DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS

FIELD OF THE INVENTION

[0001] This application claims the benefit of U.S. Provisional Application No. 61/289,692, filed on December 23, 2009, which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

[0002] The present invention provides methods to promote the differentiation of pluripotent stem cells into insulin producing cells. In particular, the present invention provides a method to increase the expression of NGN3 and NKX6.1 in populations of cells expressing markers characteristic of the pancreatic endocrine lineage.

BACKGROUND

[0003] Advances in cell-replacement therapy for Type I diabetes mellitus and a shortage of transplantable islets of Langerhans have focused interest on developing sources of insulin-producing cells, or β cells, appropriate for engraftment. One approach is the generation of functional β cells from pluripotent stem cells, such as, for example, embryonic stem cells.

[0004] During vertebrate embryonic development, a pluripotent cell gives rise to a group of cells comprising three germ layers (ectoderm, mesoderm, and endoderm) in a process known as gastrulation. Tissues such as, for example, thyroid, thymus, pancreas, gut, and liver, will develop from the endoderm, via an intermediate stage. The intermediate stage in this process is the formation of definitive endoderm. Definitive endoderm cells express a number of markers, such as, HNF3 beta, GATA4, MIXL1, CXCR4 and SOX17.

[0005] Formation of the pancreas arises from the differentiation of definitive endoderm into pancreatic endoderm. Cells of the pancreatic endoderm express the pancreatic-duodenal homeobox gene, PDX1. In the absence of PDX1, the pancreas fails to develop beyond the formation of ventral and dorsal buds. Thus, PDX1 expression marks a critical step in pancreatic organogenesis. The mature pancreas contains,

among other cell types, exocrine tissue and endocrine tissue. Exocrine and endocrine tissues arise from the differentiation of pancreatic endoderm.

[0006] Cells bearing the features of islet cells have reportedly been derived *in vitro* from embryonic cells of the mouse. For example, Lumelsky *et al.* (Science 292:1389, 2001) report differentiation of mouse embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Soria *et al.* (Diabetes 49:157, 2000) report that insulin-secreting cells derived from mouse embryonic stem cells normalize glycemia when implanted in streptozotocin-induced diabetic mice.

[0007] In one example, Hori *et al.* (PNAS 99: 16105, 2002) disclose that treatment of mouse embryonic stem cells with inhibitors of phosphoinositide 3-kinase (LY294002) produced cells that resembled β cells.

[0008] In another example, Blyszczuk *et al.* (PNAS 100:998, 2003) reports the generation of insulin-producing cells from mouse embryonic stem cells constitutively expressing Pax4.

[0009] Micallef *et al.* reports that retinoic acid can regulate the commitment of embryonic stem cells to form PDX1 positive pancreatic endoderm. Retinoic acid is most effective at inducing PDX1 expression when added to cultures at day four of embryonic stem cell differentiation during a period corresponding to the end of gastrulation in the embryo (Diabetes 54:301, 2005).

[0010] Miyazaki *et al.* reports a mouse embryonic stem cell line over-expressing Pdx1. Their results show that exogenous Pdx1 expression clearly enhanced the expression of insulin, somatostatin, glucokinase, neurogenin3, p48, Pax6, and HNF6 in the resulting differentiated cells (Diabetes 53: 1030, 2004).

[0011] Skoudy *et al.* reports that activin A (a member of the TGF-β superfamily) upregulates the expression of exocrine pancreatic genes (p48 and amylase) and endocrine genes (Pdx1, insulin, and glucagon) in mouse embryonic stem cells. The maximal effect was observed using 1nM activin A. They also observed that the expression level of insulin and Pdx1 mRNA was not affected by retinoic acid; however, 3nM FGF7 treatment resulted in an increased level of the transcript for Pdx1 (Biochem. J. 379: 749, 2004).

[0012] Shiraki *et al.* studied the effects of growth factors that specifically enhance differentiation of embryonic stem cells into PDX1 positive cells. They observed that TGF-β2 reproducibly yielded a higher proportion of PDX1 positive cells (Genes Cells. 2005 Jun; 10(6): 503-16.).

[0013] Gordon *et al.* demonstrated the induction of brachyury [positive]/ HNF3 beta [positive] endoderm cells from mouse embryonic stem cells in the absence of serum and in the presence of activin along with an inhibitor of Wnt signaling (US 2006/0003446A1).

[0014] Gordon *et al.* (PNAS, Vol 103, page 16806, 2006) states "Wnt and TGF-beta/ nodal/ activin signaling simultaneously were required for the generation of the anterior primitive streak."

[0015] However, the mouse model of embryonic stem cell development may not exactly mimic the developmental program in higher mammals, such as, for example, humans.

[0016] Thomson *et al.* isolated embryonic stem cells from human blastocysts (Science 282:114, 1998). Concurrently, Gearhart and coworkers derived human embryonic germ (hEG) cell lines from fetal gonadal tissue (Shamblott *et al.*, Proc. Natl. Acad. Sci. USA 95:13726, 1998). Unlike mouse embryonic stem cells, which can be prevented from differentiating simply by culturing with Leukemia Inhibitory Factor (LIF), human embryonic stem cells must be maintained under very special conditions (U.S. Pat. No. 6,200,806; WO 99/20741; WO 01/51616).

[0017] D'Amour *et al.* describes the production of enriched cultures of human embryonic stem cell-derived definitive endoderm in the presence of a high concentration of activin and low serum (Nature Biotechnology 2005). Transplanting these cells under the kidney capsule of mice resulted in differentiation into more mature cells with characteristics of some endodermal organs. Human embryonic stem cell-derived definitive endoderm cells can be further differentiated into PDX1 positive cells after addition of FGF-10 (US 2005/0266554A1).

[0018] D'Amour *et al.* (Nature Biotechnology - 24, 1392 - 1401 (2006)) states: "We have developed a differentiation process that converts human embryonic stem (hES) cells to endocrine cells capable of synthesizing the pancreatic hormones insulin, glucagon,

somatostatin, pancreatic polypeptide and ghrelin. This process mimics *in vivo* pancreatic organogenesis by directing cells through stages resembling definitive endoderm, gut-tube endoderm, pancreatic endoderm and endocrine precursor en route to cells that express endocrine hormones".

[0019] In another example, Fisk *et al.* reports a system for producing pancreatic islet cells from human embryonic stem cells (US2006/0040387A1). In this case, the differentiation pathway was divided into three stages. Human embryonic stem cells were first differentiated to endoderm using a combination of sodium butyrate and activin A. The cells were then cultured with TGF-β antagonists such as Noggin in combination with EGF or betacellulin to generate PDX1 positive cells. The terminal differentiation was induced by nicotinamide.

[0020] In one example, Benvenistry *et al.* states: "We conclude that over-expression of PDX1 enhanced expression of pancreatic enriched genes, induction of insulin expression may require additional signals that are only present *in vivo*" (Benvenistry *et al*, Stem Cells 2006; 24:1923–1930).

[0021] In another example, Grapin-Botton *et al.* states: "Early activation of Ngn3 almost exclusively induced glucagon [positive] cells while depleting the pool of pancreas progenitors. As from E11.5, PDX-1 progenitors became competent to differentiate into insulin [positive] and PP [positive] cells" (Johansson KA *et al*, Developmental Cell 12, 457 – 465, March 2007).

[0022] The expression of NGN3 in cells expressing markers characteristic of the pancreatic endoderm lineage may reduce the ability of the cells to further differentiate into insulin expressing cells. Previous studies have showed that cells expressing markers characteristic of the pancreatic endoderm lineage that express NGN3 are more likely to produce glucagon expressing cells than insulin expressing cells, when subjected to further differentiation. However, NGN3 expression is required to form pancreatic endocrine cells, or pancreatic endocrine precursor cells (cells that can form, for example glucagon, or insulin expressing cells). Therefore, the temporal regulation of NGN3 is important in guiding the ultimate fate of pancreatic endocrine precursor cells toward insulin expressing cells.

[0023] Therefore, there still remains a significant need to develop conditions for establishing pluripotent stem cell lines that can be expanded to address the current clinical needs, while retaining the potential to differentiate into insulin expressing cells. The present invention takes an alternative approach to improve the efficiency of differentiating human embryonic stem cells toward insulin expressing cells, by providing a method to increase the expression of NGN3 and NKX6.1 in cells expressing markers characteristic of the pancreatic endocrine lineage.

SUMMARY

[0024] In one embodiment, the present invention provides a method to increase the expression of NGN3 and NKX6.1 in a population of cells expressing markers characteristic of the pancreatic endocrine lineage, comprising the steps of:

a) culturing pluripotent stem cells,

b) differentiating the pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage,

c) differentiating the cells expressing markers characteristic of the definitive endoderm lineage into cells expressing markers characteristic of the pancreatic endoderm lineage, supplementing the medium used to differentiate the cells expressing markers characteristic of the definitive endoderm lineage with a compound selected from the group consisting of H-9, H-89, GF 109203X, HA-1004, PP2, PP1, LY 294002, Wortmannin, SB-203580, SB-202190, Tyrphostin 25, Tyrphostin, AG1478, Tyrphostin 46, GW 5074, Kenpaullone, HNMPA, AG490, Y27632, and ML-7, and

d) differentiating the cells expressing markers characteristic of the pancreatic endoderm lineage into cells expressing markers characteristic of the pancreatic endocrine lineage.

[0025] In one embodiment, the medium used to differentiate the cells expressing markers characteristic of the pancreatic endoderm lineage is supplemented with a compound selected from the group consisting of H-9, H-89, GF 109203X, HA-1004, PP2, PP1, LY 294002, Wortmannin, SB-203580, SB-202190, Tyrphostin 25, Tyrphostin,

AG1478, Tyrphostin 46, GW 5074, Kenpaullone, HNMPA, AG490, Y27632, and ML-7.

DETAILED DESCRIPTION

[0026] For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the following subsections that describe or illustrate certain features, embodiments or applications of the present invention.

Definitions

[0027] Stem cells are undifferentiated cells defined by their ability at the single cell level to both self-renew and differentiate to produce progeny cells, including self-renewing progenitors, non-renewing progenitors, and terminally differentiated cells. Stem cells are also characterized by their ability to differentiate *in vitro* into functional cells of various cell lineages from multiple germ layers (endoderm, mesoderm and ectoderm), as well as to give rise to tissues of multiple germ layers following transplantation and to contribute substantially to most, if not all, tissues following injection into blastocysts.

[0028] Stem cells are classified by their developmental potential as: (1) totipotent, meaning able to give rise to all embryonic and extraembryonic cell types; (2) pluripotent, meaning able to give rise to all embryonic cell types; (3) multipotent, meaning able to give rise to a subset of cell lineages but all within a particular tissue, organ, or physiological system (for example, hematopoietic stem cells (HSC) can produce progeny that include HSC (self- renewal), blood cell restricted oligopotent progenitors, and all cell types and elements (e.g., platelets) that are normal components of the blood); (4) oligopotent, meaning able to give rise to a more restricted subset of cell lineages than multipotent stem cells; and (5) unipotent, meaning able to give rise to a single cell lineage (e.g., spermatogenic stem cells).

[0029] Differentiation is the process by which an unspecialized ("uncommitted") or less specialized cell acquires the features of a specialized cell such as, for example, a nerve cell or a muscle cell. A differentiated or differentiation-induced cell is one that has taken on a more specialized ("committed") position within the lineage of a cell. The term “committed”, when applied to the process of differentiation, refers to a cell

that has proceeded in the differentiation pathway to a point where, under normal circumstances, it will continue to differentiate into a specific cell type or subset of cell types, and cannot, under normal circumstances, differentiate into a different cell type or revert to a less differentiated cell type. De-differentiation refers to the process by which a cell reverts to a less specialized (or committed) position within the lineage of a cell. As used herein, the lineage of a cell defines the heredity of the cell, i.e., which cells it came from and what cells it can give rise to. The lineage of a cell places the cell within a hereditary scheme of development and differentiation. A lineage-specific marker refers to a characteristic specifically associated with the phenotype of cells of a lineage of interest and can be used to assess the differentiation of an uncommitted cell to the lineage of interest.

[0030] "Cells expressing markers characteristic of the definitive endoderm lineage", or "Stage 1 cells", or "Stage 1", as used herein, refers to cells expressing at least one of the following markers: SOX17, GATA4, HNF3 beta, GSC, CER1, Nodal, FGF8, Brachyury, Mix-like homeobox protein, FGF4 CD48, eomesodermin (EOMES), DKK4, FGF17, GATA6, CXCR4, C-Kit, CD99, or OTX2. Cells expressing markers characteristic of the definitive endoderm lineage include primitive streak precursor cells, primitive streak cells, mesendoderm cells and definitive endoderm cells.

[0031] "Cells expressing markers characteristic of the pancreatic endoderm lineage", as used herein, refers to cells expressing at least one of the following markers: PDX1, HNF1 beta, PTF1 alpha, HNF6, NKX6.1, or HB9. Cells expressing markers characteristic of the pancreatic endoderm lineage include pancreatic endoderm cells, primitive gut tube cells, and posterior foregut cells.

[0032] "Definitive endoderm", as used herein, refers to cells which bear the characteristics of cells arising from the epiblast during gastrulation and which form the gastrointestinal tract and its derivatives. Definitive endoderm cells express the following markers: HNF3 beta, GATA4, SOX17, Cerberus, OTX2, goosecoid, C-Kit, CD99, and MIXL1.

[0033] "Markers", as used herein, are nucleic acid or polypeptide molecules that are differentially expressed in a cell of interest. In this context, differential expression means an increased level for a positive marker and a decreased level for a negative marker. The detectable level of the marker nucleic acid or polypeptide is sufficiently

higher or lower in the cells of interest compared to other cells, such that the cell of interest can be identified and distinguished from other cells using any of a variety of methods known in the art.

[0034] "Pancreatic endocrine cell", or "pancreatic hormone expressing cell", as used herein, refers to a cell capable of expressing at least one of the following hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide.

Isolation, Expansion and Culture of Pluripotent Stem Cells

Characterization of Pluripotent Stem Cells

[0035] Pluripotent stem cells may express one or more of the stage-specific embryonic antigens (SSEA) 3 and 4, and markers detectable using antibodies designated Tra-1-60 and Tra-1-81 (Thomson *et al.*, Science 282:1145, 1998). Differentiation of pluripotent stem cells *in vitro* results in the loss of SSEA-4, Tra 1-60, and Tra 1-81 expression (if present) and increased expression of SSEA-1. Undifferentiated pluripotent stem cells typically have alkaline phosphatase activity, which can be detected by fixing the cells with 4% paraformaldehyde, and then developing with Vector Red as a substrate, as described by the manufacturer (Vector Laboratories, Burlingame Calif.). Undifferentiated pluripotent stem cells also typically express OCT4 and TERT, as detected by RT-PCR.

[0036] Another desirable phenotype of propagated pluripotent stem cells is a potential to differentiate into cells of all three germinal layers: endoderm, mesoderm, and ectoderm tissues. Pluripotency of pluripotent stem cells can be confirmed, for example, by injecting cells into severe combined immunodeficient (SCID) mice, fixing the teratomas that form using 4% paraformaldehyde, and then examining them histologically for evidence of cell types from the three germ layers. Alternatively, pluripotency may be determined by the creation of embryoid bodies and assessing the embryoid bodies for the presence of markers associated with the three germinal layers.

[0037] Propagated pluripotent stem cell lines may be karyotyped using a standard G-banding technique and compared to published karyotypes of the corresponding primate species. It is desirable to obtain cells that have a "normal karyotype," which means

that the cells are euploid, wherein all human chromosomes are present and not noticeably altered.

Sources of Pluripotent Stem Cells

[0038] The types of pluripotent stem cells that may be used include established lines of pluripotent cells derived from tissue formed after gestation, including pre-embryonic tissue (such as, for example, a blastocyst), embryonic tissue, or fetal tissue taken any time during gestation, typically but not necessarily before approximately 10-12 weeks gestation. Non-limiting examples are established lines of human embryonic stem cells or human embryonic germ cells, such as, for example the human embryonic stem cell lines H1, H7, and H9 (WiCell). Also contemplated is use of the compositions of this disclosure during the initial establishment or stabilization of such cells, in which case the source cells would be primary pluripotent cells taken directly from the source tissues. Also suitable are cells taken from a pluripotent stem cell population already cultured in the absence of feeder cells. Also suitable are mutant human embryonic stem cell lines, such as, for example, BG01v (BresaGen, Athens, GA).

[0039] In one embodiment, human embryonic stem cells are prepared as described by Thomson *et al.* (U.S. Pat. No. 5,843,780; Science 282:1145, 1998; Curr. Top. Dev. Biol. 38:133 ff., 1998; Proc. Natl. Acad. Sci. U.S.A. 92:7844, 1995).

Culture of Pluripotent Stem Cells

[0040] In one embodiment, pluripotent stem cells are typically cultured on a layer of feeder cells that support the pluripotent stem cells in various ways. Alternatively, pluripotent stem cells are cultured in a culture system that is essentially free of feeder cells, but nonetheless supports proliferation of pluripotent stem cells without undergoing substantial differentiation. The growth of pluripotent stem cells in feeder-free culture without differentiation is supported using a medium conditioned by culturing previously with another cell type. Alternatively, the growth of pluripotent stem cells in feeder-free culture without differentiation is supported using a chemically defined medium.

[0041] For example, Reubinoff *et al* (Nature Biotechnology 18: 399 - 404 (2000)) and Thompson *et al* (Science 6 November 1998: Vol. 282. no. 5391, pp. 1145 – 1147) disclose the culture of pluripotent stem cell lines from human blastocysts using a mouse embryonic fibroblast feeder cell layer.

[0042] Richards *et al*, (Stem Cells 21: 546-556, 2003) evaluated a panel of 11 different human adult, fetal and neonatal feeder cell layers for their ability to support human pluripotent stem cell culture. Richards *et al*, states: "human embryonic stem cell lines cultured on adult skin fibroblast feeders retain human embryonic stem cell morphology and remain pluripotent".

[0043] US20020072117 discloses cell lines that produce media that support the growth of primate pluripotent stem cells in feeder-free culture. The cell lines employed are mesenchymal and fibroblast-like cell lines obtained from embryonic tissue or differentiated from embryonic stem cells. US20020072117 also discloses the use of the cell lines as a primary feeder cell layer.

[0044] In another example, Wang *et al* (Stem Cells 23: 1221-1227, 2005) discloses methods for the long-term growth of human pluripotent stem cells on feeder cell layers derived from human embryonic stem cells.

[0045] In another example, Stojkovic *et al* (Stem Cells 2005 23: 306-314, 2005) disclose a feeder cell system derived from the spontaneous differentiation of human embryonic stem cells.

[0046] In a further example, Miyamoto *et al* (Stem Cells 22: 433-440, 2004) disclose a source of feeder cells obtained from human placenta.

[0047] Amit *et al* (Biol. Reprod 68: 2150-2156, 2003) discloses a feeder cell layer derived from human foreskin.

[0048] In another example, Inzunza *et al* (Stem Cells 23: 544-549, 2005) disclose a feeder cell layer from human postnatal foreskin fibroblasts.

[0049] US6642048 discloses media that support the growth of primate pluripotent stem (pPS) cells in feeder-free culture, and cell lines useful for production of such media. US6642048 states: "This invention includes mesenchymal and fibroblast-like cell

lines obtained from embryonic tissue or differentiated from embryonic stem cells. Methods for deriving such cell lines, processing media, and growing stem cells using the conditioned media are described and illustrated in this disclosure."

[0050] In another example, WO2005014799 discloses conditioned medium for the maintenance, proliferation and differentiation of mammalian cells. WO2005014799 states: "The culture medium produced in accordance with the present invention is conditioned by the cell secretion activity of murine cells; in particular, those differentiated and immortalized transgenic hepatocytes, named MMH (Met Murine Hepatocyte)."

[0051] In another example, Xu *et al* (Stem Cells 22: 972-980, 2004) discloses conditioned medium obtained from human embryonic stem cell derivatives that have been genetically modified to over express human telomerase reverse transcriptase.

[0052] In another example, US20070010011 discloses a chemically defined culture medium for the maintenance of pluripotent stem cells.

[0053] An alternative culture system employs serum-free medium supplemented with growth factors capable of promoting the proliferation of embryonic stem cells. For example, Cheon *et al* (BioReprod DOI:10.1095/biolreprod.105.046870, October 19, 2005) disclose a feeder-free, serum-free culture system in which embryonic stem cells are maintained in unconditioned serum replacement (SR) medium supplemented with different growth factors capable of triggering embryonic stem cell self-renewal.

[0054] In another example, Levenstein *et al* (Stem Cells 24: 568-574, 2006) disclose methods for the long-term culture of human embryonic stem cells in the absence of fibroblasts or conditioned medium, using media supplemented with bFGF.

[0055] In another example, US20050148070 discloses a method of culturing human embryonic stem cells in defined media without serum and without fibroblast feeder cells, the method comprising: culturing the stem cells in a culture medium containing albumin, amino acids, vitamins, minerals, at least one transferrin or transferrin substitute, at least one insulin or insulin substitute, the culture medium essentially free of mammalian fetal serum and containing at least about 100 ng/ml of a fibroblast growth factor capable of activating a fibroblast growth factor signaling receptor,

wherein the growth factor is supplied from a source other than just a fibroblast feeder layer, the medium supported the proliferation of stem cells in an undifferentiated state without feeder cells or conditioned medium.

[0056] In another example, US20050233446 discloses a defined medium useful in culturing stem cells, including undifferentiated primate primordial stem cells. In solution, the medium is substantially isotonic as compared to the stem cells being cultured. In a given culture, the particular medium comprises a base medium and an amount of each of bFGF, insulin, and ascorbic acid necessary to support substantially undifferentiated growth of the primordial stem cells.

[0057] In another example, US6800480 states "In one embodiment, a cell culture medium for growing primate-derived primordial stem cells in a substantially undifferentiated state is provided which includes a low osmotic pressure, low endotoxin basic medium that is effective to support the growth of primate-derived primordial stem cells. The basic medium is combined with a nutrient serum effective to support the growth of primate-derived primordial stem cells and a substrate selected from the group consisting of feeder cells and an extracellular matrix component derived from feeder cells. The medium further includes non-essential amino acids, an anti-oxidant, and a first growth factor selected from the group consisting of nucleosides and a pyruvate salt."

[0058] In another example, US20050244962 states: "In one aspect the invention provides a method of culturing primate embryonic stem cells. One cultures the stem cells in a culture essentially free of mammalian fetal serum (preferably also essentially free of any animal serum) and in the presence of fibroblast growth factor that is supplied from a source other than just a fibroblast feeder layer. In a preferred form, the fibroblast feeder layer, previously required to sustain a stem cell culture, is rendered unnecessary by the addition of sufficient fibroblast growth factor."

[0059] In a further example, WO2005065354 discloses a defined, isotonic culture medium that is essentially feeder-free and serum-free, comprising: a. a basal medium; b. an amount of bFGF sufficient to support growth of substantially undifferentiated mammalian stem cells; c. an amount of insulin sufficient to support growth of substantially undifferentiated mammalian stem cells; and d. an amount of ascorbic

acid sufficient to support growth of substantially undifferentiated mammalian stem cells.

[0060] In another example, WO2005086845 discloses a method for maintenance of an undifferentiated stem cell, said method comprising exposing a stem cell to a member of the transforming growth factor-beta (TGF-β) family of proteins, a member of the fibroblast growth factor (FGF) family of proteins, or nicotinamide (NIC) in an amount sufficient to maintain the cell in an undifferentiated state for a sufficient amount of time to achieve a desired result.

[0061] The pluripotent stem cells may be plated onto a suitable culture substrate. In one embodiment, the suitable culture substrate is an extracellular matrix component, such as, for example, those derived from basement membrane or that may form part of adhesion molecule receptor-ligand couplings. In one embodiment, the suitable culture substrate is MATRIGEL® (Becton Dickenson). MATRIGEL® is a soluble preparation from Engelbreth-Holm Swarm tumor cells that gels at room temperature to form a reconstituted basement membrane.

[0062] Other extracellular matrix components and component mixtures are suitable as an alternative. Depending on the cell type being proliferated, this may include laminin, fibronectin, proteoglycan, entactin, heparan sulfate, and the like, alone or in various combinations.

[0063] The pluripotent stem cells may be plated onto the substrate in a suitable distribution and in the presence of a medium that promotes cell survival, propagation, and retention of the desirable characteristics. All these characteristics benefit from careful attention to the seeding distribution and can readily be determined by one of skill in the art.

[0064] Suitable culture media may be made from the following components, such as, for example, Dulbecco's modified Eagle's medium (DMEM), Gibco # 11965-092; Knockout Dulbecco's modified Eagle's medium (KO DMEM), Gibco #10829-018; Ham's F12/50% DMEM basal medium; 200 mM L-glutamine, Gibco # 15039-027; non-essential amino acid solution, Gibco 11140-050; β-mercaptoethanol, Sigma # M7522; human recombinant basic fibroblast growth factor (bFGF), Gibco # 13256-029.

Formation of a Population of Cells Expressing Markers Characteristic of the Pancreatic Endocrine Lineage with Increased Expression of NGN3 and NKX6.1

[0065] In one embodiment, the present invention provides a method to increase the expression of NGN3 and NKX6.1 in a population of cells expressing markers characteristic of the pancreatic endocrine lineage, comprising the steps of:

a) culturing pluripotent stem cells,

b) differentiating the pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage,

c) differentiating the cells expressing markers characteristic of the definitive endoderm lineage into cells expressing markers characteristic of the pancreatic endoderm lineage, supplementing the medium used to differentiate the cells expressing markers characteristic of the definitive endoderm lineage with a compound selected from the group consisting of H-9, H-89, GF 109203X, HA-1004, PP2, PP1, LY 294002, Wortmannin, SB-203580, SB-202190, Tyrphostin 25, Tyrphostin, AG1478, Tyrphostin 46, GW 5074, Kenpaullone, HNMPA, AG490, Y27632, and ML-7, and

d) differentiating the cells expressing markers characteristic of the pancreatic endoderm lineage into cells expressing markers characteristic of the pancreatic endocrine lineage.

Differentiation of Pluripotent Stem Cells into Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage

[0066] Formation of cells expressing markers characteristic of the definitive endoderm lineage may be determined by testing for the presence of the markers before and after following a particular protocol. Pluripotent stem cells typically do not express such markers. Thus, differentiation of pluripotent cells is detected when cells begin to express them.

[0067] Pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by any method in the art or by any method proposed in this invention.

[0068] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in D'Amour *et al*, Nature Biotechnology 23, 1534 – 1541 (2005).

[0069] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in Shinozaki *et al*, Development 131, 1651 - 1662 (2004).

[0070] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in McLean *et al*, Stem Cells 25, 29 - 38 (2007).

[0071] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in D'Amour *et al*, Nature Biotechnology 24, 1392 – 1401 (2006).

[0072] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by culturing the pluripotent stem cells in medium containing activin A in the absence of serum, then culturing the cells with activin A and serum, and then culturing the cells with activin A and serum of a different concentration. An example of this method is disclosed in Nature Biotechnology 23, 1534 - 1541 (2005).

[0073] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by culturing the pluripotent stem cells in medium containing activin A in the absence of serum, then culturing the cells with activin A with serum of another concentration. An example of this method is disclosed in D' Amour *et al*, Nature Biotechnology, 2005.

[0074] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by culturing the pluripotent stem cells in medium containing activin A and a Wnt ligand in the absence of serum, then removing the Wnt ligand and culturing the cells with activin A with serum. An example of this method is disclosed in Nature Biotechnology 24, 1392 - 1401 (2006).

[0075] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in US patent application Ser. No. 11/736,908, assigned to LifeScan, Inc.

[0076] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in US patent application Ser. No. 11/779,311, assigned to LifeScan, Inc.

[0077] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in US patent application Ser. No. 60/990,529.

[0078] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in US patent application Ser. No. 61/076,889.

[0079] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in US patent application Ser. No. 61/076,900.

[0080] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in US patent application Ser. No. 61/076,908.

[0081] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in US patent application Ser. No. 61/076,915.

Differentiation of Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage into Cells Expressing Markers Characteristic of the Pancreatic Endoderm Lineage

[0082] Cells expressing markers characteristic of the definitive endoderm lineage may be differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage by any method in the art or by any method proposed in this invention.

[0083] For example, cells expressing markers characteristic of the definitive endoderm lineage may be differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage according to the methods disclosed in D'Amour *et al.*, Nature Biotechnol. 24:1392-1401, 2006.

[0084] For example, cells expressing markers characteristic of the definitive endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage, by treating the cells expressing markers characteristic of the definitive endoderm lineage with a fibroblast growth factor and the hedgehog signaling pathway inhibitor KAAD-cyclopamine, then removing the medium containing the fibroblast growth factor and KAAD-cyclopamine and subsequently culturing the cells in medium containing retinoic acid, a fibroblast growth factor and KAAD-cyclopamine. An example of this method is disclosed in Nature Biotechnology 24, 1392 - 1401 (2006).

[0085] In one aspect of the present invention, cells expressing markers characteristic of the definitive endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage, by treating the cells expressing markers characteristic of the definitive endoderm lineage with retinoic acid and at least one fibroblast growth factor for a period of time, according to the methods disclosed in US patent application Ser. No. 11/736,908, assigned to LifeScan, Inc.

[0086] In one aspect of the present invention, cells expressing markers characteristic of the definitive endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage, by treating the cells expressing markers characteristic of the definitive endoderm lineage with retinoic acid and at least one fibroblast growth factor for a period of time, according to the methods disclosed in US patent application Ser. No. 11/779,311, assigned to LifeScan, Inc.

[0087] In one aspect of the present invention, cells expressing markers characteristic of the definitive endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage, by treating the cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in US patent application Ser. No. 60/990,529.

[0088] The efficiency of differentiation may be determined by exposing a treated cell population to an agent (such as an antibody) that specifically recognizes a protein marker expressed by cells expressing markers characteristic of the definitive endoderm lineage.

[0089] Methods for assessing expression of protein and nucleic acid markers in cultured or isolated cells are standard in the art. These include quantitative reverse transcriptase polymerase chain reaction (RT-PCR), Northern blots, *in situ* hybridization (see, e.g., Current Protocols in Molecular Biology (Ausubel *et al.*, eds. 2001 supplement)), and immunoassays, such as immunohistochemical analysis of sectioned material, Western blotting, and for markers that are accessible in intact cells, such as flow cytometric analysis (FACS) (see, e.g., Harlow and Lane, Using Antibodies: A Laboratory Manual, New York: Cold Spring Harbor Laboratory Press (1998)).

[0090] Characteristics of pluripotent stem cells are well known to those skilled in the art, and additional characteristics of pluripotent stem cells continue to be identified. Pluripotent stem cell markers include, for example, the expression of one or more of the following: ABCG2, cripto, FOXD3, CONNEXIN43, CONNEXIN45, OCT4, SOX2, Nanog, hTERT, UTF1, ZFP42, SSEA-3, SSEA-4, Tra 1-60, Tra 1-81.

[0091] After treating pluripotent stem cells with the methods of the present invention, the differentiated cells may be purified by exposing a treated cell population to an agent (such as an antibody) that specifically recognizes a protein marker, such as CXCR4, expressed by cells expressing markers characteristic of the definitive endoderm lineage.

[0092] Pluripotent stem cells suitable for use in the present invention include, for example, the human embryonic stem cell line H9 (NIH code: WA09), the human embryonic stem cell line H1 (NIH code: WA01), the human embryonic stem cell line H7 (NIH code: WA07), and the human embryonic stem cell line SA002 (Cellartis, Sweden).

Also suitable for use in the present invention are cells that express at least one of the following markers characteristic of pluripotent cells: ABCG2, cripto, CD9, FOXD3, CONNEXIN43, CONNEXIN45, OCT4, SOX2, Nanog, hTERT, UTF1, ZFP42, SSEA-3, SSEA-4, Tra 1-60, and Tra 1-81.

[0093] Markers characteristic of the definitive endoderm lineage are selected from the group consisting of SOX17, GATA4, HNF3 beta, GSC, CER1, Nodal, FGF8, Brachyury, Mix-like homeobox protein, FGF4 CD48, eomesodermin (EOMES), DKK4, FGF17, GATA6, CXCR4, C-Kit, CD99, and OTX2. Suitable for use in the present invention is a cell that expresses at least one of the markers characteristic of the definitive endoderm lineage. In one aspect of the present invention, a cell expressing markers characteristic of the definitive endoderm lineage is a primitive streak precursor cell. In an alternate aspect, a cell expressing markers characteristic of the definitive endoderm lineage is a mesendoderm cell. In an alternate aspect, a cell expressing markers characteristic of the definitive endoderm lineage is a definitive endoderm cell.

[0094] Markers characteristic of the pancreatic endoderm lineage are selected from the group consisting of PDX1, HNF1 beta, PTF1 alpha, HNF6, HB9 and PROX1. Suitable for use in the present invention is a cell that expresses at least one of the markers characteristic of the pancreatic endoderm lineage. In one aspect of the present invention, a cell expressing markers characteristic of the pancreatic endoderm lineage is a pancreatic endoderm cell.

[0095] In one embodiment, the cells expressing markers characteristic of the pancreatic endoderm lineage are further differentiated into cells expressing markers of the characteristic of the pancreatic endocrine lineage. The present invention provides methods to increase the expression of NGN3 and NKX6.1 in populations of cells expressing markers characteristic of the pancreatic endocrine lineage.

[0096] Increasing the expression of NGN3 and NKX6.1 in populations of cells expressing markers characteristic of the pancreatic endocrine lineage may be achieved by treating cells expressing markers expressing markers characteristic of the definitive endoderm lineage with a compound selected from the group consisting of H-9, H-89, GF 109203X, HA-1004, PP2, PP1, LY 294002, Wortmannin, SB-203580, SB-202190,

Tyrphostin 25, Tyrphostin, AG1478, Tyrphostin 46, GW 5074, Kenpaullone, HNMPA, AG490, Y27632, and ML-7. Alternatively, increasing the expression of NGN3 and NKX6.1 in populations of cells expressing markers characteristic of the pancreatic endocrine lineage may be achieved by treating cells expressing markers expressing markers characteristic of the pancreatic endoderm lineage with a compound selected from the group consisting of H-9, H-89, GF 109203X, HA-1004, PP2, PP1, LY 294002, Wortmannin, SB-203580, SB-202190, Tyrphostin 25, Tyrphostin, AG1478, Tyrphostin 46, GW 5074, Kenpaullone, HNMPA, AG490, Y27632, and ML-7.

[0097] In the case where cells expressing markers expressing markers characteristic of the definitive endoderm lineage are treated with a compound selected from the group consisting of X, Y, and Z, the cells are treated by supplementing the medium used to differentiate the cells to cells expressing markers characteristic of the pancreatic endoderm lineage with a compound selected from the group consisting of H-9, H-89, GF 109203X, HA-1004, PP2, PP1, LY 294002, Wortmannin, SB-203580, SB-202190, Tyrphostin 25, Tyrphostin, AG1478, Tyrphostin 46, GW 5074, Kenpaullone, HNMPA, AG490, Y27632, and ML-7.

[0098] In the case where cells expressing markers expressing markers characteristic of the pancreatic endoderm lineage are treated with a compound selected from the group consisting of X, Y, and Z, the cells are treated by supplementing the medium used to differentiate the cells to cells expressing markers characteristic of the pancreatic endocrine lineage with a compound selected from the group consisting of H-9, H-89, GF 109203X, HA-1004, PP2, PP1, LY 294002, Wortmannin, SB-203580, SB-202190, Tyrphostin 25, Tyrphostin, AG1478, Tyrphostin 46, GW 5074, Kenpaullone, HNMPA, AG490, Y27632, and ML-7.

Differentiation of Cells Expressing Markers Characteristic of the Pancreatic Endoderm Lineage into Cells Expressing Markers Characteristic of the Pancreatic Endocrine Lineage with an Increased Expression of NGN3 and NKX6.1

[0099] Cells expressing markers characteristic of the pancreatic endoderm lineage may be differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage by any method in the art or by any method proposed in this invention.

[0100] For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by culturing the cells expressing markers characteristic of the pancreatic endoderm lineage in medium containing exendin 4, then removing the medium containing exendin 4 and subsequently culturing the cells in medium containing exendin 1, IGF-1 and HGF. An example of this method is disclosed in D' Amour *et al*, Nature Biotechnology, 2006.

[0101] For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by culturing the cells expressing markers characteristic of the pancreatic endoderm lineage in medium containing DAPT (Sigma-Aldrich, MO) and exendin 4. An example of this method is disclosed in D' Amour *et al*, Nature Biotechnology, 2006.

[0102] For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by culturing the cells expressing markers characteristic of the pancreatic endoderm lineage in medium containing exendin 4. An example of this method is disclosed in D' Amour *et al*, Nature Biotechnology, 2006.

[0103] For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the cells expressing markers characteristic of the pancreatic endoderm lineage with a factor that inhibits the Notch signaling pathway, according to the methods disclosed in US patent application Ser. No. 11/736,908, assigned to LifeScan, Inc.

[0104] For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention are further differentiated into cells expressing markers characteristic of the pancreatic endocrine

lineage, by treating the cells expressing markers characteristic of the pancreatic endoderm lineage with a factor that inhibits the Notch signaling pathway, according to the methods disclosed in US patent application Ser. No. 11/779,311, assigned to LifeScan, Inc.

[0105] For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the cells expressing markers characteristic of the pancreatic endoderm lineage with a factor that inhibits the Notch signaling pathway, according to the methods disclosed in US patent application Ser. No. 60/953,178, assigned to LifeScan, Inc.

[0106] For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the cells expressing markers characteristic of the pancreatic endoderm lineage with a factor that inhibits the Notch signaling pathway, according to the methods disclosed in US patent application Ser. No. 60/990,529, assigned to LifeScan, Inc.

[0107] Markers characteristic of the pancreatic endocrine lineage are selected from the group consisting of NGN3, NEUROD, ISL1, PDX1, NKX6.1, PAX4, NGN3, and PTF-1 alpha. In one embodiment, a pancreatic endocrine cell is capable of expressing at least one of the following hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide. Suitable for use in the present invention is a cell that expresses at least one of the markers characteristic of the pancreatic endocrine lineage. In one aspect of the present invention, a cell expressing markers characteristic of the pancreatic endocrine lineage is a pancreatic endocrine cell. The pancreatic endocrine cell may be a pancreatic hormone-expressing cell. Alternatively, the pancreatic endocrine cell may be a pancreatic hormone-secreting cell.

[0108] In one aspect of the present invention, the pancreatic endocrine cell is a cell expressing markers characteristic of the β cell lineage. A cell expressing markers characteristic of the β cell lineage expresses PDX1 and at least one of the following

transcription factors: NGN3, NKX2.2, NKX6.1, NEUROD, ISL1, HNF3 beta, MAFA, PAX4, and PAX6. In one aspect of the present invention, a cell expressing markers characteristic of the β cell lineage is a β cell.

[0109] The present invention provides methods to increase the expression of NGN3 and NKX6.1 in populations of cells expressing markers characteristic of the pancreatic endocrine lineage.

[0110] In one embodiment, increasing the expression of NGN3 and NKX6.1 in populations of cells expressing markers characteristic of the pancreatic endocrine lineage may be achieved by treating cells expressing markers expressing markers characteristic of the pancreatic endoderm lineage with a compound selected from the group consisting of H-9, H-89, GF 109203X, HA-1004, PP2, PP1, LY 294002, Wortmannin, SB-203580, SB-202190, Tyrphostin 25, Tyrphostin, AG1478, Tyrphostin 46, GW 5074, Kenpaullone, HNMPA, AG490, Y27632, and ML-7.

[0111] In the case where cells expressing markers expressing markers characteristic of the pancreatic endoderm lineage are treated with a compound selected from the group consisting of H-9, H-89, GF 109203X, HA-1004, PP2, PP1, LY 294002, Wortmannin, SB-203580, SB-202190, Tyrphostin 25, Tyrphostin, AG1478, Tyrphostin 46, GW 5074, Kenpaullone, HNMPA, AG490, Y27632, and ML-7, the cells are treated by supplementing the medium used to differentiate the cells to cells expressing markers characteristic of the pancreatic endocrine lineage with a compound selected from the group consisting of H-9, H-89, GF 109203X, HA-1004, PP2, PP1, LY 294002, Wortmannin, SB-203580, SB-202190, Tyrphostin 25, Tyrphostin, AG1478, Tyrphostin 46, GW 5074, Kenpaullone, HNMPA, AG490, Y27632, and ML-7.

[0112] The present invention is further illustrated, but not limited by, the following examples.

EXAMPLES

Example 1

Screening for Small Molecule Analogues that Mediate NGN3 Expression

[0113] Expression of the transcription factor NGN3 is required during the progression of progenitor cells towards an endocrine cell fate. Enhancing the efficiency of this process is a desirable outcome. A screen of small molecule compounds was performed on the assumption that enzymatic inhibitors may regulate cellular signals transmitted during differentiation and have direct or indirect effects on the gene expression of critical transcription factors such as NGN3.

[0114] *Preparation of cells for assay:* Stock cultures of human embryonic stem cells (H1 human embryonic stem cell line) were maintained in an undifferentiated, pluripotent state on reduced growth factor MATRIGEL (BD Biosciences; Cat # 356231)-coated dishes in MEF conditioned medium with passage on average every four days. Passage was performed by exposing cell cultures to a solution of 1 mg/ml dispase (Invitrogen, Cat #: 17105-041) for 5 to 7 minutes at 37°C followed by rinsing the monolayer with MEF conditioned culture medium and gentle scraping to recover cell clusters. Clusters were centrifuged at low speed to collect a cell pellet and remove residual dispase. Cell clusters were split at a 1:3 or 1:4 ratio for routine maintenance culture. All human embryonic stem cell lines were maintained at passage numbers less than 50 and routinely evaluated for normal karyotype and absence of mycoplasma. For screens in miniaturized assay format, clusters of H1 human embryonic stem cells were harvested from culture with dispase treatment as described and plated with even dispersal at a ratio of 1:2 (surface area) on reduced growth factor MATRIGEL (BD Biosciences; Cat # 356231)-coated 96-well black plates (Packard ViewPlates; PerkinElmer; Cat #6005182) using volumes of 100 µl/well. Cells were allowed to attach and then recover log phase growth over a 1 to 3 day time period, feeding daily with MEF conditioned medium supplemented with 8ng/ml bFGF (R&D Systems; Cat # 233-FB). Plates were maintained at 37°C, 5% CO_2 in a humidified box throughout the duration of assay.

[0115] *Preparation of compounds:* Screening was conducted using two commercial libraries of small molecule kinase inhibitors (BioMol Intl; Cat # 2832A(V2.2) and EMD Biosciences: Cat # 539745). Table 1 and Table 2 describe the compounds in these BioMol and EMD kinase inhibitor libraries, respectively. Compounds from these libraries were made available as 10 mM stocks in 96-well plate format, solubilized in 100% DMSO and stored at –80°C. The library compounds were further diluted to an

intermediate concentration of 2.5mM in 100% DMSO (Sigma; Cat # D2650), also stored at –80°C until use. On the day of assay, compounds were diluted 1:12.5 into DMEM high glucose medium to yield a 200uM working stock in 8% DMSO and then further diluted 1:80 into each assay test well for a final concentration of 2.5μM compound and 0.1% DMSO.

[0116] *Differentiation and Screening Assay:* Step 1 of the differentiation protocol was conducted over three days, feeding daily by aspirating the medium from each well and replacing with a fresh aliquot (100μl). On the first day of assay, wells were fed using RPMI-1640 medium (Invitrogen; Cat #: 22400) containing 2% Albumin Bovine Fraction V, Fatty Acid Free (FAF BSA) (Proliant Inc.; Cat #: SKU 68700), 100ng/ml Activin A (PeproTech; Cat #120-14), 20ng/ml Wnt3a (R&D Systems; Cat # 1324-WN/CF), and 8ng/ml bFGF (R&D Systems; Cat # 233-FB). On the second and third day of assay, wells were fed with the same medium except that Wnt3a was removed. All wells were fed and treated identically.

[0117] Step 2 of the differentiation protocol was conducted over two days. Cells were fed daily by aspirating the medium from each well and replacing with a fresh aliquot (100μl) of DMEM:F12 medium (Invitrogen; Cat # 11330-032) containing 2% FAF BSA, 50ng/ml FGF7 (PeproTech; Cat # 100-19), and 250nM KAAD-cyclopamine (Calbiochem; Cat # 239804). All wells were fed and treated identically.

[0118] Step 3 of the differentiation protocol was conducted over four days. Cells were fed on alternating days by aspirating medium from each well and replacing with a fresh aliquot (200ul) of DMEM-high glucose (Invitrogen; Cat # 10569) supplemented with 0.1% Albumax (Invitrogen; Cat #: 11020-021), 0.5x Insulin-Transferrin-Selenium (ITS-X; Invitrogen; Cat # 51500056), 50 ng/ml FGF7, 100 ng/ml Noggin (R&D Systems; Cat # 3344-NG), 250 nM KAAD-cyclopamine, 2 μM all-trans retinoic acid (RA) (Sigma-Aldrich; Cat # R2625), and 30ng/ml Activin A. During step 3, test samples of kinase inhibitors were added to single wells in two individual plates (Plates A and B); a third plate (Plate C) was left untreated. In each plate, a total of 16 control wells were treated with an equivalent amount of 0.1% DMSO without any test compound.

[0119] Step 4 of the differentiation protocol was conducted over three days. Cells were fed on days 1 and 2, not day 3, by aspirating the medium from each well and replacing with a fresh aliquot (200ul) of DMEM-high glucose supplemented with 0.1% Albumax, 0.5x Insulin-Transferrin-Selenium, 100 ng/ml Noggin, and 1μM Alk 5 inhibitor (Axxora; Cat # ALX-270-445). During step 4, test samples of kinase inhibitors were added to single wells in two individual plates (Plates B and C); a third plate was left untreated (Plate A). In each plate, a total of 16 control wells were treated with an equivalent amount of 0.1% DMSO without any test compound.

[0120] *High Content Analysis:* At the conclusion of step 4, medium from all assay plates was aspirated followed by fixing at room temperature for 20 minutes with 4% paraformaldehyde (Sigma-Aldrich; Cat # 158127) diluted in PBS without divalent cations (Invitrogen; Cat # 14190), then washing once with PBS. Sample wells were permeabilized with 0.5% Triton X-100 (VWR; Cat # VW3929-2) for 20 minutes at room temperature, washed two times with PBS, and blocked with 5% donkey serum (Jackson ImmunoResearch; Cat # 017-000-121) in PBS for 30 minutes at room temperature. Primary antibody (sheep anti-NGN3; R&D Systems; AF3444) was diluted 1:300 in 5% donkey serum and added to each well for one hour at room temperature. After washing two times in PBS, Alexa Fluor 647 donkey anti-sheep secondary antibody (Invitrogen; Cat # A21448) was diluted 1:100 and added to each sample well for 30 minutes at room temperature, followed by two washes in PBS. To counterstain nuclei, 4μg/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for ten minutes at room temperature. Plates were washed once with PBS and left in 100μl/well PBS for imaging.

[0121] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Flour 647. Exposure times were optimized from positive control wells stained with secondary antibody alone. Images from 15 fields per well were acquired to compensate for any cell loss during the bioassay and subsequent staining procedures. Measurements for total cell number and total NGN3 intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Total NGN3 protein expression was reported as total intensity or integrated intensity,

defined as total fluorescence of the cell multiplied by the area of the cell. Background was eliminated based on acceptance criteria of gray-scale ranges between 200 to 3500. Total intensity data were normalized by dividing total intensities for each well by the average total intensity for the positive control.

[0122] Screening results are shown in Table 3 from the combination of two kinase inhibitor libraries used to treat six assay plates in this single experiment. Data shown are a representative ratio of the intensity of NGN3 staining for individual compound treated wells relative to staining in wells with DMSO vehicle alone. Intensity ratios as well as rank order comparisons are shown for individual compounds dosed during stage 3 alone or stage 4 alone or combined stages 3 and 4. Compounds with ratio intensities >1.4 relative to a vehicle treated control were tagged as hits for confirmation and additional evaluation. Of special interest, as summarized in Table 4, these compounds appear to target several cell signaling pathways that may be involved in the optimal expression pattern of NGN3 during endocrine differentiation.

Example 2

Screening for Small Molecule Analogues that Mediate NKX6.1 and NGN3 Expression

[0123] Expression of NKX6.1, along with NGN3 is required during the progression of progenitor cells towards an endocrine cell fate. A screen of kinase inhibitors was conducted to determine if any could up-regulate the expression of one or both markers during differentiation. In this example, the HDAC inhibitor Trichostatin A was also included in the differentiation protocol to modulate chromatin remodeling and possibly enhance gene transcription.

[0124] *Preparation of cells for assay:* Stock cultures of human embryonic stem cells (H1 human embryonic stem cell line) were maintained in an undifferentiated, pluripotent state on reduced growth factor MATRIGEL (BD Biosciences; Cat # 356231)-coated dishes in MEF conditioned medium with passage on average every four days. Passage was performed by exposing cell cultures to a solution of 1 mg/ml dispase (Invitrogen, Cat #: 17105-041) for 5 to 7 minutes at 37°C followed by rinsing the monolayer with MEF conditioned culture medium and gentle scraping to recover cell clusters. Clusters were centrifuged at low speed to collect a cell pellet and remove

residual dispase. Cell clusters were split at a 1:3 or 1:4 ratio for routine maintenance culture. All human embryonic stem cell lines were maintained at passage numbers less than 50 and routinely evaluated for normal karyotype and absence of mycoplasma. For screens in miniaturized assay format, clusters of H1 human embryonic stem cells were harvested from culture with dispase treatment as described and plated with even dispersal at a ratio of 1:2 (surface area) on reduced growth factor MATRIGEL (BD Biosciences; Cat # 356231)-coated 96-well black plates (Packard ViewPlates; PerkinElmer; Cat #6005182) using volumes of 100 µl/well. Cells were allowed to attach and then recover log phase growth over a 1 to 3 day time period, feeding daily with MEF conditioned medium supplemented with 8ng/ml bFGF (R&D Systems; Cat # 233-FB). Plates were maintained at 37°C, 5% CO_2 in a humidified box throughout the duration of assay.

[0125] *Preparation of compounds:* Screening was conducted using a single commercial library of small molecule kinase inhibitors (BioMol Intl; Cat # 2832A(V2.2) as defined in Table 1. Compounds from this library were made available as 10 mM stocks in 96-well plate format, solubilized in 100% DMSO and stored at –80°C. The library compounds were further diluted to an intermediate concentration of 2.5mM in 100% DMSO (Sigma; Cat # D2650), also stored at –80°C until use. On the day of assay, compounds were diluted 1:12.5 into DMEM high glucose medium to yield a 200uM working stock in 8% DMSO and then further diluted 1:80 into each assay test well for a final concentration of 2.5µM compound and 0.1% DMSO.

[0126] *Differentiation and Screening Assay:* Step 1 of the differentiation protocol was conducted over three days, feeding daily by aspirating the medium from each well and replacing with a fresh aliquot (100µl). On the first day of assay, wells were fed using RPMI-1640 medium (Invitrogen; Cat #: 22400) containing 2% Albumin Bovine Fraction V, Fatty Acid Free (FAF BSA) (Proliant Inc.; Cat #: SKU 68700), 100ng/ml Activin A (PeproTech; Cat #120-14), 20ng/ml Wnt3a (R&D Systems; Cat # 1324-WN/CF), and 8ng/ml bFGF (R&D Systems; Cat # 233-FB). On the second and third day of assay, wells were fed with the same medium except that Wnt3a was removed. All wells were fed and treated identical.

[0127] Step 2 of the differentiation protocol was conducted over two days. Cells were fed daily by aspirating the medium from each well and replacing with a fresh aliquot

(100µl) of DMEM:F12 medium (Invitrogen; Cat # 11330-032) containing 2% FAF BSA, 50ng/ml FGF7 (PeproTech; Cat # 100-19), and 250nM KAAD-cyclopamine (Calbiochem; Cat # 239804). All wells were fed and treated identically.

[0128] Step 3 of the differentiation protocol was conducted over five days. Cells were fed on alternating days by aspirating medium from each well and replacing with a fresh aliquot (200µl) of DMEM-high glucose (Invitrogen; Cat # 10569) supplemented with 0.1% Albumax (Invitrogen; Cat #: 11020-021), 0.5x Insulin-Transferrin-Selenium (ITS-X; Invitrogen; Cat # 51500056), 50 ng/ml FGF7, 100 ng/ml Noggin (R&D Systems; Cat # 3344-NG), 250 nM KAAD-cyclopamine, 2 µM all-trans retinoic acid (RA) (Sigma-Aldrich; Cat # R2625), 30ng/ml Activin A, and 100nM Trichostatin A (TsA; Sigma; Cat # T8552). During step 3, test samples of kinase inhibitors were added to single wells on days 2 and 4. In each plate, a total of 16 control wells were treated with an equivalent amount of 0.1% DMSO without any test compound.

[0129] Step 4 of the differentiation protocol was conducted over three days. Cells were fed daily by aspirating the medium from each well and replacing with a fresh aliquot (200µl) of DMEM-high glucose supplemented with 0.1% Albumax, 0.5x Insulin-Transferrin-Selenium, 100 ng/ml Noggin, 1µM Alk 5 inhibitor (Axxora; Cat # ALX-270-445), and 1ug/ml DAPT (Sigma; Cat #D5942). During step 4, test samples of kinase inhibitors were added to single wells on the first day along with 100nM Trichostatin A, then both test samples of kinase inhibitors and TsA were omitted during feeding on days 2 and 3. In each plate, a total of 16 control wells were treated with an equivalent amount of 0.1% DMSO without any test compound.

[0130] *High Content Analysis:* At the conclusion of step 4, medium from all wells was aspirated followed by fixing at room temperature for 20 minutes with 4% paraformaldehyde (Sigma-Aldrich; Cat # 158127) diluted in PBS without divalent cations (Invitrogen; Cat # 14190), then washing once with PBS. Sample wells were permeabilized with 0.5% Triton X-100 (VWR; Cat # VW3929-2) for 20 minutes at room temperature, washed two times with PBS, and blocked with 5% donkey serum (Jackson ImmunoResearch; Cat # 017-000-121) in PBS for 30 minutes at room temperature. Primary antibodies (sheep anti-NGN3; R&D Systems; AF3444 or mouse anti-NKX6.1; University of Iowa; Cat # F55A12) were diluted (1:300 for anti-

NGN3; 1:500 for anti-NKX6.1) in 5% donkey serum and added to each well for one hour at room temperature. After washing two times in PBS, Alexa Fluor 647 donkey anti-sheep secondary antibody (Invitrogen; Cat # A21448) and Alexa Fluor 488 donkey anti-mouse secondary antibody (Invitrogen; Cat #A21202) were diluted 1:100 (both secondary antibodies) and added to each sample well for 30 minutes at room temperature, followed by two washes in PBS. To counterstain nuclei, 4μg/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for ten minutes at room temperature. Plates were washed once with PBS and left in 100μl/well PBS for imaging.

[0131] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488 and Alexa Flour 647. Exposure times were optimized from positive control wells stained with each secondary antibody alone. Images from 15 fields per well were acquired to compensate for any cell loss during the bioassay and subsequent staining procedures. Measurements for total cell number and total NGN3 or NKX6.1 intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Total NGN3 or NKX6.1 protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell multiplied by the area of the cell. Background was eliminated based on acceptance criteria of gray-scale ranges between 200 to 3500. Total intensity data were normalized by dividing total intensities for each well by the average total intensity for the positive control.

[0132] Results from this screen are summarized in Table 5, Table 6, and Table 7. Data in Table 5 depict a representative ratio of NGN3 and NKX6.1 staining for each well treated with an individual compound relative to average staining in wells with DMSO alone. In addition, the rank order for each compound's effect on protein expression for either NGN3 or NKX6.1 is also shown. Table 6 lists ordered rankings for the top 16 hits having a positive effect on NGN3 and/or NKX6.1 expression. Table 7 summarizes the targets and signal transduction pathways that correspond to these top hits. Pathways with multiple hits from this screen would appear to have greatest

validity for having an impact on expression on these two transcription factors critical for endocrine fate determination.

Example 3

Confirmations for Small Molecule Analogues that Mediate NGN3 and NKX6.1 Expression

[0133] Expression of a NKX6.1, along with NGN3 is required during the progression of progenitor cells towards an endocrine cell fate. A screen of kinase inhibitors was repeated to determine if any small molecule compounds could up-regulate expression of one or both markers during differentiation. In this example, the HDAC inhibitor Trichostatin A was also included in the differentiation protocol to modulate chromatin remodeling and possibly enhance gene transcription.

[0134] *Preparation of cells for assay:* Stock cultures of human embryonic stem cells (H1 human embryonic stem cell line) were maintained in an undifferentiated, pluripotent state on reduced growth factor MATRIGEL (BD Biosciences; Cat # 356231)-coated dishes in MEF conditioned medium with passage on average every four days. Passage was performed by exposing cell cultures to a solution of 1 mg/ml dispase (Invitrogen, Cat #: 17105-041) for 5 to 7 minutes at 37°C followed by rinsing the monolayer with MEF conditioned culture medium and gentle scraping to recover cell clusters. Clusters were centrifuged at low speed to collect a cell pellet and remove residual dispase. Cell clusters were split at a 1:3 or 1:4 ratio for routine maintenance culture. All human embryonic stem cell lines were maintained at passage numbers less than 50 and routinely evaluated for normal karyotype and absence of mycoplasma. For screens in miniaturized assay format, clusters of H1 human embryonic stem cells were harvested from culture with dispase treatment as described and plated with even dispersal at a ratio of 1:2 (surface area) on reduced growth factor MATRIGEL (BD Biosciences; Cat # 356231)-coated 96-well black plates (Packard ViewPlates; PerkinElmer; Cat #6005182) using volumes of 100 µl/well. Cells were allowed to attach and then recover log phase growth over a 1 to 3 day time period, feeding daily with MEF conditioned medium supplemented with 8ng/ml bFGF (R&D Systems; Cat # 233-FB). Plates were maintained at 37°C, 5% CO_2 in a humidified box throughout the duration of assay.

[0135] *Preparation of compounds:* Confirmation screening was conducted using a single commercial library of small molecule kinase inhibitors (BioMol Intl; Cat # 2832A(V2.2) as defined in Table 1. Compound hits of interest from this library were made available as 10 mM stocks in 96-well plate format, solubilized in 100% DMSO and stored at –80°C. Individual library compounds of interest were further diluted to an intermediate concentration of 2.5mM in 100% DMSO (Sigma; Cat # D2650), also stored at –80°C until use. On the day of assay, these individual compounds of interest were diluted 1:12.5 into DMEM high glucose medium to yield a 200μM working stock in 8% DMSO and then further diluted 1:80 into each assay test well for a final concentration of 2.5μM compound and 0.1% DMSO.

[0136] *Differentiation and Screening Assay:* Step 1 of the differentiation protocol was conducted over three days, feeding daily by aspirating the medium from each well and replacing with a fresh aliquot (100μl). On the first day of assay, wells were fed using RPMI-1640 medium (Invitrogen; Cat #: 22400) containing 2% Albumin Bovine Fraction V, Fatty Acid Free (FAF BSA) (Proliant Inc.; Cat #: SKU 68700), 100ng/ml Activin A (PeproTech; Cat #120-14), 20ng/ml Wnt3a (R&D Systems; Cat # 1324-WN/CF), and 8ng/ml bFGF (R&D Systems; Cat # 233-FB). On the second and third day of assay, wells were fed with the same medium except that Wnt3a was removed. All wells were fed and treated identically.

[0137] Step 2 of the differentiation protocol was conducted over two days. Cells were fed daily by aspirating the medium from each well and replacing with a fresh aliquot (100μl) of DMEM:F12 medium (Invitrogen; Cat # 11330-032) containing 2% FAF BSA, 50ng/ml FGF7 (PeproTech; Cat # 100-19), and 250nMKAAD-cyclopamine (Calbiochem; Cat # 239804). All wells were fed and treated identically.

[0138] Step 3 of the differentiation protocol was conducted over four days. Cells were fed on alternating days by aspirating medium from each well and replacing with a fresh aliquot (200μl) of DMEM-high glucose (Invitrogen; Cat # 10569) supplemented with 0.1% Albumax (Invitrogen; Cat #: 11020-021), 0.5x Insulin-Transferrin-Selenium (ITS-X; Invitrogen; Cat # 51500056), 50 ng/ml FGF7, 100 ng/ml Noggin (R&D Systems; Cat # 3344-NG), 250 nM KAAD-cyclopamine, 2 μM all-trans retinoic acid (RA) (Sigma-Aldrich; Cat # R2625), and 20ng/ml Activin A. During step 3, triplicate

test samples of kinase inhibitors were added to wells at the time of feeding on days 1 and 3. In each plate, a total of 16 control wells were treated with an equivalent amount of 0.1% DMSO without any test compound.

[0139] Step 4 of the differentiation protocol was conducted over four days. Cells were fed on alternating days by aspirating the medium from each well and replacing with a fresh aliquot (200µl) of DMEM-high glucose supplemented with 0.1% Albumax, 0.5x Insulin-Transferrin-Selenium, 100 ng/ml Noggin, and 1µM Alk 5 inhibitor (Axxora; Cat # ALX-270-445). During step 4, triplicate test samples of kinase inhibitors were added to wells at the time of feeding on days 1 and 3. In each plate, a total of 16 control wells were treated with an equivalent amount of 0.1% DMSO without any test compound.

[0140] *High Content Analysis:* At the conclusion of step 4, medium from all wells was aspirated followed by fixing at room temperature for 20 minutes with 4% paraformaldehyde (Sigma-Aldrich; Cat # 158127) diluted in PBS without divalent cations (Invitrogen; Cat # 14190), then washing once with PBS. Sample wells were permeabilized with 0.5% Triton X-100 (VWR; Cat # VW3929-2) for 20 minutes at room temperature, washed two times with PBS, and blocked with 5% donkey serum (Jackson ImmunoResearch; Cat # 017-000-121) in PBS for 30 minutes at room temperature. Primary antibodies (sheep anti-NGN3; R&D Systems; AF3444 or mouse anti-NKX6.1 ; University of Iowa; Cat # F55A12) were diluted (1:300 for anti-NGN3; 1:500 for anti-NKX6.1) in 5% donkey serum and added to each well for one hour at room temperature. After washing two times in PBS, Alexa Fluor 647 donkey anti-sheep secondary antibody (Invitrogen; Cat # A21448) and Alexa Fluor 488 donkey anti-mouse secondary antibody (Invitrogen; Cat #A21202) were diluted 1:100 (both secondary antibodies) and added to each sample well for 30 minutes at room temperature, followed by two washes in PBS. To counterstain nuclei, 4µg/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for ten minutes at room temperature. Plates were washed once with PBS and left in 100µl/well PBS for imaging.

[0141] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488 and Alexa Flour 647. Exposure times were optimized from positive control wells stained

with each secondary antibody alone. Images from 15 fields per well were acquired to compensate for any cell loss during the bioassay and subsequent staining procedures. Measurements for total cell number and total NGN3 or NKX6.1 intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Total NGN3 or NKX6.1 protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell multiplied by the area of the cell. Background was eliminated based on acceptance criteria of gray-scale ranges between 200 to 3500. Total intensity data were normalized by dividing total intensities for each well by the average total intensity for the positive control.

[0142] Results for these studies are shown in Table 8. Two compounds (Kenpaullon and BML-259) did not confirm and have no enhancing effects on either NGN3 or NKX6.1 expression relative to a control treatment. The remaining compounds in this assay show a positive impact on one or both transcription factors, confirming earlier results and highlighting the importance of these associated signaling pathways.

Table 1: BioMol KINASE INHIBITOR LIBRARY (Cat # 2832, v2.2)

PLATE LOCATION	CAS #	COMPOUND NAME OR ID NUMBER	M.W.	TARGET
B1	167869-21-8	PD-98059	267.3	MEK
B2	109511-58-2	U-0126	380.5	MEK
B3	152121-47-6	SB-203580	377.4	p38 MAPK
B4	84477-87-2	H-7	364.3	PKA, PKG, MLCK, and PKC.
B5	84468-17-7	H-9	324.3	PKA, PKG, MLCK, and PKC.
B6	62996-74-1	Staurosporine	466.5	Pan-specific

B7	133550-35-5	AG-494	280.3	EGFRK, PDGFRK
B8		AG-825	397.5	HER1-2
B9	125697-92-9	Lavendustin A	381.4	EGFRK
B10	136831-49-7	RG-14620	274.1	EGFRK
B11	118409-57-7	Tyrphostin 23	186.1	EGFRK
B12	118409-58-8	Tyrphostin 25	202.1	EGFRK
C1	122520-85-8	Tyrphostin 46	204.2	EGFRK, PDGFRK
C2	122520-86-9	Tyrphostin 47	220.2	EGFRK
C3	122520-90-5	Tyrphostin 51	268.2	EGFRK

C4	2826-26-8	Tyrphostin 1	184.2	Negative control for tyrosine kinase inhibitors.
C5	116313-73-6	Tyrphostin AG 1288	231.2	Tyrosine kinases
C6	63177-57-1	Tyrphostin AG 1478	315.8	EGFRK
C7	71897-07-9	Tyrphostin AG 1295	234.3	Tyrosine kinases
C8	10537-47-0	Tyrphostin 9	282.4	PDGFRK
C9		HNMPA (Hydroxy-2-naphthalenylmethylphosphonic acid)	238.2	IRK
C10	120685-11-2	PKC-412	570.6	PKC inhibitor

C11	10083-24-6	Piceatannol	244.3	Syk
C12	172889-26-8	PP1	281.4	Src family
D1	133550-35-3	AG-490	294.3	JAK-2
D2		AG-126	215.2	IRAK
D3		AG-370	259.3	PDGFRK
D4		AG-879	316.5	NGFRK
D5	154447-36-6	LY 294002	307.4	PI 3-K
D6	19545-26-7	Wortmannin	428.4	PI 3-K
D7	133052-90-1	GF 109203X	412.5	PKC

D8	548-04-9	Hypericin	504.4	PKC
D9	138489-18-6	Ro 31-8220	553.7	PKC
D10	123-78-4	Sphingosine	299.5	PKC
D11	127243-85-0	H-89	519.2	PKA
D12	84478-11-5	H-8	338.3	PKA, PKG
E1	91742-10-8	HA-1004	329.8	PKA, PKG
E2	103745-39-7	HA-1077	327.8	PKA, PKG
E3		HDBA (2-Hydroxy-5-(2,5-dihydroxybenzylamino)benzoic acid)	275.3	EGFRK, CaMK II

E4	127191-97-3	KN-62	721.9	CaMK II
E5		KN-93	501	CaMK II
E6	109376-83-2	ML-7	452.7	MLCK
E7	105637-50-1	ML-9	361.3	MLCK
E8	452-06-2	2-Aminopurine	135.1	p58 PITSLRE beta1
E9	158982-15-1	N9-Isopropyl-olomoucine	326.4	CDK
E10	101622-51-9	Olomoucine	298.3	CDK
E11	101622-50-8	iso-Olomoucine	298.4	Negative control for olomoucine.
E12	186692-46-6	Roscovitine	354.5	CDK

F1	24386-93-4	5-Iodotubercidin	392.2	ERK2, adenosine kinase, CK1, CK2,
F2	62004-35-7	LFM-A13	360	BTK
F3	152121-30-7	SB-202190	331.3	p38 MAPK
F4	172889-27-9	PP2	301.8	Src family
F5	208260-29-1	ZM 336372	389.4	cRAF
F6	5812-07-7	SU 4312	264.3	Flk1
F7	146535-11-7	AG-1296	266.3	PDGFRK
F8	220904-83-6	GW 5074	520.9	cRAF
F9	6865-14-1	Palmitoyl-DL-carnitine Cl	436.1	PKC

F10	82-08-6	Rottlerin	516.6	PKC delta
F11	446-72-0	Genistein	270.2	Tyrosine Kinases
F12	486-66-8	Daidzein	254.2	Negative control for Genistein.
G1	63177-57-1	Erbstatin analog	194	EGFRK
G2	6151-25-3	Quercetin dihydrate	338.3	PI 3-K
G3		SU1498	390.5	Flk1
G4	4452-06-6	ZM 449829	182.2	JAK-3
G5	195462-67-7	BAY 11-7082	207.3	IKK pathway
G6	53-85-0	DRB (5,6-Dichloro-1-b-D-	319.1	CK II

		ribofuranosylbenzimidazole)		
G7		HBDDE (2,2',3,3',4,4'-Hexahydroxy-1,1'-biphenyl-6,6'-dimethanol dimethyl ether)	338.4	PKC alpha, PKC gamma
G8	129-56-6	SP 600125	220.2	JNK
G9	479-41-4	Indirubin	262	GSK-3beta, CDK5
G10	160807-49-8	Indirubin-3'-monoxime	277.3	GSK-3beta
G11	146986-50-7	Y-27632	338.3	ROCK
G12	142273-20-9	Kenpaullone	327.2	GSK-3beta
H1	121-40-4	Terreic acid	154.1	BTK

H2	35943-35-2	Triciribine	320.3	Akt signaling pathway
H3		BML-257	326.4	Akt
H4		SC-514	224.3	IKK2
H5		BML-259	260.4	Cdk5/p25
H6	520-36-5	Apigenin	270.2	CK-II
H7		BML-265 (Erlotinib analog)	305.4	EGFRK
H8	53123-88-9	Rapamycin	914.2	mTOR

Table 2: EMD Calbiochem KINASE INHIBITOR LIBRARY (Cat # 539745)

PLATE LOCATION	CAS #	COMPOUND NAME OR ID NUMBER	M.W.
A2	127191-97-3	KN-62	721.9
A3	587871-26-9	ATM Kinase Inhibitor	395.5
A4	905973-89-9	ATM/ATR Kinase Inhibitor	555.8
A5	237430-03-4	Alsterpaullone	293.3
A6	852527-97-0	Alsterpaullone, 2-Cyanoethyl	346.3
A7	496864-16-5	Aloisine A, RP107	267.3

A8	496864-15-4	Aloisine, RP106	281.4
A9	220792-57-4	Aminopurvalanol A	403.9
A10	866405-64-3	AMPK Inhibitor, Compound C	399.5
A11	879127-16-9	Aurora Kinase Inhibitor III	413.4
B2	443797-96-4	Aurora Kinase/Cdk Inhibitor	435.4
B3	160807-49-8	Indirubin-3′-monoxime	277.3
B4	19542-67-7	BAY 11-7082	207.2
B5	189232-42-6	Bohemine	340.4
B6	220749-41-7	Cdk1 Inhibitor	294.7

B7	190654-01-4	Cdk1 Inhibitor, CGP74514A	385.9
B8	443798-55-8	Cdk1/2 Inhibitor III	425.4
B9	40254-90-8	Cdk1/5 Inhibitor	185.2
B10	301836-43-1	Casein Kinase I Inhibitor, D4476	398.4
B11	934358-00-6	Casein Kinase II Inhibitor III, TBCA	463.8
C2	546102-60-7	Cdk4 Inhibitor	404.2
C3	141992-47-4	Cdk4 Inhibitor II, NSC 625987	271.3

C4	265312-55-8	Cdk4 Inhibitor III	284.3
C5	300801-52-9	Cdc2-Like Kinase Inhibitor, TG003	249.3
C6	516480-79-8	Chk2 Inhibitor II	363.8
C7	212779-48-1	Compound 52	346.8
C8	199986-75-9	Cdk2 Inhibitor III	400.5
C9	444723-13-1	Cdk2 Inhibitor IV, NU6140	422.5
C10	784211-09-2	Cdk/Crk Inhibitor	473.4
C11		ERK Inhibitor III	318.3

D2	146986-50-7	ROCK Inhibitor, Y-27632	338.3
D3	865362-74-9	ERK Inhibitor II, FR180204	327.3
D4		ERK Inhibitor II, Negative control	328.3
D5		Fascaplysin, Synthetic	306.8
D6		GSK-3b Inhibitor I	222.3
D7	478482-75-6	GSK-3b Inhibitor II	395.2
D8	487021-52-3	GSK-3b Inhibitor VIII	308.3
D9	667463-62-9	GSK-3 Inhibitor IX	356.2
D10		GSK-3 Inhibitor X	398.2

D11	626604-39-5	GSK-3b Inhibitor XI	349.3
E2	330161-87-0	SU6656	371.5
E3	404828-08-6	GSK-3 Inhibitor XIII	301.4
E4	244148-46-7	Isogranulatimide	276.3
E5	186611-52-9	IC261	311.3
E6	507475-17-4	IKK-2 Inhibitor IV	279.3
E7		Indirubin Derivative E804	365.4
E8	129-56-6	JNK Inhibitor II	220.2
E9		JNK Inhibitor, Negative Control	234.2

E10	345987-15-7	JNK Inhibitor V	372.5
E11	312917-14-9	JNK Inhibitor IX	350.4
F2	41179-33-3	MK2a Inhibitor	349.4
F3	894804-07-0	JNK Inhibitor VIII	356.4
F4	97161-97-2	K-252a, Nocardiopsis sp.	467.5
F5	142273-20-9	Kenpaullone	327.2
F6	139298-40-1	KN-93	501.0
F7		MEK Inhibitor I	374.5
F8	623163-52-0	MEK Inhibitor II	289.7

F9	305350-87-2	MEK1/2 Inhibitor	335.4
F10	522629-08-9	MNK1 Inhibitor	244.2
F11	545380-34-5	NF-kB Activation Inhibitor	356.4
G2	581098-48-8	p38 MAP Kinase Inhibitor III	404.5
G3	219138-24-6	p38 MAP Kinase Inhibitor	365.8
G4	167869-21-8	PD 98059	267.3
G5	152121-53-4	PD 169316	360.3
G6	165806-53-1	SB220025	338.4
G7	212844-53-6	Purvalanol A	388.9

G8		GSK3b Inhibitor XII, TWS119	318.3
G9	127243-85-0	H-89, Dihydrochloride	519.3
G10		SB 202474, Negative control for p38 MAPK inhibition studies	279.3
G11	152121-30-7	SB 202190	331.3
H2	152121-47-6	SB 203580	377.4
H3	103745-39-7	HA 1077, Dihydrochloride Fasudil	364.3
H4	135897-06-2	SB 218078	393.4

H5	318480-82-9	SC-68376	236.3
H6	72873-74-6	SKF-86002	297.4
H7		Sphingosine Kinase Inhibitor	339.2
H8	62996-74-1	Staurosporine, Streptomyces sp.	466.5
H9	52029-86-4	STO-609	374.4
H10	666837-93-0	SU9516	241.3
H11	871307-18-5	Tpl2 Kinase Inhibitor	404.8

Table 3: EMDII and BioMol Kinase Inhibitor Libraries

				NGN3 Intensity					
				Dose Stage 3		Dose Stage 4		Dose Stages 3&4	
LIBRARY	**WELL**	**TARGET**	**INHIBITOR**	**RANK**	**RATIO**	**RANK**	**RATIO**	**RANK**	**RATIO**
BioMol Kin INH	F - 4	Src family	PP2	**8**	**1.81**	**2**	**2.50**	**1**	**2.49**
BioMol Kin INH	D - 5	PI 3-K	LY 294002	**7**	**1.82**	**9**	**1.63**	**2**	**2.46**
BioMol Kin INH	B - 3	p38 MAPK	SB-203580	**3**	**2.23**	**7**	**1.88**	**3**	**2.34**
EMDII Kin INH	H - 2	p38	SB 203580	**19**	**1.53**	52	1.08	**4**	**2.22**

EMDII Kin INH	H - 5	p38	SC-68376	**29**	**1.41**	121	0.69	**5**	**2.12**
BioMol Kin INH	B - 4	PKA, PKG, MLCK, and PKC.	H-7	**5**	**1.93**	**5**	**2.18**	**6**	**2.08**
EMDII Kin INH	G - 10	NOT a p38 Inh	SB 202474, Neg control for p38 MAPK	116	0.88	62	1.03	**7**	**2.02**
EMDII Kin INH	A - 7	(CDK1/2/5)(GSK3i)	Aloisine A, RP107	58	1.25	55	1.06	**8**	**1.97**
EMDII Kin INH	H - 3	ROCK	HA 1077, Fasudil	47	1.32	**20**	**1.40**	**9**	**1.93**
BioMol Kin INH	F - 3	p38 MAPK	SB-202190	**11**	**1.72**	58	1.05	**10**	**1.83**
EMDII Kin	A - 2	CAMKII	KN-62	110	0.93	107	0.81	**11**	**1.82**

INH									
BioMol Kin INH	G - 11	ROCK	Y-27632	120	0.84	**1**	**2.67**	**12**	**1.78**
BioMol Kin INH	D - 11	PKA	H-89	**2**	**2.57**	**4**	**2.23**	**13**	**1.75**
BioMol Kin INH	C - 6	EGFRK	Tyrphostin AG 1478	**15**	**1.62**	**10**	**1.57**	**14**	**1.74**
EMDII Kin INH	A - 3	ATM	ATM Kinase Inhibitor	54	1.28	88	0.91	**15**	**1.70**
BioMol Kin INH	C - 12	Src family	PP1	**4**	**2.05**	**12**	**1.48**	**16**	**1.67**
BioMol Kin INH	E - 1	PKA, PKG	HA-1004	**6**	**1.82**	**3**	**2.30**	**17**	**1.63**

EMDII Kin INH	B - 9	CDK1/5	Cdk1/5 Inhibitor	83	1.12	100	0.85	**18**	**1.61**
EMDII Kin INH	C - 7	CDC28	Compound 52	67	1.22	122	0.68	**19**	**1.61**
EMDII Kin INH	F - 9	MEK1/2	MEK1/2 Inhibitor	94	1.03	110	0.77	**20**	**1.60**
BioMol Kin INH	H - 7	EGFRK	BML-265 (Erlotinib analog)	**16**	**1.58**	30	1.25	**21**	**1.59**
EMDII Kin INH	G - 4	MEK1/2	PD 98059	71	1.21	78	0.96	**22**	**1.57**
EMDII Kin INH	F - 2	MK2a	MK2a Inhibitor	107	0.95	80	0.95	**23**	**1.54**

EMDII Kin INH	H - 6	p38	SKF-86002	46	1.32	92	0.89	**24**	**1.49**
BioMol Kin INH	C - 9	IRK	HNMPA	93	1.06	39	1.19	25	1.38
BioMol Kin INH	B - 5	PKA, PKG, MLCK, and PKC.	H-9	**1**	**2.62**	**6**	**1.97**	26	1.36
BioMol Kin INH	H - 6	CK-II	Apigenin	38	1.35	120	0.70	27	1.32
BioMol Kin INH	E - 2	PKA, PKG	HA-1077	**12**	**1.69**	35	1.21	28	1.30
BioMol Kin INH	D - 12	PKA, PKG	H-8	**28**	**1.43**	**16**	**1.45**	29	1.30
BioMol Kin	E - 10	CDK	Olomoucine	**25**	**1.46**	41	1.17	30	1.30

INH									
EMDII Kin INH	F - 8	MEK	MEK Inhibitor II	105	0.96	96	0.88	31	1.28
EMDII Kin INH	H - 4	CHK1	SB 218078	61	1.23	29	1.25	32	1.27
BioMol Kin INH	C - 7	Tyrosine kinases	Tyrphostin AG 1295	90	1.07	48	1.12	33	1.24
BioMol Kin INH	F - 7	PDGFRK	AG-1296	43	1.33	82	0.94	34	1.24
BioMol Kin INH	C - 3	EGFRK	Tyrphostin 51	79	1.16	28	1.26	35	1.22
BioMol Kin INH	F - 5	cRAF	ZM 336372	**23**	**1.48**	114	0.76	36	1.19

BioMol Kin INH	C - 2	EGFRK	Tyrphostin 47	40	1.35	47	1.12	37	1.18
BioMol Kin INH	F - 6	Flk1	SU 4312	37	1.35	65	1.01	38	1.13
BioMol Kin INH	H - 4	IKK2	SC-514	**20**	**1.52**	64	1.01	39	1.13
EMDII Kin INH	D - 11	GSK3	GSK-3b Inhibitor XI	132	0.62	44	1.15	40	1.13
EMDII Kin INH	C - 6	CHK2	Chk2 Inhibitor II	63	1.23	66	1.00	41	1.11
EMDII Kin INH	D - 3	ERK	ERK Inhibitor II, FR180204	55	1.27	33	1.22	42	1.11
EMDII Kin	A - 10	AMPK	AMPK Inhibitor, Compound C	32	1.38	87	0.92	43	1.08

INH									
EMDII Kin INH	E - 8	JNK	JNK Inhibitor II	48	1.31	115	0.75	44	1.08
BioMol Kin INH	B - 8	HER1-2	AG-825	106	0.96	37	1.20	45	1.06
EMDII Kin INH	B - 10	CK1/ALK/p38	Casein Kinase I Inhibitor, D4476	100	0.99	45	1.13	46	1.05
BioMol Kin INH	B - 11	EGFRK	Tyrphostin 23	36	1.36	**13**	**1.47**	47	1.05
BioMol Kin INH	H - 1	BTK	Terreic acid	**10**	**1.78**	**15**	**1.46**	48	1.05
EMDII Kin INH	F - 11	NF-kappaB	NF-kB Activation Inhibitor	121	0.84	125	0.65	49	1.04

EMDII Kin INH	E - 5	CHK1	IC261	118	0.87	131	0.55	50	1.04
BioMol Kin INH	D - 7	PKC	GF 109203X	101	0.99	26	1.29	51	1.04
EMDII Kin INH	A - 11	Auroroa/LCK/BMX/IGF1R/SYK	Aurora Kinase Inhibitor III	87	1.10	67	1.00	52	1.03
BioMol Kin INH	G - 12	GSK-3beta	Kenpaullone	**14**	**1.66**	23	1.32	53	1.03
EMDII Kin INH	B - 4	NF-kappaB	BAY 11-7082	123	0.83	79	0.96	54	1.03
BioMol Kin INH	C - 4	Negative control for tyrosine kinase inhibitors.	Tyrphostin 1	131	0.66	49	1.12	55	1.02
BioMol Kin	F - 12	Negative control for	Daidzein	62	1.23	**19**	**1.41**	56	1.01

INH		Genistein.							
BioMol Kin INH	D - 10	PKC	Sphingosine	72	1.20	25	1.30	57	1.00
BioMol Kin INH	E - 8	p58 PITSLRE beta1	2-Aminopurine	111	0.93	60	1.04	58	1.00
BioMol Kin INH	G - 3	Flk1	SU1498	68	1.21	59	1.04	59	0.99
BioMol Kin INH	B - 10	EGFRK	RG-14620	114	0.89	**17**	**1.44**	60	0.97
EMDII Kin INH	H - 11	TPL2	Tpl2 Kinase Inhibitor	99	0.99	76	0.97	61	0.96
BioMol Kin INH	G - 8	JNK	SP 600125	31	1.38	89	0.91	62	0.95

EMDII Kin INH	G - 11	p38	SB 202190	50	1.30	38	1.20	63	0.94
BioMol Kin INH	D - 3	PDGFRK	AG-370	70	1.21	70	0.98	64	0.94
BioMol Kin INH	F - 8	cRAF	GW 5074	39	1.35	111	0.77	65	0.94
BioMol Kin INH	D - 6	PI 3-K	Wortmannin	**9**	**1.80**	50	1.10	66	0.93
EMDII Kin INH	D - 2	ROCK	ROCK Inhibitor, Y-27632	122	0.83	**14**	**1.46**	67	0.93
BioMol Kin INH	D - 2	IRAK	AG-126	**18**	**1.53**	85	0.93	68	0.92
BioMol Kin	C - 5	Tyrosine kinases	Tyrphostin	104	0.98	34	1.22	69	0.91

INH			AG 1288						
EMDII Kin INH	F - 7	MEK	MEK Inhibitor I	126	0.74	102	0.85	70	0.91
EMDII Kin INH	H - 10	CDK1/2/4	SU9516	89	1.09	132	0.54	71	0.91
BioMol Kin INH	G - 9	GSK-3beta, CDK5	Indirubin	115	0.88	124	0.66	72	0.91
EMDII Kin INH	A - 4	ATM/ATR	ATM/ATR Kinase Inhibitor	82	1.14	43	1.15	73	0.89
EMDII Kin INH	G - 2	p38	p38 MAP Kinase Inhibitor III	**22**	**1.51**	**18**	**1.43**	74	0.89
EMDII Kin INH	G - 9	PKA	H-89, Dihydrochloride	57	1.26	24	1.31	75	0.89

EMDII Kin INH	D - 6	GSK3	GSK-3b Inhibitor I	96	1.03	75	0.97	76	0.89
EMDII Kin INH	G - 3	p38	p38 MAP Kinase Inhibitor	53	1.28	94	0.89	77	0.88
BioMol Kin INH	E - 11	Negative control for olomoucine.	iso-Olomoucine	34	1.38	22	1.37	78	0.88
BioMol Kin INH	E - 9	CDK	N9-Isopropyl-olomoucine	73	1.19	93	0.89	79	0.87
BioMol Kin INH	E - 12	CDK	Roscovitine	52	1.28	108	0.79	80	0.86
BioMol Kin INH	H - 3	Akt	BML-257	33	1.38	51	1.09	81	0.86
BioMol Kin	F - 11	Tyrosine Kinases	Genistein	**27**	**1.44**	**11**	**1.56**	82	0.85

INH									
BioMol Kin INH	E - 3	EGFRK, CaMK II	HDBAd)	85	1.11	95	0.89	83	0.84
EMDII Kin INH	C - 11	ERK	ERK Inhibitor III	78	1.16	27	1.28	84	0.84
BioMol Kin INH	C - 1	EGFRK, PDGFRK	Tyrphostin 46	66	1.22	**21**	**1.40**	85	0.84
BioMol Kin INH	F - 2	BTK	LFM-A13	51	1.29	105	0.83	86	0.83
EMDII Kin INH	E - 3	GSK	GSK-3 Inhibitor XIII	**17**	**1.56**	98	0.87	87	0.82
BioMol Kin INH	B - 7	EGFRK, PDGFRK	AG-494	84	1.12	63	1.03	88	0.82

BioMol Kin INH	F - 9	PKC	Palmitoyl-DL-carnitine Cl	92	1.06	74	0.97	89	0.82
BioMol Kin INH	H - 5	Cdk5/p25	BML-259	41	1.34	77	0.96	90	0.80
BioMol Kin INH	G - 10	GSK-3beta	Indirubin-3'-monoxime	86	1.10	53	1.08	91	0.80
EMDII Kin INH	D - 7	GSK3	GSK-3b Inhibitor II	109	0.94	81	0.94	92	0.77
BioMol Kin INH	G - 6	CK II	DRB	49	1.31	84	0.93	93	0.76
EMDII Kin INH	C - 5	CDC2	Cdc2-Like Kinase Inhibitor, TG003	44	1.33	61	1.04	94	0.75
BioMol Kin	B - 2	MEK	U-0126	75	1.17	**8**	**1.73**	95	0.74

INH									
EMDII Kin INH	D - 10	GSK3	GSK-3 Inhibitor X	136	0.44	56	1.05	96	0.71
BioMol Kin INH	D - 1	JAK-2	AG-490	60	1.24	71	0.98	97	0.70
EMDII Kin INH	B - 11		Casein Kinase II Inhibitor III, TBCA	69	1.21	36	1.21	98	0.70
EMDII Kin INH	H - 9		STO-609	91	1.06	126	0.62	99	0.69
BioMol Kin INH	G - 4	JAK-3	ZM 449829	59	1.24	83	0.94	100	0.68
BioMol Kin INH	C - 11	Syk	Piceatannol	65	1.22	69	0.98	101	0.65

EMDII Kin INH	A - 5		Alsterpaullone	103	0.98	119	0.72	102	0.64
EMDII Kin INH	F - 6	CAMKII	KN-93	108	0.94	113	0.76	103	0.61
EMDII Kin INH	F - 3	JNK	JNK Inhibitor VIII	42	1.34	46	1.12	104	0.60
EMDII Kin INH	D - 8	GSK3	GSK-3b Inhibitor VIII	77	1.17	103	0.85	105	0.60
BioMol Kin INH	G - 2	PI 3-K	Quercetin dihydrate	**21**	**1.51**	128	0.59	106	0.59
EMDII Kin INH	E - 9	Not a JNK Inh	JNK Inhibitor, Negative Control	74	1.18	72	0.98	107	0.59
EMDII Kin	E - 4		Isogranulatimide	76	1.17	99	0.86	108	0.59

INH									
EMDII Kin INH	B - 3		Indirubin-3′-monoxime	97	1.02	118	0.72	109	0.57
EMDII Kin INH	B - 7		Cdk1 Inhibitor, CGP74514A	119	0.86	123	0.67	110	0.55
BioMol Kin INH	B - 9	EGFRK	Lavendustin A	98	1.01	40	1.17	111	0.54
BioMol Kin INH	E - 5	CaMK II	KN-93	113	0.90	86	0.93	112	0.54
BioMol Kin INH	D - 4	NGFRK	AG-879	102	0.99	112	0.77	113	0.53
BioMol Kin INH	G - 1	EGFRK	Erbstatin analog	35	1.38	109	0.77	114	0.53

EMDII Kin INH	C - 3		Cdk4 Inhibitor II, NSC 625987	64	1.23	54	1.06	115	0.51
EMDII Kin INH	F - 5		Kenpaullone	124	0.80	106	0.82	116	0.46
BioMol Kin INH	F - 10	PKC delta	Rottlerin	133	0.61	136	0.39	117	0.45
EMDII Kin INH	B - 5	CDK1	Bohemine	30	1.39	90	0.90	118	0.44
BioMol Kin INH	E - 4	CaMK II	KN-62	125	0.80	32	1.23	119	0.44
BioMol Kin INH	G - 7	PKC alpha, PKC gamma	HBDDE	88	1.09	134	0.48	120	0.43
BioMol Kin	E - 7	MLCK	ML-9	134	0.61	73	0.97	121	0.40

INH									
EMDII Kin INH	E - 10		JNK Inhibitor V	130	0.66	101	0.85	122	0.39
EMDII Kin INH	G - 6		SB220025	95	1.03	130	0.55	123	0.36
EMDII Kin INH	A - 8		Aloisine, RP106	80	1.15	91	0.89	124	0.35
EMDII Kin INH	E - 6	IKK2	IKK-2 Inhibitor IV	**26**	**1.44**	116	0.74	125	0.35
BioMol Kin INH	E - 6	MLCK	ML-7	127	0.73	31	1.25	126	0.35
BioMol Kin INH	C - 8	PDGFRK	Tyrphostin 9	135	0.58	135	0.40	127	0.34

BioMol Kin INH	G - 5	IKK pathway	BAY 11-7082	128	0.71	127	0.60	128	0.34
BioMol Kin INH	H - 2	Akt signaling pathway	Triciribine	**13**	**1.68**	129	0.57	129	0.34
EMDII Kin INH	C - 8	CDK2	Cdk2 Inhibitor III	**24**	**1.46**	117	0.73	130	0.32
EMDII Kin INH	G - 7		Purvalanol A	112	0.91	133	0.53	131	0.32
EMDII Kin INH	G - 5	p38	PD 169316	45	1.32	97	0.87	132	0.31
EMDII Kin INH	D - 4		ERK Inhibitor II, Negative control	56	1.26	57	1.05	133	0.31
EMDII Kin	F - 10		MNK1 Inhibitor	81	1.15	42	1.16	134	0.31

INH									
EMDII Kin INH	C - 9		Cdk2 Inhibitor IV, NU6140	129	0.70	68	0.99	135	0.29
BioMol Kin INH	H - 8	mTOR	Rapamycin	117	0.87	104	0.84	136	0.25

Table 4:

		Dose Stage 3		Dose Stage 4		Dose Stages 3&4	
Target Pathway	**Inhibitor**	**RANK**	**RATIO**	**RANK**	**RATIO**	**RANK**	**RATIO**
Src family	PP2	**8**	**1.81**	**2**	**2.50**	**1**	**2.49**
PI 3-K	LY 294002	**7**	**1.82**	**9**	**1.63**	**2**	**2.46**
p38 MAPK	SB-203580	**3**	**2.23**	**7**	**1.88**	**3**	**2.34**
p38	SB 203580	**19**	**1.53**	52	1.08	**4**	**2.22**
p38	SC-68376	**29**	**1.41**	121	0.69	**5**	**2.12**
PKA, PKG, MLCK, and	H-7	**5**	**1.93**	**5**	**2.18**	**6**	**2.08**

PKC.							
NOT a p38 Inh	SB 202474, Neg control for p38 MAPK	116	0.88	62	1.03	**7**	**2.02**
(CDK1/2/5)(GSK3i)	Aloisine A, RP107	58	1.25	55	1.06	**8**	**1.97**
ROCK	HA 1077, Fasudil	47	1.32	**20**	**1.40**	**9**	**1.93**
p38 MAPK	SB-202190	**11**	**1.72**	58	1.05	**10**	**1.83**
CAMKII	KN-62	110	0.93	107	0.81	**11**	**1.82**
ROCK	Y-27632	120	0.84	**1**	**2.67**	**12**	**1.78**
PKA	H-89	**2**	**2.57**	**4**	**2.23**	**13**	**1.75**
EGFRK	Tyrphostin AG	**15**	**1.62**	**10**	**1.57**	**14**	**1.74**

	1478						
ATM	ATM Kinase Inhibitor	54	1.28	88	0.91	**15**	**1.70**
Src family	PP1	**4**	**2.05**	**12**	**1.48**	**16**	**1.67**
PKA, PKG	HA-1004	**6**	**1.82**	**3**	**2.30**	**17**	**1.63**
CDK1/5	Cdk1/5 Inhibitor	83	1.12	100	0.85	**18**	**1.61**
CDC28	Compound 52	67	1.22	122	0.68	**19**	**1.61**
MEK1/2	MEK1/2 Inhibitor	94	1.03	110	0.77	**20**	**1.60**
EGFRK	BML-265 (Erlotinib analog)	**16**	**1.58**	30	1.25	**21**	**1.59**
MEK1/2	PD 98059	71	1.21	78	0.96	**22**	**1.57**

MK2a	MK2a Inhibitor	107	0.95	80	0.95	**23**	**1.54**
p38	SKF-86002	46	1.32	92	0.89	**24**	**1.49**
PKA, PKG, MLCK, and PKC.	H-9	**1**	**2.62**	**6**	**1.97**	26	1.36
PKA, PKG	HA-1077	**12**	**1.69**	35	1.21	28	1.30
PKA, PKG	H-8	**28**	**1.43**	**16**	**1.45**	29	1.30
CDK	Olomoucine	**25**	**1.46**	41	1.17	30	1.30
cRAF	ZM 336372	**23**	**1.48**	114	0.76	36	1.19
IKK2	SC-514	**20**	**1.52**	64	1.01	39	1.13
EGFRK	Tyrphostin 23	36	1.36	**13**	**1.47**	47	1.05

BTK	Terreic acid	**10**	**1.78**	**15**	**1.46**	48	1.05
GSK-3beta	Kenpaullone	**14**	**1.66**	23	1.32	53	1.03
Negative control for Genistein.	Daidzein	62	1.23	**19**	**1.41**	56	1.01
EGFRK	RG-14620	114	0.89	**17**	**1.44**	60	0.97
PI 3-K	Wortmannin	**9**	**1.80**	50	1.10	66	0.93
ROCK	ROCK Inhibitor, Y-27632	122	0.83	**14**	**1.46**	67	0.93
IRAK	AG-126	**18**	**1.53**	85	0.93	68	0.92
p38	p38 MAP Kinase Inhibitor III	**22**	**1.51**	**18**	**1.43**	74	0.89

Tyrosine Kinases	Genistein	**27**	**1.44**	**11**	**1.56**	82	0.85
EGFRK, PDGFRK	Tyrphostin 46	66	1.22	**21**	**1.40**	85	0.84
GSK	GSK-3 Inhibitor XIII	**17**	**1.56**	98	0.87	87	0.82
MEK	U-0126	75	1.17	**8**	**1.73**	95	0.74
PI 3-K	Quercetin dehydrate	**21**	**1.51**	128	0.59	106	0.59
IKK2	IKK-2 Inhibitor IV	**26**	**1.44**	116	0.74	125	0.35
Akt signaling pathway	Triciribine	**13**	**1.68**	129	0.57	129	0.34

CDK2	Cdk2 Inhibitor III	**24**	**1.46**	117	0.73	130	0.32

Table 5:

				NKX6.1 Intensity		NGN3 Intensity	
WELL	**Target Activity**	**COMPOUND**	**Total Nuclei**	**RATIO**	**RANK**	**RATIO**	**RANK**
B - 5	PKA, PKG, MLCK, and PKC.	H-9	1.00	7.36	1	2.24	1
D - 8	PKC	Hypericin	1.07	2.15	18	2.22	2
D - 5	PI 3-K	LY 294002	1.08	6.84	2	2.18	3
D - 11	PKA	H-89	1.09	5.39	3	2.13	4
F - 4	Src family	PP2	1.01	4.07	7	2.09	5
B - 3	p38 MAPK	SB-203580	1.07	5.05	4	1.95	6
C - 6	EGFRK	Tyrphostin AG 1478	1.10	2.41	14	1.89	7
F - 8	cRAF	GW 5074	1.06	3.48	10	1.78	8
D - 6	PI 3-K	Wortmannin	1.10	3.87	9	1.67	9
E - 1	PKA, PKG	HA-1004	1.08	3.88	8	1.55	10

D - 7	PKC	GF 109203X	1.05	4.54	6	1.53	11
C - 12	Src family	PP1	0.99	1.90	22	1.43	12
F - 3	p38 MAPK	SB-202190	1.08	1.90	23	1.40	13
G - 11	ROCK	Y-27632	1.08	1.31	40	1.40	14
G - 12	GSK-3beta	Kenpaullone	0.99	3.18	11	1.32	15
C - 9	IRK	HNMPA	1.01	2.31	15	1.32	16
B - 4	PKA, PKG, MLCK, and PKC.	H-7	1.02	1.99	21	1.26	17
B - 7	EGFRK, PDGFRK	AG-494	1.10	1.57	30	1.26	18
C - 7	Tyrosine kinases	Tyrphostin AG 1295	1.08	1.43	36	1.26	19
H - 7	EGFRK	BML-265 (Erlotinib analog)	1.06	2.15	17	1.25	20
B - 12	EGFRK	Tyrphostin 25	1.02	4.68	5	1.24	21
E - 2	PKA, PKG	HA-1077	1.02	1.46	34	1.20	22

F - 7	PDGFRK	AG-1296	1.09	1.74	25	1.18	23
E - 9	CDK	N9-Isopropyl-olomoucine	1.05	1.42	37	1.18	24
B - 11	EGFRK	Tyrphostin 23	1.03	1.43	35	1.17	25
D - 12	PKA, PKG	H-8	0.99	1.21	45	1.16	26
C - 5	Tyrosine kinases	Tyrphostin AG 1288	1.04	1.63	27	1.15	27
E - 11	Negative control for olomoucine.	iso-Olomoucine	1.04	1.51	31	1.14	28
F - 6	Flk1	SU 4312	1.07	1.01	55	1.12	29
C - 11	Syk	Piceatannol	1.07	1.32	39	1.10	30
G - 8	JNK	SP 600125	1.04	1.67	26	1.09	31
H - 11		DMSO	1.03	1.04	53	1.09	32
E - 5	CaMK II	KN-93	1.04	1.10	51	1.03	33
E - 12	CDK	Roscovitine	1.03	0.67	75	1.03	34
F - 11	Tyrosine	Genistein	1.03	1.10	52	1.02	35

	Kinases						
B - 8	HER1-2	AG-825	1.05	1.12	50	1.00	36
B - 10	EGFRK	RG-14620	1.02	1.26	41	1.00	37
F - 9	PKC	Palmitoyl-DL-carnitine Cl	1.05	1.19	47	0.99	38
E - 10	CDK	Olomoucine	1.04	1.22	44	0.99	39
D - 10	PKC	Sphingosine	1.04	1.62	28	0.99	40
G - 6	CK II	DRB	1.01	1.50	32	0.98	41
C - 3	EGFRK	Tyrphostin 51	1.04	1.46	33	0.97	42
F - 2	BTK	LFM-A13	0.97	1.82	24	0.96	43
E - 8	p58 PITSLRE beta1	2-Aminopurine	1.07	0.93	61	0.93	44
B - 9	EGFRK	Lavendustin A	1.02	0.98	57	0.92	45
G - 7	PKC alpha, PKC gamma	HBDDE	1.07	0.92	63	0.91	46

D - 2	IRAK	AG-126	0.99	1.21	46	0.86	47
H - 12		DMSO	1.02	0.75	69	0.86	48
H - 6	CK-II	Apigenin	1.05	0.77	68	0.84	49
F - 5	cRAF	ZM 336372	1.06	1.36	38	0.83	50
F - 12	Negative control for Genistein.	Daidzein	1.03	0.92	62	0.83	51
C - 1	EGFRK, PDGFRK	Tyrphostin 46	1.05	2.54	13	0.82	52
H - 9		DMSO	1.03	0.95	60	0.81	53
B - 1	MEK	PD-98059	0.92	2.02	20	0.81	54
H - 1	BTK	Terreic acid	1.02	1.24	42	0.81	55
H - 5	Cdk5/p25	BML-259	1.04	0.83	67	0.80	56
C - 2	EGFRK	Tyrphostin 47	1.00	1.13	49	0.79	57
H - 2	Akt signaling pathway	Triciribine	0.73	2.06	19	0.78	58

D - 3	PDGFRK	AG-370	1.01	1.15	48	0.77	59
G - 10	GSK-3beta	Indirubin-3'-monoxime	0.96	0.92	64	0.74	60
C - 4	Negative control for tyrosine kinase inhibitors.	Tyrphostin 1	1.00	0.95	59	0.73	61
G - 4	JAK-3	ZM 449829	1.01	1.23	43	0.73	62
D - 1	JAK-2	AG-490	1.04	2.26	16	0.72	63
D - 4	NGFRK	AG-879	1.03	0.39	80	0.72	64
G - 2	PI 3-K	Quercetin dihydrate	0.97	1.03	54	0.71	65
G - 5	IKK pathway	BAY 11-7082	0.93	1.00	56	0.68	66
H - 10		DMSO	0.99	0.88	65	0.67	67
G - 9	GSK-3beta, CDK5	Indirubin	1.01	0.59	77	0.66	68
E - 6	MLCK	ML-7	0.98	2.84	12	0.65	69

B - 2	MEK	U-0126	0.87	0.68	73	0.65	70
G - 3	Flk1	SU1498	1.01	0.70	72	0.65	71
E - 3	EGFRK, CaMK II	HDBA	0.99	0.88	66	0.64	72
H - 4	IKK2	SC-514	0.95	0.71	71	0.62	73
E - 7	MLCK	ML-9	1.06	1.60	29	0.59	74
F - 10	PKC delta	Rottlerin	0.24	0.96	58	0.56	75
E - 4	CaMK II	KN-62	0.96	0.71	70	0.56	76
H - 3	Akt	BML-257	0.98	0.68	74	0.55	77
H - 8	mTOR	Rapamycin	0.47	0.65	76	0.39	78
C - 10	PKC inhibitor	PKC-412	0.17	0.45	79	0.39	79
G - 1	EGFRK	Erbstatin analog	0.82	0.38	81	0.34	80
D - 9	PKC	Ro 31-8220	0.09	0.48	78	0.30	81
B - 6	Pan-specific	Staurosporine	0.13	0.17	83	0.30	82

C - 8	PDGFRK	Tyrphostin 9	0.28	0.29	82	0.29	83
F - 1	ERK2, adenosine kinase, CK1, CK2,	5-Iodotubercidin	0.08	0.13	84	0.27	84

Table 6:

RANK	NKX6.1 RANK	NGN3 RANK
1	**H-9**	**H-9**
2	**LY 294002**	Hypericin
3	**H-89**	**LY 294002**
4	**SB-203580**	**H-89**
5	Tyrphostin 25	**PP2**
6	**GF 109203X**	**SB-203580**
7	**PP2**	**Tyrphostin AG 1478**
8	**HA-1004**	**GW 5074**
9	**Wortmannin**	**Wortmannin**
10	**GW 5074**	**HA-1004**
11	**Kenpaullone**	**GF 109203X**
12	ML-7	PP1
13	Tyrphostin 46	SB-202190

14	**Tyrphostin AG 1478**	Y-27632
15	**HNMPA**	**Kenpaullone**
16	AG-490	**HNMPA**

Table 7:

Pathway	**Compounds**
PKC/PKA/PKG	H-9, Hypericin, H-89, GF 109203X, HA-1004
SRC kinase	PP2, PP1
PI3 Kinase	LY 294002, Wortmannin
p38 MAP kinase	SB-203580, SB-202190
EGF receptor kinase	Tyrphostin 25, Tyrphostin AG1478, Tyrphostin 46
cRAF	GW 5074
GSK3 beta	Kenpaullone
IRK	HNMPA
JAK2	AG490
ROCK	Y27632
MLCK	ML-7

Table 8:

		Total Nuclei	NKX6.1		NGN3	
Plate	**Treatment [conc]**	**Ratio**	**Cell Count Ratio**	**Intensity Ratio**	**Cell Count Ratio**	**Intensity Ratio**
Plate 1	PD-98059 [2.5uM]	0.96	2.08	2.37	2.21	2.41
Plate 1	SB-203580 [2.5uM]	1.05	2.93	2.58	5.26	4.74
Plate 1	H-7 [2.5uM]	1.09	2.02	1.88	2.75	2.44
Plate 1	H-9 [2.5uM]	1.13	1.93	1.76	2.85	2.47
Plate 1	AG-490 [2.5uM]	0.99	3.78	4.20	2.48	2.35
Plate 1	LY 294002 [2.5uM]	1.03	6.54	6.60	4.93	4.43
Plate 1	GF109203X [2.5uM]	0.82	5.20	3.57	4.17	3.33
Plate 1	H-89 [2.5uM]	1.08	2.41	2.30	4.00	3.74
Plate 1	KN-62	1.02	0.69	0.61	0.81	0.77

	[1uM]					
Plate 1	KN-93 [1uM]	1.05	0.59	0.55	0.84	0.79
Plate 1	Control Treatment	1.00	1.00	1.00	1.00	1.00
Plate 2	HA-1004 [2.5uM]	1.08	2.83	2.66	2.77	2.49
Plate 2	HA-1077[2.5uM]	1.07	1.48	1.31	2.57	2.27
Plate 2	SB-202190 [2.5uM]	1.08	2.12	1.90	4.53	3.91
Plate 2	PP2 [2.5uM]	1.04	2.06	1.71	6.21	6.12
Plate 2	GW 5074 [2.5uM]	1.11	2.77	2.32	3.15	2.79
Plate 2	Kenpaullone [2.5uM]	1.02	0.53	0.45	1.52	1.40
Plate 2	BML-259 [2.5uM]	0.99	1.08	1.02	1.21	1.23
Plate 2	BML-265 [2.5uM]	0.97	6.12	6.34	4.50	4.65
Plate 2	KN-62	1.03	0.71	0.67	0.72	0.71

	[1uM]					
Plate 2	KN-93 [1uM]	1.04	0.75	0.76	0.93	0.93
Plate 2	Control Treatment	1.00	1.00	1.00	1.00	1.00

[0143] Publications cited throughout this document are hereby incorporated by reference in their entirety. Although the various aspects of the invention have been illustrated above by reference to examples and preferred embodiments, it will be appreciated that the scope of the invention is defined not by the foregoing description but by the following claims properly construed under principles of patent law.

What is claimed is:

1. A method to increase the expression of NGN3 and NKX6.1 in a population of cells expressing markers characteristic of the pancreatic endocrine lineage, comprising the steps of:

    a) culturing pluripotent stem cells,

    b) differentiating the pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage,

    c) differentiating the cells expressing markers characteristic of the definitive endoderm lineage into cells expressing markers characteristic of the pancreatic endoderm lineage, supplementing the medium used to differentiate the cells expressing markers characteristic of the definitive endoderm lineage with a compound selected from the group consisting of H-9, H-89, GF 109203X, HA-1004, PP2, PP1, LY 294002, Wortmannin, SB-203580, SB-202190, Tyrphostin 25, Tyrphostin, AG1478, Tyrphostin 46, GW 5074, Kenpaullone, HNMPA, AG490, Y27632, and ML-7, and

    d) differentiating the cells expressing markers characteristic of the pancreatic endoderm lineage into cells expressing markers characteristic of the pancreatic endocrine lineage.

2. The method of claim 1, wherein the medium used to differentiate the cells expressing markers characteristic of the pancreatic endoderm lineage is supplemented with a compound selected from the group consisting of H-9, H-89, GF 109203X, HA-1004, PP2, PP1, LY 294002, Wortmannin, SB-203580, SB-202190, Tyrphostin 25, Tyrphostin, AG1478, Tyrphostin 46, GW 5074, Kenpaullone, HNMPA, AG490, Y27632, and ML-7.