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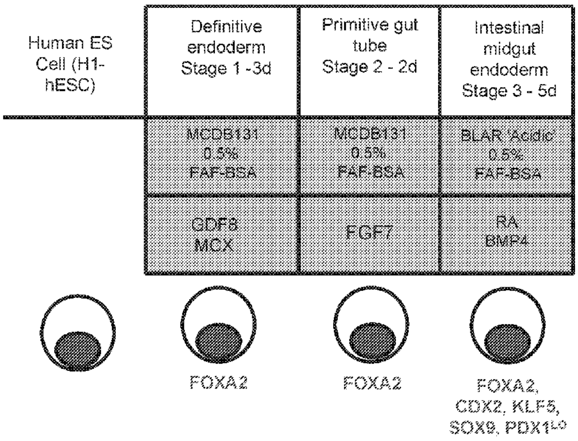
(54) **Title**: DIFFERENTIATION OF PLURIPOTENT STEM CELLS TO INTESTINAL MIDGUT ENDODERM CELLS

FIG. 1A



(57) **Abstract**: Cell populations of intestinal midgut endoderm cells and methods of generating the cells expressing markers characteristic of intestinal endoderm lineage are disclosed. Methods of treating conditions such as diabetes are also disclosed.

WO 2017/180361 A1

DIFFERENTIATION OF PLURIPOTENT STEM CELLS TO INTESTINAL MIDGUT ENDODERM CELLS

FIELD OF INVENTION

[0001] The invention relates to the field of cell-based therapy for conditions such as diabetes. In particular, the invention relates to cell differentiation, including directing differentiation of human pluripotent stem cells to generate a population of intestinal midgut endoderm cells. The invention provides cells or a cell population and methods of producing the cells that express markers characteristic of intestinal midgut endoderm.

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 (HAND1), and forkhead box F1 (FOXF1), 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] As embodied and fully described, the invention provides cells, cell populations and methods of generating the cells by differentiating human pluripotent stem cells. In particular, the invention features 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] One aspect of the invention is a method of producing a population of intestinal midgut endoderm cells comprising culturing human pluripotent stem cells in culture media. In embodiments, the method comprises inducing differentiation of human pluripotent stem cells to intestinal midgut endoderm cells. In some embodiments, a population of intestinal midgut endoderm cells is produced. In some embodiments, a population of substantially intestinal midgut endoderm cells is produced. In embodiments of the invention, the intestinal midgut endoderm cells form and are stable as a monolayer in culture. In embodiments, 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. In embodiments, differentiated cells express markers characteristic of intestinal midgut endoderm are intestinal midgut endoderm cells. In embodiments, the intestinal midgut endoderm cells express CDX2 and

FOXA2. In all embodiments, the intestinal midgut endoderm cells express transcription factors selected from SOX9, PDX1, KLF5 and HOXC5. In embodiments, the intestinal midgut endoderm cells do not express transcription factors selected from SOX2, ALB, PTF1A, HOXA13 and LGR5.

[0009] In embodiments of the invention, human pluripotent stem cells are 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 embodiments, acidic conditions is culture with BLAR medium. The pH of the acidic culture can range from 6.8 to 7.2. In the embodiments of the invention, the intestinal midgut endoderm cells form a monolayer in culture. In embodiments, the monolayer of intestinal midgut endoderm cells is maintained in culture.

[0010] Another embodiment of the invention 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. In embodiments, diabetes is Type 1 or Type 2. In embodiments, administering the cells may be via implantation, injection or otherwise administration directly or indirectly to the site of treatment. In some embodiments, the intestinal midgut endoderm cells are implanted in the body, such as in subcutaneous space, omentum, liver, kidney, etc. Further embodiments encompass encapsulated delivery of the cells including encapsulation of macro- or micro-encapsulation devices.

[0011] A further embodiments of the invention is a method of producing intestinal midgut endoderm cells comprising inducing differentiation of definitive endoderm cells in culture to primitive gut tube cells. In embodiments, the definitive endoderm cells are cultured in culture media containing ascorbic acid and FGF7. In further embodiments, the primitive gut tube cells are cultured in culture media containing retinoic acid and BMP2 or BMP4. The primitive gut tube cells are differentiated to intestinal midgut endoderm cells. In some embodiments, primitive gut tube cells are differentiated to intestinal midgut endoderm cells in acidic conditions (acidic culture media). In particular embodiments, acidic conditions is culture with BLAR medium. The pH of the acidic culture can range from 6.8 to 7.2. In the embodiments, the intestinal midgut endoderm cells form and maintain a monolayer in culture.

[0012] In each of the embodiments discussed above, human pluripotent stem cells are human embryonic stem cells or induced pluripotent stem cells. In each of the embodiments above, the intestinal midgut endoderm cells express CDX2 and FOXA2. In all embodiments, the intestinal midgut endoderm cells express transcription factors selected from SOX9, PDX1, KLF5 and HOXC5. In the embodiments, the intestinal midgut endoderm cells do not express transcription factors selected from SOX2, ALB, PTF1A, HOXA13 and LGR5. In the embodiments above, the intestinal midgut endoderm cells express CDX2, FOXA2, SOX9, PDX1, KLF5 and HOXC5. In the embodiments above, the intestinal midgut endoderm cells do not express SOX2, ALB, PTF1A, HOXA13 and LGR5. In embodiments, 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. In embodiments, differentiated cells express markers characteristic of intestinal midgut endoderm are intestinal midgut endoderm cells. In embodiments, the intestinal midgut endoderm cells express CDX2 and FOXA2. In embodiments, the intestinal midgut endoderm cells express transcription factors selected from SOX9, PDX1, KLF5 and HOXC5. In embodiments, the intestinal midgut endoderm cells do not express transcription factors selected from SOX2, ALB, PTF1A, HOXA13 and LGR5. In the embodiments, intestinal midgut endoderm cells do not express HAND1.

[0013] In the embodiments discussed above, the population of intestinal midgut endoderm cells is substantially intestinal midgut endoderm cells. In some embodiments, the population of intestinal midgut endoderm cells comprises greater than 70% intestinal midgut endoderm cells, preferably greater than 80%, greater than 90%, and greater than 95% of intestinal midgut endoderm cells. In some embodiments, the population of intestinal midgut endoderm cells comprises 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%. In embodiments, intestinal midgut endoderm cells lack expression of HAND1.

[0014] In some embodiments of the invention described above, differentiation is induced *in vitro*. In other embodiments, intestinal midgut endoderm cells differentiate further *in vivo*. Another embodiment relates to the intestinal midgut endoderm cells further differentiating into enteroendocrine cells *in vivo*. The enteroendocrine cells express or secrete incretin hormones. In embodiments, the incretin hormones are GLP1 and GIP.

[0015] In a further embodiment, intestinal midgut endoderm cells 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).

[0017] 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.

[0018] 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.

[0019] 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 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).

[0020] 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.

[0021] 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).

[0022] 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 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).

[0023] 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 (*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 HAND1 (FIG. 8F). KLF5 was not induced (FIG. 8E).

DETAILED DESCRIPTION OF THE INVENTION

[0024] 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.

[0025] The present invention pertains to the generation of intestinal midgut endoderm cells. The cells were generated using a specific culturing sequence. Accordingly, the present invention provides 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. The invention further provides a method for obtaining and maintaining such cells in a monolayer via an *in vitro* cell culture. In certain embodiments, the invention is 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, the present invention provides methods of differentiating pluripotent stem cells to generate intestinal midgut endoderm cells that express CDX2 and FOXA2.

Definitions

[0026] 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. In describing and claiming the present invention, the following terminology will be used.

[0027] 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.

[0028] 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).

[0029] 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.

[0030] 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).

[0031] 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.

[0032] 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.

[0033] “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.

[0034] 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%.

[0035] 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.

[0036] To differente 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”).

[0037] 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 illustrating the present invention, characteristics of the various cell types associated with the above-identified stages are described herein.

[0038] "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.

[0039] "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.

[0040] "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.

[0041] "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.

[0042] "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.

[0043] “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.

[0044] 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.

[0045] 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.

[0046] 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 semi-solid 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). Liquid or fluid pharmaceutical compositions may be administered to a more general location.

[0047] 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.

[0048] 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.

[0049] 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;

HAND1- 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.

Detailed Description

[0050] 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 may be used 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 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). 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 also be used. The human embryonic stem cells used in the methods of the invention 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.), or cells derived from adult human somatic cells, such as, cells disclosed in Takahashi *et al.*, *Cell* 131: 1-12 (2007) may also be used. In certain embodiments, pluripotent stem cells suitable for use in the present invention 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. In certain embodiments, the pluripotent stem cells may be of non-embryonic origins. All of these references, patents, and patent applications are herein incorporated by reference in their entirety, in particular, as they pertain to the isolation, culture, expansion and differentiation of pluripotent cells.

[0051] 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

[0052] 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.

[0053] Exemplary pluripotent stem cells 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). Also 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.

[0054] Also suitable for use in the present invention is a cell that expresses at least one of the markers characteristic of the definitive endoderm lineage. In one embodiment of the invention, a cell expressing markers characteristic of the definitive endoderm lineage is a primitive streak precursor cell. In an alternate embodiment, a cell expressing markers characteristic of the definitive endoderm lineage is a mesendoderm cell. In an alternate embodiment, a cell expressing markers characteristic of the definitive endoderm lineage is a definitive endoderm cell.

[0055] Also suitable for use in the present invention is a cell that expresses at least one of the markers characteristic of the intestinal midgut endoderm lineage. In one embodiment of the present invention, a cell expressing markers characteristic of the intestinal endoderm lineage is an intestinal midgut endoderm cell in which the cell expresses FOXA2 and CDX2. In some embodiments, the cell does not express SOX2, ALB, PTF1A, HOXA13 or LGR5. In embodiments, a cell expressing markers characteristic of the intestinal endoderm lineage is an intestinal midgut endoderm cell in which the cell expresses each of FOXA2, CDX2, SOX9, PDX1, KLF5 and HOXC5. In embodiments, a cell expressing markers characteristic of the intestinal endoderm lineage is an intestinal midgut endoderm cell in which the cell does not express any of SOX2, ALB, PTF1A, HOXA13 and LGR5.

[0056] The invention provides for staged, directed differentiation of pluripotent stem cells toward intestinal midgut endoderm cells using cell culture conditions and media. In embodiments of the invention, 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, is 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.

[0057] 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*

[0058] 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 proposed in this invention. In one embodiment of the invention, pluripotent stem cells are 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; incorporated herein by reference in its entirety) 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 GSK3β 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.

[0059] 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. In one embodiment, the pluripotent cells are 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. In an alternate embodiment, 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.

[0060] 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. In the embodiments, cells expressing markers characteristic of definitive endoderm cells are definitive endoderm cells.

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

[0061] The cells expressing markers characteristic of definitive endoderm cells may be further differentiated into cells expressing markers characteristic of primitive gut tube cells. In one embodiment, 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.

[0062] In another embodiment, differentiation into cells expressing markers characteristic of primitive gut tube cells includes 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^{TM} 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. In one embodiment, cells expressing markers characteristic of definitive endoderm cells are 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. In the embodiments, cells expressing markers characteristic of primitive gut tube cells are 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*

[0063] Cells expressing markers characteristic of primitive gut tube cells may be further differentiated into cells expressing markers characteristic of intestinal midgut endoderm cells. In one embodiment, primitive gut tube cells are 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. In a preferred embodiment, the medium is 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^{TM}; 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. In one embodiment, cells expressing markers characteristic of primitive gut cells are 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).

[0064] The invention relates to 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. In embodiments, the method induces differentiation of human pluripotent stem cell to intestinal midgut endoderm cells in a staged process. In embodiments, a population of intestinal midgut endoderm cells is produced. In some embodiments, a population of substantially intestinal midgut endoderm cells is produced. In the embodiments, the intestinal midgut endoderm cells form and maintain a monolayer on planar culture. In embodiments, the intestinal midgut endoderm cells are 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.

[0065] In embodiments, greater than 50% of the differentiated cells express markers characteristic of intestinal midgut endoderm. In embodiments, greater than 60%, greater than 70%, greater than 80%, greater than 90% or greater than 95% of the differentiated cells express markers characteristic of intestinal midgut endoderm. In embodiments, differentiated cells express markers characteristic of intestinal midgut endoderm are intestinal midgut endoderm cells. In embodiments, the intestinal midgut endoderm cells express CDX2 and FOXA2 as determined by FACS analysis and qPCR. In some embodiments, intestinal midgut endoderm cells express transcription factors selected from SOX9, PDX1, KLF5 and HOXC5 as determined by IF analysis and qPCR. In embodiments, the intestinal midgut endoderm cells do not express transcription factors selected from SOX2, ALB, PTF1A as determined by IF analysis and qPCR, and HOXA13 and LGR5 as determined by qPCR.

[0066] A further embodiment of the invention is a method of producing intestinal midgut endoderm cells comprising inducing differentiation of definitive endoderm cells in culture to primitive gut tube cells. In embodiments, the definitive endoderm cells are cultured in culture media containing ascorbic acid and FGF7. In further embodiments, the primitive gut tube cells are cultured in culture media containing retinoic acid and BMP2 or BMP4. The primitive gut tube cells are differentiated to intestinal midgut endoderm cells. In some embodiments, primitive gut tube cells are differentiated to intestinal midgut endoderm cells in acidic conditions (acidic culture media). In particular embodiments, acidic conditions is 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). In the embodiments, the intestinal midgut endoderm cells form and maintain a monolayer in culture.

[0067] In each of the embodiments discussed herein, human pluripotent stem cells are human hESC or iPS cells. In each of the embodiments above and herein, the intestinal midgut endoderm cells express CDX2 and FOXA2 as determined by FACS analysis and qPCR. In all embodiments, the intestinal midgut endoderm cells express transcription factors selected from SOX9, PDX1, KLF5 and HOXC5 as determined by IF analysis and qPCR. In the embodiments above and herein, the intestinal midgut endoderm cells do not express transcription factors selected from SOX2, ALB, PTF1A as determined by IF analysis and qPCR, and HOXA13 and LGR5 as determined by qPCR. In the embodiments above and herein, the intestinal midgut endoderm cells express CDX2, FOXA2, SOX9, PDX1, KLF5 and HOXC5 by IF analysis and qPCR. In each of the embodiments, 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.

[0068] 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 HAND1 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.

[0069] In certain embodiments, the population of intestinal midgut endoderm cells is substantially intestinal midgut endoderm cells. In some embodiments, the population of intestinal midgut endoderm cells comprises greater than 70% intestinal midgut endoderm cells, preferably greater than 80%, greater than 90%, and greater than 95% of intestinal midgut endoderm cells. In some embodiments, the population of intestinal midgut endoderm cells comprises 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%. In embodiments, intestinal midgut endoderm cells lack expression of HAND1.

Use of Differentiated Intestinal Midgut Endoderm Cells

[0070] In another embodiment of the invention, 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. In such embodiments, differentiated intestinal midgut endoderm cells, or mixtures thereof, may be administered to a patient having diabetes, for example Type 1 or Type 2 diabetes. In embodiments, intestinal midgut endoderm cells differentiate and mature to enteroendocrine cells. In embodiments, intestinal midgut endoderm cells differentiate and mature to enteroendocrine cells, and the enteroendocrine cells express or secrete incretin type hormones. In embodiments, incretin hormones include GLP1 and GIP. Administration of the cells may be via implantation or injection in the body, in particular implantation into subcutaneous space, omentum, liver, kidney, etc.

[0071] In some embodiments of the invention described above, differentiation of intestinal midgut endoderm cells is induced *in vitro*. In other embodiments, intestinal midgut endoderm cells further differentiate and mature *in vivo*. Another embodiment relates to the intestinal midgut endoderm cells further differentiating into enteroendocrine cells *in vivo* or in a mixture with enteroendocrine cells *in vivo*. Such enteroendocrine cells express or secrete incretin hormones. In embodiments, the enteroendocrine cell-secreted incretin hormones include GLP1 and GIP.

[0072] In a further embodiment, intestinal midgut endoderm cells 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.

[0073] Cells and cell populations and mixtures such as those described herein may be micro- or macro-encapsulated and subsequently transplanted into a mammalian host. 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

[0074] The invention can be further understood in view of the following non-limiting examples.

Example 1

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

[0075] 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

[0076] **Cell culture**: Cells of the human embryonic stem cell line H1 ("H1-hESC") (WA01 cells, WiCell Research Institute, Madison, Wisconsin) cultured with EZ8 media (Cat#A1516901 Gibco, Thermo Fisher Scientific) at passage 28 were seeded as single cells at 0.094 x 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™ (Life Technologies Corporation, Carlsbad, California, Catalog No. 35050-079)	1:100 dilution ("1X concentration")
Ascorbic acid (Sigma Aldrich Co. LLC, St. Louis, Missouri, Catalog No. A4544)	0.25mM

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

[0077] 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.

[0078] **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, which is incorporated by reference in its entirety; 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 (Sigma-Aldrich Co. LLC, St. Louis, Missouri, Catalog No. 5761)	2.7 g/1000 ml
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 OR BMP2 compound (R&D Systems, Inc., Minneapolis, Minnesota, Catalog No. 355-BM),	50 ng/ml

[0079] 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
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 ($NaHCO_3$)	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

[0080] 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, incorporated herein by reference in its entirety, 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"). IgG FACS 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

Antigen	Species	Source/Catalog Number	Dilution
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

[0081] 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 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. ab16667)	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	Rabbit	Life Technologies (Catalog No. A10040)	1:200

Antigen	Species	Source	Dilution
Antibody, Alexa Fluor 546			
Donkey anti-goat IgG (H+L) Secondary Antibody, Alexa Fluor 546	Goat	Life Technologies (Catalog No. A11056)	1:50-1:100
Goat anti-rabbit IgG, HRP-Labeled	Rabbit	Perkin Elmer, Waltham, MA (Catalog No. NEF812001)	1:250

[0082] For quantification of gene expression at various stages, Caco-2, H1-hESC, 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

	Gene	**Assay ID**
10	KLF5	Hs00156145_m1
11	LGR5	Hs00969422_m1
12	HOXA13	Hs00426284_ml
13	HAND1	Hs00231848_ml

Results

[0083] 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).

[0084] 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.

[0085] 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).

[0086] 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).

[0087] 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 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).

[0088] 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.

[0089] 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).

[0090] 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 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).

EXAMPLE 2

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

[0091] 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

[0092] Cell culture: H1-hESC cells were cultured and maintained as described in Example 1.

[0093] 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:

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

[0094] 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%

[0095] 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%

[0096] 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 (R&D Systems, Inc., Minneapolis, Minnesota, Catalog No. 235-F4)	500 ng/ml
Defined Fetal Bovine Serum (FBS)	2.0%
WNT3A (R&D Systems, Inc., Minneapolis, Minnesota, Catalog No. 5036-WN)	500 ng/ml

OR Chiron99201 (Stemgent, Catalog No. 0400041)	3μM

[0097] **Quantification:** Phase contrast imaging and quantification of gene expression followed the procedures in Example 1.

Results

[0098] 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 (HAND1) 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 (*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).

[0099] 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 HAND1 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 HAND1; only endodermal CDX2$^+$ FOXA2$^+$ population was induced.

[0100] In describing the present invention and its various embodiments, specific terminology is employed for the sake of clarity. However, the invention is not intended to be limited to the specific terminology so selected. A person skilled in the relevant art will recognize that other equivalent components can be employed and other methods developed without departing from the broad concepts of the current invention. All references cited anywhere in this specification are incorporated by reference as if each had been individually incorporated.

CLAIMS

1. A method of producing a population of intestinal midgut endoderm cells comprising culturing human pluripotent stem cells in culture media to induce differentiation to intestinal midgut endoderm cells that express at least transcription factors CDX2 and FOXA2, wherein a population of substantially intestinal midgut endoderm cells is produced.

2. A method of producing a population of intestinal midgut endoderm cells comprising culturing human pluripotent stem cells in culture media comprising an effective amount of a transforming growth factor (TGF)-beta receptor signaling molecule, a glycogen synthase kinase 3 beta (GSK3beta) inhibitor, fibroblast growth factor (FGF), ascorbic acid, retinoic acid and a bone morphogenic protein (BMP), to induce differentiation to intestinal midgut endoderm cells,

wherein the differentiated cells are intestinal midgut endoderm cells, and wherein the intestinal midgut endoderm cells form and remain stable as a monolayer,

wherein method comprises the steps of:

a. culturing the human pluripotent stem cells in a first culture media containing GDF-8 and a GSK3β inhibitor compound to definitive endoderm cells;

b. culturing the definitive endoderm cells in a second culture media containing ascorbic acid and FGF7 to primitive gut tube cells; and

c. culturing primitive gut tube cells in a third culture media containing retinoic acid and BMP2 or BMP4 to intestinal midgut endoderm cells.

3. The method of claim 1 or claim 2, wherein the intestinal midgut endoderm cells express CDX2 and FOXA2.

4. The method of claim 1 or claim 2, wherein the intestinal midgut endoderm cells express transcription factors selected from the group consisting of SOX9, PDX1, KLF5 and HOXC5.

5. The method of claim 1 or claim 2, wherein the intestinal midgut endoderm cells do not express transcription factors selected from the group consisting of SOX2, ALB, PTF1A, HOXA13 and LGR5.

6. The method of claim 1, wherein the intestinal midgut endoderm cells form and maintain a monolayer in culture.

7. The method of claim 1 or claim 2, wherein the population of cells does not include mesenchymal cells.

8. The method of claim 1 or claim 2, wherein the population of cells does not express HAND1.

9. The method of claim 1 or claim 2, wherein differentiation is induced *in vitro*.

10. 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 implanting the differentiated cells in the patient.

11. The method of claim 10, further comprising differentiating the implanted intestinal midgut endoderm cells *in vivo*.

12. The method of claim 10, wherein the diabetes is Type 1 or Type 2.

13. The method of claim 11, wherein the intestinal midgut endoderm cells are further differentiated *in vivo* into enteroendocrine cells, wherein the enteroendocrine cells secrete incretin hormones.

14. The method of claim 13, wherein the incretin hormones are GLP1 and GIP.

15. The method of claim 10, wherein the intestinal midgut endoderm cells express CDX2 and FOXA2.

16. The method of claim 10, wherein the intestinal midgut endoderm cells express transcription factors selected from the group consisting of SOX9, PDX1, KLF5 and HOXC5.

17. The method of claim 10, wherein the intestinal midgut endoderm cells do not express transcription factors selected from the group consisting of SOX2, ALB, PTF1A, HOXA13 and LGR5.

18. A method of producing intestinal midgut endoderm cells comprising inducing differentiation of definitive endoderm cells in culture to primitive gut tube cells, wherein the definitive endoderm cells are cultured in culture media containing ascorbic acid and FGF7.

19. The method of claim 18, wherein the primitive gut tube cells are cultured in culture media containing retinoic acid and BMP2 or BMP4 to differentiate to intestinal midgut endoderm cells.

20. The method of claim 18, wherein the culture media is acidic.

21. The method of claim 18, wherein the intestinal midgut endoderm cells form and maintain a monolayer in culture.

22. Intestinal midgut endoderm cells when produced by the process of any one of claims 1 to 9 or 18 to 21.

FIG. 1A

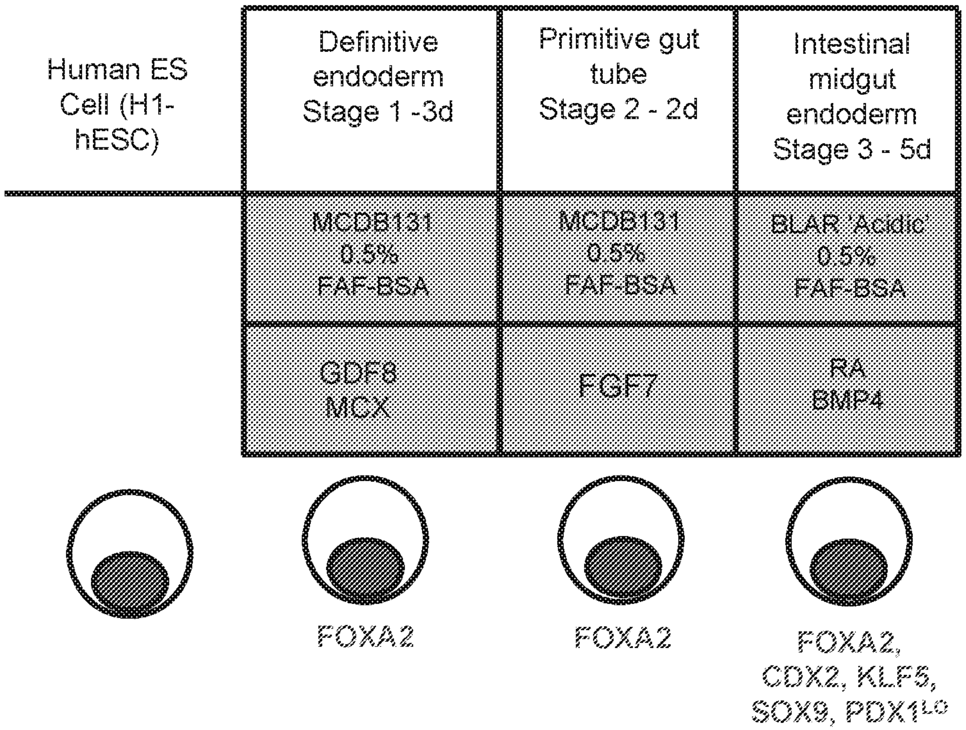

Human ES Cell (H1-hESC)
Definitive endoderm Stage 1 -3d
Primitive gut tube Stage 2 - 2d
Intestinal midgut endoderm Stage 3 - 5d
MCDB131 0.5% FAF-BSA
MCDB131 0.5% FAF-BSA
BLAR 'Acidic' 0.5% FAF-BSA
GDF8 MCX
FGF7
RA BMP4
FOXA2
FOXA2
FOXA2, CDX2, KLF5, SOX9, PDX1^LO

FIG. 1B

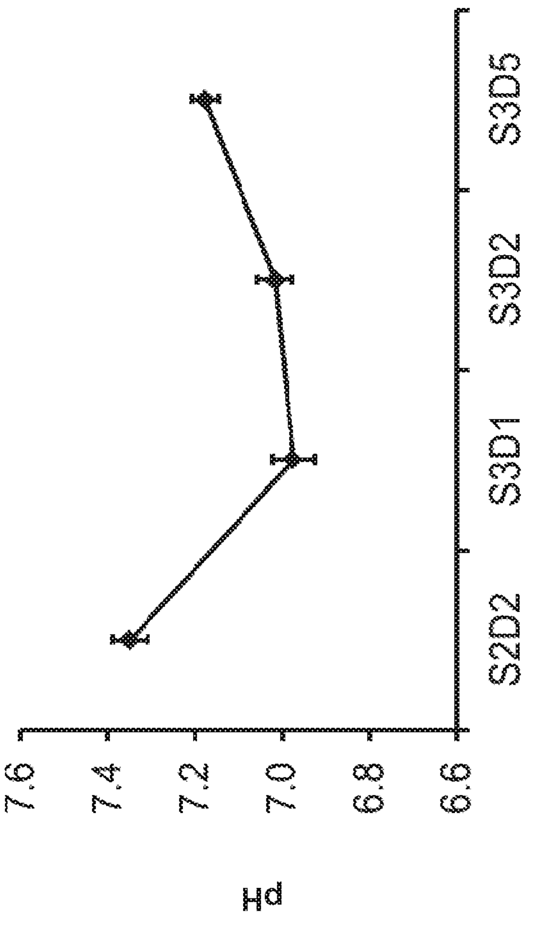


FIG. 1C

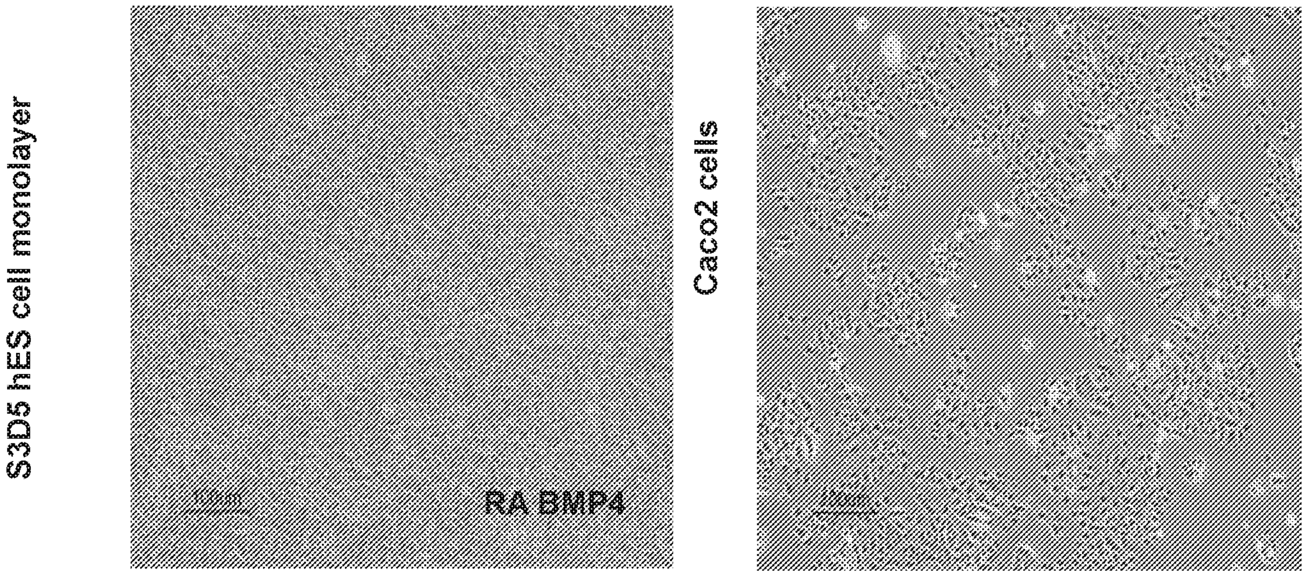


FIG. 1D

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

FIG. 2A

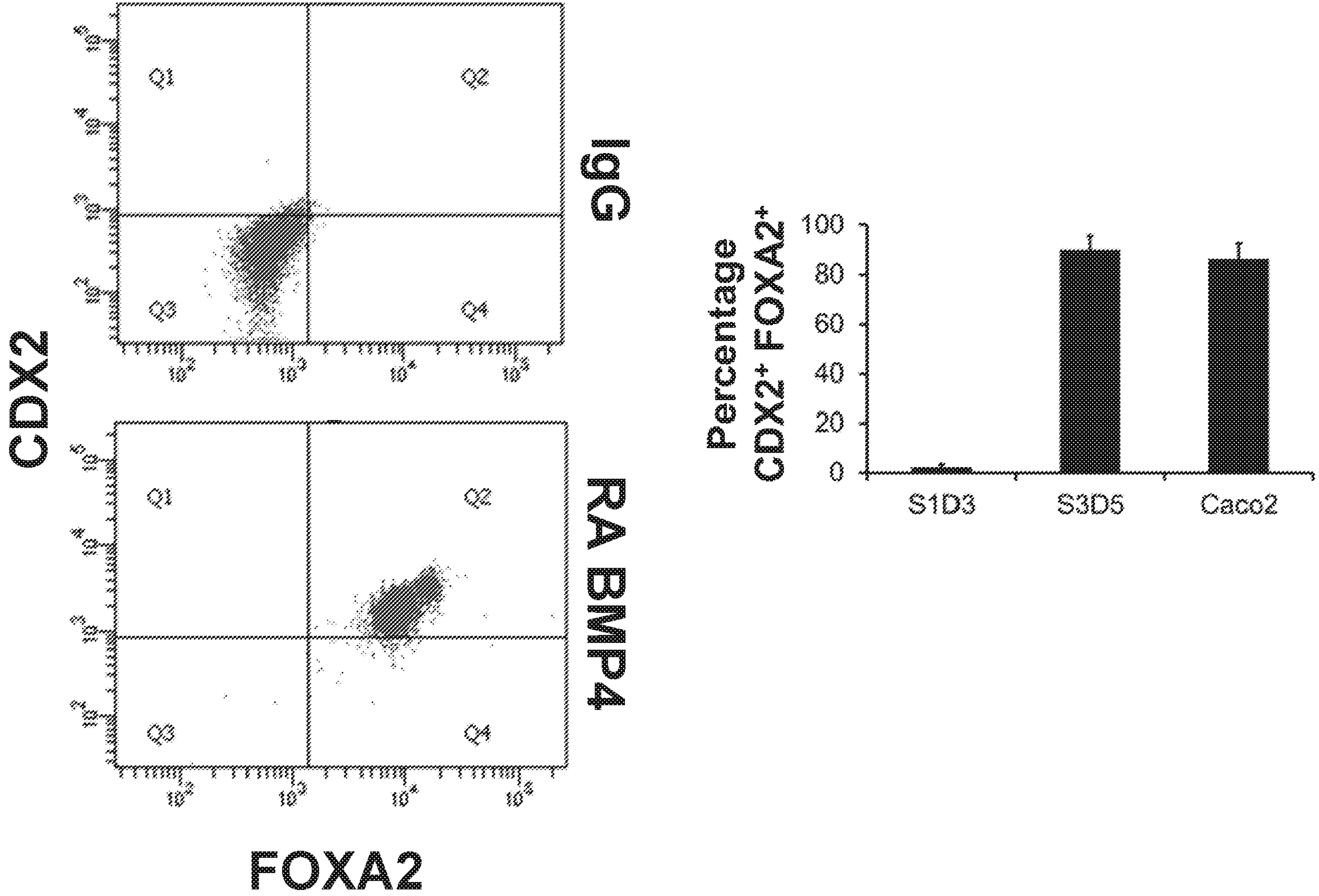

IgG
RA BMP4
CDX2
FOXA2
Q1
Q2
Q3
Q4
10^2
10^3
10^4
10^5
Percentage CDX2⁺ FOXA2⁺
0
20
40
60
80
100
S1D3
S3D5
Caco2

FIG. 2B

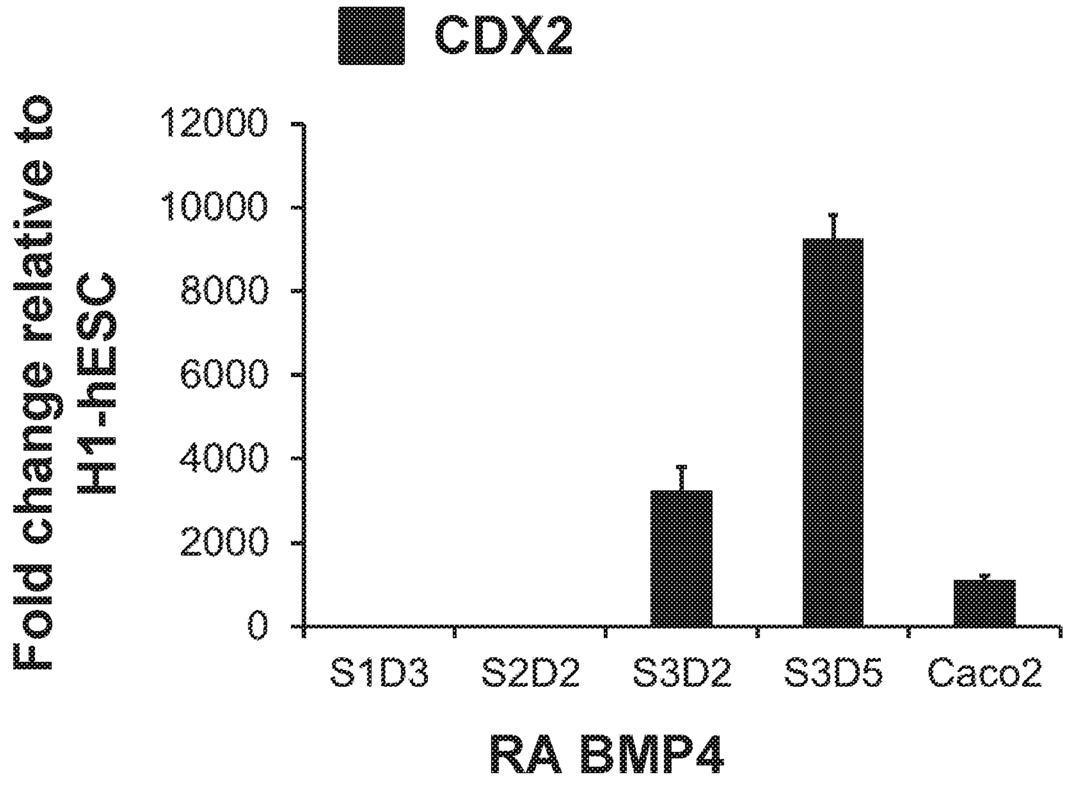

CDX2
Fold change relative to H1-hESC
12000
10000
8000
6000
4000
2000
0
S1D3
S2D2
S3D2
S3D5
Caco2
RA BMP4

FIG. 2C

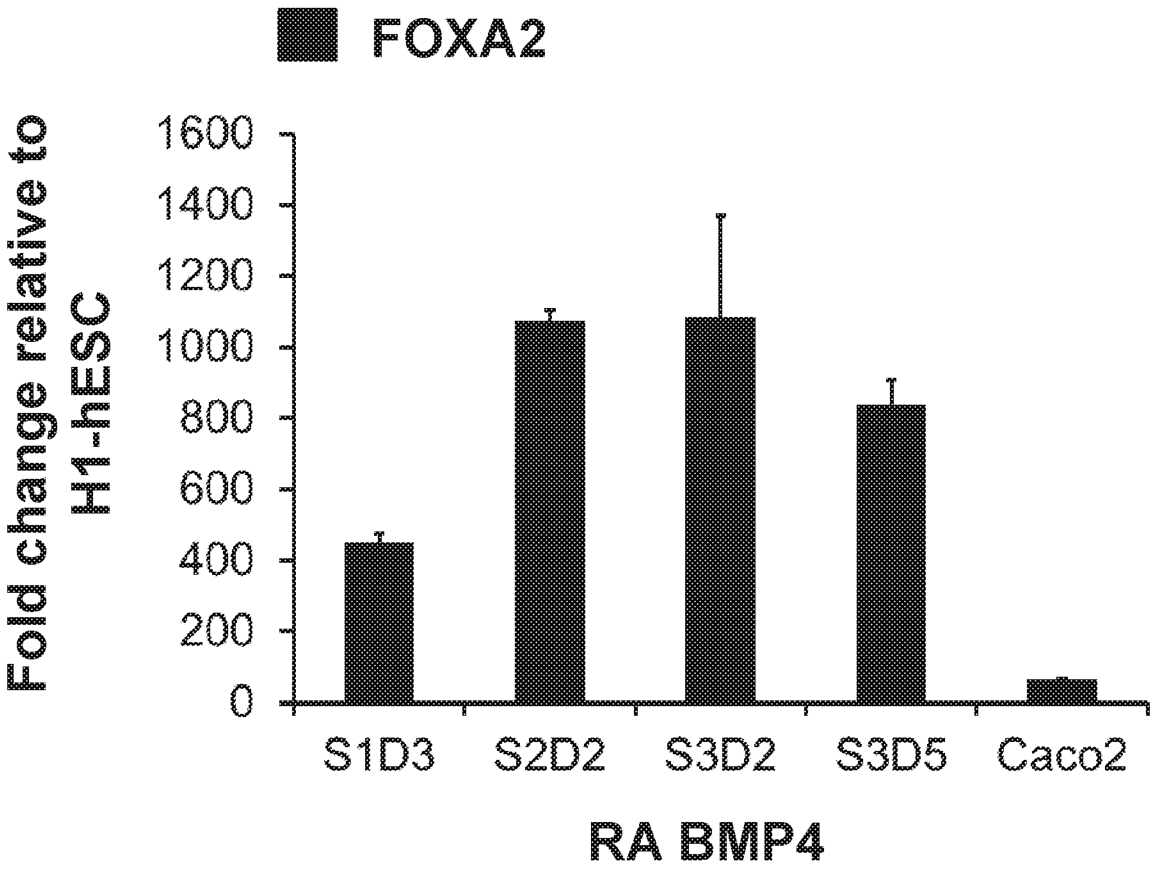

FOXA2
Fold change relative to H1-hESC
1600
1400
1200
1000
800
600
400
200
0
S1D3
S2D2
S3D2
S3D5
Caco2
RA BMP4

FIG. 2D

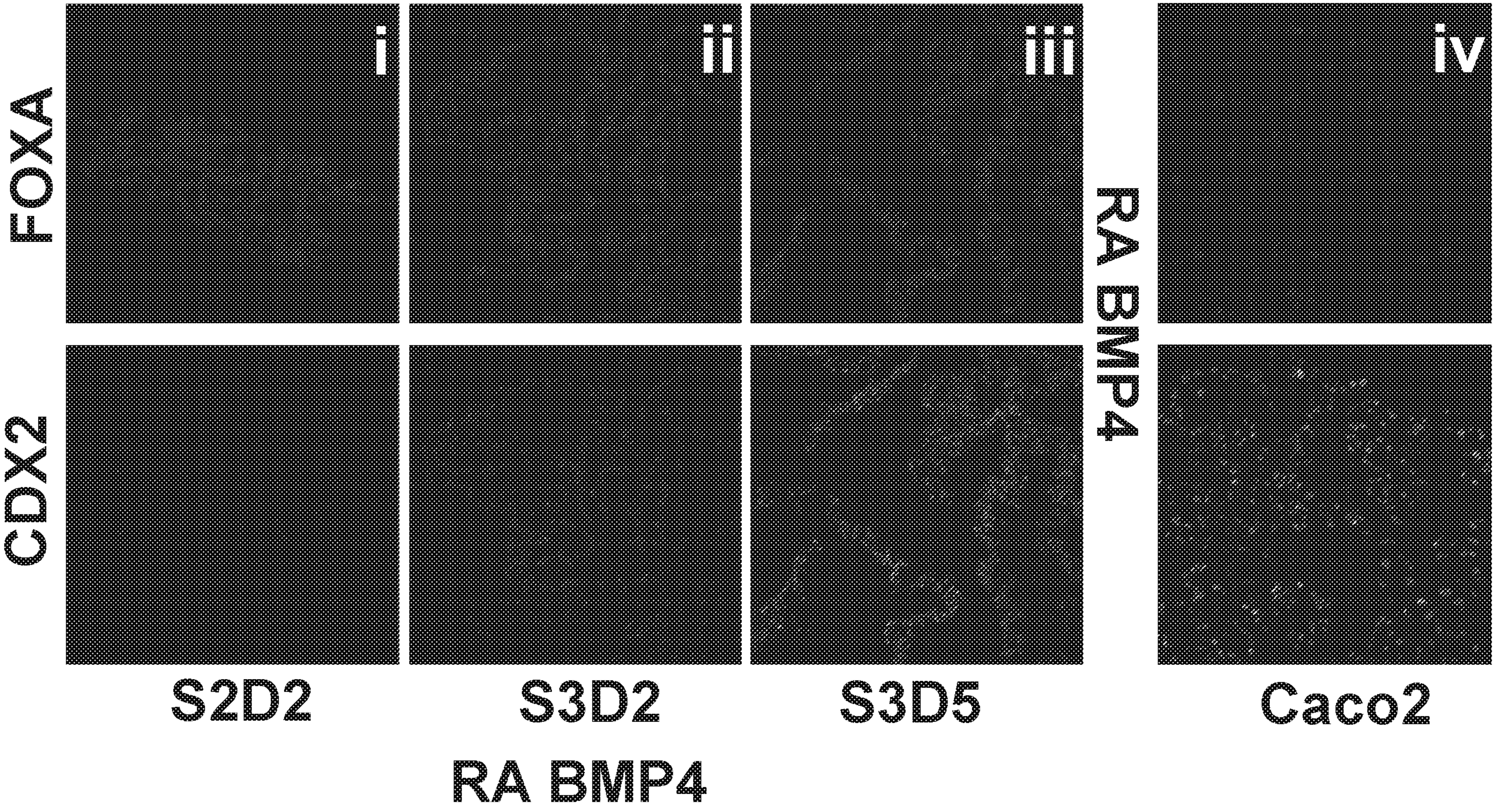


FIG. 3A

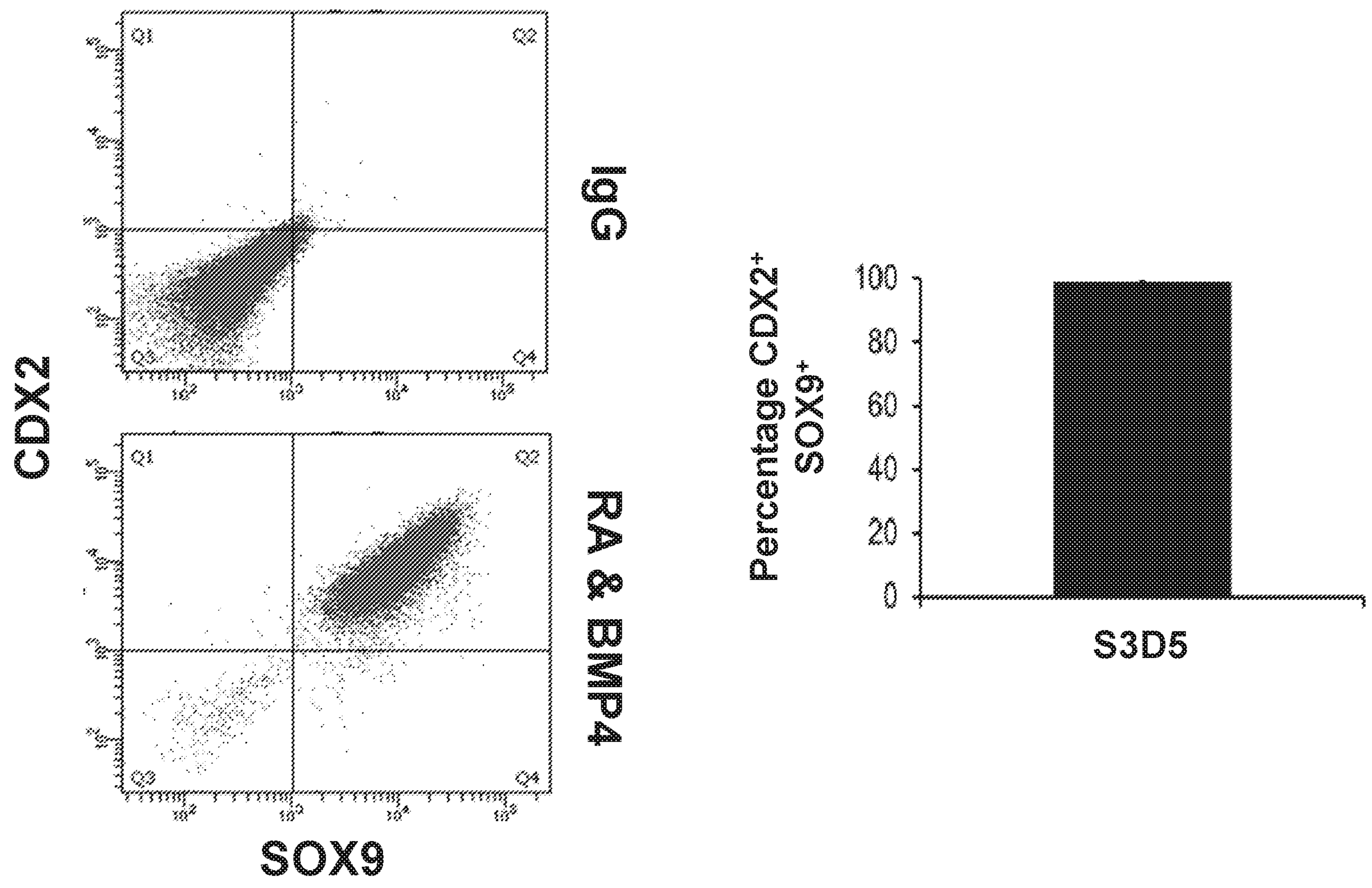
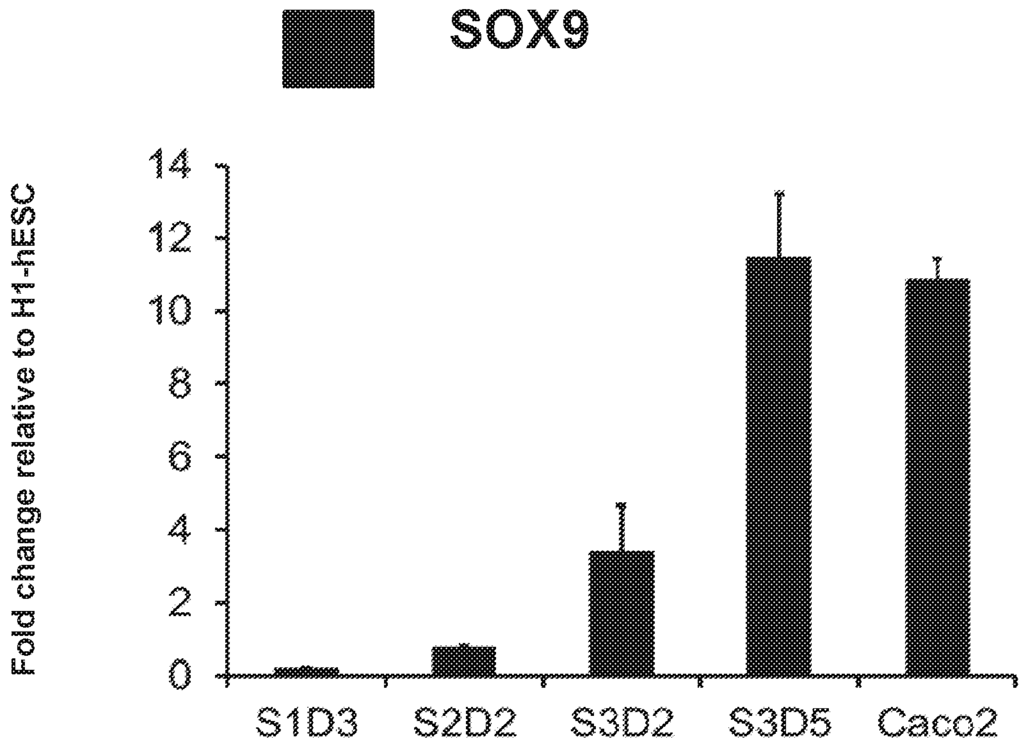

IgG
RA & BMP4
Q1
Q2
Q3
Q4
CDX2
SOX9
10^2
10^3
10^4
10^5
Percentage CDX2⁺ SOX9⁺
100
80
60
40
20
0
S3D5

FIG. 3B


▨ SOX9
Fold change relative to H1-hESC
14
12
10
8
6
4
2
0
S1D3
S2D2
S3D2
S3D5
Caco2

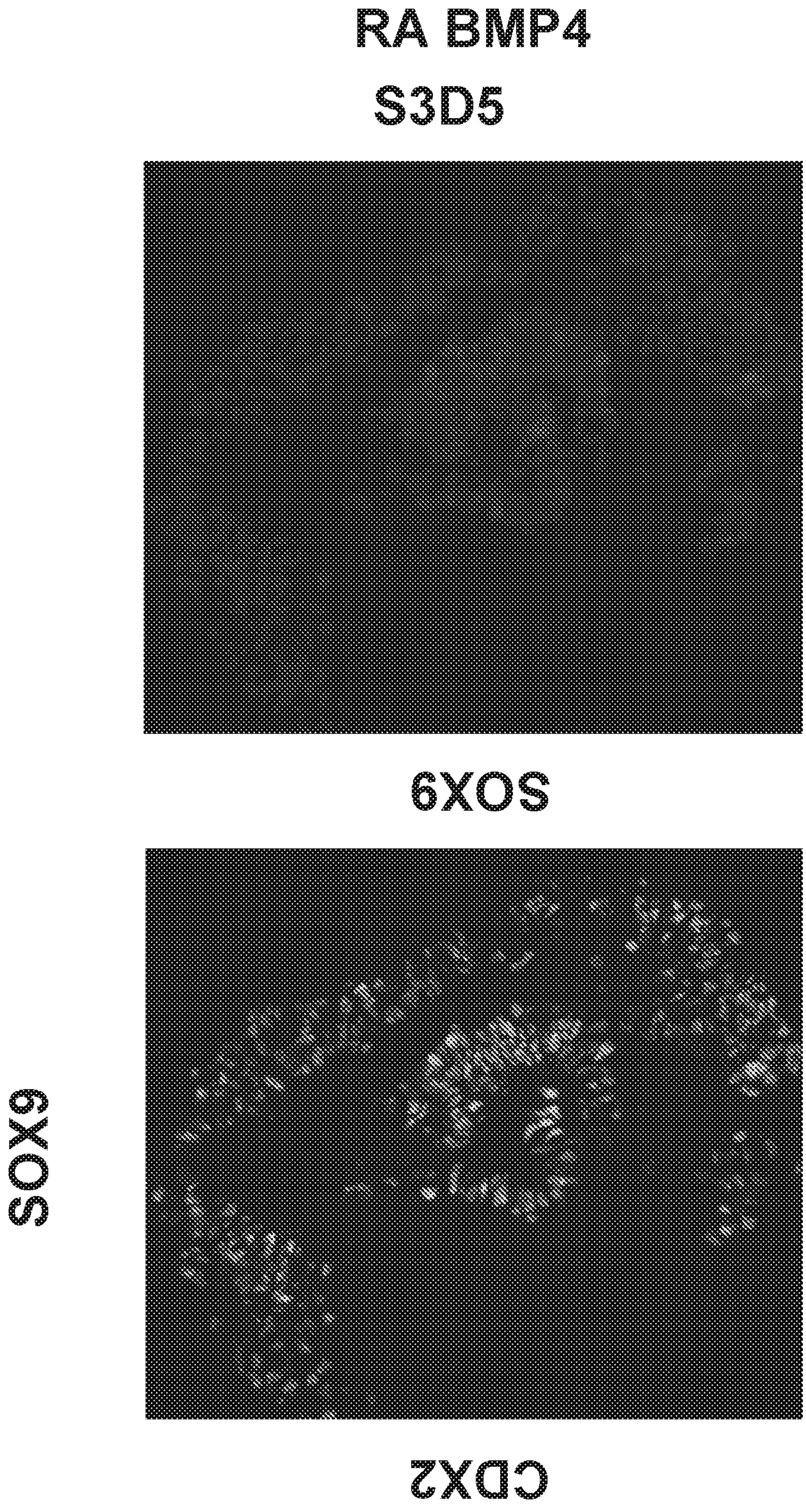


FIG. 3C

FIG. 3D

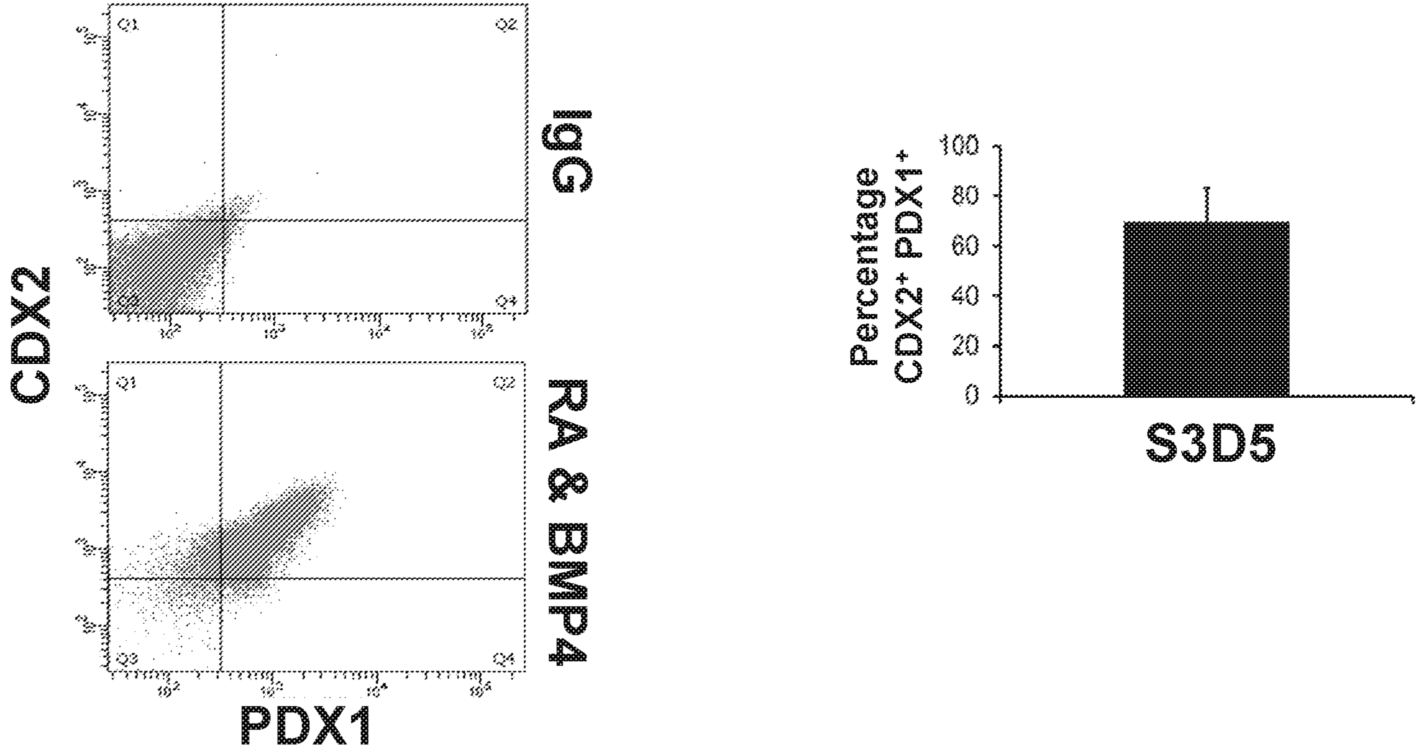

IgG
RA & BMP4
CDX2
PDX1
Q1
Q2
Q3
Q4
10^2
10^3
10^4
10^5
Percentage CDX2⁺ PDX1⁺
100
80
60
40
20
0
S3D5

FIG. 3E

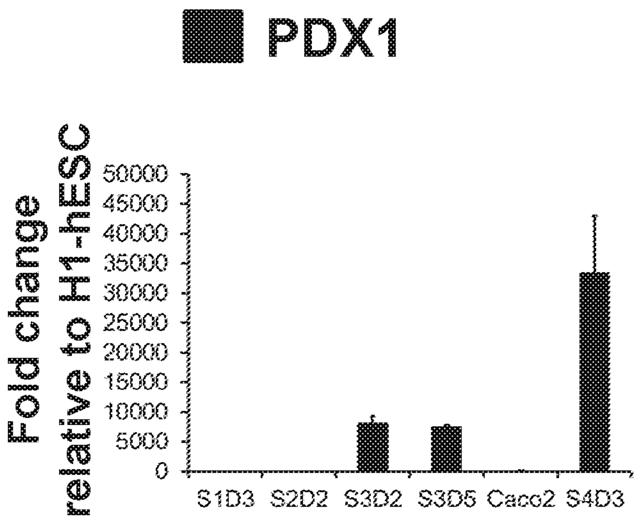

PDX1
Fold change relative to H1-hESC
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
S1D3
S2D2
S3D2
S3D5
Caco2
S4D3

FIG. 3F

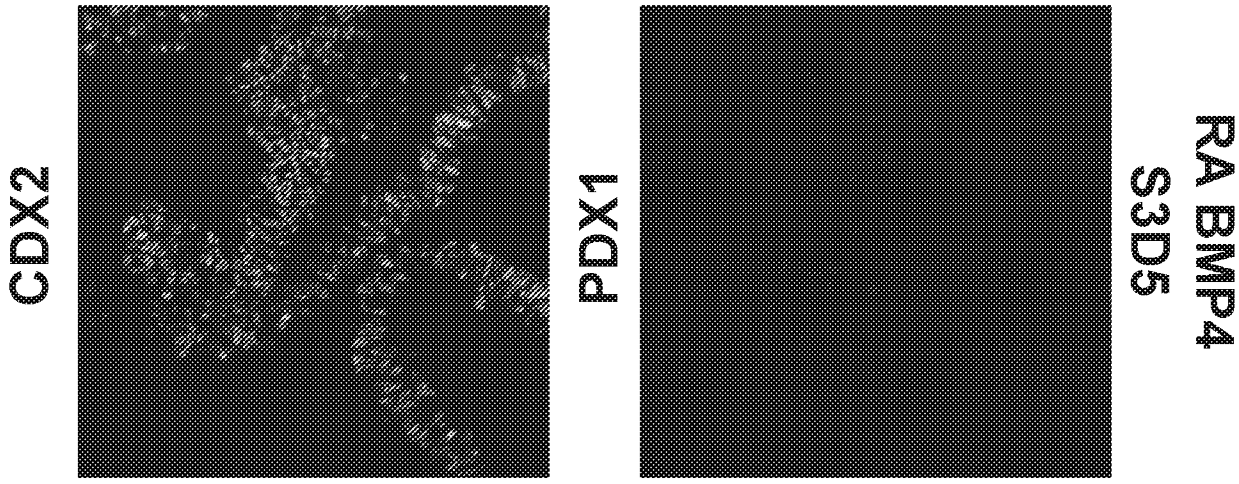

CDX2
PDX1
RA BMP4
S3D5

FIG. 3G

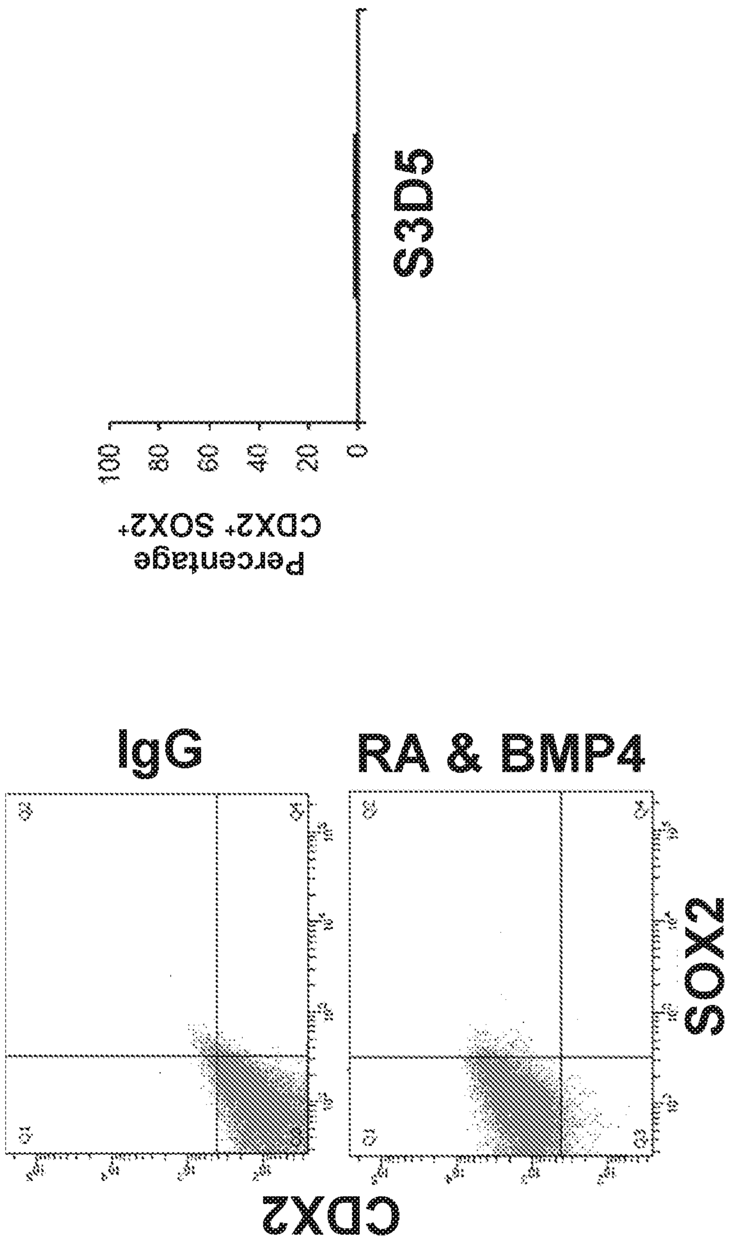

Percentage
CDX2+ SOX2+
100
80
60
40
20
0
S3D5
IgG
RA & BMP4
SOX2
CDX2

FIG. 3H

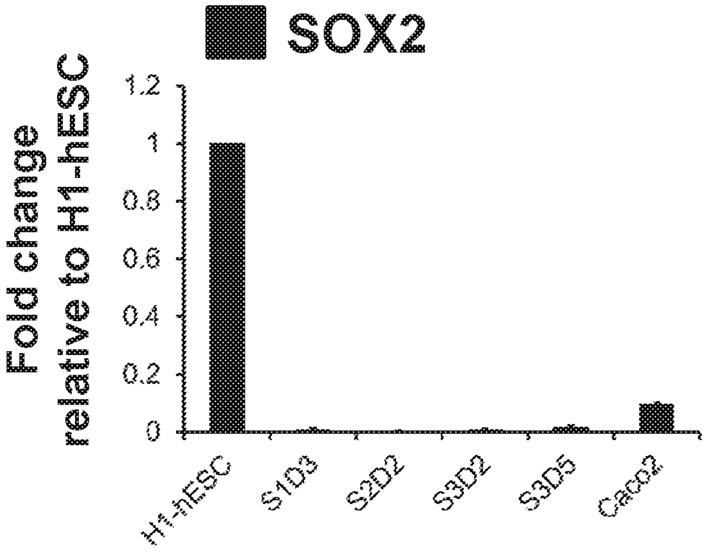

SOX2
Fold change relative to H1-hESC
1.2
1
0.8
0.6
0.4
0.2
0
H1-hESC
S1D3
S2D2
S3D2
S3D5
Caco2

FIG. 3I

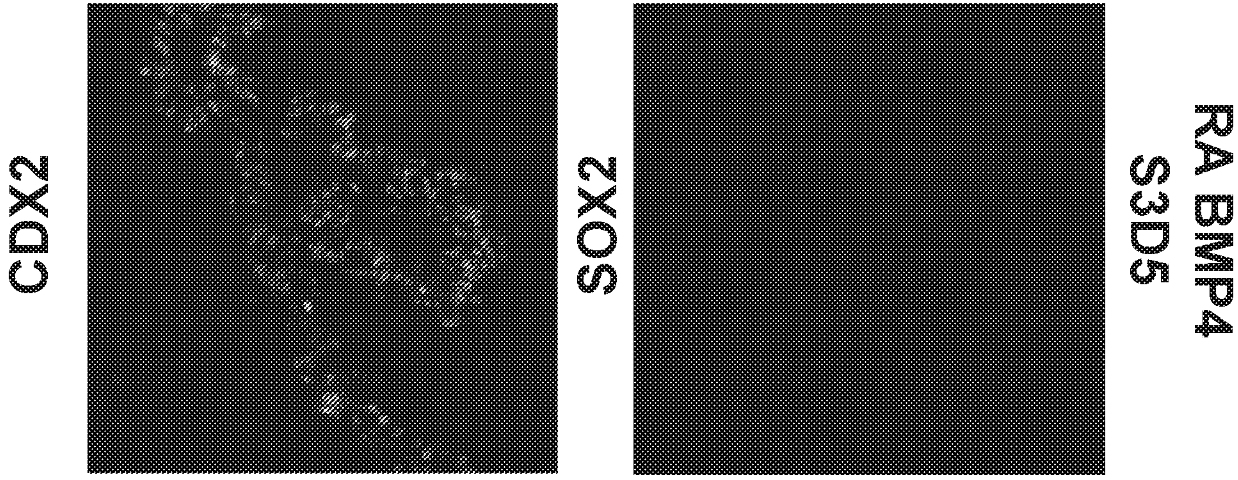

RA BMP4
S3D5
SOX2
CDX2

FIG. 3J

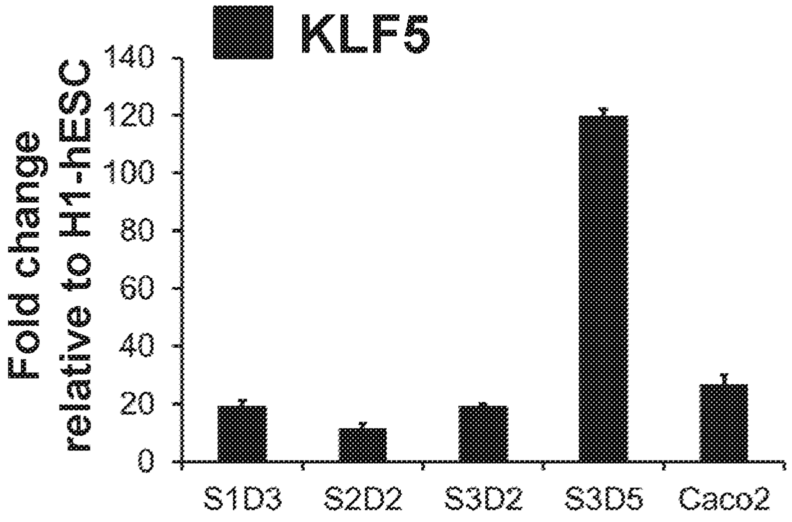

KLF5
Fold change relative to H1-hESC
140
120
100
80
60
40
20
0
S1D3
S2D2
S3D2
S3D5
Caco2

FIG. 3K

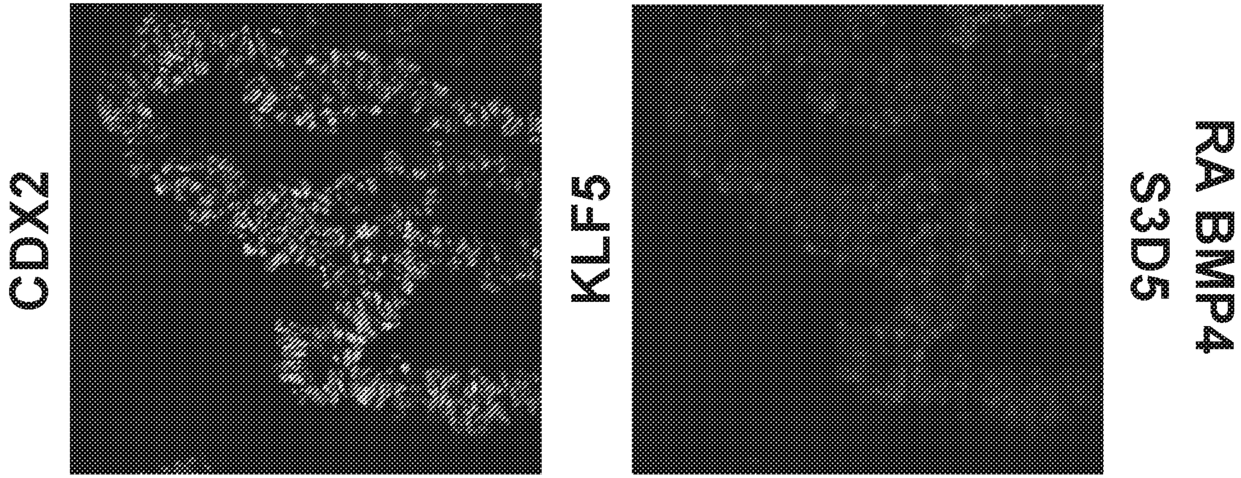

CDX2
KLF5
RA BMP4
S3D5

FIG. 3L

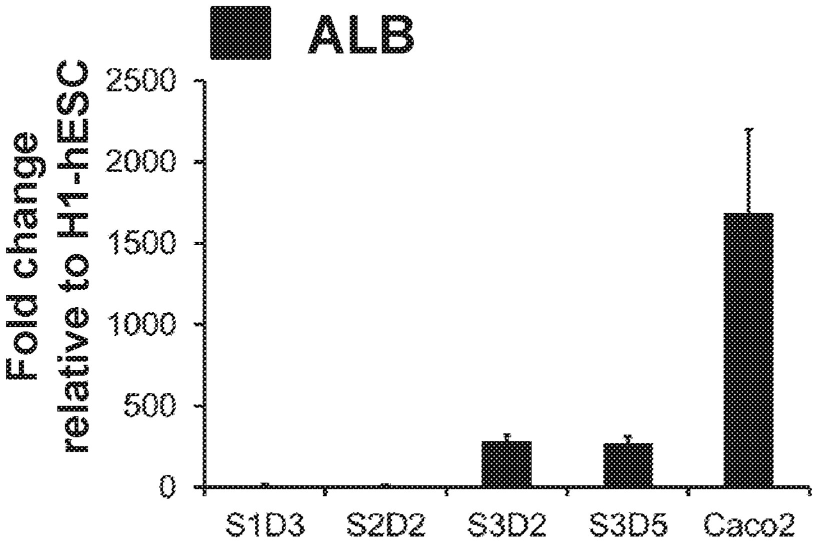

ALB
Fold change relative to H1-hESC
2500
2000
1500
1000
500
0
S1D3
S2D2
S3D2
S3D5
Caco2

FIG. 3M

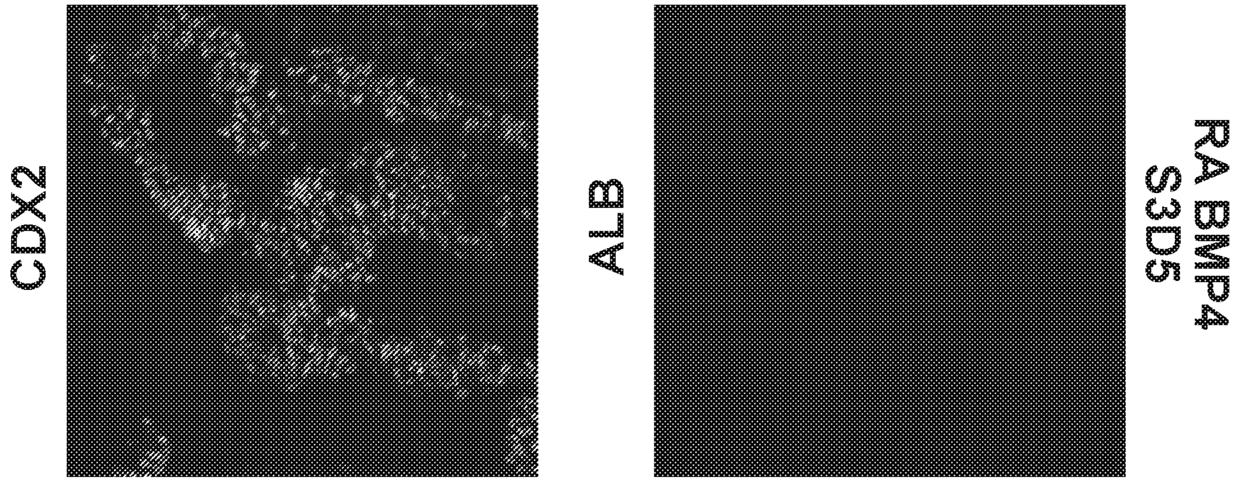

CDX2
ALB
RA BMP4
S3D5

FIG. 3N

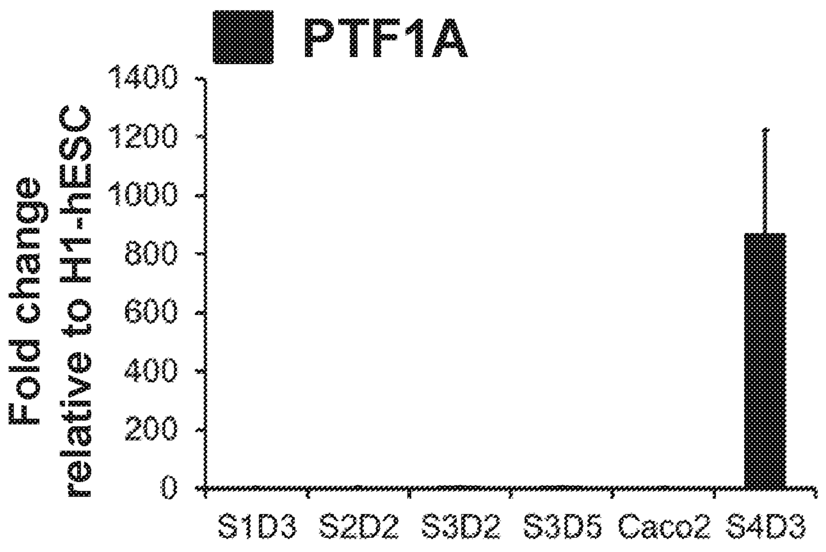

▨ PTF1A
Fold change relative to H1-hESC
1400
1200
1000
800
600
400
200
0
S1D3
S2D2
S3D2
S3D5
Caco2
S4D3

FIG. 3O

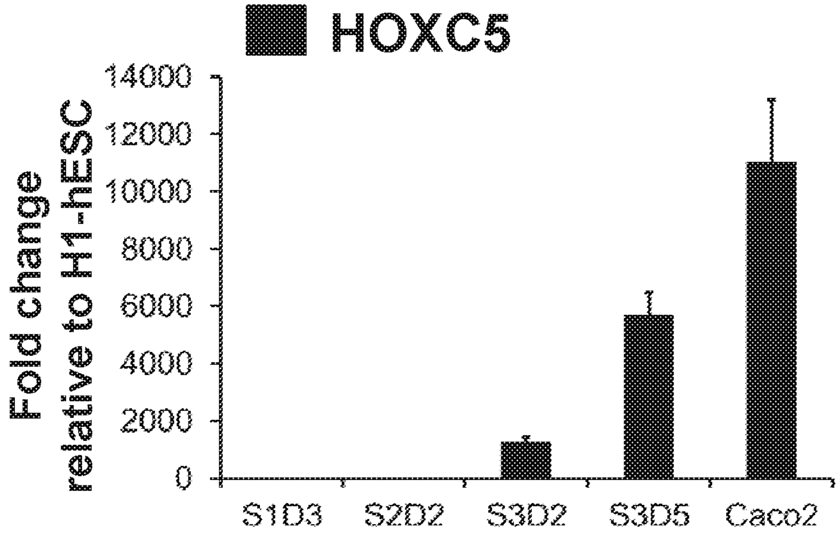

▨ HOXC5
Fold change relative to H1-hESC
14000
12000
10000
8000
6000
4000
2000
0
S1D3
S2D2
S3D2
S3D5
Caco2

FIG. 3P

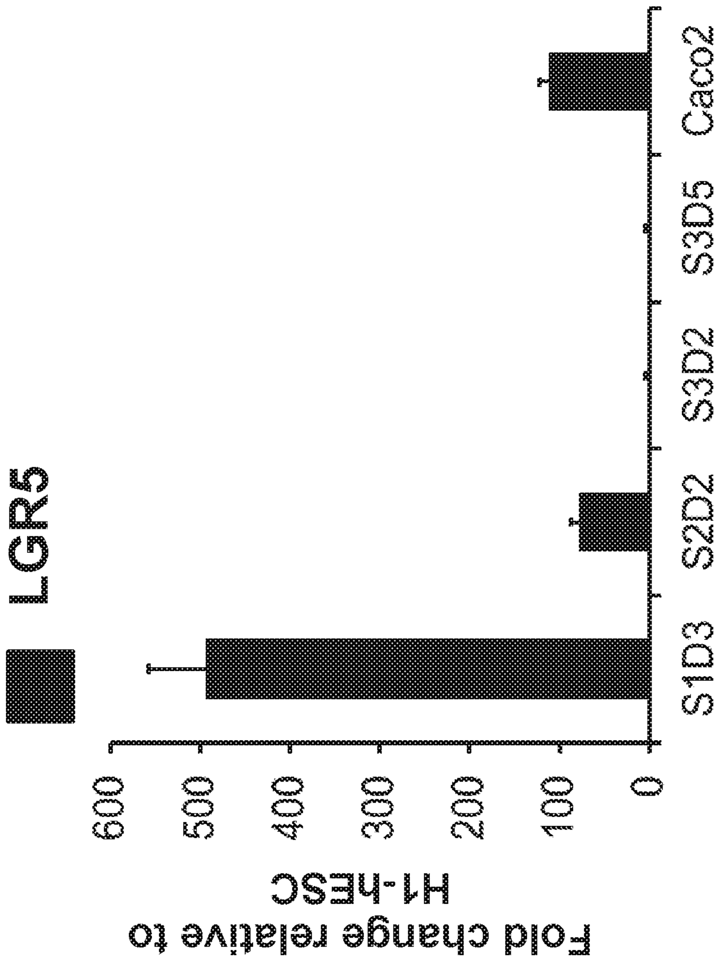

▪ LGR5
Fold change relative to H1-hESC
600
500
400
300
200
100
0
S1D3
S2D2
S3D2
S3D5
Caco2

FIG. 3Q

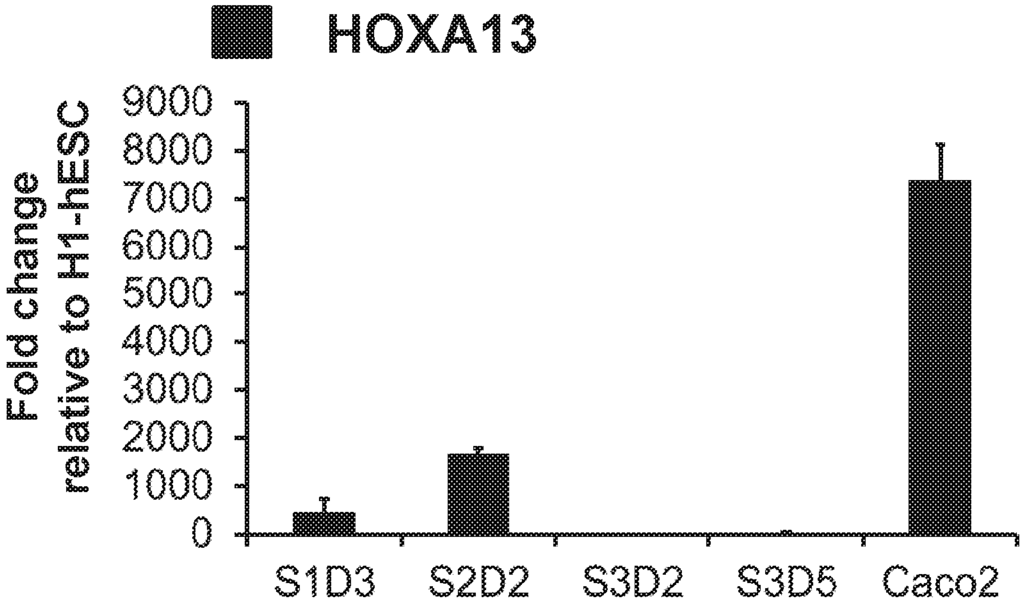

HOXA13
Fold change relative to H1-hESC
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
S1D3
S2D2
S3D2
S3D5
Caco2

FIG. 4A

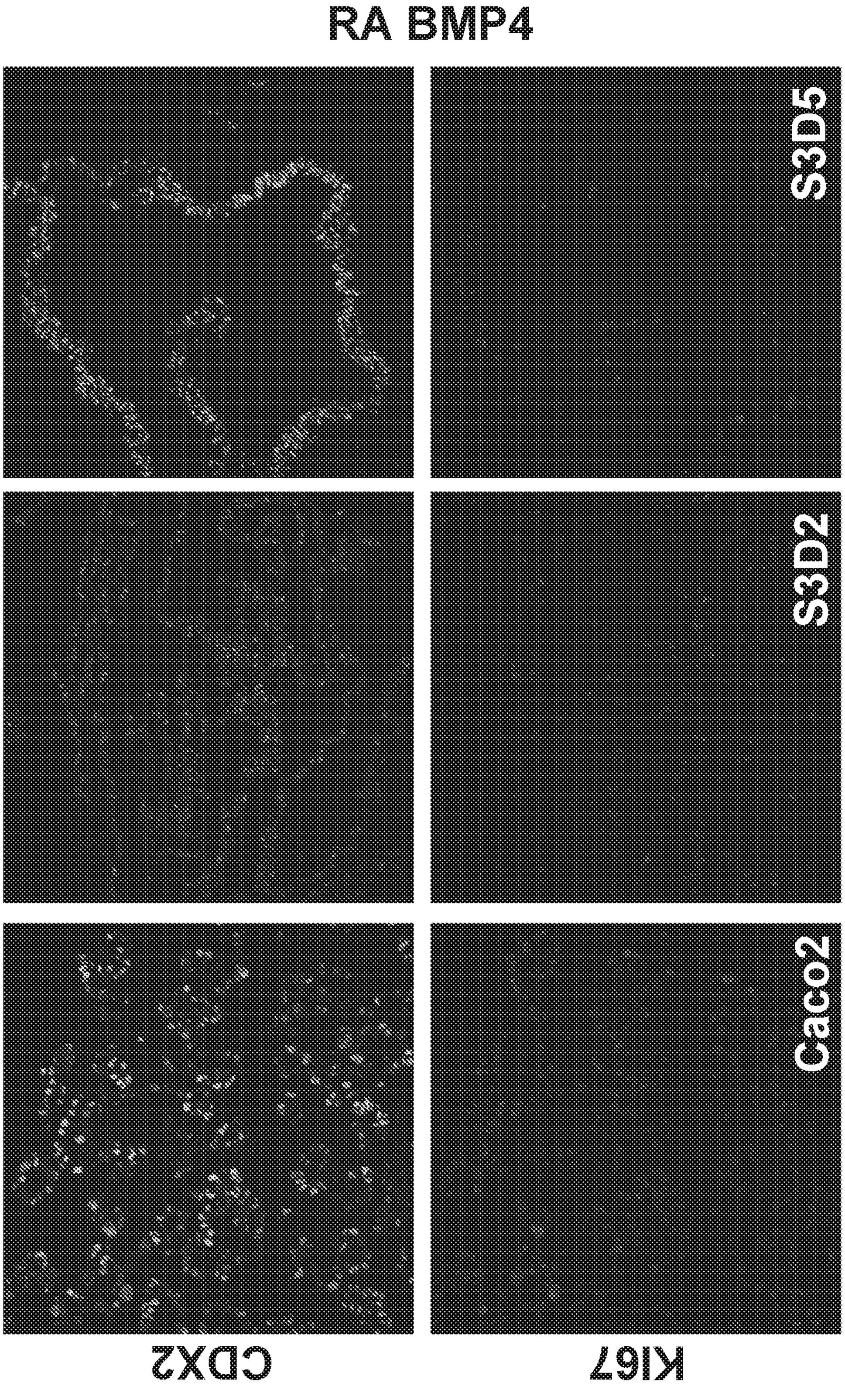


FIG. 4B

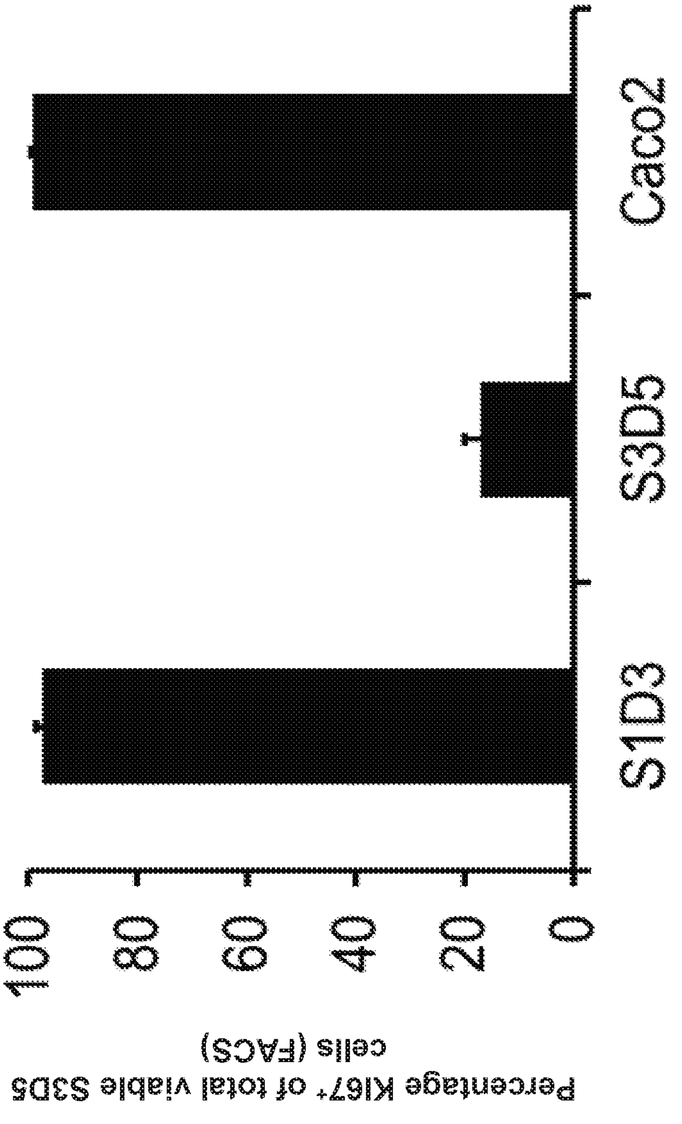

Percentage Ki67+ of total viable S3D5 cells (FACS)
100
80
60
40
20
0
S1D3
S3D5
Caco2

FIG. 5A

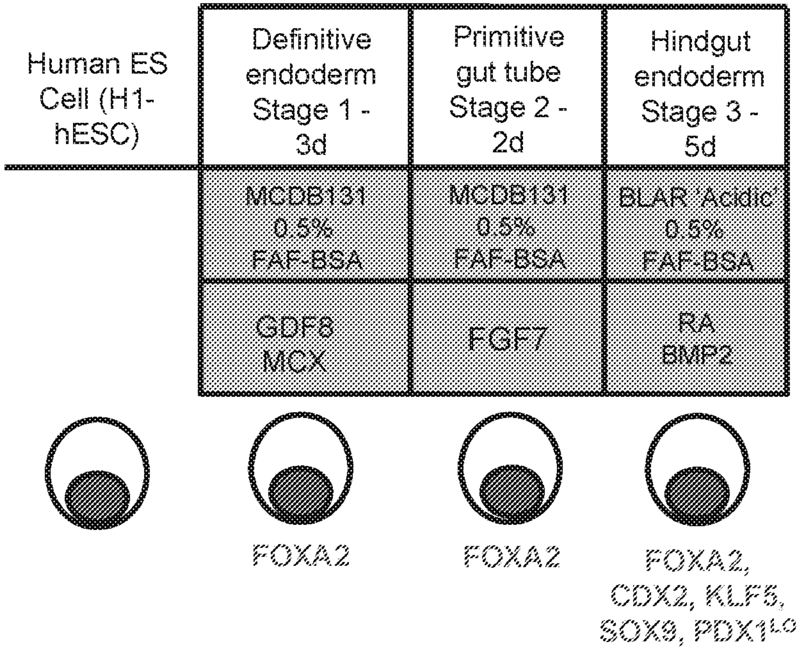
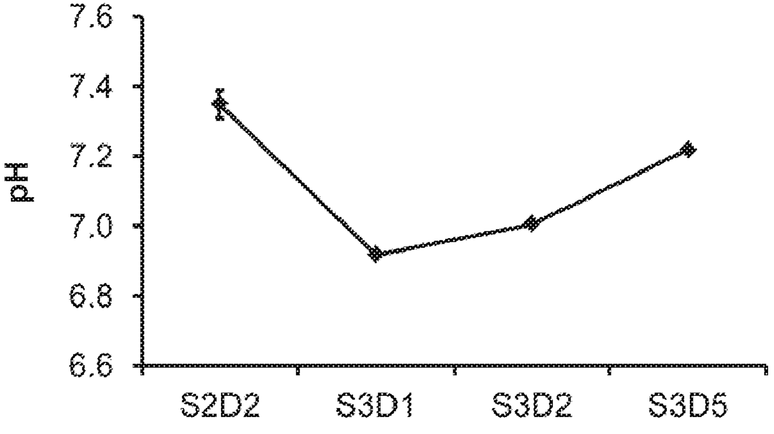
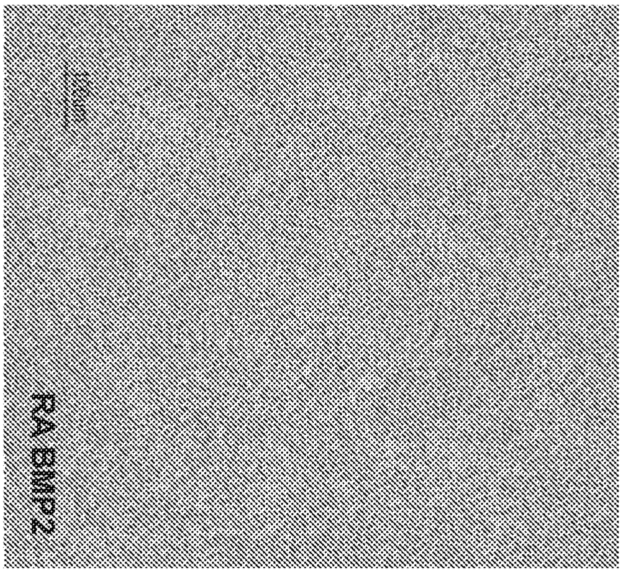

Human ES Cell (H1-hESC)
Definitive endoderm Stage 1 - 3d
Primitive gut tube Stage 2 - 2d
Hindgut endoderm Stage 3 - 5d
MCDB131 0.5% FAF-BSA
MCDB131 0.5% FAF-BSA
BLAR 'Acidic' 0.5% FAF-BSA
GDF8 MCX
FGF7
RA BMP2
FOXA2
FOXA2
FOXA2, CDX2, KLF5, SOX9, PDX1LO

FIG. 5B


pH
7.6
7.4
7.2
7.0
6.8
6.6
S2D2
S3D1
S3D2
S3D5

S3D5 hES cell monolayer



Caco2 cells

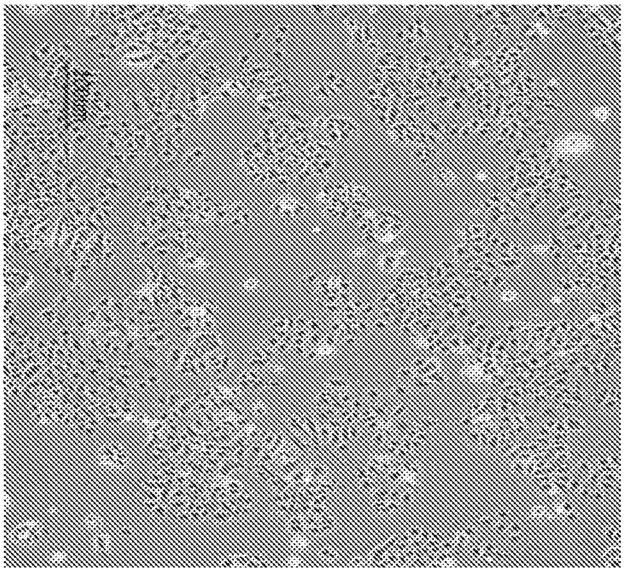


FIG. 5C

FIG. 6A

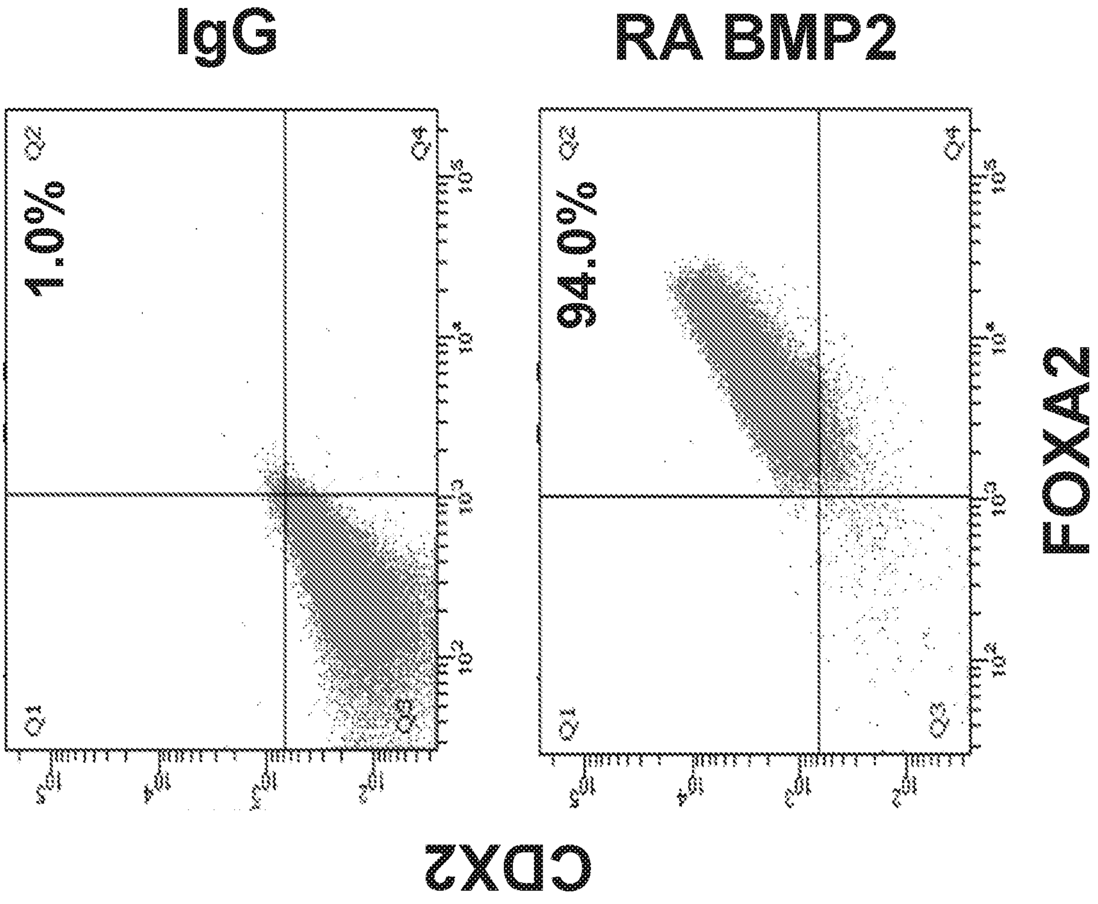

IgG
1.0%
Q1
Q2
Q3
Q4
RA BMP2
94.0%
Q1
Q2
Q3
Q4
10^2
10^3
10^4
10^5
FOXA2
CDX2

FIG. 6B

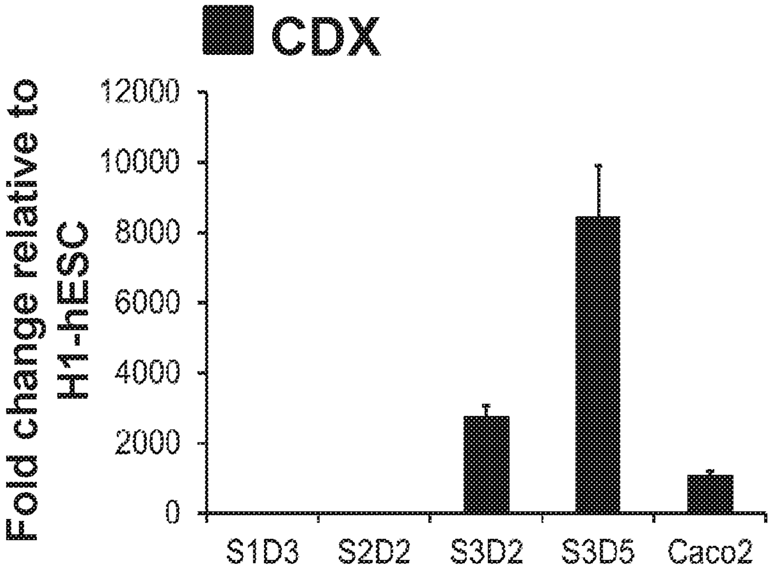

CDX
Fold change relative to H1-hESC
12000
10000
8000
6000
4000
2000
0
S1D3
S2D2
S3D2
S3D5
Caco2

FIG. 6C

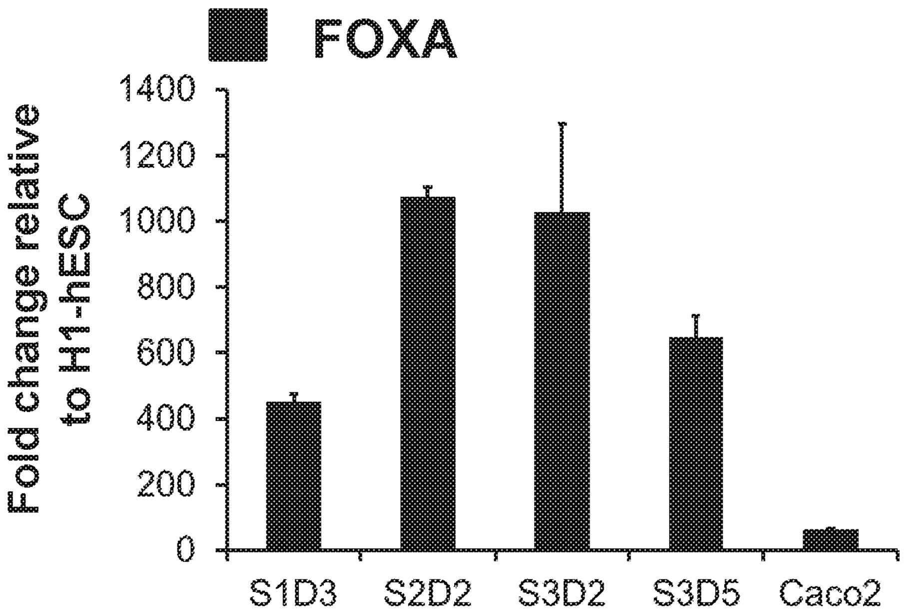

FOXA
Fold change relative to H1-hESC
1400
1200
1000
800
600
400
200
0
S1D3
S2D2
S3D2
S3D5
Caco2

FIG. 6D

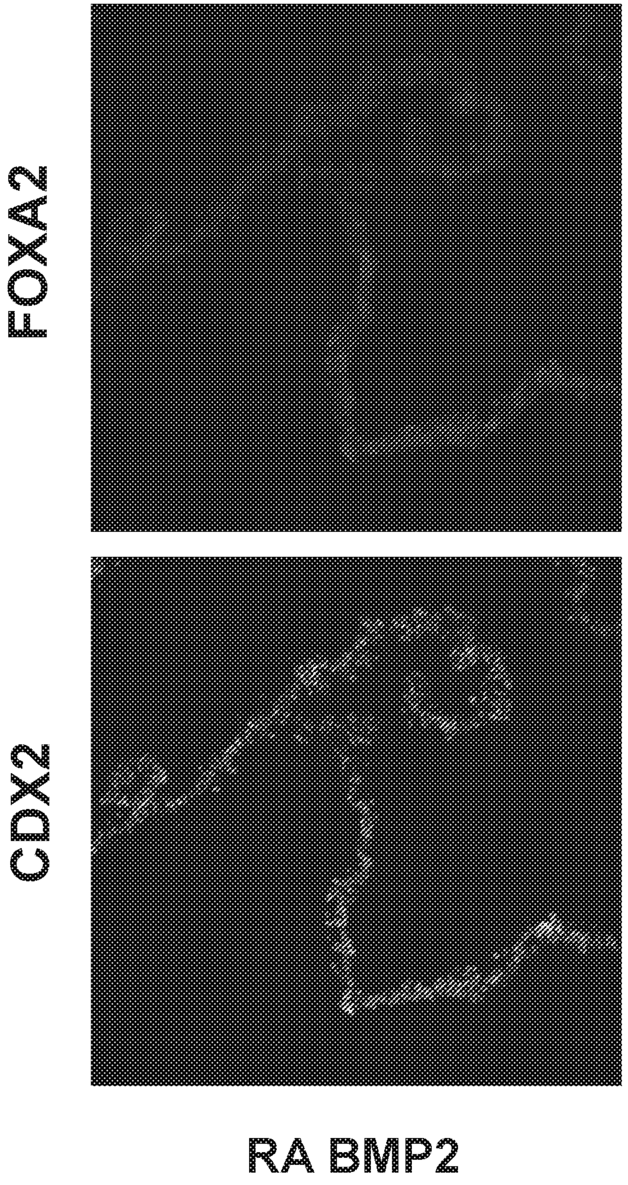


FIG. 6E

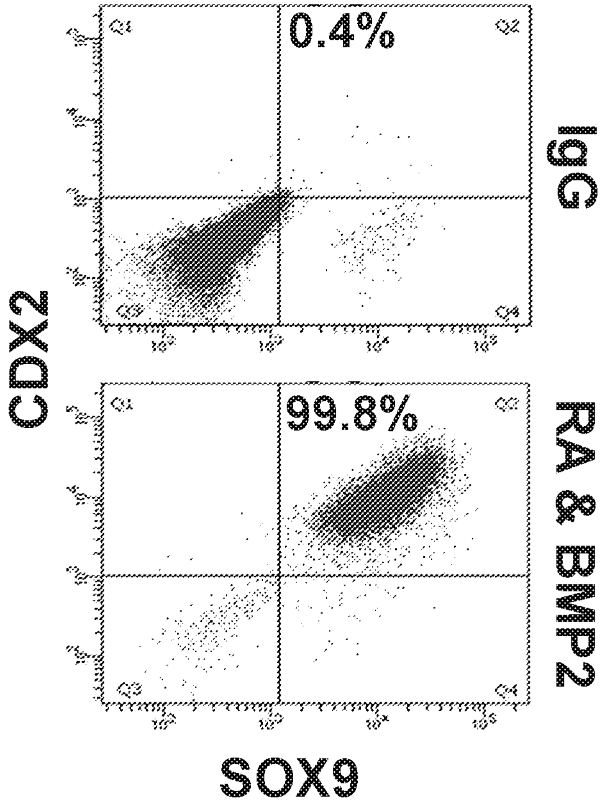
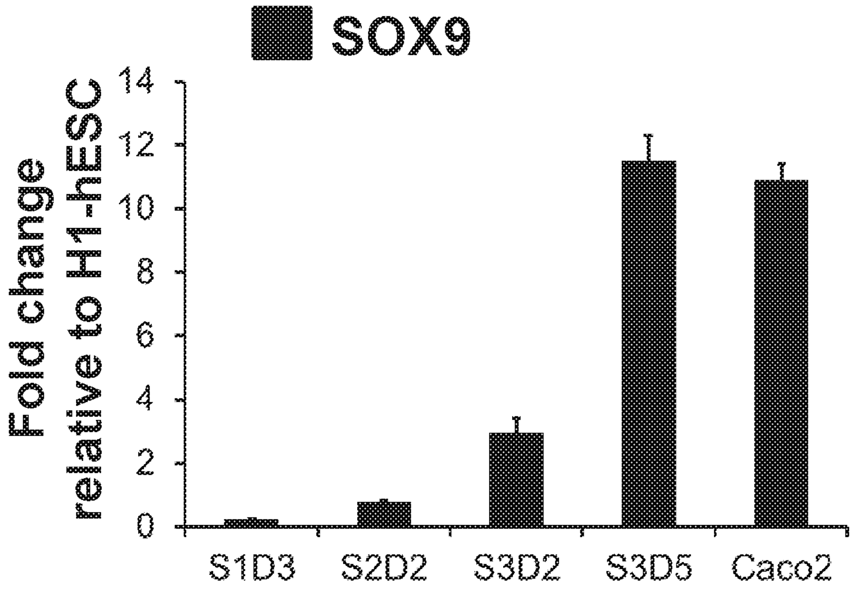

0.4%
IgG
99.8%
RA & BMP2
CDX2
SOX9
Q1
Q2
Q3
Q4

FIG. 6F


SOX9
Fold change relative to H1-hESC
0
2
4
6
8
10
12
14
S1D3
S2D2
S3D2
S3D5
Caco2

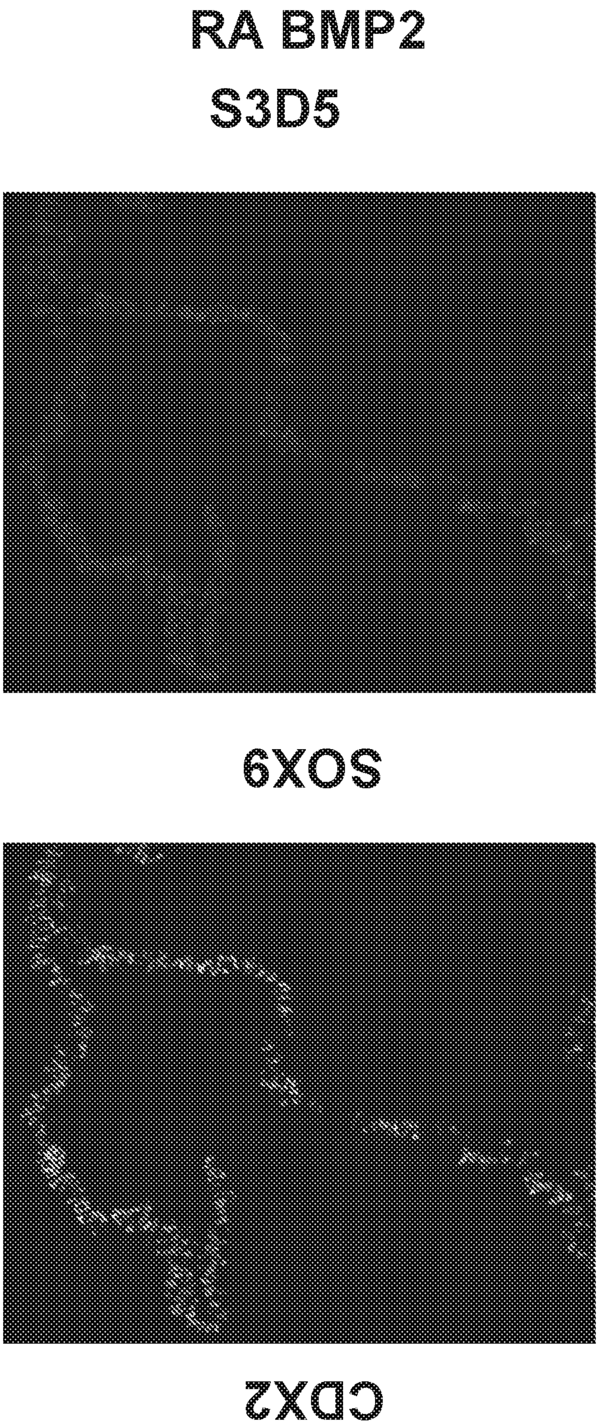


FIG. 6G

FIG. 6H

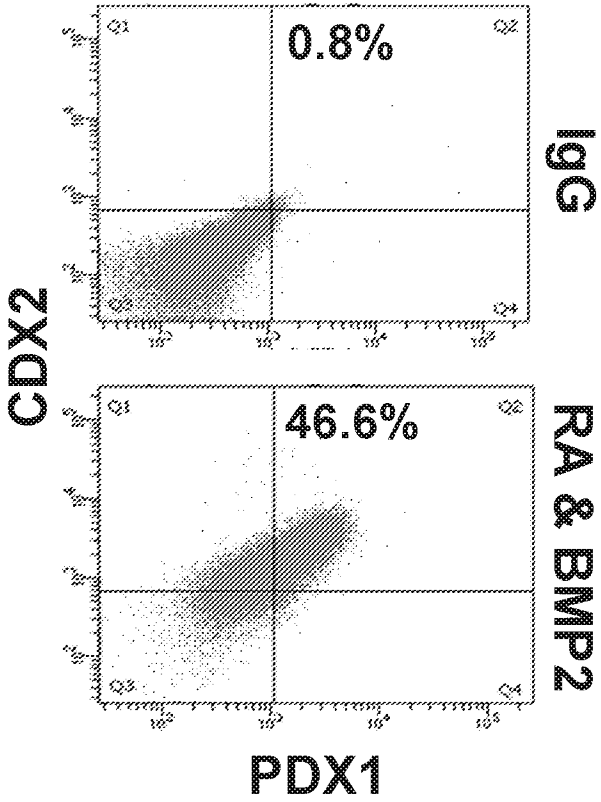
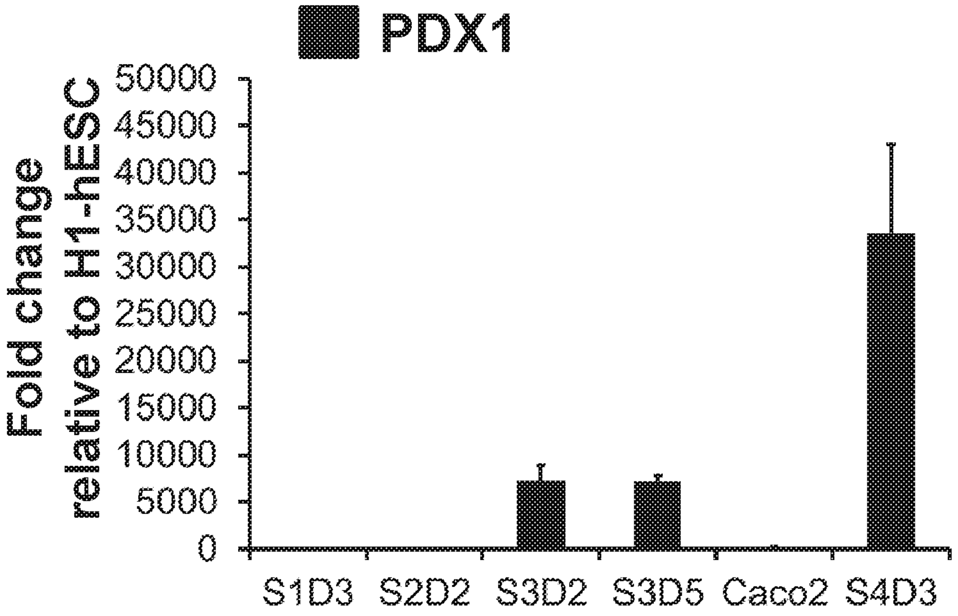

0.8%
IgG
46.6%
RA & BMP2
CDX2
PDX1
Q1
Q2
Q3
Q4

FIG. 6I


PDX1
Fold change relative to H1-hESC
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
S1D3
S2D2
S3D2
S3D5
Caco2
S4D3

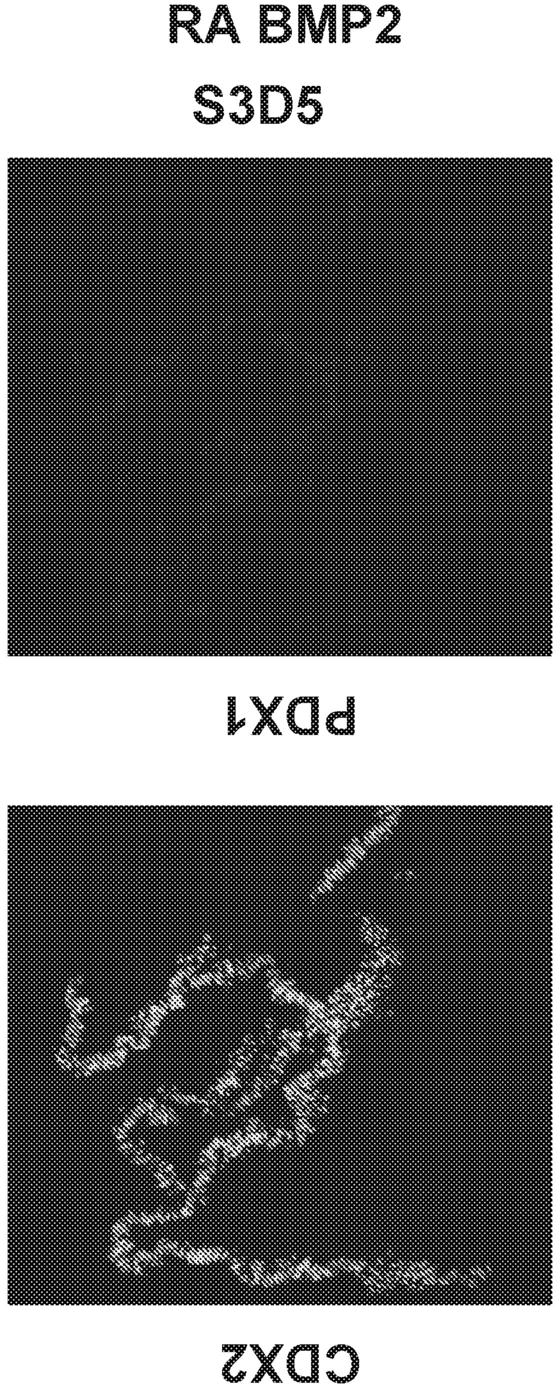


FIG. 6J

FIG. 6K

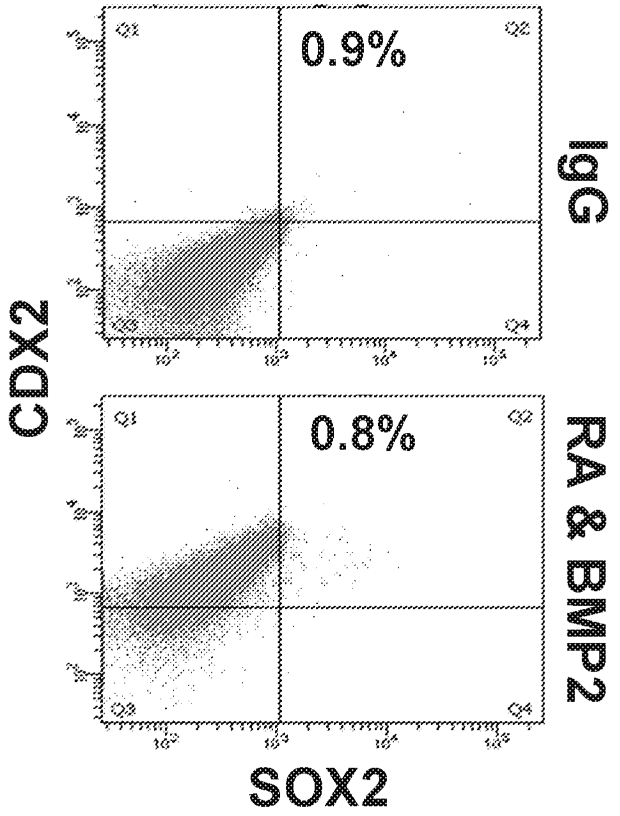
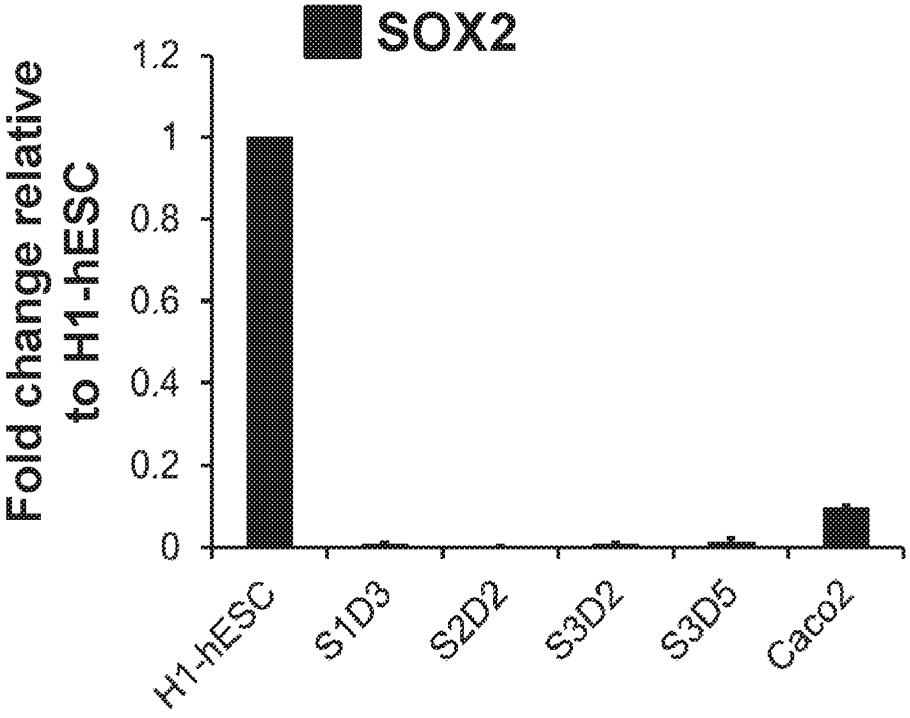

IgG
RA & BMP2
0.9%
0.8%
Q1
Q2
Q3
Q4
CDX2
SOX2
10^2
10^3
10^4
10^5

FIG. 6L


SOX2
Fold change relative to H1-hESC
1.2
1
0.8
0.6
0.4
0.2
0
H1-hESC
S1D3
S2D2
S3D2
S3D5
Caco2

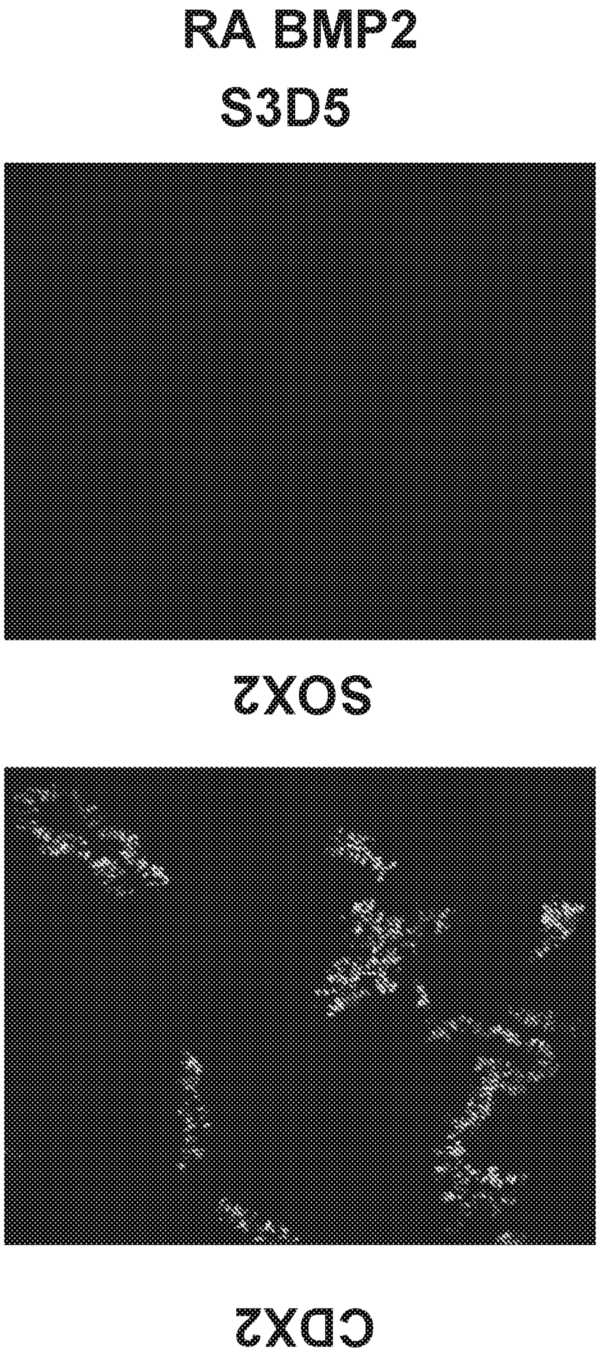


FIG. 6M

FIG. 6N

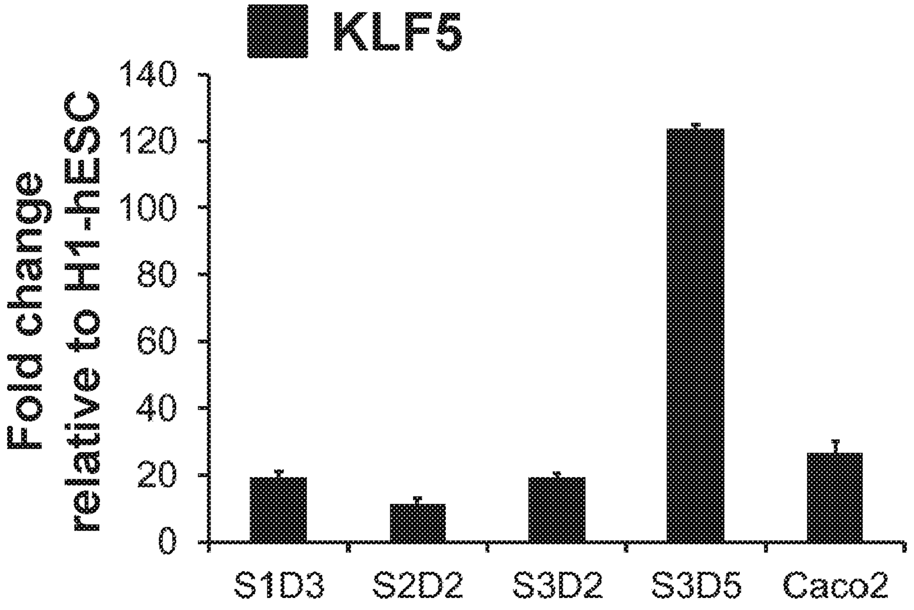

KLF5
Fold change relative to H1-hESC
0
20
40
60
80
100
120
140
S1D3
S2D2
S3D2
S3D5
Caco2

FIG. 6O

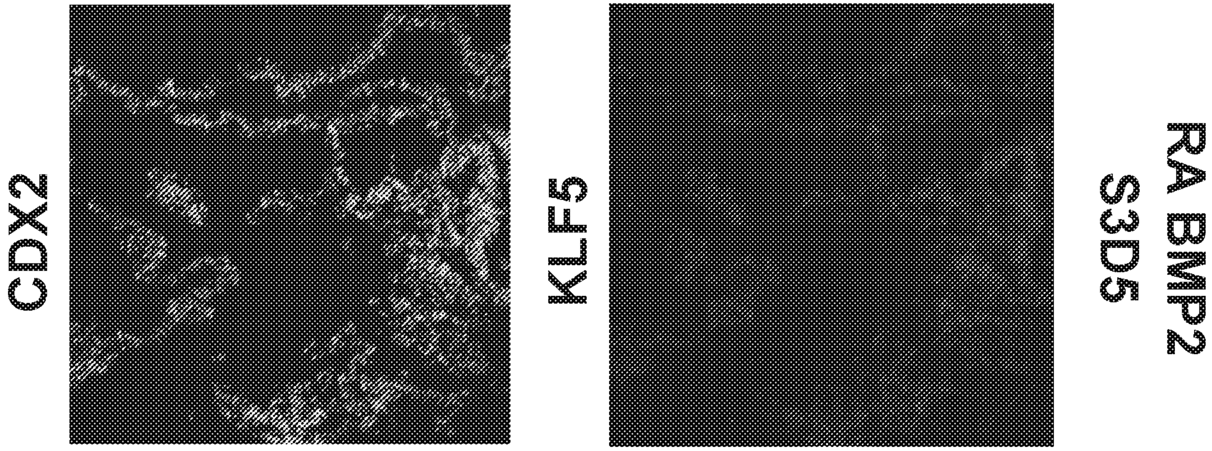

CDX2
KLF5
RA BMP2
S3D5

FIG. 6P

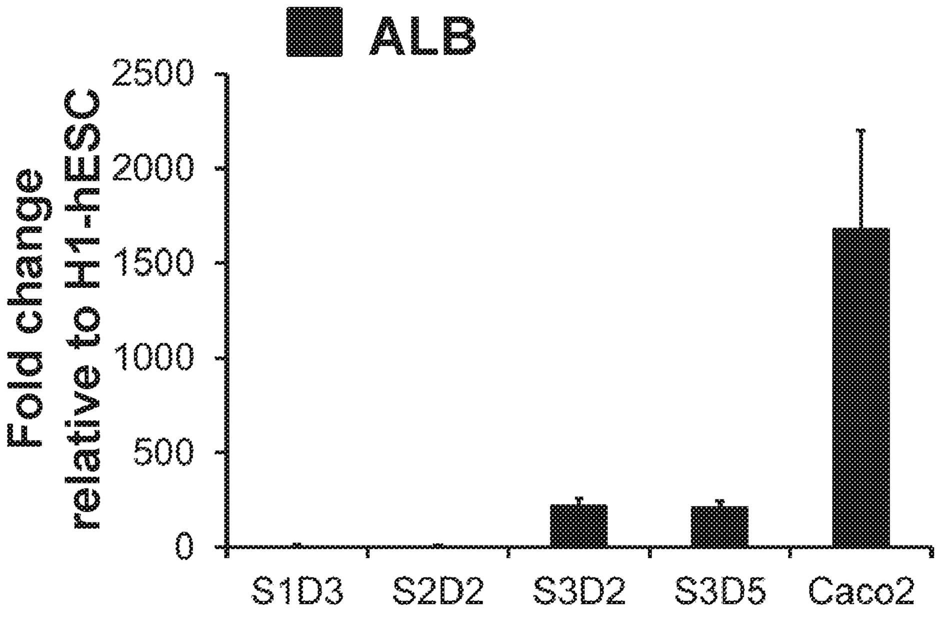

ALB
Fold change relative to H1-hESC
2500
2000
1500
1000
500
0
S1D3
S2D2
S3D2
S3D5
Caco2

FIG. 6Q

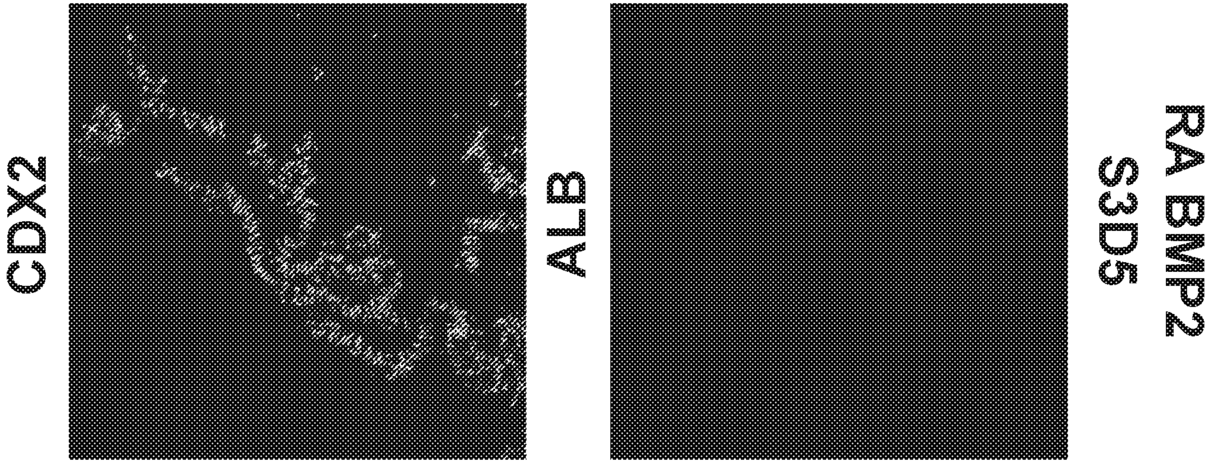

CDX2
ALB
RA BMP2
S3D5

FIG. 6R

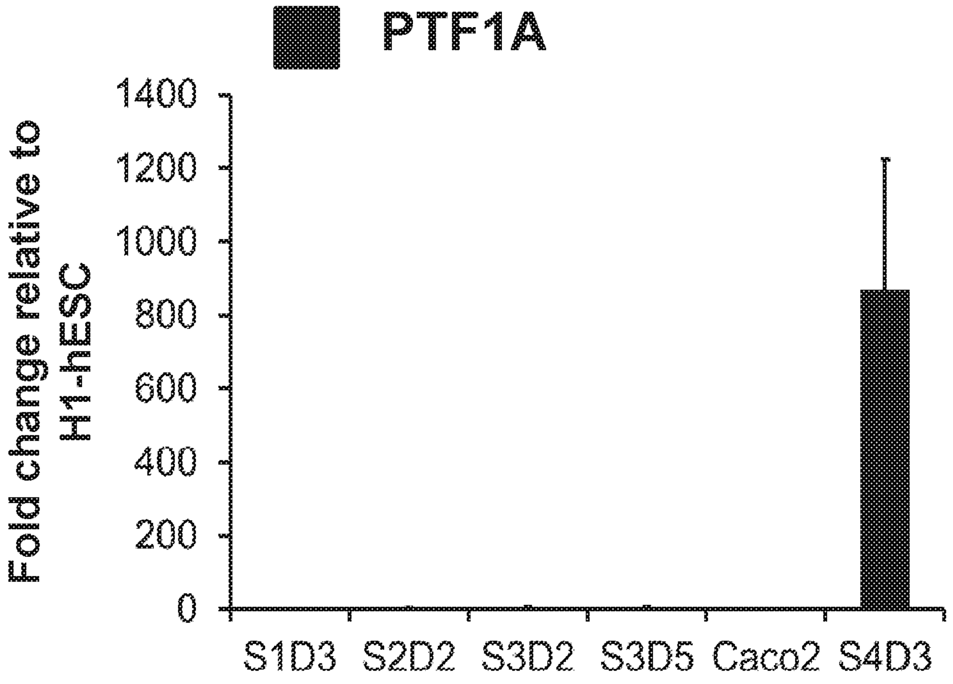

■ PTF1A
Fold change relative to H1-hESC
1400
1200
1000
800
600
400
200
0
S1D3
S2D2
S3D2
S3D5
Caco2
S4D3

FIG. 6S

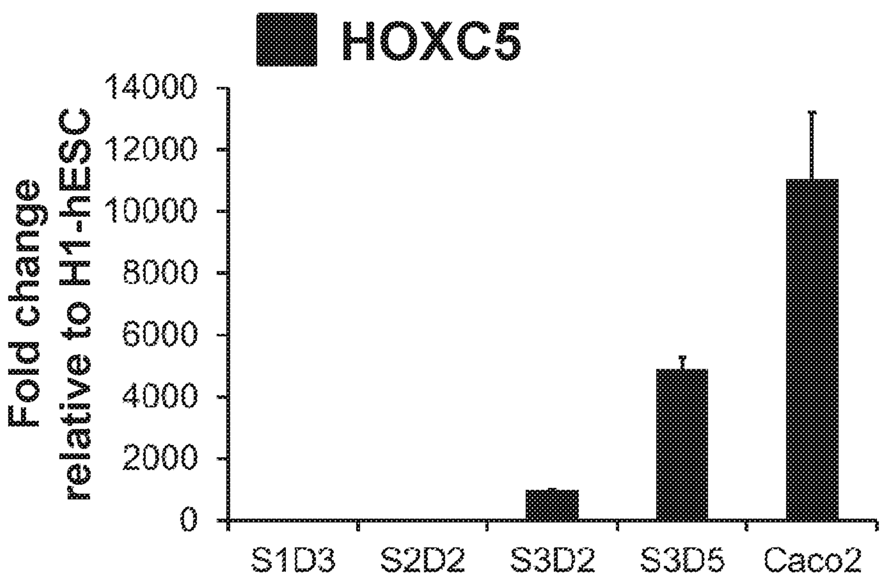

▨ HOXC5
Fold change relative to H1-hESC
14000
12000
10000
8000
6000
4000
2000
0
S1D3
S2D2
S3D2
S3D5
Caco2

FIG. 6T

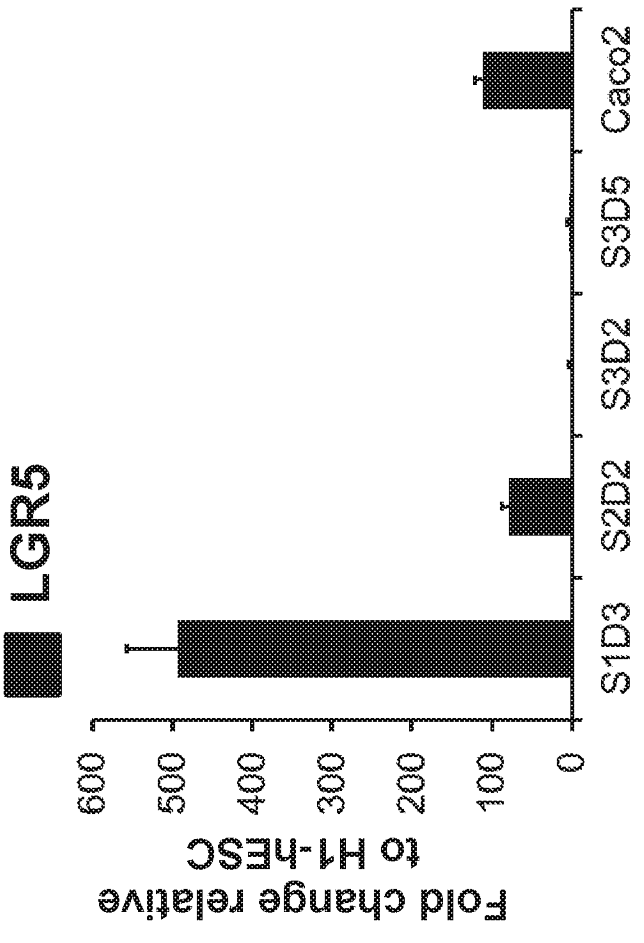
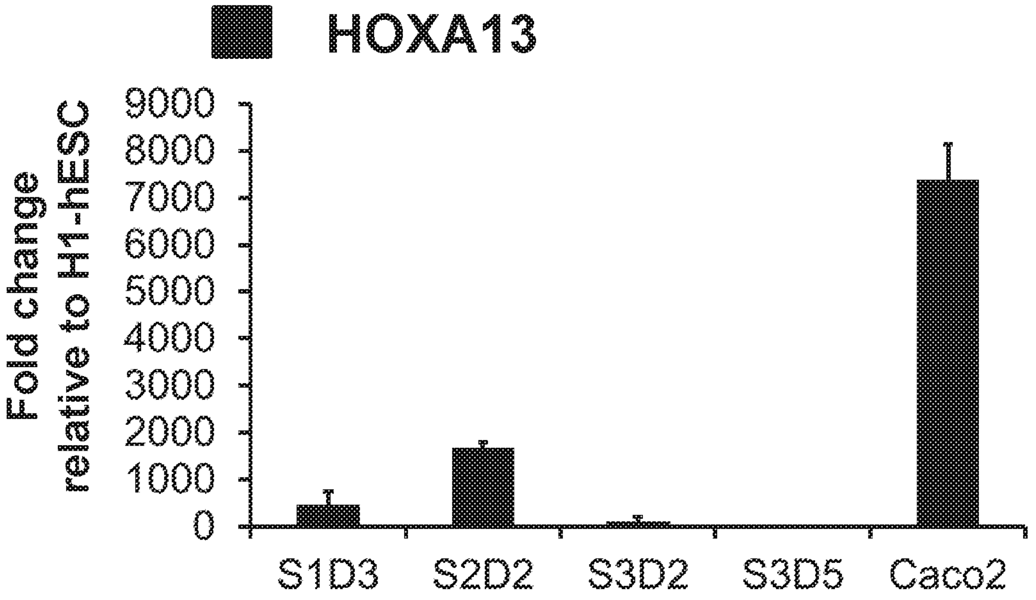

LGR5
Fold change relative to H1-hESC
600
500
400
300
200
100
0
S1D3
S2D2
S3D2
S3D5
Caco2

FIG. 6U


HOXA13
Fold change relative to H1-hESC
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
S1D3
S2D2
S3D2
S3D5
Caco2

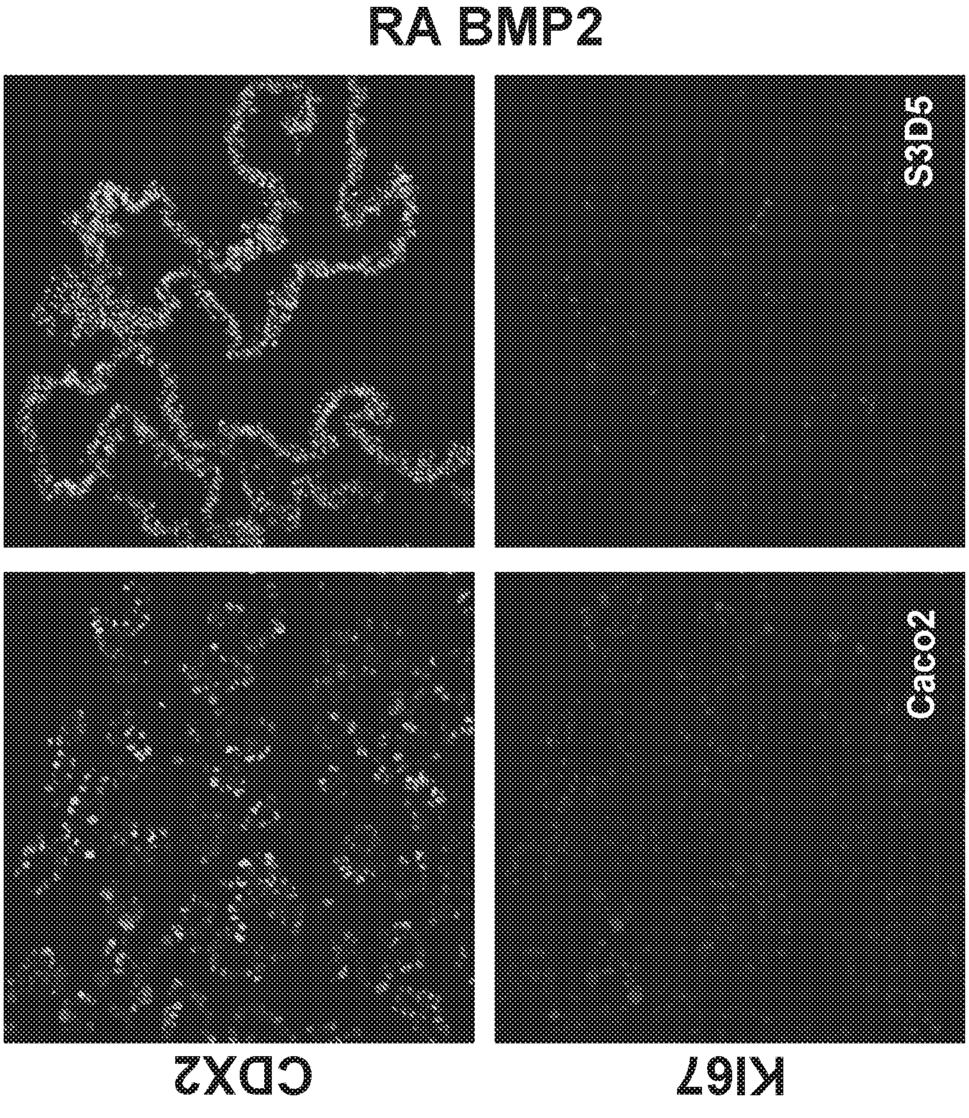


FIG. 7

FIG. 8A

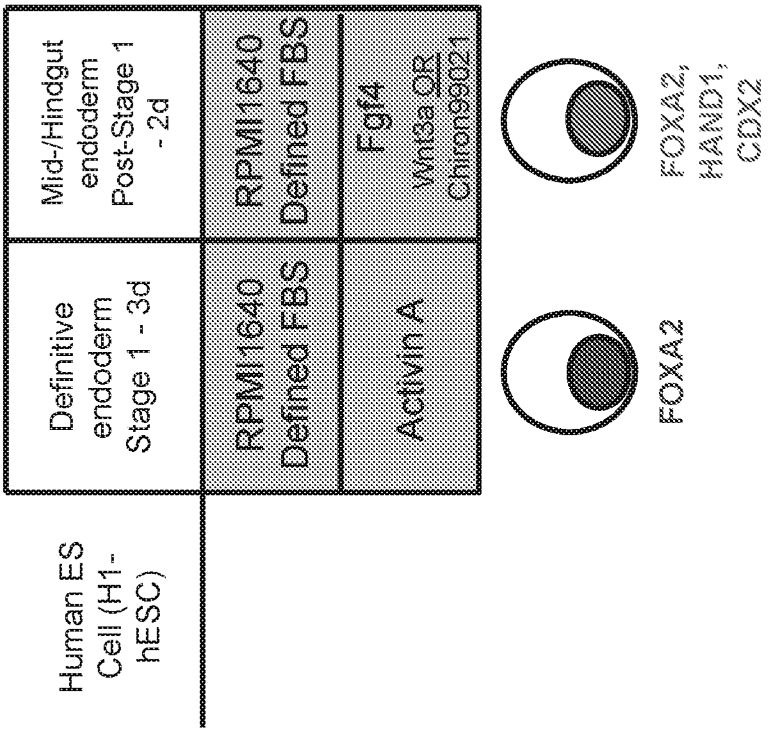

Human ES Cell (H1-hESC)
Definitive endoderm Stage 1 - 3d
RPMI1640 Defined FBS
Activin A
FOXA2
Mid-/Hindgut endoderm Post-Stage 1 - 2d
RPMI1640 Defined FBS
Fgf4
Wnt3a OR Chiron99021
FOXA2, HAND1, CDX2

FIG. 8B

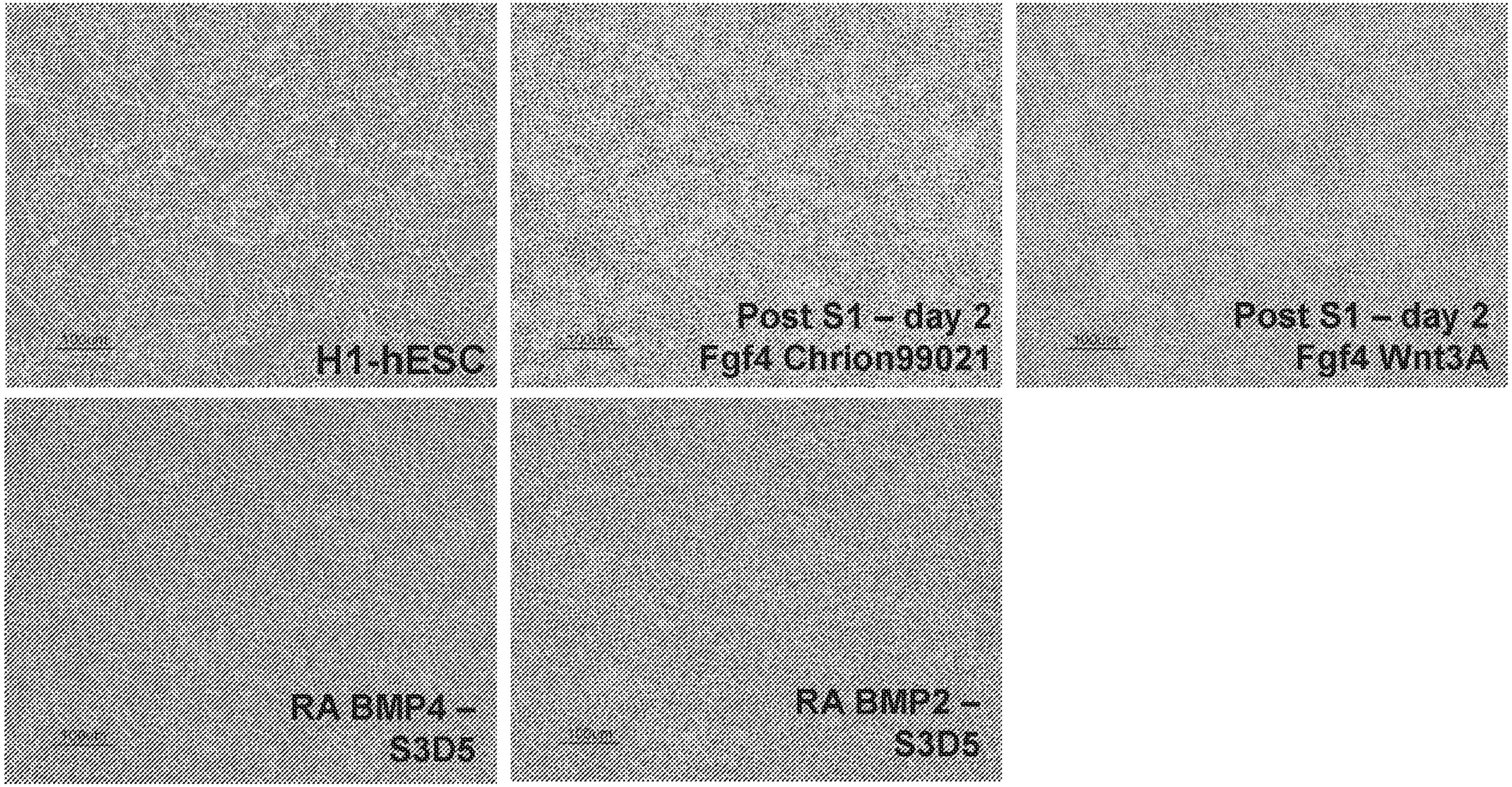

H1-hESC
Post S1 – day 2
Fgf4 Chrion99021
Post S1 – day 2
Fgf4 Wnt3A
RA BMP4 –
S3D5
RA BMP2 –
S3D5

FIG. 8C

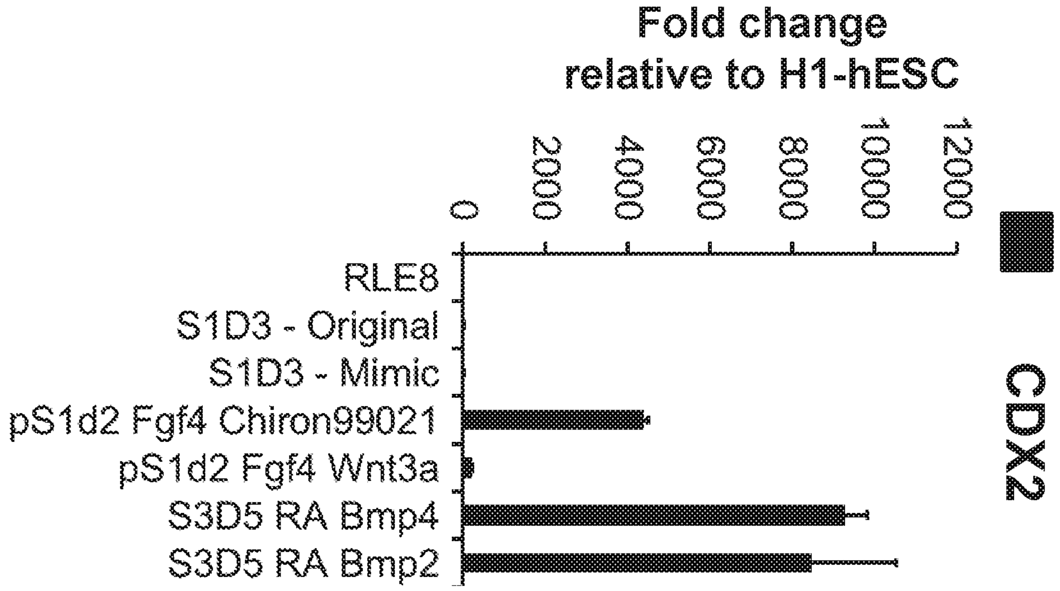

CDX2
Fold change relative to H1-hESC
0
2000
4000
6000
8000
10000
12000
RLE8
S1D3 - Original
S1D3 - Mimic
pS1d2 Fgf4 Chiron99021
pS1d2 Fgf4 Wnt3a
S3D5 RA Bmp4
S3D5 RA Bmp2

FIG. 8D

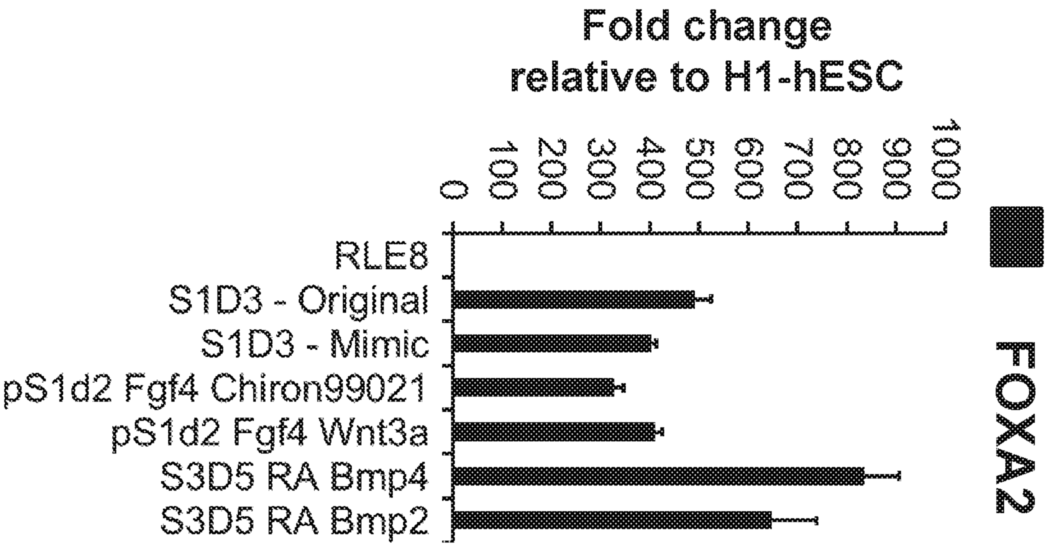

Fold change
relative to H1-hESC
0
100
200
300
400
500
600
700
800
900
1000
FOXA2
RLE8
S1D3 - Original
S1D3 - Mimic
pS1d2 Fgf4 Chiron99021
pS1d2 Fgf4 Wnt3a
S3D5 RA Bmp4
S3D5 RA Bmp2

FIG. 8E

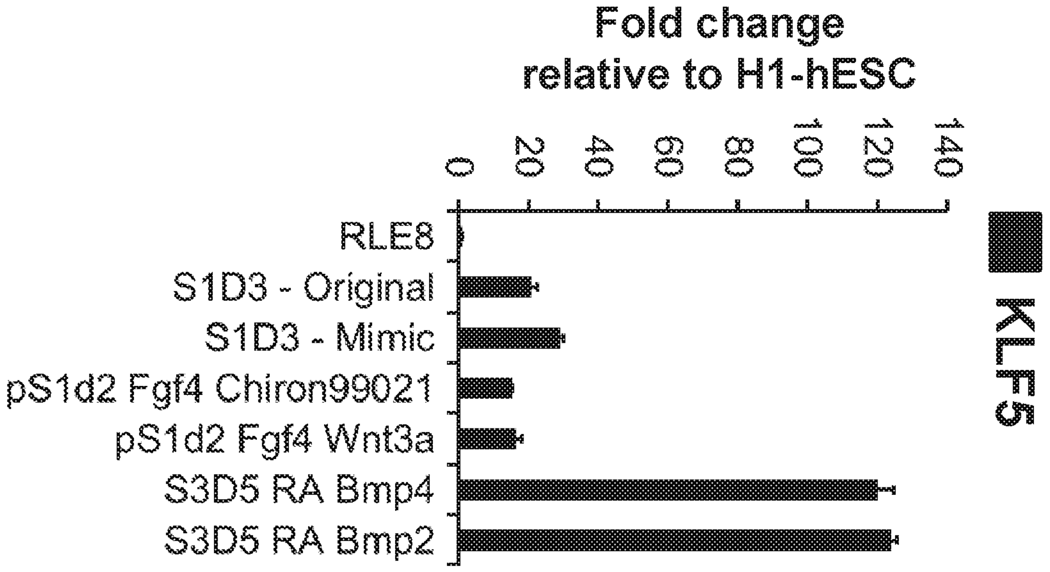
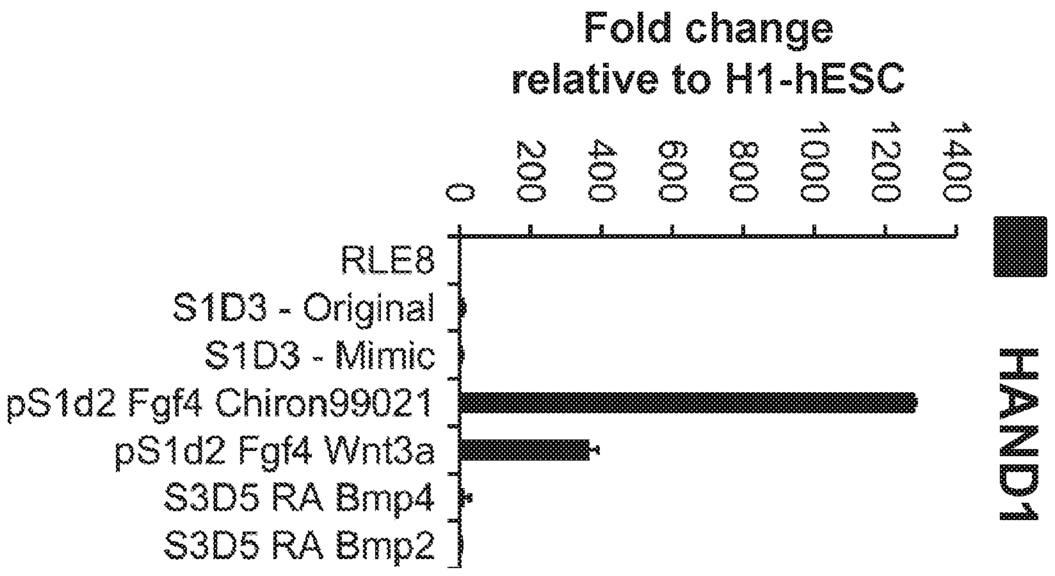

Fold change
relative to H1-hESC
0
20
40
60
80
100
120
140
RLE8
S1D3 - Original
S1D3 - Mimic
pS1d2 Fgf4 Chiron99021
pS1d2 Fgf4 Wnt3a
S3D5 RA Bmp4
S3D5 RA Bmp2
KLF5

FIG. 8F


HAND1
Fold change relative to H1-hESC
0
200
400
600
800
1000
1200
1400
RLE8
S1D3 - Original
S1D3 - Mimic
pS1d2 Fgf4 Chiron99021
pS1d2 Fgf4 Wnt3a
S3D5 RA Bmp4
S3D5 RA Bmp2