METHODS FOR INCREASING DEFINITIVE ENDODERM PRODUCTION

SEQUENCE LISTING

[0001] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled CYTHERA_60VPC_SEQ.TXT, created September 24, 2007, which is 9 Kb in size. The information in the electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention relates to the fields of medicine and cell biology. In particular, the present invention relates to methods comprising efficient production of definitive endoderm and compositions thereof.

BACKGROUND

[0003] Human pluripotent stem cells, such as embryonic stem (ES) cells and embryonic germ (EG) cells, were first isolated in culture without fibroblast feeders in 1994 (Bongso et al., 1994) and with fibroblast feeders (Hogan, 1997). Later, Thomson, Reubinoff and Shamblott established continuous cultures of human ES and EG cells using mitotically inactivated mouse feeder layers (Reubinoff et al., 2000; Shamblott et al., 1998; Thomson et al., 1998).

[0004] Two properties that make human embryonic stem cells (hESCs) uniquely suited to cell therapy applications are pluripotence and the ability to maintain these cells in culture for prolonged periods. Pluripotency is defined by the ability of hESCs to differentiate to derivatives of all three (3) primary germ layers (endoderm, mesoderm, ectoderm) which, in turn, form all somatic cell types of the mature organism in addition to extraembryonic tissues (e.g. placenta) and germ cells. Owing to the large variety of cell types that may arise in differentiating hESC cultures, the vast majority of cell types are produced at very low efficiencies. Hence, improving the efficiency of directed differentiation of hESCs, or conversion of the hESCs to various differentiated derivatives, is advantageous.

SUMMARY OF THE INVENTION

[0005] In one embodiment of the invention, there is provided an *in vitro* method for increasing the definitive endoderm production by contacting an agent with a human embryonic stem cell (hESC) culture in a first medium, wherein the culture comprises at least one E-cadherin expressing cell, and wherein the agent selectively binds to E-cadherin on the E-cadherin expressing cell and inhibits adhesion of the E-cadherin expressing cell to another cell; and differentiating the hESC culture in a second medium comprising a growth factor of the Nodal/Activin subgroup of the TGFβ superfamily, thereby increasing definitive endoderm (DE) production.

[0006] In another embodiment of the invention, there is provided a method for identifying an agent capable of increasing production of a cell derived from a human embryonic stem cell (hESC) by contacting a hESC in the presence of an agent in a culture medium, wherein the agent binds to extracellular calcium ions in the medium; differentiating the hESC culture in the culture; measuring production of the differentiated cell in the presence of the agent, wherein production of the differentiated cell in the presence of the agent is increased as compared to production of the differentiated cell in the absence of the agent, thereby indicating an agent capable of increasing production of a human embryonic derived cell.

[0007] Still, in another embodiment of the invention, there is provided an *in vitro* composition containing an antagonist specifically binding to an E-cadherin expressing cell, wherein the cell comprises a human embryonic stem cell (hESC), and wherein binding of the antagonist to the cell inhibits cellular adhesion of the E-cadherin expressing cell.

[0008] In another embodiment of the invention, there is provided a cell culture containing calcium binding agent and an E-cadherin expressing cell in a culture medium, wherein the cell comprises a human embryonic stem cell (hESC), and wherein the agent binds to extracellular calcium ions in the culture medium.

[0009] In still another embodiment of the invention, there is provided a method of identifying an E-cadherin agonist or antagonist by providing a peptide library based on hESCs and an E-cadherin peptide; screening said peptide library for agents having high affinity binding to the E-cadherin peptide; and selecting a member of the peptide library binding to the E-cadherin peptide wherein the affinity of the member is equivalent or higher than that of a native

homotypic E-cadherin peptide.

[0010] Other aspects of the present invention are set forth in the numbered paragraphs below:

[0011] 1. An *in vitro* method for increasing definitive endoderm production comprising providing an agent to a cell culture comprising E-cadherin-expressing human embryonic stem cells, thereby inhibiting adhesion of the E-cadherin-expressing cells to each other; and differentiating said E-cadherin-expressing human embryonic stem cells by contacting said cells with a medium comprising a growth factor of the Nodal/Activin subgroup of the TGFβ superfamily of growth factors, thereby increasing the production of definitive endoderm.

[0012] 2. The method of paragraph 1, wherein the agent binds E-cadherin.

[0013] 3. The method of paragraph 2, wherein the agent is an antagonist of E-cadherin.

[0014] 4. The method of paragraph 3, wherein the antagonist is a polyclonal or a monoclonal E-cadherin antibody. wherein the agent is a peptide or peptide analog.

[0015] 5. The method of paragraph 1 wherein the agent is a peptide or a peptide analog.

[0016] 6. The method of paragraph 5, wherein the peptide is an E-cadherin peptide corresponding to an E-cadherin extracellular domain at amino acid residues 600-700 of human E-cadherin (SEQ ID NO:1).

[0017] 7. The method of paragraph 1, wherein the agent is a calcium ion chelator.

[0018] 8. The method of paragraph 7, wherein the calcium ion chelator is selected from the group consisting of ethylenediaminetetraacetic acid (EDTA), ethyleneglycoltetraacetic acid (EGTA), 1,10 phenanthroline, diethylenetriaminepentaacetate (DTPA), hydroxyethylethylenediaminetriacetic acid (HEEDTA), diaminocyclohexanetetraacetic acid (CDTA), 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), and pharmaceutically acceptable salts thereof.

[0019] 9. The method of paragraph 1, wherein said E-cadherin-expressing human embryonic stem cells are provided with said agent in a first medium, and wherein said E-cadherin-expressing human embryonic stem cells are differentiated in a second medium.

[0020] 10. The method of paragraph 1, wherein said first medium is different from said second medium.

[0021] 11. A method for identifying an agent capable of increasing production of a cell

derived from a human embryonic stem cell, said method comprising providing a candidate agent to a human embryonic stem cell culture; differentiating the human embryonic stem cell in a culture medium comprising a differentiation factor known to be capable of promoting the differentiation of said human embryonic stem cells; and determining whether the candidate agent increases the production of cells differentiated from said human embryonic stem cells by comparing the production of differentiated cells in said cell culture provided with the candidate agent to the production of differentiated cells in a human embryonic stem cell culture that has not been provided with said candidate agent but has been treated with the same differentiation factor as the cell culture provided with the candidate agent, wherein greater production of differentiated cells in the cell culture provided with the candidate agent as compared to the production of differentiated cells in the cell culture not provided with the candidate agent indicates that the candidate agent increases the production of a cell derived from a human embryonic cell.

[0022] 12. The method of paragraph 11, wherein the differentiation factor is a growth factor of the Nodal/Activin subgroup of the TGFβ superfamily.

[0023] 13. The method of paragraph 11, wherein the differentiated cell is a definitive endoderm cell or derivative thereof.

[0024] 14. The method of paragraph 11, wherein the agent is an antagonist of human E-cadherin.

[0025] 15. The method of paragraph 11, wherein the agent is a synthetic compound.

[0026] 16. The method of paragraph 15, wherein the agent is a natural product.

[0027] 17. An *in vitro* composition comprising an antagonist of E-cadherin specifically bound to an E-cadherin-expressing human embryonic stem cells, wherein the binding of the antagonist inhibits adhesion between said embryonic stem cells.

[0028] 18. The composition of paragraph 17, wherein the antagonist is a polyclonal or a monoclonal E-cadherin antibody.

[0029] 19. The composition of paragraph 17, wherein at least 10% of the human embryonic stem cells are not adhered to other human embryonic stem cells.

[0030] 20. The composition of paragraph 17, wherein at least 50% of the human embryonic stem cells are not adhered to other human embryonic stem cells.

[0031] 21. A cell culture comprising a calcium-binding agent and E-cadherin-expressing

human embryonic stem cells in a culture medium, wherein the calcium-binding agent is bound to calcium ions in the culture medium, thereby inhibiting adhesion between said embryonic stem cells.

[0032] 22. The cell culture of paragraph 19, wherein the calcium binding agent is selected from the group consisting of ethylenediaminetetraacetic acid (EDTA), ethyleneglycoltetraacetic acid (EGTA), 1,10 phenanthroline, diethylenetriaminepentaacetate (DTPA), hydroxyethylethylenediaminetriacetic acid (HEEDTA), diaminocyclohexanetetraacetic acid (CDTA), 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), and pharmaceutically acceptable salts thereof.

[0033] 23. The composition of paragraph 21, wherein at least 10% of the human embryonic stem cells are not adhered to other human embryonic stem cells.

[0034] 24. The composition of paragraph 21, wherein at least 50% of the human embryonic stem cells are not adhered to other human embryonic stem cells.

[0035] 25. A method of identifying an E-cadherin agonist or antagonist comprising providing a peptide library of peptides derived from hESCs and an E-cadherin peptide; screening said peptide library for peptides having high affinity binding to the E-cadherin peptide; and selecting a member of the peptide library binding to the E-cadherin peptide wherein the affinity of the member is equivalent to or higher than that of a native homotypic E-cadherin peptide.

[0036] 26. An *in vitro* method for increasing definitive endoderm (DE) production comprising contacting an agent with a human embryonic stem cell (hESC) culture in a first medium, wherein the culture comprises at least one E-cadherin expressing cell, and wherein the agent selectively binds to E-cadherin on the E-cadherin expressing cell and inhibits contact of the E-cadherin expressing cell to another cell; and differentiating the hESC culture in a second medium comprising a growth factor of the Nodal/Activin subgroup of the TGFβ superfamily, thereby increasing DE production.

[0037] 27. The method of paragraph 26, wherein the agent is an agonist or an antagonist.

[0038] 28. The method of paragraph 26, wherein the agent is a peptide or peptide analog.

[0039] 29. The method of paragraph 27, wherein the antagonist is a polyclonal or a monoclonal E-cadherin antibody.

[0040] 30. The method of paragraph 28, wherein the peptide is an E-cadherin peptide

corresponding to an E-cadherin extracellular domain at amino acid residues 600-700 of human E-cadherin (SEQ ID NO:1).

[0041] 31. A method for identifying an agent capable of increasing production of a cell derived from a human embryonic stem cell (hESC) comprising contacting a hESC in the presence of an agent in a culture medium, wherein the agent binds to extracellular calcium ions in the medium; differentiating the hES cells in the culture medium; and measuring production of the differentiated cell in the presence of the agent, wherein production of the differentiated cell in the presence of the agent is increased as compared to production of the differentiated cell in the absence of the agent, thereby indicating an agent capable of increasing production of a differentiated cell derived from a human embryonic cell.

[0042] 32. The method of paragraph 31, wherein the culture medium further comprises a growth factor of the Nodal/Activin subgroup of the TGFβ superfamily.

[0043] 33. The method of paragraph 31, wherein the differentiated cell is a definitive endoderm cell or derivative thereof.

[0044] 34. The method of paragraph 31, wherein the agent is a calcium ion chelator.

[0045] 35. The method of paragraph 34, wherein the calcium ion chelator is selected from the group consisting of ethylenediaminetetraacetic acid (EDTA), Ethyleneglycoltetraacetic acid (EGTA), Diethylenetriaminepentaacetate (DTPA), Hydroxyethylethylenediaminetriacetic acid (HEEDTA), Diaminocyclohexanetetraacetic acid (CDTA), 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), and pharmaceutically acceptable salts thereof.

[0046] 36. An *in vitro* composition comprising an antagonist specifically binding to an E-cadherin expressing cell, wherein the cell comprises a human embryonic stem cell (hESC), and wherein binding of the antagonist to the cell inhibits cellular adhesion of the E-cadherin expressing cell.

[0047] 37. The composition of paragraph 36, wherein the antagonist is a polyclonal or a monoclonal E-cadherin antibody.

[0048] 38. A cell culture comprising a calcium binding agent and an E-cadherin expressing cell in a culture medium, wherein the cell comprises a human embryonic stem cell (hESC), and wherein the agent binds to extracellular calcium ions in the culture medium.

[0049] 39. The cell culture of paragraph 38, wherein the calcium binding agent is selected

from the group consisting of ethylenediaminetetraacetic acid (EDTA), Ethyleneglycoltetraacetic acid (EGTA), Diethylenetriaminepentaacetate (DTPA), Hydroxyethylethylenediaminetriacetic acid (HEEDTA), Diaminocyclohexanetetraacetic acid (CDTA), 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), and pharmaceutically acceptable salts thereof.

[0050] 40. A method of identifying an E-cadherin agonist or antagonist comprising providing a peptide library based on hESCs and an E-cadherin peptide; screening said peptide library for agents having high affinity binding to the E-cadherin peptide; selecting a member of the peptide library binding to the E-cadherin peptide wherein the affinity of the member is equivalent or higher than that of a native homotypic E-cadherin peptide.

[0051] Additional embodiments of the present invention may also be found in United States Provisional Patent Application No. 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 11/165,305, entitled METHODS FOR IDENTIFYING FACTORS FOR DIFFERENTIATING DEFINITIVE ENDODERM, filed June 23, 2005; and U.S. Provisional Patent Application No. 60/736,598, entitled MARKERS OF DEFINITIVE ENDODERM, filed November 14, 2005.

[0052] These and other aspects of the invention, as well as various advantages and utilities, will be more apparent with reference to the detailed description of the preferred embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

[0053] Figure 1 is the deduced amino acid sequence of human E-cadherin. Signature E-cadherin motifs are bolded and underlined. The first amino acid of the mature protein is underlined twice.

[0054] Figures 2A- C are flow cytometry dot plots of hESC-derived cells that have been

treated to differentiate to definitive endoderm cells post-treatment with anti-human E-cadherin (5μg/mL) and labeled using a fluorescently conjugated CXCR4 antibody. Figures 2A, B & C (top left quadrant) show the proportion of cells that are CXCR4 positive following analysis.

[0055] Figures 3A-D are bar charts showing the mRNA levels of certain markers as detected by QPCR in hESC derived cells that have been treated to differentiate to definitive endoderm cells along with treatment with anti-human E-cadherin. Specifically shown are the mRNA levels of CXCR4 (FIG. 3A), Cerberus (CER; FIG. 3B), NANOG (FIG. 3C), and OCT4 (FIG. 3D). The abbreviations are indicated as follows: 1d and 3d – days 1 and 3, respectively; no AB – no anti-human E-cadherin antibody.

[0056] Figures 4A-C are bar charts showing the mRNA levels of certain markers as detected by QPCR in hESC derived cells that have been treated to differentiate to definitive endoderm cells along with treatment with EDTA. Specifically shown are the mRNA levels of NODAL (FIG. 4A), Brachyury (BRACH; FIG. 4B) and Cerberus (CER; FIG. 4C). The abbreviations are indicated as follows: ESC – embryonic stem cell; $PBS^{-/-}$ - PBS with no calcium and no magnesium; $PBS^{+/+}$ - PBS with calcium and magnesium; A100 – 100 ng/ml activin A; and W25 – 25 ng/ml Wnt3a.

DETAILED DESCRIPTION OF THE INVENTION

[0057] 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., *Curr Opin Genet Dev* 11, 384-392 (2001); Schoenwolf and Smith, *Methods Mol Biol* 135, 113-125 (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, *Trends Genet* 16, 124-130 (2000); Kimelman and Griffin, *Curr Opin Genet Dev* 10, 350-356 (2000); Tremblay et al., *Development* 127, 3079-3090 (2000); Wells and Melton, *Annu Rev Cell Dev*

Biol 15, 393-410 (1999); Wells and Melton, *Development* 127, 1563-1572 (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.

[0058] 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. 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.

[0059] While not intending to be bound by any particular theory, it is believed that E-cadherin causes hESCs to be tightly associated thereby inhibiting efficient directed differentiation of the cells *in vitro*. Accordingly, certain aspects of the present invention relate to agents which bind to E-cadherin on at least one E-cadherin expressing cell, for example, an hESC. In some embodiments, the agents bind selectively and/or specifically to E-cadherin. It is believed that the binding of E-cadherin decreases cellular adhesion and/or results in cell signaling events, thereby increasing the efficiency of directed differentiation of definitive endoderm (DE) derived from the hESCs. Other aspects of the present invention relate to methods for identifying agents which selectively or specifically bind to E-cadherin and/or an E-cadherin expressing cell, and which binding increases the efficiency of directed differentiation of DE from hESCs.

[0060] Although the following description is directed to a preferred embodiment of the present invention, namely, compositions and methods for increasing the efficiency of DE production from hESCs, it should be understood that this description is illustrative only and is not intended to limit the scope of the present invention. Thus, in its broadest sense, the present invention relates to the discovery that agents (e.g., antagonists), which disrupt epithelial interactions and facilitate differentiation. For example, antagonists that selectively and specifically bind to E-cadherin, modulate cellular adhesion by inhibiting E-cadherin homophilic or heterophilic cell-to-cell contacts, and increase differentiation efficiency (e.g., increase

differentiation efficiency of definitive endoderm).

[0061] The adhesive interactions of cells with other cells (homotypic and heterotypic) and between cells and the extracellular matrix play critical roles in a wide variety of processes including, for example, regulation of developmental processes, modulation of the immune system, and tumor progression and metastasis. Cellular adhesion is the binding of a cell to another cell (homotypic and heterotypic) or to a surface or matrix. Cellular adhesion is regulated by specific adhesion molecules, which transduce information from the extracellular to the intracellular matrix. e.g., cadherins that interact with molecules on the opposing cell or surface. Such adhesion molecules are also termed "receptors" and the molecules they recognize are termed "ligands" (and sometimes "counter-receptors"). Therefore, the study of cell adhesion involves cell adhesion proteins and the molecules that they bind to.

[0062] At least three families of adhesion molecules mediate these interactions: the integrins, the cadherins and the selectins. In general, adhesion molecules are transmembrane proteins which contain an extracellular domain for interacting with an extracellular matrix or cellular component, a transmembrane domain spanning the cell membrane and a cytoplasmic domain for interacting with one or more cytoskeletal components.

[0063] The integrins represent one of the best characterized families of adhesion receptors. Integrins are glycoprotein heterodimers which contain a noncovalently-associated α and β subunit. There are at least fourteen known α subunits and eight known β subunits which can pair to form at least twenty different integrin molecules. Still, several distinct integrin α chains are capable of pairing with one type of β chain to form a β chain subfamily.

[0064] Selectins are a family of transmembrane molecules, expressed on the surface of leukocytes and activated endothelial cells. Selectins contain an N-terminal extracellular domain with structural homology to calcium-dependent lectins, followed by a domain homologous to epidermal growth factor, and two to nine consensus repeats (CR) similar to sequences found in complement regulatory proteins. Each of these adhesion receptors is inserted via a hydrophobic transmembrane domain and possesses a short cytoplasmic tail.

[0065] The cadherins constitute a superfamily that share a basic structure. They play an important role in the establishment and maintenance of intercellular connections between cells of the same type (homotypic; reviewed in Geiger B. et al. (1992) *Annual Review of Cell Biology*

8:307; Kemler R. (1993) *Trends in Gastroenterology* 9:317; Takeichi M. (1990) *Annual Review of Biochem.* 59:237; Takeichi M. (1991) *Science* 251: 1451; Bussemakers, M. et al (1993) Mol. *Biol. Rep.* 17 (2), 123-128). The cadherins are synthesized as precursors that are cleaved during post-translational processing. The mature cadherins are single chain molecules which include relatively large extracellular domains, a single transmembrane region and a cytoplasmic tail. The members of the cadherin family share homology to each other. For example, epithelial cadherin, or E-cadherin, is a 120-kDa transmembrane glycoprotein expressed mainly on the surface of epithelial cells. Bussamakers et al. *supra* describe that the mature human E-cadherin amino acid sequence (FIG. 1; SEQ ID NO: 1) is about 76% homologous to human P-cadherin and about 67% to N-cadherin. Within the extracellular domains, characteristic sequences of four to five amino acids, LDRE and DXNDN, which are bolded/underlined in FIG.1) are well conserved among all cadherins. In particular, the sequence DXNDNXP (bolded/underlined in FIG.1), is thought to bind divalent calcium and is generally believed to be essential for cadherin function. Two additional, less well conserved domains are located proximal to the membrane. Among the classical cadherins (i.e., P-(placenta), E-(epithelial), and N-(neural) cadherin), the cytoplasmic domain contains the highest degree of homology, followed by the first extracellular, or ecto-, domain (Takeichi M. (1990) *Annual Review of Biochem.* 59:237). The cytoplasmic or intracellular domain contains a highly phosphorylated region vital to β-catenin binding and therefore to cadherin function. Beta-catenin can also bind to α-catenin, which participates in regulation of actin-containing cytoskeletal filaments. There are also various calcium binding sites throughout the amino acid sequence (Bussemakers, M. et al *supra*; Kemler R. (1993) *Trends in Gastroenterology* 9:317).

[0066] The best characterized function of E-cadherin are homotypic interactions, i.e. each class will only bind to members of the same class such as, N-cadherin will bind only to another N-cadherin molecule. Because of this specificity, groups of cells that express the same type of cadherin molecule tend to cluster and "stick" together during development, while cells expressing different types of cadherin binding molecules (heterotypic) tend to separate. It is generally believed that sequences in the EC-1 extracellular domain are necessary to mediate homotypic (i.e., cadherin-to-cadherin) binding. Swapping experiments in which part of the E-cadherin molecule is replaced with a corresponding portion of the P-cadherin molecule have been used to

identify the amino acid portions of post-translationally processed cadherin that are required for biological activity. In particular, Nose et al. (1990) report that an HAV tripeptide sequence is essential for homotypic cadherin binding. Nose et al. (1990) *Cell* 61:147. Further, Takeichi report that the amino acid residues flanking the HAV tripeptide sequence also contribute to homotypic binding specificity. (Takeichi M. (1991) *Science* 251:1451). A review of the literature indicates that research directed to understanding cadherin-mediated adhesion has focused on efforts to elucidate the mechanism underlying cadherin-mediated homotypic cell adhesion. Although homotypic E-cadherin interactions are well-characterized, little attention has been directed to studying, if any, heterotypic E-cadherin interactions. Whittard et al. showed that E-cadherin is capable of heterotypic interactions, i.e., an E-cadherin expressing cell binding to a non-E-cadherin ligand on a different, and non-E-cadherin expressing cell type (*Matrix Biol.* 2002 21(6):525-32). Whittard et al. demonstrated that E-cadherin interacts with integrins expressed on non-leukocytic- cells based on cell adhesion. Whittard et al. suggests that heterotypic interactions between E-cadherins and integrins, for example, may be more common than originally thought.

[0067] Accordingly, although not intending to be bound by any particular theory, it is believed that E-cadherin mediated binding of hESCs to each other inhibits their directed differentiation to other cell types. Improved and/or increased efficiency of directed differentiation of DE and other cell types derived from hESCs is facilitated, at least in part, by inhibiting the intercellular adhesion of hESCs. Accordingly, certain aspects of the present invention provide compositions and methods for increasing the efficiency of DE production from hESCs by inhibiting their cell-to-cell adhesions.

Definitions

[0068] In one embodiment of the invention, there is provided isolated agents which antagonize and/or inhibit *in vitro* cellular adhesion between E-cadherin expressing cells, e.g., hESCs, or matrix. In some embodiments, the agents can be homotypic and selectively and specifically bind to E-cadherin expressing cells, or they can be non-E-cadherin ligands which selectively and specifically bind to E-cadherin (heterotypic). According to one aspect of the invention, the agent is a polypeptide, functional peptide fragment or a portion of a polypeptide

that has one or more sequences related to, or derived from, the amino acid sequence of the extracellular domain of E-cadherin. For example, an E-cadherin derived peptide can bind to an E-cadherin-expressing cell (homotypic) or to an E-cadherin cognate of a non-E-cadherin expressing cell (heterotypic).

[0069] When used in connection with an E-cadherin, the term "fragment" or "portion" means any non-zero amount of the full length E-cadherin polypeptide. In preferred embodiments, the term "fragment" or "portion" means at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 11%, at least 12%, at least 13%, at least 14%, at least 15%, at least 16%, at least 17%, at least 18%, at least 19%, at least 20%, at least 21%, at least 22%, at least 23%, at least 24%, at least 25%, at least 26%, at least 27%, at least 28%, at least 29%, at least 30%, at least 31%, at least 32%, at least 33%, at least 34%, at least 35%, at least 36%, at least 37%, at least 38%, at least 39%, at least 40%, at least 41%, at least 42%, at least 43%, at least 44%, at least 45%, at least 46%, at least 47%, at least 48%, at least 49%, at least 50%, at least 51%, at least 52%, at least 53%, at least 54%, at least 55%, at least 56%, at least 57%, at least 58%, at least 59%, at least 60%, at least 61%, at least 62%, at least 63%, at least 64%, at least 65%, at least 66%, at least 67%, at least 68%, at least 69%, at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 96%, at least 97%, at least 98%, and at least 99% of the full length E-cadherin polypeptide.

[0070] As used herein, reference to a polypeptide, peptide, or functional fragment thereof, embraces peptides of the extracellular E-cadherin domain(s), as well as functionally equivalent peptide analogs of the foregoing peptide fragments. For example, an isolated E-cadherin peptide is obtained by isolating the extracellular cleavage product of E-cadherin that results following exposure of epithelial cells to trypsin in the presence of divalent calcium. Trypsin cleavage yields an approximately 80 kD fragment of E-cadherin containing a portion of the extracellular domain. Thus, this particularly preferred peptide has an amino acid sequence corresponding to the naturally occurring proteolytic cleavage site of E-cadherin.

[0071] In some embodiments, the phrase "isolated peptides" refers to a cloned expression

product of a nucleic acid or oligonucleotide, a peptide which is isolated following cleavage from a larger polypeptide or a peptide that is synthesized, e.g., using solution and/or solid phase peptide synthesis methods as disclosed in, for example, U.S. Pat. No. 5,120,830, the entire contents of which are incorporated herein by reference.

[0072] As used herein, the term "peptide analog" refers to a peptide which shares a common structural feature with the molecule to which it is deemed to be an analog. Peptide analogs include "unique fragments" which are related to, or derived from, functional domain(s) of E-cadherin, polymers of functional domain(s) or polymers of unique fragments of functional domain(s). A unique fragment of a protein or nucleic acid sequence can include a fragment which is not currently known to occur elsewhere in nature (except in allelic or allelomorphic variants). Unique fragments act as a "signature" of the gene or protein from which they are derived. A unique fragment will generally be at least about 9, 12, 15, 18, 21, 24, and 27 nucleotides, or 3, 4, 5, 6, 7, 8 and 9 amino acids in length, respectively.

[0073] In addition to the foregoing, tagged E-Cadherin peptides are encompassed in the present invention, for example, recombinant fusion protein fragments corresponding to specific amino acid regions of E-cadherin are commercially available. For example, E-cadherin peptide fragments corresponding to amino acids about 600-707 (AbCam); smaller synthetic peptides, such as S A L L L L L Q V S S W L (SEQ ID NO: 2), corresponding to amino acid residues 9-21 (MA1-06303), and the peptide P G F D A E S Y T F T V P R (SEQ ID NO: 3), corresponding to amino acid residues 30-43 (MA1-06304) from Affinity BioReagents. Also included are MA1-06301 and MA1-06302 immunogens (Affinity BioReagents), which are affinity purified ~80 kD extracellular fragments of E-cadherin derived from tryptic digestion of A-431 human vulva carcinoma cells. Antibodies to MA1-06302 detect an approximately 120 kDa E-cadherin protein. Furthermore, there is data demonstrating that cleavage of the 80-kDa extracellular domain of E-cadherin from the cell surface may provide an innate form of pathogen defense by acting as a decoy receptor. Fernanda da Silva et al., (2003) *Infect Immun.* 71(3): 1580–1583. Accordingly, commercially available E-cadherin peptides can be used in the methods describe herein. Alternatively, one of ordinary skill in the art can readily identify unique fragments by searching available computer databases of nucleic acid and protein sequences such as Genbank, (Los Alamos National Laboratories, USA), EMBL, or SWISS-PROT. A unique fragment is

particularly useful, for example, in generating monoclonal antibodies or in screening genomic DNA or cDNA libraries.

[0074] It will be appreciated by those skilled in the art that various modifications of the foregoing peptide and or peptide analogs can be made without departing from the essential nature of the invention. For example, the peptides of the invention can be specifically reactive with a cadherin, e.g., an E-cadherin, and thereby preventing E-cadherin from binding to its cognate or ligand. Accordingly, it is intended that peptides include conservative substitutions, as well as those peptides coupled to other proteins and/or matrices, e.g., coupled to a solid support (e.g., polymeric bead, microtiter plates or beads), a carrier molecule (e.g., keyhole limpet hemocyanin), a toxin (e.g., ricin) or a reporter group (e.g., a radiolabel or other tag), are embraced within the teachings of the invention. These and other methods of coupling a peptide are known and available to one of ordinary skill in the art.

[0075] As used herein, the term "functionally equivalent peptide analog" refers to a peptide analog that retains function, for example, such an analog is capable of inhibiting the binding of an E-cadherin expressing cell *in vitro* by competing with E-cadherin for binding to another cell. Functionally equivalent peptide analogs of E-cadherin are identified, for example, in *in vitro* screening assays that measure the ability of the peptide analog to inhibit E-cadherin-mediated adhesion between cells. Such assays are predictive of the ability of a molecule to inhibit this adhesion *in vivo*. Accordingly, a "functionally equivalent peptide analog" of E-cadherin includes, but is not limited to, the extracellular domain of E-cadherin, fragments of the extracellular domain and peptide analogs of the extracellular domain, provided that the peptide fragments and analogs are capable of inhibiting adhesion between at least one E-cadherin expressing cell *in vitro*. As used herein, "conservative amino acid substitution" refers to an amino acid substitution which does not alter the relative charge or size characteristics of the peptide in which the amino acid substitution is made. Conservative substitutions of amino acids include substitutions made amongst the individual amino acids within the following groups: i) M, I, L and V; ii) F, Y, and W; iii) K, R, and H; iv) A and G; v) S and T; vi) Q and N; and vii) E and D.

[0076] Additionally, one skilled in the art can review structure-function studies identifying residues in similar polypeptides that are important for activity or structure. In view of

such a comparison, the skilled artisan can predict the importance of amino acid residues in a protein that correspond to amino acid residues important for activity or structure in similar proteins. One skilled in the art may opt for chemically similar amino acid substitutions for such predicted important amino acid residues.

[0077] One skilled in the art can also analyze the three-dimensional structure and amino acid sequence in relation to that structure in similar polypeptides. In view of such information, one of ordinary skill in the art may predict the alignment of amino acid residues of a polypeptide with respect to its three dimensional structure. In certain embodiments, one skilled in the art may choose to not make radical changes to amino acid residues predicted to be on the surface of the protein, since such residues may be involved in important interactions with other molecules. Moreover, one skilled in the art may generate test variants containing a single amino acid substitution at each desired amino acid residue. The variants can then be screened using activity assays known in the art. Such variants can be used to gather information about suitable variants. For example, if one discovered that a change to a particular amino acid residue resulted in destroyed, undesirably reduced, or unsuitable activity, variants with such a change can be avoided. In other words, based on information gathered from such routine experiments, one skilled in the art can readily determine the amino acids where further substitutions should be avoided either alone or in combination with other mutations.

[0078] A number of scientific publications have been devoted to the prediction of secondary structure. See Moult, 1996, *Curr. Op. in Biotech.* 7:422-427; Chou et al, 1974, *Biochemistry* 13:222-245; Chou et al, 1974, *Biochemistry* 113:211-222; Chou et al, 1978, *Adv. Enzymol Relat. Areas Mol. Biol* 47:45-148; Chou et al, 1979, *Ann. Rev. Biochem.* 47:251-276; and Chou et al, 1979, *Biophys. J.* 26:367-384. Moreover, computer programs are currently available to assist with predicting secondary structure. One method of predicting secondary structure is based upon homology modeling. For example, two polypeptides or proteins that have a sequence identity of greater than about 30%, or similarity greater than 40% often have similar structural topologies. The recent growth of the protein structural database has provided enhanced predictability of secondary structure, including the potential number of folds within a polypeptide's or protein's structure. See Holm et al, 1999, *Nucl. Acid. Res.* 27:244-247. It has been suggested (Brenner et al, 1997, *Curr. Op. Struct. Biol.* 7:369-376) that there are a limited

number of folds in a given polypeptide or protein and that once a critical number of 5 structures have been resolved, structural prediction will become dramatically more accurate.

[0079] Still, a peptide analog includes non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or "peptidomimetics". See Fauchere, 1986, *Adv. Drug Res.* 15:29; Veber & Freidinger, 1985, *TINS* p.392; and Evans et al., 1987, *J. Med. Chem.* 30:1229, which are incorporated herein by reference for any purpose. Such compounds are often developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce a similar therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biochemical property or pharmacological activity), such as human antibody, but have one or more peptide linkages optionally replaced by a linkage selected from: -CH2-NH-, -CH2-S-, -CH2-CH2-, -CH=CH-(cis and trans), -COCH2-, -CH(OH)CH2-, and -CH2SO-, by methods well known in the art. Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) may be used in certain embodiments to generate more stable peptides. In addition, constrained peptides comprising a consensus sequence or a substantially identical consensus sequence variation may be generated by methods known in the art (Rizo & Gierasch, 1992, *Ann. Rev. Biochem.* 61:387, incorporated herein by reference for any purpose); for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.

[0080] As used herein, "a heterotypic cognate of E-cadherin" refers to a peptide or protein that is present in, or derived from a specific cell type, and which specifically recognizes and binds to E-cadherin, but is not the same cell type as the E-cadherin expressing cell. For example, Whittard et al. *supra* describe that $\alpha^E\beta_7$ integrin is an exemplary heterotypic cognate of E-cadherin. Heterotypic cognates of E-cadherin are useful as reagents in *in vitro* adhesion assays for screening molecular libraries. Such adhesion assays assess the ability of a molecule (e.g., a molecular library member) to modulate the interaction of two binding partners. Typically, the binding partners are cells which specifically bind to one another via a ligand-receptor mediated mechanism. The cell can be a cell which naturally expresses a binding partner, or can be a cell

which is transfected or otherwise genetically altered to express the binding partner.

[0081] The agent or peptide described herein can also be a "ligand", which refers to any molecule that binds to another, e.g., a soluble molecule that binds to a receptor. A ligand as encompassed herein can be a heterotypic E-cadherin cognate, a functionally equivalent peptide fragment or analog of the isolated heterotypic E-cadherin cognate, or a cell extracellularly expressing the isolated heterotypic cognate or its functionally equivalent peptide fragment or analog. Simply, at least one ligand is E-cadherin. As used herein, a cell expressing E-cadherin on the cell surface also functions as a "receptor E-cadherin". Hence, a ligand as described herein binds to the receptor E-cadherin. In a preferred embodiment, the receptor binds selectively and/or specifically.

[0082] In some embodiments, the agent described herein includes a nucleic acid and its deduced amino acid sequence. For example, a nucleic acid which corresponds to the extracellular domain of E-cadherin, may be used in computer-based modeling systems to predict the secondary and tertiary structure of the extracellular domain. Such computer-based systems are well known to those of ordinary skill in the art of rational drug design. Based upon the tertiary structure of a receptor protein, it is often possible to identify a binding region which is involved in its biological activity. From this information, peptides or other compounds which include or mimic this structure and/or which are capable of binding to it can be rationally designed. In this way, new compounds may be designed which mimic the activity of the receptor or ligand or which will act as competitive inhibitors of the receptor or ligand.

Production of Definitive Endoderm

[0083] In some processes, differentiation to definitive endoderm is achieved by providing to the stem cell culture a growth factor of the TGFβ superfamily in an amount sufficient to promote differentiation to definitive endoderm. Growth factors of the TGFβ superfamily which are useful for the production of definitive endoderm are selected from the Nodal/Activin or BMP subgroups. In some preferred differentiation processes, the growth factor is selected from the group consisting of Nodal, activin A and activin B. Additionally, the growth factor Wnt3a and other Wnt family members are useful for the production of definitive endoderm cells. In certain differentiation processes, combinations of any of the above-mentioned growth factors can be

used.

[0084] Also, as used herein, "exogenously added," compounds such as growth factors, differentiation factors, and the like, in the context of cultures or conditioned media, refers to growth factors that are added to the cultures or media to supplement any compounds or growth factors that may already be present in the culture or media. For example, growth factors of the invention include but are not limited to a "retinoid", which refers to retinol, retinal or retinoic acid as well as derivatives of any of these compounds. In a preferred embodiment, the retinoid is retinoic acid. A growth factor also includes a "FGF family growth factor," "a fibroblast growth factor" or "member of the fibroblast growth factor family" is meant an FGF selected from the group consisting of FGF1, FGF2, FGF3, FGF4, FGF5, FGF6, FGF7, FGF8, FGF9, FGF10, FGF11, FGF12, FGF13, FGF14, FGF15, FGF16, FGF17, FGF18, FGF19, FGF20, FGF21, FGF22 and FGF23. In some embodiments, "FGF family growth factor," "a fibroblast growth factor" or "member of the fibroblast growth factor family" means any growth factor having homology and/or function similar to a known member of the fibroblast growth factor family.

[0085] With respect to some of the processes for the differentiation of pluripotent stem cells to definitive endoderm cells, the above-mentioned growth factors are provided to the cells so that the growth factors are present in the cultures at concentrations sufficient to promote differentiation of at least a portion of the stem cells to definitive endoderm cells. In some processes, the above-mentioned growth factors are present in the cell culture at a concentration of at least about 5 ng/ml, at least about 10 ng/ml, at least about 25 ng/ml, at least about 50 ng/ml, at least about 75 ng/ml, at least about 100 ng/ml, at least about 200 ng/ml, at least about 300 ng/ml, at least about 400 ng/ml, at least about 500 ng/ml, at least about 1000 ng/ml, at least about 2000 ng/ml, at least about 3000 ng/ml, at least about 4000 ng/ml, at least about 5000 ng/ml or more than about 5000 ng/ml.

[0086] In certain processes for the differentiation of pluripotent stem cells to definitive endoderm cells, the above-mentioned growth factors are removed from the cell culture subsequent to their addition. For example, the growth factors can be removed within about one day, about two days, about three days, about four days, about five days, about six days, about seven days, about eight days, about nine days or about ten days after their addition. In a preferred process, the growth factors are removed about four days after their addition.

[0087] Cultures of definitive endoderm cells can be produced from embryonic stem cells 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 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. In other embodiments, the definitive endoderm cells are grown in the absence of B27.

[0088] In some processes for differentiating human definitive endoderm cells from hESCs, differentiation is initiated in the absence of serum and in the absence of insulin and/or insulin-like growth factor. During the course of differentiation, the serum concentration may be gradually increased in order to promote adequate cell survival. In preferred embodiments, differentiation of hESCs to definitive endoderm cells is initiated in the absence of serum and in the absence of any supplement comprising insulin or insulin-like growth factors. The absence of serum and absence of supplement comprising insulin or insulin-like growth factors is maintained for about 1 to about 2 days, after which, serum is gradually added to the differentiating cell culture over the course of differentiation. In preferred embodiments, the concentration of serum does not exceed about 2% during the course of differentiation.

[0089] Definitive endoderm cell cultures and cell populations as well as detailed processes for the production of definitive endoderm cells from embryonic stem cells are further described in U.S. Patent Application Number 11/021,618, entitled DEFINITIVE ENDODERM, filed December 23, 2004 and U.S. Patent Application Number 11/317,387, entitled EXPANSION OF DEFINITIVE ENDODERM CELLS, filed December 22, 2005, the disclosures of which are incorporated herein by reference in their entireties.

Methods for increasing definitive endoderm production and/or increasing production of hESC-derived cell population

[0090] As used herein, "derived from hESCs," "produced from hESCs," "differentiated from hESCs" and/or "hESC-derived cell population" or equivalent expressions refer to the production of a differentiated cell type from hESCs *in vitro* rather than *in vivo*.

[0091] One embodiment of the present invention relates to an *in vitro* method for increasing definitive endoderm production by providing an agent to a cell culture comprising E-cadherin-expressing human embryonic stem cells. In such embodiments, the agent inhibits adhesion of the E-cadherin-expressing cells to each other. Under such conditions, the E-cadherin-expressing human embryonic stem cells are then differentiated by contacting the cells with a medium comprising a growth factor of the Nodal/Activin subgroup of the TGFβ superfamily of growth factors. As compared to identically or similarly differentiated human embryonic cell cultures that have not been contacted with the agent, the production of definitive endoderm will have been increased in the cultures having been contacted with the agent.

[0092] In some embodiments, the agent that is used binds to E-cadherin. As described further below, in certain preferred embodiments, the agent selectively and/or specifically binds to E-cadherin and acts so as to antagonize the E-cadherin adhesion function. In other preferred embodiments, the E-cadherin antagonist is a peptide or a peptide analog. For example, the peptide can be an E-cadherin peptide corresponding to an E-cadherin extracellular domain at amino acid residues 600-700 of human E-cadherin (SEQ ID NO:1).

[0093] Other embodiments of the present invention relate to *in vitro* methods for increasing the production of definitive endoderm as described above wherein the provided agent is a metal ion chelator. In a preferred embodiment, the metal ion chelator is a calcium ion chelator. Calcium ion chelators are able to bind calcium in a selective way. That is, the calcium chelators have higher affinity for calcium than for any other metal ions. Binding to calcium is performed through carboxylic groups, so it can be affected by pH, other ions or co-ordination to proteins, lipids, etc. The process is a reversible equilibrium. The calcium, chelator and complex concentrations are related by a dissociation constant, $K_d = ([Ca^{2+}] \cdot [Chelator]) / [\{Chelator\text{-}Ca\}_{complex}]$. When the K_d is very low, it is a high-affinity chelator (i.e., the chelator has a high

tendency to bind calcium). If the K_d is high (μM or higher), it is a low-affinity chelator. Preferred calcium chelators include, ethylenediaminetetraacetic acid (EDTA) and ethyleneglycoltetraacetic acid (EGTA); however, it will be appreciated that other metal ion chelators, such as those selected from the group consisting of 1,10 phenanthroline, diethylenetriaminepentaacetate (DTPA), hydroxyethylethylenediaminetriacetic acid (HEEDTA), diaminocyclohexanetetraacetic acid (CDTA), 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) can also be used. Furthermore, combinations of such chelators and salts of such chelators can also be used. Example 3 describes directed differentiation of DE from hESCs in the presence of EDTA. The presence of EDTA in the culture media reduces the concentration of Ca^{2+} ions present. More selective chelation of Ca^{2+} can be achieved by using EGTA. Similar methods of chelating or binding or capturing calcium ions, extra- or intra-cellularly, using other calcium chelators is well known to one skilled in the art. High-affinity chelators trap calcium efficiently and the calcium chelators described herein are only illustrative examples and are not meant to be limiting. Binding of calcium disrupts E-cadherin function, which decreases cell-cell binding and/or affects cell signaling.

[0094] In some embodiments of the present invention, the step of providing the agent to the E-cadherin-expressing human embryonic stem cells and the differentiation step can be performed in the same medium. However, in preferred embodiments, the E-cadherin-expressing human embryonic stem cells are provided with the agent in a first medium and then differentiated in a second medium. In some embodiments, the first medium is different from the second medium. In other embodiments, the first medium is the same as the second medium except that the second medium does not comprise exogenously added agent.

[0095] In still other embodiments of the invention, there is provided an *in vitro* method for increasing definitive endoderm production and/or increasing production of a cell derived from a human embryonic stem cell (hESC) by contacting an agent with a human embryonic stem cell (hESC) culture in a first medium, wherein the culture comprises at least one E-cadherin expressing cell, and wherein the agent selectively binds to E-cadherin on the E-cadherin expressing cell and inhibits adhesion of the E-cadherin expressing cell to another cell; and differentiating the hESC culture in a second medium comprising a growth factor of the Nodal/Activin subgroup of the TGFβ superfamily, thereby increasing definitive endoderm

production and/or production of a cell derived from a human embryonic stem cell (hESC).

[0096] Accordingly, in one aspect of the invention, the agent capable of selectively binding or specifically binding to an E-cadherin expressing cell is an E-cadherin polyclonal or a monoclonal antibody. As used herein, the term "selectively binds" or "specifically binds," when used in reference to an antibody and an antigen or epitopic portion thereof, means that the antibody and the antigen (or epitope) have a dissociation constant of at least about 1×10^{-7}, generally at least about 1×10^{-8}, usually at least about 1×10^{-9}, and particularly at least about 1×10^{-10} or less. Methods for identifying and selecting an antibody having a desired specificity are well known and routine in the art (see, for example, Harlow and Lane, "Antibodies: A Laboratory Manual" (Cold Spring Harbor Pub. 1988), which is incorporated herein by reference.

[0097] Methods for producing antibodies those can selectively and specifically bind to one or more E-cadherin polypeptide epitopes, particularly epitopes unique to an E-cadherin polypeptide or peptide (e.g., signature or unique sequences), are disclosed herein or are otherwise well known and routine in the art. Such antibodies can be polyclonal antibodies or monoclonal antibodies (mAbs), and can be humanized or chimeric antibodies, single chain antibodies, anti idiotypic antibodies, and epitope-binding fragments of any of the above, including, for example, Fab fragments, F(ab')2 fragments or fragments produced by a Fab expression library. Such antibodies can be used, for example, in the detection of E-cadherin polypeptides, or mutant E-cadherin polypeptides, including variant E-cadherin polypeptides, which can be in a biological sample, or can be used for the inhibition of abnormal E-cadherin activity. Thus, the antibodies can be utilized to inhibit E-cadherin binding and decrease or inhibit cellular adhesion and thereby increase directed differentiation of hESCs.

[0098] For the production of antibodies that bind to E-cadherin, including an E-cadherin variant or E-cadherin mutant, various host animals can be immunized by injection with an E-cadherin polypeptide, mutant polypeptide, variant, or a portion thereof. Such host animals can include but are not limited to, rabbits, mice, and rats. Various adjuvants can be used to increase the immunological response, depending on the host species, including, but not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG

(Bacillus Calmette-Guerin) or Corynebacterium parvum.

[0099] Antibodies that bind to an E-cadherin polypeptide, or peptide portion thereof, or to a variant or mutant peptide, of the invention can be prepared using an intact polypeptide or fragments containing small peptides of interest as the immunizing antigen, e.g., LDRE (amino acids 220-223 of SEQ ID NO: 1), DXNDN (amino acids 367-371 of SEQ ID NO: 1), DXNDNXP (amino acids 367-373 of SEQ ID NO: 1), HAV, and the like (FIG.1). The polypeptide or a peptide used to immunize an animal can be derived from translated cDNA or chemical synthesis, and can be conjugated to a carrier protein, if desired. Such commonly used carriers that can be chemically coupled to the peptide include keyhole limpet hemocyanin, thyroglobulin, bovine serum albumin, tetanus toxoid and others as described above or otherwise known in the art. The coupled polypeptide or peptide is then used to immunize the animal and antiserum can be collected. If desired, polyclonal or monoclonal antibodies can be purified, for example, by binding to and elution from a matrix to which the polypeptide or a peptide to which the antibodies were raised is bound. Any of various techniques commonly used in immunology for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies, can be used (see for example, Coligan, et al., Unit 9, Current Protocols in Immunology, Wiley Interscience, 1991, which is incorporated herein by reference).

[00100] Anti-idiotype technology can be used to produce monoclonal antibodies that mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody will have a binding domain in the hyper variable region that is the image of the epitope bound by the first monoclonal antibody. Antibodies of the invention include polyclonal antibodies, monoclonal antibodies, and fragments of polyclonal and monoclonal antibodies that specifically bind to a mutant E-cadherin polypeptide or peptide portion thereof.

[00101] The preparation of polyclonal antibodies is well known to those skilled in the art (see, for example, Green et al., Production of Polyclonal Antisera, in Immunochemical Protocols (Manson, ed.), pages 1-5 (Humana Press 1992); Coligan et al., Production of Polyclonal Antisera in Rabbits, Rats, Mice and Hamsters, in Current Protocols in Immunology, section 2.4.1 (1992), which are incorporated herein by reference). The preparation of monoclonal antibodies likewise is conventional (see, for example, Kohler and Milstein, *Nature*, 256:495, 1975, which is incorporated herein by reference; see, also Coligan et al., *supra*, sections 2.5.1-2.6.7). Briefly,

monoclonal antibodies can be obtained by injecting mice with a composition comprising an antigen, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B lymphocytes, fusing the B lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures.

[00102] Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography (see Coligan et al., sections 2.7.1-2.7.12 and sections 2.9.1-2.9.3; Barnes et al., Purification of Immunoglobulin G (IgG), in *Methods in Molecular Biology*, Vol. 10, pages 79-104 (Humana Press 1992)). Methods of *in vitro* and *in vivo* multiplication of hybridoma cells expressing monoclonal antibodies are well-known to those skilled in the art. Multiplication in vitro can be carried out in suitable culture media such as Dulbecco's Modified Eagle Medium (DMEM) or RPMI 1640 medium, optionally supplemented by a mammalian serum such as fetal calf serum or trace elements and growth-sustaining supplements such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages. Production *in vitro* provides relatively pure antibody preparations and allows scale-up to yield large amounts of the desired antibodies. Large scale hybridoma cultivation can be carried out by homogenous suspension culture in an airlift reactor, in a continuous stirrer reactor, or in immobilized or entrapped cell culture. Multiplication *in vivo* can be carried out by injecting cell clones into mammals histocompatible with the parent cells, e.g., syngeneic mice, to cause growth of antibody-producing tumors. Optionally, the animals are primed with a hydrocarbon, especially oils such as pristane tetramethylpentadecane prior to injection. After one to three weeks, the desired monoclonal antibody is recovered from the body fluid of the animal.

[00103] In some embodiments, antibodies of the present invention can be derived from subhuman primate antibodies. General techniques for raising therapeutically useful antibodies in baboons can be found, for example, in Goldenberg et al., International Application Publication No. WO 91/11465, 1991; Losman et al., *Int. J. Cancer*, 46:310, 1990, which are incorporated herein by reference.

[00104] An E-cadherin antibody can also be derived from a "humanized" monoclonal

antibody. Humanized monoclonal antibodies are produced by transferring mouse complementarity determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain, and then substituting human residues in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, by Orlandi et al., *Proc. Natl. Acad. Sci. USA* 86:3833, 1989, which is incorporated herein by reference. Techniques for producing humanized monoclonal antibodies are described, for example, by Jones et al., *Nature*, 321:522, 1986; Riechmann et al., *Nature* 332:323, 1988; Verhoeyen et al., Science 239:1534, 1988; Carter et al., *Proc. Natl. Acad. Sci.* USA, 89:4285, 1992; Sandhu, *Crit. Rev. Biotech.* 12:437, 1992; and *Singer et al., J. Immunol.* 150:2844, 1993, which are incorporated herein by reference.

[00105] Antibodies of the invention also can be derived from human antibody fragments isolated from a combinatorial immunoglobulin library (see, for example, Barbas et al., Methods: *A Companion to Methods in Enzymology*, Vol. 2, page 119, 1991; Winter et al., Ann. Rev. Immunol. 12:433, 1994, which are incorporated herein by reference). Cloning and expression vectors that are useful for producing a human immunoglobulin phage library can be obtained.

[00106] In addition, antibodies of the present invention can be derived from a human monoclonal antibody. Such antibodies are obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain loci are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described by Green et al., *Nature Genet.*, 7:13 (1994); Lonberg et al., *Nature*, 368:856 (1994); Taylor et al., *Int. Immunol.*, 6:579 (1994), each of which is incorporated herein by reference.

[00107] Antibody fragments of the invention can be prepared by proteolytic hydrolysis of an antibody or by expression in *E. coli* of DNA encoding the fragment. Antibody fragments can

be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted $F(ab')_2$. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647, and references contained therein, each of which in incorporated herein by reference (see, also, Nisonhoff et al., *Arch. Biochem. Biophys*,. 89:230, 1960; Porter, *Biochem. J.* 73:119, 1959; Edelman et al., Meth. Enzymol. 1:422, 1967; and Coligan et al., at sections 2.8.1-2.8.10 and 2.10.1-2.10.4). Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques can also be used, provided the fragments bind to the antigen that is recognized by the intact antibody.

[00108] Fv fragments comprise an association of VH and VL chains, for example, which can be noncovalent (see Inbar et al., *Proc. Natl. Acad. Sci. USA* 69:2659, 1972). The variable chains also can be linked by an intermolecular disulfide bond, can be crosslinked by a chemical such as glutaraldehyde (Sandhu, supra, 1992), or Fv fragments comprising VH and VL chains can be connected by a peptide linker. These single chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the VH and VL domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as E. coli. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by Whitlow et al., Methods: A Companion to Meth. Enzymol., 2:97, 1991; Bird et al., *Science* 242:423, 1988; Ladner et al., U.S. Patent No. 4,946,778; Pack et al., *BioTechnology* 11:1271, 1993; and Sandhu, supra, 1992).

[00109] Another form of an antibody fragment is a peptide coding for a single complementarity determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region

from RNA of antibody-producing cells. See, for example, Larrick et al., Methods: *A Companion to Meth. Enzymol.*, 2:106, (1991).

[00110] The above methods are merely illustrative and other methods of antibody production are known in the art and available to one of ordinary skill in the art, which methods are embodied in the present invention.

Methods of monitoring the production of definitive endoderm and/or a hESC-derived cell population

[00111] Also provided herein, is a method of monitoring the production of definitive endoderm and/or a hESC-derived cell population. As hESCs differentiate to definitive endoderm they down regulate E-cadherin and transition from an epithelial epiblast state to a mesenchymal definitive endoderm cell (D'Amour et al. *Nat. Biotech.* **23**, 1534-1541, (2005)). 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 be 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 quantitative PCR (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 definitive endoderm as well as the lack of significant expression of marker genes characteristic of hESCs and other cell types is determined.

[00112] As described further in the Examples below, the definitive endoderm cells produced by the processes described herein express the CXCR4 and CER marker gene, thereby producing the CXCR4 and CER gene product. As explained in our previous U.S. Patent Application No. 11/021,618, the principal markers defining the early DE cell include but are not limited to FOXA2, CER, GSC, N-cadherin, CXCR4 and SOX17, and by the absence of

significant expression of certain other markers, such as SOX1, SOX7, thrombomodulin (TM), SPARC and alpha fetoprotein (AFP)[D'Amour et al. 2005 *supra*]. Other markers of definitive endoderm are MIXL1, GATA4, HNF3b, GSC, FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1.

[00113] As stated above, at least one marker of definitive endoderm is the CXCR4 gene. The CXCR4 gene encodes a cell surface chemokine receptor whose ligand is the chemoattractant SDF-1. The principal roles of the CXCR4 receptor in the adult are believed to be the migration of hematopoetic cells to the bone marrow, lymphocyte trafficking and the differentiation of various B cell and macrophage blood cell lineages [Kim, C., and Broxmeyer, H. J. *Leukocyte Biol.* 65, 6-15 (1999)]. The CXCR4 receptor also functions as a co-receptor for the entry of HIV-1 into T-cells [Feng, Y., et al. *Science*, 272, 872-877 (1996)]. In an extensive series of studies [McGrath, K.E. et al. *Dev. Biology* 213, 442-456 (1999)], the expression of the chemokine receptor CXCR4 and its unique ligand, SDF-1 [Kim, C., and Broxmyer, H., J. *Leukocyte Biol.* 65, 6-15 (1999)], were delineated during early development and adult life in the mouse. The CXCR4/SDF1 interaction in development became apparent when it was demonstrated that if either gene was disrupted in transgenic mice [Nagasawa et al. *Nature,* 382, 635-638 (1996)], Ma, Q., et al *Immunity*, 10, 463-471 (1999)] it resulted in late embryonic lethality. McGrath et al. demonstrated that CXCR4 is the most abundant chemokine receptor messenger RNA detected during early gastrulating embryos (E7.5) using a combination of RNase protection and in situ hybridization methodologies. In the gastrulating embryo, CXCR4/SDF-1 signaling appears to be mainly involved in inducing migration of primitive-streak germ layer cells and is expressed by definitive endoderm, mesoderm and extraembryonic mesoderm present at this time. In E7.2-7.8 mouse embryos, CXCR4 and alpha-fetoprotein are mutually exclusive indicating a lack of CXCR4 expression in visceral endoderm [McGrath, K.E. et al. *Dev. Biology* 213, 442-456 (1999)].

[00114] Since DE cells produced by differentiating pluripotent cells express the CXCR4 marker gene, expression of CXCR4 can be monitored in order to track the production of definitive endoderm cells. Additionally, definitive endoderm cells produced by the methods described herein express other markers of definitive endoderm including, but not limited to, SOX17, MIXL1, GATA4, HNF3b, GSC, FGF17, VWF, CALCR, FOXQ1, CER, CMKOR1 and

CRIP1. In other processes, expression of the both the CXCR4 marker gene and the OCT4 marker gene, is monitored. Additionally, because DE cells express the CXCR4 marker gene at a level higher than that of the AFP, SPARC or Thrombomodulin (TM) marker genes, the expression of these genes can also be monitored and requires no more experimentation that that described herein.

[00115] It will be appreciated that expression of CXCR4 in endodermal cells does not preclude the expression of SOX17. As such, definitive endoderm cells produced by the processes described herein will substantially express SOX17 and CXCR4 but will not substantially express AFP, TM, SPARC or PDX1.

[00116] Still, various methods can be utilized for quantifying the presence of the selected markers. For measuring the amount of a molecule that is present, a convenient method is to label a molecule with a detectable moiety, which may be fluorescent, luminescent, radioactive, enzymatically active, etc., particularly a molecule specific for binding to the parameter with high affinity. Fluorescent moieties are readily available for labeling virtually any biomolecule, structure, or cell type. Immunofluorescent moieties can be directed to bind not only to specific proteins but also specific conformations, cleavage products, or site modifications like phosphorylation. Individual peptides and proteins can be engineered to autofluoresce, e.g. by expressing them as green fluorescent protein chimeras inside cells (for a review see Jones et al. (1999) Trends Biotechnol. 17(12):477 81). Thus, antibodies can be genetically modified to provide a fluorescent dye as part of their structure. Depending upon the label chosen, parameters may be measured using other than fluorescent labels, using such immunoassay techniques as radioimmunoassay (RIA) or enzyme linked immunosorbance assay (ELISA), homogeneous enzyme immunoassays, and related non-enzymatic techniques. The quantitation of nucleic acids, especially messenger RNAs, is also of interest as a parameter. These can be measured by hybridization techniques that depend on the sequence of nucleic acid nucleotides. Techniques include polymerase chain reaction methods as well as gene array techniques. See, for example, Current Protocols in Molecular Biology, Ausubel et al., eds, John Wiley & Sons, New York, N.Y., 2000; Freeman et al. (1999) *Biotechniques* 26(1):112 225; Kawamoto et al. (1999) *Genome Res* 9(12):1305 12; and Chen et al. (1998) *Genomics* 51(3):313 24.

Compositions of human embryonic stem cells *in vitro*

[00117] One embodiment of the present invention relates to an *in vitro* composition comprising an antagonist of E-cadherin specifically bound to an E-cadherin-expressing human embryonic stem cells. In such compositions, the binding of the antagonist inhibits adhesion between the embryonic stem cells. Accordingly, compositions of the present invention include human embryonic stem cell cultures wherein at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least greater than 95% of the human embryonic stem cells are not adhered to other human embryonic stem cells in the cell culture. The proportion of cells adhered to other cells in the culture depends on, among other things, the antagonistic efficacy and the concentration of the agent supplied to the cell culture. In a preferred embodiment, the antagonist is a polyclonal or a monoclonal E-cadherin antibody.

[00118] Other embodiments of the present invention included cell cultures comprising a calcium-binding agent and E-cadherin-expressing human embryonic stem cells in a culture medium. In such cultures, the calcium-binding agent is bound to calcium ions in the culture medium, thereby inhibiting adhesion between the embryonic stem cells. Accordingly, compositions of the present invention include human embryonic stem cell cultures wherein at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least greater than 95% of the human embryonic stem cells are not adhered to other human embryonic stem cells in the cell culture. In preferred embodiments, the calcium binding agent is selected from the group consisting of ethylenediaminetetraacetic acid (EDTA), ethyleneglycoltetraacetic acid (EGTA), 1,10 phenanthroline, diethylenetriaminepentaacetate (DTPA), hydroxyethylethylenediaminetriacetic acid (HEEDTA), diaminocyclohexanetetraacetic acid (CDTA), 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), and pharmaceutically acceptable salts thereof.

Methods for identifying an E-cadherin an agent capable of increasing production of cell derived from a human embryonic stem cell

[00119] Some embodiments of the present invention relate to methods for identifying an agent capable of increasing production of a cell derived from a human embryonic stem cell. In these embodiments, a candidate agent is provided to a human embryonic stem cell culture. The human embryonic stem cell culture is then differentiated in a culture medium comprising a differentiation factor known to be capable of promoting the differentiation of said human embryonic stem cells. For example, human embryonic stem cells can be differentiated to definitive endoderm in medium lacking insulin like growth factor receptor agonists (or containing low levels of such molecules) and in the presence of activin A. Other factors capable of promoting the differentiation of human embryonic stem cells are known in the art. After differentiation has occurred, it can be determined whether the candidate agent increases the production of cells differentiated from the human embryonic stem cells by comparing the production of differentiated cells in the cell culture provided with the candidate agent to the production of differentiated cells in a human embryonic stem cell culture that has not been provided with the candidate agent but which has been treated with the same differentiation factor (differentiated under substantially the same conditions) as the cell culture provided with the candidate agent. Greater production of differentiated cells in the cell culture provided with the candidate agent as compared to the production of differentiated cells in the cell culture not provided with the candidate agent indicates that the candidate agent increases the production of a cell derived from a human embryonic cell.

[00120] In some embodiments of the present invention, candidate agents can be obtained from combinatorial synthetic chemical libraries. Generation of combinatorial synthetic chemical libraries is well known in the art. Alternatively, a natural product chemical library or a library of biological molecules generated by recombinant DNA or cell extraction processes can be utilized to obtain candidate agents. Procedures for generating each of the above libraries are routine in the art.

[00121] In another embodiment of the invention, there is provided a method for identifying an agent capable of increasing production of a cell derived from a human embryonic

stem cell (hESC) by contacting a hESC in the presence of an agent in a culture medium, wherein the agent binds to extracellular calcium ions in the medium; differentiating the hESC culture in the culture; measuring production of the differentiated cell in the presence of the agent, wherein production of the differentiated cell in the presence of the agent is increased as compared to production of the differentiated cell in the absence of the agent, thereby indicating an agent capable of increasing production of a human embryonic derived cell.

Methods for screening for agonists and/or antagonists of E-cadherin

[00122] The present invention also provides a method for identifying an E-cadherin agonist or antagonist by providing a peptide library based on hESCs and an E-cadherin peptide; screening said peptide library for agents having high affinity binding to the E-cadherin peptide; and selecting a member of the peptide library binding to the E-cadherin peptide wherein the affinity of the member is equivalent or higher than that of a native homotypic E-cadherin peptide.

[00123] Human ES cells are useful for *in vitro* assays and screening to detect agents that affect hESC cellular adhesion and increase production of definitive endoderm. A wide variety of assays may be used for this purpose, including toxicology testing, immunoassays for protein binding; determination of cell growth, differentiation and functional activity; production of hormones; and the like.

[00124] Agents of interest for screening include known and unknown compounds that encompass numerous chemical classes, primarily organic molecules (e.g., polypeptides, peptides, peptide analogs, peptide variants and/or mutants), which may include organometallic molecules, inorganic molecules, genetic sequences, etc. In addition to complex biological agents, candidate agents include organic molecules comprising functional groups necessary for structural interactions, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, frequently at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules, including peptides, polynucleotides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

[00125] Agents which are useful for modulation of cellular adhesion include but are not

limited to agonists and/or antagonists that bind to the receptor E-cadherin or E-cadherin. As used herein, the term "agonist" refers to an agent or analog that binds productively to a receptor and mimics its biological activity. The term "antagonist" refers to an agent that binds to receptors but does not provoke the normal biological response. An agent as described herein in detail includes polypeptides, peptides and functional fragments or portions thereof, and as described above.

[00126] Compounds, including candidate agents, are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds, including biomolecules, expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs.

[00127] Agents are screened for biological activity by adding the agent to at least one and usually a plurality of cell samples, usually in conjunction with cells lacking the agent. The change in parameters in response to the agent is measured, and the result evaluated by comparison to reference cultures, e.g. in the presence and absence of the agent, obtained with other agents, etc.

[00128] The agents are conveniently added in solution, or readily soluble form, to the medium of cells in culture. The agents may be added in a flow-through system, as a stream, intermittent or continuous, or alternatively, adding a bolus of the compound, singly or incrementally, to an otherwise static solution. In a flow-through system, two fluids are used, where one is a physiologically neutral solution, and the other is the same solution with the test compound added. The first fluid is passed over the cells, followed by the second. In a single solution method, a bolus of the test compound is added to the volume of medium surrounding the cells. The overall concentrations of the components of the culture medium should not change significantly with the addition of the bolus, or between the two solutions in a flow through method.

[00129] Preferred agent formulations do not include additional components, such as preservatives, that may have a significant effect on the overall formulation. Thus preferred formulations consist essentially of a biologically active compound and a physiologically acceptable carrier, e.g. water, ethanol, DMSO, etc. However, if a compound is liquid without a solvent, the formulation may consist essentially of the compound itself.

[00130] A plurality of assays may be run in parallel with different agent concentrations to obtain a differential response to the various concentrations. As known in the art, determining the effective concentration of an agent typically uses a range of concentrations resulting from 1:10, or other log scale, dilutions. The concentrations may be further refined with a second series of dilutions, if necessary. Typically, one of these concentrations serves as a negative control, i.e. at zero concentration or below the level of detection of the agent or at or below the concentration of agent that does not give a detectable change in the phenotype.

[00131] Having generally described this invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only, and are not intended to be limiting.

EXAMPLE 1

DISRUPTION OF CELLULAR ADHERANCE OF PLURIPOTENT CELLS INCREASES PRODUCTION OF DEFINITIVE ENDODERM

[00132] Human embryonic stem cells (hESCs; CyT203) were differentiated *in vitro* to definitive endoderm (DE) for about 2-3 days substantially as described in D'Amour et al. 2005 *Nat Biotechnol.* 23:1534-41, D'Amour et al. 2006 *Nat Biotechnol.* 24(11):1392-401 and U.S. Patent Application Publication Number 2007/0154984, which are herein incorporated in their entireties. Briefly, undifferentiated human embryonic stem (hES) cells were maintained on mouse embryo fibroblast feeder layers (Specialty Media) in DMEM/F12 (Mediatech) supplemented with 20% KnockOut serum replacement (Gibco), 1 mM nonessential amino acids (Gibco), Glutamax (Gibco), penicillin/streptomycin (Gibco), 0.55 mM 2-mercaptoethanol (Gibco) and 4 ng/ml recombinant human FGF2 (R&D Systems). Activin A was added to the growth culture medium at 10-25 ng/ml to help maintain undifferentiated growth. Cultures were manually passaged at 1:4–1:10 split ratio every 5–7 days. Before initiating differentiation, hES

cells were given a brief wash in PBS+/+ (Gibco). Cells were differentiated in RPMI (Mediatech) supplemented with Glutamax, penicillin/streptomycin, 100 ng/mL activin A and varying concentrations of defined FBS (HyClone). Additionally, 0.2% BSA and 25ng/mL Wnt3a was added on the first day (d1) of differentiation. In most differentiation experiments FBS concentrations were 0% for the first 24 h, 0.2% for the second 24 h, and 0.2% for the third 24h. Recombinant human activin A and Wnt3a were purchased from R&D Systems. Three 60mm plates were utilized. One plate was treated with a mouse anti-human E-cadherin antibody (Zymed Cat. No.13-1700) at 5 μg /mL for the first day (d1) and cultured for 3 days. The other two plates received no E-cadherin antibody treatment (controls). After the three (3) day treatment, hES-derived cells were dissociated using either TrypLE (Invitrogen #12563-011) or Accutase (Innovative Cell Technologies #AT104) at 37 °C. The cells were washed in PBS with 10% FBS to remove enzyme. Cells were pelleted and resuspended in PBS with 3%FBS (buffer) to block nonspecific antibody binding. Cells were labeled with CXCR4-PE conjugated antibody (R&D Systems) at 10 μL per approximately $1x10^6$ cells for 20 minutes at room temperature. Cells were washed in buffer and resuspended in buffer at approx. 3-5 x 10^6 cells/ml. Cells were analyzed using a FACSCalibur (BD Bioscience).

[00133] As described in D'Amour et al. 2005 and U.S. Patent Application Publication No. 2007/0154984 *supra*, CXCR4 expression permits isolation of definitive endoderm. The chemokine receptor CXCR4 is expressed in the definitive endoderm and mesoderm but not in primitive endoderm/visceral endoderm. It was previously shown that hES-derived cell cultures exposed to activin A and lower levels of FBS have an increase in CXCR4 mRNA, which corresponds to the increase in other definitive endoderm markers, for example, see Figure 5 of D'Amour et al. The percentage of CXCR4 positive cells in the two plates not receiving E-cadherin antibody treatment was approximately 70% and 76% (FIGS. 2A and 2B, respectively). In contrast, the plate receiving the E-cadherin antibody treatment had approximately 93% CXCR4 positive cells (FIG. 1C). Approximately 25,000 cells were analyzed per sample.

[00134] In view of the foregoing data, treatment of hESCs with an agent that binds E-cadherin on an E-cadherin expressing cell increases the efficiency of definitive endoderm production of the E-cadherin cell in the presence of strong nodal agonists, for example, Activin A.

EXAMPLE 2

ANTI-HUMAN E-CADHERIN TREATMENT INCREASES THE EXPRESSION OF DEFINITIVE ENDODERM CELL SURFACE MARKERS

[00135] Cell cultures and culture conditions were substantially similar to those described above in Example 1 and in D'Amour et al. 2005 *supra*, including addition of 0.2% BSA. Nine tissue culture plates (35mm) of hESCs (CyT203) were differentiated to definitive endoderm for three (3) days. Two (2) plates were treated with anti-human E-cadherin (Zymed Cat. No. 13-1700) at 5 μg/mL on the first day (d1). One plate did not receive anti-human E-cadherin (control). Six (6) plates were treated with anti-human E-cadherin (Zymed Cat. No. 13-5700) for the first (d1) or the first two (d1 and d2) days at concentration of 5, 1, or 0.2 μg/ mL.

[00136] To determine cell surface marker expression, small samples of cells were harvested from differentiating plates, and total RNA was isolated from duplicate or triplicate samples with a 6100 nucleic acid extractor (Applied Biosystems) and 100–500 ng was used for reverse transcription with iScript cDNA synthesis kit (Bio-Rad). PCR reactions were run in duplicate using 1/40th of the cDNA per reaction and 400 nM forward and reverse primers with QuantiTect SYBR Green master mix (Qiagen). Alternatively, QuantiTect Primer Assays (Qiagen) were used according to the manufacturer's instructions. Real-time PCR was performed using the Rotor Gene 3000 (Corbett Research). Relative quantification was performed in relation to a standard curve. The standard curve was created using a mixture of total RNA samples from various fetal human endoderm tissues and differentiated hES cells, and 1 μg was used per cDNA reaction in creating the standard curve. Quantified values for each gene of interest were normalized against the input determined by two housekeeping genes (CYCG and GUSB or TBP). After normalization, the samples were plotted relative to the lowest detectable sample in the dataset and the standard deviation of four- or six- gene expression measurements was reported. Primer sequences: CXCR4 forward primer (5' to 3'), CACCGCATCTGGAGAACCA (SEQ ID NO: 3); CXCR4 reverse primer (5' to 3'), GCCCATTTCCTCGGTGTAGTT (SEQ ID NO: 4); OCT4 forward primer (5' to 3'), TGGGCTCGAGAAGGATGTG (SEQ ID NO: 5); OCT4 reverse primer (5' to 3'), GCATAGTCGCTGCTTGATCG (SEQ ID NO: 6); CER forward primer (5' to 3'), ACAACTACTTTTTCACAGCCTTCGT (SEQ ID NO: 7); CER reverse primer (5' to 3'), CCACGACTTGCCCAGCAT (SEQ ID NO: 8); NANOG forward primer (5' to 3'),

GCAAATGTCTTCTGCTGAGATGC (SEQ ID NO: 9); and NANOG Reverse primer (5' to 3'), CCATGGAGGAGGGAAGAGGA (SEQ ID NO: 10).

[00137] As can be seen in Figure 3, the levels of cell surface marker expression were dependent on the dose of antibody provided. That is, levels of expression directly depended on the amount or concentration of anti-human E-cadherin added to the culture medium (e.g., 0.2, 1 and 5 μg/mL). For example, CXCR4 levels were increased at 3 days with antibody treatments at 1 μg/mL or 5μg/mL of anti-human E-cadherin, whereas, samples treated with 0.2 μg/mL had CXCR4 expression levels substantially similar to those observed in the control samples (FIG. 3A). Another marker expressed in definitive endoderm is CERBERUS (CER). This marker was strongly upregulated after one day of E-cadherin antibody treatment and the upreguation was also dose-dependent (FIG. 3B).

[00138] To determine the relative percentage of cells expressing Oct4 and Nanog, transcription factors required to maintain the hESC pluripotency and self-renewal, OCT4 and NANOG expression in the definitive endoderm cultures were analyzed. Figures 3C and 3D demonstrate that NANOG and OCT4 hESC expression levels were decreased with anti-human E-cadherin treatment, and at time periods typical of definitive endoderm formation (D'Amour et.al 2005, *supra*).

EXAMPLE 3

DECREASED LEVELS OF EXTRACELLULAR CALCIUM INCREASES PRODUCTION OF DEFINITIVE ENDODERM CELLS

[00139] Cell cultures and culture conditions were substantially similar to that described above and in D'Amour et al. 2005 and 2006 *supra*. Briefly, three 60 mm plates of hESCs (CyT203 p36) were differentiated for about 18 hr using activin A (100 ng/ml) and Wnt3a (25 ng/ml) in RPMI without FBS. Samples of undifferentiated hESCs were taken prior to the start of the differentiation procedure. Plates were briefly washed with 2 ml of PBS either containing Ca^{2+} or Mg^{2+} or without Ca^{2+} and Mg^{2+} ($PBS^{-/-}$). In addition, the one plate which was washed with $PBS^{-/-}$ also received ethylenediamine tetraacetic acid (EDTA) at 0.075 mM to reduce the concentration of Ca^{2+} ions present in the media. Analysis by real-time PCR for expression of brachyury (BRACH), nodal (NODAL) and cerberus (CER) indicates that the addition of EDTA

facilitated the differentiation to mesendoderm, as indicated by levels of BRACH (FIG. 4A) and NODAL (FIG. 4B), and definitive endoderm, as indicated by levels of CER (FIG. 4C). There was about a two-fold increase in gene expression for mesendoderm markers, and about a three-fold increase in expression for the DE marker in EDTA-treated cells. These data indicate that the reduction of extracellular calcium in the culture media *in vitro* was sufficient to increase efficient production of definitive endoderm from hESCs as compared to the no treatment control cultures.

[00140] In addition to EDTA, it will be appreciated that other calcium ion chelators can be used, including but not limited to, ethyleneglycoltetraacetic acid (EGTA), 1,10 phenanthroline, diethylenetriaminepentaacetate (DTPA), hydroxyethylethylenediaminetriacetic acid (HEEDTA), Diaminocyclohexanetetraacetic acid (CDTA), 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), and pharmaceutically acceptable salts thereof.

[00141] The methods and compositions described herein are presently representative of preferred embodiments 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.

[00142] 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.

WE CLAIM:

1. An *in vitro* method for increasing definitive endoderm production comprising:

providing an agent to a cell culture comprising E-cadherin-expressing human embryonic stem cells, thereby inhibiting adhesion of the E-cadherin-expressing cells to each other; and

differentiating said E-cadherin-expressing human embryonic stem cells by contacting said cells with a medium comprising a growth factor of the Nodal/Activin subgroup of the TGFβ superfamily of growth factors, thereby increasing the production of definitive endoderm.

2. The method of claim 1, wherein the agent binds E-cadherin.

3. The method of claim 2, wherein the agent is an antagonist of E-cadherin.

4. The method of claim 3, wherein the antagonist is a polyclonal or a monoclonal E-cadherin antibody. wherein the agent is a peptide or peptide analog.

5. The method of claim 1 wherein the agent is a peptide or a peptide analog.

6. The method of claim 5, wherein the peptide is an E-cadherin peptide corresponding to an E-cadherin extracellular domain at amino acid residues 600-700 of human E-cadherin (SEQ ID NO:1).

7. The method of claim 1, wherein the agent is a calcium ion chelator.

8. The method of claim 7, wherein the calcium ion chelator is selected from the group consisting of ethylenediaminetetraacetic acid (EDTA), ethyleneglycoltetraacetic acid (EGTA), 1,10 phenanthroline, diethylenetriaminepentaacetate (DTPA), hydroxyethylethylenediaminetriacetic acid (HEEDTA), diaminocyclohexanetetraacetic acid (CDTA), 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), and pharmaceutically acceptable salts thereof.

9. The method of claim 1, wherein said E-cadherin-expressing human embryonic stem cells are provided with said agent in a first medium, and wherein said E-cadherin-expressing human embryonic stem cells are differentiated in a second medium.

10. The method of claim 1, wherein said first medium is different from said second medium.

11. A method for identifying an agent capable of increasing production of a cell derived from a human embryonic stem cell, said method comprising:

providing a candidate agent to a human embryonic stem cell culture;

differentiating the human embryonic stem cell in a culture medium comprising a differentiation factor known to be capable of promoting the differentiation of said human embryonic stem cells; and

determining whether the candidate agent increases the production of cells differentiated from said human embryonic stem cells by comparing the production of differentiated cells in said cell culture provided with the candidate agent to the production of differentiated cells in a human embryonic stem cell culture that has not been provided with said candidate agent but has been treated with the same differentiation factor as the cell culture provided with the candidate agent, wherein greater production of differentiated cells in the cell culture provided with the candidate agent as compared to the production of differentiated cells in the cell culture not provided with the candidate agent indicates that the candidate agent increases the production of a cell derived from a human embryonic cell.

12. The method of claim 11, wherein the differentiation factor is a growth factor of the Nodal/Activin subgroup of the TGFβ superfamily.

13. The method of claim 11, wherein the differentiated cell is a definitive endoderm cell or derivative thereof.

14. The method of claim 11, wherein the agent is an antagonist of human E-cadherin.

15. The method of claim 11, wherein the agent is a synthetic compound.

16. The method of claim 15, wherein the agent is a natural product.

17. An *in vitro* composition comprising an antagonist of E-cadherin specifically bound to an E-cadherin-expressing human embryonic stem cells, wherein the binding of the antagonist inhibits adhesion between said embryonic stem cells.

18. The composition of claim 17, wherein the antagonist is a polyclonal or a monoclonal E-cadherin antibody.

19. The composition of claim 17, wherein at least 10% of the human embryonic stem cells are not adhered to other human embryonic stem cells.

20. The composition of claim 17, wherein at least 50% of the human embryonic stem cells are not adhered to other human embryonic stem cells.

21. A cell culture comprising a calcium-binding agent and E-cadherin-expressing human embryonic stem cells in a culture medium, wherein the calcium-binding agent is bound to calcium ions in the culture medium, thereby inhibiting adhesion between said embryonic stem cells.

22. The cell culture of claim 19, wherein the calcium binding agent is selected from the group consisting of ethylenediaminetetraacetic acid (EDTA), ethyleneglycoltetraacetic acid (EGTA), 1,10 phenanthroline, diethylenetriaminepentaacetate (DTPA), hydroxyethylethylenediaminetriacetic acid (HEEDTA), diaminocyclohexanetetraacetic acid (CDTA), 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), and pharmaceutically acceptable salts thereof.

23. The cell culture of claim 22, wherein at least 10% of the human embryonic stem cells are not adhered to other human embryonic stem cells.

24. The composition of claim 22, wherein at least 50% of the human embryonic stem cells are not adhered to other human embryonic stem cells.

25. A method of identifying an E-cadherin agonist or antagonist comprising:

providing a peptide library of peptides derived from hESCs and an E-cadherin peptide;

screening said peptide library for peptides having high affinity binding to the E-cadherin peptide; and

selecting a member of the peptide library binding to the E-cadherin peptide wherein the affinity of the member is equivalent to or higher than that of a native homotypic E-cadherin peptide.