[0005] As such, in addition to achieving efficient directed differentiation of hESCs, it would be beneficial to identify markers which can be used to identify and/or segregate cells at their earliest stages of differentiation away from hESCs. Additionally, it would be beneficial to identify factors which promote the differentiation of these early precursor cells derived from hESCs to cell types useful for cell therapies.

Summary ~~of the Invention~~

[0006] In one aspect, the present invention refers to a method of producing mesendoderm cells, the method comprising the steps of: obtaining a cell population comprising human pluripotent stem cells; providing the human pluripotent stem cells with a medium comprising less than 2% serum and at least one TGFβ superfamily growth factor in an amount sufficient to promote differentiation of the human pluripotent stem cells to mesendoderm cells, said mesendoderm cells being multipotent cells that can differentiate into mesoderm or definitive endoderm cells; and allowing sufficient time for the mesendoderm cells to form, wherein the sufficient time for the mesendoderm cells to form has been determined by detecting the presence of mesendoderm cells in the cell population. In another aspect, the present invention refers to an *in vitro* method of producing human mesendoderm cells, the method comprising: obtaining a cell population comprising human pluripotent stem cells; and differentiating said cell population in a medium comprising less than 2% serum and at least one growth factor of the TGFβ superfamily, wherein the growth factor is present in the medium in an amount sufficient to promote differentiation of at least a portion of said pluripotent cells to mesendoderm. Also described herein are~~Embodiments of the present invention relate to~~ cell cultures comprising human cells. In such cell cultures, at least about 5% of the human cells are preprimitive streak cells, wherein the preprimitive streak cells are multipotent cells that can differentiate into mesendoderm cells. In other ~~embodiments~~examples, at least about 10% to at least about 90% of the human cells in culture are preprimitive streak cells. In certain examples~~embodiments~~ described herein, human feeder cells are also present in the cell cultures. In such examples~~embodiments~~, from at least about 5% to at least about 75% of human cells other than feeder cells are preprimitive streak cells. In some examples~~embodiments~~, the preprimitive streak cells express a marker, such as FGF8 and/or nuclear-localized β-catenin. In certain examples~~embodiments~~, the expression of one or both of these markers is greater than the expression of brachyury, FGF4, SNAI1, SOX17, FOXA2, SOX7 and/or SOX1. In still other examples~~embodiments~~ described herein, the cell cultures are substantially free of visceral endodermal cells, parietal endodermal cells, primitive endodermal cells, definitive endodermal cells, ectodermal cells and/or mesodermal cells.

[0007] With respect to certain examples~~embodiments~~ described herein, the preprimitive streak cell cultures comprise pluripotent human cells, such as human embryonic stem

cells (hESCs). In such examples~~embodiments~~, from at least about 2 to at least about 10 preprimitive streak cells are present for about every 1 hESC in the cell cultures. In some examples~~embodiments~~ the hESCs are derived from a morula, embryonic inner cell mass (ICM) or embryonic gonadal ridges. In some examples~~embodiments~~, the cell cultures containing human preprimitive streak cells comprise a medium comprising from less than about 2% (v/v) to from less than about 0.2% (v/v) serum. In preferred examples~~embodiments~~, such cell cultures comprise a medium that lacks serum or serum replacement. In other examples~~embodiments~~, the cell cultures containing human preprimitive streak cells comprise a growth factor of the Nodal/Activin subgroup of the TGFβ superfamily. In preferred embodiments, the growth factor is activin A.

[0008] Additional examples~~embodiments~~ described herein relate to cell cultures comprising mesendoderm cells, wherein the mesendoderm cells are multipotent cells that can differentiate into mesoderm or definitive endoderm cells. In such examples~~embodiments~~, the cell cultures comprise human cells, wherein at least about 5% of the human cells are mesendoderm cells. In other examples~~embodiments~~, at least about 10% to at least about 90% of the human cells in culture are

mesendoderm cells. In certain examples~~embodiments~~ described herein, human feeder cells are also present in the cell cultures. In such examples~~embodiments~~, from at least about 5% to at least about 75% of human cells other than feeder cells are mesendoderm cells. In some examples~~embodiments~~, the mesendoderm cells express a marker, such as brachyury, FGF4 and/or SNAI1. In certain examples~~embodiments~~, the expression of one or more of these markers is greater than the expression of OCT4, SOX17, CXCR4, FOXA2, SOX7 and/or SOX1. In still other examples~~embodiments~~ described herein, the cell cultures are substantially free of visceral endodermal cells, parietal endodermal cells, primitive endodermal cells, definitive endodermal cells, ectodermal cells and/or mesodermal cells.

[0009] With respect to certain examples~~embodiments~~ described herein, the mesendoderm cell cultures comprise pluripotent human cells, such as human embryonic stem cells (hESCs). In such examples~~embodiments~~, from at least about 2 to at least about 10 mesendoderm cells are present for about every 1 hESC in the cell cultures. In some examples~~embodiments~~ the hESCs are derived from a morula, embryonic inner cell mass (ICM) or embryonic gonadal ridges. In some examples~~embodiments~~, the cell cultures containing human mesendoderm cells comprise a medium comprising from less than about 2% (v/v) to from less than about 0.2% (v/v) serum. In preferred examples~~embodiments~~, such cell cultures comprise a medium that lacks serum or serum replacement. In other examples~~embodiments~~, the cell cultures containing human mesendoderm cells comprise a growth factor of the Nodal/Activin subgroup of the TGFβ superfamily. In preferred examples~~embodiments~~, the growth factor is activin A.

[0010] Further examples~~embodiments~~ described herein relate to cell populations comprising cells wherein at least about 90% of the cells are human preprimitive streak cells. In these ~~embodiment~~examples, the preprimitive streak cells are multipotent cells that can differentiate into mesendoderm cells. In other ~~embodiment~~examples, at least about 95% to at least about 98% of the human cells in the population are preprimitive streak cells. In some ~~embodiment~~examples, preprimitive streak cells express a marker, such as FGF8 and/or nuclear-localized β-catenin. In certain ~~embodiment~~examples, the expression of one or both of these markers is greater than the expression of brachyury, FGF4, SNAI1, SOX17, FOXA2, SOX7 and/or SOX1. In still other ~~embodiment~~examples described herein, the cell populations are substantially free of visceral endodermal cells, parietal endodermal cells, primitive endodermal cells, definitive endodermal cells, ectodermal cells and/or mesodermal cells.

[0011] Still other ~~embodiment~~examples described herein relate to cell populations comprising cells wherein at least about 90% of the cells are human mesendoderm cells. In these ~~embodiment~~examples, the mesendoderm cells are multipotent cells that can differentiate into mesoderm cells and/or definitive endoderm cells. In other ~~embodiment~~examples, at least about 95% to at least about 98% of the human cells in the population are mesendoderm cells. In some ~~embodiment~~examples, mesendoderm cells express a marker, such as brachyury, FGF4 and/or -SNAI1. In certain ~~embodiment~~examples, the expression of one or both of these markers is greater than the expression of OCT4, SOX17, CXCR4, FOXA2, SOX7 and/or SOX1. In still other ~~embodiment~~examples described

herein, the cell populations are substantially free of visceral endodermal cells, parietal endodermal cells, primitive endodermal cells, definitive endodermal cells, ectodermal cells and/or mesodermal cells.

[0012] Additional ~~embodiment~~examples described herein relate to methods of producing preprimitive streak cells. In such methods, a cell population comprising pluripotent human cells, such as hESCs, is obtained. Pluripotent human cells within the cell population are differentiated in a medium comprising less than about 2% serum and at least one growth factor of the TGFβ superfamily, wherein the growth factor is present in the medium in an amount sufficient to promote differentiation of at least a portion of said pluripotent cells to preprimitive streak cells which are multipotent and can differentiate into mesendoderm cells. Some ~~embodiment~~examples include a further step that comprises allowing sufficient time for preprimitive streak cells to form, wherein said sufficient time for preprimitive streak cells to form has been determined by detecting the presence of preprimitive streak cells in said cell population. In some ~~embodiment~~examples, sufficient time is at least about 6 hours. In other ~~embodiment~~examples, detecting the presence of preprimitive streak cells in the cell population comprises detecting the expression of at least one marker selected from the group consisting of FGF8 and nuclear-localized β-catenin and at least one marker from the group consisting of brachyury, FGF4, SNAI1, SOX17, FOXA2, SOX7 and SOX1 in cells of the cell population, wherein the expression of a marker selected from the group consisting of FGF8 and nuclear-localized β-catenin is greater than the expression of a marker selected from the group consisting of brachyury, FGF4, SNAI1, SOX17, FOXA2, SOX7 and SOX1 in said preprimitive streak cells. In such ~~embodiment~~examples, marker detection can be by quantitative polymerase chain reaction (Q-PCR), immunocytochemistry or other comparable method.

[0013] With respect to certain methods described herein, from at least about 5% to at least about 90% of the human cells in culture differentiate into preprimitive streak cells. In some ~~embodiment~~examples of the methods described herein, the growth factor present in the medium is a growth factor of the Nodal/Activin subgroup of the TGFβ superfamily. In preferred ~~embodiment~~examples, the growth factor is activin A. In other preferred ~~embodiment~~examples, the growth factor is present in the medium at a concentration ranging from at least about 10 ng/ml to at least about 1000 ng/ml. In certain ~~embodiment~~examples, the growth factor is withdrawn after about 6 hours, 12 hours or 18 hours. In additional ~~embodiment~~examples, the medium comprises from less than about 1% (v/v) to less than about 0.2% (v/v) serum. In other ~~embodiment~~examples, the medium is low serum RPMI. In still other ~~embodiment~~examples, the cell population is differentiated in the absence of serum or serum replacement.

[0014] Other methods described herein are methods of producing mesendoderm cells. In such methods, a cell population comprising pluripotent human cells, such as hESCs, is obtained. Pluripotent human cells within the cell population are differentiated in a medium

comprising less than about 2% serum and at least one growth factor of the TGFβ superfamily, wherein the growth factor is present in the medium in an amount sufficient to promote differentiation of at least a portion of said pluripotent cells to mesendoderm cells which are multipotent and can differentiate into mesoderm cells and/or definitive endoderm cells. Some ~~embodiment~~examples include a further step that comprises allowing sufficient time for mesendoderm cells to form, wherein said sufficient time for mesendoderm cells to form has been determined by detecting the presence of mesendoderm cells in said cell population. In some ~~embodiment~~examples, sufficient time is at least about 24 hours. In other ~~embodiment~~examples, detecting the presence of mesendoderm in the cell population comprises detecting the expression of at least one marker selected from the group consisting of brachyury, FGF4 and/or SNAI1 and at least one marker from the group consisting of OCT4, SOX17, CXCR4, FOXA2, SOX7 and/or SOX1 in cells of the cell population, wherein the expression of a marker selected from the group consisting of brachyury, FGF4 and/or SNAI1 is greater than the expression of a marker selected from the group consisting of OCT4, SOX17, CXCR4, FOXA2, SOX7 and/or SOX1 in said mesendoderm cells. In such ~~embodiment~~examples, marker detection can be by quantitative polymerase chain reaction (Q-PCR), immunocytochemistry or other comparable method.

[0015] With respect to certain methods described herein, from at least about 5% to at least about 90% of the human cells in culture differentiate into mesendoderm cells. In some ~~embodiment~~examples of the methods described herein, the growth factor present in the medium is a growth factor of the Nodal/Activin subgroup of the TGFβ superfamily. In preferred ~~embodiment~~examples, the growth factor is activin A. In other preferred ~~embodiment~~examples, the growth factor is present in the medium at a concentration ranging from at least about 10 ng/ml to at least about 1000 ng/ml. In certain ~~embodiment~~examples, the growth factor is withdrawn after about 24 hours, 36 hours or 48 hours. In additional ~~embodiment~~examples, the medium comprises from less than about 1% (v/v) to less than about 0.2% (v/v) serum. In other ~~embodiment~~examples, the medium is low serum RPMI. In still other ~~embodiment~~examples, the cell population is differentiated in the absence of serum or serum replacement.

[0016] Still other ~~embodiment~~examples described herein relate to methods for producing a cell population that is enriched in preprimitive streak cells. Such methods comprise the steps of (a) obtaining a population of pluripotent cells, such as hESCs, wherein at least a one cell of the pluripotent cell population comprises a copy of a nucleic acid sequence encoding green fluorescent protein (GFP) or a biologically active fragment thereof under the control of the FGF8 promoter, (b) differentiating the pluripotent cells so as to produce preprimitive streak cells which are multipotent cells that can differentiate into mesendoderm cells, and (c) separating the preprimitive streak cells from cells that do not express GFP. In some ~~embodiment~~examples, the cell population comprises at least about 95% to at least about 98% preprimitive streak cells. In some

~~embodiment~~examples, the differentiating step of the methods described herein comprises providing a pluripotent cell population with at least one growth factor of the TGFβ superfamily, such as activin A. Preferred concentrations of activin A range from at least about 50 ng/ml to at least about 500 ng/ml. In additional ~~embodiment~~examples, the cell population is differentiated in a medium comprising from less than about 1% (v/v) to less than about 0.1% (v/v) serum. In other ~~embodiment~~examples, the medium is low serum RPMI. In still other ~~embodiment~~examples, the cell population is differentiated in the absence of serum or serum replacement.

[0017] Other ~~embodiment~~examples described herein relate to methods for producing a cell population that is enriched in mesendoderm cells. Such methods comprise the steps of (a) obtaining a populations of pluripotent cells, such as hESCs, wherein at least a one cell of the pluripotent cell population comprises a copy of a nucleic acid sequence encoding green fluorescent protein (GFP) or a biologically active fragment thereof under the control of the brachyury, FGF4 or SNAI1 promoter, (b) differentiating the pluripotent cells so as to produce mesendoderm cells which are multipotent cells that can differentiate into mesoderm cells and/or definitive endoderm cells, and (c) separating the mesendoderm cells from cells that do not express GFP. In some ~~embodiment~~examples, the cell population comprises at least about 95% to at least about 98% mesendoderm cells. In some ~~embodiment~~examples, the differentiating step of the methods described herein comprises providing a pluripotent cell population with at least one growth factor of the TGFβ superfamily, such as activin A. Preferred concentrations of activin A range from at least about 50 ng/ml to at least about 500 ng/ml. In additional ~~embodiment~~examples, the cell population is differentiated in a medium comprising from less than about 1% (v/v) to less than about 0.1% (v/v) serum. In other ~~embodiment~~examples, the medium is low serum RPMI. In still other ~~embodiment~~examples, the cell population is differentiated in the absence of serum or serum replacement.

[0018] Some ~~embodiment~~examples described herein are screening methods for identifying a differentiation factor capable of promoting the differentiation of preprimitive streak cells in a cell population comprising human cells. Such methods comprise the steps of (a) obtaining a cell population comprising human preprimitive streak cells, (b) providing a candidate differentiation factor to the cell population, (c) determining expression of a marker in the cell population at a first time point, determining expression of the same marker in the cell population at a second time point, wherein the second time point is subsequent to the first time point and wherein the second time point is subsequent to providing the population with the candidate differentiation factor, (d) and determining if expression of the marker in the cell population at the second time point is increased or decreased as compared to the expression of the marker in the cell population at the first time point, wherein an increase or decrease in expression of the marker in the cell population indicates that the candidate differentiation factor is capable of promoting the differentiation of the preprimitive streak cells. In certain ~~embodiment~~examples, the first time point is prior to or at

approximately the same time as providing the candidate differentiation factor. In other ~~embodiment~~examples, the first time point is subsequent to providing the candidate differentiation factor. In some ~~embodiment~~examples of the screening methods described herein, the human preprimitive streak cells differentiate into cells, such as mesendoderm cells, mesoderm cells and/or definitive endoderm cells, in response to the candidate differentiation factor. In some ~~embodiment~~examples, mesendoderm is indicated by the expression of markers, such as brachyury, FGF4 and/or SNAI1. In other ~~embodiment~~examples, mesoderm is indicated by the expression of markers, such as FOXF1, FLK1, BMP4, MOX1 and SDF1. In still other ~~embodiment~~examples, definitive endoderm is indicated by the expression of markers, such as CXCR4 and/or SOX17.

[0019] With respect to the screening methods described herein, certain ~~embodiment~~examples relate to providing a candidate differentiation factor, such as at least one growth factor from the TGFβ superfamily, such as activin A. In other ~~embodiment~~examples, the candidate differentiation factor is a small molecule or a polypeptide. In still other ~~embodiment~~examples, the candidate differentiation factor is not a factor of the TGFβ superfamily. In still other ~~embodiment~~examples, the candidate differentiation factor is a factor that is not known to cause the differentiation of preprimitive streak cells.

[0020] Other ~~embodiment~~examples described herein are screening methods for identifying a differentiation factor capable of promoting the differentiation of mesendoderm cells in a cell population comprising human cells. Such methods comprise the steps of (a) obtaining a cell population comprising human mesendoderm cells, (b) providing a candidate differentiation factor to the cell population, (c) determining expression of a marker in the cell population at a first time point, determining expression of the same marker in the cell population at a second time point, wherein the second time point is subsequent to the first time point and wherein the second time point is subsequent to providing the population with the candidate differentiation factor, (d) and determining if expression of the marker in the cell population at the second time point is increased or decreased as compared to the expression of the marker in the cell population at the first time point, wherein an increase or decrease in expression of the marker in the cell population indicates that the candidate differentiation factor is capable of promoting the differentiation of the mesendoderm cells. In certain ~~embodiment~~examples, the first time point is prior to or at approximately the same time as providing the candidate differentiation factor. In other ~~embodiment~~examples, the first time point is subsequent to providing the candidate differentiation factor. In some ~~embodiment~~examples of the screening methods described herein, the human mesendoderm cells differentiate into cells, such as mesoderm cells and/or definitive endoderm cells, in response to the candidate differentiation factor. In some ~~embodiment~~examples, mesoderm is indicated by the expression of markers, such as FOXF1, FLK1, BMP4, MOX1 and SDF1. In other ~~embodiment~~examples, definitive endoderm is indicated by the expression of markers, such as CXCR4 and/or SOX17.

[0021] With respect to the screening methods described herein, certain ~~embodiment~~examples relate to providing a candidate differentiation factor, such as at least one growth factor from the TGFβ superfamily, such as activin A. In other ~~embodiment~~examples, the candidate differentiation factor is a small molecule or a polypeptide. In still other ~~embodiment~~examples, the candidate differentiation factor is not a factor of the TGFβ superfamily. In still other ~~embodiment~~examples, the candidate differentiation factor is a factor that is not known to cause the differentiation of mesendoderm cells.

[0022] Additional ~~embodiment~~examples relate to a method of increasing the expression of the FGF8 gene product in a human embryonic stem cell (hESC) in vitro. The method comprises obtaining an hESC in a medium comprising less than about 2% (v/v) serum and contacting the hESC with a differentiation factor in an amount sufficient to increase expression of the FGF8 gene product. In some ~~embodiment~~examples, the differentiation factor is at least one growth factor from the TGFβ superfamily, such as activin A. In some ~~embodiment~~examples, the medium does not comprise serum replacement.

[0023] Still other ~~embodiment~~examples relate to a method of increasing the expression of a gene product selected from the group consisting of brachyury, FGF4 and SNAI1 in a human embryonic stem cell (hESC) in vitro. The method comprises obtaining an hESC in a medium comprising less than about 2% (v/v) serum and contacting the hESC with a differentiation factor in an amount sufficient to increase expression of a gene product selected from the group consisting of brachyury, FGF4 and SNAI1. In some ~~embodiment~~examples, the differentiation factor is at least one growth factor from the TGFβ superfamily, such as activin A. In some ~~embodiment~~examples, the medium does not comprise serum replacement.

[0024] Some ~~embodiment~~examples described herein relate to a cell culture comprising human embryonic stem cells (hESCs) and a medium comprising less than about 2% (v/v) serum, wherein the hESCs begin differentiating at a reference time point such that expression of FGF8 mRNA is substantially upregulated as compared to baseline FGF8 mRNA expression in the hESCs by about 6 hours from the reference time point. In some ~~embodiment~~examples, the expression of β-catenin polypeptide begins to become localized to the cell nucleus by about 17 hours from the reference time point. In other ~~embodiment~~examples, the expression of brachyury, FGF4 and/or SNAI1 mRNA is substantially upregulated by about 24 hours from the reference time point. In still other ~~embodiment~~examples, the expression of E-cadherin mRNA begins to be downregulated by about 12 hours from the reference time point. Additionally, in some ~~embodiment~~examples, the expression of SOX17 mRNA is substantially upregulated by about 48 hours from the reference time point and/or the expression of FOXA2 mRNA is substantially upregulated by about 96 hours from the reference time point. In certain ~~embodiment~~examples of the cell cultures described herein, the medium comprises from less than about 1% (v/v) to less than about 0.2% (v/v) serum. In other ~~embodiment~~examples, the

medium comprises about 0% (v/v) serum. In still other ~~embodiment~~examples, the medium does not comprise serum replacement.

[0025] Additional ~~embodiment~~examples described herein relate to cells culture comprising human embryonic stem cells, a differentiation factor of the TGFβ superfamily and a medium comprising less than about 2% (v/v) serum, wherein a first set of marker genes is upregulated or downregulated prior to, or at about the same time as, the upregulation or peak expression of a second set and/or a third set of marker genes. In some ~~embodiment~~examples the medium does not include serum or serum replacement.

[0026] Still other ~~embodiment~~examples relate to methods of differentiating cells in a cell culture by contacting a cell culture comprising human embryonic stem cells with a medium comprising less that about 2% serum, providing the hESCs with a differentiation factor of the TGFβ superfamily, and permitting differentiation of the hESCs to occur. In some ~~embodiment~~examples, such methods produce cells having a first set of marker genes that is upregulated or downregulated prior to, or at about the same time as, the upregulation or peak expression of a second set and/or a third set of marker genes. In some ~~embodiment~~examples the medium does not include serum or serum replacement.

[0027] In certain jurisdictions, there may not be any generally accepted definition of the term "comprising." As used herein, the term "comprising" is intended to represent "open" language which permits the inclusion of any additional elements. With this in mind, additional ~~embodiment~~examples ~~of the present inventions~~ are described with reference to the numbered paragraphs below:

[0028] 1. A cell culture comprising human cells wherein at least about 5% of said human cells are preprimitive streak cells, said preprimitive streak cells being multipotent cells that can differentiate into mesendoderm cells.

[0029] 2. The cell culture of paragraph 1, wherein at least about 10% of said human cells are preprimitive streak cells.

[0030] 3. The cell culture of paragraph 1, wherein at least about 20% of said human cells are preprimitive streak cells.

[0031] 4. The cell culture of paragraph 1, wherein at least about 30% of said human cells are preprimitive streak cells.

[0032] 5. The cell culture of paragraph 1, wherein at least about 40% of said human cells are preprimitive streak cells.

[0033] 6. The cell culture of paragraph 1, wherein at least about 50% of said human cells are preprimitive streak cells.

[0034] 7. The cell culture of paragraph 1, wherein at least about 60% of said human cells are preprimitive streak cells.

consisting of FZD10, FGF5, Nanog and OCT4 is upregulated or expression of a marker gene selected from the group consisting of GBX2, ZFP42 and SOX2 is downregulated prior to upregulation of expression of a marker gene selected from the group consisting of FGF8, Nodal, HEG, HEY1, GATA2, BIK and ID1.

[0360] 333. The method of paragraph 332, wherein expression of a marker gene selected from the group consisting of FZD10, FGF5, Nanog and OCT4 is upregulated or expression of a marker gene selected from the group consisting of GBX2, ZFP42 and SOX2 is downregulated prior to upregulation of expression of a marker gene selected from the group consisting of brachyury, FGF4, SNAI1, Wnt3, MIXL1, DKK4, NETO1, T, DACT1, FLJ22662, SLIT2, GAD1 and GRM4.

[0361] 334. The method of paragraph 332, wherein said medium comprises less than about 1% (v/v) serum.

[0362] 335. The method of paragraph 332, wherein said medium comprises less than about 0.2% (v/v) serum.

[0363] 336. The method of paragraph 332, wherein said medium comprises about 0% (v/v) serum.

[0364] 337. The method of paragraph 332, wherein said medium lacks serum or lacks serum replacement.

[0365] 338. The method of paragraph 332 further comprising a differentiation factor of the TGFβ superfamily.

[0366] 339. The method of paragraph 338, wherein said differentiation factor comprises activin A.

[0367] 340. The method of paragraph 339, wherein said activin A is present at a concentration of about 100 ng/ml.

[0368] It will be appreciated that the methods and compositions described above relate to cells cultured *in vitro*. However, the above-described *in vitro* differentiated cell compositions may be used for *in vivo* applications.

[0369] Additional ~~embodiments~~ examples in connection with~~of~~ the present invention may also be found in United States Provisional Patent Application Number 60/532,004, entitled DEFINITIVE ENDODERM, filed December 23, 2003; U.S. Provisional Patent Application Number 60/566,293, entitled PDX1 EXPRESSING ENDODERM, filed April 27, 2004; U.S. Provisional Patent Application Number 60/586,566, entitled CHEMOKINE CELL SURFACE RECEPTOR FOR THE ISOLATION OF DEFINITIVE ENDODERM, filed July 9, 2004; U.S. Provisional Patent Application Number 60/587,942, entitled CHEMOKINE CELL SURFACE RECEPTOR FOR THE ISOLATION OF DEFINITIVE ENDODERM, filed July 14, 2004; U.S. Patent Application Number 11/021,618, entitled DEFINITIVE ENDODERM, filed December 23, 2004, U.S. Patent Application Number 11/115,868, entitled PDX1 EXPRESSING ENDODERM, filed

Detailed Description

[0382] A crucial stage in early human development termed gastrulation occurs 2-3 weeks after fertilization. Gastrulation is extremely significant because it is at this time that the three primary germ layers are first specified and organized (Lu et al., 2001; Schoenwolf and Smith, 2000). The ectoderm is responsible for the eventual formation of the outer coverings of the body and the entire nervous system whereas the heart, blood, bone, skeletal muscle and other connective tissues are derived from the mesoderm. Definitive endoderm is defined as the germ layer that is responsible for formation of the entire gut tube which includes the esophagus, stomach and small and large intestines, and the organs which derive from the gut tube such as the lungs, liver, thymus, parathyroid and thyroid glands, gall bladder and pancreas (Grapin-Botton and Melton, 2000; Kimelman and Griffin, 2000; Tremblay et al., 2000; Wells and Melton, 1999; Wells and Melton, 2000). A very important distinction should be made between the definitive endoderm and the completely separate lineage of cells termed primitive endoderm. The primitive endoderm is primarily responsible for formation of extra-embryonic tissues, mainly the parietal and visceral endoderm portions of the placental yolk sac and the extracellular matrix material of Reichert's membrane.

[0383] During gastrulation, the process of definitive endoderm formation begins with a cellular migration event in which mesendoderm cells (cells competent to form mesoderm or endoderm) migrate through a structure called the primitive streak. As the mesendoderm cells ingress through the primitive streak, they undergo an epithelial to mesenchymal transition (EMT) and become either mesoderm or definitive endoderm. Definitive endoderm is derived from cells, which migrate through the anterior portion of the streak and through the node (a specialized structure at the anterior-most region of the streak). As migration occurs, definitive endoderm populates first the most anterior gut tube and culminates with the formation of the posterior end of the gut tube.

[0384] Both primitive streak precursor cells (preprimitive streak cells) and primitive streak cells (mesendoderm cells) are early stage precursor cells the give rise to the mesoderm and the definitive endoderm. In fact, preprimitive streak cells and mesendoderm cells may be the earliest precursors in the developmental process from pluripotency to terminally differentiated cells, tissues and/or organs made from the mesoderm and definitive endoderm lineages. Until now, neither cell populations enriched in human preprimitive streak cells nor cell populations enriched in human mesendoderm cells have been obtained. Furthermore, the cells of such cell populations have not been previously characterized *in vitro*.

[0385] In view of the foregoing, some ~~embodiments of the invention~~examples described herein relate to cell cultures and/or enriched cell populations comprising human preprimitive streak cells and cell cultures and/or enriched cell populations comprising human mesendoderm cells. In such ~~embodiment~~examples, the preprimitive streak cells and the mesendoderm cells are capable

of further differentiation into mesoderm cells and/or definitive endoderm cells as well as cells, tissues and/or organs derived from these lineages.

[0386] Other ~~embodiments of the invention~~examples relate to methods for producing cell cultures and/or enriched cell populations comprising human preprimitive streak cells as well as methods for producing cell cultures and/or enriched cell populations comprising human mesendoderm cells.

[0387] Still other ~~embodiment~~examples described herein relate to screening methods for identifying one or more differentiation factors that are useful for differentiating cells in a cell population comprising preprimitive streak cells or mesendoderm cells. Such factors are useful for promoting the differentiation of these cell types to mesoderm and/or definitive endoderm cells as well as cells, tissues and/or organs derived from either of these cell lineages.

[0388] ~~Certain other aspects of the present invention~~Other examples relate to methods for increasing the expression of certain early stage cell markers. Further ~~aspects~~ examples relate to cell compositions comprising cells expressing certain markers during the course of differentiation.

Definitions

[0389] Certain terms and phrases as used throughout this application have the meanings provided as follows:

[0390] As used herein, "embryonic" refers to a range of developmental stages of an organism beginning with a single zygote and ending with a multicellular structure that no longer comprises pluripotent or totipotent cells other than developed gametic cells. In addition to embryos derived by gamete fusion, the term "embryonic" refers to embryos derived by somatic cell nuclear transfer.

[0391] As used herein, "multipotent" or "multipotent cell" refers to a cell type that can give rise to a limited number of other particular cell types.

[0392] As used herein, "expression" refers to the production of a material or substance as well as the level or amount of production of a material or substance. Thus, determining the expression of a specific marker refers to detecting either the relative or absolute amount of the marker that is expressed or simply detecting the presence or absence of the marker.

[0393] As used herein, "marker" refers to any molecule that can be observed or detected. For example, a marker can include, but is not limited to, a nucleic acid, such as a transcript of a specific gene, a polypeptide product of a gene, a non-gene product polypeptide, a glycoprotein, a carbohydrate, a glycolipd, a lipid, a lipoprotein or a small molecule (for example, molecules having a molecular weight of less than 10,000 amu)

[0394] When used in connection with cell cultures and/or cell populations, the term "portion" means any non-zero amount of the cell culture or cell population, which ranges from a single cell to the entirety of the cell culture or cells population.

[0395] With respect to cells in cell cultures or in cell populations, the phrase "substantially free of" means that the specified cell type of which the cell culture or cell population is free, is present in an amount of less than about 5% of the total number of cells present in the cell culture or cell population.

[0396] With respect to cell culture medium, as used herein, "low serum RPMI" refers to a low serum containing medium, wherein the serum concentration is gradually increased over a defined time period. For example, in one ~~embodiment~~example, low serum RPMI comprises a concentration of about 0.2% fetal bovine serum (FBS) on the first day of cell growth, about 0.5% FBS on the second day of cell growth and about 2% FBS on the third through fifth day of cell growth. In another ~~embodiment~~example, low serum RPMI comprises a concentration of about 0% on day one, about 0.2% on day two and about 2% on the third and subsequent days.

[0397] As used herein, "serum replacement" refers to serum substitute comprising IGF or insulin.

Model of Early Events in the Differentiation of hESCs to Definitive Endoderm Cells

[0398] Figure 1 displays a model summarizing the early transitions of human embryonic stem cells (hESCs) *in vitro*. Differentiation of hESCs through a process that closely recapitulates gastrulation can be orchestrated by the application of high dose activin A in the context of low serum supplementation. The expression of FGF8 and nuclear localization of β-catenin, events that occur in the proximal epiblast prior to primitive streak formation, is evident prior to about 24 hours (preprimitive streak cells). High level expression of the primitive streak-expressed genes (brachyury and FGF4) occurs at about 24 hours. If maintained in high dose activin A, the primitive streak cells (mesendoderm cells) are efficiently converted into definitive endoderm. In contrast, in the absence of activins, these mesendoderm precursors become mesoderm. Treatment of hESCs with BMP4 and SU5402 induces gene expression associated with primitive endoderm and trophectoderm.

Preprimitive Streak Cells and Mesendoderm Cells and Processes Related Thereto

[0399] ~~Embodiment~~Examples described herein relate to novel, defined processes for the production of preprimitive streak cells and/or mesendoderm cells in culture by differentiating pluripotent cells, such as stem cells into preprimitive streak cells and/or mesendoderm cells. As described above, preprimitive streak cells are capable of differentiating into mesendoderm cells as well as cells, tissues and/or organs derived therefrom. Mesendoderm cells are capable of differentiating into mesoderm cells and/or definitive endoderm cells as well as cells, tissues and/or organs derived from either of these lineages. In some ~~embodiment~~examples, the preprimitive steak cells are converted, through a mesendoderm intermediate, into terminally differentiated cells of either the mesoderm or definitive endoderm lineages. As will be described in further detail

below, such processes can provide the basis for efficient production of a variety of human endodermal and mesodermal derived tissues. For example, such processes can provide the basis for efficient production of human endodermal derived tissues, such as pancreas, liver, lungs, stomach, intestine, thyroid, thymus, pharynx, gallbladder and urinary bladder. Importantly, production of preprimitive streak cells and/or mesendoderm cells is an early step in differentiation of a stem cell to a functional insulin-producing β-cell. As another example, preprimitive streak cell and/or mesendoderm cell differentiation can provide the basis for efficient production of human mesodermal derived tissues, such as blood cells, cardiovascular tissues, skeletal tissues as well as other structural and connective tissues. To obtain useful quantities of any of the above-described cell or tissue types, high efficiency differentiation is desirable for each of the differentiation steps that occur prior to reaching the terminally differentiated cell fate. Since differentiation of stem cells to preprimitive streak cells and/or mesendoderm cells represents very early steps towards the production of functional terminally differentiated cells of the mesoderm and definitive endoderm cell lineages (as shown in Figure 1), high efficiency differentiation at this step is particularly desirable.

[0400] In view of the desirability of efficient differentiation of pluripotent cells to preprimitive streak cells and/or mesendoderm cells, some aspects of the differentiation processes described herein relate to *in vitro* methodology that results in approximately 5-90% conversion of pluripotent cells to preprimitive streak cells and/or mesendoderm cells. Typically, such methods encompass the application of culture and growth factor conditions in a defined and temporally specified fashion. Further enrichment of the cell population for preprimitive streak cells and/or mesendoderm cells can be achieved by isolation and/or purification of the preprimitive streak cells and/or mesendoderm cells from other cells in the population by sorting cells based on differential fluorescent marker expression. As such, some ~~embodiment~~examples described herein relate to preprimitive streak cells as well as methods for producing and isolating and/or purifying such cells. Other ~~embodiment~~examples relate to mesendoderm cells as well as methods for producing and isolating and/or purifying such cells.

[0401] In order to determine the amount of preprimitive streak cells in a cell culture or cell population, a method of distinguishing this cell type from the other cells in the culture or in the population is desirable. Accordingly, certain ~~embodiment~~examples described herein relate to cell markers whose presence, absence and/or relative expression levels are specific for preprimitive streak cells and methods for detecting and determining the expression of such markers.

[0402] In some ~~embodiment~~examples described herein, the presence, absence and/or level of expression of a marker is determined by quantitative PCR (Q-PCR). For example, the amount of transcript produced by certain genetic markers, such as OCT4, ECAD, FGF8, β-catenin, brachyury, FGF4, SNAI1, SOX17, CXCR4, GSC, MIXL1, FOXA2, SOX7, FOXF1, FLK1,

BML4, MOX1, SDF1 and other markers described herein is determined by quantitative Q-PCR. In other ~~embodiment~~examples, immunohistochemistry is used to detect the proteins expressed by the above-mentioned genes. In still other ~~embodiment~~examples, immunohistochemistry/ immunocytochemistry is used to detect cell compartmental localization of certain polypeptide markers, such as the nuclear localization of β-catenin. In yet other ~~embodiment~~examples, Q-PCR and immunohistochemical techniques are both used to identify and determine the amount or relative proportions of such markers.

[0403] By using methods, such as those described above, to determine the expression of one or more appropriate markers, it is possible to identify preprimitive streak cells and/or mesendoderm cells, as well as determine the proportion of preprimitive streak cells and/or mesendoderm cells in a cell culture or cell population. For example, in some ~~embodiments of the present invention~~examples, the preprimitive streak cells or cell populations that are produced express the FGF8 marker and/or nuclear-localized β-catenin at a level of about 2 orders of magnitude greater than non- preprimitive streak cell types or cell populations. In other ~~embodiment~~examples, the preprimitive streak cells or cell populations that are produced express the FGF8 marker and/or nuclear-localized β-catenin at a level of more than 2 orders of magnitude greater than non-preprimitive streak cell types or cell populations. In other ~~embodiments of the present invention~~examples, the mesendoderm cells or cell populations that are produced express the brachyury, FGF4 and/or SNAI1 markers at a level of about 2 orders of magnitude greater than non- mesendoderm cell types or cell populations. In other ~~embodiment~~examples, the mesendoderm cells or cell populations that are produced express the brachyury, FGF4 and/or SNAI1 markers at a level of more than 2 orders of magnitude greater than non-mesendoderm cell types or cell populations.

[0404] ~~Embodiment~~Examples described herein also relate to preprimitive streak and/or mesendoderm compositions. For example, some ~~embodiment~~examples relate to cell cultures comprising preprimitive streak cells and/or mesendoderm cells, whereas others relate to cell populations enriched in preprimitive streak cells and/or mesendoderm cells. Some preferred ~~embodiment~~examples relate to cell cultures which comprise preprimitive streak cells and/or mesendoderm cells, wherein at least about 5-90% of the cells in culture are preprimitive streak cells and/or mesendoderm cells. An especially preferred ~~embodiment~~example relates to cells cultures comprising human cells, wherein at least about 5-90% of the human cells in culture are preprimitive streak cells and/or mesendoderm cells. Because the efficiency of the differentiation procedure can be adjusted by modifying certain parameters, which include but are not limited to, cell growth conditions, growth factor concentrations and the timing of culture steps, the differentiation procedures described herein can result in about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or greater than about 95% conversion of pluripotent

cells to preprimitive streak cells and/or mesendoderm cells. In other preferred ~~embodiment~~examples, conversion of a pluripotent cell population, such as a stem cell population, to substantially pure preprimitive streak cell and/or mesendoderm cell population is contemplated.

[0405] The compositions and methods described herein have several useful features. For example, the cell cultures and cell populations comprising preprimitive streak cells and/or mesendoderm cells as well as the methods for producing such cell cultures and cell populations are useful for modeling the early stages of human development. Furthermore, the compositions and methods described herein can also serve for therapeutic intervention in disease states, such as diabetes mellitus. For example, since preprimitive streak cells and/or mesendoderm cells serve as the source for only a limited number of tissues, they can be used in the development of pure tissue or cell types.

Production of Preprimitive Streak Cells from Pluripotent Cells

[0406] In some processes for producing preprimitive streak cells, the pluripotent cells used as starting material are stem cells. In certain processes, preprimitive streak cell cultures and enriched cell populations comprising preprimitive streak cells are produced from embryonic stem cells. A preferred method for deriving preprimitive streak cells utilizes human embryonic stem cells as the starting material for preprimitive streak cell production. Such pluripotent cells can be cells that originate from the morula, embryonic inner cell mass or those obtained from embryonic gonadal ridges. Human embryonic stem cells can be maintained in culture in a pluripotent state without substantial differentiation using methods that are known in the art. Such methods are described, for example, in US Patent Nos. 5,453,357, 5,670,372, 5,690,926 5,843,780, 6,200,806 and 6,251,671 the disclosures of which are incorporated herein by reference in their entireties.

[0407] In some processes for producing preprimitive streak cells, hESCs are maintained on a feeder layer. In such processes, any feeder layer which allows hESCs to be maintained in a pluripotent state can be used. One commonly used feeder layer for the cultivation of human embryonic stem cells is a layer of mouse fibroblasts. More recently, human fibroblast feeder layers have been developed for use in the cultivation of hESCs (see US Patent Application No. 2002/0072117, the disclosure of which is incorporated herein by reference in its entirety). Alternative processes for producing preprimitive streak cells permit the maintenance of pluripotent hESC without the use of a feeder layer. Methods of maintaining pluripotent hESCs under feeder-free conditions have been described in US Patent Application No. 2003/0175956, the disclosure of which is incorporated herein by reference in its entirety.

[0408] The human embryonic stem cells used herein can be maintained in culture either with or without serum. In some embryonic stem cell maintenance procedures, serum replacement is used. In others, serum free culture techniques, such as those described in US

(v/v), less than about 0.9% (v/v), less than about 1% (v/v), less than about 2% (v/v), less than about 3% (v/v), less than about 4% (v/v), less than about 5% (v/v), less than about 6% (v/v), less than about 7% (v/v), less than about 8% (v/v), less than about 9% (v/v) or less than about 10% (v/v). In some processes, preprimitive streak cells are grown without serum or without serum replacement. In still other processes, preprimitive streak cells are grown in the presence of B27. In such processes, the concentration of B27 supplement can range from about 0.1% (v/v) to about 20% (v/v).

Monitoring the Differentiation of Pluripotent Cells to Preprimitive Streak Cells

[0413] The progression of the hESC culture to preprimitive streak cells can be monitored by determining the temporal expression of markers characteristic of preprimitive streak cells. In some processes, the expression of certain markers is determined by detecting the presence or absence of the marker. Alternatively, the expression of certain markers can be determined by measuring the level at which the marker is present in the cells of the cell culture or cell population at one or more time points subsequent to the addition of the differentiation factor. In such processes, the measurement of marker expression can be qualitative or quantitative. One method of quantitating the expression of markers that are produced by marker genes is through the use of quantitative polymerase chain reaction (Q-PCR). Methods of performing Q-PCR are well known in the art. Other methods which are known in the art can also be used to quantitate marker gene expression. For example, the expression of a marker gene product can be detected by using antibodies specific for the marker gene product of interest. In certain processes, the expression of marker genes characteristic of preprimitive streak cells as well as the lack of significant expression of marker genes characteristic of hESCs and other cell types is determined. In still other processes, both the timing and amount of expression of marker genes characteristic of preprimitive streak cells at one or more time points subsequent to the addition of the differentiation factor is determined.

[0414] As described further in the Examples below, markers of preprimitive streak cells are FGF8 and β-catenin. As such, the preprimitive streak cells produced by the processes described herein express the FGF8 and β-catenin marker genes, thereby producing the FGF8 and β-catenin marker gene products. In some ~~embodiment~~examples, the FGF8 mRNA is substantially expressed in preprimitive streak cells but not in hESCs. Substantial upregulation of the FGF8 mRNA, to near peak levels, can be observed in a differentiating hESC culture by 6 hours after contacting the hESCs with an appropriate differentiation factor, such as activin A. At this time, expression of markers indicative of other cells types, such as mesendoderm, primitive endoderm, definitive endoderm, mesoderm and ectoderm (see Table 1), is still comparatively low. In some ~~embodiment~~examples, markers indicative of mesendoderm, primitive endoderm, definitive endoderm, mesoderm and ectoderm are not substantially expressed by 6 hours after contacting the hESCs

with the differentiation factor. FGF8 mRNA expression is maintained at high levels for at least about 24 hours after contacting the hESCs with the differentiation factor but begins to decline thereafter. Additionally, by about 17 hours after contacting the hESCs with an appropriate differentiation factor, such as activin A, nuclear localization of the β-catenin polypeptide (the expression of nuclear-localized β-catenin) is observed by immunocytochemistry. In hESCs, the β-catenin polypeptide is present at the cell periphery but not in the nucleus.

[0415] It will be appreciated that FGF8 and nuclear-localized β-catenin expression is induced over a range of different levels in preprimitive streak cells depending on the differentiation conditions. As such, in some ~~embodiment~~examples described herein, the expression of the FGF8 marker and/or the nuclear-localized β-catenin marker in preprimitive streak cells or cell populations is at least about 2-fold higher to at least about 10,000-fold higher than the expression of these markers in non-preprimitive streak cells or cell populations, during about the first 6 to 18 hours of differentiation from hESCs. In other ~~embodiment~~examples, the expression of the FGF8 marker and/or the nuclear-localized β-catenin marker in preprimitive streak cells or cell populations is at least about 4-fold higher, at least about 6-fold higher, at least about 8-fold higher, at least about 10-fold higher, at least about 15-fold higher, at least about 20-fold higher, at least about 40-fold higher, at least about 80-fold higher, at least about 100-fold higher, at least about 150-fold higher, at least about 200-fold higher, at least about 500-fold higher, at least about 750-fold higher, at least about 1000-fold higher, at least about 2500-fold higher, at least about 5000-fold higher, at least about 7500-fold higher or at least about 10,000-fold higher than the expression of the FGF8 marker and/or the nuclear-localized β-catenin marker in non-preprimitive streak cells or cell populations, during about the first 6 to 18 hours of differentiation from hESCs. In some ~~embodiment~~examples, the expression of the FGF8 marker and/or the nuclear-localized β-catenin marker in preprimitive streak cells or cell populations is infinitely higher than the expression of the FGF8 marker and/or the nuclear-localized β-catenin marker in non-preprimitive streak cells or cell populations, during about the first 6 to 18 hours of differentiation from hESCs.

[0416] Additionally, it will be appreciated that there is a range of differences between the expression level of the FGF8 marker and the expression levels of the brachyury, FGF4, SNAI1, SOX17, FOXA2, SOX7 and/or SOX1 markers in preprimitive streak cells. Similarly, there exists a range of differences between the expression level of the nuclear-localized β-catenin marker and the expression levels of the brachyury, FGF4, SNAI1, SOX17, FOXA2, SOX7 and/or SOX1 markers in preprimitive streak cells. As such, in some ~~embodiment~~examples described herein, the expression of the FGF8 marker and/or the nuclear-localized β-catenin marker is at least about 2-fold higher to at least about 10,000-fold higher than the expression of the brachyury, FGF4, SNAI1, SOX17, FOXA2, SOX7 and/or SOX1 markers. In other ~~embodiment~~examples, the expression of the FGF8 marker and/or the nuclear-localized β-catenin marker is

at least about 4-fold higher, at least about 6-fold higher, at least about 8-fold higher, at least about 10-fold higher, at least about 15-fold higher, at least about 20-fold higher, at least about 40-fold higher, at least about 80-fold higher, at least about 100-fold higher, at least about 150-fold higher, at least about 200-fold higher, at least about 500-fold higher, at least about 750-fold higher, at least about 1000-fold higher, at least about 2500-fold higher, at least about 5000-fold higher, at least about 7500-fold higher or at least about 10,000-fold higher than the expression of the brachyury, FGF4, SNAI1, SOX17, FOXA2, SOX7 and/or SOX1 markers. In some ~~embodiment~~examples, the brachyury, FGF4, SNAI1, SOX17, FOXA2, SOX7 and/or SOX1 markers are not significantly expressed in preprimitive streak cells.

Enrichment, Isolation and/or Purification of Preprimitive Streak Cells

[0417] With respect to additional aspects of the processes described herein, preprimitive streak cells can be enriched, isolated and/or purified. In some ~~embodiment~~examples, cell populations enriched for preprimitive streak cells are produced by isolating such cells from cell cultures.

[0418] In some ~~embodiment~~examples of the processes described herein, preprimitive streak cells are fluorescently labeled then isolated from non-labeled cells by using a fluorescence activated cell sorter (FACS). In such ~~embodiment~~examples, a nucleic acid encoding green fluorescent protein (GFP) or another nucleic acid encoding an expressible fluorescent marker gene is used to label preprimitive streak cells. For example, in some ~~embodiment~~examples, at least one copy of a nucleic acid encoding GFP or a biologically active fragment thereof is introduced into a pluripotent cell, preferably a human embryonic stem cell, downstream of the FGF8 promoter such that the expression of the GFP gene product or biologically active fragment thereof is under control of the FGF8 promoter. In some ~~embodiment~~examples, the entire coding region of the nucleic acid, which encodes FGF8, is replaced by a nucleic acid encoding GFP or a biologically active fragment thereof. In other ~~embodiment~~examples, the nucleic acid encoding GFP or a biologically active fragment thereof is fused in frame with at least a portion of the nucleic acid encoding FGF8, thereby generating a fusion protein. In such ~~embodiment~~examples, the fusion protein retains a fluorescent activity similar to GFP.

[0419] Fluorescently marked cells, such as the above-described pluripotent cells, are differentiated to preprimitive streak cells as described previously above. Because preprimitive streak cells express the fluorescent marker gene, whereas non-preprimitive streak cells do not, these two cell types can be separated. In some ~~embodiment~~examples, cell suspensions comprising a mixture of fluorescently-labeled preprimitive streak cells and unlabeled non-preprimitive streak cells are sorted using a FACS. Preprimitive streak cells are collected separately from non-preprimitive streak cells, thereby resulting in the isolation of such cell types. If desired, the

isolated cell compositions can be further purified by additional rounds of sorting using the same or different markers that are specific for preprimitive streak cells.

[0420] In addition to the procedures just described, preprimitive streak cells may also be isolated by other techniques for cell isolation. Additionally, preprimitive streak cells may also be enriched or isolated by methods of serial subculture in growth conditions which promote the selective survival or selective expansion of the preprimitive streak cells.

[0421] It will be appreciated that the above-described enrichment, isolation and purification procedures can be used with such cultures at any stage of differentiation.

[0422] Using the methods described herein, enriched, isolated and/or purified populations of preprimitive streak cells and/or tissues can be produced *in vitro* from hESC cultures or cell populations which have undergone differentiation for from about 1 hour to about 24 hours. In some ~~embodiment~~examples, the cells undergo random differentiation. In a preferred ~~embodiment~~example, however, the cells are directed to differentiate primarily into preprimitive streak cells. Some preferred enrichment, isolation and/or purification methods relate to the *in vitro* production of preprimitive streak cells from human embryonic stem cells.

[0423] Using the methods described herein, cell populations or cell cultures can be enriched in preprimitive streak cell content by at least about 2- to about 1000-fold as compared to untreated or unenriched cell populations or cell cultures. In some ~~embodiment~~examples, preprimitive streak cells can be enriched by at least about 5- to about 500-fold as compared to untreated or unenriched cell populations or cell cultures. In other ~~embodiment~~examples, preprimitive streak cells can be enriched from at least about 10- to about 200-fold as compared to untreated or unenriched cell populations or cell cultures. In still other ~~embodiment~~examples, preprimitive streak cells can be enriched from at least about 20- to about 100-fold as compared to untreated or unenriched cell populations or cell cultures. In yet other ~~embodiment~~examples, preprimitive streak cells can be enriched from at least about 40- to about 80-fold as compared to untreated or unenriched cell populations or cell cultures. In certain ~~embodiment~~examples, preprimitive streak cells can be enriched from at least about 2- to about 20-fold as compared to untreated or unenriched cell populations or cell cultures.

Compositions Comprising Preprimitive Streak Cells

[0424] Cell compositions produced by the above-described methods include cell cultures comprising preprimitive streak cells and cell populations enriched in preprimitive streak cells. For example, cell cultures which comprise preprimitive streak cells, wherein at least about 5-90% of the cells in culture are preprimitive streak cells, can be produced. Because the efficiency of the differentiation process can be adjusted by modifying certain parameters, which include but are not limited to, cell growth conditions, growth factor concentrations and the timing of culture steps, the differentiation procedures described herein can result in about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about

50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or greater than about 95% conversion of pluripotent cells to preprimitive streak cells. In processes in which isolation of preprimitive streak cells is employed, a substantially pure preprimitive streak cell population can be recovered.

[0425] Some ~~embodiment~~examples described herein relate to compositions, such as cell populations and cell cultures, that comprise both pluripotent cells, such as stem cells, and preprimitive streak cells. For example, using the methods described herein, compositions comprising mixtures of hESCs and preprimitive streak cells can be produced. In some ~~embodiment~~examples, compositions comprising at least about 5 preprimitive streak cells for about every 95 pluripotent cells are produced. In other ~~embodiment~~examples, compositions comprising at least about 95 preprimitive streak cells for about every 5 pluripotent cells are produced. Additionally, compositions comprising other ratios of preprimitive streak cells to pluripotent cells are contemplated. For example, compositions comprising at least about 1 preprimitive streak cell for about every 1,000,000 pluripotent cells, at least about 1 preprimitive streak cell for about every 100,000 pluripotent cells, at least about 1 preprimitive streak cell for about every 10,000 pluripotent cells, at least about 1 preprimitive streak cell for about every 1000 pluripotent cells, at least about 1 preprimitive streak cell for about every 500 pluripotent cells, at least about 1 preprimitive streak cell for about every 100 pluripotent cells, at least about 1 preprimitive streak cell for about every 10 pluripotent cells, at least about 1 preprimitive streak cell for about every 5 pluripotent cells, at least about 1 preprimitive streak cell for about every 2 pluripotent cells, at least about 2 preprimitive streak cells for about every 1 pluripotent cell, at least about 5 preprimitive streak cells for about every 1 pluripotent cell, at least about 10 preprimitive streak cells for about every 1 pluripotent cell, at least about 20 preprimitive streak cells for about every 1 pluripotent cell, at least about 50 preprimitive streak cells for about every 1 pluripotent cell, at least about 100 preprimitive streak cells for about every 1 pluripotent cell, at least about 1000 preprimitive streak cells for about every 1 pluripotent cell, at least about 10,000 preprimitive streak cells for about every 1 pluripotent cell, at least about 100,000 preprimitive streak cells for about every 1 pluripotent cell and at least about 1,000,000 preprimitive streak cells for about every 1 pluripotent cell are contemplated. In some ~~embodiment~~examples, the pluripotent cells are human pluripotent stem cells. In certain ~~embodiment~~examples, the stem cells are derived from a morula, the inner cell mass of an embryo or the gonadal ridges of an embryo. In certain other ~~embodiment~~examples, the pluripotent cells are derived from the gonadal or germ tissues of a multicellular structure that has developed past the embryonic stage.

[0426] Some ~~embodiment~~examples described herein relate to cell cultures or cell populations comprising from at least about 5% preprimitive streak cells to at least about 99% preprimitive streak cells. In some ~~embodiment~~examples the cell cultures or cell populations comprise mammalian cells. In preferred ~~embodiment~~examples, the cell cultures or cell populations comprise human cells. For

example, certain specific ~~embodiment~~examples relate to cell cultures comprising human cells, wherein from at least about 5% to at least about 99% of the human cells are preprimitive streak cells. Other ~~embodiment~~examples relate to cell cultures comprising human cells, wherein at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99%, or greater than 99% of the human cells are preprimitive streak cells. In ~~embodiment~~examples where the cell cultures or cell populations comprise human feeder cells, the above percentages are calculated without respect to the human feeder cells in the cell cultures or cell populations.

[0427] Further ~~embodiment~~examples described herein relate to compositions, such as cell cultures or cell populations, comprising human cells, such as human preprimitive streak cells, wherein the expression of the FGF8 marker and/or the nuclear-localized β-catenin marker is greater than the expression of the brachyury, FGF4, SNAI1, SOX17, FOXA2, SOX7 and/or SOX1 markers in at least about 5% of the human cells. In other ~~embodiment~~examples, the expression of either the FGF8 marker and/or the nuclear-localized β-catenin marker is greater than the expression of the brachyury, FGF4, SNAI1, SOX17, FOXA2, SOX7 and/or SOX1 marker in at least about 10% of the human cells, in at least about 15% of the human cells, in at least about 20% of the human cells, in at least about 25% of the human cells, in at least about 30% of the human cells, in at least about 35% of the human cells, in at least about 40% of the human cells, in at least about 45% of the human cells, in at least about 50% of the human cells, in at least about 55% of the human cells, in at least about 60% of the human cells, in at least about 65% of the human cells, in at least about 70% of the human cells, in at least about 75% of the human cells, in at least about 80% of the human cells, in at least about 85% of the human cells, in at least about 90% of the human cells, in at least about 95% of the human cells, in at least about 98% of the human cells, in at least about 99% of the human cells or in greater than 99% of the human cells. In ~~embodiment~~examples where the cell cultures or cell populations comprise human feeder cells, the above percentages are calculated without respect to the human feeder cells in the cell cultures or cell populations.

[0428] Additional ~~embodiment~~examples described herein relate to compositions, such as cell cultures or cell populations, comprising human hESCs and human preprimitive streak cells, wherein substantial upregulation of the expression of FGF8 mRNA occurs in cells of the cell culture or cell population by about 1 hour, by about 2 hours, by about 3 hours, by about 4 hours, by about 5 hours, by about 6 hours, by about 7 hours, by about 8 hours, by about 9 hours, by about 10 hours, by about 11 hours, by about 12 hours, by about 13 hours, by about 14 hours, by about 15 hours, by about 16 hours, by about 17 hours, by about 18 hours, by about 19 hours, by

about 20 hours, by about 21 hours, by about 22 hours, by about 23 hours, by about 24 hours, or by greater than about 24 hours after contacting hESCs in the culture with an appropriate differentiation factor, such as activin A. In such ~~embodiment~~examples, substantial upregulation of the expression of FGF8 mRNA occurs in at least about 5% of the human cells, at least about 10% of the human cells, in at least about 15% of the human cells, in at least about 20% of the human cells, in at least about 25% of the human cells, in at least about 30% of the human cells, in at least about 35% of the human cells, in at least about 40% of the human cells, in at least about 45% of the human cells, in at least about 50% of the human cells, in at least about 55% of the human cells, in at least about 60% of the human cells, in at least about 65% of the human cells, in at least about 70% of the human cells, in at least about 75% of the human cells, in at least about 80% of the human cells, in at least about 85% of the human cells, in at least about 90% of the human cells, in at least about 95% of the human cells, in at least about 98% of the human cells, in at least about 99% of the human cells or in greater than 99% of the human cells. In ~~embodiment~~examples where the cell cultures or cell populations comprise human feeder cells, the above percentages are calculated without respect to the human feeder cells in the cell cultures or cell populations.

[0429] Further ~~embodiment~~examples described herein relate to compositions, such as cell cultures or cell populations, comprising human hESCs and human preprimitive streak cells, wherein substantial nuclear localization of the β-catenin polypeptide (expression of nuclear localized β-catenin marker) occurs in cells of the cell culture or cell population by about 1 hour, by about 2 hours, by about 3 hours, by about 4 hours, by about 5 hours, by about 6 hours, by about 7 hours, by about 8 hours, by about 9 hours, by about 10 hours, by about 11 hours, by about 12 hours, by about 13 hours, by about 14 hours, by about 15 hours, by about 16 hours, by about 17 hours, by about 18 hours, by about 19 hours, by about 20 hours, by about 21 hours, by about 22 hours, by about 23 hours, by about 24 hours, or by greater than about 24 hours after contacting hESCs in the culture with an appropriate differentiation factor, such as activin A. In such ~~embodiment~~examples, expression of nuclear-localized β-catenin occurs in at least about 5% of the human cells, at least about 10% of the human cells, in at least about 15% of the human cells, in at least about 20% of the human cells, in at least about 25% of the human cells, in at least about 30% of the human cells, in at least about 35% of the human cells, in at least about 40% of the human cells, in at least about 45% of the human cells, in at least about 50% of the human cells, in at least about 55% of the human cells, in at least about 60% of the human cells, in at least about 65% of the human cells, in at least about 70% of the human cells, in at least about 75% of the human cells, in at least about 80% of the human cells, in at least about 85% of the human cells, in at least about 90% of the human cells, in at least about 95% of the human cells, in at least about 98% of the human cells, in at least about 99% of the human cells or in greater than 99% of the human cells. In ~~embodiment~~examples where the cell cultures or cell populations comprise human feeder cells,

the above percentages are calculated without respect to the human feeder cells in the cell cultures or cell populations.

[0430] Using the methods described herein, compositions comprising preprimitive streak cells substantially free of other cell types can be produced. In some ~~embodiment~~examples described herein, the preprimitive streak cell populations or cell cultures produced by the methods described herein are substantially free of cells that significantly express the brachyury, FGF4, SNAI1, SOX17, FOXA2, SOX7 and/or SOX1 marker genes.

[0431] In one ~~embodiment~~example, a description of a preprimitive streak cell based on the expression of marker genes is, FGF8 high, nuclear-localized β-catenin high, brachyury low, FGF4 low, SNAI1 low, SOX17 low, FOXA2 low, SOX7 low and SOX1 low.

Production of Mesendoderm Cells from Pluripotent Cells

[0432] In some processes for producing mesendoderm cells, the pluripotent cells used as starting material are stem cells. In certain processes, mesendoderm cell cultures and enriched cell populations comprising mesendoderm cells are produced from embryonic stem cells. A preferred method for deriving mesendoderm cells utilizes human embryonic stem cells as the starting material for mesendoderm cell production. Such pluripotent cells can be cells that originate from the morula, embryonic inner cell mass or those obtained from embryonic gonadal ridges. Human embryonic stem cells can be maintained in culture in a pluripotent state without substantial differentiation using methods that are known in the art. Such methods are described, for example, in US Patent Nos. 5,453,357, 5,670,372, 5,690,926 5,843,780, 6,200,806 and 6,251,671 the disclosures of which are incorporated herein by reference in their entireties.

[0433] In some processes for producing mesendoderm cells, hESCs are maintained on a feeder layer. In such processes, any feeder layer which allows hESCs to be maintained in a pluripotent state can be used. One commonly used feeder layer for the cultivation of human embryonic stem cells is a layer of mouse fibroblasts. More recently, human fibroblast feeder layers have been developed for use in the cultivation of hESCs (see US Patent Application No. 2002/0072117, the disclosure of which is incorporated herein by reference in its entirety). Alternative processes for producing mesendoderm cells permit the maintenance of pluripotent hESC without the use of a feeder layer. Methods of maintaining pluripotent hESCs under feeder-free conditions have been described in US Patent Application No. 2003/0175956, the disclosure of which is incorporated herein by reference in its entirety.

[0434] The human embryonic stem cells used herein can be maintained in culture either with or without serum. In some embryonic stem cell maintenance procedures, serum replacement is used. In others, serum free culture techniques, such as those described in US Patent Application No. 2003/0190748, the disclosure of which is incorporated herein by reference in its entirety, are used.

than about 0.9% (v/v), less than about 1% (v/v), less than about 2% (v/v), less than about 3% (v/v), less than about 4% (v/v), less than about 5% (v/v), less than about 6% (v/v), less than about 7% (v/v), less than about 8% (v/v), less than about 9% (v/v) or less than about 10% (v/v). In some processes, mesendoderm cells are grown without serum or without serum replacement. In still other processes, mesendoderm cells are grown in the presence of B27. In such processes, the concentration of B27 supplement can range from about 0.1% (v/v) to about 20% (v/v).

Monitoring the Differentiation of Pluripotent Cells to Mesendoderm Cells

[0439] The progression of the hESC culture to mesendoderm cells can be monitored by determining the temporal expression of markers characteristic of mesendoderm cells. In some processes, the expression of certain markers is determined by detecting the presence or absence of the marker. Alternatively, the expression of certain markers can be determined by measuring the level at which the marker is present in the cells of the cell culture or cell population at one or more time points subsequent to the addition of the differentiation factor. In such processes, the measurement of marker expression can be qualitative or quantitative. One method of quantitating the expression of markers that are produced by marker genes is through the use of Q-PCR. Other methods which are known in the art can also be used to quantitate marker gene expression. For example, the expression of a marker gene product can be detected by using antibodies specific for the marker gene product of interest. In certain processes, the expression of marker genes characteristic of mesendoderm cells as well as the lack of significant expression of marker genes characteristic of hESCs and other cell types is determined. In still other processes, both the timing and amount of expression of marker genes characteristic of mesendoderm cells at one or more time points subsequent to the addition of the differentiation factor is determined.

[0440] As described further in the Examples below, markers of mesendoderm cells are brachyury, FGF4 and SNAI1. As such, the mesendoderm cells produced by the processes described herein express the brachyury, FGF4 and SNAI1 marker genes, thereby producing the brachyury, FGF4 and SNAI1 marker gene products. In some ~~embodiment~~examples, the brachyury, FGF4 and/or SNAI1 mRNA is substantially expressed in mesendoderm cells but not in hESCs. Substantial upregulation of the brachyury, FGF4 and/or SNAI1 mRNA, to near peak levels, can be observed in a differentiating hESC culture by 24 hours after contacting the hESCs with an appropriate differentiation factor, such as activin A. At this time, expression of certain markers indicative of other cells types, such as primitive endoderm, definitive endoderm, mesoderm and ectoderm (see Table 1), is still comparatively low. In some ~~embodiment~~examples, certain markers indicative of primitive endoderm, definitive endoderm, mesoderm and ectoderm are not substantially expressed by 24 hours after contacting the hESCs with the differentiation factor. In some ~~embodiment~~examples, brachyury, FGF4 and/or SNAI1 mRNA expression begins to decline after about 24 hours subsequent to contacting the hESCs with the differentiation factor. In some

~~embodiment~~examples, brachyury, FGF4 and/or SNAI1 mRNA expression begins to decline after about 30 hours, after about 36 hours, after about 42 hours, after about 48 hours or after more than about 48 hours subsequent to contacting the hESCs with the differentiation factor.

[0441] It will be appreciated that brachyury, FGF4 and/or SNAI1 expression is induced over a range of different levels in mesendoderm cells depending on the differentiation conditions. As such, in some ~~embodiment~~examples described herein, the expression of the brachyury, FGF4 and/or SNAI1 marker in mesendoderm cells or cell populations is at least about 2-fold higher to at least about 10,000-fold higher than the expression of these markers in non-mesendoderm cells or cell populations, after about 24 hours of differentiation from hESCs. In other ~~embodiment~~examples, the expression of the brachyury, FGF4 and/or SNAI1 marker in mesendoderm cells or cell populations is at least about 4-fold higher, at least about 6-fold higher, at least about 8-fold higher, at least about 10-fold higher, at least about 15-fold higher, at least about 20-fold higher, at least about 40-fold higher, at least about 80-fold higher, at least about 100-fold higher, at least about 150-fold higher, at least about 200-fold higher, at least about 500-fold higher, at least about 750-fold higher, at least about 1000-fold higher, at least about 2500-fold higher, at least about 5000-fold higher, at least about 7500-fold higher or at least about 10,000-fold higher than the expression of the brachyury, FGF4 and/or SNAI1 in non-mesendoderm cells or cell populations, after about 24 hours of differentiation from hESCs. In some ~~embodiment~~examples, the expression of the brachyury, FGF4 and/or SNAI1 marker in mesendoderm cells or cell populations is infinitely higher than the expression of the brachyury, FGF4 and/or SNAI1 marker in non-mesendoderm cells or cell populations, after about 24 hours of differentiation from hESCs.

[0442] Additionally, it will be appreciated that there is a range of differences between the expression level of the brachyury, FGF4 and/or SNAI1 markers and the expression levels of the OCT4, SOX17, CXCR4, FOXA2, SOX7 and/or SOX1 markers in mesendoderm cells. As such, in some ~~embodiment~~examples described herein, the expression of the brachyury, FGF4 and/or SNAI1 markers is at least about 2-fold higher to at least about 10,000-fold higher than the expression of the OCT4, SOX17, CXCR4, FOXA2, SOX7 and/or SOX1 markers. In other ~~embodiment~~examples, the expression of the brachyury, FGF4 and/or SNAI1 marker is at least about 4-fold higher, at least about 6-fold higher, at least about 8-fold higher, at least about 10-fold higher, at least about 15-fold higher, at least about 20-fold higher, at least about 40-fold higher, at least about 80-fold higher, at least about 100-fold higher, at least about 150-fold higher, at least about 200-fold higher, at least about 500-fold higher, at least about 750-fold higher, at least about 1000-fold higher, at least about 2500-fold higher, at least about 5000-fold higher, at least about 7500-fold higher or at least about 10,000-fold higher than the expression of the OCT4, SOX17, CXCR4, FOXA2, SOX7 and/or SOX1 markers. In some ~~embodiment~~examples, the OCT4, SOX17,

CXCR4, FOXA2, SOX7 and/or SOX1 markers are not significantly expressed in mesendoderm cells.

Enrichment, Isolation and/or Purification of Mesendoderm Cells

[0443] With respect to additional aspects of the processes described herein, mesendoderm cells can be enriched, isolated and/or purified. In some ~~embodiment~~examples, cell populations enriched for mesendoderm cells are produced by isolating such cells from cell cultures.

[0444] In some ~~embodiment~~examples of the processes described herein, mesendoderm cells are fluorescently labeled then isolated from non-labeled cells by using a FACS. In such ~~embodiment~~examples, a nucleic acid encoding green fluorescent protein (GFP) or another nucleic acid encoding an expressible fluorescent marker gene is used to label mesendoderm cells. For example, in some ~~embodiment~~examples, at least one copy of a nucleic acid encoding GFP or a biologically active fragment thereof is introduced into a pluripotent cell, preferably a human embryonic stem cell, downstream of the brachyury, FGF4 or SNAI1 promoter such that the expression of the GFP gene product or biologically active fragment thereof is under control of the brachyury, FGF4 or SNAI1 promoter. In some ~~embodiment~~examples, the entire coding region of the nucleic acid, which encodes brachyury, FGF4 or SNAI1, is replaced by a nucleic acid encoding GFP or a biologically active fragment thereof. In other ~~embodiment~~examples, the nucleic acid encoding GFP or a biologically active fragment thereof is fused in frame with at least a portion of the nucleic acid encoding brachyury, FGF4 or SNAI1, thereby generating a fusion protein. In such ~~embodiment~~examples, the fusion protein retains a fluorescent activity similar to GFP.

[0445] Fluorescently marked cells, such as the above-described pluripotent cells, are differentiated to mesendoderm cells as described previously above. Because mesendoderm cells express the fluorescent marker gene, whereas non-mesendoderm cells do not, these two cell types can be separated. In some ~~embodiment~~examples, cell suspensions comprising a mixture of fluorescently-labeled mesendoderm cells and unlabeled non-mesendoderm cells are sorted using a FACS. Mesendoderm cells are collected separately from non-mesendoderm cells, thereby resulting in the isolation of such cell types. If desired, the isolated cell compositions can be further purified by additional rounds of sorting using the same or different markers that are specific for mesendoderm cells.

[0446] In addition to the procedures just described, mesendoderm cells may also be isolated by other techniques for cell isolation. Additionally, mesendoderm cells may also be enriched or isolated by methods of serial subculture in growth conditions which promote the selective survival or selective expansion of the mesendoderm cells.

[0447] It will be appreciated that the above-described enrichment, isolation and purification procedures can be used with such cultures at any stage of differentiation.

[0448] Using the methods described herein, enriched, isolated and/or purified populations of mesendoderm cells and/or tissues can be produced *in vitro* from hESC cultures or cell populations which have undergone differentiation for from about 18 hours to about 48 hours. In some ~~embodiment~~examples, the cells undergo random differentiation. In a preferred ~~embodiment~~example, however, the cells are directed to differentiate primarily into mesendoderm cells. Some preferred enrichment, isolation and/or purification methods relate to the *in vitro* production of mesendoderm cells from human embryonic stem cells.

[0449] Using the methods described herein, cell populations or cell cultures can be enriched in mesendoderm cell content by at least about 2- to about 1000-fold as compared to untreated or unenriched cell populations or cell cultures. In some ~~embodiment~~examples, mesendoderm cells can be enriched by at least about 5- to about 500-fold as compared to untreated or unenriched cell populations or cell cultures. In other ~~embodiment~~examples, mesendoderm cells can be enriched from at least about 10- to about 200-fold as compared to untreated or unenriched cell populations or cell cultures. In still other ~~embodiment~~examples, mesendoderm cells can be enriched from at least about 20- to about 100-fold as compared to untreated or unenriched cell populations or cell cultures. In yet other ~~embodiment~~examples, mesendoderm cells can be enriched from at least about 40- to about 80-fold as compared to untreated or unenriched cell populations or cell cultures. In certain ~~embodiment~~examples, mesendoderm cells can be enriched from at least about 2- to about 20-fold as compared to untreated or unenriched cell populations or cell cultures.

Compositions Comprising Mesendoderm Cells

[0450] Cell compositions produced by the above-described methods include cell cultures comprising mesendoderm cells and cell populations enriched in mesendoderm cells. For example, cell cultures which comprise mesendoderm cells, wherein at least about 5-90% of the cells in culture are mesendoderm cells, can be produced. Because the efficiency of the differentiation process can be adjusted by modifying certain parameters, which include but are not limited to, cell growth conditions, growth factor concentrations and the timing of culture steps, the differentiation procedures described herein can result in about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or greater than about 95% conversion of pluripotent cells to mesendoderm cells. In processes in which isolation of mesendoderm cells is employed, a substantially pure mesendoderm cell population can be recovered.

[0451] Some ~~embodiment~~examples described herein relate to compositions, such as cell populations and cell cultures, that comprise both pluripotent cells, such as stem cells, and mesendoderm cells. For example, using the methods described herein, compositions comprising mixtures of hESCs and mesendoderm cells can be produced. In some ~~embodiment~~examples,

compositions comprising at least about 5 mesendoderm cells for about every 95 pluripotent cells are produced. In other ~~embodiment~~examples, compositions comprising at least about 95 mesendoderm cells for about every 5 pluripotent cells are produced. Additionally, compositions comprising other ratios of mesendoderm cells to pluripotent cells are contemplated. For example, compositions comprising at least about 1 mesendoderm cell for about every 1,000,000 pluripotent cells, at least about 1 mesendoderm cell for about every 100,000 pluripotent cells, at least about 1 mesendoderm cell for about every 10,000 pluripotent cells, at least about 1 mesendoderm cell for about every 1000 pluripotent cells, at least about 1 mesendoderm cell for about every 500 pluripotent cells, at least about 1 mesendoderm cell for about every 100 pluripotent cells, at least about 1 mesendoderm cell for about every 10 pluripotent cells, at least about 1 mesendoderm cell for about every 5 pluripotent cells, at least about 1 mesendoderm cell for about every 2 pluripotent cells, at least about 2 mesendoderm cells for about every 1 pluripotent cell, at least about 5 mesendoderm cells for about every 1 pluripotent cell, at least about 10 mesendoderm cells for about every 1 pluripotent cell, at least about 20 mesendoderm cells for about every 1 pluripotent cell, at least about 50 mesendoderm cells for about every 1 pluripotent cell, at least about 100 mesendoderm cells for about every 1 pluripotent cell, at least about 1000 mesendoderm cells for about every 1 pluripotent cell, at least about 10,000 mesendoderm cells for about every 1 pluripotent cell, at least about 100,000 mesendoderm cells for about every 1 pluripotent cell and at least about 1,000,000 mesendoderm cells for about every 1 pluripotent cell are contemplated. In some ~~embodiment~~examples, the pluripotent cells are human pluripotent stem cells. In certain ~~embodiment~~examples, the stem cells are derived from a morula, the inner cell mass of an embryo or the gonadal ridges of an embryo. In certain other ~~embodiment~~examples, the pluripotent cells are derived from the gonadal or germ tissues of a multicellular structure that has developed past the embryonic stage.

[0452] Some ~~embodiment~~examples described herein relate to cell cultures or cell populations comprising from at least about 5% mesendoderm cells to at least about 99% mesendoderm cells. In some ~~embodiment~~examples the cell cultures or cell populations comprise mammalian cells. In preferred ~~embodiment~~examples, the cell cultures or cell populations comprise human cells. For example, certain specific ~~embodiment~~examples relate to cell cultures comprising human cells, wherein from at least about 5% to at least about 99% of the human cells are mesendoderm cells. Other ~~embodiment~~examples relate to cell cultures comprising human cells, wherein at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99%, or greater than 99% of the human cells are mesendoderm cells. In ~~embodiment~~examples where the cell cultures or cell

populations comprise human feeder cells, the above percentages are calculated without respect to the human feeder cells in the cell cultures or cell populations.

[0453] Further ~~embodiment~~examples described herein relate to compositions, such as cell cultures or cell populations, comprising human cells, such as human mesendoderm cells, wherein the expression of the brachyury, FGF4 and/or SNAI1 markers is greater than the expression of the OCT4, SOX17, CXCR4, FOXA2, SOX7 and/or SOX1 markers in at least about 5% of the human cells. In other ~~embodiment~~examples, the expression of either the brachyury, FGF4 and/or SNAI1 marker is greater than the expression of the OCT4, SOX17, CXCR4, FOXA2, SOX7 and/or SOX1 marker in at least about 10% of the human cells, in at least about 15% of the human cells, in at least about 20% of the human cells, in at least about 25% of the human cells, in at least about 30% of the human cells, in at least about 35% of the human cells, in at least about 40% of the human cells, in at least about 45% of the human cells, in at least about 50% of the human cells, in at least about 55% of the human cells, in at least about 60% of the human cells, in at least about 65% of the human cells, in at least about 70% of the human cells, in at least about 75% of the human cells, in at least about 80% of the human cells, in at least about 85% of the human cells, in at least about 90% of the human cells, in at least about 95% of the human cells, in at least about 98% of the human cells, in at least about 99% of the human cells or in greater than 99% of the human cells. In ~~embodiment~~examples where the cell cultures or cell populations comprise human feeder cells, the above percentages are calculated without respect to the human feeder cells in the cell cultures or cell populations.

[0454] Additional ~~embodiment~~examples described herein relate to compositions, such as cell cultures or cell populations, comprising human hESCs and human mesendoderm cells, wherein substantial upregulation of the expression of brachyury, FGF4 and/or SNAI1 mRNA occurs in cells of the cell culture or cell population by about 18 hours, by about 19 hours, by about 20 hours, by about 21 hours, by about 22 hours, by about 23 hours, by about 24 hours, by about 25 hours, by about 26 hours, by about 27 hours, by about 28 hours, by about 29 hours, by about 30 hours, by about 31 hours, by about 32 hours, by about 33 hours, by about 34 hours, by about 35 hours, by about 36 hours, by about 37 hours, by about 38 hours, by about 39 hours, by about 40 hours, by about 41 hours, by about 42 hours, by about 43 hours, by about 44 hours, by about 45 hours, by about 46 hours, by about 47 hours, by about 48 hours or by greater than about 48 hours after contacting hESCs in the culture with an appropriate differentiation factor, such as activin A. In such ~~embodiment~~examples, substantial upregulation of the expression of brachyury, FGF4 and/or SNAI1 mRNA occurs in at least about 5% of the human cells, at least about 10% of the human cells, in at least about 15% of the human cells, in at least about 20% of the human cells, in at least about 25% of the human cells, in at least about 30% of the human cells, in at least about 35% of the human cells, in at least about 40% of the human cells, in at least about 45% of the human cells, in at least about 50% of the human cells, in at least about 55% of the human cells, in at least

about 60% of the human cells, in at least about 65% of the human cells, in at least about 70% of the human cells, in at least about 75% of the human cells, in at least about 80% of the human cells, in at least about 85% of the human cells, in at least about 90% of the human cells, in at least about 95% of the human cells, in at least about 98% of the human cells, in at least about 99% of the human cells or in greater than 99% of the human cells. In ~~embodiment~~examples where the cell cultures or cell populations comprise human feeder cells, the above percentages are calculated without respect to the human feeder cells in the cell cultures or cell populations.

[0455] Using the methods described herein, compositions comprising mesendoderm cells substantially free of other cell types can be produced. In some ~~embodiment~~examples described herein, the mesendoderm cell populations or cell cultures produced by the methods described herein are substantially free of cells that significantly express the OCT4, SOX17, CXCR4, FOXA2, SOX7 and/or SOX1 marker genes.

[0456] In one ~~embodiment~~example, a description of a mesendoderm cell based on the expression of marker genes is, brachyury high, FGF4 high, SNAI1 high, SOX17 low, CXCR4 low, FOXA2 low, SOX7 low and SOX1 low.

Production of Definitive Endoderm Cells

[0457] Processes for differentiating pluripotent cells to produce cell cultures and enriched cell populations comprising definitive endoderm is described briefly below and in detail in US Patent No. 11/021,618, entitled DEFINITIVE ENDODERM, filed December 23, 2004, the disclosure of which is incorporated herein by reference in its entirety. In some of these processes, the pluripotent cells used as starting material are stem cells. In certain processes, definitive endoderm cell cultures and enriched cell populations comprising definitive endoderm cells are produced from embryonic stem cells. A preferred method for deriving definitive endoderm cells utilizes human embryonic stem cells as the starting material for definitive endoderm production. Such pluripotent cells can be cells that originate from the morula, embryonic inner cell mass or those obtained from embryonic gonadal ridges. Human embryonic stem cells can be maintained in culture in a pluripotent state without substantial differentiation using methods that are known in the art. Such methods are described, for example, in US Patent Nos. 5,453,357, 5,670,372, 5,690,926 5,843,780, 6,200,806 and 6,251,671 the disclosures of which are incorporated herein by reference in their entireties.

[0458] In some processes for producing definitive endoderm cells, hESCs are maintained on a feeder layer. In such processes, any feeder layer which allows hESCs to be maintained in a pluripotent state can be used. One commonly used feeder layer for the cultivation of human embryonic stem cells is a layer of mouse fibroblasts. More recently, human fibroblast feeder layers have been developed for use in the cultivation of hESCs (see US Patent Application

[0463] Cultures of definitive endoderm cells can be grown in medium containing reduced serum or no serum. Under certain culture conditions, serum concentrations can range from about 0.05% v/v to about 20% v/v. For example, in some differentiation processes, the serum concentration of the medium can be less than about 0.05% (v/v), less than about 0.1% (v/v), less than about 0.2% (v/v), less than about 0.3% (v/v), less than about 0.4% (v/v), less than about 0.5% (v/v), less than about 0.6% (v/v), less than about 0.7% (v/v), less than about 0.8% (v/v), less than about 0.9% (v/v), less than about 1% (v/v), less than about 2% (v/v), less than about 3% (v/v), less than about 4% (v/v), less than about 5% (v/v), less than about 6% (v/v), less than about 7% (v/v), less than about 8% (v/v), less than about 9% (v/v), less than about 10% (v/v), less than about 15% (v/v) or less than about 20% (v/v). In some processes, definitive endoderm cells are grown without serum or without serum replacement. In some ~~embodiment~~examples, definitive endoderm cells are grown with serum replacement. In still other processes, definitive endoderm cells are grown in the presence of B27. In such processes, the concentration of B27 supplement can range from about 0.1% v/v to about 20% v/v.

Monitoring the Differentiation of Pluripotent Cells to Definitive Endoderm Cells

[0464] The progression of the hESC culture to definitive endoderm can be monitored by determining the expression of markers characteristic of definitive endoderm. In some processes, the expression of certain markers is determined by detecting the presence or absence of the marker. Alternatively, the expression of certain markers can determined by measuring the level at which the marker is present in the cells of the cell culture or cell population. In such processes, the measurement of marker expression can be qualitative or quantitative. One method of quantitating the expression of markers that are produced by marker genes is through the use of Q-PCR. Other methods which are known in the art can also be used to quantitate marker gene expression. For example, the expression of a marker gene product can be detected by using antibodies specific for the marker gene product of interest. In certain processes, the expression of marker genes characteristic of definitive endoderm as well as the lack of significant expression of marker genes characteristic of hESCs and other cell types is determined.

[0465] As described further in the Examples below, a reliable marker of definitive endoderm is the SOX17 gene. As such, the definitive endoderm cells produced by the processes described herein express the SOX17 marker gene, thereby producing the SOX17 gene product. Other markers of definitive endoderm are MIXL1, GATA4, HNF3b, GSC, FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1. Since definitive endoderm cells express the SOX17 marker gene at a level higher than that of the SOX7 marker gene, which is characteristic of primitive and visceral endoderm, in some processes, the expression of both SOX17 and SOX7 is monitored. In other processes, expression of the both the SOX17 marker gene and the OCT4 marker gene, which is characteristic of hESCs, is monitored. Additionally, because definitive

described herein will substantially express SOX17 and CXCR4 but will not substantially express AFP, TM, SPARC or PDX1.

Enrichment, Isolation and/or Purification of Definitive Endoderm Cells

[0469] Definitive endoderm cells produced by any of the above-described processes can be enriched, isolated and/or purified by using an affinity tag that is specific for such cells. Examples of affinity tags specific for definitive endoderm cells are antibodies, ligands or other binding agents that are specific to a marker molecule, such as a polypeptide, that is present on the cell surface of definitive endoderm cells but which is not substantially present on other cell types that would be found in a cell culture produced by the methods described herein. In some processes, an antibody which binds to CXCR4 is used as an affinity tag for the enrichment, isolation or purification of definitive endoderm cells. In other processes, the chemokine SDF-1 or other molecules based on SDF-1 can also be used as affinity tags. Such molecules include, but not limited to, SDF-1 fragments, SDF-1 fusions or SDF-1 mimetics.

[0470] Methods for making antibodies and using them for cell isolation are known in the art and such methods can be implemented for use with the antibodies and definitive endoderm cells described herein. In one process, an antibody which binds to CXCR4 is attached to a magnetic bead and then allowed to bind to definitive endoderm cells in a cell culture which has been enzymatically treated to reduce intercellular and substrate adhesion. The cell/antibody/bead complexes are then exposed to a movable magnetic field which is used to separate bead-bound definitive endoderm cells from unbound cells. Once the definitive endoderm cells are physically separated from other cells in culture, the antibody binding is disrupted and the cells are replated in appropriate tissue culture medium.

[0471] Additional methods for obtaining enriched, isolated or purified definitive endoderm cell cultures or populations can also be used. For example, in some ~~embodiment~~examples, the CXCR4 antibody is incubated with a definitive endoderm-containing cell culture that has been treated to reduce intercellular and substrate adhesion. The cells are then washed, centrifuged and resuspended. The cell suspension is then incubated with a secondary antibody, such as an FITC-conjugated antibody that is capable of binding to the primary antibody. The cells are then washed, centrifuged and resuspended in buffer. The cell suspension is then analyzed and sorted using a fluorescence activated cell sorter (FACS). CXCR4-positive cells are collected separately from CXCR4-negative cells, thereby resulting in the isolation of such cell types. If desired, the isolated cell compositions can be further purified by using an alternate affinity-based method or by additional rounds of sorting using the same or different markers that are specific for definitive endoderm.

~~embodiment~~examples where the cell cultures or cell populations comprise human feeder cells, the above percentages are calculated without respect to the human feeder cells in the cell cultures or cell populations.

Identification of Factors Capable of Promoting the Differentiation of Preprimitive Streak and/or Mesendoderm

[0477] Certain screening methods described herein relate to methods for identifying at least one differentiation factor that is capable of promoting the differentiation of preprimitive streak and/or mesendoderm cells. In some ~~embodiment~~examples of these methods, a cell population comprising preprimitive streak and/or mesendoderm cells, such as human preprimitive streak and/or mesendoderm cells, is obtained. The cell population is then provided with a candidate differentiation factor. At a first time point, which is prior to or at approximately the same time as providing the candidate differentiation factor, expression of a marker is determined. Alternatively, expression of the marker can be determined after providing the candidate differentiation factor. At a second time point, which is subsequent to the first time point and subsequent to the step of providing the candidate differentiation factor to the cell population, expression of the same marker is again determined. Whether the candidate differentiation factor is capable of promoting the differentiation of the definitive endoderm cells is determined by comparing expression of the marker at the first time point with the expression of the marker at the second time point. If expression of the marker at the second time point is increased or decreased as compared to expression of the marker at the first time point, then the candidate differentiation factor is capable of promoting the differentiation of definitive endoderm cells.

[0478] Some ~~embodiment~~examples of the screening methods described herein utilize cell populations or cell cultures which comprise human preprimitive streak and/or mesendoderm cells. For example, the cell population can be a substantially purified population of human preprimitive streak and/or mesendoderm cells. Alternatively, the cell population can be an enriched population of human preprimitive streak and/or mesendoderm cells, wherein at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% or greater than at least about 99% of the human cells in the cell population are human preprimitive streak and/or mesendoderm cells. In other ~~embodiment~~examples described herein, the cell population comprises human cells wherein at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85% or greater than at least about 85% of the human cells are human preprimitive streak and/or mesendoderm cells. In some ~~embodiment~~examples, the cell population includes non-human cells such as non-human feeder cells. In other ~~embodiment~~examples, the cell

population includes human feeder cells. In such ~~embodiment~~examples, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95% or greater than at least about 95% of the human cells, other than said feeder cells, are human preprimitive streak and/or mesendoderm cells.

[0479] In ~~embodiment~~examples of the screening methods described herein, the cell population is contacted or otherwise provided with a candidate (test) differentiation factor. The candidate differentiation factor can comprise any molecule that may have the potential to promote the differentiation of human preprimitive streak and/or mesendoderm cells. In some ~~embodiment~~examples described herein, the candidate differentiation factor comprises a molecule that is known to be a differentiation factor for one or more types of cells. In alternate ~~embodiment~~examples, the candidate differentiation factor comprises a molecule that in not known to promote cell differentiation. In preferred ~~embodiment~~examples, the candidate differentiation factor comprises molecule that is not known to promote the differentiation of human preprimitive streak and/or mesendoderm cells.

[0480] In some ~~embodiment~~examples of the screening methods described herein, the candidate differentiation factor comprises a small molecule. In preferred ~~embodiment~~examples, a small molecule is a molecule having a molecular mass of about 10,000 amu or less. In some ~~embodiment~~examples the small molecule is a retinoid, such as retinoic acid.

[0481] In other ~~embodiment~~examples described herein, the candidate differentiation factor comprises a polypeptide. The polypeptide can be any polypeptide including, but not limited to, a glycoprotein, a lipoprotein, an extracellular matrix protein, a cytokine, a chemokine, a peptide hormone, an interleukin or a growth factor. Preferred polypeptides include growth factors. In some preferred ~~embodiment~~examples, the candidate differentiation factors comprises one or more growth factors selected from the group consisting of FGF10, FGF4, FGF2 and Wnt3B.

[0482] In some ~~embodiment~~examples of the screening methods described herein, the candidate differentiation factors comprise one or more growth factors selected from the group consisting of Amphiregulin, B-lymphocyte stimulator, IL-16, Thymopoietin, TRAIL/Apo-2, Pre B cell colony enhancing factor, Endothelial differentiation-related factor 1 (EDF1), Endothelial monocyte activating polypeptide II, Macrophage migration inhibitory factor (MIF), Natural killer cell enhancing factor (NKEFA), Bone mophogenetic protein 2, Bone mophogenetic protein 8 (osteogeneic protein 2), Bone morphogenic protein 6, Bone morphogenic protein 7, Connective tissue growth factor (CTGF), CGI-149 protein (neuroendocrine differentiation factor), Cytokine A3 (macrophage inflammatory protein 1-alpha), Gliablastoma cell differentiation-related protein (GBDR1), Hepatoma-derived growth factor, Neuromedin U-25 precursor, Vascular endothelial growth factor (VEGF), Vascular endothelial growth factor B (VEGF-B), T-cell specific RANTES

precursor, thymic dendritic cell-derived factor 1, Transferrin, Interleukin-1 (IL 1), Interleukin-2 (IL 2), Interleukin-3 (IL 3), Interleukin-4 (IL 4), Interleukin-5 (IL 5), Interleukin-6 (IL 6), Interleukin-7 (IL 7), Interleukin-8 (IL 8), Interleukin-9 (IL 9), Interleukin-10 (IL 10), Interleukin-11 (IL 11), Interleukin-12 (IL 12), Interleukin-13 (IL 13), Granulocyte-colony stimulating factor (G-CSF), Granulocyte macrophage colony stimulating factor (GM-CSF), Macrophage colony stimulating factor (M-CSF), Erythropoietin, Thrombopoietin, Vitamin D_3, Epidermal growth factor (EGF), Brain-derived neurotrophic factor, Leukemia inhibitory factor, Thyroid hormone, Basic fibroblast growth factor (bFGF), aFGF, FGF-4, FGF-6, Keratinocyte growth factor (KGF), Platelet-derived growth factor (PDGF), Platelet-derived growth factor-BB, beta nerve growth factor, activin A, Transforming growth factor beta 1 (TGF-β1), Interferon-α, Interferon-β, Interferon-γ, Tumor necrosis factor- α, Tumor necrosis factor- β, Burst promoting activity (BPA), Erythroid promoting activity (EPA), PGE_2, insulin growth factor-1 (IGF-1), IGF-II, Neutrophin growth factor (NGF), Neutrophin-3, Neutrophin 4/5, Ciliary neurotrophic factor, Glial-derived nexin, Dexamethasone, β-mercaptoethanol, Retinoic acid, Butylated hydroxyanisole, 5-azacytidine, Amphotericin B, Ascorbic acid, Ascrorbate, isobutylxanthine, indomethacin, β-glycerolphosphate, nicotinamide, DMSO, Thiazolidinediones, TWS119, oxytocin, vasopressin, melanocyte-stimulating hormone, corticortropin, lipotropin, thyrotropin, growth hormone, prolactin, luteinizing hormone, human chorionic gonadotropin, follicle stimulating hormone, corticotropin-releasing factor, gonadotropin-releasing factor, prolactin-releasing factor, prolactin-inhibiting factor, growth-hormone releasing factor, somatostatin, thyrotropin-releasing factor, calcitonin gene-related peptide, parathyroid hormone, glucagon-like peptide 1, glucose-dependent insulinotropic polypeptide, gastrin, secretin, cholecystokinin, motilin, vasoactive intestinal peptide, substance P, pancreatic polypeptide, peptide tyrosine tyrosine, neuropeptide tyrosine, , insulin, glucagon, placental lactogen, relaxin, angiotensin II, calctriol, atrial natriuretic peptide, and melatonin. thyroxine, triiodothyronine, calcitonin, estradiol, estrone, progesterone, testosterone, cortisol, corticosterone, aldosterone, epinephrine, norepinepherine, androstiene, calcitriol, collagen, Dexamethasone, β-mercaptoethanol, Retinoic acid, Butylated hydroxyanisole, 5-azacytidine, Amphotericin B, Ascorbic acid, Ascrorbate, isobutylxanthine, indomethacin, β-glycerolphosphate, nicotinamide, DMSO, Thiazolidinediones, and TWS119.

[0483] In some ~~embodiment~~examples of the screening methods described herein, the candidate differentiation factor is provided to the cell population in one or more concentrations. In some ~~embodiment~~examples, the candidate differentiation factor is provided to the cell population so that the concentration of the candidate differentiation factor in the medium surrounding the cells ranges from about 0.1 ng/ml to about 10 mg/ml. In some ~~embodiment~~examples, the concentration of the candidate differentiation factor in the medium surrounding the cells ranges from about 1 ng/ml to about 1 mg/ml. In other ~~embodiment~~examples, the concentration of the candidate differentiation factor in the medium surrounding the cells ranges from about 10 ng/ml to about 100 μg/ml. In still other

~~embodiment~~examples, the concentration of the candidate differentiation factor in the medium surrounding the cells ranges from about 100 ng/ml to about 10 μg/ml. In preferred ~~embodiment~~examples, the concentration of the candidate differentiation factor in the medium surrounding the cells is about 5 ng/ml, about 25 ng/ml, about 50 ng/ml, about 75 ng/ml, about 100 ng/ml, about 125 ng/ml, about 150 ng/ml, about 175 ng/ml, about 200 ng/ml, about 225 ng/ml, about 250 ng/ml, about 275 ng/ml, about 300 ng/ml, about 325 ng/ml, about 350 ng/ml, about 375 ng/ml, about 400 ng/ml, about 425 ng/ml, about 450 ng/ml, about 475 ng/ml, about 500 ng/ml, about 525 ng/ml, about 550 ng/ml, about 575 ng/ml, about 600 ng/ml, about 625 ng/ml, about 650 ng/ml, about 675 ng/ml, about 700 ng/ml, about 725 ng/ml, about 750 ng/ml, about 775 ng/ml, about 800 ng/ml, about 825 ng/ml, about 850 ng/ml, about 875 ng/ml, about 900 ng/ml, about 925 ng/ml, about 950 ng/ml, about 975 ng/ml, about 1 μg/ml, about 2 μg/ml, about 3 μg/ml, about 4 μg/ml, about 5 μg/ml, about 6 μg/ml, about 7 μg/ml, about 8 μg/ml, about 9 μg/ml, about 10 μg/ml, about 11 μg/ml, about 12 μg/ml, about 13 μg/ml, about 14 μg/ml, about 15 μg/ml, about 16 μg/ml, about 17 μg/ml, about 18 μg/ml, about 19 μg/ml, about 20 μg/ml, about 25 μg/ml, about 50 μg/ml, about 75 μg/ml, about 100 μg/ml, about 125 μg/ml, about 150 μg/ml, about 175 μg/ml, about 200 μg/ml, about 250 μg/ml, about 300 μg/ml, about 350 μg/ml, about 400 μg/ml, about 450 μg/ml, about 500 μg/ml, about 550 μg/ml, about 600 μg/ml, about 650 μg/ml, about 700 μg/ml, about 750 μg/ml, about 800 μg/ml, about 850 μg/ml, about 900 μg/ml, about 950 μg/ml, about 1000 μg/ml or greater than about 1000 μg/ml.

[0484] In certain ~~embodiment~~examples of the screening methods described herein, the cell population is provided with a candidate differentiation factor which comprises any molecule other than foregut differentiation factor. For example, in some ~~embodiment~~examples, the cell population is provided with a candidate differentiation factor which comprises any molecule other than a retinoid, a member of the TGFβ superfamily of growth factors, FGF10 or FGF4. In some ~~embodiment~~examples, the cell population is provided with a candidate differentiation factor which comprises any molecule other than retinoic acid.

[0485] In some ~~embodiment~~examples, steps of the screening methods described herein comprise determining expression of at least one marker at a first time point and a second time point. In some of these ~~embodiment~~examples, the first time point can be prior to or at approximately the same time as providing the cell population with the candidate differentiation factor. Alternatively, in some ~~embodiment~~examples, the first time point is subsequent to providing the cell population with the candidate differentiation factor. In some ~~embodiment~~examples, expression of a plurality of markers is determined at a first time point.

[0486] In addition to determining expression of at least one marker at a first time point, some ~~embodiment~~examples of the screening methods described herein contemplate determining expression of at least one marker at a second time point, which is subsequent to the first time point and which is subsequent to providing the cell population with the candidate differentiation

factor. In such ~~embodiment~~examples, expression of the same marker is determined at both the first and second time points. In some ~~embodiment~~examples, expression of a plurality of markers is determined at both the first and second time points. In such ~~embodiment~~examples, expression of the same plurality of markers is determined at both the first and second time points. In some ~~embodiment~~examples, marker expression is determined at a plurality of time points, each of which is subsequent to the first time point, and each of which is subsequent to providing the cell population with the candidate differentiation factor. In certain ~~embodiment~~examples, marker expression is determined by Q-PCR. In other ~~embodiment~~examples, marker expression is determined by immunocytochemistry.

[0487] In certain ~~embodiment~~examples of the screening methods described herein, the marker having its expression is determined at the first and second time points is a marker that is associated with the differentiation of human preprimitive streak cells and/or mesendoderm cells to cells which are the precursors of cells which make up tissues and/or organs that are derived from the gut tube. In some ~~embodiment~~examples, the tissues and/or organs that are derived from the gut tube comprise terminally differentiated cells. In some ~~embodiment~~examples, the marker is indicative of pancreatic cells or pancreatic precursor cells. In preferred ~~embodiment~~examples, the marker is pancreatic-duodenal homeobox factor-1 (PDX1). In other ~~embodiment~~examples, the marker is homeobox A13 (HOXA13) or homeobox C6 (HOXC6). Additionally, in other ~~embodiment~~examples, the marker is indicative of liver cells or liver precursor cells. In certain preferred ~~embodiment~~examples, the marker is albumin, hepatocyte specific antigen (HSA) or prospero-related homeobox 1 (PROX1). In other ~~embodiment~~examples, the marker is indicative of lung or lung precursor cells. In some preferred ~~embodiment~~examples, the marker is thyroid transcription factor 1 (TITF1). In yet other ~~embodiment~~examples, the marker is indicative of intestinal or intestinal precursor cells. In additional preferred ~~embodiment~~examples, the marker is villin or caudal type homeobox transcription factor 2 (CDX2). In still other ~~embodiment~~examples, the marker is indicative of stomach or stomach precursor cells. In additional preferred ~~embodiment~~examples, the marker is VCAM1, VWF or CXCR4. In other ~~embodiment~~examples, the marker is indicative of thyroid or thyroid precursor cells. In such ~~embodiment~~examples, the marker is TITF1. In still other ~~embodiment~~examples, the marker is indicative of thymus or thymus precursor cells.

[0488] In some ~~embodiment~~examples of the screening methods described herein, sufficient time is allowed to pass between providing the cell population with the candidate differentiation factor and determining marker expression at the second time point. Sufficient time between providing the cell population with the candidate differentiation factor and determining expression of the marker at the second time point can be as little as from about 1 hour to as much as about 10 days. In some ~~embodiment~~examples, the expression of at least one marker is determined multiple times subsequent to providing the cell population with the candidate differentiation factor. In some ~~embodiment~~examples, sufficient time is at least about 1 hour, at least about 6 hours, at least about 12 hours, at least about 18 hours, at least about 24 hours, at least about 30 hours, at least about 36 hours, at least about 42 hours, at least about 48 hours, at least about 54 hours, at least about 60

hours, at least about 66 hours, at least about 72 hours, at least about 78 hours, at least about 84 hours, at least about 90 hours, at least about 96 hours, at least about 102 hours, at least about 108 hours, at least about 114 hours, at least about 120 hours, at least about 126 hours, at least about 132 hours, at least about 138 hours, at least about 144 hours, at least about 150 hours, at least about 156 hours, at least about 162 hours, at least about 168 hours, at least about 174 hours, at least about 180 hours, at least about 186 hours, at least about 192 hours, at least about 198 hours, at least about 204 hours, at least about 210 hours, at least about 216 hours, at least about 222 hours, at least about 228 hours, at least about 234 hours or at least about 240 hours.

[0489] In some ~~embodiment~~examples of the methods described herein, it is further determined whether the expression of the marker at the second time point has increased or decreased as compared to the expression of this marker at the first time point. An increase or decrease in the expression of the at least one marker indicates that the candidate differentiation factor is capable of promoting the differentiation of the definitive endoderm cells. Similarly, if expression of a plurality of markers is determined, it is further determined whether the expression of the plurality of markers at the second time point has increased or decreased as compared to the expression of this plurality of markers at the first time point. An increase or decrease in marker expression can be determined by measuring or otherwise evaluating the amount, level or activity of the marker in the cell population at the first and second time points. Such determination can be relative to other markers, for example housekeeping gene expression, or absolute. In certain ~~embodiment~~examples, wherein marker expression is increased at the second time point as compared with the first time point, the amount of increase is at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 60-fold, at least about 70-fold, at least about 80-fold, at least about 90-fold, at least about 100-fold or more than at least about 100-fold. In some ~~embodiment~~examples, the amount of increase is less than 2-fold. In ~~embodiment~~examples where marker expression is decreased at the second time point as compared with the first time point, the amount of decrease is at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 60-fold, at least about 70-fold, at least about 80-fold, at least about 90-fold, at least about 100-fold or more than at least about 100-fold. In some ~~embodiment~~examples, the amount of decrease is less than 2-fold.

[0490] In some ~~embodiment~~examples of the screening methods described herein, after providing the cell population with a candidate differentiation factor, the human preprimitive streak and/or mesendoderm cells differentiate into one or more cell types of the definitive endoderm lineage. In some ~~embodiment~~examples, after providing the cell population with a candidate differentiation factor, the human preprimitive streak and/or mesendoderm cells differentiate into cells that are derived from the gut tube. Such cells include, but are not limited to, cells of the pancreas, liver, lungs, stomach, intestine, thyroid, thymus, pharynx, gallbladder and urinary

bladder as well as precursors of such cells. Additionally, these cells can further develop into higher order structures such as tissues and/or organs.

[0491] In other ~~embodiment~~examples of the screening methods described herein, after providing the cell population with a candidate differentiation factor, the human preprimitive streak and/or mesendoderm cells differentiate into one or more cell types of the mesoderm lineage. In some ~~embodiment~~examples, after providing the cell population with a candidate differentiation factor, the human preprimitive streak and/or mesendoderm cells differentiate into cells which include, but are not limited to, blood cells, cells of the cardiovascular system, skeletal tissues and other structural and connective tissues as well as precursors of each of the aforementioned cell types. Additionally, these cells can further develop into higher order structures such as tissues and/or organs.

Methods of Increasing the Expression of the FGF8 Gene Product

[0492] Some ~~embodiment~~examples of the methods described herein relate to a method of increasing the expression of the FGF8 gene product in a human embryonic stem cell *in vitro*. Such methods comprise the step of obtaining said hESC in a medium comprising less than about 2% (v/v) serum. For example, the medium can comprise serum at a concentration of about 0% (v/v), of about 0.05% (v/v), about 0.1% (v/v), about 0.2% (v/v), about 0.3% (v/v), about 0.4% (v/v), about 0.5% (v/v), about 0.6% (v/v), about 0.1% (v/v), about 0.2% (v/v), about 0.3% (v/v), about 0.4% (v/v), about 0.5% (v/v), about 0.6% (v/v), about 0.7% (v/v), about 0.8% (v/v), about 0.9% (v/v), about 1% (v/v), about 1.1% (v/v), about 1.2% (v/v), about 1.3% (v/v), about 1.4% (v/v), about 1.5% (v/v), about 1.6% (v/v), about 1.7% (v/v), about 1.8% (v/v) or about 1.9% (v/v). In some ~~embodiment~~examples, the medium does not comprise serum replacement. The hESCs are contacted with a differentiation factor in an amount sufficient to increase expression of the FGF8 gene product. In some ~~embodiment~~examples, the differentiation factor is at least one growth factor of the TGFβ superfamily. In preferred ~~embodiment~~examples, the growth factor of the TGFβ superfamily is activin A. The concentration of differentiation factor that is used to contact the hESCs ranges from about 1 ng/ml to about 1 mg/ml. For example, the hESCs can be contacted with a differentiation factor at a concentration of 1 ng/ml, about 5 ng/ml, about 25 ng/ml, about 50 ng/ml, about 75 ng/ml, about 100 ng/ml, about 125 ng/ml, about 150 ng/ml, about 175 ng/ml, about 200 ng/ml, about 225 ng/ml, about 250 ng/ml, about 275 ng/ml, about 300 ng/ml, about 325 ng/ml, about 350 ng/ml, about 375 ng/ml, about 400 ng/ml, about 425 ng/ml, about 450 ng/ml, about 475 ng/ml, about 500 ng/ml, about 525 ng/ml, about 550 ng/ml, about 575 ng/ml, about 600 ng/ml, about 625 ng/ml, about 650 ng/ml, about 675 ng/ml, about 700 ng/ml, about 725 ng/ml, about 750 ng/ml, about 775 ng/ml, about 800 ng/ml, about 825 ng/ml, about 850 ng/ml, about 875 ng/ml, about 900 ng/ml, about 925 ng/ml, about 950 ng/ml, about 975 ng/ml, about 1 μg/ml, about 2 μg/ml, about 3 μg/ml, about 4 μg/ml, about 5 μg/ml, about 6 μg/ml, about 7 μg/ml, about

8 μg/ml, about 9 μg/ml, about 10 μg/ml, about 11 μg/ml, about 12 μg/ml, about 13 μg/ml, about 14 μg/ml, about 15 μg/ml, about 16 μg/ml, about 17 μg/ml, about 18 μg/ml, about 19 μg/ml, about 20 μg/ml, about 25 μg/ml, about 50 μg/ml, about 75 μg/ml, about 100 μg/ml, about 125 μg/ml, about 150 μg/ml, about 175 μg/ml, about 200 μg/ml, about 250 μg/ml, about 300 μg/ml, about 350 μg/ml, about 400 μg/ml, about 450 μg/ml, about 500 μg/ml, about 550 μg/ml, about 600 μg/ml, about 650 μg/ml, about 700 μg/ml, about 750 μg/ml, about 800 μg/ml, about 850 μg/ml, about 900 μg/ml, about 950 μg/ml, about 1000 μg/ml or greater than about 1000 μg/ml.

Methods of Increasing the Expression of the Brachyury, FGF4 and/or SNAI1 Gene Product

[0493] Other ~~embodiment~~examples of the methods described herein relate to a method of increasing the expression of the brachyury, FGF4 and/or SNAI1 gene product in a human embryonic stem cell *in vitro*. Such methods comprise the step of obtaining said hESC in a medium comprising less than about 2% (v/v) serum. For example, the medium can comprise serum at a concentration of about 0% (v/v), of about 0.05% (v/v), about 0.1% (v/v), about 0.2% (v/v), about 0.3% (v/v), about 0.4% (v/v), about 0.5% (v/v), about 0.6% (v/v), about 0.1% (v/v), about 0.2% (v/v), about 0.3% (v/v), about 0.4% (v/v), about 0.5% (v/v), about 0.6% (v/v), about 0.7% (v/v), about 0.8% (v/v), about 0.9% (v/v), about 1% (v/v), about 1.1% (v/v), about 1.2% (v/v), about 1.3% (v/v), about 1.4% (v/v), about 1.5% (v/v), about 1.6% (v/v), about 1.7% (v/v), about 1.8% (v/v) or about 1.9% (v/v). In some ~~embodiment~~examples, the medium does not comprise serum replacement. The hESCs are contacted with a differentiation factor in an amount sufficient to increase expression of the brachyury, FGF4 and/or SNAI1 gene product. In some ~~embodiment~~examples, the differentiation factor is at least one growth factor of the TGFβ superfamily. In preferred ~~embodiment~~examples, the growth factor of the TGFβ superfamily is activin A. The concentration of differentiation factor that is used to contact the hESCs ranges from about 1 ng/ml to about 1 mg/ml. For example, the hESCs can be contacted with a differentiation factor at a concentration of 1 ng/ml, about 5 ng/ml, about 25 ng/ml, about 50 ng/ml, about 75 ng/ml, about 100 ng/ml, about 125 ng/ml, about 150 ng/ml, about 175 ng/ml, about 200 ng/ml, about 225 ng/ml, about 250 ng/ml, about 275 ng/ml, about 300 ng/ml, about 325 ng/ml, about 350 ng/ml, about 375 ng/ml, about 400 ng/ml, about 425 ng/ml, about 450 ng/ml, about 475 ng/ml, about 500 ng/ml, about 525 ng/ml, about 550 ng/ml, about 575 ng/ml, about 600 ng/ml, about 625 ng/ml, about 650 ng/ml, about 675 ng/ml, about 700 ng/ml, about 725 ng/ml, about 750 ng/ml, about 775 ng/ml, about 800 ng/ml, about 825 ng/ml, about 850 ng/ml, about 875 ng/ml, about 900 ng/ml, about 925 ng/ml, about 950 ng/ml, about 975 ng/ml, about 1 μg/ml, about 2 μg/ml, about 3 μg/ml, about 4 μg/ml, about 5 μg/ml, about 6 μg/ml, about 7 μg/ml, about 8 μg/ml, about 9 μg/ml, about 10 μg/ml, about 11 μg/ml, about 12 μg/ml, about 13 μg/ml, about 14 μg/ml, about 15 μg/ml, about 16 μg/ml, about 17 μg/ml, about 18 μg/ml, about 19 μg/ml, about 20 μg/ml, about 25

μg/ml, about 50 μg/ml, about 75 μg/ml, about 100 μg/ml, about 125 μg/ml, about 150 μg/ml, about 175 μg/ml, about 200 μg/ml, about 250 μg/ml, about 300 μg/ml, about 350 μg/ml, about 400 μg/ml, about 450 μg/ml, about 500 μg/ml, about 550 μg/ml, about 600 μg/ml, about 650 μg/ml, about 700 μg/ml, about 750 μg/ml, about 800 μg/ml, about 850 μg/ml, about 900 μg/ml, about 950 μg/ml, about 1000 μg/ml or greater than about 1000 μg/ml.

Temporal Expression of Gene Products in Cell Cultures

[0494] Further ~~embodiments of the present invention~~examples relate to cell cultures having certain temporal patterns of gene expression. In some ~~embodiment~~examples, the cell culture comprise human embryonic stem cells (hESCs) and a medium comprising less than about 2% (v/v) serum. For example, the medium can comprise serum at a concentration of about 0% (v/v), of about 0.05% (v/v), about 0.1% (v/v), about 0.2% (v/v), about 0.3% (v/v), about 0.4% (v/v), about 0.5% (v/v), about 0.6% (v/v), about 0.1% (v/v), about 0.2% (v/v), about 0.3% (v/v), about 0.4% (v/v), about 0.5% (v/v), about 0.6% (v/v), about 0.7% (v/v), about 0.8% (v/v), about 0.9% (v/v), about 1% (v/v), about 1.1% (v/v), about 1.2% (v/v), about 1.3% (v/v), about 1.4% (v/v), about 1.5% (v/v), about 1.6% (v/v), about 1.7% (v/v), about 1.8% (v/v) or about 1.9% (v/v). In some ~~embodiment~~examples, the medium does not comprise serum replacement. In some ~~embodiment~~examples, the medium is low serum RPMI.

[0495] In some ~~embodiment~~examples described herein, hESCs in culture begin differentiating at a reference time point. The reference time point is the point at which a differentiation factor is provided to the cells. In some ~~embodiment~~examples, the differentiation factor is at least one growth factor of the TGFβ superfamily. In preferred ~~embodiment~~examples, the growth factor of the TGFβ superfamily is activin A. The concentration of differentiation factor that is provided to the hESCs ranges from about 1 ng/ml to about 1 mg/ml. For example, the hESCs can be contacted with a differentiation factor at a concentration of 1 ng/ml, about 5 ng/ml, about 25 ng/ml, about 50 ng/ml, about 75 ng/ml, about 100 ng/ml, about 125 ng/ml, about 150 ng/ml, about 175 ng/ml, about 200 ng/ml, about 225 ng/ml, about 250 ng/ml, about 275 ng/ml, about 300 ng/ml, about 325 ng/ml, about 350 ng/ml, about 375 ng/ml, about 400 ng/ml, about 425 ng/ml, about 450 ng/ml, about 475 ng/ml, about 500 ng/ml, about 525 ng/ml, about 550 ng/ml, about 575 ng/ml, about 600 ng/ml, about 625 ng/ml, about 650 ng/ml, about 675 ng/ml, about 700 ng/ml, about 725 ng/ml, about 750 ng/ml, about 775 ng/ml, about 800 ng/ml, about 825 ng/ml, about 850 ng/ml, about 875 ng/ml, about 900 ng/ml, about 925 ng/ml, about 950 ng/ml, about 975 ng/ml, about 1 μg/ml, about 2 μg/ml, about 3 μg/ml, about 4 μg/ml, about 5 μg/ml, about 6 μg/ml, about 7 μg/ml, about 8 μg/ml, about 9 μg/ml, about 10 μg/ml, about 11 μg/ml, about 12 μg/ml, about 13 μg/ml, about 14 μg/ml, about 15 μg/ml, about 16 μg/ml, about 17 μg/ml, about 18 μg/ml, about 19 μg/ml, about 20 μg/ml, about 25 μg/ml, about 50 μg/ml, about 75 μg/ml, about 100 μg/ml,

about 125 μg/ml, about 150 μg/ml, about 175 μg/ml, about 200 μg/ml, about 250 μg/ml, about 300 μg/ml, about 350 μg/ml, about 400 μg/ml, about 450 μg/ml, about 500 μg/ml, about 550 μg/ml, about 600 μg/ml, about 650 μg/ml, about 700 μg/ml, about 750 μg/ml, about 800 μg/ml, about 850 μg/ml, about 900 μg/ml, about 950 μg/ml, about 1000 μg/ml or greater than about 1000 μg/ml.

[0496] After providing the differentiation factor to the hESCs, the expression of FGF8 mRNA is substantially upregulated as compared to baseline FGF8 mRNA expression in the hESCs. Baseline FGF8 expression in hESCs is the expression of FGF8 gene product, such as mRNA, present in an hESC cell culture that is maintained in it undifferentiated state. In some ~~embodiment~~examples, FGF8 mRNA expression is substantially upregulated by about 6 hours from the reference time point. In further ~~embodiment~~examples described herein, expression of FGF8 mRNA is downregulated after about 24 hours from the reference time point. In other ~~embodiment~~examples, peak expression of FGF8 mRNA is reached at a time between about 6 hours and about 24 hours from the reference time point. In other ~~embodiment~~examples, peak expression of FGF8 may be reached at a time less than about 6 hours from the reference time point.

[0497] Other ~~embodiment~~examples described herein relate to cell cultures exhibiting increased nuclear localization of the β-catenin polypeptide. In such ~~embodiment~~examples, the β-catenin polypeptide begins to become localized to the cell nucleus by about 17 hours from the reference time point. In some ~~embodiment~~examples, the β-catenin polypeptide begins to become localized to the cell nucleus by less than about 17 hours from the reference time point. In still other ~~embodiment~~examples, the β-catenin polypeptide becomes predominantly localized to the cell nucleus by about 17 hours from the reference time point.

[0498] Still other ~~embodiment~~examples of the cell cultures described herein relate to cell cultures having increased expression of brachyury mRNA. In such cell cultures, brachyury mRNA expression is substantially upregulated by about 24 hours from the reference time point. In some ~~embodiment~~examples, brachyury mRNA expression is substantially upregulated prior to about 24 hours from the reference time point. In some ~~embodiment~~examples, expression of brachyury mRNA is substantially downregulated by about 48 hours from the reference time point. In certain ~~embodiment~~examples, peak expression of brachyury mRNA is reached at a time between about 12 hours and about 48 hours from the reference time point. In preferred ~~embodiment~~examples, the brachyury mRNA in not substantially expressed by about 72 hours from the reference time point. In other preferred ~~embodiment~~examples, brachyury mRNA is substantially upregulated by about 24 hours from the reference time point and is not substantially expressed by about 72 hours from the reference time point.

[0499] Still further ~~embodiment~~examples of the cell cultures described herein relate to cell cultures having increased expression of FGF4 mRNA. In such cell cultures, FGF4 mRNA

expression is substantially upregulated by about 24 hours from the reference time point. In some ~~embodiment~~examples, FGF4 mRNA expression is substantially upregulated prior to about 24 hours from the reference time point. In some ~~embodiment~~examples, expression of FGF4 mRNA is substantially downregulated by about 48 hours from the reference time point. In certain ~~embodiment~~examples, peak expression of FGF4 mRNA is reached at a time between about 12 hours and about 48 hours from the reference time point. In preferred ~~embodiment~~examples, the FGF4 mRNA in not substantially expressed by about 72 hours from the reference time point. In other preferred ~~embodiment~~examples, FGF4 mRNA is substantially upregulated by about 24 hours from the reference time point and is not substantially expressed by about 72 hours from the reference time point.

[0500] Preferred ~~embodiment~~examples of the cell cultures described herein relate to cell cultures having increased expression of brachyury and FGF4 mRNA. In such cell cultures, brachyury and FGF4 mRNA expression is substantially upregulated by about 24 hours from the reference time point. In some ~~embodiment~~examples, brachyury and FGF4 mRNA expression is substantially upregulated prior to about 24 hours from the reference time point. In some ~~embodiment~~examples, expression of brachyury and FGF4 mRNA is substantially downregulated by about 48 hours from the reference time point. In certain ~~embodiment~~examples, peak expression of brachyury and FGF4 mRNA is reached at a time between about 12 hours and about 48 hours from the reference time point. In preferred ~~embodiment~~examples, the brachyury and FGF4 mRNA in not substantially expressed by about 72 hours from the reference time point. In other preferred ~~embodiment~~examples, brachyury and FGF4 mRNA is substantially upregulated by about 24 hours from the reference time point and is not substantially expressed by about 72 hours from the reference time point.

[0501] Additional ~~embodiment~~examples of the cell cultures described herein relate to cell cultures having increased expression of SNAI1 mRNA. In such cell cultures, SNAI1 mRNA expression is substantially upregulated by about 24 hours from the reference time point. In some ~~embodiment~~examples, SNAI1 mRNA expression is substantially upregulated prior to about 24 hours from the reference time point. In some ~~embodiment~~examples, expression of SNAI1 mRNA is downregulated by about 48 hours from the reference time point. In certain ~~embodiment~~examples, peak expression of SNAI1 mRNA is reached at a time between about 12 hours and about 48 hours from the reference time point.

[0502] ~~Embodiment~~Examples of the cell cultures described herein also relate to cell cultures having a specific temporal expression of the E-cadherin (ECAD) gene product. In such ~~embodiment~~examples, expression of E-cadherin mRNA begins to be downregulated by about 12 hours from the reference time point. In other ~~embodiment~~examples, expression of E-cadherin mRNA can be downregulated by about less than 12 hours from the reference time point. In preferred ~~embodiment~~examples, expression of E-cadherin mRNA is substantially downregulated by about 48 hours from the reference time point.

[0503] Further ~~embodiment~~examples described herein relate to cell cultures that express the SOX17 and/or FOXA2 marker. In some ~~embodiment~~examples, expression of SOX17 mRNA is substantially upregulated by about 48 hours from the reference time point. In other ~~embodiment~~examples, expression of FOXA2 mRNA is substantially upregulated by about 96 hours from the reference time point.

[0504] Some ~~embodiment~~examples described herein relate to cell cultures that comprise cells having certain specified patterns of gene expression. In such ~~embodiment~~examples, the cell cultures comprise hESCs, a differentiation factor, such as a differentiation factor of the TGFβ superfamily, and a medium comprising less than about 2% (v/v) serum. In alternative ~~embodiment~~examples, the medium comprises greater than 2% (v/v) serum. In some ~~embodiment~~examples, the medium lacks serum replacement.

[0505] In some ~~embodiment~~examples, at the time the cell culture is provided with the differentiation factor, the cell culture comprises all or predominantly all hESCs. During the course of differentiation, at least a portion of the hESCs differentiate into other cell types as indicated by the expression of the products (mRNA and/or polypeptides) of certain marker genes.

[0506] In some ~~embodiment~~examples of the cell cultures described herein, the expression of a first set of marker genes is upregulated prior to the upregulation of a second and/or a third set of marker genes. In some ~~embodiment~~examples, each set of marker genes can include one or more marker genes. Upregulation of gene expression can range from slight to substantial. For example, expression of a marker gene can be upregulated by at least about 10% as compared to the expression of the same marker gene in undifferentiated hESCs. In other ~~embodiment~~examples, expression of a marker gene can be upregulated by at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or greater than at least about 90% as compared to the expression of the same marker gene in undifferentiated hESCs. In still other ~~embodiment~~examples, the marker gene expression can be upregulated by at least 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least about 8-fold, at least about 9-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 60-fold, at least about 70-fold, at least about 80-fold, at least about 90-fold, at least about 100-fold or greater than at least about 100-fold as compared to the expression of the same marker gene in undifferentiated hESCs.

[0507] In other ~~embodiment~~examples of the cell cultures described herein, the expression of a first set of marker genes is downregulated regulated prior to the upregulation of a second and/or a third set of marker genes. In such ~~embodiment~~examples, the each set of marker genes can include one or more marker genes. As with upregulation of gene expression, downregulation can range from slight to substantial. For example, expression of a marker gene can be downregulated by at least about 10% as compared to the expression of the same marker gene in undifferentiated hESCs. In

other ~~embodiment~~examples, expression of a marker gene can be downregulated by at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or greater than at least about 90% as compared to the expression of the same marker gene in undifferentiated hESCs. In still other ~~embodiment~~examples, the marker gene expression can be downregulated by at least 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least about 8-fold, at least about 9-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 60-fold, at least about 70-fold, at least about 80-fold, at least about 90-fold, at least about 100-fold or greater than at least about 100-fold as compared to the expression of the same marker gene in undifferentiated hESCs.

[0508] In still other ~~embodiment~~examples of the cell cultures described herein, the expression of a first set of marker genes is upregulated prior to or at about the same time as the peak expression of a second set and/or a third set of marker genes. In such ~~embodiment~~examples, each set of marker genes can comprise one or more marker genes. In other ~~embodiment~~examples, the expression of a first set of marker genes is downregulated prior to or at about the same time as the peak expression of a second set and/or a third set of marker genes. As described above, in such ~~embodiment~~examples, each set of marker genes can comprise one or more marker genes. Furthermore, in the above-described ~~embodiment~~examples, both upregulation and downregulation of gene expression can range from slight to substantial. For example, expression of a marker gene can be upregulated or downregulated by at least about 10% as compared to the expression of the same marker gene in undifferentiated hESCs. In other ~~embodiment~~examples, expression of a marker gene can be upregulated or downregulated by at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or greater than at least about 90% as compared to the expression of the same marker gene in undifferentiated hESCs. In still other ~~embodiment~~examples, the marker gene expression can be upregulated or downregulated by at least 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least about 8-fold, at least about 9-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 60-fold, at least about 70-fold, at least about 80-fold, at least about 90-fold, at least about 100-fold or greater than at least about 100-fold as compared to the expression of the same marker gene in undifferentiated hESCs.

[0509] In some ~~embodiment~~examples described herein, in at least some cells of the cell culture, expression of a marker gene selected from the group consisting of FGF8, Nodal, HEG, HEY1, GATA2, BIK and ID1 is upregulated prior to upregulation of expression of a marker gene selected from the group consisting of brachyury, FGF4, SNAI1, Wnt3, MIXL1, DKK4, NETO1, T, DACT1, FLJ22662, SLIT2, GAD1 and GRM4. In other ~~embodiment~~examples, in at least some cell of

the cell culture, expression of a marker gene selected from the group consisting of FGF8, Nodal, HEG, HEY1, GATA2, BIK and ID1 is upregulated prior to peak expression of a marker gene selected from the group consisting of brachyury, FGF4, SNAI1, Wnt3, MIXL1, DKK4, NETO1, T, DACT1, FLJ22662, SLIT2, GAD1 and GRM4. In still other ~~embodiment~~examples, in at least some cell of the cell culture, expression of a marker gene selected from the group consisting of HEY1, GATA2, BIK and ID1 is downregulated prior to or at about the same time as peak expression of a marker gene selected from the group consisting of brachyury, FGF4, SNAI1, Wnt3, MIXL1, DKK4, NETO1, T, DACT1, FLJ22662, SLIT2, GAD1 and GRM4. In further ~~embodiment~~examples, in at least some cell of the cell culture, expression of a marker gene selected from the group consisting of FGF8, Nodal, HEG, HEY1, GATA2, BIK and ID1 is upregulated prior to upregulation of expression of a marker gene selected from the group consisting of SOX17, FOXA2, CXCR4 and MIXL1. In still other ~~embodiment~~examples, in at least some cell of the cell culture, expression of a marker gene selected from the group consisting of brachyury, FGF4, SNAI1, Wnt3, MIXL1, DKK4, NETO1, T, DACT1, FLJ22662, SLIT2, GAD1 and GRM4 is upregulated prior to or at about the same time as upregulation of expression of a marker gene selected from the group consisting of SOX17, FOXA2, CXCR4 and MIXL1. In yet other ~~embodiment~~examples, in at least some cell of the cell culture, peak expression of a marker gene selected from the group consisting of brachyury, FGF4, SNAI1, Wnt3, MIXL1, DKK4, NETO1, T, DACT1, FLJ22662, SLIT2, GAD1 and GRM4 is reached prior to or at about the same time as upregulation of expression of a marker gene selected from the group consisting of SOX17, FOXA2, CXCR4 and MIXL1. In additional ~~embodiment~~examples, in at least some cell of the cell culture, expression of a marker gene selected from the group consisting of FGF8, Nodal, HEG, HEY1, GATA2, BIK and ID1 is upregulated prior to upregulation of expression of a marker gene selected from the group consisting of brachyury, FGF4, SNAI1, Wnt3, MIXL1, DKK4, NETO1, T, DACT1, FLJ22662, SLIT2, GAD1 and GRM4.

[0510] Some of the cell cultures having one or more of the temporal gene expression patterns described above include a medium comprising less than about 2% (v/v) serum. In some ~~embodiment~~examples, the medium does not include serum. In other ~~embodiment~~examples, the medium lacks serum replacement. Some of the media used in the cell cultures described herein include serum at a concentration of less than about 1.9% (v/v), less than about 1.8% (v/v), less than about 1.7% (v/v), less than about 1.6% (v/v), less than about 1.5% (v/v), less than about 1.4% (v/v), less than about 1.3% (v/v), less than about 1.2% (v/v), less than about 1.1% (v/v), less than about 1% (v/v), less than about 0.9 % (v/v), less than about 0.8% (v/v), less than about 0.7% (v/v), less than about 0.6% (v/v), less than about 0.5% (v/v), less than about 0.4% (v/v), less than about 0.3% (v/v), less than about 0.2% (v/v), less than about 0.1% (v/v) or less than about 0.05% (v/v).

[0511] Some of the cell cultures having one or more of the temporal gene expression patterns described above comprise at least one differentiation factor of the TGFβ superfamily. In

some ~~embodiment~~examples, the growth factor is nodal, activin A and/or activin B. In preferred ~~embodiment~~examples, the differentiation factor is activin A. In more preferred ~~embodiment~~examples, the activin A is present in the medium at a concentration of about 100 ng/ml.

[0512] It will be appreciated, however, that the differentiation factor of the TGFβ superfamily can be supplied to the cell culture at concentrations ranging from about 1 ng/ml to about 1 mg/ml. In some ~~embodiment~~examples, the differentiation factor of the TGFβ superfamily is supplied to the cell culture at about 5 ng/ml, about 25 ng/ml, about 50 ng/ml, about 75 ng/ml, about 100 ng/ml, about 125 ng/ml, about 150 ng/ml, about 175 ng/ml, about 200 ng/ml, about 225 ng/ml, about 250 ng/ml, about 275 ng/ml, about 300 ng/ml, about 325 ng/ml, about 350 ng/ml, about 375 ng/ml, about 400 ng/ml, about 425 ng/ml, about 450 ng/ml, about 475 ng/ml, about 500 ng/ml, about 525 ng/ml, about 550 ng/ml, about 575 ng/ml, about 600 ng/ml, about 625 ng/ml, about 650 ng/ml, about 675 ng/ml, about 700 ng/ml, about 725 ng/ml, about 750 ng/ml, about 775 ng/ml, about 800 ng/ml, about 825 ng/ml, about 850 ng/ml, about 875 ng/ml, about 900 ng/ml, about 925 ng/ml, about 950 ng/ml, about 975 ng/ml, about 1 μg/ml, about 2 μg/ml, about 3 μg/ml, about 4 μg/ml, about 5 μg/ml, about 6 μg/ml, about 7 μg/ml, about 8 μg/ml, about 9 μg/ml, about 10 μg/ml, about 11 μg/ml, about 12 μg/ml, about 13 μg/ml, about 14 μg/ml, about 15 μg/ml, about 16 μg/ml, about 17 μg/ml, about 18 μg/ml, about 19 μg/ml, about 20 μg/ml, about 25 μg/ml, about 50 μg/ml, about 75 μg/ml, about 100 μg/ml, about 125 μg/ml, about 150 μg/ml, about 175 μg/ml, about 200 μg/ml, about 250 μg/ml, about 300 μg/ml, about 350 μg/ml, about 400 μg/ml, about 450 μg/ml, about 500 μg/ml, about 550 μg/ml, about 600 μg/ml, about 650 μg/ml, about 700 μg/ml, about 750 μg/ml, about 800 μg/ml, about 850 μg/ml, about 900 μg/ml, about 950 μg/ml, about 1000 μg/ml or greater than about 1000 μg/ml.

[0513] Methods of differentiating hESCs so as to produce cells having certain temporal marker gene expression patterns are also contemplated herein. For example, some ~~embodiment~~examples relate to a method of differentiating human embryonic stem cells (hESCs), by contacting the hESCs with a medium comprising less that about 2% serum, providing the hESCs with a differentiation factor of the TGFβ superfamily, and then permitting differentiation of the hESCs to occur.

[0514] In some ~~embodiment~~examples of the above-described methods of differentiating hESCs, the expression of a first set of marker genes is upregulated prior to the upregulation of a second and/or a third set of marker genes. In some ~~embodiment~~examples, each set of marker genes can include one or more marker genes. Upregulation of gene expression can range from slight to substantial. For example, expression of a marker gene can be upregulated by at least about 10% as compared to the expression of the same marker gene in undifferentiated hESCs. In other ~~embodiment~~examples, expression of a marker gene can be upregulated by at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least

about 80%, at least about 90% or greater than at least about 90% as compared to the expression of the same marker gene in undifferentiated hESCs. In still other ~~embodiment~~examples, the marker gene expression can be upregulated by at least 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least about 8-fold, at least about 9-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 60-fold, at least about 70-fold, at least about 80-fold, at least about 90-fold, at least about 100-fold or greater than at least about 100-fold as compared to the expression of the same marker gene in undifferentiated hESCs.

[0515] In other ~~embodiment~~examples of methods of differentiating hESCs described herein, the expression of a first set of marker genes is downregulated regulated prior to the upregulation of a second and/or a third set of marker genes. In such ~~embodiment~~examples, the each set of marker genes can include one or more marker genes. As with upregulation of gene expression, downregulation can range from slight to substantial. For example, expression of a marker gene can be downregulated by at least about 10% as compared to the expression of the same marker gene in undifferentiated hESCs. In other ~~embodiment~~examples, expression of a marker gene can be downregulated by at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or greater than at least about 90% as compared to the expression of the same marker gene in undifferentiated hESCs. In still other ~~embodiment~~examples, the marker gene expression can be downregulated by at least 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least about 8-fold, at least about 9-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 60-fold, at least about 70-fold, at least about 80-fold, at least about 90-fold, at least about 100-fold or greater than at least about 100-fold as compared to the expression of the same marker gene in undifferentiated hESCs.

[0516] In still other ~~embodiment~~examples of methods of differentiating hESCs described herein, the expression of a first set of marker genes is upregulated prior to or at about the same time as the peak expression of a second set and/or a third set of marker genes. In such ~~embodiment~~examples, each set of marker genes can comprise one or more marker genes. In other ~~embodiment~~examples, the expression of a first set of marker genes is downregulated prior to or at about the same time as the peak expression of a second set and/or a third set of marker genes. As described above, in such ~~embodiment~~examples, each set of marker genes can comprise one or more marker genes. Furthermore, in the above-described ~~embodiment~~examples, both upregulation and downregulation of gene expression can range from slight to substantial. For example, expression of a marker gene can be upregulated or downregulated by at least about 10% as compared to the expression of the same marker gene in undifferentiated hESCs. In other ~~embodiment~~examples, expression of a marker gene can be upregulated or downregulated by at least about 20%, at least about 30%,

at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or greater than at least about 90% as compared to the expression of the same marker gene in undifferentiated hESCs. In still other ~~embodiment~~examples, the marker gene expression can be upregulated or downregulated by at least 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least about 8-fold, at least about 9-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 60-fold, at least about 70-fold, at least about 80-fold, at least about 90-fold, at least about 100-fold or greater than at least about 100-fold as compared to the expression of the same marker gene in undifferentiated hESCs.

[0517] In some ~~embodiment~~examples described herein, in at least some cells of the cell culture, expression of a marker gene selected from the group consisting of FGF8, Nodal, HEG, HEY1, GATA2, BIK and ID1 is upregulated prior to upregulation of expression of a marker gene selected from the group consisting of brachyury, FGF4, SNAI1, Wnt3, MIXL1, DKK4, NETO1, T, DACT1, FLJ22662, SLIT2, GAD1 and GRM4. In other ~~embodiment~~examples, in at least some cell of the cell culture, expression of a marker gene selected from the group consisting of FGF8, Nodal, HEG, HEY1, GATA2, BIK and ID1 is upregulated prior to peak expression of a marker gene selected from the group consisting of brachyury, FGF4, SNAI1, Wnt3, MIXL1, DKK4, NETO1, T, DACT1, FLJ22662, SLIT2, GAD1 and GRM4. In still other ~~embodiment~~examples, in at least some cell of the cell culture, expression of a marker gene selected from the group consisting of HEY1, GATA2, BIK and ID1 is downregulated prior to or at about the same time as peak expression of a marker gene selected from the group consisting of brachyury, FGF4, SNAI1, Wnt3, MIXL1, DKK4, NETO1, T, DACT1, FLJ22662, SLIT2, GAD1 and GRM4. In further ~~embodiment~~examples, in at least some cell of the cell culture, expression of a marker gene selected from the group consisting of FGF8, Nodal, HEG, HEY1, GATA2, BIK and ID1 is upregulated prior to upregulation of expression of a marker gene selected from the group consisting of SOX17, FOXA2, CXCR4 and MIXL1. In still other ~~embodiment~~examples, in at least some cell of the cell culture, expression of a marker gene selected from the group consisting of brachyury, FGF4, SNAI1, Wnt3, MIXL1, DKK4, NETO1, T, DACT1, FLJ22662, SLIT2, GAD1 and GRM4 is upregulated prior to or at about the same time as upregulation of expression of a marker gene selected from the group consisting of SOX17, FOXA2, CXCR4 and MIXL1. In yet other ~~embodiment~~examples, in at least some cell of the cell culture, peak expression of a marker gene selected from the group consisting of brachyury, FGF4, SNAI1, Wnt3, MIXL1, DKK4, NETO1, T, DACT1, FLJ22662, SLIT2, GAD1 and GRM4 is reached prior to or at about the same time as upregulation of expression of a marker gene selected from the group consisting of SOX17, FOXA2, CXCR4 and MIXL1. In additional ~~embodiment~~examples, in at least some cell of the cell culture, expression of a marker gene selected from the group consisting of FGF8, Nodal, HEG, HEY1, GATA2, BIK and ID1 is upregulated prior to upregulation of expression of a marker gene selected from the group consisting of brachyury,

FGF4, SNAI1, Wnt3, MIXL1, DKK4, NETO1, T, DACT1, FLJ22662, SLIT2, GAD1 and GRM4.

[0518] In some ~~embodiment~~examples of the differentiation methods described herein, cells of the cell culture are contacted or otherwise provided with a medium that comprises less than about 2% (v/v) serum. In some ~~embodiment~~example the medium does not include serum. In other ~~embodiment~~examples, the medium lacks serum replacement. Some of the media used in the methods described herein include serum at a concentration of less than about 1.9% (v/v), less than about 1.8% (v/v), less than about 1.7% (v/v), less than about 1.6% (v/v), less than about 1.5% (v/v), less than about 1.4% (v/v), less than about 1.3% (v/v), less than about 1.2% (v/v), less than about 1.1% (v/v), less than about 1% (v/v), less than about 0.9 % (v/v), less than about 0.8% (v/v), less than about 0.7% (v/v), less than about 0.6% (v/v), less than about 0.5% (v/v), less than about 0.4% (v/v), less than about 0.3% (v/v), less than about 0.2% (v/v), less than about 0.1% (v/v) or less than about 0.05% (v/v).

[0519] Some of the methods for differentiating hESCs to produce the above-described temporal patterns of gene expression include providing the cells in culture, such as hESCs, with at least one differentiation factor of the TGFβ superfamily. In some ~~embodiment~~examples, the growth factor is nodal, activin A and/or activin B. In preferred ~~embodiment~~examples, the differentiation factor is activin A. In more preferred ~~embodiment~~examples, the activin A is present in the medium at a concentration of about 100 ng/ml.

[0520] It will be appreciated, however, that the differentiation factor of the TGFβ superfamily can be supplied to the cell culture at concentrations ranging from about 1 ng/ml to about 1 mg/ml. In some ~~embodiment~~examples, the differentiation factor of the TGFβ superfamily is supplied to the cell culture at about 5 ng/ml, about 25 ng/ml, about 50 ng/ml, about 75 ng/ml, about 100 ng/ml, about 125 ng/ml, about 150 ng/ml, about 175 ng/ml, about 200 ng/ml, about 225 ng/ml, about 250 ng/ml, about 275 ng/ml, about 300 ng/ml, about 325 ng/ml, about 350 ng/ml, about 375 ng/ml, about 400 ng/ml, about 425 ng/ml, about 450 ng/ml, about 475 ng/ml, about 500 ng/ml, about 525 ng/ml, about 550 ng/ml, about 575 ng/ml, about 600 ng/ml, about 625 ng/ml, about 650 ng/ml, about 675 ng/ml, about 700 ng/ml, about 725 ng/ml, about 750 ng/ml, about 775 ng/ml, about 800 ng/ml, about 825 ng/ml, about 850 ng/ml, about 875 ng/ml, about 900 ng/ml, about 925 ng/ml, about 950 ng/ml, about 975 ng/ml, about 1 μg/ml, about 2 μg/ml, about 3 μg/ml, about 4 μg/ml, about 5 μg/ml, about 6 μg/ml, about 7 μg/ml, about 8 μg/ml, about 9 μg/ml, about 10 μg/ml, about 11 μg/ml, about 12 μg/ml, about 13 μg/ml, about 14 μg/ml, about 15 μg/ml, about 16 μg/ml, about 17 μg/ml, about 18 μg/ml, about 19 μg/ml, about 20 μg/ml, about 25 μg/ml, about 50 μg/ml, about 75 μg/ml, about 100 μg/ml, about 125 μg/ml, about 150 μg/ml, about 175 μg/ml, about 200 μg/ml, about 250 μg/ml, about 300 μg/ml, about 350 μg/ml, about 400 μg/ml, about 450 μg/ml, about 500 μg/ml, about 550 μg/ml, about 600 μg/ml, about 650

[0590] The methods, compositions, and devices described herein are presently representative of preferred ~~embodiment~~examples and are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are ~~encompassed within the spirit of the invention and are~~ defined by the scope of the disclosure. Accordingly, it will be apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope ~~and spirit~~ of the invention.

[0591] As used in the claims below and throughout this disclosure, by the phrase "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

References

[0592] Numerous literature and patent references have been cited in the present patent application. Each and every reference that is cited in this patent application is incorporated by reference herein in its entirety.

[0593] For some references, the complete citation is in the body of the text. For other references the citation in the body of the text is by author and year, the complete citation being as follows:

[0594] Alexander, J., Rothenberg, M., Henry, G. L., and Stainier, D. Y. (1999). Casanova plays an early and essential role in endoderm formation in zebrafish. Dev Biol *215*, 343-357.

[0595] Alexander, J., and Stainier, D. Y. (1999). A molecular pathway leading to endoderm formation in zebrafish. Curr Biol *9*, 1147-1157.

[0596] Ang, S.L. et al. The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins. Development 119, 1301-1315 (1993).

[0597] Ang, S.L. & Rossant, J. HNF-3 beta is essential for node and notochord formation in mouse development. Cell 78, 561-574 (1994).

[0598] Aoki, T. O., Mathieu, J., Saint-Etienne, L., Rebagliati, M. R., Peyrieras, N., and Rosa, F. M. (2002). Regulation of nodal signalling and mesendoderm formation by TARAM-A, a TGFbeta-related type I receptor. Dev Biol *241*, 273-288.

[0599] Arnold, S.J. et al. Brachyury is a target gene of the Wnt/beta-catenin signaling pathway. Mech Dev 91, 249-258 (2000).

WHAT IS CLAIMED IS:

1. A method of producing mesendoderm cells, the method comprising the steps of:
obtaining a cell population comprising human pluripotent stem cells;
providing the human pluripotent stem cells with a medium comprising less than 2% serum and at least one TGFβ superfamily growth factor in an amount sufficient to promote differentiation of the human pluripotent stem cells to mesendoderm cells, said mesendoderm cells being multipotent cells that can differentiate into mesoderm or definitive endoderm cells; and
allowing sufficient time for the mesendoderm cells to form, wherein the sufficient time for the mesendoderm cells to form has been determined by detecting the presence of mesendoderm cells in the cell population.

2. The method of claim 1, wherein, the TGFβ superfamily growth factor is selected from the group consisting of Nodal, activin A and activin B.

3. The method of claim 2, wherein the TGFβ superfamily growth factor is activin A.

4. The method of claim 3, wherein at least 10 ng/ml activin A is provided.

5. The method of claim 3, wherein at least 100 ng/ml activin A is provided.

6. The method of claim 1, wherein the human pluripotent stem cells comprise human embryonic stem cells.

7. The method of claim 1, wherein the human embryonic stem cells are derived from a tissue selected from the group consisting of the morula or the ICM of an embryo.

8. The method of claim 1 or 2 further comprising the step of providing serum to the human pluripotent stem cells.

~~9. The method of claim 1 further comprising a medium that comprises less than 2% (v/v) serum.~~

~~10~~9. The method of claim 1 or 2, wherein the expression of the marker selected from the group consisting of brachyury, FGF4 and SNAI1 is greater than the expression of the marker selected from the group consisting of OCT4, SOX17, CXCR4, FOXA2, SOX7 and SOX1 in the population of mesendoderm cells.

~~11~~10. The method of claim 1, wherein at least 15% of the pluripotent human stem cells differentiate into mesendoderm cells.

~~12~~11. The method of claim 1, wherein at least 50% of the pluripotent human stem cells differentiate into mesendoderm cells.

~~13~~12. The method of claim 1, wherein at least 90% of the pluripotent human stem cells differentiate into mesendoderm cells.

~~14~~13. The method of claim 1, wherein the said mesendoderm cells are further provided with one or more growth factors selected from the group consisting of FGF10, FGF4, FGF2 and Wnt3B.

~~15~~14. An *in vitro* method of producing human mesendoderm cells, the method comprising: obtaining a cell population comprising human pluripotent stem cells; and differentiating said cell population in a medium comprising less than 2% serum and at least one growth factor of the TGFβ superfamily, wherein the growth factor is present in the medium in an amount sufficient to promote differentiation of at least a portion of said pluripotent cells to mesendoderm.

~~16~~15. The method of claim ~~15~~14, further comprising allowing sufficient time for the population of mesendoderm cells to form.