive endoderm cells present at about embryonic day 8.5 ("E8.5") during mouse development, or at about the 3-4 week time point during human embryonic development.

Materials and Methods

[0069] **Cell culture:** Cells of the human embryonic stem cell line H1 ("H1-hESC") (WA01 cells, WiCell Research Institute, Madison, Wisconsin), disclosed for reference only, cultured with EZ8 media (Cat#A1516901 Gibco, Thermo Fisher Scientific) at passage 28 were seeded as single cells at 0.094×10^6 cells/cm^2 on MATRIGEL™, at a 1:30 dilution, (Corning Incorporated, Corning, New York, Catalog # 356231) coated dishes in a media of Dulbecco's Modified Eagle's Medium Nutrient mixture F-12 ("DMEM-F12") (Life Technologies Corporation, Carlsbad, California, Catalog No. 11330-032), with the following:

GlutaMAX™	1:100 dilution ("1X concentration")
(Life Technologies Corporation, Carlsbad, California, Catalog No. 35050-079)	

Ascorbic acid	0.25mM
(Sigma Aldrich Co. LLC, St. Louis, Missouri, Catalog No. A4544)	
FGF2	100ng/ml
(R & D Systems Inc., Minneapolis, Minnesota, Catalog No. 233-FB-025)	
Transforming Growth Factor beta ("TGFβ")	1 ng/ml
(R & D Systems Inc., Minneapolis, Minnesota, Catalog No. 240-B-002)	
insulin-transferrin-selenium-ethanolamine ("ITS-X")	1:200 dilution
(Life Technologies, Carlsbad, California, Catalog No. 51500056) at a 1:100 dilution	
FAF-BSA	2%
(Proliant, Inc., Boone, Idaho, Catalog No. 68700)	
Insulin-like Growth Factor-1 ("IGF-1")	20 ng/ml
(R & D Systems Inc., Minneapolis, Minnesota, Catalog No. 291-G1-200)	
Rock Inhibitor Y-27632 ("Y-compound")	10μM
(Sigma Aldrich Co. LLC, St. Louis, Missouri, Catalog No. Y-0503)	

[0070] About forty-eight hours post-seeding, the cultures were washed in incomplete PBS (phosphate buffered saline without magnesium or calcium) (Life Technologies, Carlsbad, California, Catalog No. 14190). Rock Inhibitor Y-27632 (Y compound) was used only for the first 24 hours of culture.

[0071] **Differentiation:** The cultures were differentiated using the following protocol. During Stages 1 through 3 of the protocol, cultures were maintained on planar adherent cultures. Others, however, have described differentiation using suspension culture including US US2014/0242693; the protocol described herein can be modified and performed in suspension, which provides for scalability of manufacturing. The following nomenclature, S#D#, specifies exact time during Stages 1 through 3. For example, S1D3 is stage 1 day 3. Briefly, each stage defines differentiation towards definitive endoderm (stage 1), primitive gut tube (stage 2), and intestinal midgut endoderm (stage 3).

a. Stage 1 (3 days): Cells were cultured for one day in the following Stage 1 media:

MCDB-131 medium	
(Life Technologies, Carlsbad, California, Catalog No. ME120219L2)	
Sodium bicarbonate	2.7 g/1000 ml
(Sigma-Aldrich Co. LLC, St. Louis, Missouri, Catalog No.	

5761)	
FAF-BSA	0.5%
GlutaMAX™	1:100 dilution ("1X concentration")
D-Glucose (Sigma-Aldrich Co. LLC, St. Louis, Missouri, Catalog No. G8769)	4.5mM to obtain a concentration of 10mM D-Glucose
GDF8 (Peprotech, Rocky Hill, New Jersey, Catalog No. 120-00)	100 ng/ml
14-Prop-2-en-1-yl-3,5,7,14,17,23,27-heptaazatetracyclo [19.3.1.1~2,6~1~8,12~]heptacosa-1 (25),2(27),3, 5, 8(26),9,11,21,23-nonaen-16-one ("MCX compound").	1.5 µM

Cells were then cultured for an additional day in the following media:

MCDB-131 medium	
Sodium bicarbonate	2.7g/1000ml
FAF-BSA	0.5%
GlutaMAX™	1X concentration
D-Glucose	4.5mM to obtain a concentration of 10mM D-Glucose
GDF8	100 ng/ml
MCX compound	0.1 µM

Cells were then cultured for an additional day in the same media as day 2 above but without MCX compound.

b. Stage 2 (2 days): Cells were treated for two days with the following medium:

MCDB-131 medium	
Sodium bicarbonate	2.7g/1000ml
FAF-BSA	0.5%
GlutaMAX™	1X concentration
D-Glucose	4.5mM to obtain a concentration of 10mM D-Glucose
Ascorbic Acid	0.25 mM
FGF7 (R&D Systems, Inc., Minneapolis, Minnesota, Catalog No. 251-K	50 ng/ml

c. Stage 3 (5 days): Cells were treated for five day with BLAR 001 custom medium:

BLAR medium	
(custom manufactured by Life Technologies, Corporation, Carlsbad, California, Catalog No. ME120123L2, components listed on Table I)	

FAF-BSA	0.5%
GlutaMAX™	1X concentration
D-Glucose	4.5mM to obtain a concentration of 10mM D-Glucose
Retinoic Acid (Sigma Aldrich, St. Louis, Missouri, Catalog No. R2625)	1 µM
BMP4 compound (R&D Systems, Inc., Minneapolis, Minnesota, Catalog No. 314-BP	50 ng/ml
OR	
BMP2 compound (R&D Systems, Inc., Minneapolis, Minnesota, Catalog No. 355-BM),	

[0072] Either BMP4 or BMP2 can be used during Stage 3 in this method to achieve intestinal midgut endoderm cells in monolayer with CDX2 and FOXA2 protein co-presence, as illustrated in Figures 1A - 4B (BMP4-based), and Figures 5A - 7 (BMP2-based).

Table I: List of components of BLAR medium.

Amino Acids	Concentration (mM)
Glycine	3.00E-02
Alanine	3.00E-02
Arginine	3.00E-01
Aspargine	1.00E-01
Aspartic Acid	1.00E-01
Cysteine	1.99E-01
Glutamic acid	3.00E-02
Histidine	1.10E-01
Isoleucine	1.00E-02
Leucine	9.00E-02
Lysine hydrochloride	1.50E-01
Methiane	3.00E-02
Phenylalanine	3.00E-02
Proline	1.00E-01
Serine	1.00E-01
Theronine	3.00E-02
Tryptophan	2.00E-03
Tyrosine disodium	1.00E-02

Amino Acids	Concentration (mM)
Valine	3.00E-02
Vitamins	
Biotin	3.00E-05
Choline chloride	5.00E-03
D-Calcium pantothenate	1.50E-03
Folinic Acid Calcium salt	2.30E-03
Niacinamide	4.90E-03
Pyridoxine hydrochloride	9.70E-04
Riboflavin	1.00E-05
Thiamine hydrochloride	3.00E-03
Vitamin B12	3.70E-06
i-Inositol	2.80E-03
Salts/Minerals	
Calcium Chloride ($CaCl_2$-$2H_2O$)	3.00E-01
Cupric sulfate ($CuSO_4$-$5H_2O$)	4.80E-06
Ferric sulfate ($FeSO_4$-$7H_2O$)	1.00E-03
Magnesium Sulfate ($MgSO_4$-$7H_2O$)	4.10E-01
Potassium Chloride (KCl)	3.80E+00
Sodium Bicarbonate (NaHCOs)	1.40E+01
Sodium Chloride (NaCl)	1.10E+02
Sodium Phosphate dibasic (Na_2HPO_4-$7H_2O$)	5.00E-01
Zinc Sulfate (ZnSO4-H2O)	1.00E-04
Other	
Adenine	1.00E-03
D-Glucose (Dextrose)	5.00E+00
Lipoic Acid	1.20E-05
Phenol Red	1.00E-02
Sodium Pyruvate	1.00E+00
Thymidine	9.80E-05

[0073] **Quantification of differentiated cells:** For quantification of protein presence co-localization, S3D5 cells were harvested and analyzed by FACS. FACS staining was conducted as described in Nature Biotechnology, 2014 (32) 11, 1121-1133, and using the antibodies listed in Table II. In brief, differentiated cells were incubated in TrypLE™ Express (Life Technologies,

Carlsbad, California, Catalog No. 12604) for 5-10 minutes at 37°C, released into a single-cell suspension, after which they were washed twice with a staining buffer of PBS containing 0.2% BSA (BD Biosciences, San Jose, California, Catalog No. 554657). Intracellular antibody staining was accomplished by utilizing the LIVE/DEAD Violet Fluorescent reactive dye (Life Technologies, Carlsbad, California, Catalog No. L34955) at 4°C for 30 minutes followed by a single wash in cold PBS. Fixing of cells was in 300µl of Cytofix/Cytoperm Buffer (BD Biosciences, San Jose, California, Catalog No. 554723) followed by two washes in Perm/Wash Buffer (BD Biosciences, San Jose, California, Catalog No. 554722). Cells were then incubated with the appropriate antibodies at 4°C for 30 minutes (for unconjugated antibodies) or 1 hour (for conjugated antibodies), and then washed twice prior to analysis on the BD FACS Canto II using BD FACS Diva Software with at least 30,000 events being acquired. Non-viable cells were excluded during FACS analysis, and gating was determined by using isotype antibodies ("IgG"). IgGFACS data is shown as the top panel for each FACS experiment presented. Antibodies were tested for specificity using positive controls, such as Caco-2 cells, or negative controls, such as S1D3 definitive endoderm ("DE") cells.

Table II: List of antibodies used for FACS analysis.

Antigen	Species	Source/Catalog Number	Dilution
PE IgG1, κ Isotype control	Mouse	BD Cat# 555749	1:5
Alexa Fluor 647 IgG1, κ Isotype control	Mouse	BD Cat# 557732	Neat
PE anti-Human FOXA2	Mouse	BD Cat# 561589	Neat
PE Anti-human CDX2	Mouse	BD Cat# 563428	Neat
Alexa Fluor 647 anti-CDX2	Mouse	BD Cat#560395	Neat
Alexa Fluor 647 anti-KI67	Mouse	BD Cat# 561126	Neat
PE anti-PDX1	Mouse	BD Cat# 562161	Neat
PE anti-SOX2	Mouse	BD Cat# 560291	Neat
Anti-Mouse IgG(H+L) Secondary Antibody, Alexa Fluor 647 conjugate	Goat	Life technology Cat# A21235	1:4000
F(ab')2 anti-rabbit IgG(H+L) Secondary Antibody, RPE conjugate	Goat	Life technology Cat# A10542	Neat
Anti-SOX9	Rabbit	Millipore Cat# AB5535	1:10
Anti-CDX2 [CDX2-88]	Mouse	BioGenex Cat# MU392A-UC	1:10

[0074] For quantification of protein co-localization at various stages, Caco-2, S2D2, S3D2 and S3D5 cells were harvested as a monolayer and analyzed by immunofluorescence ("IF"). Note that the morphology seen in IF images was caused by the method of cell scraping from the monolayer of adherent cultures. H1-hESC-derived cells, disclosed for reference only, were prepared and stained as described in Nature Biotechnology, 2014 (32) 11, 1121-1133, and

using the antibodies listed in Table III. For cryosectioning, cells were rinsed with PBS followed by overnight fixation in 4% PFA (Sigma Aldrich, St. Louis, Missouri, Catalog No. 158127) at 4°C. Following fixation, 4% PFA was removed, cells rinsed twice with PBS, and incubated overnight at 4°C in 30% sucrose solution (Amresco, Solon, Ohio, Catalog No. 0335). The samples were cryopreserved in OCT solution (Sakura Finetek USA Inc., Torrance, California, Catalog No. 4583), and 5 μm sections placed on Superfrost plus slides (VWR International, LLC, Radnor, PA, Catalog No. 48311-703). For IF-staining, primary antibodies were added at appropriate dilutions overnight at 4 °C, while secondary antibodies were added for 30 min at room temperature followed by rinsing with PBS and adding Vectastain mounting reagent with DAPI (Vector Laboratories Inc., Burlingame, California, Catalog No. H-1200). The sections were visualized using a Nikon Ti fluorescence microscope (Nikon Instruments, Inc., Melville, NY).

Table III: List of antibodies used for IF analysis.

Antigen	Species	Source	Dilution
CDX2	Mouse	BioGenex (Catalog No. MU392A-UC)	1:50
FOXA2	Rabbit	Seven Hills (Catalog No. WRAB 1200)	1:500
SOX9	Rabbit	Millipore EMD (Catalog No. AB5535)	1:100
PDX1	Goat	R&D Systems (Catalog No. AF2419)	1:33
SOX2	Goat	Sigma (Catalog No. sc-17320)	1:50
KLF5	Rabbit	Abcam (Catalog No. ab24331)	1:150 (Tyramide amplification - Perkin Elmer, Waltham, MA, NEL744001KT)
ALB	Rabbit	Sigma (Catalog No. A0433)	1:250
KI67	Rabbit	Abcam (Catalog No. ab 16667)	1:100
Donkey anti-mouse IgG (H+L) Secondary antibody, Alexa Fluor 488	Mouse	Life Technologies (Catalog No. A21202)	1:100
Donkey anti-rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 546	Rabbit	Life Technologies (Catalog No. A10040)	1:200
Donkey anti-goat IgG (H+L) Secondary	Goat	Life Technologies (Catalog No. A11056)	1:50-1:100

Antigen	Species	Source	Dilution
Antibody, Alexa Fluor 546			
Goat anti-rabbit IgG, HRP-Labeled	Rabbit	Perkin Elmer, Waltham, MA (Catalog No. NEF812001)	1:250

[0075] For quantification of gene expression at various stages, Caco-2, H1-hESC (disclosed for reference only), S1D3, S2D2, S3D2 and S3D5 cells were harvested, as described in Nature Biotechnology, 2014 (32) 11, 1121-1133. Briefly, gene expression was assessed in cells using custom Taqman® Arrays (Applied Biosystems, Foster City, California); Open Array® (OA) was used for CDX2, FOXA2, SOX2, SOX9, PDX1, ALB, PTF1A, and Taqman® Low Density Array (TLDA) was used for KLF5, HOXC5, and LGR5, with housekeeping gene GAPDH used for both tests. Data were analyzed using Sequence Detection Software (Applied Biosystems, Foster City, California), and normalized using GAPDH as a housekeeping gene to undifferentiated H1-hESC using the ΔΔCt method. Primer details are outlined in Table IV.

Table IV: List of RT-qPCR primers.

	Gene	Assay ID
1	ALB	Hs00609411_m1
2	CDX2	Hs00230919_m1
3	FOXA2	Hs00232764_m1
4	GAPDH	Hs99999905_m1
5	PDX1	Hs00236830_m1
6	PTF1A	Hs00603586_g1
7	SOX2	Hs01053049_s1
8	SOX9	Hs00165814_m1
9	HOXC5	Hs00232747_m1
10	KLF5	Hs00156145_m1
11	LGR5	Hs00969422_m1
12	HOXA13	Hs00426284_ml
13	HAND1	Hs00231848_ml

Results

[0076] A summary of a differentiation method, including the important medium components, growth factors and small molecules that were added to each stage, and key stage-specific

markers of the differentiating intestinal midgut endoderm cells (FOXA2; CDX2; KLF5; SOX9; PDX1^{LO}) is depicted in Figure 1A. As compared to the neutral pH noted for S2D2 (7.35±0.04), cells were exposed to slightly acidic conditions in the BLAR medium, during the entirety of stage 3 (pH; S3D1, 6.98±0.05; S3D2, 7.02±0.04; S3D5, 7.18±0.03) (Figure 1B), and as a result of lower sodium bicarbonate levels in BLAR medium. The pH of the culture can range from about 6.8 to 7.2 during the five days of stage 3. Figure 1C depicts representative phase-contrast images of a S3D5 monolayer (*left*), and human epithelial colon adenocarcinoma cell line ("Caco-2") (*right*), which was used as a benchmark for characterization of differentiation. A uniform morphology at S3D5 was observed. Characterization of cell number using the Nucleocounter® NC-100 (Chemometec, Alleroed, Denmark, Catalog No. 900-004) shows that one hESC differentiated into 4.56±2.60 S3D5 hindgut endoderm cells (Figure 1D).

[0077] The differentiation method, utilizing BMP4, efficiently generates and maintains intestinal midgut endoderm cells in monolayer, each comprising both CDX2 and FOXA2 on transcript and protein levels. Figure 2A (*bottom*) shows that 90.0±5.85 percent of S3D5 cells were co-present for both CDX2 and FOXA2 protein, similar to percentage observed in Caco-2 cells (86.0±6.67). Conversely, definitive endoderm (DE - S1D3) cells were devoid of CDX2 and FOXA2 co-presence (2.3±1.2). Gene expression analysis shows CDX2 induced (Figure 2B), and FOXA2 maintained (Figure 2C) during Stage 3. Figure 2D shows that the induction of CDX2 protein levels and CDX2/FOXA2 protein co-presence after the establishment of the FOXA2-positive primitive gut endoderm stage, S2D2 (Figure 2D-i), progressively increased through S3D2 (Figure 2D-ii), and at S3D5 (Figure 2D-iii) reached similar levels as seen in Caco-2 cells (Figure 2D-iv). CDX2 protein is depicted on the bottom row, and FOXA2 protein is depicted on the top row.

[0078] Transcript and protein levels of additional TFs are found at S3D5, which constitute robust intestinal midgut endoderm induction. Figures 3A - 3P show that proper intestinal midgut endoderm was achieved. In addition to CDX2 and FOXA2 co-presence, S3D5 cells also exhibited co-presence of SOX9, PDX1, KLF5, HOXC5, but did not express SOX2, ALB, PTF1A, and LGR5. The protein presence of all TFs is depicted in separate single channel images. Figure 3A (*bottom*) shows that 98.7±0.25 percent of cells were co-present for both CDX2 and SOX9 at S3D5. Strong induction of SOX9 gene expression was comparable to levels observed in Caco-2 cells (Figure 3B), and protein presence as assessed by IF-analysis were observed (Figure 3C). 69.4±14.2 percent of cells were co-positive for both CDX2 and PDX1 (Figure 3D - *bottom*). PDX1 gene expression was induced at low levels, as compared to pancreas-biased S4D3 cells (See, e.g., US2014/0242693) (Figure 3E), and this was reflected low to absent protein levels in the IF-analysis (Figure 3F).

[0079] S3D5 cells did not express the anterior endoderm TF SOX2, and only 1.45±0.15 of S3D5 cells exhibited SOX2 and CDX2 co-presence (Figure 3G - *bottom;* 3I), and gene expression was below levels seen in hESC and Caco-2 cells (Figure 3H). Gene expression of KLF5, essential for proper development of hindgut endoderm, was upregulated at S3D5 (Figure 3J). Protein co-presence of KLF5 within CDX2-positive cells at S3D5 was observed

(Figure 3K). ALB gene expression (Figure 3L), and protein presence (Figure 3M) was not observed in S3D5 cells. Similarly, the gene expression of PTF1A, a pancreas lineage marker, was not induced in S3D5 cells, as compared to pancreas-biased S4D3 cells (Figure 3N). The homeobox gene, HOXC5, present in the embryonic intestinal midgut endoderm was strongly induced in S3D5 cells (Figure 3O). Figure 3P shows that LGR5, a marker of embryonic intestinal endoderm beginning at mid-gestation in the mouse, was not induced in S3D5 cells (Figure 3P). FIG. 3Q shows that HOXA13, a marker of the intestinal hindgut endoderm, was not induced in S3D5 cells (FIG. 3P).

[0080] Figures 4A - 4B illustrate the proliferative profile of differentiating S3D5 cells. Figure 4A depicts Caco-2 cells, where most of CDX2-protein positive cells were in active cell cycle (as indicated by the co-expression with KI67 protein) *(left)*, and the proliferative index of the H1-hESC-derived cells (disclosed for reference only) during Stage 3 that decreased over time (S3D2 - *middle;* S3D5 - *right).* CDX2 *(top* row) and KI67 *(bottom* row) protein levels are depicted as single channel images. The percentage KI67-protein positive cells of total S3D5 cells (total cells are >90% CDX2-positive), assessed by FACS, was 16.8±3.12, in contrast with percentage seen at S1D3 (97.3±1.3), and in Caco-2 cells (99.2±0.2) (Figure 4B).

[0081] BMP2 can be used as an alternative to BMP4, during Stage 3 in this method to produce a monolayer of intestinal midgut endoderm cells with CDX2 and FOXA2 protein co-presence. Figure 5A depicts a summary of a differentiation method, including the medium components, growth factors and small molecules that were added to each stage, and key stage-specific markers of the differentiating intestinal midgut endoderm cells (FOXA2, CDX2, KLF5, SOX9, and PDX1^{LO}). Compared to the neutral pH noted at S2D2 (7.35±0.04), cells were exposed to slightly acidic conditions in BLAR medium, during the entirety of stage 3 (pH; S3D1, 6.92; S3D2, 7.01; S3D5, 7.22) (Figure 5B), and as a result of lower sodium bicarbonate levels in BLAR acidic medium. Figure 5C depicts representative phase-contrast images of a S3D5 monolayer *(left),* and Caco-2 cells *(right).* A uniform morphology at S3D5 was observed.

[0082] The differentiation method generates and maintains proper intestinal midgut endoderm cells in monolayer, each comprising of CDX2, FOXA2, KLF5, SOX9, PDX1^{LO} and HOXC5 on transcript and protein levels. All TF protein levels are depicted as single channel IF images. Figure 6A *(bottom)* shows that 94 percent of S3D5 cells were co-present for both CDX2 and FOXA2 protein, similar to percentage seen in Caco-2 cells (86.0±6.67). Gene expression analysis shows that CDX2 was induced (Figure 6B), and FOXA2 maintained (Figure 6C) during Stage 3. Figure 6D shows that CDX2 protein levels and complete CDX2/FOXA2 protein co-presence were induced at S3D5 (Figure 6D), which is comparable to levels observed in Caco-2 cells (Figure 2D-iv). Figure 6E *(bottom)* shows that 99.8 percent of cells were co-present for both CDX2 and SOX9 at S3D5. Strong induction of SOX9 gene expression was observed similar to levels in Caco-2 cells (Figure 6F), and protein presence as assessed by IF-analysis was observed (Figure 6G). 45.5 percent of cells were co-positive for both CDX2 and PDX1 (Figure 6H - *bottom).* PDX1 gene expression was induced at low levels, when compared to pancreas-biased S4D3 cells (Figure 6I), and low to absent protein levels was reflected in the IF-analysis (Figure 6J). Anterior endoderm TF SOX2 was not observed in S3D5 cells, as 0.8

percent of S3D5 cells exhibited SOX2 and CDX2 co-presence (Figure 6K - *bottom;* 6M), and gene expression was below levels seen in hESC and Caco-2 cells (Figure 6L). The gene expression of KLF5, an essential marker for demonstrating proper development of hindgut endoderm, was strongly upregulated at S3D5 (Figure 6N). Protein co-presence of KLF5 within CDX2-positive cells at S3D5 was observed (Figure 6O). ALB gene expression (Figure 6P), and protein presence (Figure 6Q) was not observed in S3D5 cells. The gene expression of pancreas lineage allocating TF, PTF1A, was not induced in S3D5 cells, as compared to pancreas-biased S4D3 cells (Figure 6R). The homeobox gene HOXC5, present in the embryonic intestinal midgut endoderm, was strongly induced in S3D5 cells (Figure 6S). Figure 6T demonstrates that LGR5, a marker of embryonic intestinal endoderm beginning at mid-gestation, was not induced in S3D5 cells. FIG. 6U demonstrates that HOXA13, a marker of the intestinal hindgut endoderm, was not induced in S3D5 cells (FIG. 6U).

[0083] Figure 7 characterizes the proliferative profile of differentiating S3D5 cells, showing Caco-2 cells, where most of CDX2-protein positive cells were in active cell cycle (as indicated by the co-expression with KI67 protein) *(left),* compared to the proliferative index of the H1-hESC-derived cells (disclosed for reference only) during Stage 3 that was lower (S3D5 - *right).* CDX2 *(top* row) and KI67 *(bottom* row) protein levels are depicted as single channel images. The percentage KI67-protein positive cells of total S3D5 cells (total cells are >90% CDX2-positive), as assessed by FACS, was 14.1 percent, in contrast with percentage seen at S1D3 (97.3±1.3), compared to Caco-2 cells (99.2±0.2) (Figure 7; 4B).

REFERENCE EXAMPLE 2

[0084] **Intestinal culturing starting from the definitive endoderm, and using FGF4 and WNT-agonists, generates an endoderm-mesenchyme mixture of CDX2+ mid-/hindgut cells**

[0085] This example demonstrates the endoderm-mesenchyme-mixed quality of the CDX2+ mid-/hindgut cells generated from intestinal culturing beginning at the definitive endoderm stage using FGF4 and WNT-agonists (Spence et al., Nature, 2011; 470:105-109; Watson et al. Nature Med, 2014; 11:1310-1314). To examine the induction to midgut/hindgut endoderm cells described in Spence *et al., infra,* hESCs were differentiated using the protocol below. Note that the differentiation conditions outlined in this Example differ from Example 1 by the following: (i) intestinal condition starting point begins at the definitive endoderm stage; (ii) different growth factors and small molecules are used than RA and BMP4 or BMP2; and (iii) acidic culture conditions are not used.

Materials and Methods

[0086] **Cell culture:** H1-hESC cells, which are disclosed for reference only, were cultured and

maintained as described in Example 1.

[0087] **Differentiation:** The cultures were differentiated using the following protocol.

Stage 1-Mimic (3 days): Cells were cultured for one day in the following Stage 1 medium:

[0088]

RMPI 1640 medium	
(Thermo Fisher Scientific, Catalog No. 11875)	
Penicillin-Streptomycin	1X concentration (1:100 dilution from stock concentration)
(Thermo Fisher Scientific, Catalog No. 15140122)	
L-Glutamine	2mM
(Thermo Fisher Scientific, Catalog No. 25030081)	
Activin A	100 ng/ml
(R&D Systems, Inc., Minneapolis, Minnesota, Catalog No. 338-AC)	

[0089] Cells were then cultured for an additional day in the following media:

RMPI 1640 medium	
Penicillin-Streptomycin	1X concentration (1:100 dilution from stock concentration)
L-Glutamine	2mM
Activin A	100 ng/ml
Defined Fetal Bovine Serum (FBS) (Hyclone, Catalog No. SH30070.02)	0.2%

[0090] Cells were then cultured for an additional day in the following media:

RMPI 1640 medium	
Penicillin-Streptomycin	1X concentration (1:100 dilution from stock concentration)
L-Glutamine	2mM
Activin A	100 ng/ml
Defined FBS	2.0%

[0091] Post-Stage 1 (2 days): For example, pS1d1 is post-Stage 1 day 1 and pS1d2 is post-Stage 1 day 2. Cells were cultured for two days in the following Post-Stage 1 medium :

RMPI 1640 medium	
Penicillin-Streptomycin	1X concentration (1:100 dilution from stock concentration)
L-Glutamine	2mM
FGF4	500 ng/ml
(R&D Systems, Inc., Minneapolis, Minnesota, Catalog No. 235-F4)	
Defined Fetal Bovine Serum (FBS)	2.0%
WNT3A	500 ng/ml
(R&D Systems, Inc., Minneapolis, Minnesota, Catalog No. 5036-WN)	
OR	3µM
Chiron99201 (Stemgent, Catalog No. 0400041)	

[0092] Quantification: Phase contrast imaging and quantification of gene expression followed the procedures in Example 1.

Results

[0093] A summary of the differentiation methods, including medium components, growth factors and small molecules added to each stage, and signature or key stage-specific markers of the differentiating intestinal mid-/hindgut endoderm cells (HANOI) is depicted in FIG. 8A. Intestinal conditioning (post-Stage 1), started at the definitive endoderm stage, with 500 ng/ml FGF4, and either 3µM Chiron99021 (Watson *et al.*), or 500 ng/ml Wnt3A (Spence *et al.*). The term "Stage 1-mimic" refers to the definitive endoderm differentiation protocol in this example that differs from the "S1D3-Original" that refers to Stage 1 conditioning described in Example 1. FIG. 8B shows phase-contrast images of H1-hESC cells (disclosed for reference only) *(top row, left)*, post-Stage 1 cells conditioned two days with 500 ng/ml FGF4 and 3µM Chiron99021 *(top row, middle)*, post-Stage 1 cells conditioned two days with 500 ng/ml FGF4 and 500 ng/ml Wnt3A *(top row, right)*, along with a S3D5 monolayer conditioned by RA/BMP4 *(bottom row, left)*, and a S3D5 monolayer conditioned by RA/BMP2 *(bottom row, right)* (from Example 1).

[0094] Induction of CDX2 gene expression was achieved after two days of conditioning, but at much lower levels compared to RA/BMP2 or RA/BMP4 S3D5 (FIG. 8C). However, the gene expression of the endoderm marker FOXA2 was maintained (FIG. 8D), and

mesoderm/mesenchyme marker HANOI was strongly induced (FIG. 8F). Also, KLF5 was not induced at the two day time point unlike by RA/BMP4 and RA/BMP2 conditioning (FIG. 8E). As conclusively shown in FIG. 8F, this gene expression pattern is reflective of the heterogeneous cell population seen in Watson *et al.* and Spence *et al.* containing not only a CDX2$^+$ FOXA2$^+$ endodermal population, but also a significant mesenchymal CDX2$^+$ cell population. In contrast, RA/BMP4 or RA/BMP2 post-Stage 2 (primitive gut tube cell) conditioning did not induce the mesoderm/mesenchyme marker HAND 1; only endodermal CDX2$^+$ FOXA2$^+$ population was induced.

REFERENCES CITED IN THE DESCRIPTION

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This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

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Patentkrav

1. Population af humane celler i dyrkningsmedium, hvor mere end 50% af cellerne udtrykker markører, der er karakteristiske for intestinal mellemtarm-endoderm,

5 hvor cellerne, der udtrykker markører, der er karakteristiske for intestinal mellemtarmendoderm, udtrykker CDX2, FOXA2, SOX9, PDX1, KLF5 og HOXC5, og ikke udtrykker en transkriptionsfaktor valgt fra SOX2, ALB, PTF1A, HOXA13 og LGR5.

10 **2.** Populationen ifølge krav 1, hvor mere end 60% af cellerne udtrykker markører, der er karakteristiske for intestinal mellemtarmendoderm.

3. Populationen ifølge krav 1, hvor mere end 70% af cellerne udtrykker markører, der er karakteristiske for intestinal mellemtarmendoderm.

15
4. Populationen ifølge krav 1, hvor mere end 80% af cellerne udtrykker markører, der er karakteristiske for intestinal mellemtarmendoderm.

5. Populationen ifølge krav 1, hvor mere end 90% af cellerne udtrykker markører,

20 der er karakteristiske for intestinal mellemtarmendoderm.

6. Populationen ifølge et hvilket som helst af kravene 1 til 5, hvor dyrkningsmediet yderligere omfatter retinsyre og BMP4.

25 **7.** Populationen ifølge et hvilket som helst af kravene 1 til 5, hvor dyrkningsmediet yderligere omfatter retinsyre og BMP2.

8. Populationen ifølge et hvilket som helst af kravene 1 til 7, hvor dyrkningsmediet yderligere omfatter 0,2% serum.

30
9. Populationen ifølge et hvilket som helst af kravene 1 til 7, hvor dyrkningsmediet yderligere omfatter 2% serum.

10. Populationen ifølge et hvilket som helst af kravene 1 til 7, hvor

35 dyrkningsmediet er serumfrit.

DRAWINGS

Drawing

FIG. 1A

Definitive endoderm Stage 1 -3d	Primitive gut tube Stage 2 - 2d	Intestinal midgut endoderm Stage 3 - 5d
MCDB131 0.5% FAF-BSA	MCDB131 0.5% FAF-BSA	BLAR Acidic 0.5% FAF-BSA
GDF8 MCX	FGF7	RA BMP4

Human ES Cell (H1-hESC)

FOXA2

FOXA2

FOXA2

FOXA2, CDX2, KLF5, SOX9, PDX1-o

FIG. 1B

FIG. 1C

Caco2 cells

RA BMP4

S3D5 hES cell monolayer
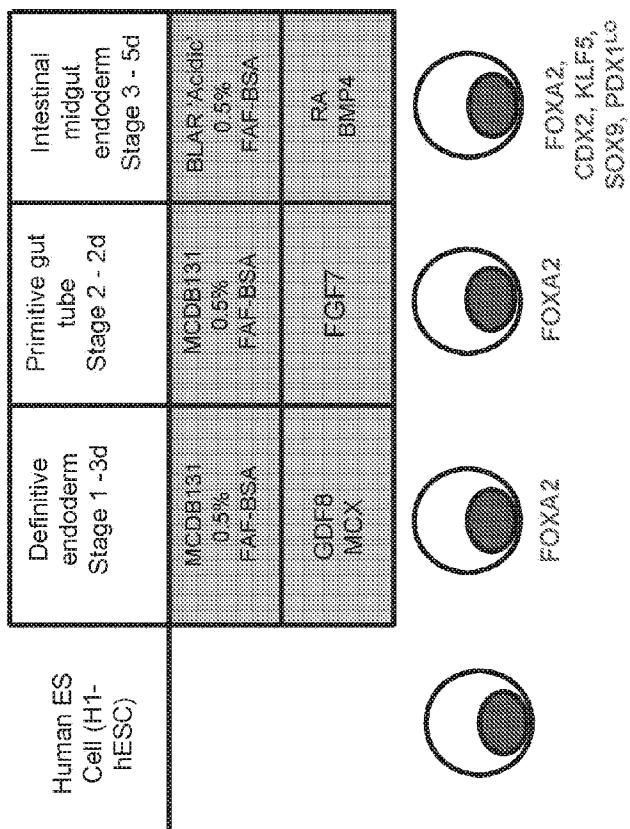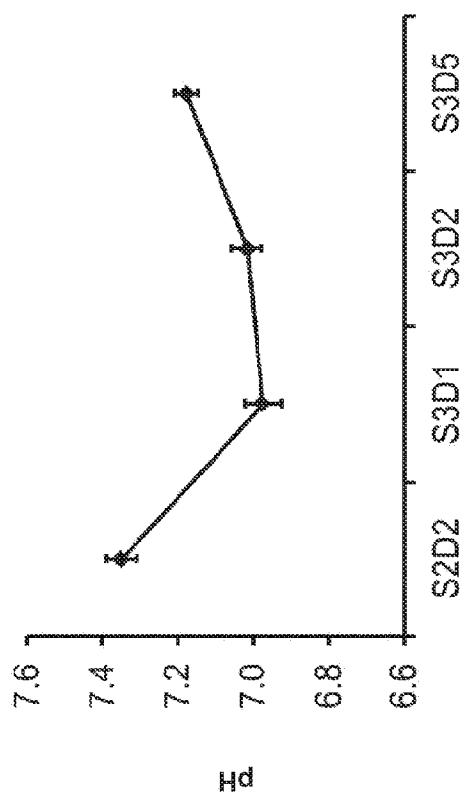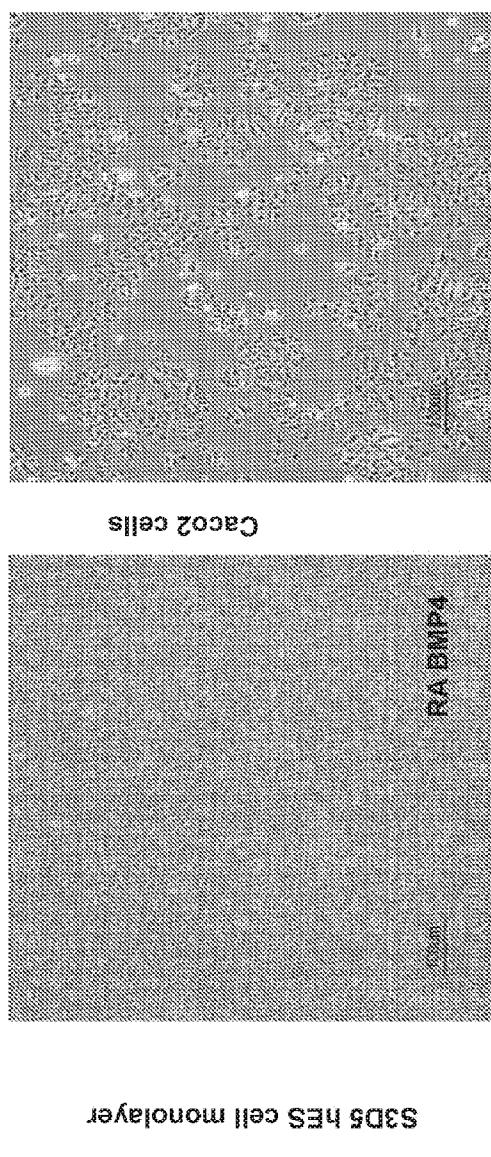
FIG. 1D

H1-hESC [cells/cm^2]	337969 ± 156017
S3D5 [cells/cm^2]	905208 ± 184586
hESC::S3D5	4.56 ± 2.60

FIG. 2A
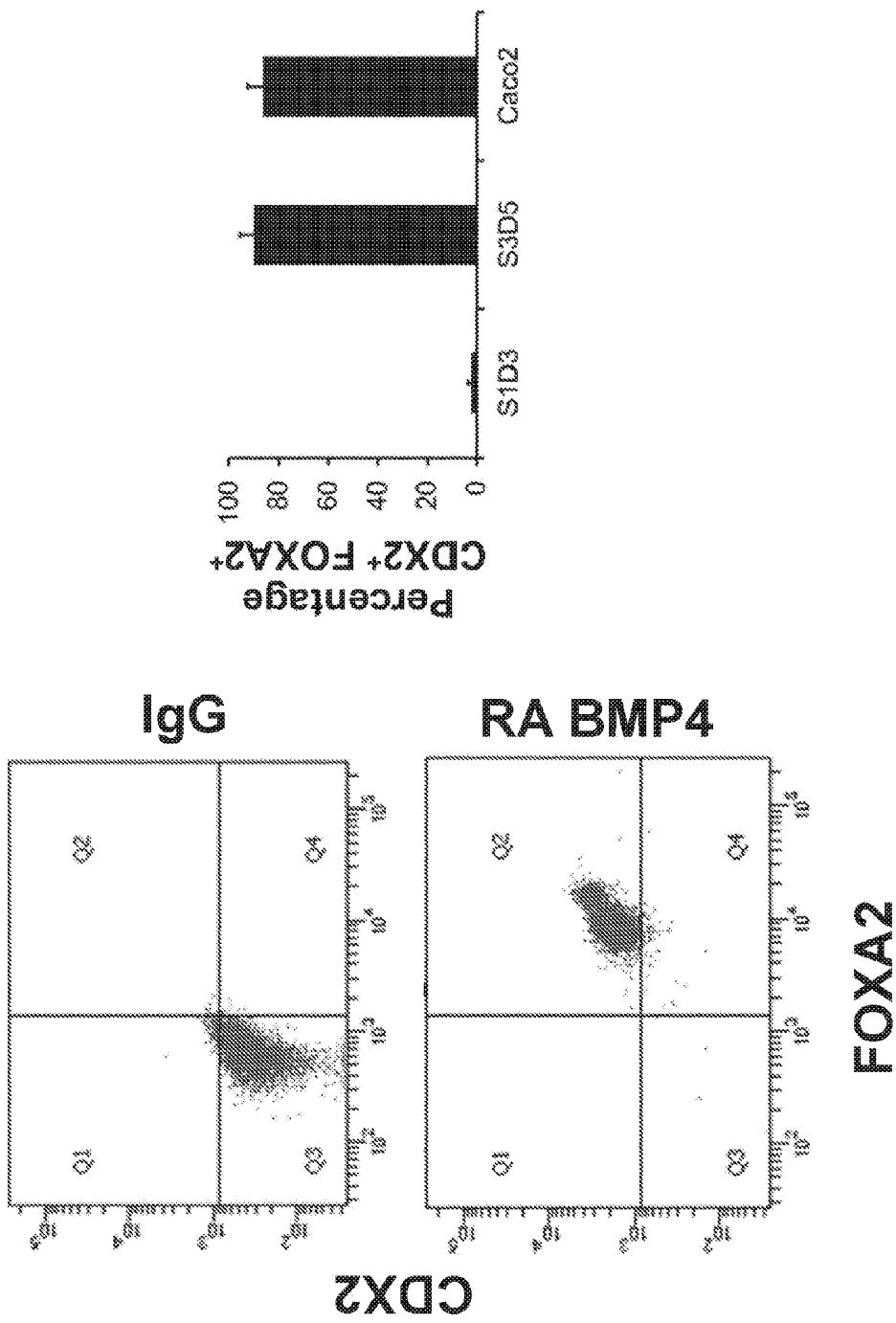
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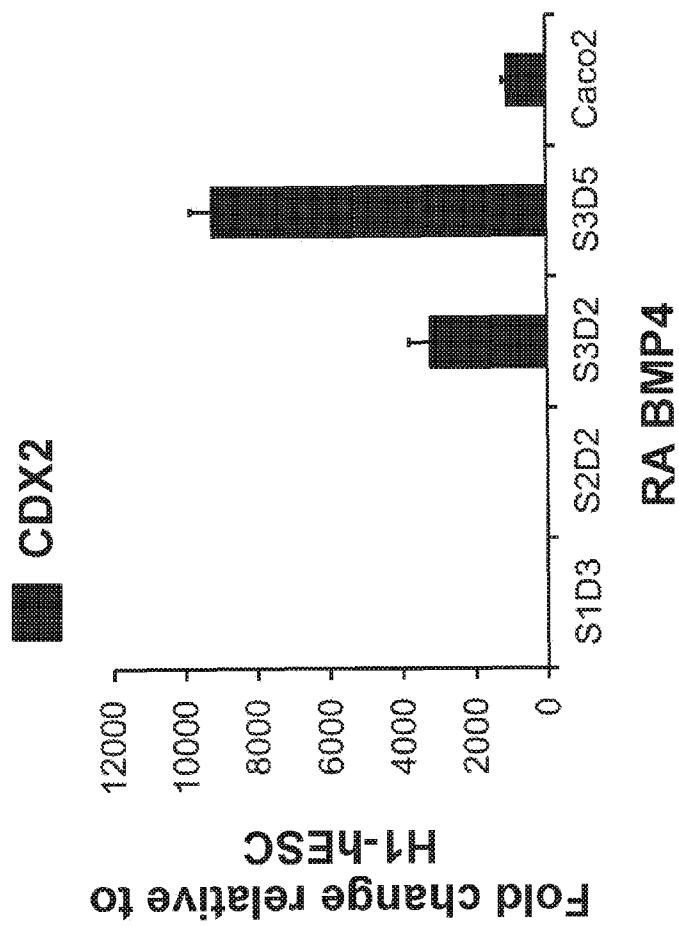
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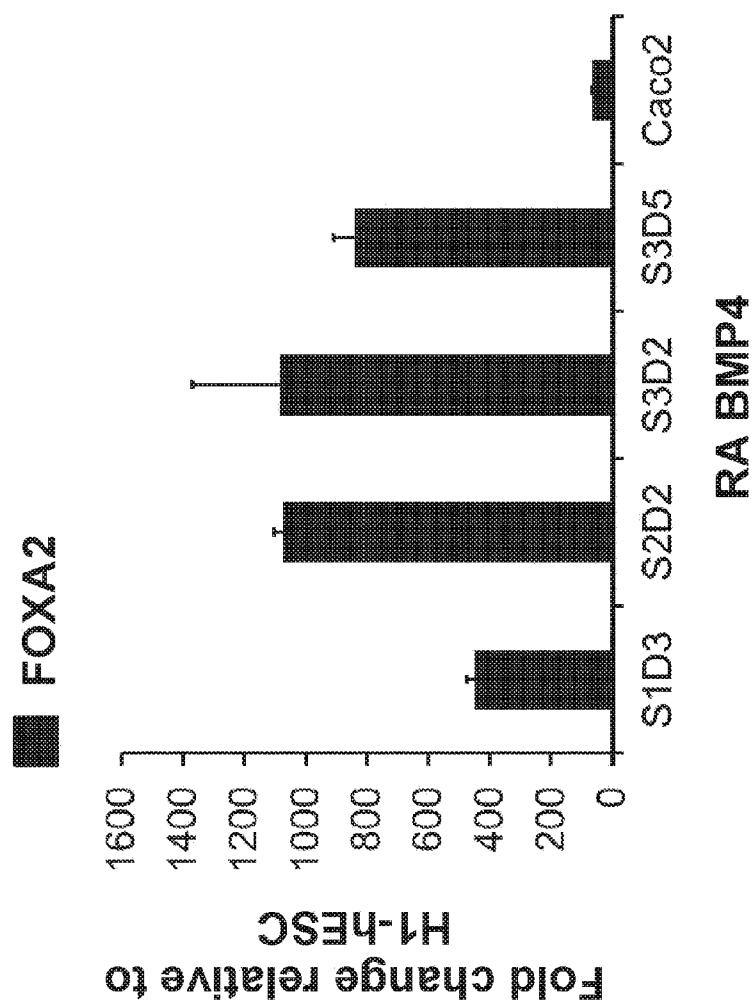
FIG. 2D

FIG. 3A
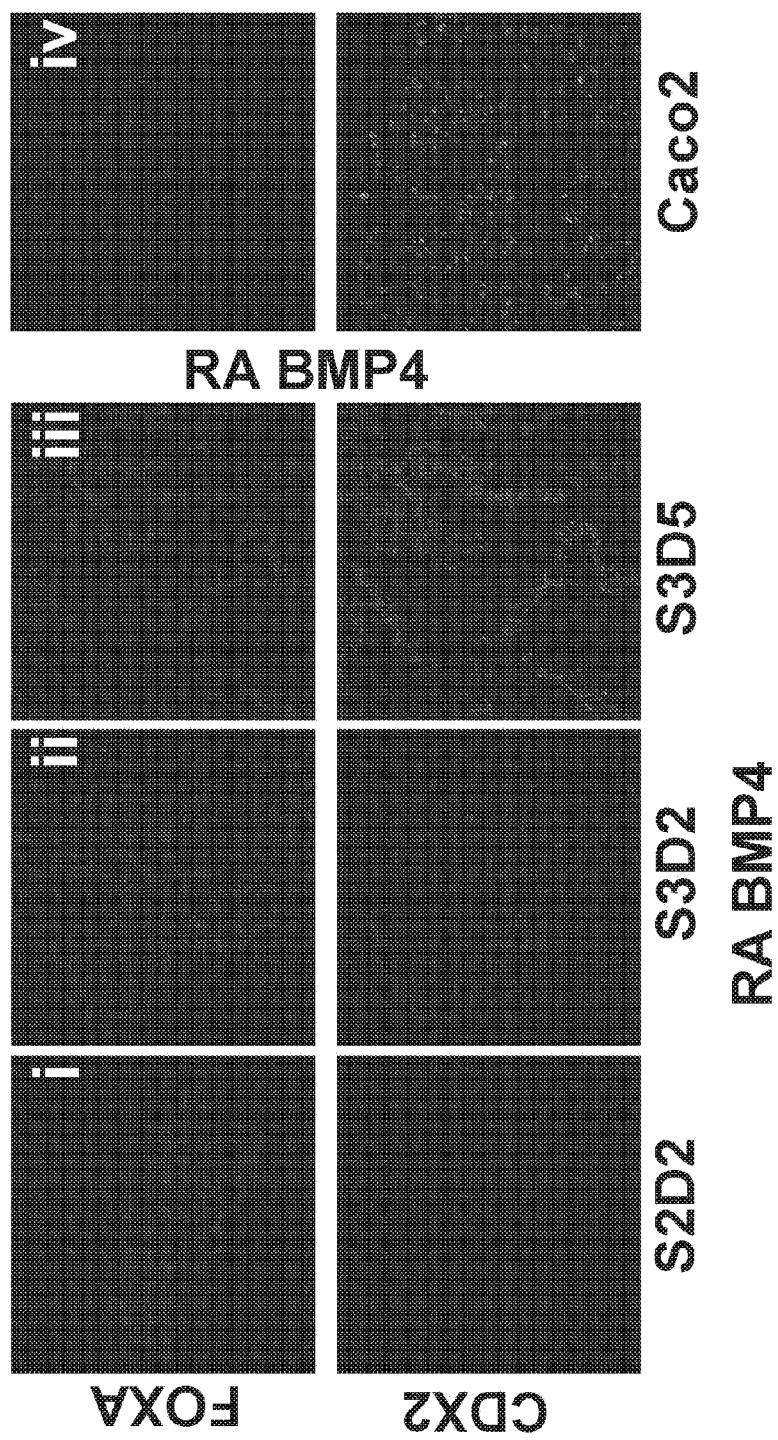
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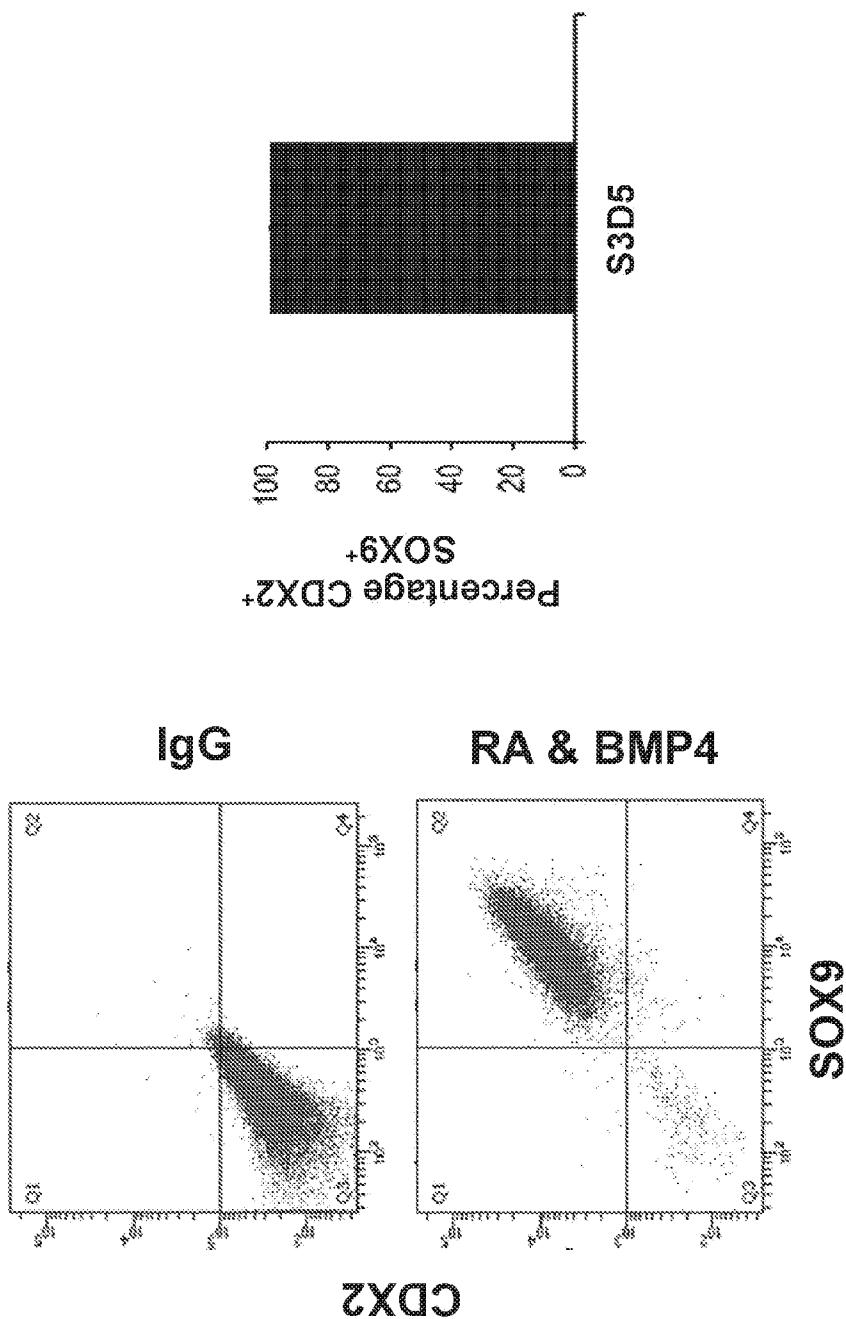
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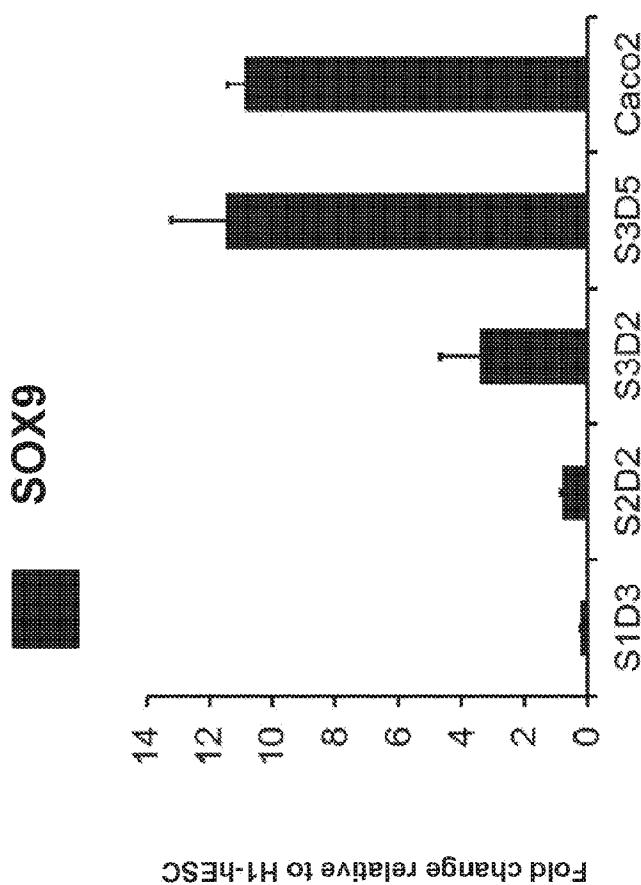
RA BMP4
S3D5
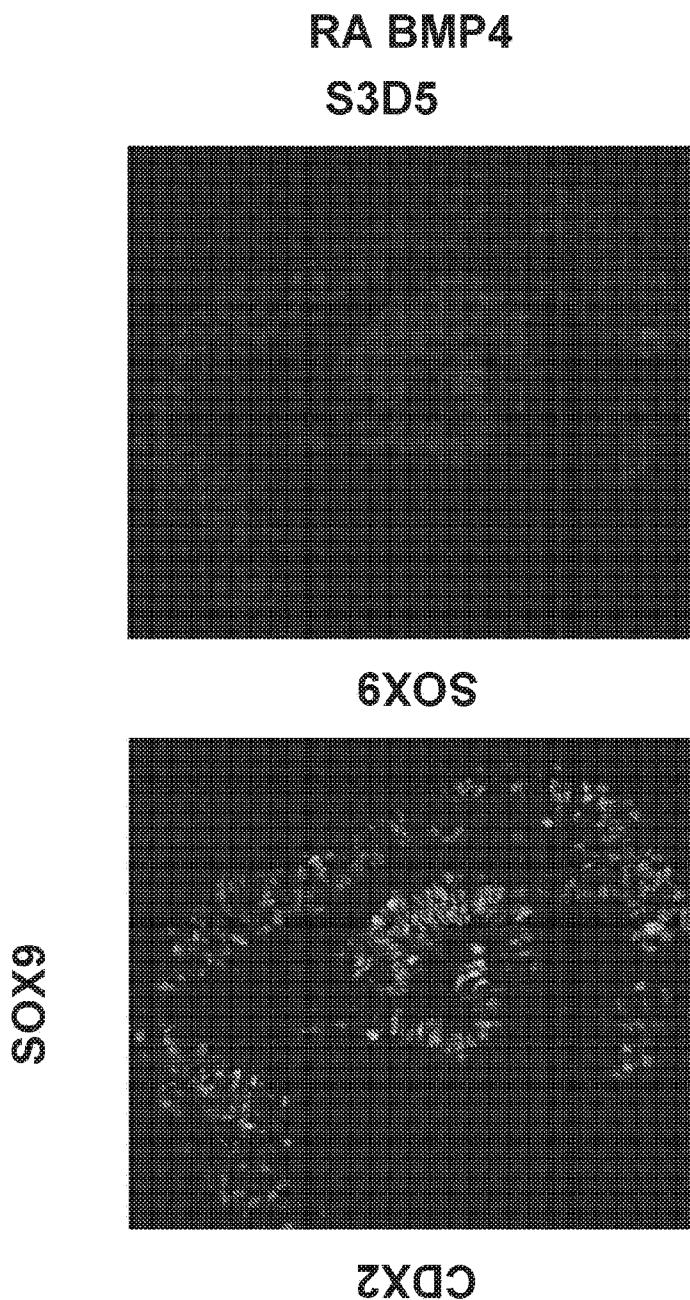
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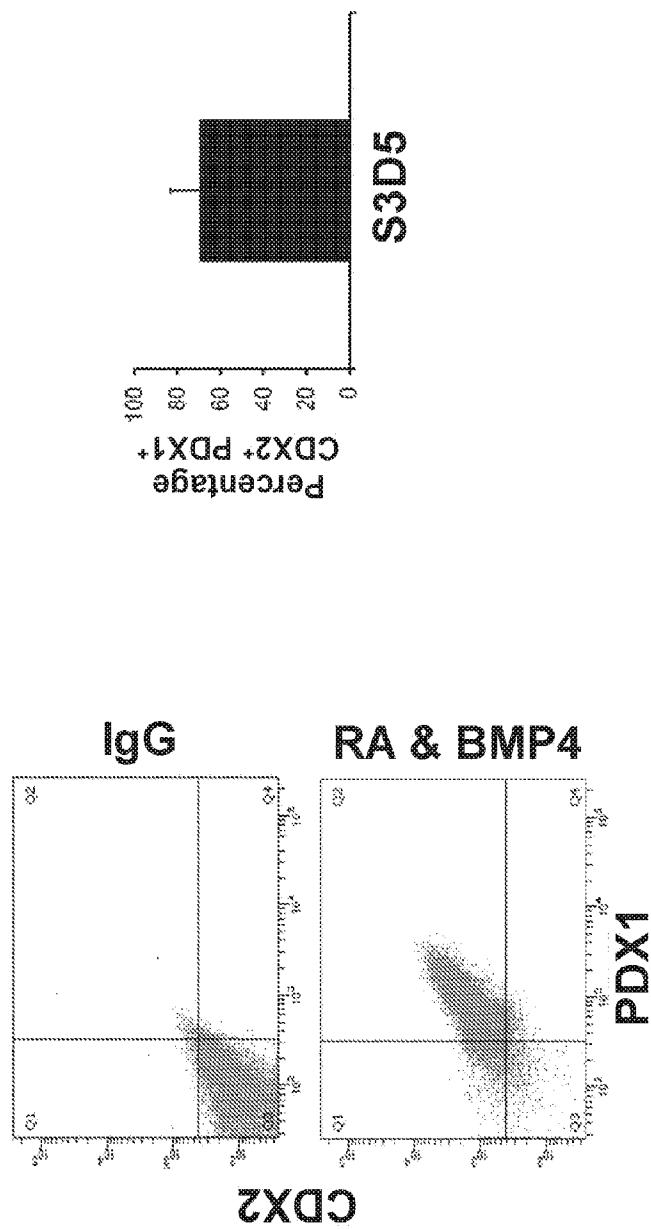
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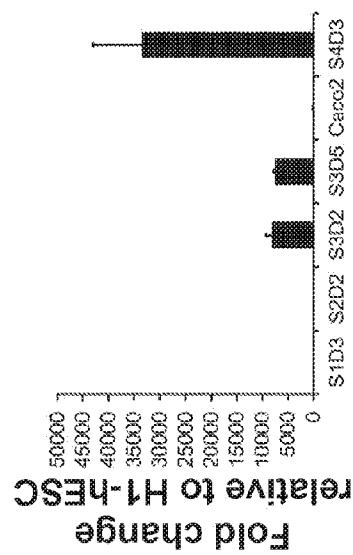
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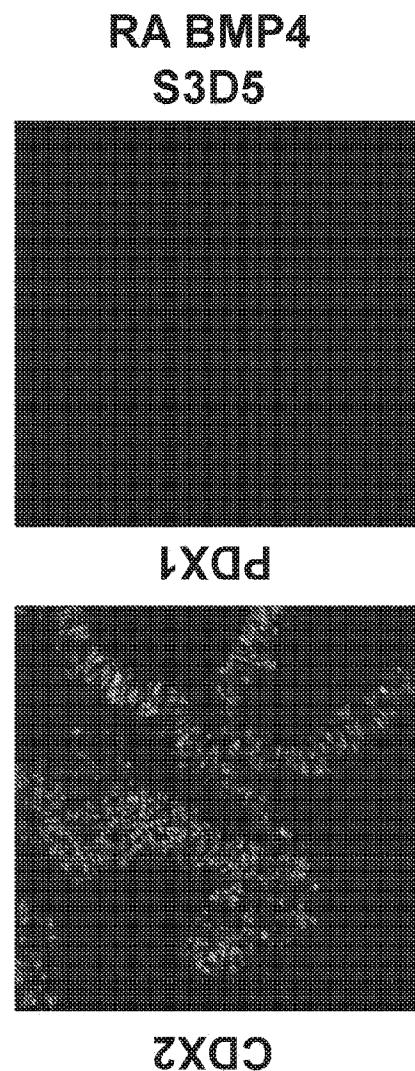
RA BMP4
S3D5

PDX1

CDX2

FIG. 3G
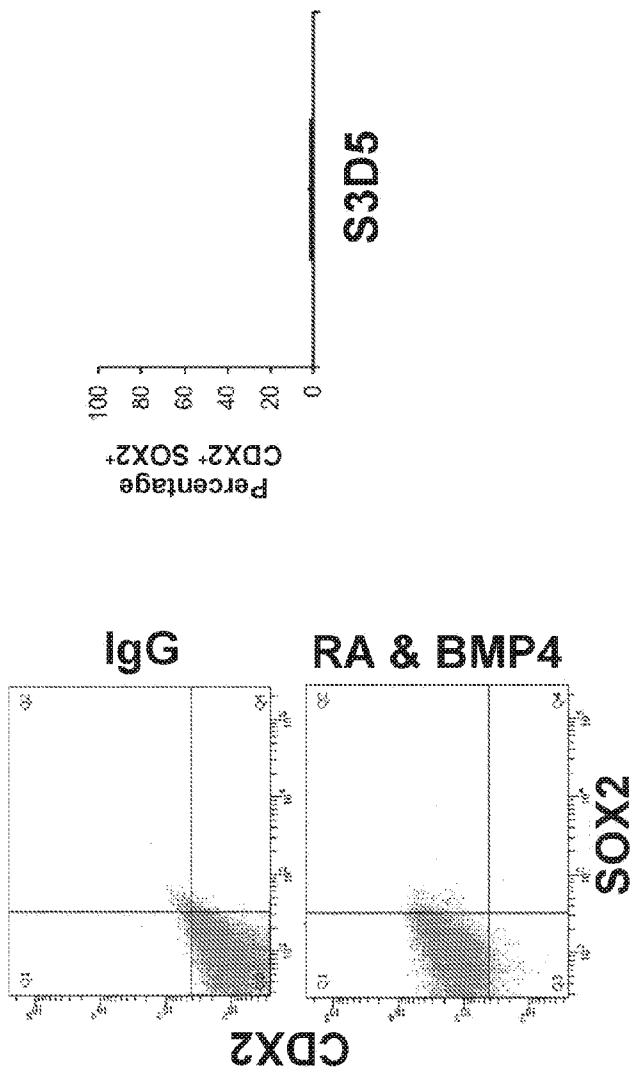
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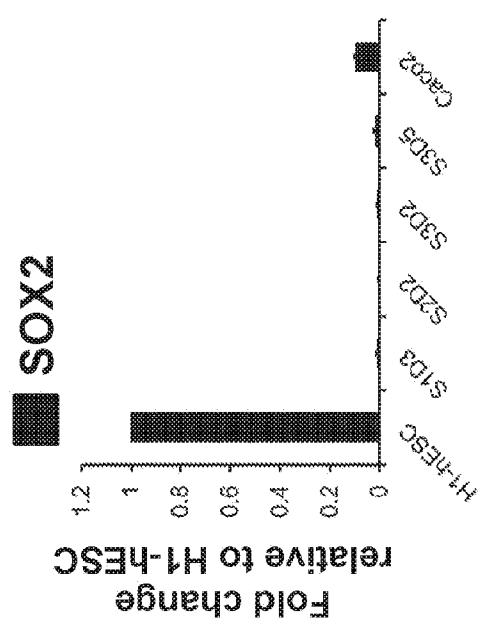
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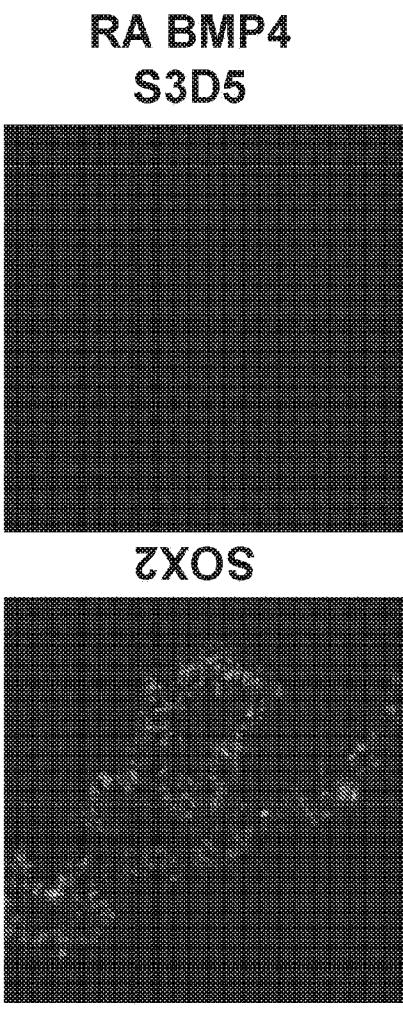
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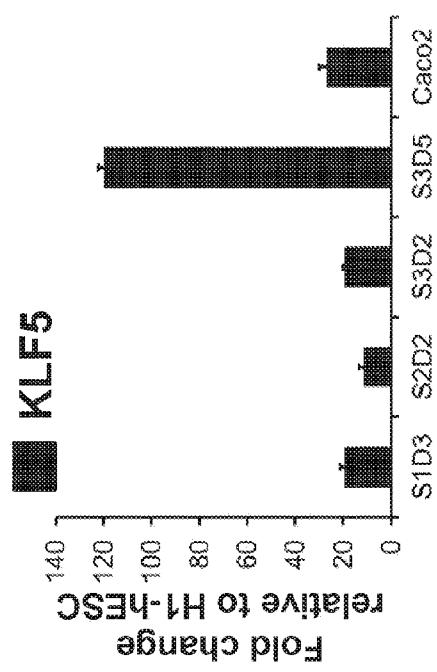
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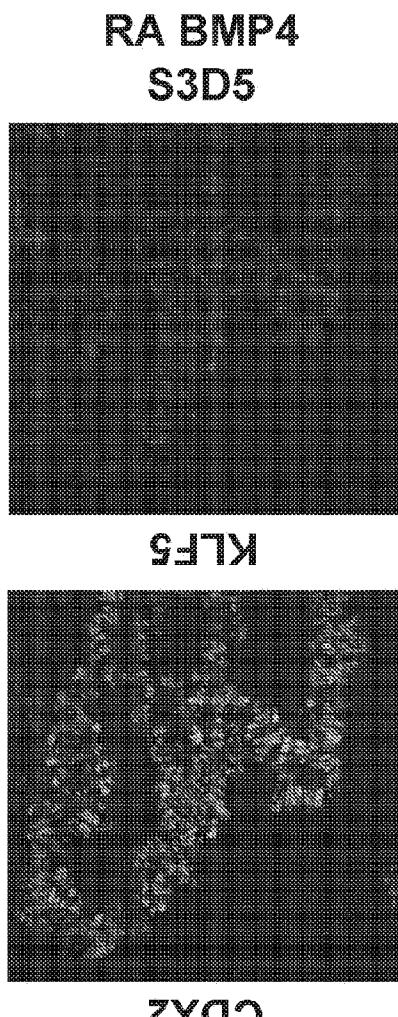
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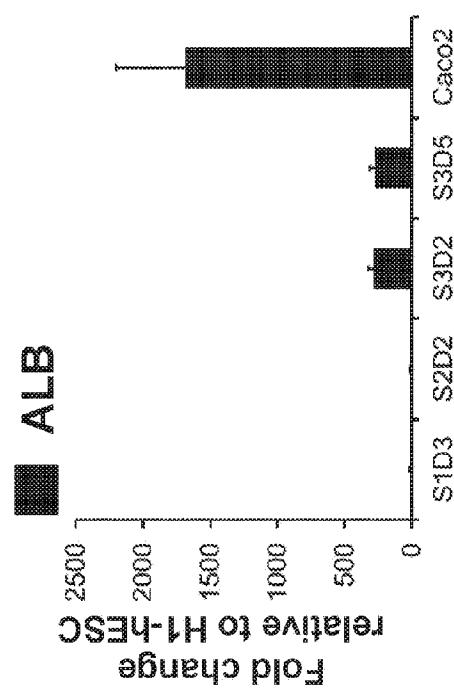
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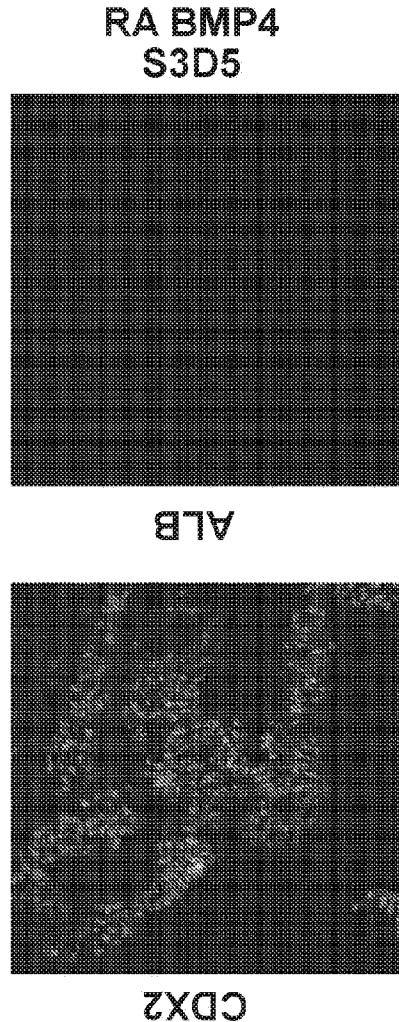
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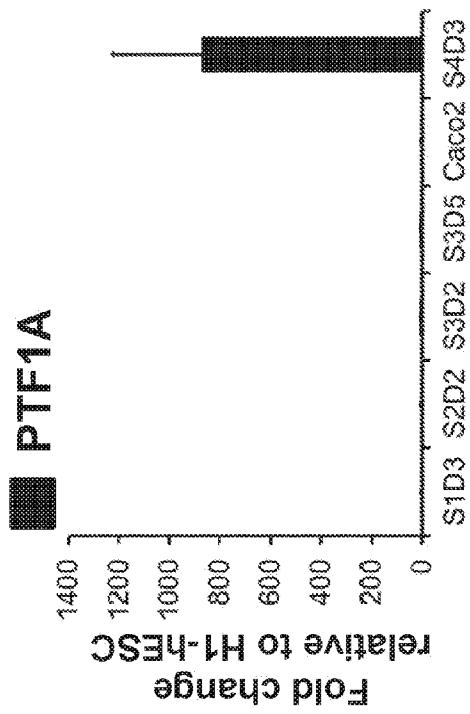
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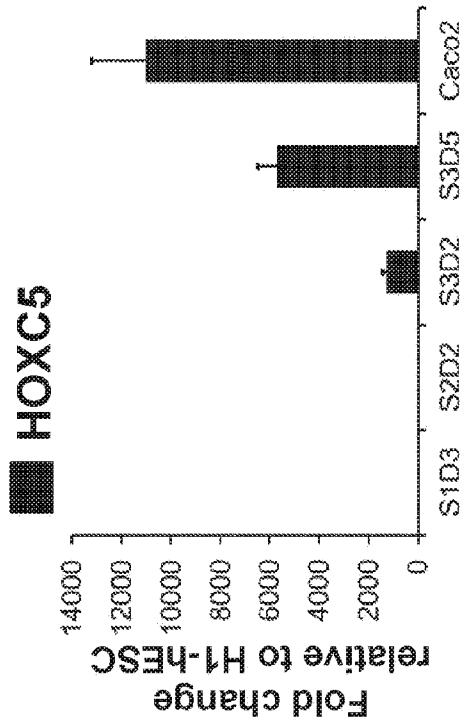
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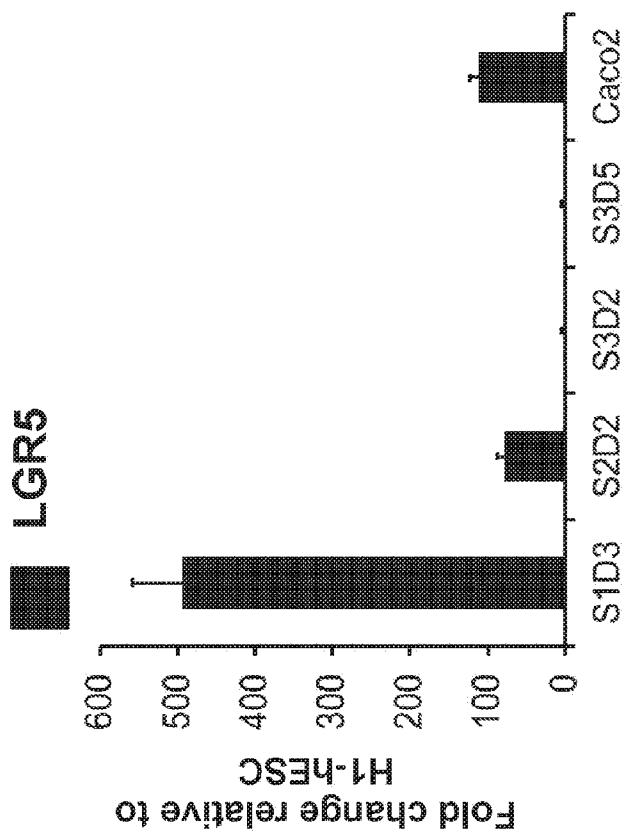
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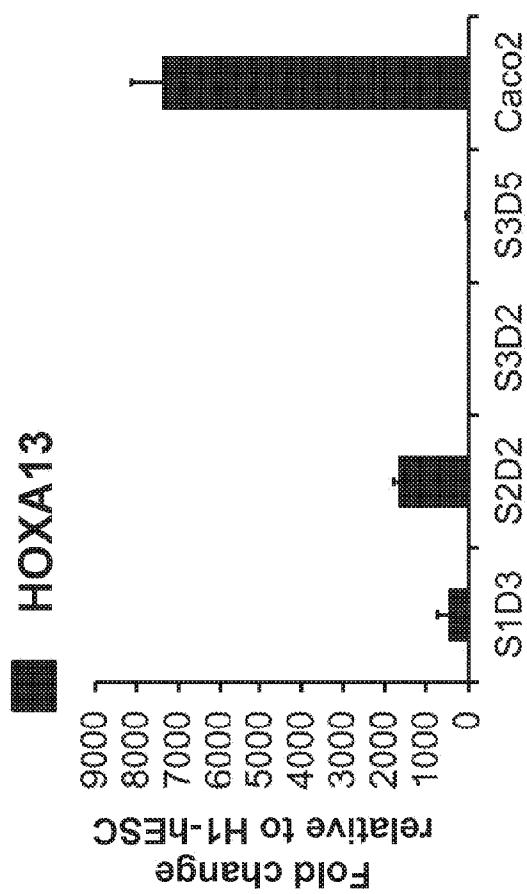
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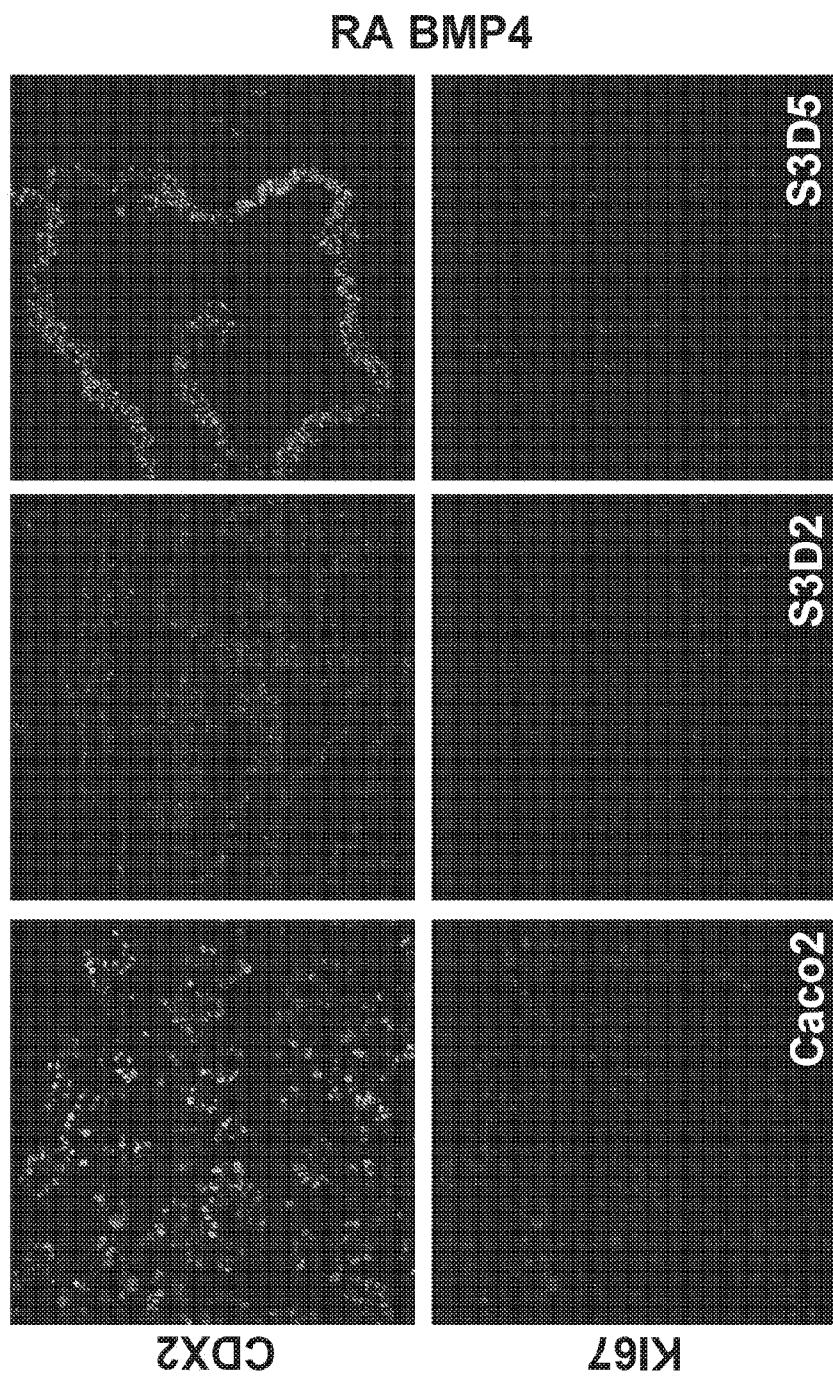
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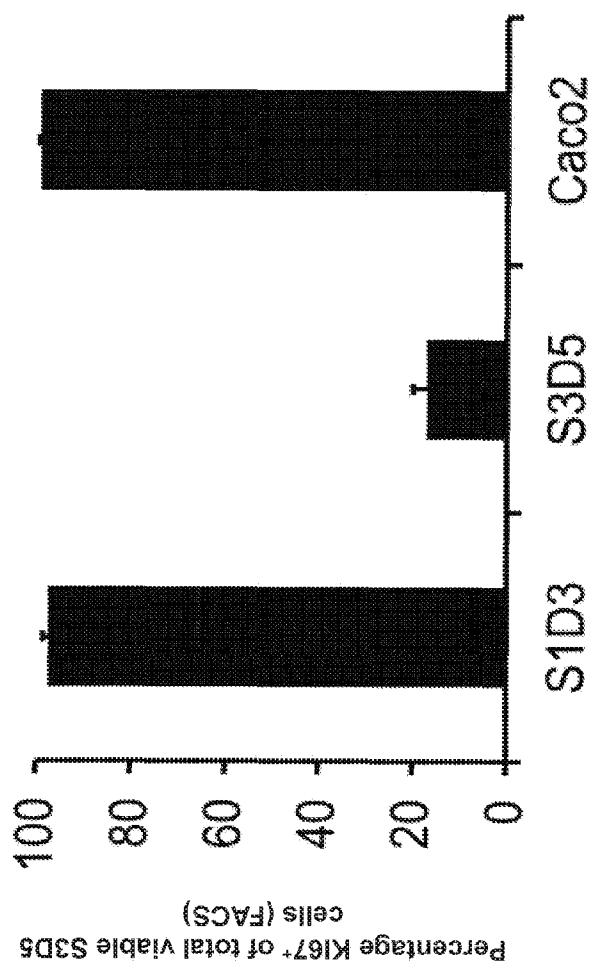
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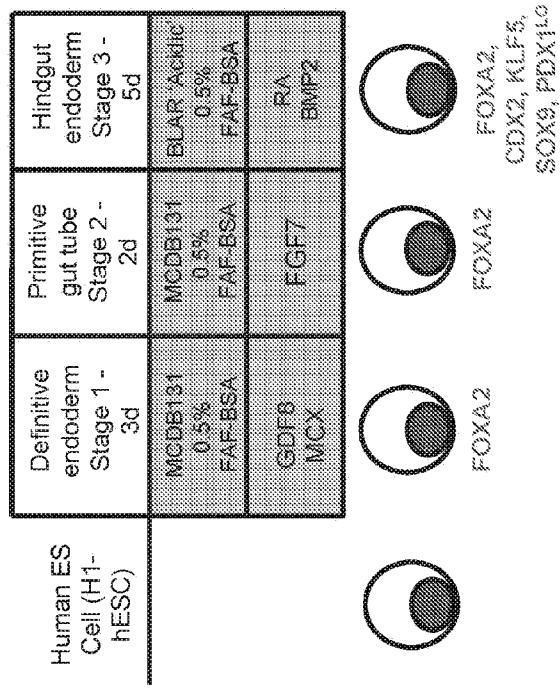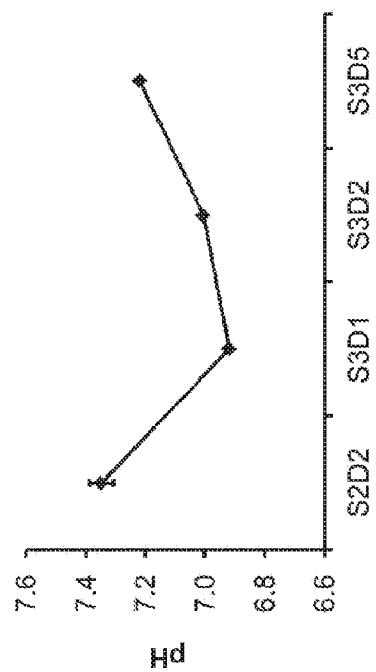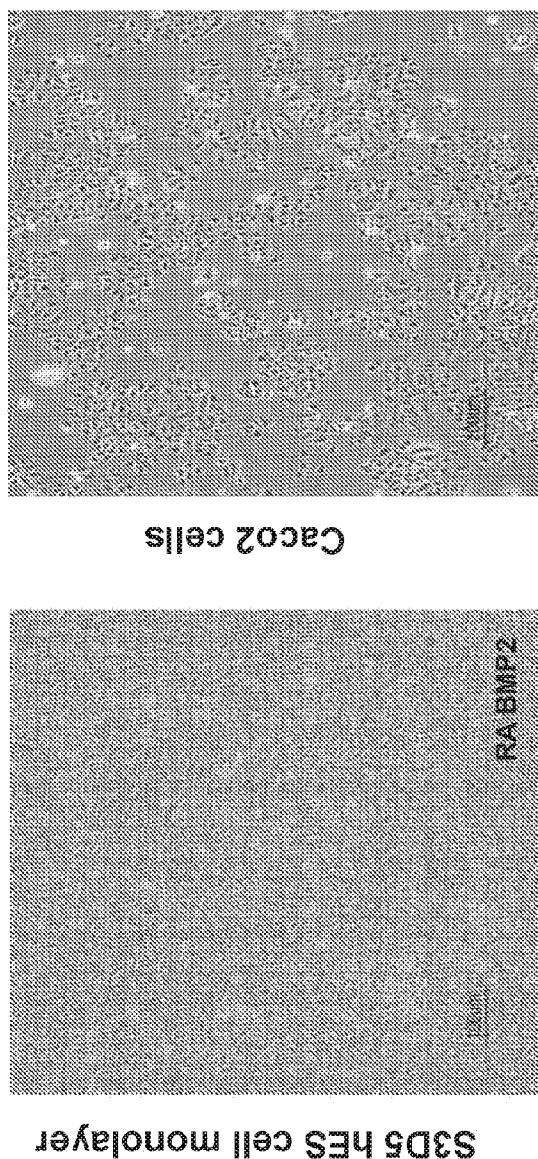
Human ES Cell (H1-hESC)	Definitive endoderm Stage 1 - 3d	Primitive gut tube Stage 2 - 2d	Hindgut endoderm Stage 3 - 5d
	MCDB131 0.5% FAF-BSA	MCDB131 0.5% FAF-BSA	BLAR Acidic 0.5% FAF-BSA
	GDF8 MCX	FGF7	RA, BMP2

FOXA2   FOXA2   FOXA2, CDX2, KLF5, SOX9, PDX1,0

FIG. 5B

FIG. 5C

S3D5 hES cell monolayer

Caco2 cells

FIG. 6A

FIG. 6C

FIG. 6B

FIG. 6D

FIG. 6F

FIG. 6E

RA BMP2
S3D5

FIG. 6G

SOX6

CDX2

FIG. 6I

FIG. 6H

FIG. 6J
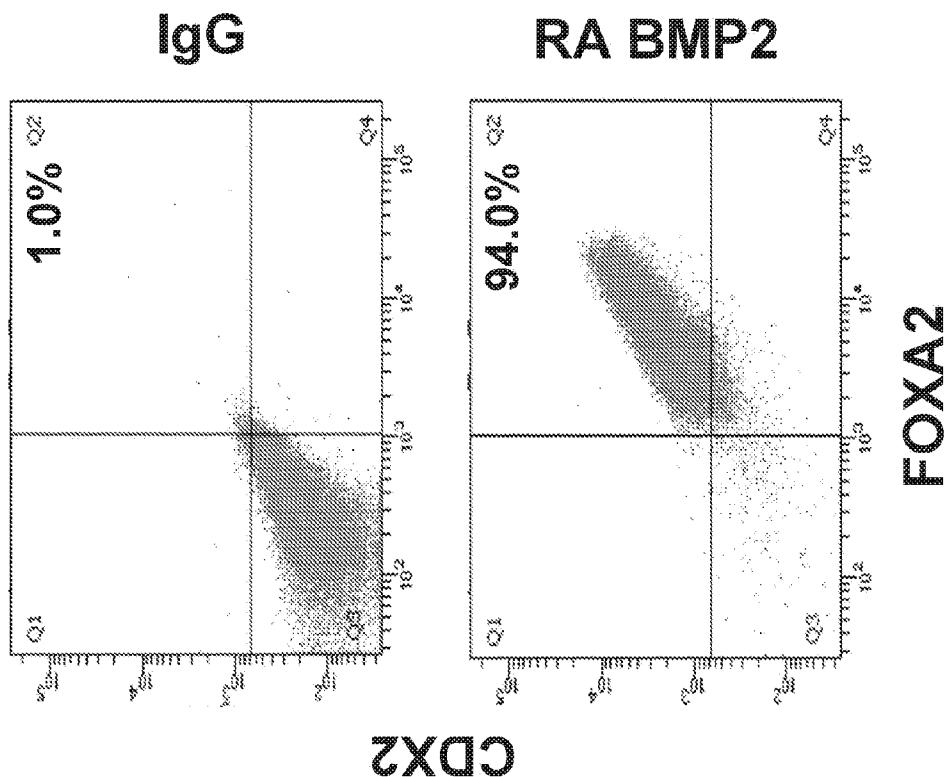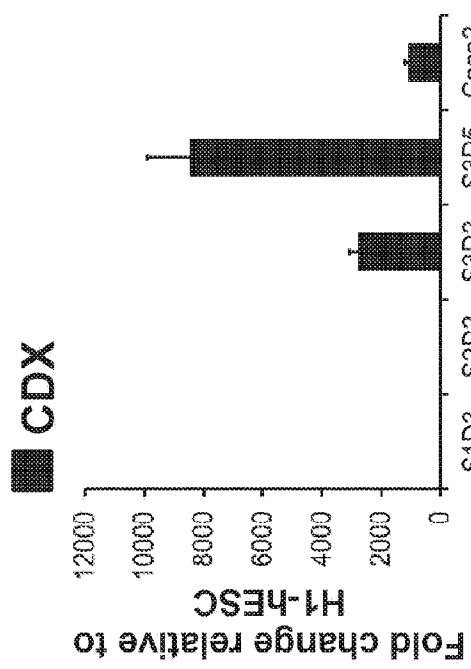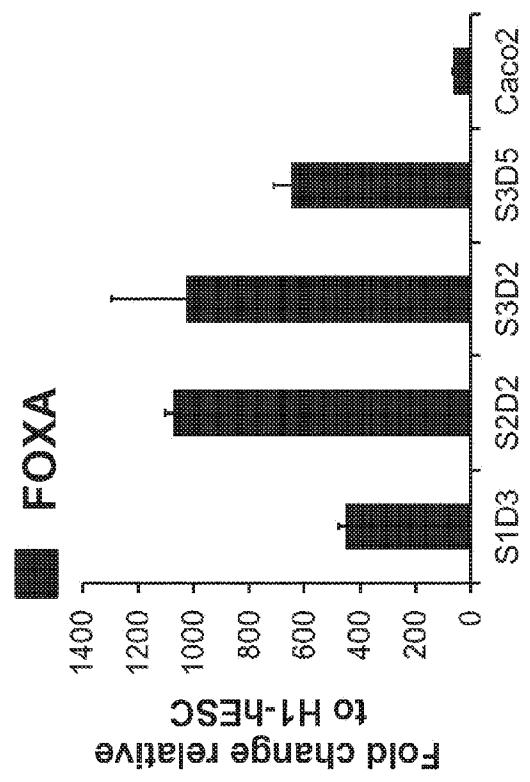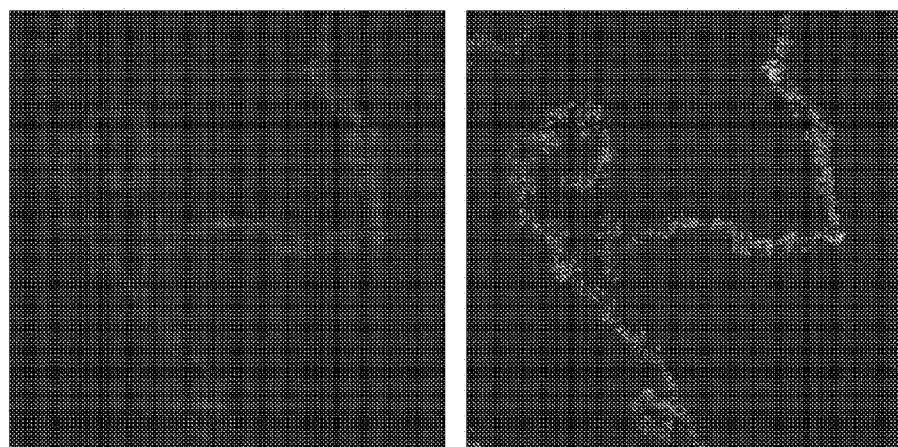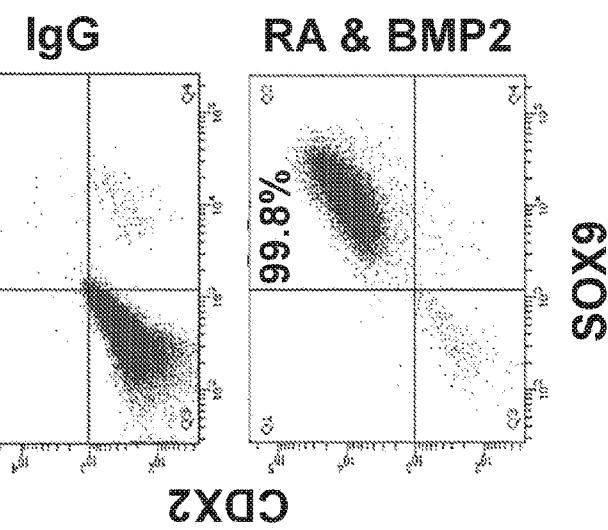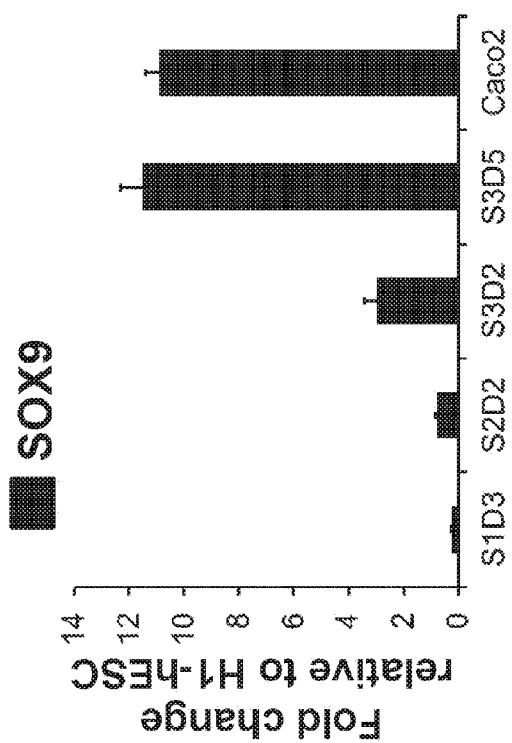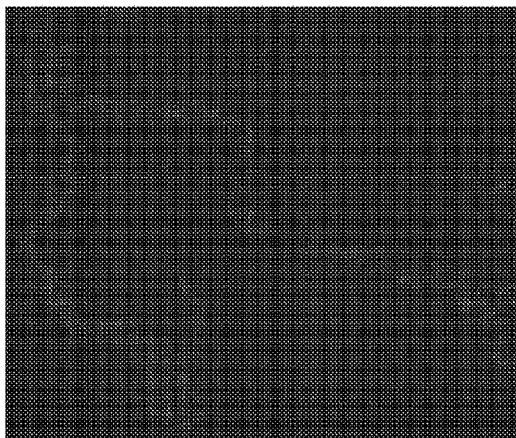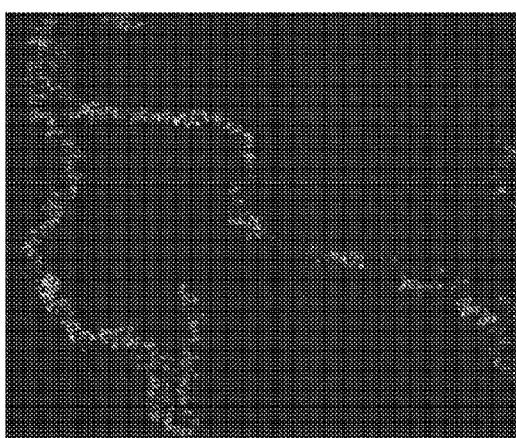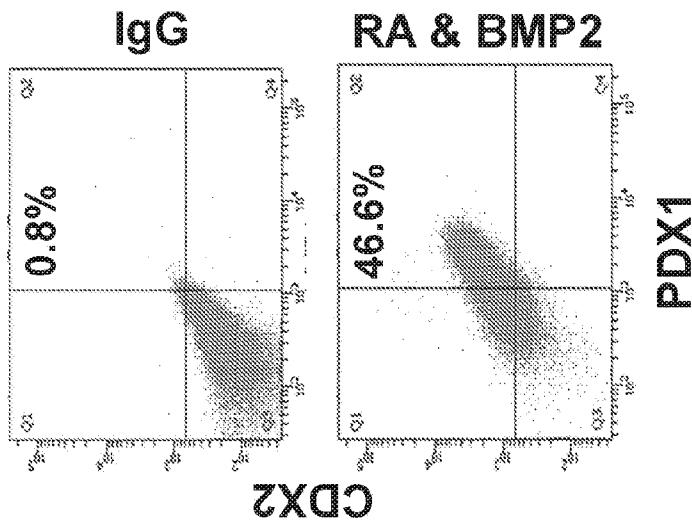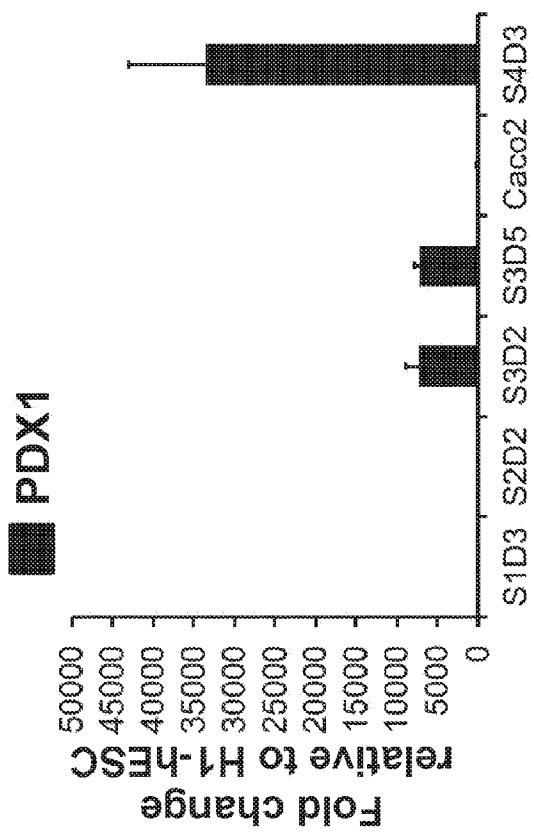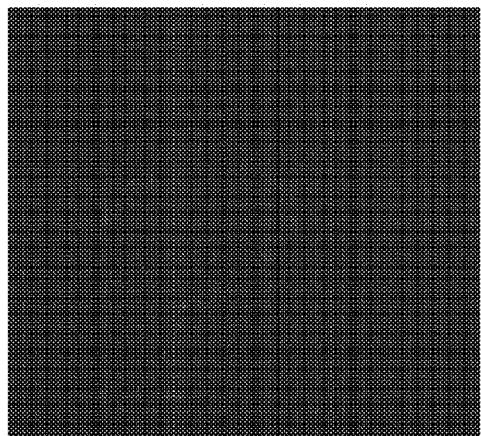
RA BMP2
S3D5

PDX1
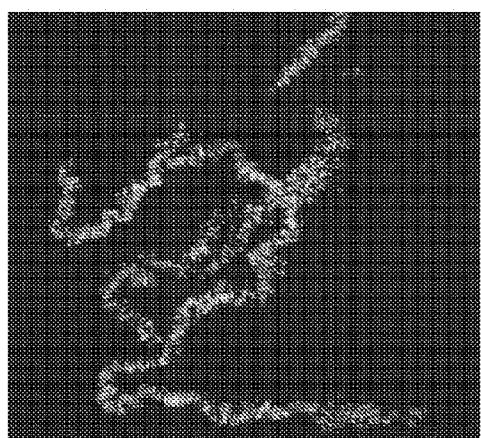
CDX2

FIG. 6L
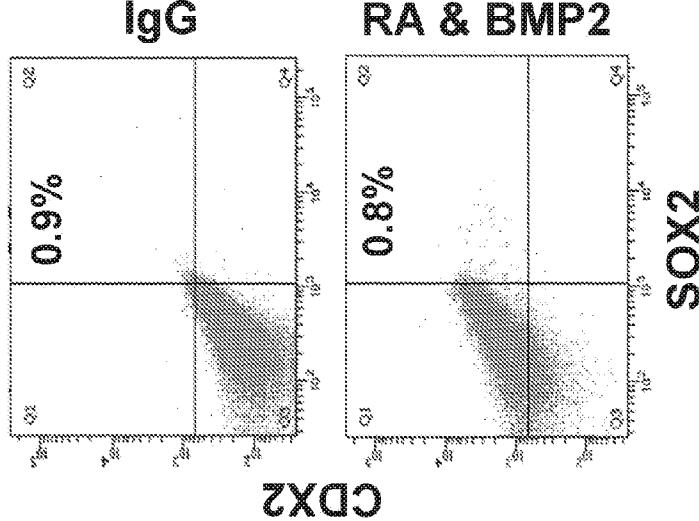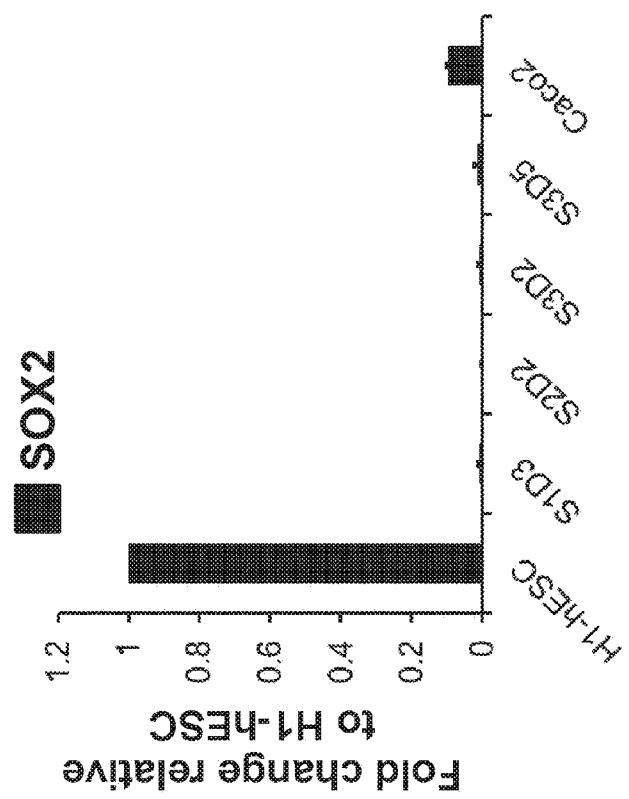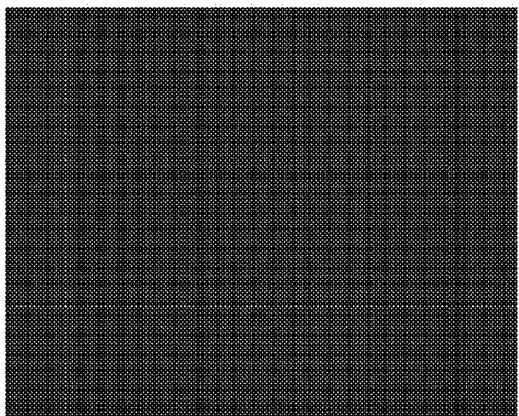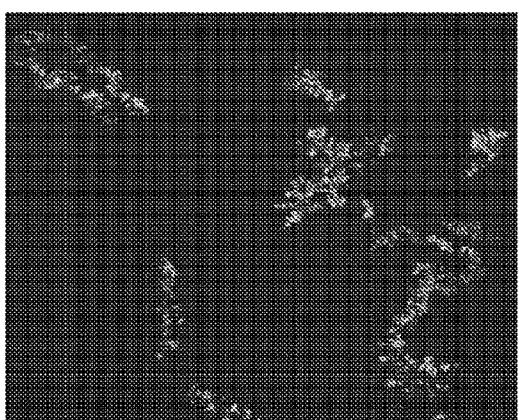
FIG. 6K

RA BMP2
S3D5

FIG. 6M

SOX2

CDX2

FIG. 6N
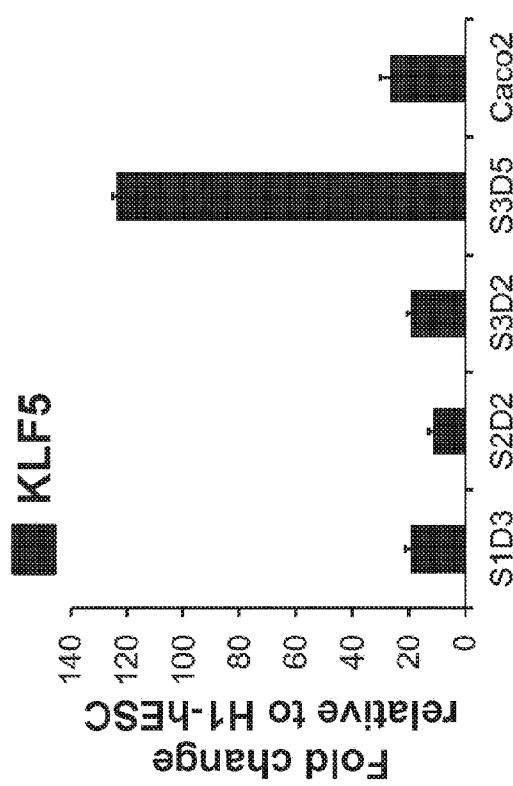
KLF5

FIG. 6O
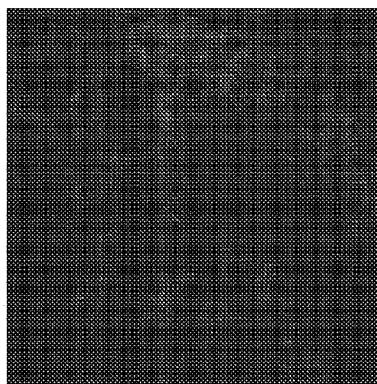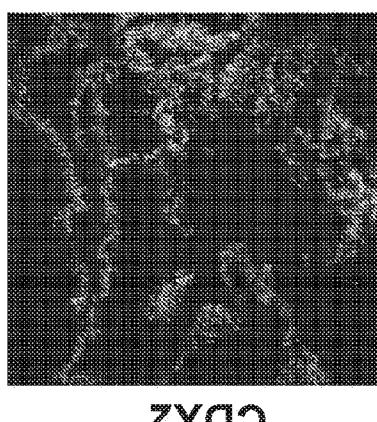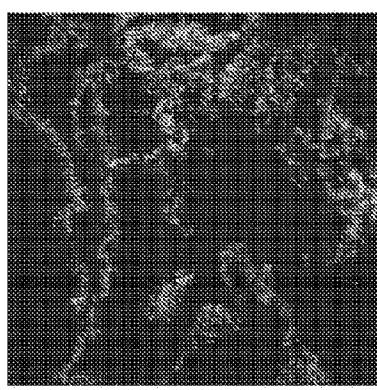
RA BMP2
S3D5

FIG. 6P
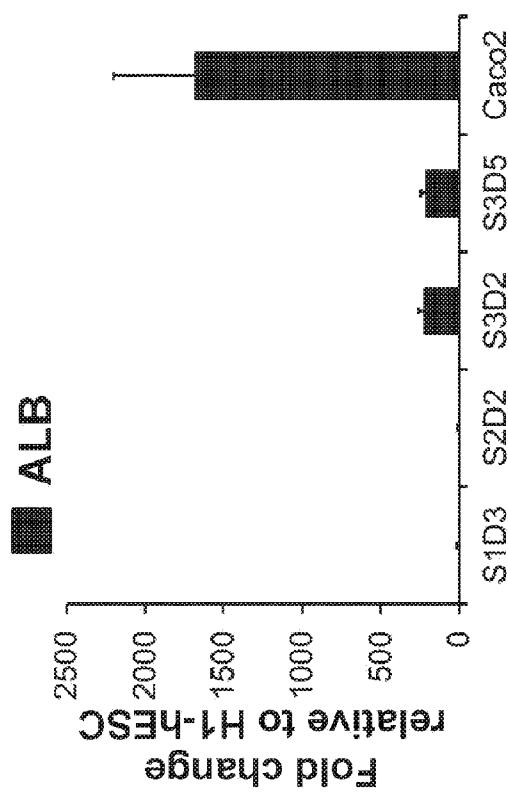
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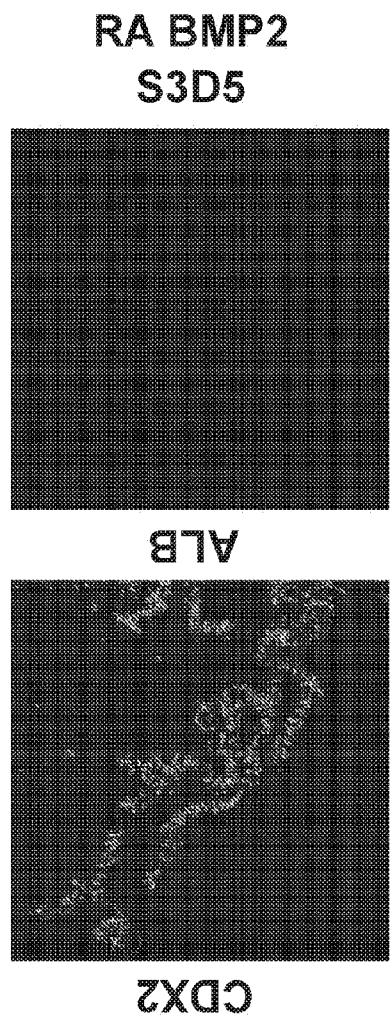
FIG. 6R

FIG. 6S

FIG. 6T

FIG. 6U

FIG. 7

RA BMP2

FIG. 8A

FIG. 8B

Post S1 – day 2
Fgf4 Wnt3A

Post S1 – day 2
Fgf4 Chrion99021

RA BMP2 –
S3D5

H1-hESC

RA BMP4 –
S3D5

FIG. 8C

CDX2

FIG. 8D

FOXA2

Fold change relative to H1-hESC

1000 900 800 700 600 500 400 300 200 100 0

RLE8
S1D3 - Original
S1D3 - Mimic
pS1d2 Fgf4 Chiron99021
pS1d2 Fgf4 Wnt3a
S3D5 RA Bmp4
S3D5 RA Bmp2

FIG. 8E

FIG. 8F