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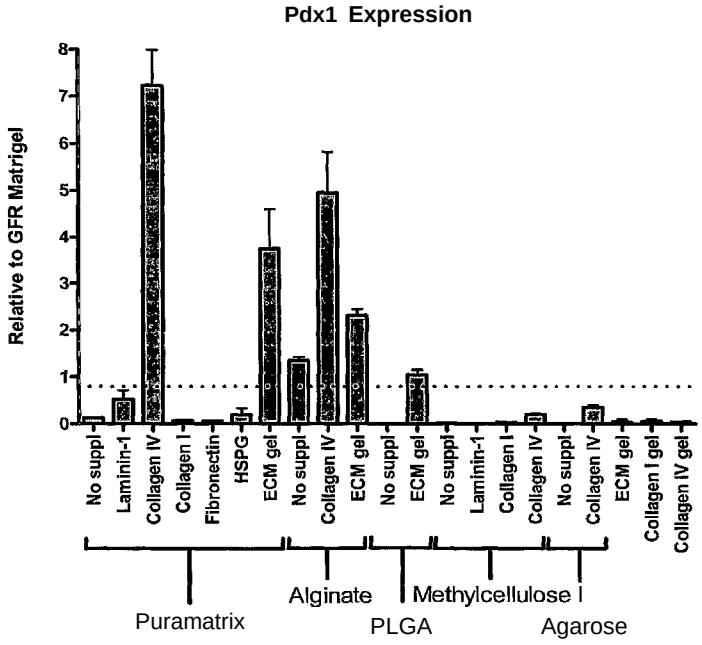
(54) **Title:** METHODS FOR THE DIRECTED DIFFERENTIATION OF EMBRYONIC STEM CELL



(57) **Abstract:** The present invention provides compositions and methods for the directed differentiation of embryonic stem cells. The invention further provides the use of cells differentiated from the ES cells for treating diseases and conditions.

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METHODS FOR THE DIRECTED DIFFERENTIATION OF EMBRYONIC STEM CELL

Reference to Related Applications

This application claims the benefit of the filing date of U.S. Provisional Application Serial Nos. 60/752,214, filed on December 20, 2005. The entire teachings of the referenced application are incorporated herein by reference.

Background of the Invention

Over the last decade, tremendous excitement in the stem cell field has fueled the hope that various stem cell populations will form the basis of treatments for a diverse array of degenerative diseases / disorders. Embryonic stem cells have attracted particular excitement for their seemingly unprecedented ability to differentiate to tissues derived from all three germ layers. Accordingly, embryonic stem cells may form the basis of a wider range of therapeutics than adult *stem cells* derived from any particular tissue.

However, despite the excitement generated by the limitless potential of embryonic stem cells to differentiate along ectodermal, mesodermal, and endodermal lineages, effective therapeutics require the ability to control and direct the differentiation of embryonic stem cells to a particular cell type. Furthermore, effective therapeutics require that this directed differentiation efficiently yields a particular differentiated cell type. In other words, it is advantageous for methods of directed differentiation to yield a high percentage of a particular differentiated cell type or to yield a high percentage of cell types that comprise a particular tissue or organ. Such efficient methods of differentiation represent a substantial leap from prior art methods which either fail to consistently yield particular cell types or yield an exceedingly low percentage of a particular differentiated cell type.

There exists a tremendous need to supply realistic therapeutic alternatives to the wide range of degenerative diseases and injuries affecting tissues derived from the ectoderm, mesoderm, or endoderm. Embryonic stem cells are a particularly attractive resource for developing such diverse therapies.

The extra-cellular matrix formulation or basement membrane matrix (or

"BMM") marketed by BD Biosciences (San Jose, CA) as MATRIGEL™ or reduced growth factor (RGF) MATRIGEL™) supports the growth and differentiation of embryonic stem cells (ESC). Due to its xenobiotic and complex nature, however, the use of BMM may be an impediment towards the generation of clinically compliant cell products. One major reason is that BMM is prepared from extracts of the Engelbreth-Holm-Swarm (EHS) mouse sarcoma containing a complex mixture of extra-cellular matrix proteins,' proteoglycans and a host of growth factors and secreted enzymes. In addition to being a xenobiotic product, the precise composition of each BMM preparation cannot be controlled.

Accordingly, there is a need to provide composition and methods that promote directed differentiation of embryonic stem cells to particular endodermally derived cell types, and the use of such composition and methods to generate cultures of partially and/or terminally differentiated cells, which can be used therapeutically to treat or prophylactically treat injuries and diseases of endodermally derived tissues and organs.

Summary of the Invention

The invention provides compositions and methods of using such compositions for the directed differentiation of ES cells to one or more desired endodermal lineage cell types, such as pancreatic lineage cells. Endodermal cell types differentiated using the subject compositions, and according to the subject methods can be used to treat or prophylactically treat injuries and diseases of endodermally derived tissues and organs.

More specifically, the present invention provides a composition comprising a chemically defined three-dimensional polymer matrix supplemented with a collagen IV polypeptide, wherein said composition supports directed differentiation of embryonic stem (ES) cells to one or more desired endodermal lineage cells, such as pancreatic lineage cells.

In certain embodiments, the methods and compositions of the invention lead to the production, from ES cells, of *pdx-1*$^+$ cells indicative of cells that have begun differentiation to a pancreatic lineage cell fate. In certain other embodiments, the methods and compositions of the invention lead to the production, from ES cells, of

insulin-producing cells. In still other embodiments, the methods and compositions of the invention lead to the production, from ES cells, of cells that express insulin and secrete C-peptide, and/or are optionally glucose-responsive.

This in one aspect, the invention provides a composition comprising a chemically defined three-dimensional polymer matrix supplemented with a collagen IV polypeptide, wherein said composition supports directed differentiation of embryonic stem (ES) cells to one or more desired endodermal lineage cells.

In certain embodiments, the polymer matrix comprises one or more synthetic or naturally occurring polymers. The chemically defined matrices are free, or substantially free, of any undefined, or unpurified, or undesirable xenobiotic components.

The polymer matrix may be biodegradable. Biodegradable polymer matrix may comprise poly(lactic acid) (PLA), poly(glycolic acid) (PGA), PLA-PGA co-polymer (PLGA), poly(anhydride), poly(hydroxy acid), poly(orthoester), poly(propylfumarate), poly(caprolactone), polyamide, polyamino acid, polyacetal, biodegradable polycyanoacrylate, biodegradable polyurethane, or polysaccharide.

In certain embodiments, the polymer matrix is non-biodegradable. Non-biodegradable polymer matrix comprises polypyrrole, polyaniline, polythiophene, polystyrene, polyester, non-biodegradable polyurethane, polyurea, poly(ethylene vinyl acetate), polypropylene, polymethacrylate, polyethylene, polycarbonate, or poly(ethylene oxide).

In certain embodiments, the polymer matrix is a gel, such as one comprising methylcellulose, agarose, or alginate.

In certain embodiments, the polymer matrix is a copolymer, a mixture, or an adduct of one or more of: poly(lactic acid) (PLA), poly(glycolic acid) (PGA), PLA-PGA co-polymer (PLGA), poly(anhydride), poly(hydroxy acid), poly(orthoester), poly(propylfumarate), poly(caprolactone), polyamide, polyamino acid, polyacetal, biodegradable polycyanoacrylate, biodegradable polyurethane, polysaccharide, polypyrrole, polyaniline, polythiophene, polystyrene, polyester, non-biodegradable polyurethane, polyurea, poly(ethylene vinyl acetate), polypropylene, polymethacrylate, polyethylene, polycarbonate, poly(ethylene oxide), methylcellulose, agarose, peptide hydrogel, or alginate.

In certain embodiments, the polymer matrix is peptide hydrogel, alginate, or PLGA.

In certain embodiments, the polymer matrix excludes one or more components of a solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma.

In certain embodiments, the solubilized basement membrane preparation is a basement membrane matrix or a growth factor-reduced (GFR) basement membrane matrix.

In certain embodiments, the polymer matrix consists of or consists essentially of a mixture of: (1) peptide hydrogel, alginate, or PLGA, and (2) purified collagen IV.

In certain embodiments, the collagen IV is from mouse or human.

In certain embodiments, the polymer matrix is formed as a particle, a sponge, a tube, or a sphere.

In certain embodiments, the composition further comprises ES cells and/or one or more endodermal lineage cells in various stages of differentiation from said ES cells.

In certain embodiments, the ES cells are human or mouse cells.

In certain embodiments, the composition provides a suitable growth environment for the production of three-dimensional tissue structure that limits the formation of the visceral endoderm while stimulating the formation of the definitive endoderm.

In certain embodiments, the cells of the definitive endoderm express one or more of: *cerberus*, *soxJ7, vHNFl, hex, GΛTΛ6*, *HNF3*β, and substantially lack expression of the visceral endodermal marker *Hl9*.

In certain embodiments, the endodermal lineage cells include pancreatic lineage cells that are *pdxl* $^{+}$ and/or secrete C-peptide extracellularly.

In certain embodiments, the composition produces at least about 50% as many of a desired differentiated endodermal lineage cells as compared to that produced by solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, or a growth factor-reduced (GFR) version thereof.

In certain embodiments, the composition produces at least about twice as many of a desired differentiated endodermal lineage cells as compared to that produced by solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, or a growth factor-reduced (GFR) version thereof.

In certain embodiments, the composition produces a desired differentiated endodermal lineage cells with similar or substantially the same phenotypes as are produced using solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, or a growth factor-reduced (GFR) version thereof.

In certain embodiments, the phenotypes include one or more of: expression of one or both *of pdx I* and *insulin,* secretion of C-peptide extracellularly, or morphology.

In certain embodiments, the composition produces pancreatic lineage cells that (1) express both *pdxl* and *insulin,* and/or (2) secrete C-peptide, at levels at least as high as, or higher than that produced by cells obtained using solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, or a growth factor-reduced (GFR) version thereof.

In certain embodiments, the composition produces a population of differentiated endodermal lineage cells that are at least about 25% homogeneous without purification with respect to at least one differentiation marker.

Another aspect of the invention comprises a method for directed differentiation of an increased number of embryonic stem (ES) cells to endodermal cell types, comprising ciilturing the ES cells in a composition of any of the subject compositions.

In certain embodiments, the ES cells are differentiated into pancreatic lineage cells that express pancreatic lineage marker(s), and/or exhibit a pancreatic lineage function, such as expressing insulin, secreting C-peptide and/or becoming glucose-responsive.

In certain embodiments, the method further comprises contacting the ES cells for a sufficient period of time with a sufficient amount of one or more early factors (EFs) selected from activin A, BMP-2, BMP-4, or nodal.

In certain embodiments, the pancreatic lineage cells express Pdx-1 and/or insulin, and/or are responsive to glucose, and/or secret C-peptide. Such pancreatic lineage cells may be insulin-producing cells, such as pancreatic β-cells.

In certain embodiments, the ES cells are cultured as embryoid bodies (EBs) plated directly onto a support matrix (such as the subject composition or the BMM marketed as MATRIGEL™), and/or plated directly onto tissue culture plates. For example, the EBs may be cultured in a floating suspension culture, in a support matrix (such as the subject composition, the BMM marketed as MATRIGEL™ BMM, or other similar matrix), and/or on a filter.

Supporting matrices other than BMM marketed as MATRIGEL™ are known in the art, including basement membrane extractable from placenta as described in Kawaguchi *et al, Proc. Natl. Acad. ScL* 95(3): 1062-66, 1998; BD Biosciences' and 3DM Inc.'s PURAMATRIX synthetic peptide scaffold; or fibronectin matrix, *etc.*

In certain embodiments, the EBs are cultured in a support matrix (such as the subject composition), only during the period when the EBs are in contact with the EFs.

In certain embodiments, the EBs are generated from ES cells grown on mouse embryonic feeder (MEF) or other feeder layers, or from ES cells grown under feeder-free conditions.

In a preferred embodiment, the ES cells are xeno-free, preferably also CGMP- and GTCP-compliant (CGMP: Current Good Manufacturing Practice; GTCP: Good Tissue Culture Practice).

ES cells from many different species of animals may be used in the methods of the invention. In certain embodiments, the ES cells are human ES cells. In other embodiments, the ES cells are from non-human mammals, such as ES cells from rodents (rats, mice, rabbits, hamsters, *etc.);* primates *{e.g.*, monkey, apes, *etc.),* pets (cats, dogs, *etc.);* livestock animals (cattle, pigs, horses, sheep, goats, *etc.).*

In certain embodiments, the human ES cells are from the hESl, hES2, hES3, hES4, hES5, hES6 or DM ES cell lines.

In certain embodiments, the ES cells are partially or terminally differentiated into the pancreatic lineage.

In other embodiments, the ES cells are contacted with the EFs for about 15

days, preferably about 10 days, or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or about 14 days.

In certain embodiments, the EFs comprise activin A and BMP-4. In certain embodiments, the EFs comprise about 50 ng/mL *(e.g.,* about 10-200 ng/mL, or about 20-100 ng/mL, or about 30-70 ng/mL, or about 40-60 ng/mL) of activin A and about 50 ng/mL *(e.g.,* about 10-200 ng/mL, or about 20-100 ng/mL, or about 30-70 ng/mL, or about 40-60 ng/mL) of BMP-4.

In certain embodiments, the EFs comprise about 50 ng/mL of activin A and about 50 ng/mL of BMP-4.

In certain embodiments, the method further comprises contacting the ES cells, subsequent to contacting the ES cells with the EFs, with a sufficient amount of one or more late factors (LFs) for a second sufficient period of time. For example, the LFs may be HGF, exendin4, betacellulin, and nicotinamide. In certain embodiments, the one or more LFs include about 50 ng/mL *(e.g.,* about 10-200 ng/mL, or about 20- 100 ng/mL, or about 30-70 ng/mL, or about 40-60 ng/mL) of HGF, about 10 ng/mL (e.g., about 2-50 ng/mL, or about 5-20 ng/mL) of exendin4, and about 50 ng/mL *(e.g.,* about 10-200 ng/mL, or about 20-100 ng/mL, or about 30-70 ng/mL, or about 40-60 ng/mL) of β-cellulin.

In certain embodiments, the one or more LFs are HGF, exendin-4, betacellulin, and nicotinamide.

In certain embodiments, the one or more LFs include about 50 ng/mL of HGF, about 10 ng/mL of exendin-4, and about 50 ng/mL of β-cellulin.

In certain embodiments, the ES cells are contacted with the EFs for about 10 days, and are subsequently contacted with the LFs for about 10 days.

In certain embodiments, the EFs comprise about 50 ng/mL of activin A and about 50 ng/mL of BMP-4, and the LFs include about 50 ng/mL of HGF, about 10 ng/mL of exendin-4, and about 50 ng/mL of β-cellulin.

In certain embodiments, the method further comprises contacting the ES cells, subsequent to the initiation protocol and during a maturation protocol, consecutively with: (1) a basal medium for about 6 days; (2) about 20 ng/ml (e.g., about 5-100 ng/mL, or about 10-40 ng/mL) FGF-18, and about 2 μg/ml *(e.g.,* about 0.5-10 μg/ml, or about 1-5 μg/ml) heparin in the basal medium for about 5-6 days;

(3) about 20 ng/m! *(e.g.,* about 5-100 ng/niL, or about 10-40 ng/mL) FGF-1 8, about 2 μg/ml *(e.g.,* about 0.5-10 μg/ml, or about 1-5 μg/ml) heparin, about 10 ng/ml EGF (e.g., about 2-50 ng/mL, or about 5-20 ng/mL), about 4 ng/ml TGF-α (e.g., about 1-20 ng/mL, or about 2-10 ng/mL), about 30 ng/ml (e.g., about 5-150 ng/mL, or about 15-60 ng/mL) IGFl, about 30 ng/ml (e.g., about 5-150 ng/mL, or about 15-60 ng/mL) IGF2, and about 10 ng/ml (e.g., about 2-50 ng/mL, or about 5-20 ng/mL) VEGF in the basal medium for about 4-5 days; (4) about 10 μM (e.g., about 2-50 μM, or 5-20 μM) forskolin, about 40 ng/ml (e.g., about 10-150 ng/mL, or about 20-80 ng/mL) HGF, and about 200 ng/ml (e.g., about 50-800 ng/mL, or about 100-400 ng/mL) PYY for about 3-4 days; and, (5) about 100 ng/ml (e.g., about 25-400 ng/mL, or about 50-200 ng/mL) exendin-4, and about 5 mM (e.g., about 1-20 mM, or 2-10 mM) nicotinamide for about 3-4 days.

In certain embodiments, steps (1) - (3) use DMEM/F12 medium or equivalents. In certain embodiments, step (4) uses RPMI 1640 or equivalent medium. In certain embodiments, step (5) uses CMRL medium. In certain embodiments, the ES cells are not dissociated by dispase between step (1) and (2). In certain embodiments, FBS (if any is used) in the medium is replaced with a chemically defined serum replacer (SR).

In certain embodiments, the method further comprises contacting the ES cells, subsequent to the EF and LF treatment, and during a maturation protocol, with about 10 μM (e.g., about 2-50 μM, or 5-20 μM) forskolin, about 40 ng/ml (e.g., about 10-150 ng/mL, or about 20-80 ng/mL) HGF, and about 200 ng/ml (e.g., about 50-800 ng/mL, or about 100-400 ng/mL) PYY for about 3-4 days.

In certain embodiments, the ES cells are not dissociated by dispase between steps (1) and (2).

In certain embodiments, FBS (if any) in the medium is replaced with a chemically defined serum replacer (SR).

In certain embodiments, the ES cells are grown on fibronectin-coated tissue culture surfaces during the maturation protocol.

In certain embodiments, the differentiated cells release C-peptide and/or are responsive to glucose stimulation.

In certain embodiments, the method further comprises contacting the ES

cells, subsequent to contacting the ES cells with the EFs and during a maturation protocol, consecutively with: (1) about 20 ng/ml FGF-18, and about 2 μg/ml heparin in a basal medium for about 8 days; (2) about 20 ng/ml FGF-18, about 2 μg/ml heparin, about 10 ng/ml EGF, about 4 ng/ml TGFα, about 30 ng/ml IGFl, about 30 ng/ml IGF2, and about 10 ng/ml VEGF in the basal medium for about 6 days; and (3) about 10 μM forskolin, about 40 ng/ml HGF, and about 200 ng/ml PYY for about 5 days. It is contemplated that the range of concentrations of the factors are as those described above.

In certain embodiments, the differentiated cells release C-peptide.

In certain embodiments, step (1) lasts 6 days, and steps (2) and (3) last 4 days each.

Another aspect of the invention provides cells and cell clusters differentiated from embryonic stem cells, using the subject composition, and/or by the subject methods. In one embodiment, the cells or cell clusters express *pdx-1*. In another embodiment, the cells or cell clusters express insulin. In still another embodiment, the cells or cell clusters express and secrete C-peptide. In yet another embodiment, the cells or cell clusters express both insulin and C-peptide. In any of the foregoing, exemplary cells or cell clusters may also be glucose-responsive.

Thus this aspect of the invention provides differentiated pancreatic lineage cells or cell cultures obtained through any of the compositions and methods of the invention.

In certain embodiments, the differentiated pancreatic lineage cells or cell cultures are partially differentiated.

In certain embodiments, the differentiated pancreatic lineage cells or cell cultures are terminally differentiated.

In certain embodiments, the differentiated pancreatic lineage cells or cell cultures mimic the function, in whole or in part, of insulin-producing cells, such as pancreatic beta islet cells.

pdx-J^{+} cells and/or insulin-producing cells produced using the composition and methods of the present invention can be delivered to human or animal patients and used for the treatment or prophylaxis of conditions of the pancreas.

Thus another aspect of the invention provides a method for the treatment or

prophylaxis, in an individual, of diseases, injuries, or conditions of the pancreas characterized by impaired pancreatic function, comprising administering to the individual the subject differentiated pancreatic lineage cells.

In certain embodiments, the impaired pancreatic function includes impaired ability to properly regulate glucose metabolism in an affected individual.

In certain embodiments, the condition is type I or type 11 diabetes.

In certain embodiments, the method is in conjunction with one or more additional therapies effective for the treatment or prophylaxis of the diseases, injuries, or conditions.

In various embodiments, compositions, differentiation protocols, such as initiation protocols, maturation protocols, and combinations of initiation and maturation protocols may be used in accordance with the compositions / methods described herein for directed differentiation of embryonic stem cells along a pancreatic lineage. In one embodiment, the initiation protocol, maturation protocol, or combination thereof promotes expression of *pdx-1*, insulin, and/or C-peptide. In another embodiment, using the subject composition, the initiation protocol, maturation protocol, or a combination thereof promotes induction of glucose-responsive cells or cell clusters that mimic the function, in whole or in part, of beta islet cells.

Thus this aspect of the invention provides a method for the treatment or prophylaxis, in an individual, of diseases, injuries, or conditions of the pancreas characterized by impaired pancreatic function, comprising administering to the individual the subject differentiated pancreatic lineage cells.

. In another aspect, the invention provides initiation protocols, maturation protocols, and combinations of initiation and maturation protocols for the directed differentiation of embryonic stem cells along a pancreatic lineage using the subject composition. In one embodiment, the initiation protocol, maturation protocol, or combination thereof promotes expression of *pdx-* /, insulin, and/or C-peptide. In another embodiment, the initiation protocol, maturation protocol, or combination thereof promotes induction of glucose responsive cells or cell clusters that mimic the function, in whole or in part, of beta islet cells.

In any of the foregoing, the invention contemplates that these compositions /

methods can be used to direct the differentiation of other adult and fetal stem cell populations to endodermal cell types.

The embodiments of the invention, even when described for different aspects of the invention, are contemplated to be applicable for all aspects of the invention where appropriate.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, virology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, Molecular Cloning: A Laboratory Manual, 3rd Ed., ed. by Sambrook and Russell (Cold Spring Harbor Laboratory Press: 2001); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Using Antibodies, Second Edition by Harlow and Lane, Cold Spring Harbor Press, New York, 1999; Current Protocols in Cell Biology, ed. by Bonifacino, Dasso, Lippincott-Schwartz, Harford, and Yamada, John Wiley and Sons, Inc., New York, 1999.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Detailed Description of the Drawings

Figure 1 shows the results of RT-PCR analysis of human embryonic stem cells allowed to spontaneously differentiate via embryoid body formation. Expression analysis confirmed that human embryonic stem cells can spontaneously differentiate along ectodermal, mesodermal, and endodermal lineages.

Figure 2 shows a schematic representation of methods of directing differentiation of a stem cell to a differentiated pancreatic cell. The method proceeds in two stages. In the first stage, the stem cells are directed to differentiate along a particular lineage, for example the pancreatic lineage, by promoting expression of a marker indicative of partial differentiation down a particular lineage. In the second stage, the partially differentiated cells are terminally differentiated to express one or more markers of a particular differentiated cell type. Note that either partially or terminally differentiated cells may be therapeutically useful, and thus the method contemplates variations in which the end point *(e.g.,* the goal of a particular

differentiation protocol) is either generation of partially differentiated cells or the generation of terminally differentiated cells.

Figure 3 summarizes the results of experiments in which hES3 were cultured as embryoid bodies suspended in 3D in BMM marketed as MATRIGEL™. The cells were cultured for 10 days in medium containing the early factors and then for 10 days in medium containing the late factors. Following culture, cells were assayed for expression of pdx-1. For each bar depicted in Figure 3, the embryoid bodies were cultured, except as indicated, with the following early and late factors: early factors were activin A, BMP-2, BMP-4, and nodal; late factors were HGF, exendin4, betacellulin, and nicotinamide. The particular factor omitted is indicated under each bar.

Figures 4A and 4B show the directed differentiation of a mouse embryonic stem cell along a particular endodermal lineage. A mouse embryonic stem cell line with lacZ reporter knocked into the pdx-1 locus was used to differentiate into pancreatic cells. Figure 4A shows a cluster of cells expressing β-galactosidase (indicating pdx-1 expression) after EB formation and subsequent plating. Figure 4B shows quantitative RT-PCR data for pdx-1 for mouse embryoid bodies at various stages of culture. Pdx-1 expression increased over time up to 24 days of EB formation.

Figures 5A and 5B show that expression of the early pancreatic marker, pdx-1, increased over time in embryoid bodies formed from human embryonic stem cell line hES2. Figure 5A shows that *pdx-1* expression increased between 0 - 24 days of embryoid body formation, as measured by RT-PCR. As a control, actin expression was measured and this expression did not change significantly over time. Figure 5B shows an ethidium bromide stained gel of the *pdx-1* RT-PCR product, indicating that a single band of the predicted size was detected.

Figure 6 shows that addition of TGFβ family growth factors to embryoid bodies, in culture, increased expression of pdx-1. Human ES cell line 3 (hES3) derived embryoid bodies were cultured in BMM (marketed as MATRIGEL™) in RPMI media supplemented with serum replacement. Expression of *pdx-1* by RT-PCR was measured after 20 days in culture. Expression is expressed as fg per ng actin. Addition of TGF-β family growth factors resulted in a 9-fold increase *m.pdx-1*

expression.

Figures 7A and 7B show the directed differentiation along a particular endodermal lineage. Figure 7A shows a cluster of embryonic stem cells expressing the hepatocyte marker albumin. Figure 7B shows quantitative RT-PCR data examining markers of endodermal differentiation in two different human embryonic stem cell lines undergoing any of several differentiation protocols.

Figure 8 shows *pdx-1* expression in embryonic stem cells at various time points during culture as embryoid bodies suspended in 3D culture in BMM marketed as MATRJGEL™. The cells were cultured for 10 days in medium containing the early factors and then for 10 days in medium containing the late factors.

Figures 9A and 9B show expression *of pdx-1* and insulin in embryonic stem cells differentiated under a combination of conditions. Cells were cultured as embryoid bodies in 3D cultures for about three weeks (20 days) in the presence of early and late factors, and were then subjected to a 24-day, multi-step differentiation protocol. Figure 9A shows expression of *pdx-1,* and Figure 9B shows expression of insulin.

Figures 1OA and 1OB show expression of *pdx-1* and *insulin* in embryonic stem cells differentiated under a combination of conditions. Cells were cultured as embryoid bodies in 3D cultures for 10 days in the presence of early factors only, and then were subjected to a 24-day, multi-step differentiation protocol. Figure 1OA shows expression of *pdx-1,* and Figure 1OB shows expression of insulin.

Figure 11 shows the kinetics of endodermal and pancreatic gene expression during *in vitro,* directed differentiation of embryonic stem cells.

Figure 12 shows a detailed analysis of the temporal pattern of gene expression during *in vitro,* directed differentiation of embryonic stem cells. The data summarized in Figures 11 and 12 demonstrate that gene expression during the directed differentiation of embryonic stem cells along a pancreatic lineage mimics that which occurs during normal pancreatic development.

Figures 13A and 13B summarize the results of experiments designed to examine the effect on induction of *pdx-1* expression of different combinations of early and late factors. Note that the results depicted in Figure 13A represent normalized expression, and the results depicted in Figure 13B represent expression

as % of actin input.

Figure 14 shows the effect of *nodal* on induction of *pdx-1* expression. The 2 EF-3 LF protocol was performed in the presence or absence of 50 ng/ml of recombinant Nodal protein.

Figure 15 shows the effect of activin and BMP-4 protein concentrations on induction of *pdx-1* expression. The data were determined by *pdx-1* and actin standard curves, and are expressed as % actin.

Figure 16 shows the effect of activin and BMP-4 protein concentrations on induction of expression of a number of endocrine genes, *pdx-1* and insulin gene expression were calculated based on standard curves and expressed as *%* actin. *pax4, somatostatin,* and *glucagon* were calculated as relative values.

Figures 17A-17D show that the 2 EF-3 LF initial differentiation protocol (Figures 17C and 17D) more effectively induces *pdx-1* expression than the 4 EF-4 LF initial differentiation protocol (Figures 17A and 17B).

Figures 18A and 18B show *pdx~l* expression followed by release of C-peptide from representative cultures of embryonic stem cells differentiated using an extended differentiation protocol including both an initial differentiation phase and a maturation phase. Figure 18A is a schematic representation of the combined protocol. The left panel of Figure 18B shows the expression of *pdx-1* by quantitative PCR following the first 20 days of differentiation (the initial differentiation protocol). The right panel of Figure 18B shows release of C-peptide at day 36 of differentiation. Day 36 is approximately half-way through the maturation portion of the extended differentiation protocol.

Figures 19A and 19B show release of C-peptide from cultures assayed at various stages during the extended differentiation protocol.

Figures 20A-20F show insulin expression by *in situ* hybridization. Figures 2OA and 2OB show that after 20 days in the initial differentiation protocol, embryoid bodies contain a few isolated insulin^{+} cells. Figures 2OC and 2OD show that further differentiation using the maturation protocol induces insulin expression in a higher percentage of cells in the embryoid body. Additionally, following the maturation protocol, the insulin^{+} cells appear most prevalent within sectors/clusters within the embryoid body. Figure 2OE shows a cryosection of a day 20 embryoid body. Figure

2OF shows a sense strand negative control.

Figures 21A-21F show C-peptide protein expression by immunocytochemistry in Day 45 embryoid bodies.

Figures 22A-22E show *pdx-1* expression by *in situ* hybridization. Figures 22A and 22B show *pdx-1* expression in embryoid bodies cultured in the initial differentiation protocol for 20 days. Figure 22C shows that embryoid bodies cultured for 20 days in the absence of growth factors fail to express *pdx-1*. Figure 22D summarizes the results of the experiments depicted in Figures 22A and 22C, and confirms robust *pdx-1* expression in cells cultured for 20 days in the presence (left) versus the absence (right) of growth factors. Figure 22E shows that after 43 days in a combination of the initial and maturation protocols, embryoid bodies robustly express *pdx-1*. *pdx-1* expression is generally clustered to a portion of a particular embryoid body.

Figure 23 shows two variations of the multi-step maturation protocol that result in C-peptide release. The top diagram is identical to that shown in Figure 19. The middle and bottom diagrams show two variations, each more efficient than the top diagram protocol.

Figure 24 shows the release of C-peptide when variations of the multi-step protocol was used.

Figures 25A-25C show the effect of forskolin in Step 4 of the multi-step maturation protocol on the release of C-peptide.

Figures 26A and 26B show the effect of fetal bovine serum (FBS) in Step 4 of the multi-step protocol on the release of C-peptide.

Figures 27A-27D show the protocol used (Figure 27A), the effect of glucose concentration on differentiated HES3 cells measured by the release of C-peptide (Figure 27B), *pdx-1* mRNA (Figure 27C) and insulin mRNA (Figure 27D).

Figure 28 shows the expression *of pdx-1* and C-peptide by single and double immunohistochemistry in differentiated HES3 embryoid bodies.

Figure 29 shows the expression of *pdx-1* on Day 20 of differentiation in BMM marketed as MATR1GEL™ in the presence and absence of growth factors, various late factors and early factors.

Figure 30 shows the expression of *pdx-1* in the presence and absence of

BMM marketed as MATRIGEL™ between Day 0 and Day 10.

Figure 31 shows the release of C-peptide on day 26 and day 29 when a simplified multi-step maturation protocol was used.

Figure 32 shows the presence of insulin and C-peptide by double immunofluorescence in sectioned embryoid bodies. The top panels are high magnification images and the bottom panels are low magnification images.

Figure 33 shows the expression of *Pdx-1* and *Nkx6.1*, both differentiation markers of β-cell endocrine lineage.

Figures 34A-34E show quantitative RT-PCR of RNA harvested from hES3 cells at the indicated time points. Cells were cultured as free-floating EBs (Free Floating), plated on a thin layer of GFR BMM marketed as MATRIGEL™ (2D MATRIGEL™), or embedded within gelatinous GFR BMM marketed as MATRIGEL™ (3D MATRIGEL™). Gene expression is represented relative to expression of undifferentiated hES3 cultures (day 0). Tbra: Brachyury, SHH: sonic hedgehog. Error bars indicate the standard deviation (SD) of three replicate experiments, each of two independent samples.

Figures 35A-35J show quantitative RT-PCR of RNA harvested from hES3 cells at the indicated time points. Cells were cultured as free-floating EBs (Free Floating), plated on a thin layer of GFR BMM marketed as GFR MATRTGEL™ (2D MATRIGEL™), or embedded within gelatinous GFR BMM marketed as GFR MATRIGEL™ (3D MATRIGEL™). Gene expression is represented relative to expression of undifferentiated hES3 cultures (day 0). AFP: Alpha feto protein, TTR: transthyretin. Error bars indicate the standard deviation (SD) of three replicate experiments, each of two independent samples.

Figure 36 shows quantitative RT-PCR of RNA harvested at day 20 from hES3 cells cultured according to differentiation protocol and embedded either in complete or growth factor reduced (GFR) formulations of BMM marketed as GFR MATRIGEL™. Gene expression is represented relative to expression detected in human adult pancreas. Error bars indicate the standard deviation (SD) of three replicate experiments, each of two independent samples.

Figure 37 shows quantitative RT-PCR of RNA harvested at day 20 from hES3 cells cultured according to differentiation protocol and embedded in the

scaffold and matrix supplement as indicated. Gene expression is represented relative to expression of samples embedded in gelatinous GFR BMM marketed as GFR MATR1GEL™. No suppl: no supplement. HSPG: Heparan sulfate proteoglycan. All matrix supplements were used at a final concentration of 100 μg/ml except for HSPG, which was used at 10 μg/ml. ECM gel: matrix formulation derived from EngelBreth-Holm-Swarm cells (Sigma). PLGA: poly(lactic-co-glycolic acid) and poly(L-lactic acid). Error bars indicate the standard deviation (SD) of three replicate experiments, each of two independent samples.

Figure 38 shows a comparison of the protocols used to seed hES cells into BMM marketed as MATRJGEL™, alginate, and synthetic peptide hydrogels *(e.g.,* marketed as PURAM ATRIX™). Collagen IV was added to alginate or synthetic peptide hydrogel *(e.g.,* marketed as PURAMATRIX™) solution for a final concentration of 50 or 100 μg/ml.

Figures 39A-39B show that hESC cultures embedded in synthetic peptide hydrogel or SPH *(e.g.,* marketed as PURAMATRIX™) supplemented with collagen IV differentiate into cells which express *pdx-1* and insulin mRNA, and secrete C-peptide into the medium. HESC cultures (hES3) were embedded in GFR BMM marketed as GFR MATRIGEL™, or SPH *(e.g.,* marketed as PURAMATRIX™) supplemented with collagen IV and differentiated using growth conditions included in Figure 18A. (Figure 39A) Total RNA was harvested on day 37 and analyzed by quantitative PCR for expression of the *pdx-1* and insulin transcripts. Both transcripts were expressed at higher levels in the SPH *(e.g.,* marketed as PURAM ATRIX™)-embedded cultures. (Figure 39B) Three-day-old media was harvested from day 37 cultures and analyzed by ELISA for the presence of human C-peptide. Media from SPH *(e.g.,* marketed as PURAMATRIX™)-embedded cultures expressed contained more C-peptide than GFR BMM marketed as GFR MATRIGEL™ embedded cultures. Error bars indicate standard deviation.

Figures 40A-40D show that hESC clusters embedded in SPH *(e.g.,* marketed as PURAMATRIX™)-sxipplemented with collagen IV resemble hESC clusters embedded in GFR BMM marketed as GFR MATRIGEL™. (Figures 4OA & 40B) hESC clusters were embedded in GFR BMM marketed as GFR MATRIGEL™ (A) or SPH (e.g., marketed as PURAM ATRIX™)-supplemented with collagen IV

(Figure 40B) and imaged by bright field microscopy after ten days of differentiation (original magnification: 10X). (Figures 4OC and 40D) hESC clusters were embedded in GFR BMM marketed as GFR MATRIGEL™ (Figure 40C) or SPH *(e.g.,* marketed as PURAMATRIX™)-supplemented with collagen IV (Figure 40C) and allowed to differentiate using growth conditions included in Figure 18A. Cell clusters were harvested on day 34, cryosectioned, and visualized by indirect immunofluorescence microscopy using antibodies which targeted Pdx-1 (red), C-peptide (green), and nuclear stain (DAPI, Blue). Differentiated hESC cells cultured in both conditions contained large patches of cells with nuclear staining of Pdx-1 and smaller clusters of Pdx- 1/ C-peptide double positive cells.

Detailed Description of the Invention

(i) Overview

The expansion and differentiation *in vitro* of ESC into functional mature cells may provide a new source of human tissue for transplantation. To achieve this goal, efficient and controlled methods must be developed to direct ESC differentiation. Culture of ESC in contact with the extra-cellular matrix formulation or basement membrane matrix (or "BMM," such as those marketed by BD Biosciences under the trademark MATRIGEL™) can enrich the development of endothelial, hepatic, neuronal, and mesenchymal, and polarized epithelial cells (1-3). Without being bound by theory, BMM likely exerts these effects by providing an adhesive structure which aids in tissue organization, and by stimulating biological signaling through a combination of adhesive receptors and the presentation of embedded growth factors (4-8).

For the purpose of reproducibly generating clinically compliant cells for human transplantation, however, the use of BMM (including those marketed as MATRIGEL™) may prove to be an impediment. This is partly because BMM is prepared from extracts of the Engelbreth-Holm-Swarm (EHS) mouse sarcoma containing a complex mixture of extra-cellular matrix proteins, proteoglycans and a host of growth factors and secreted enzymes (4, 9). In addition to being a xenobiotic product, the precise composition of each BMM preparation cannot be controlled. Thus identification of a defined composition with the desired biological properties of

BMM would provide an advantage to protocols designed to direct the differentiation of ESCs.

To this end, Applicants have shown that, in conjunction with a directed differentiation protocol, growth factor reduced BMM *(e.g.,* marketed by BD Biosciences as GFR MATRIGEL™) contributes to the differentiation of pancreatic beta cells from human ESC. Applicants have also show that GFR BMM *(e.g.,* GFR MATRIGEL™) is sufficient to enrich the differentiation of human ESC towards cells of the definitive endoderm, progenitors of mature pancreas.

However, as discussed earlier, due to its xenobiotic and complex nature, the use of BMM *(e.g.,* MATRIGEL™ or GFR MATRTGEL™) may be an impediment towards the generation of clinically compliant cell products.

Applicants have tested and discovered that human ESC grown in several scaffolds of chemically defined polymer matrices supplemented with collagen IV, such as synthetic peptide hydrogel or SPH *(e.g.,* marketed as PURAMATR1X™ by 3DM Inc., Cambridge, MA, and BD Biosciences), alginate, or poly(lactic-co-glycolic acid) and poly(L-lactic acid) (PLGA), can differentiate towards the endodermal lineages, such as the pancreatic lineage, as effectively as (if not more effective) when grown embedded in GFR BMM *(e.g.,* those marketed as GFR MATRIGEL™).

Thus the invention, in one aspect, provides a composition comprising a chemically defined three-dimensional polymer matrix supplemented with a collagen IV polypeptide, wherein the composition supports directed differentiation of embryonic stem (ES) cells to one or more desired endodermal lineage cells, such as pancreatic lineage cells. The composition of the invention may be used to provide controlled and clinically compliant conditions for the differentiation of human or animal cell products, which may be used to treat a number of diseases or conditions.

In certain embodiments, the polymer matrix comprises one or more synthetic or naturally occurring polymers. Optionally, the polymers are biodegradable. Biodegradable polymers of the invention may be embedded directly into a host (e.g., human or non-human animal), without the need to first isolate or purify desired cells differentiated from the ES cells embedded in the subject polymer matrix.

Exemplary biodegradable polymer matrices of the invention include (but are

not limited to) one or more of: poly(lactic acid) (PLA), poly(glycolic acid) (PGA), PLA-PGA co-polymer (PLGA), poly(anhydride), poly(hydroxy acid), poly(orthoester), poly(propylfumarate), poly(caprolactone), polyamide, polyamino acid, polyacetal, biodegradable polycyanoacrylate, biodegradable polyurethane, or polysaccharide.

In certain embodiments, the degradation products of the polymer matrix are non-toxic, such as amino acids, oligo- or mono-saccharides, *etc.*

In certain embodiments, the subject polymer matrix is non-biodegradable. Exemplary non-biodegradable polymer matrices of the invention include (but are not limited to) one or more of: polypyrrole, polyaniline, polythiophene, polystyrene, polyester, non-biodegradable polyurethane, polyurea, poly(ethylene vinyl acetate), polypropylene, polymethacrylate, polyethylene, polycarbonate, or poly(ethylene oxide).

In certain embodiments, the subject polymer matrix is a gel, such as a gel comprising methylcellulose, agarose, and/or alginate.

In certain embodiments, the subject polymer matrix is a copolymer, a mixture, or an adduct of one or more of: poly(lactic acid) (PLA), poly(glycolic acid) (PGA), PLA-PGA co-polymer (PLGA), poly(anhydride), poly(hydroxy acid), poly(orthoester), poly(propylfumarate), poly(caprolactone), polyamide, polyamino acid, polyacetal, biodegradable polycyanoacrylate, biodegradable polyurethane, polysaccharide, polypyrrole, polyaniline, polythiophene, polystyrene, polyester, non-biodegradable polyurethane, polyurea, poly(ethylene vinyl acetate), polypropylene, polymethacrylate, polyethylene, polycarbonate, poly(ethylene oxide), methylcellulose, agarose, peptide hydrogel *(e.g.,* those marked as PURAMATRIX™ by 3DM Inc., Cambridge, MA and BD Biosciences), or alginate.

In certain embodiments, the subject polymer matrix is non-antigenic *{e.g.,* does not provoke / stimulate host immune response against the polymer matrix).

In certain preferred embodiments, the subject polymer matrix is peptide hydrogel *(e.g.,* those marked as PURAMATRIX™ by 3DM Inc., Cambridge, MA and BD Biosciences), alginate, or PLGA.

In certain embodiments, the polymer matrix excludes one or more components of a solubilized basement membrane preparation extracted from

Engelbreth-Holm-Swarm (EHS) mouse sarcoma. The industry standard polymer matrix, *e.g.*, those BMM marketed as MATRIGEL™ by BD Biosciences, is a solubilized basement membrane preparation extracted from EHS mouse sarcoma - a tumor rich in extracellular matrix (ECM) proteins. At room temperature, such matrix polymerizes to produce biologically active matrix material resembling the mammalian cellular basement membrane. It provides a physiologically relevant environment for studies of cell morphology, biochemical function, migration or invasion, and gene expression. The major component of such BMM preparation is laminin, followed by collagen IV, heparan sulfate proteoglycans, and entactin 1. Such BMM may be prepared as described in Kleinman *et al. Biochemistry* 25: 312-318, 1986 (incorporated by reference). However, there may also be several uncharacterized minor components in the BMM, the amount of which could vary from lot to lot. Applicants have tested and shown that certain unidentified components present in complete BMM marketed as MATRIGEL™ indeed suppress the formation of the definitive endoderm (DE) or pancreatic lineages *{see* Example 18).

Thus the subject polymer matrix, being a chemically defined three-dimensional staicture, is advantageous compared to the complete BMM marketed as MATRIGEL™, in that it lacks or excludes at least one component of a solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, which component may inhibit the differentiation of ES cells embedded within the matrix towards the DE lineage or pancreatic lineage. Although the exact identity of the component / inhibitor need not be known, the presence or absence of such component / inhibitor can be tested by, for example, conducting a side-by-side experiment using the subject matrix and the BMM marketed as MATRIGEL™, to determine whether the subject matrix can better support the desired differentiation (which may be measured by the expression of lineage specific markers, such *aspdx-1* or *insulin,* and/or the number of differentiating embryonic bodies).

In certain embodiments, partly due to the exclusion of one or more components of a solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, the subject composition with the

subject polymer matrix is at least about 10%, 20%, 30%, 50%, 75%, 100%, 2-fold, 5-fold, 10-fold or more better than BMM marketed as MATRTGEL™ (BD Biosciences) or its growth factor reduced version marketed as GFR MATRIGEL™ (BD Biosciences), as measured by the extent of stimulating the expression of a given differentiation marker, such as *pdx-1* or *insulin*, and/or the number of differentiating embryonic bodies (EBs).

In certain embodiments, in the subject composition, the polymer matrix consists of or consists essentially of a mixture of: (1) peptide hydrogel *{e.g.*, those marketed as PURAMATRIX™ by 3DM Inc., Cambridge, MA and BD Biosciences), alginate, or PLGA, and (2) purified collagen IV *(e.g.*, from human, non-human animal such as mouse, or other sources).

In certain embodiments, the subject composition and polymer matrix has a microstructure similar to that of an extracellular matrix, such as the BMM marketed by BD Biosciences as MATRIGEL™ or GFR MATRIGEL™, or the SPH marketed by BD Biosciences and 3DM Inc. as PURAMATRIX™. The SPH marketed as PURAMATRIX™ is described in detail in U.S. Pat. Nos. 5,670,483, 5,955,343, 6,548,630, 6,800,481, 7,098,028, and U.S. patent application publication US 2004-0087013 A1 (all incorporated herein by reference).

For example, the SPH may be formed by formed by self-assembly of amphiphilic peptides in an aqueous solution containing monovalent metal cations, wherein the peptides contain 12 or more amino acids, have alternating hydrophobic and hydrophilic amino acids and are complementary and structurally compatible. Conditions under which the peptides self-assemble into macroscopic membranes and methods for producing the matrix are also described. One of the exemplary peptides named EAK 16 was observed to form a membranous structure with the appearance of a piece of transparent, thin (about 10-20 μm) plastic membrane when viewed under 100x magnification by phase-contrast microscopy. The membrane was formed in phosphate-buffered saline (PBS) and is colorless and isobuoyant. At low magnifications (50-100x), the structure looks like a flat membrane. At high magnifications (30,00Ox), scanning electron microscope (SEM) revealed that the membrane is made up of individual filaments that are interwoven. The architecture of the structure appears to resemble high density felt or cloth. The diameters of the

filaments are approximately 10-20 nm and the distance between the fibers is approximately 50-80 nm. The membranes have β-sheet secondary structure (confirmed by circular dichroism (CD) spectroscopy - a typical β-sheet CD spectrum with an absorbance minimum at 218 nm and a maximum at 195 nm was detected).

The effect of length and sequence on membrane formation was also examined using several peptides. These results indicate that the peptide length should be more than 12 amino acids and preferably at least 16 residues. Very long peptides, *e.g.*, of about 200 amino acids, may encounter problems due to insolubility and intramolecular interactions which destabilize membrane formation. Furthermore, peptides with a large amount of hydrophobic residues may have insolubility problems. The optimal lengths for membrane formation will probably vary with the amino acid composition. In addition, alternating hydrophobic and hydrophilic sequence of the peptide is important to membrane formation.

The criteria of amphiphilic sequence, length, complementarity and structural compatibility also apply to heterogeneous mixtures of peptides. Membranes can also be formed of heterogeneous mixtures of peptides, each of which alone would not form membranes, if they are complementary and structurally compatible to each other. Peptides, which are not perfectly complementary or structurally compatible, can be thought of as containing mismatches analogous to mismatched base pairs in the hybridization of nucleic acids. Peptides containing mismatches can form membranes if the disruptive force of the mismatched pair is dominated by the overall stability of the interpeptide interaction. Functionally, such peptides can also be considered as complementary or structurally compatible. Mismatched peptides can be tested for ability to self-assemble into macroscopic membranes using the methods described therein.

In general, peptides expected to form macroscopic membranes have alternating hydrophobic and hydrophilic amino acids, are more than 12 amino acids and preferably at least 16 amino acids long, are complementary and structurally compatible. The hydrophobic amino acids include Ala, VaI, He, Met, Phe, Tyr, Trp, Ser, Thr and GIy. The hydrophilic amino acids can be basic amino acids, *e.g.*, Lys, Arg, His, Orn; acidic amino acids, *e.g.*, GIu, Asp; or amino acids which form hydrogen bonds, *e.g.*, Asn, GIn. Acidic and basic amino acids can be clustered on a

peptide. The carboxyi and amino groups of the terminal residues can be protected or not protected. Membranes can be formed in a homogeneous mixture of self-cornplementary and self-compatible peptides or in a heterogeneous mixture of peptides which are complementary and structurally compatible to each other. Peptides fitting the above criteria can self-assemble into macroscopic membranes under suitable conditions.

Salt appears to play an important role in the self-assembiy process. Various metal cations have been tested for effectiveness at inducing membrane formation from exemplary peptides (such as EAKl 6, AEAEAKAKAEAEAKAK, SEQ ID NO: 29). The results indicate that monovalent metal cations induce membrane formation, but divalent cations primarily induce unstructured aggregates. The order of effectiveness of the monovalent cations appears to be $Li^+ > Na^+ > K^+ > Cs^+$. Cs^+ produces the least amount of membranes, and in addition, yields nonmembranous precipitates. The effectiveness of the monovalent cations appears to correlate inversely with the crystal radii of the ions: Li^+ (0.6.ANG.), Na^+ (0.95.ANG.), K^+ (1.33.ANG.), and Cs^+ (1.69.ANG.) (Pauling, 1960). A correlation is also seen with the hydrated radii of the ions: Li^+ (3AANG.), Na^+ (2.76.ANG.), K^+ (2.32.ANG.), and Cs^+ (2.28.ANG.), and with the order of enthalpies of the monovalent cations (Pauling, I960). The size of the filaments (10-20 nm) and interfilament distance (50-80 nm) in the membranes formed from EAK 16 suggest that hydrated ions may stabilize the intermolecular interaction.

Concentrations of monovalent metal cations (NaCl) as low as 5 mM and as high as 5M have been found to induce membrane formation within a few minutes. Thus, membrane formation appears to be independent of salt concentration over this wide range. Salt concentrations of less than 5 mM may also induce membrane formation, but at a slower rate. The initial concentration of the peptide is a significant factor in the size and thickness of the membrane formed. In general, the higher the peptide concentration, the higher the extent of membrane formation. Membranes can form from initial peptide concentrations as low as 0.5 mM or 1 mg/ml. However, membranes formed at higher initial peptide concentrations (about 10 mg/ml) are thicker and thus, likely to be stronger. Therefore, it is preferable when producing the membranes to add peptide to a salt solution, rather than to add salt to a

peptide solution.

Formation of the membranes is very fast, on the order of a few minutes, and seems to be irreversible. The process is unaffected by pH<12 (the peptides tend to precipitate out at pH above 12), and by temperature. The membranes can form at temperatures in the range of 4 ^{0}C to 90 °C.

Formation of the membranes is inhibited by the presence of divalent metal cations at concentrations equal to or greater than 100 μM, which promote unstructured aggregation rather than membrane formation, and by sodium dodecyl sulfate (SDS) at a concentration of at least 0.1 %.

Once formed, the macroscopic membranes are stable in a variety of aqueous solutions, including water, phosphate-buffered saline (PBS), tissue culture medium, serum, and also in ethanol, and can be transferred to and stored in any of these liquids. Membranes formed have been found to be stable in water or PBS for at least a week without any sign of deterioration. The membranes can be transferred from one solution to another using a solid support such as a spatula. They can be broken by cutting, tearing or shearing. Membranes formed of EAKl 6 were found to be unusually stable under various conditions expected to disrupt them. Circular dichroism (CD) spectroscopy measurements further demonstrated the unusual stability of the β-sheet secondary structure of the peptide EAK16. The β-sheets can be thought of as the building blocks for the macroscopic membrane structures and their unusual stability confirms the strength of the peptide interactions holding the membrane together.

The stability of the membranes was also tested under a range of temperature, pH and chemical conditions. For these experiments, membranes were formed by adding 20 μï of a 0.5 mM stock solution of EAKl 6 to 0.5 ml of PBS, and transferred into water or other solutions at test conditions. The membranes were found to be stable in water over a wide range of temperatures: up to 95 ^{0}C and at 4 ^{0}C, -20 ^{0}C, and -80 ^{0}C. The membranes could be frozen and thawed, although care was required in handling the frozen membranes which were brittle. In boiling water, the membranes tended to be sheared by the mechanical agitation. The CD spectra of EAK 16 were also found to be unaffected over the range of 25-90 ^{0}C. The EAK 16 membranes were also tested in water at pH 1.5, 3, 7 and 11 at room temperature for

at least a week and at 95 ^{0}C for about 4 hours. The membranes were unaffected at these pH values. The β-sheet structure of the peptide was also unaffected over this pH range; the pH profile shows a less than 10% decrease of ellipticity. Precipitation of the peptide was observed at pH above 12.5.

The membranes also appear to be non-cytotoxlc, since the peptides and resultant membranes did not affect the appearance or rate of growth of the cells.

The subject polymer matrices are formed to resemble one or more of the characteristics of the commercially available SPH or BMM in terms of molecular weight, tacticity, integrity, durability, hydration, and/or cross-linkage, such that the polymers can be formulated accordingly to control the mechanical properties of the matrix and/or the degradation rate for degradable scaffolds. Any art-recognized methods, such as SEM imaging and/or biological function testing, may be used to verify that a specific polymer matrix has similar consistency of the matrix in terms of durability, hydration, and/or cross-linkage as that of the BMM marketed as MATRIGEL™ or SPH marketed as PURAMATR1X™.

The polymer matrix of the subject composition may adopt a variety of shapes and sizes. For example, the subject polymer matrix may be formed as a particle, a sponge, a tube, a sphere, a strand, a coiled strand, a capillary network, a film, a fiber, a mesh, and/or a sheet.

In certain embodiments, the subject composition may further comprise one or more embryonic stem (ES) cells and/or one or more endodermal lineage cells in various stages of differentiation from the ES cells.

Although any ES cells may be used with the subject composition, exemplary ES cells include human and mouse ES cells.

In certain embodiments, the subject composition may also be used for the differentiation of adult stem cells, such as hematopoietic stem cells and bone marrow stromal cells *(e.g.,* human, non-human animal, *etc.).*

In certain embodiments, the subject composition provides a suitable growth environment for the production of three-dimensional tissue structure that limits the formation of the visceral endoderm while stimulating the formation of the definitive endoderm.

The differentiation towards visceral endoderm or definitive endoderm may

be detected by, for example, the expression of marker genes. For example, cells of the definitive endoderm may express one or more of: *cerberus, sox!7, vHNFJ, hex, GATA6, HNF3*β, and substantially lack expression of the visceral endodermal marker *Hl9*. As used herein, "substantially lack," in various embodiments, refers to less than 50%, 40%, 30%, 20%, 10%, 5%, 1%, or nearly no expression, when compared to a suitable control. For example, in certain embodiments, cells of the definitive endoderm express less than 20% of *H19* when compared to cells differentiating towards the visceral endodermal lineage.

In certain embodiments, the endodermal lineage cells include pancreatic lineage cells that *axepdxl* $^{+}$ and/or secrete C-peptide extracellularly.

In certain embodiments, the composition produces at least about 50%, 75%, 100%, twice, 3-times, 5-times, 10-times or more as many of a desired differentiated endodermal lineage cells (such as pancreatic lineage cells) as compared to that produced by solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma *(e.g.,* BD™ Biosciences MATRIGEL™), or a growth factor-reduced (GFR) version thereof (e.g., BD™ Biosciences GFR MATRIGEL™).

The extent of the desired differentiation towards endodermal lineage may be measured / quantitated by, for example, the expression of certain endodermal lineage markers (such *a.spdx-1* or *insulin),* the number of EBs, *etc.*

In certain embodiments, the composition produces a desired differentiated endodermal lineage cells with similar or substantially the same phenotypes as are produced using solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma *(e.g.,* BD™ Biosciences MATRIGEL™), or a growth factor-reduced (GFR) version thereof *(e.g.,* BD™ Biosciences GFR MATRIGEL™).

Exemplary phenotypes may include one or more of: expression of endodermal differentiation markers or pancreatic differentiation markers (e.g., one or both of *pdxl* and *insulin),* secretion of C-peptide extracellularly, or morphology.

In certain embodiments, the subject composition produces pancreatic lineage cells that (1) express both *pdxl* and *insulin,* and/or (2) secrete C-peptide, at levels at least as high as, or at least 10%, 20%, 30%, 50%, 75%, 100%, twice, 3-times, 5-

times,or 10-times higher than that produced by cells obtained using solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma *(e.g.*, BD™ Biosciences MATRIGEL™), or a growth factor-reduced (GFR) version thereof (e.g., BD™ Biosciences GFR MATRIGEL™).

In certain embodiments, the subject composition produces a population of differentiated endodermal lineage cells that are at least about 25%, 50%, 60%, 70%, 80%, 90%, 95% homogeneous without purification with respect to at least one differentiation marker. As used herein, "x% homogeneous" refers to the fact that the stated percentage of cells are the same with respect to at least one phenotype, such as expression of one desired differentiation marker. For example, in the context of differentiating ES cells towards pancreatic lineage cells, a population of differentiated endodermal lineage cells *(e.g.*, pancreatic cells) are at least about 25% homogeneous, if at least about 25% of the cells express *pdx-1* or *insulin*, although individual cells within this 25% of the cells may have different *levels of pdx-1* or *insulin* expression, or are heterogeneous with respect to other phenotypes, such as the expression of other markers.

Another aspect of the invention relates to a method for directed differentiation of an increased number of ES cells to endodermal cell types, comprising culturing the ES cells in a composition of any of the subject compositions described herein.

In certain embodiments, the ES cells are differentiated into pancreatic lineage cells that express one or more pancreatic lineage marker(s), such as those described herein, and/or exhibit a pancreatic lineage function, such as secrete C-peptide, become glucose-responsive, *etc.*

Type IV Collagen is a ubiquitous component of basement membranes. The type IV collagen that can be used in the subject composition may be obtained from any source, such as from human or non-human animal. Collagen IV may also be synthetic *(e.g.*, produced by recombinant DNA technology).

Collagen IV is also commercially available. See, for example, Sigma C-0543.

For example, collagen IV may be obtained from discarded human tissue, such as placenta. Preferably, the human source material is tested for blood-born

pathogens, including hepatitis B virus *(e.g.,* detecting the presence of HBV antigen or antibody against the antigen) and/or HIV-I virus *(e.g.,* detecting the presence of HIV-I antibody).

If the collagen IV is obtained from mouse, Engelbreth-Holm-Swarm Iathrytic mouse tumor may be used as the source material.

Collagen IV obtained from the various sources may also be tested for the presence of bacteria, fungi, and mycoplasma using art-recognized means. Only collagen IV isolated from sources free of these pathogens are used in the subject composition.

The purity of the collagen IV may be measured using art-recognized means, such as by SDS-PAGE. In certain embodiments, collagen IV used in the subject composition is at least about 50%, 60%, 70%, 80%, 90%, 95%, 99% pure (electrophoretically homogeneous), as judged by SDS-PAGE.

In a preferred embodiment, collagen IV used in the subject composition is 100% pure *(i.e.,* has no detectable impurity based on SDS-PAGE).

In certain embodiments, the subject composition is supplemented with about 10 μg/ml, 20 μg/ml, 50 μg/ml, 75 μg/ml, 100 μg/ml, 150 μg/ml, 200 μg/ml, 250 μg/ml equivalent of collagen IV.

Various differentiation protocols may be used in conjunction with the subject compositions to differentiate ES cells.

For example, in certain embodiments, the method of the invention may comprise contacting the ES cells for a sufficient period of time with a sufficient amount of one or more early factors (EFs) selected from activin A, BMP-2, BMP-4, or nodal.

In certain embodiments, the differentiated pancreatic lineage cells express *pd.x-J* and/or *insulin,* and/or are responsive to glucose, and/or secret C-peptide. Preferably, the pancreatic lineage cells are insulin-producing cells.

In certain embodiments, as part of the protocol for generating insulin-secreting cells, the methods of the invention include steps to culture ES cells as embryoid bodies (EBs) plated directly onto a support matrix and/or plated directly onto tissue culture plates. For example, the EBs may be cultured in a floating suspension culture, in a support matrix, and/or on a filter.

In certain embodiments, using the subject composition results in at least 10%, 20%, 30%, 50%, 75%, 100%, twice, 3-times, 5-times, 10-times or more endoderm lineage formation than using the BMM marketed as MATRIGEL™ or GFR MATRIGEL™. In certain embodiments, the using the subject composition results in at least 10%, 20%, 30%, 50%, 75%, 100%, twice, 3-times, 5-times, 10-times or more insulin-producing cells than using the BMM marketed as MATRIGEL™ or GFR MATRIGEL™.

In certain embodiments, the EBs are cultured in a support matrix *(e.g.,* BMM marketed as MATRIGEL™ or GFR MATRIGEL™) only during the period when the EBs are in contact with the EFs.

In certain embodiments, the EBs are generated from ES cells grown on mouse embryonic feeder (MEF) or other feeder layers, or from ES cells grown under feeder-free conditions.

The ES cells may be human ES cells, or mouse ES cells.

In certain embodiments, the ES cells are partially or terminally differentiated into the pancreatic lineage.

In certain embodiments, the ES cells are contacted with the EFs for about 10 days, or about 15 days.

In certain embodiments, the EFs comprise activin A and BMP-4, such as about 50 ng/mL of activin A and about 50 ng/mL of BMP-4.

In certain embodiments, the methods of the invention further comprise contacting the ES cells, subsequent to contacting the ES cells with the EFs, with a sufficient amount of one or more late factors (LFs) for a second sufficient period of time.

In certain embodiments, the one or more LFs may be HGF, exendin4, betacellulin, and nicotinamide, such as about 50 ng/mL of HGF, about 10 ng/mL of exendin4, and about 50 ng/mL of β-cellulin.

In a preferred embodiment, the ES cells are contacted with the EFs for about 10 days, and are subsequently contacted with the LFs for about 10 days. Preferably, the EFs comprise about 50 ng/mL of activin A and about 50 ng/mL of BMP4, and the LFs include about 50 ng/mL of HGF, about 10 ng/mL of exendin4, and about 50 ng/mL of β-cellulin.

In certain embodiments, the methods of the invention further comprise contacting the ES cells, subsequent to the initiation protocol and during a maturation protocol, consecutively with: (1) a basal medium for about 6 days; (2) about 20 ng/ml FGF-18, and about 2 μg/ml heparin in the basal medium for about 5-6 days; (3) about 20 ng/ml FGF-18, about 2 μg/ml heparin, about 10 ng/ml EGF, about 4 ng/ml TGFα, about 30 ng/ml IGFl, about 30 ng/ml IGF2, and about 10 ng/ml VEGF in the basal medium for about 4-5 days; (4) about 10 μM forskolin, about 40 ng/ml HGF, and about 200 ng/ml PYY for about 3-4 days; and, (5) about 100 ng/ml exendin-4, and about 5 mM nicotinamide for about 3-4 days.

In certain embodiments, the ES cells are not dissociated by dispase between steps (1) and (2).

In certain embodiments, FBS (if any) in the medium is replaced with a chemically defined serum replacer (SR).

In certain embodiments, the methods further comprise contacting the ES cells, subsequent to the EF and LF treatment, and during a maturation protocol, with about 10 μM forskolin, about 40 ng/ml HGF, and about 200 ng/ml PYY for about 3-4 days. Preferably, the ES cells are grown on fibronectin-coated tissue culture surfaces during the maturation protocol.

In certain embodiments, the differentiated cells release C-peptide and/or are responsive to glucose stimulation.

In certain embodiments, the methods further comprise contacting the ES cells, subsequent to contacting the ES cells with the EFs and during a maturation protocol, consecutively with: (1) about 20 ng/ml FGF-18, and about 2 μg/ml heparin in a basal medium for about 8 days; (2) about 20 ng/ml FGF-18, about 2 μg/ml heparin, about 10 ng/ml EGF, about 4 ng/ml TGFα, about 30 ng/ml IGFl, about 30 ng/ml IGF2, and about 10 ng/ml VEGF in the basal medium for about 6 days; and (3) about 10 μM forskolin, about 40 ng/ml HGF, and about 200 ng/ml PYY for about 5 days. Preferably, step (1) lasts 6 days, steps (2) and (3) last 4 days each.

In certain embodiments, the differentiated cells release C-peptide.

Another aspect of the invention provides differentiated pancreatic lineage cells or cell cultures obtained through any of the subject methods described herein. The differentiated pancreatic lineage cells or cell cultures may be partially

differentiated, or terminally differentiated. Preferably, the differentiated pancreatic lineage cells or cell cultures mimic the function, in whole or in part, of insulin-producing cells.

Another aspect of the invention provides a method for the treatment or prophylaxis, in an individual, of diseases, injuries, or conditions of the pancreas characterized by impaired pancreatic function, comprising administering to the individual the differentiated pancreatic lineage cells, such as those produced using the subject compositions and methods.

In certain embodiments, the impaired pancreatic function includes impaired ability to properly regulate glucose metabolism in an affected individual.

In certain embodiments, the condition is type I or type II diabetes.

In certain embodiments, the method is in conjunction with one or more additional therapies effective for the treatment or prophylaxis of the diseases, injuries, or conditions.

One exemplary disease treatable using the cells differentiated by the subject composition / methods is diabetes mellitus. Diabetes mellitus is a common disease characterized by the inability to regulate circulating glucose levels due to problems with insulin production or utilization. Type I diabetes (about 5% of all diabetes cases) is caused by the autoimmune destruction of the pancreatic beta cell that produces insulin. The more common type 2 diabetes, associated with obesity, has many causes related to either a decreased insulin output by the pancreas or to inefficient utilization of insulin at the target organs (insulin resistance). Collectively, diabetes can be considered a global epidemic, affecting as many as 7.9% of the American people. The very nature of the diabetic pathology, namely the autoimmune destruction or the decreased efficiency of the pancreatic beta cell, makes it an ideal candidate for cell therapy. Recent breakthroughs in islet transplantation that draw upon improved islet isolation techniques and immunosuppression regimes have been very successful at keeping patients free from insulin dependency for extended periods of time. However, the limited supply of cadaver pancreatic tissue makes this approach inadequate to meet the global patient demand for treatment. Researchers are therefore focusing on locating other sources of beta cells, of which embryonic stem cells are an attractive choice.

Several labs have reported the differentiation of insulin-producing cells from mouse ES cells. One such protocol depends on the isolation and purification of cells that are nestin-positive, and is based on the assumption that beta cells might first pass through a neuronal intermediate. However, the insulin release observed is likely caused by insulin uptake from the cell culture media. Furthermore, several labs have demonstrated that morphologically, the cells produced by these protocols are quite different from a *bonefide* beta cell, and in fact are more neuron-like with the acquired capacity for insulin uptake and release.

The work in human ES cells has lagged behind that in mouse, due in large part to the infancy of the human ES field in general and international legislature against public hESC research. The published reports detailing beta cell differentiation from mouse ES cells are worthy pioneering efforts, and have fostered optimism for the field, yet are menaced by reports of irreproducibility or poor efficiency. This leaves a huge opening in the field of human ES cells to develop a robust directed differentiation protocol that efficiently produces large numbers of beta cells for eventual cell therapy.

Both the rise in cases of diabetes and the recent success of the Edmonton protocol as a method of treating diabetes have placed great optimism on cell therapeutic methods to cure the disease. Though a highly competitive field, there are not yet any efficient, reproducible protocols available to direct differentiation of pluripotent human embryonic stem cells (hESCs) towards a pancreatic beta cell-like phenotype.

The present invention, using the subject compositions, provides a variety of methods to direct the differentiation of embryonic stem cells to a pancreatic cell fate. These include methods comprising the use of several early and late factors (EF and LF) administered over an approximately 20 day time frame. This initial differentiation methodology promotes expression of *pdx-1* and promotes differentiation of embryonic stem cells along a pancreatic lineage. Additionally, this initial differentiation methodology promotes expression of markers of terminal pancreatic differentiation, such as insulin and somatostatin, though at a lower level than that of *pdx-1*. Also using the subject compositions, the present invention provides a variety of experiments identifying factors and optimized sub-sets of early

and late factors that help promote differentiation of embryonic stem cells along a pancreatic lineage.

In addition to a variety of initial differentiation methods, the present invention provides maturation protocols designed to further promote the differentiation of stem cells along a pancreatic lineage. Specifically, the invention provides maturation protocols that can be used to promote terminal differentiation of embryonic stem cells that were previously directed along a pancreatic lineage using the initial differentiation protocols detailed herein. Using the maturation protocols, embryonic stem cells can be further differentiated to induce and/or increase expression of terminal differentiation markers including, but not limited to, insulin and C-peptide. Furthermore, such maturation protocols can be used to produce cell or cell clusters that are glucose responsive *(e.g.,* mimic a function of pancreatic beta cells).

(H) Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

The articles "a" and "an" are used herein to refer to one or to more than one *(i.e.,* to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

As used herein, "protein" is a polymer consisting essentially of any of the 20 amino acids. Although "polypeptide" is often used in reference to relatively large polypeptides, and "peptide" is often used in reference to small polypeptides, usage of these terms in the art overlaps and is varied.

The terms "peptide(s)", "protein(s)" and "polypeptide(s)" are used interchangeably herein.

The terms "polynucleotide sequence" and "nucleotide sequence" are also used interchangeably herein.

"Recombinant," as used herein, means that a protein is derived from a prokaryotic or eukaryotic expression system.

The term "wild type" refers to the naturally-occurring polynucleotide

sequence encoding a protein, or a portion thereof, or protein sequence, or portion thereof, respe'ctively, as it normally exists *in vivo.*

The term "mutant" refers to any change in the genetic material of an organism, in particular a change *{i.e.,* deletion, substitution, addition, or alteration) in a wild-type polynucleotide sequence or any change in a wild-type protein sequence. The term "variant" is used interchangeably with "mutant". Although it is often assumed that a change in the genetic material results in a change of the function of the protein, the terms "mutant" and "variant" refer to a change in the sequence of a wild-type protein regardless of whether that change alters the function of the protein (e.g., increases, decreases, imparts a new function), or whether that change has no effect on the function of the protein *(e.g.,* the mutation or variation is silent).

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides.

As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid comprising an open reading frame encoding a polypeptide, including both exon and (optionally) intron sequences.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors".

A polynucleotide sequence (DNA, RNA) is "operatively linked" to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that polynucleotide sequence. The term "operatively linked" includes having an appropriate start signal *(e.g.,* ATG) in front of the polynucleotide sequence to be expressed, and maintaining the correct reading frame to permit expression of the polynucleotide sequence under the control of the expression control sequence, and production of the desired polypeptide encoded by

the polynucleotide sequence.

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to nucleic acid sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which they are operably linked. In some examples, transcription of a recombinant gene is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended. It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring form of a protein.

As used herein, the term "tissue-specific promoter" means a nucleic acid sequence that serves as a promoter, *i.e.,* regulates expression of a selected nucleic acid sequence operably linked to the promoter, and which affects expression of the selected nucleic acid sequence in specific cells of a tissue, such as cells of neural origin, *e.g.* neuronal cells. The term also covers so-called "leaky" promoters, which regulate expression of a selected nucleic acid primarily in one tissue, but cause expression in other tissues as well.

"Homology" and "identity" are used synonymously throughout and refer to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous or identical at that position. A degree of homology or identity between sequences is a function of the number of matching or homologous positions shared by the sequences.

A "chimeric protein" or "fusion protein" is a fusion of a first amino acid sequence encoding a polypeptide with a second amino acid sequence defining a domain *(e.g.* polypeptide portion) foreign to and not substantially homologous with any domain of the first polypeptide. A chimeric protein may present a foreign domain which is found (albeit in a different protein) in an organism which also expresses the first protein, or it may be an "interspecies", "intergenic", *etc.* fusion of

protein structures expressed by different kinds of organisms.

As used herein, "small organic molecule" refers to compounds smaller than proteins that are generally characterized by the ability to transit cellular membranes more easily than proteins. Preferred small organic molecules are characterized as having a size less than 1,000 AMU, more preferably between 100-1,000 AMU. Most preferably, the small organic molecules are characterized as having a size less than 500 AMU, 250 AMU, 150 AMU, 100 AMU, 50 AMU or lower.

The "non-human animals" of the invention include mammals such as rats, rriice, rabbits, sheep, cats, dogs, cows, pigs, and non-human primates.

As used herein, "proliferating" and "proliferation" refer to cells undergoing mitosis.

"Differentiation" in the present context means the formation of cells expressing markers known to be associated with cells that are more specialized and closer to becoming terminally differentiated cells incapable of further division or differentiation. The pathway along which cells progress from a less committed cell, to a cell that is increasingly committed to a particular cell type, and eventually to a terminally differentiated cell is referred to as progressive differentiation or progressive commitment. Cell which are more specialized *{e.g.,* have begun to progress along a path of progressive differentiation) but not yet terminally differentiated are referred to as partially differentiated.

The term "progenitor cell" is used synonymously with "stem cell". Both terms refer to an undifferentiated cell which is capable of proliferation and giving rise to more progenitor cells having the ability to generate a large number of mother cells that can in turn give rise to differentiated or differentiable daughter cells. In a preferred embodiment, the term progenitor or stem cell refers to a generalized mother cell whose descendants (progeny) specialize, often in different directions, by differentiation, *e.g.,* by acquiring completely individual characters, as occurs in progressive diversification of embryonic cells and tissues. Cellular differentiation is a complex process typically occurring through many cell divisions. A differentiated cell may derive from a multipotent cell which itself is derived from a multipotent cell, and so on. While each of these multipotent cells may be considered stem cells, the range of cell types each can give rise to may vary considerably. Some

differentiated cells also have the capacity to give rise to cells of greater developmental potential. Such capacity may be natural or may be induced artificially upon treatment with various factors.

The term "embryonic stem cell" is used to refer to the piuripotent stem cells of the inner cell mass of the embryonic blastocyst (see US Patent Nos. 5843780, 6200806). Such cells can similarly be obtained from the inner cell mass of blastocysts derived from somatic cell nuclear transfer (see, for example, US Patent Nos 5945577, 5994619, 6235970). The distinguishing characteristics of an embryonic stem cell define an embryonic stem cell phenotype. Accordingly, a cell has the phenotype of an embryonic stem cell if it possesses one or more of the unique characteristics of an embryonic stem cell such that that cell can be distinguished from other cells. Exemplary distinguishing embryonic stem cell characteristics include, without limitation, gene expression profile, proliferative capacity, differentiation capacity, karyotype, responsiveness to particular culture conditions, and the like.

The term "adult stem cell" is used to refer to any multipotent stem cell derived from non-embryonic tissue, including fetal, juvenile, and adult tissue. Stem cells have been isolated from a wide variety of adult tissues including blood, bone marrow, brain, olfactory epithelium, skin, pancreas, skeletal muscle, and cardiac muscle. Each of these stem cells can be characterized based on gene expression, factor responsiveness, and morphology in culture. Exemplary adult stem cells include neural stem cells, neural crest stem cells, mesenchymal stem cells, hematopoietic stem cells, and pancreatic stem cells. As indicated above, stem cells have been found resident in virtually every tissue. Accordingly, the present invention appreciates that stem cell populations can be isolated from virtually any animal tissue.

The term "tissue" refers to a group or layer of similarly specialized cells which together perform certain special functions.

The term "substantially pure", with respect to a particular cell population, refers to a population of cells that is at least about 75%, preferably at least about 85%, more preferably at least about 90%, and most preferably at least about 95% pure, with respect to the cells making up a total cell population. Recast, the terms

"substantially pure" or "essentially purified", with regard to a preparation of one or more partially and/or terminally differentiated cell types, refer to a population of cells that contain fewer than about 20%, more preferably fewer than about 15%, 10%, 8%, 7%, most preferably fewer than about 5%, 4%, 3%, *2%,* 1%, or less than 1%, of cells that are either undifferentiated, are differentiated to a non-endodermal cell type, or are differentiated to an endodermal tissue type that is not functionally or structurally related to that of the essentially purified population of cells.

The term "purified", with respect to small molecule, polypeptide, or polymer, refers to an object species that is the predominant species present *(i.e.,* on a molar basis it is more abundant than any other individual species in the composition). In making the determination of the purity of a species in solution or dispersion, the solvent or matrix in which the species is dissolved or dispersed is usually not included in such determination; instead, only the species (including the one of interest) dissolved or dispersed are taken into account. Generally, a purified composition will have one species that comprises more than about 80 percent of all species present in the composition, more than about 85%, 90%, 95%, 99% or more of all species present. The object species may be purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single species.

A "marker" is used to determine the state of a cell. Markers are characteristics, whether morphological or biochemical (enzymatic), particular to a cell type, or molecules expressed by the cell type. Preferably, such markers are proteins, and more preferably, possess an epitope for antibodies or other binding molecules available in the art. However, a marker may consist of any molecule found in a cell including, but not limited to, proteins (peptides and polypeptides), lipids, polysaccharides, nucleic acids and steroids. Additionally, a marker may comprise a morphological or functional characteristic of a cell. Examples of morphological traits include, but are not limited to, shape, size, and nuclear to cytoplasmic ratio. Examples of functional traits include, but are not limited to, the ability to adhere to particular substrates, ability to incorporate or exclude particular dyes, ability to migrate under particular conditions, and the ability to differentiate along particular lineages.

Markers may be detected by any method available to one of skill in the art. In addition to antibodies (and all antibody derivatives) that recognize and bind at least one epitope on a marker molecule, markers may be detected using analytical techniques, such as by protein dot blots, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), or any other gel system that separates proteins, with subsequent visualization of the marker (such as Western blots), gel filtration, affinity column purification; morphologically, such as fluorescent-activated cell sorting (FACS), staining with dyes that have a specific reaction with a marker molecule (such as ruthenium red and extracellular matrix molecules), specific morphological characteristics (such as the presence of microvilli in epithelia, or the pseudopodia/filopodia in migrating cells, such as fibroblasts and mesenchyme); and biochemically, such as assaying for an enzymatic product or intermediate, or the overall composition of a cell, such as the ratio of protein to lipid, or lipid to sugar, or even the ratio of two specific lipids to each other, or polysaccharides. In the case of nucleic acid markers, any known method may be used. If such a marker is a nucleic acid, PCR, RT-PCR, *in situ* hybridization, dot blot hybridization, Northern blots, Southern blots and the like may be used, coupled with suitable detection methods. If such a marker is a morphological and/or functional trait, suitable methods include visual inspection using, for example, the unaided eye, a stereomicroscope, a dissecting microscope, a confocal microscope, or an electron microscope. The invention contemplates methods of analyzing the progressive or terminal differentiation of a cell employing a single marker, as well as any combination of molecular and/or non-molecular markers.

Differentiation is a developmental process whereby cells assume a specialized phenotype, *e.g.*, acquire one or more characteristics or functions distinct from other cell types. In certain embodiments, differentiation refers to the formation of cells expressing markers known to be associated with cells that are more specialized and closer to becoming terminally differentiated cells incapable of further division or differentiation. In some cases, the differentiated phenotype refers to a cell phenotype that is at the mature endpoint *in* some developmental pathway (a so called terminally differentiated cell). In many but not all tissues, the process of differentiation is coupled with exit from the cell cycle. In these cases, the terminally

differentiated cells lose or greatly restrict their capacity to proliferate. However, we note that the term "differentiation" or "differentiated" refers to cells that are more specialized in their fate or function than at a previous point in their development, and includes both cells that are terminally differentiated and cells that, although not terminally differentiated, are more specialized than at a previous point in their development. The development of a cell from an uncommitted cell (for example, a stem cell), to a cell with an increasing degree of commitment to a particular differentiated cell type, and finally to a terminally differentiated cell is known as progressive differentiation or progressive commitment. Cells which have become more specialized but are not yet terminally differentiated are referred to as partially differentiated.

The terms "initiation protocol" or "initiation method" are used interchangeably to refer to any of the various methods of the invention used to begin biasing embryonic stem cells and embryoid bodies along a pancreatic lineage. The initiation protocol is typically approximately 20 days and includes addition of early factors (EF) and late factors (LF). However, initiation protocols of shorter durations, for example 10 days in the presence of only EFs, are also contemplated. Exemplary initiation protocols include, but are not limited to, the eight factor protocol comprising addition of 4 EFs and 4 LFs, as well as the 2 EF-3 LF protocol. Throughout the application, particular initiation protocols are also referred to more specifically according to the number or combination of early and late factors used to help promote initial differentiation of embryonic stem cells along a pancreatic lineage.

The term "maturation protocol" is used to refer to any of the various methods used to further differentiate embryonic stem cells and embryoid bodies previously subjected to the initiation protocol. The maturation protocol can be subdivided into various stages, and the term maturation protocol will be used to refer to methods where the cells are subjected to any or all of these various phases. Specific reference to the number of days in culture, the stage of the protocol, or the factors added will be used to help distinguish the various permutations and stages of the maturation protocol(s).

The phrases "parenteral administration" and "administered parenterally" as

used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intraventricular, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, intracerebrospinal, and intrasternal injection and infusion.

The phrases "systemic administration," "administered systemically," "peripheral administration" and "administered peripherally" as used herein mean the administration of a compound, drug or other material other than directly into the central nervous system, such that it enters the animal's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically acceptable carrier" as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject agents. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation.

The terms "hedgehog signaling," "hedgehog signal transduction," and "hedgehog signaling pathway" are used interchangeably throughout the application to refer to the mechanism whereby hedgehog proteins (Sonic, Desert, Indian hedgehog) influence proliferation, differentiation, migration, and survival of diverse cell types (see, for example, Allendoerfer (2003) Current Opinion Investig. Drugs 3: 1742-1 744; Ingham (2001) Genes & Dev 15: 3059-3087). Agents that promote hedgehog signal transduction are referred to as "hedgehog agonists" or "agonists of hedgehog signaling." Agents that inhibit hedgehog signal transduction are referred to as "hedgehog antagonists" or "antagonists of hedgehog signaling." Hedgehog signal transduction may be influenced by hedgehog proteins, or by agents that agonize or antagonize hedgehog signaling at any point in the pathway

(extracellularly, at the cell surface, or intracellularly). For further examples see U.S. Patent No. 6,444,793; U.S. Patent No. 6,683,108; U.S. Patent No. 6,683,198; U.S. Patent No. 6,686,388; WO 02/30421; WO 02/30462; WO 03/01 1219; WO 03/027234; WO 04/020599. Each of the foregoing references are hereby incorporated by reference in their entirety.

The terms "BMP signaling," "BMP signal transduction," and "BMP signaling pathway" are used interchangeably throughout the application to refer to the mechanism whereby BMP proteins influence proliferation, differentiation, migration, and survival of diverse cell types (see, for example, Balemans (2002) Developmental Biology 250: 23 1-250; US Patent No. 6498142; Miyazawa *et al.* (2002) Genes Cell 7: 1191-1204). Agents that promote BMP signal transduction are referred to as "BMP agonists" or "agonists of BMP signaling." Agents that inhibit BMP signal transduction are referred to as "BMP antagonists" or "antagonists of BMP signaling." BMP signal transduction may be influenced by BMP proteins, or by agents that agonize or antagonize BMP signaling at any point in the pathway (extracellularly, at the cell surface, or intracellularly).

The terms "Wnt signaling," "Wnt signal transduction," and "Wnt signaling pathway" are used interchangeably throughout the application to refer to the mechanism whereby Wnt proteins influence proliferation, differentiation, migration, and survival of diverse cell types (see, for example, WO 02/44378; Wharton, Developmental Biology 253: 1-17, 2003). Agents that promote Wnt signal transduction are referred to as "Wnt agonists" or "agonists of Wnt signaling." Agents that inhibit Wnt signal transduction are referred to as "Wnt antagonists" or "antagonists of Wnt signaling." Wnt signal transduction may be influenced by Wnt proteins, or by agents that agonize or antagonize Wnt signal transduction at any point in the pathway (extracellularly, at the cell surface, or intracellularly).

The terms "Notch signaling," "Notch signal transduction," and "Notch signaling pathway" are used interchangeably throughout the application to refer to the mechanism whereby Notch proteins influence proliferation, differentiation, migration, and survival of diverse cell types (see, for example, Baron, Stem Cell Dev. Bio. 14: 113-1 19, 2003). Agents that promote Notch signal transduction are referred to as "Notch agonists" or "agonists of Notch signaling." Agents that inhibit

Notch signal transduction are referred to as "Notch antagonists" or "antagonists of Notch signaling." Notch signal transduction may be influenced by a Notch protein, or by agents that agonize or antagonize Notch signal transduction at any point in the pathway (extracellularly, at the cell surface, or intracellularly).

The term "adherent matrix" refers to any matrix that promotes adherence of cells in culture *{e.g.,* fibronectin, collagen, laminins, superfibronectin). Exemplary matrices include MATRIGEL™ (Beckton-Dickinson), HTB9 matrix, and superfibronectin. MATRIGEL™ is derived from a mouse sarcoma cell line. HTB9 is derived from a bladder cell carcinoma line (US Patent 5,874,306).

The term "pancreas" is art recognized, and refers generally to a large, elongated, racemose gland situated transversely behind the stomach, between the spleen and duodenum. The pancreatic exocrine function, *e.g.,* external secretion, provides a source of digestive enzymes. Indeed, "pancreatin" refers to a substance from the pancreas containing enzymes, principally amylase, protease, and lipase, which substance is used as a digestive aid. The exocrine portion is composed of several serous cells surrounding a lumen. These cells synthesize and secrete digestive enzymes such as tripsinogen, chymotrypsinogen, carboxypeptidase, ribonuclease, deoxyribonuclease, triacylglycerol lipase, phospholipase A_2, elastase, and amylase.

The endocrine portion of the pancreas is composed of the islets of Langerhans. The islets of Langerhans appear as rounded clusters of cells embedded within the exocrine pancreas. Four different types of cells- α, β, δ, and ϕ-have been identified in the islets. The α cells constitute about 20% of the cells found in pancreatic islets and produce the hormone glucagon. Glucagon acts on several tissues to make energy available in the intervals between feeding. In the liver, glucagon causes breakdown of glycogen and promotes gluconeogenesis from amino acid precursors. The δ cells produce somatostatin which acts in the pancreas to inhibit glucagon release and to decrease pancreatic exocrine secretion. The hormone pancreatic polypeptide (PP) is produced in the ϕ cells. This hormone inhibits pancreatic exocrine secretion of bicarbonate and enzymes, causes relaxation of the gallbladder, and decreases bile secretion. The most abundant cell in the islets, constituting 60-80% of the cells, is the β cell, which produces insulin. Insulin is

known to cause the storage of excess nutrients arising during and shortly after feeding. The major target organs for insulin are the liver, muscle, and fat-organs specialized for storage of energy.

The term "pancreatic duct" includes the accessory pancreatic duct, dorsal pancreatic duct, main pancreatic duct and ventral pancreatic duct. Serous glands have extensions of the lumen between adjacent secretory cells, and these are called intercellular canaliculi. The term "interlobular ducts" refers to intercalated ducts and striated ducts found within lobules of secretory units in the pancreas. The "intercalated ducts" refers to the first duct segment draining a secretory acinus or tubule. Intercalated ducts often have carbonic anhydrase activity, such that bicarbonate ion may be added to the secretions at this level. "Striated ducts" are the largest of the intralobular duct components and are capable of modifying the ionic composition of secretions.

As used herein, "islet equivalents" or "IEs" is a measure used to compare total insulin content across a population or cluster of cells. An islet equivalent is defined based on total insulin content and an estimate of cell number which is typically quantified as total protein content. This allows standardization of the measure of insulin content based on the total number of cells within a cell cluster, culture, sphere, or other population of cells. The standard rat and human islet is approximately 150 μm in diameter and contains 40-60 ng insulin/μg of total protein. On average, human islet-like structures differentiated by the methods of the present invention contain approximately 50 ng insulin/μg of total protein.

The term "reporter construct" is used to refer to constructs that 'report' or 'identify' the presence of particular cells. Typically reporter constructs include portions of the promoter, enhancer, or other regulatory sequences of a particular gene sufficient to regulate expression in a developmentally relevant manner. Such regulatory sequences are operably linked to a nucleic acid sequence encoding a marker that can be readily detectable (the 'reporter gene'). In this way, expression of a readily detectable product can be monitored, and this product is regulated in a manner consistent with the promoter or enhancer to which it is operably linked. Reporter genes may be introduced into cells by any of a number of ways including transfection, electroporation, micro-injection, *etc.* Exemplary reporter genes include,

but are not limited to, green fluorescent protein (GFP), recombinantly engineered variants of GFP, red fluorescent protein, yellow fluorescent protein, cyan fluorescent protein, LacZ, luciferase, firefly Remmila protein. Further exemplary reporter genes encode antibiotic resistance proteins including, but not limited to, neomycin, hygromycin, zeocine, and puromycin.

The term "xeπo-free" refers to clinically compliant ES cells or cell lines or CGMP-compliant hES cells or cell lines. All current 78 U.S. National Institutes of Health (NlH)-listed human embryonic stem cell (hESC) lines approved for U.S. government federal research funding have been derived and propagated on mouse embryonic fibroblasts (MEFs) and in the presence of culture medium containing animal-based ingredients. The use of a feeder layer of animal origin and animal components in the culture media may potentially substantially elevate the risk of the cross-transfer of viruses and other pathogens to the embryonic stem (ES) cells. Hence, safer current good manufacturing practice (CGMP) and good tissue culture practice (GTCP)-compliant hESC lines and differentiated hESC progenitors are more suitable for clinical application.

Several attempts at improving hESC culture conditions have been reported. These advances include the use of conditioned media together with BMM marketed as MATRJGEL™ as an attachment substrate for hESC culture, and the derivation and propagation of hESC lines on human feeder layers. These improvements are important steps forward in developing a CGMP-compliant protocol for the establishment of xeno-free clinically compliant hESC lines. The derivation of xeno-free CGMP-compliant hES lines also necessitates the development of a cryopreservation protocol that is effective and minimizes or restricts the possibility of cell line contamination in long-term liquid nitrogen (LN_2) storage. At least two freezing protocols are currently used for hESCs. These include (a) the conventional slow stepwise programmed freezing method using cryovials (CVs) and storage in LN_2, and (b) a snap-freezing vitrification method using an open pulled straw (OPS) and storage in LN_2. Another effective, safe, and sterile cryopreservation protocol is described by Richards *et al.* (Stem Cells 22: 779-789, 2004). These protocols can be used for generation and long-term storage of CGMP- and GTCP-compliant xeno-free hESC lines useful for the instant invention.

(Ui) Exemplary Methods

Methods of isolating and maintaining undifferentiated cultures of embryonic stem cells from any of a variety of species are well known in the art. Exemplary species include, but are not limited to, mice, non-human primates, and humans. Furthermore, under a variety of circumstances, many have observed the differentiation of embryonic stem cells to any of a number of partially or terminally differentiated cell types. For example, embryonic stem cells aggregated to form embryoid bodies may produce embryoid bodies that include small regions or foci of beating tissue. This beating tissue indicates that a small percentage of cells in the embryoid body have differentiated to form cardiomyocytes.

However, the challenge is not to wait patiently as embryonic stem cells randomly differentiate along particular lineages. Nor is the challenge to devise methods of differentiating embryonic stem cells that produce a disparate "mixed bag" of cell types across a culture. At this point, the challenge is to develop efficient methods to direct the differentiation of embryonic stem cells to particular cell types or along particular developmental lineages. Such methods are essential to increase our understanding of stem cell biology, to produce substantially purified cultures of differentiated cell types, and to develop therapeutics based upon differentiated cells.

The present invention addresses the limitations in the prior art and offers methodologies for directing the differentiation of embryonic stem cells to endodermal cell types. Specifically, the methods of the present invention can be used to direct the differentiation of embryonic stem cells to produce various partially and/or terminally differentiated cells or cell clusters. By way of example, the methods of the present invention *{e.g.,* the initiation protocols, the maturation protocols, and combinations thereof) can be used to direct the differentiation of embryonic stem cells to partially and terminally differentiated pancreatic cell types.

Partially and terminally differentiated cell types, for example pancreatic cell types, induced by the initiation and/or maturation protocols of the present invention can be further expanded and/or purified to produce essentially purified cultures of one or more partially and/or terminally differentiated endodermal cell types. By way of non-limiting example, the methods of the present invention can be used to produce, from embryonic stem cells, (i) essentially purified populations of

terminally differentiated pancreatic cell types *{e.g.,* either a single terminally differentiated pancreatic cell type or multiple terminally differentiated pancreatic cell types); (ii) essentially purified populations of partially differentiated pancreatic cell types *{e.g.,* either a single partially differentiated pancreatic cell type or multiple partially differentiated pancreatic cell types); or (iii) essentially purified populations of one or more partially and/or terminally differentiated pancreatic cell types.

Cultures of embryonic stem cells (e.g., human, mouse, non-human primate, *etc.)* can be differentiated using methods that include a step involving formation of embryoid bodies or directly *{e.g.,* without a step involving the formation of embryoid bodies). In one embodiment of the present invention, an early step in the differentiation process comprises the aggregation of embryonic stem cells to form embryoid bodies.

Differentiation *via* Embryoid Body Formation

Embryonic stem (ES) cells can be differentiated by removing the cells from the feeder layer and aggregating them in suspension to form embryoid bodies (EBs). EBs can be made by plating dissociated ES cells in bulk on low-attachment plates or by the hanging drop method. ES cells may be dissociated fully into single cells or partially into small clumps by a number of methods including trypsin, collagenase, dispase, EDTA, or mechanical disruption. The method of dissociation can be readily selected by one of skill in the art and may vary depending on the species from which the cells are derived, as well as the overall health of the cells. For example, human ES cells do not survive as well following dissociation to the single cell level. Accordingly, when the methods of the present invention are performed using human ES cells, the dissociation technique can be selected so as to remove the ES cells from the feeder layer without dissociating the cells to the single cell level prior to EB formation. Following formation of EBs, the EBs can be cultured in suspension *{e.g.,* as floating aggregates of cells, on filters, or embedded in gel-like matrices) for a period of time, preferably ranging from 3 days to 3 weeks. In certain embodiments, EBs can be cultured in suspension for less than 3 days, for example, for 6 hours, 12 hours, 18 hours, 24 hours, 36 hours, or 48 hours. In certain other embodiments, the EBs can be cultured for more than 3 weeks.

Although general methods of aggregating ES cells to form EBs are known in

the art, ES cells from certain species appear to be more sensitive to the level of dissociation achieved prior to EB formation. Accordingly, in addition to the above outlined approach in which certain ES cells are dissociated less completely *(e.g.*, not dissociated to single cells) prior to EB formation, the present invention contemplates methods of EB formation in which ES cells are dissociated in the presence of agents that block apoptosis or otherwise promote cell survival. Exemplary agents include, but are not limited to, caspase inhibitors.

Following EB formation, EBs can be cultured in a variety of media including, but not limited to, basal media BME, CMRLl 066, MEM, DMEM, DMEM/F 12, RPMI, Glasgow MEM with or without alpha modification, IMDM, Leibovitz's L-15, McCoys 5A, Media 199, Ham's F-10, Ham's F-12, F-12K, NCTC-1 09 medium, Waymouth's media, William's Media E, or a combination of any of the above. One of skill in the art can readily select, based on cost, species, availability, *etc.*, from amongst these and similar media designed for the culture of EBs. Any of the foregoing media can be supplemented with varying concentration of glucose (1-50 mM), sodium pyruvate, non-essential amino acids, nucleosides, N-2 supplement, G-5 supplement, and B27 supplement. The media may or may not contain phenol-red. Furthermore, the media can be buffered with an appropriate amount of buffering salt. Exemplary buffering salts include, but are not limited to, sodium bicarbonate, Tris, HEPES, sodium acetate. The pH of the EB media can vary between 5 and 9.

In addition to the foregoing basal components of the EB media, in certain embodiments, the culture media may be supplemented with different amounts of animal serum. Exemplary animal sera commonly used in the art include, but are not limited to, fetal bovine serum (FBS), bovine serum (BS), horse serum (HS), chicken serum (CS), goat serum (GS). The serum may or may not be heat-activated. Alternatively, the media may be supplemented with a chemically defined serum replacement, such as Knockout DMEM with Knockout Serum Replacement. In one embodiment, the concentration of animal serum or serum replacement in the media is selected in the range from 0% to 20%. In other embodiments, the concentration of serum or serum replacement in the media is greater than 20%, for example, is between 20% - 40%.

Although, in certain embodiments, the EBs can be cultured in basal media supplemented with serum or serum replacement alone, EBs may also be cultured in media further containing media conditioned by another cell line. Alternatively, in another embodiment, EBs can be cultured in basal media lacking serum or serum replacement, but containing media conditioned by another cell line. Exemplary cell lines from which conditioned media can be obtained include, but are not limited to, mouse embryonic fibroblasts (MEFs); mouse or human insulinomas *(e.g.,* RIN-5, beta-TC, NIT-I, INS-I, INS-2); hepatomas *(e.g.,* HepG2, Huh-7, HepG3); HT-1080; endothelial cells *(e.g.,* HUVEC); bone marrow stromal cells; visceral endoderm-Iike cells such as end-2; or mesenchymal cells such as HEPM or 7F2. Alternatively, conditioned media can be obtained from cultured embryonic, fetal, or adult tissues *(e.g.,* derived from human, non-human primate, mouse, or other animals), or from a primary cell line established from a particular tissue type *(e.g.,* pancreas, liver, bone marrow, lung, skin, blood, *etc.)* and derived from an animal *(e.g.,* human, non-human primate, mouse, or other animal).

Embryonic tissues include endoderm, mesoderm, ectoderm, and/or extraembryonic tissue such as trophectodeπn and visceral endoderm. In certain embodiments employing conditioned media from embryonic tissue, the embryonic tissue is chosen based on the ability of that tissue to send instructive signals to the embryonic pancreas during development. Such instructive tissues include notochord and dorsal aorta. Exemplary primary cell lines include endothelial cells, aortic smooth muscle cells, mesenchymal cells, endocrine or exocrine cells of the pancreas, hepatocytes, intestinal epithelial cells, and ductal cells. Media can also be conditioned from any cells derived from mouse embryonic stem cells. In any of the foregoing embodiments in which EBs are cultured in the presence of conditioned media, the conditioned media may be derived from a cell line, tissue, *etc.* of the same species as the EBs or from a different species.

The foregoing media constitutes the starting point for directing the differentiation of the cells to a particular differentiated endodermal cell type. Any of the foregoing media can now be further supplemented with appropriate differentiation factors to direct cells in the EBs to differentiate along particular endodermal lineages such as the pancreatic lineage, hepatic lineage, lung lineage,

etc. By way of example, EBs can be cultured in media supplemented with particular differentiation factors to direct the differentiation of cells in the EBs to pancreatic cell types including insulin-producing cells. Such factors include but are not limited to activin A, activin B, BMP-2, BMP-4, nodal, TGFβ, sonic hedgehog, desert hedgehog, EGF, HGF, FGF2, FGF4, FGF8, FGFl 8, PDGF, Wnt proteins, retinoic acid, sodium butyrate, NGF, HGF, GDF, growth hormone, PYY, cardiotropin, GLP-1, exendin-4, betacellulin, nicotinamide, tri-iodothyroxine, insulin, IGF-I, IGF-II, placental lactogen, VEGF, wortmannin, gastrin, cholecystokinin, sphingosine-1-phosphate, FGF-10, FGF inhibitors, growth hormone, KGF, islet neogenesis-associated protein (INGAP), Reg, and factors that increase cAMP levels such as forskolin and IBMX. Most of the factors can be added into the media from a purified stock, or if they are protein factors, can be presented in the form of conditioned media taken from cells recombinantly expressing the factors. Additionally, the invention contemplates that, for certain of the above referenced protein factors, small molecule agonists that mimic the bioactivity of the protein are known in the art. Such small molecule mimics may function in any of a number of ways to produce similar biological consequences as the protein. Accordingly, the invention contemplates methods in which the EBs are cultured in the presence of small molecule agonists/mimics of any of the foregoing proteins.

Without being bound by theory, certain of these factors may influence cell fate by binding to receptors on the surface of the cells, and thereby modulating one or more signal transduction pathways functional in the cells. Alternatively, certain of these factors may influence cell fate by transiting the cell membrane and acting intraceHularly to modulate one or more signal transduction pathways functional in the cells.

The invention contemplates using one or more of these factors to help promote the differentiation of cells to pancreatic cell types. In one embodiment, the one or more factors influence the cells by modulating the same signal transduction pathway *(e.g.,* Sonic hedgehog protein in combination with Desert hedgehog protein). In another embodiment, the one or more factors influence the cells by modulating different signal transduction pathways *(e.g.,* one or more hedgehog proteins in combination with one or more Wnt proteins). In another embodiment,

one or more factors influence cells via mechanisms that may or may not overlap. Regardless of the precise mechanism of action, the invention contemplates that one or more of the above differentiation factors can be added to a culture of EBs to help promote their differentiation to pancreatic cell types. When more than one differentiation factor is added to the culture, the invention contemplates that the differentiation factors can be added concomitantly or concurrently.

The foregoing are exemplary of the factors and conditions that can be used to direct the differentiation of embryonic stem cells along particular lineages. By way of further specific example, the experiments summarized herein provide multiple examples of initiation protocols that bias embryonic stem cells along a pancreatic lineage. Furthermore, the experiments summarized herein provide multiple examples of maturation protocols that, when used in combination with an initiation protocol, help promote the further differentiation of biased embryonic stem cells to terminally differentiated pancreatic cell type *{e.g.,* produce cells that express one or more markers indicative of a terminally differentiated pancreatic cell type).

Following a period of suspension as EBs in culture, which in one embodiment ranges from 3 days —3 weeks, the EBs can be replated on a matrix as described further below. EBs can be replated directly onto the matrix or EBs can be dissociated prior to replating onto the matrix.

Following a period of suspension as EBs in culture, which in one embodiment ranges from 3 days - 3 weeks, the EBs can be replated on an adherent matrix. Exemplary adherent matrices include, but are not limited to, gelatin, BMM marketed as MATRIGEL™ or GFR MATRIGEL™; collagen IV-supplemented SPH *{e.g.,* marketed as PURAMATRIX™), alginate, or PLGA; various types of collagens, laminins, fibronectins, or a combination of any of the foregoing. EBs can be replated directly onto the adherent matrix or EBs can be dissociated prior to replating onto the adherent matrix.

Directed Differentiation

In another embodiment, ES cells can be differentiated without a step including EB formation. For example, to initiate differentiation towards pancreatic cells types, ES cells can be plated directly on an appropriate adherent matrix without first forming cultures of EBs. The ES cells can also be differentiated either as a

monolayer in culture or on feeder cells. The ES cells can be plated in any of the above referenced combination of media appropriate for the culture of EBs and further supplemented with one or more of the differentiation factors outlined above. Exemplary adherent matrices include, but are not limited to, gelatin, BMM marketed as MATRIGEL™ or GFR MATRIGEL™; collagen IV-supplemented SPH *(e.g.*, marketed as PURAMATRIX™), alginate, or PLGA; various types of collagens, laminins, fibronectins, or a combination of any of the foregoing.

Regardless of whether the ES cells are differentiated directly or differentiated via EB formation, the invention contemplates that the ES cells, differentiating ES cells, or EBs can be cultured either under standard tissue culture conditions of oxygen and carbon dioxide, or in an incubator where oxygen tension can be varied.

In one embodiment of any of the foregoing, EBs can be cultured either in suspension in liquid media or in suspension by embedding in a 2D or 3D gel or matrix. Exemplary matrices include, but are not limited to, gelatin, BMM marketed as MATRIGEL™ or GFR MATRIGEL™; collagen IV-supplemented SPH (e.g., marketed as PURAMATRIX™), alginate, or PLGA; various types of collagens, laminins, fibronectins, or a combination of any of the foregoing. When EBs are cultured suspended in a matrix, differentiation factors can be administered either by addition to the surrounding liquid medium or by covalently or non-covalently linking the factors to the particular matrix in which the EBs are suspended. In another embodiment, EBs can be cultured on a Transwell. Culture on a Transwell may facilitate establishment of cell polarity.

In one embodiment of any of the foregoing, differentiation of ES cells or EBs can be promoted by co-culturing the cells with cells, cell lines, or tissues of the endoderm or with cells, cell lines, or tissues derived from tissues known to induce endodermal differentiation during development.

Progressive Differentiation

In any of the foregoing methods of differentiation, the invention contemplates that a single differentiation step likely will not produce the particular partially or terminally differentiated cell type desired, or will not necessarily produce them in the desired ratios or percentages. Accordingly, the invention

contemplates that ES cells and EBs can be cultured and differentiated in stages. At each successive stage, the differentiation factors and differentiation matrices may be the same or different.

Progressive differentiation of ES cells and EBs can be measured by examining markers of partially and/or terminally differentiated cells of the particular tissue of interest. For example, in methods where the goal is the differentiation of ES cells (with or without the formation of EBs) to pancreatic cell type, progressive differentiation can be monitored by assaying expression of markers of partially or terminally differentiated pancreatic cells *(e.g.,* early markers of the endodermal lineage; early markers of the pancreatic lineage; markers of partially differentiated endocrine pancreatic cells; markers of partially differentiated exocrine pancreatic cells; markers of terminally differentiated endocrine pancreatic cells; markers of terminally differentiated exocrine pancreatic cells).

By way of example, early differentiation to definitive endoderm could be monitored by assaying expression of genes including, but not limited to, soxl7, HNF l α, HNF3α, HNF3β, HNF3γ, brachyury T, goosecoid, claudins, AFP, HEX, eomesodermin, TCF2, Mixll, CXCR4, GATA5, and proxl. In contrast, differentiation into non-endodermal lineages could be monitored by assaying expression of non-endodermal genes, for example, genes indicative of extraembryonic tissue. Exemplary genes that could be used to assess the level of non-endodermal differentiation in a culture at a particular time include, but are not limited to, chorionic gonadotropin, amnionless, HNF4, GATA4, or GATA6.

As definitive endoderm is formed, partial or terminal differentiation towards the pancreatic lineage can be monitored by expression of pancreatic genes *(e.g.,* exocrine pancreatic genes and endocrine pancreatic genes) including, but not limited to, pdx-1, ngn3, HB9, HNF6, ptfl- ρ48, islet 1, nkx6.1, nkx2.2, glut2, neuroD, cytokeratin 19, IAPP, pax4, pax6, HESl, amylase, glucagon, somatostatin, insulin, hormone convertase, glucokinase, Sur-1, Kirb6.2, and pancreatic polypeptide.

In any of the foregoing, gene expression can be measured in living cells over time to provide a snapshot of differentiation in a given culture. Alternatively, samples of cells from a given culture at a given time can be taken and processed. Such cells would provide a representation of the differentiation in a particular

culture at a particular time.

Gene expression can be measured by a variety of techniques well known in the cell biological and molecular biological arts. These techniques include RT-PCR, northern blot analysis, *in situ* hybridization, microarray analysis, SAGE, or MPSS. Protein expression can similarly be analyzed using well known techniques such as western blot analysis, immunohistochemical staining, ELISA, or RIA.

Differentiation into definitive endoderm could be accomplished by activating certain pathways including but not limited to nodal, wnt, and FGF signaling. Nodal belongs to the TGF-beta superfamily of ligands that include activins and BMPs. Addition of these TGFβ related ligands could drive hES cells towards definitive endoderm. Wnt signaling could be activated by addition of any of the Wnt ligands (see, for example, WO02/44378; Wharton (2003) Developmental Biology 253: 1-17). One main consequence of wnt signaling is the stabilization of β-catenin. Stabilization of β-catenin could also be accomplished by addition of GSK3 inhibitors, including but not limited to derivatives of 6-bromoindirubin. Inhibition of FGF signaling may also help to direct ES cells down the endodermal pathway. Inhibition of FGF signaling could be accomplished by using one of several FGF receptor antagonists such as the compound SU5402. Induction of endodermal differentiation can be assessed by measuring the phosphorylation state of intra-cellular Smad2 protein.

Differentiation towards endocrine pancreas could be biased by expression of key developmental genes in ES cells. For example, expression of pdx-1 under an appropriate promoter could be used to drive ES down the pancreatic lineage and help bias the cells to respond to the differentiation factors. The promoter could be a constitutive one such as CMV, SV40, EF1α, or beta-actin. Alternatively, the promoter could be an inducible one such as metallothionin, ecdysone, or tetracycline. The recombinant protein could also be tagged to a regulatory element such as the Iigand-binding domain of the estrogen receptor or variants thereof. Such a fusion protein could be regulated by addition or withdrawal of estrogen analogs including tomaxifin. Recombinant DNA could be introduced in ES cells using a variety of methods including electroporation, lipofection, or transduction by viral agents such as adenovirus, lentivirus, herpes virus, or other pleiotropic viruses. In

addition, inhibition of certain genes may help promote differentiation and/or help promote responsiveness of the ES to the differentiation factors. For example, inhibition of the smoothed/patched receptor, RBK-JK, or HESl in hES-derived cells could help drive the ES cells toward the pancreatic lineage. Inhibition of the genes could be accomplished by antisense oligos, siRNA, deletion of endogenous alleles by homologous recombination or constitutive expression of an inhibitor or dominant negative gene.

Chemically Defined Three Dimensional Matrices

In one embodiment, the invention provides methods for differentiating ES cells using three dimensional chemically defined matrices or scaffolds of synthetic and/or naturally occurring polymers supplemented with purified collagen IV. The methods involve using scaffolds having defined compositions with desired biological properties thereby avoiding unpredictable affects or contaminating components that may be involved in complex extra-cellular matrix formulations such as MATRIGEL™. The defined scaffold compositions described herein provide a number of advantages over directed differentiation methods involving feeder cells, cellular extracts, extra-cellular matrices, xenobiotic materials, or unpurified components. For example, defined scaffold compositions may be more easily scalable to commercial production, may facilitate generation of clinically compliant cell products, may facilitate production of a consistent/homogenous cell population, and may be attractive from the perspective of regulatory scrutiny because no xenogenic components, no unpurified components, and/or no components of cancerous origin are introduced into the cell cultures.

Methods for directed differentiation of ES cells using defined matrix compositions involve seeding a population of ES cells onto a support matrix before, concurrently with, and/or after exposure to one or more agents that facilitate directed differentiation. In various embodiments, ES cells, EBs, or other adult and fetal stem cell populations may be used in association with the directed differentiation methods involving a chemically defined three-dimensional polymer matrix supplemented with collagen IV as described herein. Compositions comprising the polymer matrices supplemented with collagen IV are also provided.

In one embodiment, a method for the directed differentiation of ES cells to

endodermal cell types is provided. The method comprises seeding hES cells or EBs on a polymer matrix and then exposing the cells to one or more differentiation factors. In certain embodiments, the polymer matrix may be selected from the group consisting of: SPH *(e.g.,* marketed as PURAMATRTX™), alginate, or PLGA supplemented with collagen IV. The cells may be seeded on the polymer matrix by mixing the cells with a polymer solution and then forming a matrix or by first forming a matrix and then adding the cells to the matrix. In exemplary embodiments, the method may be used to produce pdx-l+ cells that have begun differentiation to a pancreatic cell fate. In such embodiments, ES cells are first seeded on the matrix and then exposed to one more early factors and one or more late factors. For example, ES cells may be exposed to one or more early factors for 10-15 days and then exposed to one more late factors for 10-15 days. Exemplary early factors include, for example, activin A, BMP-2, BMP-4, and nodal. Exemplary late factors include, for example, HGF, exendin4, betacellulin, and nicotinamide. In various embodiments, the seeded ES cells may be exposed to an initiation protocol, a maturation protocol and/or a differentiation protocol as described herein.

In certain embodiments, a directed differentiation method utilizing a chemically defined polymer matrix supplemented with collagen IV may provide a homogenous compositions of differentiated cells, *e.g.,* cell populations that are at least about 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, 95%, or 98% homogeneous can be generated without having to employ a sorting technique. In certain embodiments, a directed differentiation method utilizing a polymer matrix results in a higher proportion of a desired differentiated cell type as compared to cells cultured on BMM marketed as MATRIGEL™ and/or growth factor reduced (GFR) MATRIGEL™. For example, directed differentiation on a polymer matrix may produce at least a 0.5 fold, 1 fold, 2 fold, 5 fold, 7 fold, 10 fold or greater proportion of a desired differentiated cell type as compared to cells cultured on BMM marketed as MATRIGEL™ and/or GFR MATRIGEL™. Without wishing to be bound by theory, it is believed that the complex formulation of BMM marketed as MATRIGEL™ may comprise one or more compounds that inhibit or interfere with differentiation of ES cells. The chemically defined matrix compositions provided herein comprise defined and purified components and do not contain the

undefined, undesired or inhibitory compounds of BMM marketed as MATRIGEL™. Therefore the matrix compositions provided herein are more efficient at differentiation and result in a more consistent and homogenous population of differentiated or partially differentiated cells.

Exemplary polymers that may be used as matrices or scaffolds include, for example, one or more synthetic and/or naturally-occurring polymers. In certain embodiments, polymer matrices may be biodegradable or non-biodegradable. Suitable biodegradable polymers for use in accordance with the methods described herein include, for example, poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and PLA-PGA co-polymers (PLGA). Additional biodegradable materials include PLA, poly(anhydrides), poly(hydroxy acids), poly(ortho esters), poly(propylfumerates), poly(caprolactones), polyamides, polyamino acids, polyacetals, biodegradable polycyanoacrylates, biodegradable polyurethanes and polysaccharides. Suitable non-biodegradable, yet biocompatible polymers include for example, polypyrrole, polyanilines, polythiophene, polystyrene, polyesters, non-biodegradable polyurethanes, polyureas, poly(ethylene vinyl acetate), polypropylene, polymethacrylate, polyethylene, polycarbonates, and poly(ethylene oxide). Other suitable matrices include, for example, gels such as methylcellulose, agarose, and alginate. Those skilled in the art will recognize that this is an exemplary, not a comprehensive, list of polymers appropriate for directed differentiation methods described herein. Co-polymers, mixtures, and adducts of the above polymers may also be used in accordance with the methods described herein.

In exemplary embodiments, polymer matrices suitable for directed differentiation of ES cells include, for example, SPH *(e.g.,* marketed as PURAMATRIX™), alginate, or poly(lactic-co-glycolic acid) and poly(L-lactie acid) (PLGA). In certain embodiments, the polymer matrices described herein do not comprise one or more components found in BMM marketed as MATRIGEL™ and/or GFR MATRFGEL™. Tn certain embodiments, the polymer matrices comprise, consist essentially of, or consist of a mixture of (i) SPH *(e.g.,* marketed as PURAMATRIX™), alginate, or (PLGA) and (ii) purified collagen IV. The collagen IV may be from a variety sources including, for example, mouse or human.

In certain embodiments, the matrix may be formed with a microstructure

similar to that of an extracellular matrix. The molecular weight, tacticity, integrity, hydration, and/or cross-link density of the matrix may also be regulated to control both the mechanical properties of the matrix and the degradation rate (for degradable scaffolds). The matrix may be formed in any shape, for example, as particles, a sponge, a tube, a sphere, a strand, a coiled strand, a capillary network, a film, a fiber, a mesh, or a sheet.

The porosity of the matrix may be controlled by a variety of techniques known to those skilled in the art. The minimum pore size and degree of porosity is dictated by the need to provide enough room for the cells and for nutrients to filter through the matrix to the cells. The maximum pore size and porosity is limited by the ability of the matrix to maintain its mechanical stability after seeding. As the porosity is increased, use of polymers having a higher modulus, addition of stiffer polymers as a co-polymer or mixture, or an increase in the cross-link density of the polymer may all be used to increase the stability of the matrix with respect to cellular contraction.

The matrices may be made by any of a variety of techniques known to those skilled in the art. Those skilled in the art will recognize that standard polymer processing techniques may be exploited to create polymer matrices having a variety of porosities and microstructures. Exemplary methods for producing polymer matrices suitable for the directed differentiation methods described herein are provided in the Examples.

Further Purification of Differentiated Cell Types

In certain embodiments, essentially purified preparations of one or more partially or terminally differentiated cell types of a particular tissue can be generated directly from ES cells or EBs. However, it may be necessary or preferable for certain applications of the differentiated cells to further expand or select particular differentiated cells. For example, such selection can be used to further purify a preparation of cells or can be used to, for example, take a preparation that includes multiple partially and/or terminally differentiated cell types and prepare a preparation that contains fewer, or even a single, partially and/or terminally differentiated cell types.

Cells differentiated from ES cells may need to be expanded and selected.

One non-limiting approach is to express a drug resistant marker such as neo^R under the control of a particular tissue specific promoter *(e.g.,* the insulin promoter or pdx-1 promoter) in ES cells. As the cells undergo a differentiation protocol, the selection drug like G418 can be added to select for cells expressing the marker gene. Alternatively, one can tag a suicide gene such as diphtheria toxin to a particular promoter so as to eliminate cells that differentiate along an undesired lineage.

Reporter ES lines can also be used to monitor progression of differentiation. For example, a construct comprising GFP downstream of a particular promoter (e.g., the pdx-1 promoter or insulin promoter) could be introduced into ES cells. Cells that express the reporter can be readily detected. Other reporters genes that can be used include luciferase, alkaline phosphatase, lacZ, or CAT. Useful reporter lines could comprise multiple reporters to help identify cells that have differentiated along particular lineages. Cells expressing these reporters could be easily purified by FACS, antibody affinity capture, magnetic separation, or a combination thereof. The purified reporter-expressing cells can be used for genomic analysis by techniques such as microarray hybridization, SAGE, MPSS, or proteomic analysis to identify more markers that characterize the purified population. These methods can identify cells that have not differentiated along the desired lineages, as well as populations of cells that have differentiated along the desired lineages. In cultures containing too many cells that have not differentiated along the desired lineages, the desired cells may be isolated and subcultured to generate an essentially purified populations of one or more partially or terminally differentiated cell types of the desired tissue.

Reporter lines could be used in a high-throughput screening assay to rapidly screen for small molecules, growth factors, matrices, or different growth conditions that could favor differentiation along particular lineages. Screening platform could be 24, 48, 96, or even 384-wells. Detection method would depend on the type of reporter gene being expressed. For example, a luminescence plate reader could detect luciferase reporter and a fluorescent plate reader could detect GFP or even lacZ reporters. In addition, high content screening could be performed where automated microscopes would scan each well and measure several parameters including but not limited to the number of cells expressing the reporter gene, cell size, cell shape, and cell movement.

pdx-1 positive cells or progenitors thereof could be further differentiated into endocrine cells based on methods detailed in US Patent No. 6,610,535 or as described in patent application PCT/US03/23852, the disclosures of each of which arc hereby incorporated by reference in their entirety. Differentiating factors used in this protocol include but are not limited to FGFl 8, cardiotropin, PYY, forskolin, HGF, heparin, insulin, dexamethasone, follistatin, betacellulin, growth hormone, placental lactogen, EGF, KGF, IGF-I, IGF-II, VEGF, exendin-4, leptin, and nicotinamide, and notch antagonists.

ES cells could also be differentiated *in vivo* by injecting them into a SCID animal. It is well known that hES cells when injected into SCID mice could form teratomas that comprise tissues from all three germ layers. Injection into different tissues or organs may drive them down a particular lineage. The SCID mouse may also have a regenerating pancreas, where it has undergone partial pancreatectomy or treatment with streptozotocin. Alternatively, hES cells could be engrafted into different parts of an embryonic or fetal animal at various stage of development.

In one embodiment, ES cells are differentiated to produce essentially purified preparation of pancreatic cells. Such essentially purified preparations of pancreatic cells comprise one or more partially and/or terminally differentiated pancreatic cell type. In certain embodiment, the one or more partially and/or terminally differentiated pancreatic cell types include an insulin producing cell. ES-derived insulin-producing cells will preferably have the following characteristics: (i) express insulin mRNA as detected by RT-PCR, northern blot, or *in situ* hybridization; (ii) express insulin and C-peptide as detected by western blot or immunhistochemical staining; (iii) secrete insulin and C-peptide as detectable by ELISA or RIA; (iv) show glucose responsive insulin secretion; (v) rescue a diabetic animal *(e.g.,* STZ-treated NOD/SCID mouse) when implanted in a suitable site of the animal.

During the course of differentiating ES cells into preparations including insulin-producing cells, it may be desirable to enrich the progenitors at different stages and further differentiate these progenitors. The progenitors may be purified by selecting cells expressing one or more pancreatic development genes, as outlined in detail above. The markers used for selection are preferably expressed on the cell surface so they are amenable to antibody affinity capture and sorting by either flow

cytometry or magnetic cell separation. These markers may include dynein, gap junction membrane channel protein, integral membrane protein 2A, CXCR4, Sur-1, Glut-2, Kir6.2, microfibrillar-associated protein 2, procollagens, tachykinin, Thy-1.2, tenascin C, vaninl, inward rectifier K+ channel J8, adaptor protein complex AP- 1, microtubule-associated protein IB, annexin A1, CD36, CD84, clusterin, catenin delta 2, endomucin, granulin, keratin Hb5, integrin α7, lysosomal membrane glycoprotein 2, KSPG, galectin-6, lipocortin I, mannose-binding lectin, lymphocyte antigen 64, synaptotagmin 4, thrombospondin, thrombomodulin, visinin-like 1, Fabpl, Fabp2, Slc25a5, Slc2a2, Slc7a8, Ep-cam, N-cadherin, E-cadherin, CK19, and CD31.

(iv) Exemplary Compositions

The methods of the present invention can be used to differentiate (partially or terminally) ES cells to one or more cell types of a tissue derived from the endodermal lineage. By way of example, the methods of the present invention can be used to differentiate ES cells to produce essentially purified preparations of one or more partially and/or terminally differentiated cells of the pancreas or liver. Such essentially purified preparations of one or more partially and/or terminally differentiated cells can be formulated in a pharmaceutically acceptable carrier and administrated to patients suffering from a condition characterized by a decrease in functional performance of a particular endodermally derived organ.

In one embodiment, ES cells are differentiated to produce essentially purified preparation of pancreatic cells. Such essentially purified preparations of pancreatic cells comprise one or more partially and/or terminally differentiated pancreatic cell type. In certain embodiments, the one or more partially and/or terminally differentiated pancreatic cell types include an insulin producing cell. ES-derived insulin-producing cells will preferably have the following characteristics: (i) express insulin mRNA as detected by RT-PCR, northern blot, or *in situ* hybridization; (ii) express insulin and C-peptide as detected by western blot or immunhistochemical staining; (iii) secrete insulin and C-peptide as detectable by ELISA or RIA; (iv) show glucose responsive insulin secretion; (v) rescue a diabetic animal *{e.g.,* STZ-treated NOD/SCID mouse) when implanted in a suitable site of the animal.

(v) Application to Other Stem Cell Populations

The present invention provides methods for directing the differentiation of embryonic stem cells to endodermal cell types. However, the methods provided in the present application are not limited to modulating the differentiation of embryonic stem cells. Embryonic stem cells have a limitless differentiation potential. However, this limitless potential has proved challenging to researchers trying to direct the differentiation of these cells along particular lineages, in a controlled manner, and as a commercially and therapeutically useful percentage of a cell culture. In contrast, many adult stem cell populations have actually proven more amenable to directed differentiation. Accordingly, given that the methods provided herein effectively promote directed differentiation of embryonic stem cells to endodermal cell types, the invention contemplates that these methods will similarly be able to direct the differentiation of other adult stem cells, for example, stem cells derived from a fetal or adult animal tissue. Exemplary adult stem cells include, but are not limited to, hematopoietic stem cells, neuronal stem cells, neural crest stem cells, mesenchymal stem cells, myocardial stem cells, pancreatic stem cells, hepatic stem cells, and endothelial stem cells. Further exemplary adult stem cells can be derived from virtually any organ or tissue including, but not limited to, tongue, skin, esophagus, brain, spinal cord, endothelium, hair follicle, stomach, small intestine, large intestine, ovary, testes, blood, bone, bone marrow, umbilical cord, lung, gall bladder, and the like. In one embodiment, the adult stem cell is a stem cell population which can differentiate along an endodermal lineage using either the methods of the present invention or other methodologies known in the art.

(vi) Methods of Treatment

The present invention provides a variety of methods for promoting the directed differentiation of embryonic stem cells to particular differentiated cell types. In certain embodiments, the invention provides methods for promoting the directed differentiation of embryonic stem cells along a pancreatic lineage. Exemplary methods result in production of cells and cell clusters expressing pdx-l+, insulin, and/or C-peptide. Furthermore exemplary methods result in production of cells and cell clusters that release C-peptide. The present invention further provides substantially purified cultures of cells and cell clusters *{e.g.,* partially or terminally

differentiated cells) differentiated from embryonic stem cells. By substantially purified is meant that a culture of differentiated cells or cell clusters contains less than 20%, preferably less than 15%, 10%, 7%, 5%, 4%, 3%, 2%, 1%, or less than 1% of cells that are either undifferentiated or differentiated to a cell type of a different *(e.g.,* a non-pancreatic lineage).

Substantially purified cells and cell clusters differentiated along a pancreatic lineage by the methods of the present invention can be used therapeutically for treatment of various disorders associated with injury, disease, or other decrease in the functional performance of the pancreas. Substantially purified cultures of cells for use in the therapeutic methods of the invention include essentially homogenous cultures of cells *{e.g.,* essentially all of the cells are of a particular partially or terminally differentiated cell type) or heterogeneous cultures of cells. When heterogeneous cultures of cells are used essentially all of the cells are derived from a particular lineage and are related to a particular tissue type *(e.g.,* the culture comprises various partially differentiated and/or terminally differentiated pancreatic cell types such as a mixture of pdx-l+ and insulin+ cell types).

To illustrate, the methods of the present invention can be used to generate partially or terminally differentiated pancreatic cell types. Such pancreatic cell types can be used in the treatment or prophylaxis of pancreatic disorders, both exocrine and endocrine, as well as pancreatic injuries. In one embodiment, the methods of the invention can be used to produce pancreatic beta-like cells or cell clusters useful for the treatment of diabetes or other conditions of impaired glucose regulation. By pancreatic beta-like cells or cell clusters is meant that the cells or cell clusters express pancreatic genes including, but not limited to, pdx-1, insulin, and/or C-peptide. In certain embodiments, the pancreatic beta-like cells or cell clusters secrete C-peptide and are glucose responsive. Exemplary diseases or disorders that may be treated and/or prevented using the pancreatic cell types provided herein include, for example, diabetes mellitus, pancreatitis (acute and/or chronic), and pancreatic insufficiency.

In addition to particular disease states, the methods of the present invention can be used to generate partially or terminally differentiated cell types for the treatment of an injury to the particular organ or tissue. Such injuries include, but are

not limited to, blunt trauma, surgical resection, or tissue damage caused by cancer or other proliferative disorder.

In any of the foregoing, the invention also contemplates that preparations of partially and/or terminally differentiated cell types of an endodermally derived tissue may be useful for other purposes in addition to therapeutic purposes. Such cells may be useful in screens to identify non-cell agents that promote proliferation, differentiation, survival, or migration of the differentiated cells.

The present invention provides partially and/or terminally differentiated endodermal cell types that can be used to treat or prophylactically treat injury, disease, or other decrease in functional performance of an endodermally derived tissue. Such cells can be administered directly to the affected tissue (e.g., via transplantation or injection directly or adjacent to the affected tissue). Such cells can also be administered systemically *(e.g.,* via intravenous injection) and allowed to home to the site of disease or damage *(e.g.,* to home to the affected tissue following systemic administration).

Additionally, the invention contemplates that preparations of partially and/or terminally differentiated cells can be administered alone or can be administered in combination with other therapies. By way of example, the cells can be administered concurrently to or concomitantly with one or more agents that promotes one or more of proliferation, differentiation, migration, or survival. Without wishing to be bound by theory, such agents may, for example, help transplanted cells home to the site of damage. Furthermore, such agents may help promote the survival of both the endogenous tissue and the transplanted cells. Such agents can be used to treat conditions associated, in whole or in part, by loss of, injury to, or decrease in functional performance of endodermal cell types.

The following are illustrative of disease states that can be treated using preparations of cells differentiated along a pancreatic lineage from ES cells. Such diseases can be treated using (i) preparations of differentiated cells alone, (ii) preparations of differentiated cells in combination with one or more non-cell based compounds or agents, or (iii) preparation of differentiated cells in combination with one or more treatment regimens appropriate for the particular disease or injury being treated.

Exemplary **diseases**

Pancreatic diseases

1. Diabetes mellitus

Diabetes mellitus is the name given to a group of conditions affecting about 17 million people in the United States. The conditions are linked by their inability to create and/or utilize insulin. Insulin is a hormone produced by the beta cells in the pancreas. It regulates the transportation of glucose into most of the body's cells, and works with glucagon, another pancreatic hormone, to maintain blood glucose levels within a narrow range. Most tissues in the`body rely on glucose for energy production.

Diabetes disrupts the normal balance between insulin and glucose. Usually after a meal, carbohydrates are broken down into glucose and other simple sugars. This causes blood glucose levels to rise and stimulates the pancreas to release insulin into the bloodstream. Insulin allows glucose into the cells and directs excess glucose into storage, either as glycogen in the liver or as triglycerides in adipose (fat) cells. If there is insufficient or ineffective insulin, glucose levels remain high in the bloodstream. This can cause both acute and chronic problems depending on the severity of the insulin deficiency. Acutely, it can upset the body's electrolyte balance, cause dehydration as glucose is flushed out of the body with excess urination and, if unchecked, eventually lead to renal failure, loss of consciousness, and death. Over time, chronically high glucose levels can damage blood vessels, nerves, and organs throughout the body. This can lead to other serious conditions including hypertension, cardiovascular disease, circulatory problems, and neuropathy.

2. Pancreatitis

Pancreatitis can be an acute or chronic inflammation of the pancreas. Acute attacks often are characterized by severe abdominal pain that radiates from the upper stomach through to the back and can cause effects ranging from mild pancreas swelling to life-threatening organ failure. Chronic pancreatitis is a progressive condition that may involve a series of acute attacks, causing intermittent or constant pain as it permanently damages the pancreas.

Normally, the pancreatic digestive enzymes are created and carried into the duodenum (first part of the small intestine) in an inactive form. It is thought that during pancreatitis attacks, these enzymes are prevented or inhibited from reaching the duodenum, become activated while still in the pancreas, and begin to autodigest and destroy the pancreas. While the exact mechanisms of pancreatitis are not well understood, it is more frequent in men than in women and is known to be linked to and aggravated by alcoholism and gall bladder disease (gallstones that block the bile duct where it runs through the head of the pancreas and meets the pancreatic duct, just as it joins the duodenum). These two conditions are responsible for about 80% of acute pancreatitis attacks and figure prominently in chronic pancreatitis. Approximately 10% of cases of acute pancreatitis are due to idiopathic (unknown) causes. The remaining 10% of cases are due to any of the following: drugs such as valproic acid and estrogen; viral infections such as mumps, Epstein-Barr, and hepatitis A or B; hypertriglyceridemia, hyperparathyroidism, or hypercalcemia; cystic fibrosis or Reye's syndrome; pancreatic cancer; surgery in the pancreas area (such as bile duct surgery); or trauma.

Acute pancreatitis

About 75% of acute pancreatitis attacks are considered mild, although they may cause the patient severe abdominal pain, nausea, vomiting, weakness, and jaundice. These attacks cause local inflammation, swelling, and hemorrhage that usually resolves itself with appropriate treatment and does little or no permanent damage. About 25% of the time, complications develop, such as tissue necrosis, infection, hypotension (low blood pressure), difficulty breathing, shock, and kidney or liver failure.

Chronic pancreatitis

Patients with chronic pancreatitis may have recurring attacks with symptoms similar to those of acute pancreatitis. The attacks increase in frequency as the condition progresses. Over time, the pancreas tissue becomes increasingly scarred and the cells that produce digestive enzymes are destroyed, causing pancreatic insufficiency (inability to produce enzymes and digest fats and proteins), weight loss, malnutrition, ascites, pancreatic pseudocysts (fluid pools and destroyed tissue that can become infected), and fatty stool. As the cells that produce insulin and

glucagons are destroyed, the patient may become permanently diabetic.

3. Pancreatic insufficiency

Pancreatic insufficiency is the inability of the pancreas to produce and/or transport enough digestive enzymes to break down food *in* the intestine and allow its absorption. It typically occurs as a result of chronic pancreatic damage caused by any of a number of conditions. It is most frequently associated with cystic fibrosis in children and with chronic pancreatitis in adults; it is less frequently but sometimes associated with pancreatic cancer.

Pancreatic insufficiency usually presents with symptoms of malabsorption, malnutrition, vitamin deficiencies, and weight loss (or inability to gain weight in children) and is often associated with steatorrhea (loose, fatty, foul-smelling stool). Diabetes also may be present in adults with pancreatic insufficiency.

In the treatment of any of the above mentioned conditions, the dosage *(e.g.*, what constitutes a therapeutically effective amount of differentiated cells) is expected to vary from patient to patient depending on a variety of factors. The selected dosage level will depend upon a variety of factors including the specific condition to be treated, other drugs, compounds and/or materials used in combination with the particular cell-based therapy, the severity of the patient's illness, the age, sex, weight, general health and prior medical history of the patient, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the cells of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

In general, a suitable dose of cells of the invention will be that amount of the cells which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon a variety of factors including the activity of the particular compositions employed, the route of administration, the time of administration, the rate of excretion of the particular compositions being employed, the duration of the treatment, other drugs, compounds and/or materials used in

combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient, and like factors well known in the medical arts.

In the case of the present invention, the pharmaceutical composition comprises cells differentiated by the methods of the present invention and one or more pharmaceutically acceptable carriers or excipients. In certain embodiments, the pharmaceutical compositions may additional comprise one or more additional therapeutic agents. In one embodiment, the pharmaceutical compositions may comprise an immuno-suppressant and/or other anti-rejection drug. As outlined above, the pharmaceutical composition may be administered in any of a number of ways including, but not limited to, systemically, intraperitoneally, directly transplanted, and furthermore may be administered in association with hollow fibers, tubular membranes, shunts, or other biocompatible devices or scaffolds.

The term "treatment" is intended to encompass also prophylaxis, therapy and cure, and the patient receiving this treatment is any animal in need, including primates, in particular humans, and other mammals such as equines, cattle, swine and sheep; as well as poultry and pets in general.

The present invention provides methods for directing the differentiation of embryonic stem cells to produce cultures of endodermally derived cells. Such cultures can optionally be further purified to enrich for particular cell types, thereby providing an essentially purified preparation of endodermally derived cell types.

Essentially purified preparations of partially and/or terminally differentiated endodermally derived cells can be used therapeutically to treat or prophylactically treat injuries or disease of the particular organ or tissue. For example, essentially purified preparations of pancreatic cells can be formulated in a pharmaceutically acceptable carrier and delivered to patients suffering from a condition characterized by loss in functional performance of the pancreas *{e.g.,* diabetes).

When preparations of cells are delivered to patients, the invention contemplates that the therapeutic treatment additionally comprises administering other therapeutic agents. By way of example, agents that inhibit cell death, promote cell survival, or promote cell migration can be administered concurrently or concomitantly with the preparation of differentiated cells. Furthermore, the

invention contemplates therapeutic methods comprising administration of the subject cells concurrently or concomitantly with other therapeutic regimens appropriate to treat the particular condition being treated *(e.g.,* cells + insulin for the treatment of diabetes).

When the therapeutic method involves administration of cells and one or more additional agents or treatment modalities, the invention contemplates that the cells and agents can be administrated via the same method of administration or via different methods of administration. By way of non-limiting example, the invention contemplates that in certain embodiments, preparations of terminally and/or partially differentiated pancreatic cells will be surgically or laparoscopically transplanted directly to the abdominal cavity or directly to endogenous pancreatic tissue. If one or more additional agents are also part of the particular treatment protocol, such agents may be similarly delivered, or may be delivered in another manner *(e.g.,* injected intravenously, transdermally, orally, subcutaneous, *etc.).*

The pharmaceutical compositions/preparations of the present invention *(e.g.,* pharmaceutical compositions of cells and pharmaceutical compositions of non-cell agents/compounds) are formulated according to conventional pharmaceutical compounding techniques. See, for example, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA).

Pharmaceutical formulations of the invention can contain the active polypeptide and/or agent, or a pharmaceutically acceptable salt thereof. These compositions can include, in addition to an active polypeptide and/or agent, a pharmaceutically acceptable excipient, carrier, buffer, stabilizer or other material well known in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active agent. Preferable pharmaceutical compositions are non-pyrogenic. The carrier may take a wide variety of forms depending on the route of administration, *e.g.,* intravenous, intravascular, oral, intrathecal, epineural or parenteral, transdermal, *etc.* Furthermore, the carrier may take a wide variety of forms depending on whether the pharmaceutical composition is administered systemically or administered locally, as for example, via surgical transplantation, laparoscopic transplantation, or via a biocompatible device *(e.g.,* catheter, stent, wire, or other intraluminal device).

Illustrative examples of suitable carriers are water, saline, dextrose solutions, fructose solutions, ethanol, or oils of animal, vegetative or synthetic origin. The carrier may also contain other ingredients, for example, preservatives, suspending agents, solubilizing agents, buffers and the like.

In one embodiment, the pharmaceutical composition is formulated for sustained-release. An exemplary sustained-release composition has a semi permeable matrix of a solid biocompatible polymer to which the composition is attached or in which the composition is encapsulated. Examples of suitable polymers include a polyester, a hydrogel, a polylactide, a copolymer of L-glutamic acid and ethyl-L-glutamase, non-degradable ethylene-vinyl acetate, a degradable lactic acid-glycolic acid copolymer, and poly-D+- hydroxybutyric acid.

Polymer matrices can be produced in any desired form, such as a film, or microcapsules.

Other sustained-release compositions include Iiposomally entrapped modified compositions. Liposomes suitable for this purpose can be composed of various types of lipids, phospholipids, and/or surfactants. These components are typically arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

Pharmaceutical compositions according to the invention include implants, *i.e.*, compositions or device that are delivered directly to a site within the body and are, preferably, maintained at that site to provide localized delivery.

As outlined above, biocompatible devices for use in the various methods of delivery contemplated herein can be composed of any of a number of materials. The biocompatible devices include wires, stents, catheters, balloon catheters, and other intraluminal devices. Such devices can be of varying sizes and shapes depending on the intended vessel, duration of implantation, particular condition to be treated, and overall health of the patient. A skilled physician or surgeon can readily select from among available devices based on the particular application.

By way of further illustration, exemplary biocompatible, intraluminal devices are currently produced by several companies including Cordis, Boston Scientific, Guidant, and Medtronic (Detailed description of currently available catheters, stents, wires, *etc.*, are available at the websites of Cordis Corporation

(cordis.com); Medtronic, Inc. (medtronic dot com); and Boston Scientific Corporation (bostonscientific dot com)).

The invention also provides articles of manufacture including the subject pharmaceutical compositions of the invention and related kits. The invention encompasses any type of article including a pharmaceutical composition of the invention, but the article of manufacture is typically a container, preferably bearing a label identifying the composition contained therein.

The container can be formed from any material that does not react with the contained composition and can have any shape or other feature that facilitates use of the composition for the intended application. A container for a pharmaceutical composition of the invention intended for parental administration generally has a sterile access port, such as, for example, an intravenous solution bag or a vial having a stopper pierceable by an appropriate gauge injection needle.

Cell-based and/or non-cell-based compositions for use in the therapeutic methods of the present invention may be conveniently formulated for administration with a biologically acceptable medium, such as water, buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like) or suitable mixtures thereof. For therapeutic methods comprising administration of both cell-based and non-cell based compositions, the invention contemplates that such compositions may be formulated in the same or different carriers. The appropriate formulation and medium can be chosen based on the mode of administration.

Optimal concentrations of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists. As used herein, "biologically acceptable medium" includes solvents, dispersion media, and the like which may be appropriate for the desired route of administration of the one or more agents. The use of media for pharmaceutically active substances is known in the art. Except insofar as a conventional media or agent is incompatible with the activity of a particular agent or combination of agents, its use in the pharmaceutical preparation of the invention is contemplated. Suitable vehicles and their formulation inclusive of other proteins are described, for example, in the book *Remington's Pharmaceutical Sciences* (Remington's

Pharmaceutical Sciences. Mack Publishing Company, Easton, Pa., USA 1985). These vehicles include injectable "deposit formulations".

Compositions of the present invention may be given orally, parenterally, or topically. They are of course given by forms suitable for each administration route. For example, they are administered in tablets or capsule form, by injection, inhalation, ointment, controlled release device or patch, or infusion.

The effective amount or dosage level will depend upon a variety of factors including the activity of the particular compositions employed, the route of administration, the time of administration, the rate of excretion of the particular compositions being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the animal, and like factors well known in the medical arts.

The compositions (e.g., cell-based compositions alone or in combination with one or more non-cell based compositions) can be administered as such or in admixtures with pharmaceutically acceptable and/or sterile carriers and can be administered concomitantly or concurrently with other compounds.

Thus, another aspect of the present invention provides pharmaceutically acceptable compositions comprising an effective amount of cell or non-cell based compositions, formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. As described below, the pharmaceutical compositions of the present invention may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) delivery via a catheter, port or other biocompatible, intraluminal device; (2) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes for application to the tongue; (3) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension. Furthermore, the cells or cell clusters may be surgically or laparoscopically implanted either near the pancreas or in the abdominal cavity, or at a distant and more accessible site. In certain embodiments the subject compositions may be simply dissolved or suspended in sterile water. In certain embodiments, the

pharmaceutical preparation is non-pyrogenic, *i.e.*, does not elevate the body temperature of a patient.

Some examples of the pharmaceutically acceptable carrier materials that may be used include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

Compositions for administration may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of an composition, it is desirable to slow the absorption of the agent from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the composition then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered composition form is accomplished by dissolving or suspending the agent in an oil vehicle.

For any of the foregoing, the invention contemplates administration to neonatal, adolescent, and adult patients, and one of skill in the art can readily adapt the methods of administration and dosage described herein based on the age, health, size, and particular disease status of the patient. Furthermore, the invention contemplates administration in utero to treat conditions in an affected fetus.

(vii) Immune Tolerance

One issue that may arise with any therapeutic intervention involving the delivery of xenogenic cells or tissue is that of rejection. For example, despite the efforts made to minimize antigen mismatch prior to whole organ transplantation, graft rejection remains a serious limiting factor in the long-term efficacy of transplanted organs, as well as of transplantation patients.

Although some reports suggest that embryonic stem cells, even xenogenic stem cells, will not provoke an immune response, it is unclear whether this will in practice be true. Furthermore, even if embryonic stem cells themselves do not provoke an immune response, progeny of ES cells differentiated *in vitro* may provoke an immune response. Accordingly, the invention contemplates therapeutic methods comprising administration of pharmaceutical preparations concurrently or concomitantly with immunosuppressants and/or other anti-rejection drugs. As outlined in detail above, when the therapeutic methods involve administration of both cell-based and non-cell based compositions, the invention contemplates administration via the same or via a different mode of administration, and similarly contemplates that the compositions are each formulated appropriately in light of their properties and the desired route of administration.

In addition to immuno-suppression via traditional anti-rejection drugs, the invention contemplates additional methods of preventing host rejection of the differentiated cells. Such methods are based on inducing tolerance in the patient, and can be used alone or in combination with other immunosuppresants or anti-rejection drugs.

In one embodiment, tolerance is induced by first introducing into the patient dendritic cells differentiated from the same line of ES cells that will be used to differentiate the particular endodermal cells. ES cells can be differentiated into dendritic cells by first driving them down the hematopoietic lineage via addition of

one or more factors including, but not limited to, IL-I, IL-3, IL-6, GM-CSF, G-CSF, SCF, or erythropoietin. Alternatively, the ES cells can be co-cultured with cell lines such as OP-9 stromal cells or yolk-sac endodermal cells.

Following differentiation of ES cells to dendritic cells, essentially purified populations of dendritic cells can be prepared and delivered to the patient. Such dendritic cells are delivered to the patient prior to administration of the therapeutic cells *(e.g.,* the pancreatic cells or the hepatic cells). The dendritic cells are optionally delivered along with traditional immunosuppressive therapies. When the therapeutic cells are later delivered, they may optionally be delivered with the same or with a lower dose of immunosuppresants.

When the methods of the present invention are used to direct the differentiation of non-embryonic stem cells, the invention contemplates that these partially or terminally differentiated adult stem cells can be used therapeutically in all of the ways described for embryonic stem cells. When adult stem cells are used, potential graft rejection can be eliminated by using cells derived from the patient to be treated. Alternatively, the above contemplated immunosuppressive and tolerance approaches are also contemplated.

Exemplification

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention in any respect.

Example 1: Human Embryonic **Stem Cells** Spontaneously **Differentiate to Ectodermal, Mesodermal, and Endodermal Cell** Types

Figure i confirms previous experiments demonstrating that human embryonic stem (ES) cells spontaneously differentiate along all three lineages when cultured as embryoid bodies (EBs). Human embryonic stem cell lines 1 or 2 (hESl and hES2) were used to generate embryoid bodies (EBs). Briefly, ES cells were removed from the MEF feeder layer by manually cutting (M) or collagenase digestion (C). The removed ES cells were then placed in appropriate media. After 0,

5, or 9 days post-EB formation, RNA was extracted from the EBs and analyzed for expression of the indicated markers by real-time RT-PCR. Relative expression shown is normalized to that of β-actin and expression for day 0 was set equal to 1. An asterisk (*) indicates arbitrary values due to no expression at day 0. Data is shown for two hES lines - hESl and hES2 with hES2 shown in parenthesis. There was no significant change in expression for soxl7, nkxó.l, and brachyury.

Example 2: **Methods for Generating Embryoid Bodies**

One method for directing the differentiation of stem cells is to generate embryoid bodies. These embryoid bodies can be grown under a number of conditions including, but not limited to, in floating suspension culture, in BMM marketed as MATRIGEL™ or other matrix, or on a filter. However, the first step is the actual formation of an embryoid body from a culture of embryonic stem cells. We used any of the following methods for generating embryoid bodies from cultures of embryonic stem cells. These methods can be used to generate embryoid bodies from human embryonic stem cells grown on MEF feeder layers, embryonic stem cells grown on other feeder layers, and embryonic stem cells grown under feeder free conditions.

Materials: Human embryonic stem cells (e.g., lines hES 1-6 or DM lines); culture medium, PBS; collagenase IV stock solution (5 mg/ml) - preferably for use with hES 1-6; trypsin/EDTA stock solution (0.25%) - preferably for use with DM lines; ultra-low-6-well plates.

(a) Collagenase EB Protocol

The following protocol can be used to generate embryoid bodies. This protocol was specifically used to generate embryoid bodies from cell lines hES 1-6. However, the protocol can be used more generally in other ES cell or cell lines.

PlOO tissue culture plates containing hES cells grown under standard conditions were used as starting material. The medium was aspirated, and the cells were washed 2 times with PBS. After the wash, 3 ml of 1 mg/ml collagenase IV was added to each plate, and the plates were incubated for 8 minutes in a 37°C tissue culture incubator. Following incubation, the collagenase was aspirated from the cells, and the cells were washed with 10 ml of PBS. The PBS was gently aspirated,

and care was taken to avoid disturbing the colonies of ES cells.

About 8 ml of EB culture medium was gently added to the plate, and the plate was mechanically scraped and gently streaked using a 5-ml plastic pipette and cell scraper. The materials were pipetted up and down to dislodge the cells pieces — care was taken not to over-pipette and damage the cells. The embryonic stem cell clusters were transferred to ultra-low 6-well plates to promote embryoid body formation. Embryoid bodies were cultured for several days, and EB culture medium was changed every two to three days.

When experiments called for analysis of gene or protein expression in EBs, the EBs were handled as follows: EBs were collected in a tube, and EBs were allowed to either settle to the bottom of the tube, or were spun briefly to facilitate precipitation of the EBs to the bottom of the tube. At this point, EBs can be processed for immunohistochemistry studies or for RNA extraction, using standard techniques.

(b) Collagenase EB protocol for manually-passaged hESl-6

The following protocol can be used to generate embryoid bodies. This protocol was specifically used to generate embryoid bodies from cell lines hES 1-6. However, the protocol can be used more generally in other ES cell or cell lines.

Organ cultures dishes containing hES cells grown under standard conditions on MEF feeder layers were used as starting material. The medium was aspirated, and the cells were washed 2 times with PBS. Then 0.5 ml of 1 mg/ml collagenase IV was added to each dish, and the plates were incubated for 5 minutes in a 37°C tissue culture incubator. Following incubation, the collagenase was aspirated from the cells, and the cells were washed with 1 ml of PBS. The PBS was gently aspirated, and care was taken to avoid disturbing the colonies of embryonic stem cells.

About 1 ml of EB culture medium was gently added to the plate. ES cell colonies were dissociated gently using a pipet tip. Care was taken to avoid detaching MEFs from the dish. ES cell pieces were transferred to a suspension plate containing EB culture medium to promote embryoid body formation. Embryoid bodies were cultured for several days, and EB culture medium was changed every two to three days.

When experiments called for analysis of gene or protein expression in EBs,

the EBs were handled as follows. EBs were collected in a tube, and EBs were allowed to either settle to the bottom of the tube or were spun briefly to facilitate precipitation of the EBs to the bottom of the tube. At this point, EBs can be processed for immunohistochemistry studies or for RNA extraction, using standard techniques.

(c) Collagenase EB protocol for trypsin-passaged Harvard HUES-I cells.

The following protocol can be used to generate embryoid bodies. This protocol was specifically used to generate embryoid bodies from the Harvard cell line HUES- 1. However, the protocol can be used more generally in other ES cell or cell lines.

PlOO tissue culture plates containing hES cells grown under standard conditions on MEF feeder layers were used as starting material. The process begins with a stage whereby the ES cells were waned from the feeder layer. The cells were trypsinized with 0.05% trypsin and plated on collagen IV-coated plates in the splitting ratio of 1:3. The cells were cultured for 3-4 until subconfluent.

Following this waning phase, the medium was aspirated, and the cells were washed 2 times with PBS. 3 ml of 1 mg/ml collagenase IV was added to each dish, and the plates were incubated for 4 minutes in a 37°C tissue culture incubator. Following incubation, the collagenase was aspirated from the cells, and the cells were washed with 10 ml of PBS. The PBS was gently aspirated, and care was taken to avoid disturbing the colonies of embryonic stem cells.

About 8 ml of EB culture medium was gently added to the plate, and the plate was mechanically scraped using a 5-ml plastic pipette and cell scraper. The materials were pipetted up and down to dislodge the cells pieces —care was taken not to over-pipette and damage the cells. The embryonic stem cell clusters were transferred to suspension plates to promote embryoid body formation. Embryoid bodies were cultured for several days, and EB culture medium was changed every two to three days.

When experiments called for analysis of gene or protein expression in EBs, the EBs were handled as follows. EBs were collected in a tube, and EBs were allowed to either settle to the bottom of the tube or were spun briefly to facilitate

precipitation of the EBs to the bottom of the tube. At this point, EBs can be processed for immunohistochemistry studies or for RNA extraction, using standard techniques.

Example 3: Method for Directing the Differentiation of a Stem Cell to a Particular Differentiated Cell Type

The following is indicative of protocols that can be used to direct the differentiation of stern cells to a particular differentiated cell type. The particular protocol outlined here promoted differentiation of embryonic stem cells along the pancreatic lineage, as assayed by expression of the marker pdx-1.

Materials: Human embryonic stem cells; medium (RPMÏ/20% serum replacement (20SR) / pen-strep; PBS; collagenase IV (preferably for use with hES 1-6 lines); trypsin/EDTA (preferably for DM lines); ultra-low-attachment-6-well plates; (Corning / costar) growth factor reduced BMM marketed as GFR MATRIGEL™; early factors; late factors.

Exemplary **Time table**

D-1	Liquefy MATRIGEL™ on ice and keep ice box in the cold room overnight.
D0 - D10	**Early factor stage**
DO	Make MATRIGEL™ EB.
D3	Top up with RPMI/20SR (0.5 ml) + EF (quantity for 2ml medium).
D6	Top up with RPMI/20SR (0.5 ml) + EF (quantity for 2ml medium).
D10 - D20	**Late factor stage**
D10	Wash away early factors. Change to LF.
D13	Top up with RPMI/20SR (0.5 ml) + LF (quantity for 2ml medium).
D16	Top up with RPMI/20SR (0.5 ml) + LF (quantity for 2ml medium).
D20	**Harvest** MATRIGEL™ **EB for RNA-RT-PCR, immunostainirig or *in situ* hybridization.**

DO in the time table provided above indicates the point at which culture of cells as embryoid bodies begin. Alternatively, for embodiments in which the cells are differentiated without embryoid body formation, DO indicates the point at which the embryonic stem cells are plated directly onto BMM marketed as MATRIGEL™ or other tissue culture plates. Prior to DO, cultures of proliferating ES cells must be

handled according to one of the protocol outlined above to generate a starting culture of EBs.

EXEMPLARY EXPERIMENTAL PROCEDURE

Part i : Collagenase treatment of hES 1-6

PlOO tissue culture plates containing hES cells grown under standard conditions were used as starting material. The medium was aspirated, and the cells were washed 2 times with PBS. About 3 ml of 1 mg/ml collagenase IV was added to each plate, and the plates were incubated for 8 minutes in a 37°C tissue culture incubator. Following incubation, the collagenase was aspirated from the cells, and the cells were washed with 10 ml of PBS. The PBS was gently aspirated, and care was taken to avoid disturbing the colonies of embryonic stem cells.

About 8 ml of EB culture medium (RPMI/20SR) was gently added to the plate, and the plate was mechanically scraped using a 5-ml plastic pipette and cell scraper. The materials were pipetted up and down to dislodge the cells pieces —care was taken not to over-pipette and damage the cells. The pellets were transferred to a 15-ml tube, and the pellets were spun down at 2500 rpm for 4 minutes. The medium was aspirated.

Part 2: Make BMM marketed as MATRIGELTM EB

Early **factor stage**

Day 0

1:6 MATRIGEL™ medium *(e.g.,* 1 ml liquefied BMM marketed as MATRIGEL™ + 5 ml RPMI/20SR) was prepared using prechilled pipettes. Total volume is according to 2 ml for each well. The hES cell pellets were resuspended in the MATRIGEL™ medium, and 2 ml of hES pellet: medium suspension was added / well of Ultra-low plate.

For wells in which growth factors were to be added, factor cocktail (100 ng each) can either be added in the MATRIGEL™ medium before pellet resuspension procedure or immediately after suspension is plated in the Ultra-low plate. Plates were incubated in 37°C tissue culture incubator, and the MATRIGEL™ medium gels after several hours. After overnight incubation, the hES pellets formed embedded embryoid bodies.

Day 3 **and** 6

EB cultures were supplemented with 0.5 ml additional RPMI/20SR medium + early factors (100 ng of each factor).

Late factor stage

DlO

The medium was removed. Care was taken to prevent dislodgement **of** the EBs and the BMM marketed as MATRIGEL™. Fresh medium was added to the cells which were allowed to equilibrate for approximately 1 hour. This was followed by the addition of the LF cocktail and RPMI/20SR.

D13 and 16

EB cultures were supplemented with 0.5 ml additional RPMI/20SR medium + late factors.

D20

The MATRIGEL™ EBs were collected into a 15 ml FALCON™ tube. 12 ml chilled PBS was added, and the EBs were placed on ice for 10 min. The EBs were spun for 4 minutes at 2500 rpm (round per minute). The supernatant was carefully removed, and the EBs were transferred to an EPPENDORF™ tube. The EBs were then analyzed using immuno-cytochemistry or RT-PCR.

Example 4: Schematic Representation of Multi-Step Method for Differentiating Stem Cells Along Particular Endodermal Lineages

Figure 2 provides a schematic representation of a multi-step method for directing the differentiation of stem cells along particular endodermal lineages. For the particular embodiment illustrated in Figure 2, the starting material is embryonic stem cells, and the particular endodermal lineage is pancreatic - specifically beta islet cells. Embryonic stem cells can be cultured in any of a number of formats, for example, as embryoid bodies in suspension culture, as embryoid bodies embedded in BMM marketed as MATRIGEL™ or other matrix material, as embryoid bodies on a filter, as embryonic stem cells directly plated on BMM marketed as MATRIGEL™ or other matrix, or as embryonic stem cells directly plated on tissue culture plates. Regardless of the particular format, embryonic cells cultured in any of the foregoing

ways are cultured for 1-10 days (or even 1-15 days) in medium containing early factors. This period of culture directs the cells down a particular endodermal pathway. For pancreatic cell types, culture in early factors results in partial differentiation, as assessed by expression of the early marker Pdx-1. Exemplary early factors that help to induce the expression of *pdx-1* include, but are not limited to, activin A, BMP-2, BMP-4, and nodal. Such factors can be added individually or in combination. Combinations include combinations of two, three, four, or more than four factors.

After this first stage of differentiation, cells are cultured for 1-10 days (or even 1-15 days) in medium supplemented with late factors. This period of culture further promotes differentiation of cells along the particular pathway toward terminal differentiation. This may include promotion of further expression of pdx-1, promotion of expression of terminal markers of differentiation, both promotion of further expression of pdx-1 and further expression of insulin, or decrease expression of pdx-1 accompanied by an increased expression of markers of terminal differentiation. For pancreatic cell types, and specifically for beta islet cells, culture in late factors promotes further differentiation. Promotion of further differentiation can be assessed by assaying for a further increase in expression of pdx-1. Additionally or alternatively, further differentiation can be assayed by expression of late markers including insulin. Note that pdx-1 expression is maintained, although perhaps at a lower level, upon terminal differentiation of the cells. Exemplary late factors that help induce expression of markers of terminal beta islet differentiation include, but are not limited to, HGF, exendin4, betacellulin, and nicotinamide. Such factors can be added individually or in combination. Combinations include combinations of two, three, four, or more than 4 factors.

At this point, cells may optionally be further cultured to enhance terminal differentiation and functional performance.

Example 5: Multi-Step Method for Differentiating Stem Cells Along Particular Endodermal Lineages

Human embryonic stem cell 3 (hES3) were subjected to a multi-step differentiation protocol, as outlined in Figure 2. The embryonic stem cells were

cultured as embryoid bodies suspended in 3D in BMM marketed as MATRIGEL™. The cells were cultured for 10 days in medium containing the early factors and then for 10 days in medium containing the late factors. Following culture, cells were assayed for expression of pdx-1.

Figure 3 summarizes the results of these experiments. For each bar depicted in Figure 3, the embryoid bodies were cultured, except as indicated, with the following early and late factors: early factors were activin A, BMP-2, BMP-4, and nodal; late factors were HGF, exendin4, betacellulin, and nicotinamide. The particular factor omitted is indicated under each bar.

As shown in Figure 3, culture of the embryoid bodies in the presence of all of the early factors and all of the late factors resulted in an approximately four fold increase in expression of pdx-1 in comparison to culture in the absence of these growth factors. The use of all of these factors, however, was not essential to induce robust expression of *pdx-1*. For example, the inclusion of BMP-2, nodal, betacellulin, and nicotinamide appears optional.

Further, the role of BMM (e.g., those marketed as MATRIGEL™) in differentiation was investigated. Some hES3-derived embryoid bodies were cultured free-floating for 20 days in RPMI media alone (-) or in BMM marketed as MATRIGEL™ 1:6/RPMI for the times indicated in the presence of the 2EF and 3LF growth factors. On day 20, cells were analyzed for the expression of *pdx-1*. As shown in Figure 30, *pdx-1* expression was prominently enhanced in cells that were cultured in BMM marketed as MATRIGEL™ between days 0 and 10, but not so if the cells were cultured in BMM marketed as MATRIGEL™ between days 10 and 20. These data show that the requirement for BMM marketed as MATRIGEL™ is restricted to about days 0 and 10 (when the EBs are in contact with the EFs). Continued presence of BMM marketed as MATRIGEL™ from days 10-20 is only marginally beneficial, if anything at all. The lack of BMM marketed as MATRIGEL™ during days 0-10 in this protocol, even when BMM marketed as MATRIGEL™ is present during days 10-20, does not stimulate *pdx-1* expression. This experiment also emphasizes the role of the Activin / BMP-4 co-stimulation during this early window of differentiation.

Example 6: Directed Differentiation of Mouse Embryonic **Stem Cell Reporter Lines**

A mouse embryonic stem cell line with a iacZ reporter knocked into the pdx-1 locus was differentiated along the pancreatic lineage. Cultures of ES cells were used to generate EBs which were subjected to culture in the presence of early and late factors. Figure 4A shows a cluster of cells expressing β-galactosidase, thus indicating expression of pdx-1, after EB formation and subsequent plating. Figure 4B shows quantitative RT-PCR data for pdx-1 for mouse EBs at various stages of culture. It is apparent that *pdx-1* expression increased over time up **to** 24 days of EB formation.

Example 7: Directed Differentiation Along a Pancreatic Lineage Increases Over Time

Human embryonic stem cell line hES2 was used to generate EBs suspended in BMM marketed as MATRJGEL™. Cell were treated with the early and late factors, as described above. Expression of *pdx-1* in these EBs derived increased with time. Figure 5A shows that *pdx-1* rnRNA, as detected by real time RT-PCR, increased with the number of days of EB formation between 0 to 24 days. Figure 5B shows an ethidium bromide stained gel of *the pdx-1* RT-PCR product, indicating that a single band of the predicted size was detected.

Example 8: Various Growth Factor Preparations Promote Directed Differentiation Along a Pancreatic Lineage

Human embryonic stem cell line hES3 was used to generate EBs suspended in BMM marketed as MATRIGEL™. Addition of TGF-β factors increased *pdx-1* expression in hES3. hES3 EBs were cultured in BMM marketed as MATRIGEL™ in RPMI media with addition of several TGF-β related factors. Expression of *pdx-1* by RT-PCR was measure after 20 days in culture. As shown in Figure 6, expression is expressed as fg per ng actin. Addition of growth factors led to a 9-fold increase in *pdx-1* expression in comparison to culture in the absence of growth factors. Furthermore, this treatment resulted in an increase in insulin expression, as measured by RT-PCR, after 20 days in culture. Expression is expressed as fg per ng

actin. Addition of growth factors led to about a 2-fold increase in insulin expression.

Example 9: Directed Differentiation of Human Embryonic **Stem Cells Along Particular Endodermal Lineages**

The methods of the invention can be used to direct the differentiation of stem cells to particular endodermal cell types. The results summarized in Figure 7 demonstrate that hepatocyte cell types can be differentiated from embryonic stem cells. Figure 7A shows expression of the hepatocyte marker albumin in hES cells cultured by directly plating ES cells on BMM marketed as MATRIGEL™ coated tissue culture plates.

Figure 7B shows quantitative RT-PCR data for two different hES lines subjected to several differentiation protocols. Human ES cells differentiated according to condition E had the largest increase in expression of several hepatic markers. ES cells were plated on BMM marketed as MATRIGEL™ coated plates and maintained in knock-out media supplemented with 20% serum replacement and 1% DMSO. After five days, cells were treated with 2.5 mM sodium butyrate. The medium was then replaced with hES media supplemented with 100 ng/ml α-FGF, 0.1 ng/ml TGF-β, 30 ng/ml EGF, and 30 ng/ml HGF. Finally, medium was replaced with hES media supplemented with 10 ng/ml oncostatin M, 1 μM dexamethasone, 30 ng/ml HGF, and 0.1 ng/ml TGF-β.

Example 10: Directed Differentiation of Human Embryonic Stem Cells Along a Pancreatic Lineage Using a Combinatorial Approach —44-Day **Experiment**

As outlined in detail above, the differentiation of human embryonic stem cells grown in 3-dimensional culture can be directed along the pancreatic lineage, as indicated by expression *ofpdx-1*. Additional experiments were then conducted to see whether the directed differentiation of human embryonic stem cells along pancreatic lineage can be further influenced by subjecting the cells to a combination of the 3-D culture system outlined above and other culture systems shown by us to influence differentiation of cells to pancreatic cell types.

To establish a baseline for comparison, human embryonic stem cells were differentiated, as described above, in 3-dimensional culture in BMM marketed as MATRIGEL™ for 20 days. For the first 10 days the cells were cultured in the presence of early factors (Activin A: 50 ng/ml; BMP-2: 50 ng/ml; BMP-4: 50 ng/ml; Nodal: 50 ng/ml) and for the second 10 days the cells were cultured in the presence of late factors (Betacellulin: 50 ng/ml; HGF: 50 ng/ml; Exendin-4: 20 nM or 10 ng/mL; Nicotinamide: 10 mM). *pdx-1* levels were measured by RT-PCR at various time points during culture of the cells in the 3D MATRIGEL™ protocol. RNA was isolated and analyzed for *pdx-1* and actin expression by RT-PCR. Data were standardized in comparison to actin expression, and the results are expressed as absolute g *pdx-1/kg* Actin expression +/- SD. These results are summarized in Figure 8.

We then set up an experiment based on combining the above BMM marketed as MATRIGEL™, 3-dimensional culture protocol with a 24-day, 5-step differentiation protocol originally evaluated for its ability to produce insulin cells from pancreatic ductal precursors. Human embryonic stem cells were either placed directly into the 24-day protocol, or were first cultured for 1, 2, or 3 weeks in the 3 dimensional MATRIGEL™ protocol outlined above.

The method below provides details for a 44-day experiment that includes culturing cells for the first 20 days in the 3 dimensional MATRIGEL™ protocol outlined above, followed by the 24-day, 5-step differentiation protocol.

In other similar experiments, modifications have been made such that the ES cells were first cultured for 1 week, or 2 weeks in the 3-D MATRIGEL™ protocol, or not cultured (0 week) in the 3-D MATRIGEL™ protocol, before using the 24-day differentiation protocol.

Method: Day 20 was considered the first day of the 24-day protocol. Prior to that, cells were cultured in 3D culture in BMM marketed as MATRIGEL™. The methodology used for culturing cells that were first cultured for about 3 weeks (20 days) in 3D culture and then subject to the 24-day differentiation protocol is as follows:

DO-10 (Days 0-10): Day 0 is normally the set up day. Cells were cultured in KO SR medium + early growth factor cocktail (Activin A: 50 ng/ml; BMP-2: 50

ng/ml; BMP-4: 50 ng/ml; Nodal: 50 ng/ml). The medium was topped up (to feed the cells) at D3 and D6.

D 10-20 (Days 10-20): Cells were cultured in KO SR medium + late growth factor cocktail (Betacellulin: 50 ng/ml; HGF: 50 ng/ml; Exendin-4: 20 nM or 10 ng/mL; Nicotinamide: 1O mM). The medium was change at D 16.

D20: EBs were eluted from 3D MATRIGEL™ and re-plated on low attachment plates.

D20-26 (Days 20-26): Begin 24-day maturation protocol = Step 1 of 24-day protocol. Cells were cultured in basal medium for 6 days (DMEM/F12, 17 mM GIc, 2 mM Glutamax, 8 mM HEPES, 2% B27, + Penicillin/Streptomycin). Cell were fed with fresh media on D23.

D26 (Day 26): EBs were dissociated by Dispase (1 of 2 wells, the other one remains as EB) and re-plated on low attachment plates.

D26-32 (Days 26-32): Step 2 of 24 day protocol. Cells were cultured in basal medium + 20 ng/ml FGF- 18, 2 μg/ml heparin (new medium at D26, new GFs added at D29).

D32-36 (Days 32-36): Step 3 of 24 day protocol. Cells were cultured in basal medium + 20 ng/ml FGF- 18, 2 μg/ml heparin, 10 ng/ml EGF, 4 ng/ml TGF-α, 30 ng/ml IGF-I, 30 ng/ml IGF-II, 10 ng/ml VEGF (new medium at D32, new GFs added at D34).

D36 (Day 36): Cells were re-plated on Fibronectin-coated plates.

D36-40 (Days 36-40): Step 4 of 24 day protocol. Cells were cultured in RPMI medium (11 mM GIc, 5% FBS, 2 mM Glutamax, 8 mM HEPES, Penicillin/Streptomycin) + 10 μM Forskolin, 40 ng/ml HGF, 200 ng/ml PYY (new medium at D36, new GFs added at D38).

D40-44 (Day 40-44): Step 5 of 24 day protocol. Cells were cultured in CMRL medium (5 mM GIc, 5% FBS, 2 mM Glutamax, Penicillin/Streptomycin) + 100 ng/ml Exendin-4, 5 mM Nicotinamide (new medium at D40, new GFs added at D42).

D44 (Day 44). RNA was harvested for analysis of *pdx-1* and insulin expression by RT-PCR.

Note: RNA samples were harvested from cells at various points along this

process to help evaluate the directed differentiation of the cells.

Results: The 24-day protocol contains a step where the cells are / can be dissociated using dispase. In our experiments, we assessed whether this dispase dissociation step was necessary, and whether it negatively impacted the ability of cells to be differentiated along a pancreatic lineage.

As outlined above, following the standard 3D differentiation protocol, embryonic stem cells differentiate to express a high level of *pdx-1*. As shown in Figure 9A, cells subject to both the 20 day 3D differentiation protocol and the 24-day protocol continue to express *pdx-1*. However, *pdx-1* expression is at a lower level than following the first 20 days in culture (compare Figure 8 and Figure 9A). Additionally, however, these cells express very high levels of insulin mRNA (See, Figure 9B). Expression of insulin protein will be confirmed by performing a C-peptide ELISA assay.

Furthermore, as shown in both Figure 9A and Figure 9B, treatment of the cells with dispase during the 24-day differentiation protocol had a negative impact on the ability of these cells to differentiate along a pancreatic lineage. Thus, elimination of this step may be useful. Thus in one embodiment, a modified 24-day protocol does not include treating cells with dispase.

Example 11: Directed Differentiation of Human Embryonic Stem Cells Along a Pancreatic Lineage Using a Combinatorial Approach - 34-Day Experiment

As outlined in detail above, the differentiation of human embryonic stem cells grown in 3-dimensional culture can be directed along the pancreatic lineage, as indicated by expression of *pdx-1*. Additional experiments were then conducted to see whether the directed differentiation of human embryonic stem cells along the pancreatic lineage can be further influenced by subjecting the cells to a combination of the 3D culture system outlined above and other culture systems shown by us to influence differentiation of stem cells. One such other culture system was the 24-day protocol outlined in Example 10.

We additionally tested a 34-day experiment including the 24-day differentiation protocol. In this combinatorial approach, cells are subjected to culture

and differentiation in 3D culture for only 10 days. At that point, cells are than taken and subjected to a 24-day differentiation protocol. Following this combinatorial approach, expression of *pdx-1* and *insulin* was assessed by RT-PCR.

Method: This protocol from start to finish is 34 days long (10 + 24). Prior to the 24-day differentiation protocol, cells were cultured in 3D culture in BMM marketed as MATR1GEL™ for 10 days in the presence of the early growth factor cocktail (omitting the 10 days in late growth factor cocktail). The methodology for cells that were first cultured for 10 days in 3D culture and then subject to the 24-day differentiation protocol is as follows:

Dl-10 (Days 1-10): Cells were cultured in KO SR medium + early growth factor cocktail (Activin A: 50 ng/ml; BMP-4: 50 ng/ml). The medium was change at Dl, 3, 6.

DlO (Day 10): EBs were eluted from 3D BMM marketed as MATRIGEL™ and re-plated on low attachment plates.

D 10-16 (Days 10-16): Step 1 of 24-day protocol. Cells were cultured in basal medium-((DMEM / Fl2, 17 mM Glucose, 2 mM Glutamine, 8 mM HEPES, 2% B27 and Pen / strep). The cells were fed on day 13 and 16 with fresh media without growth factor.

D 16-22 (Days 16-22): Step 2 of 24-day protocol. Cells were cultured in basal medium-((DMEM / F 12, 17 mM Glucose, 2 mM Glutamine, 8 mM HEPES, 2% B27 and Pen / strep) + 20 ng/ml FGF-18, 2 μg/ml heparin. The cells were fed on days 18 and 20 with media plus growth factor top ups.

D22-26 (Days 22-26): Step 3 of 24-day protocol. Cells were cultured in basal medium + 20 ng/ml FGF-18, 2 μg/ml heparin, 10 ng/ml EGF, 4 ng/ml TGF-α, 30 ng/ml IGF-I, 30 ng/ml IGF-II, 10 ng/ml VEGF. The cells were fed fresh medium and growth factors on D24.

D26 (Day 26): Cells re-plated on Fibronectin-coated plates.

D26-30 (Days 26-30): Step 4 of 24-day protocol. Cells were cultured in RPMI medium (11 mM GIc, 5% FBS, 2 mM Glutamax, 8 mM HEPES, Penicillin/Streptomycin) + 10 μM Forskoiin, 40 ng/ml HGF, 200 ng/ml PYY. The cells were fed on Day 28 with fresh media and growth factors.

D30-34 (Days 30-34): Step 5 of 24-day protocol. Cells were cultured in

CMRL medium (5 mM GIc, 5% FBS, 2 mM Glutamax, Penicillin/Streptomycin) + 100 ng/ml Exendin-4, 5 mM Nicotinamide. The cells were fed fresh medium and growth factors on Day 32.

D34: Continue culturing cells in CMRL without growth factors and harvest the cells.

Note: RNA samples were harvested from cells at various points along this process to help evaluate the directed differentiation of the cells. Furthermore, culture medium and factors were regularly changed throughout the differentiation protocol.

Results: As outlined in Example 10 above, following the standard 3D differentiation protocol, embryonic stem cells differentiate to express a high level of *pdx-I* . We additionally directed the differentiation of embryonic stem cells along a pancreatic lineage using the approach outlined in Example 11. During the protocol outlined in Example 11, we harvested samples at various points during the differentiation protocol. Figure 10 summarizes the results for two time points: day 10 (just prior to beginning the 24-day protocol) and day 22 (approximately halfway through the 24-day differentiation protocol).

Figure 1OA and 1OB shows that at day 10 (prior to beginning the 24-day protocol), expression *oï pdx-1* is low and expression of insulin is undetectable. However, as shown in Figure 1OA and 1OB, at 22 days, the expression of *pdx-1* is very high. In fact, expression of *pdx-1* at this point is higher than after completion of the 24-day protocol (see, Figure 9A). As shown in Figure 1OB, at 22 days insulin expression can be detected. However, expression of insulin at this point is not as robust as following the completion of the 24-day protocol (see, Figure 9B). This may indicate that in this experiment, part way through the 24-day differentiation protocol, the cells are continuing to terminally differentiate along the pancreatic lineage. At approximately 22 days, expression of *pdx-1* is still relatively high, perhaps indicating that more cells are capable of but have not yet differentiated to insulin expressing cells.

Further analysis of cells using similar methods at various time points in the 34-day experiment can refine the time and conditions under which cells are directed to partially versus terminally differentiate along a pancreatic lineage.

Example 12: Directed Differentiation of Embryonic **Stem Cells to PdX-I$^+$ Cells Using the 20-Day 3D Differentiation Protocol Mimics Normal Pancreatic Development**

Much of the work aimed at the *in vitro* generation of beta cells from embryonic and adult stem cells has focused on the activation of *the pdx-1* gene due to its tissue-specific expression in early pancreatic progenitors. However, one potential criticism of a reliance on *pdx-1* expression as indicative of differentiation along a pancreatic lineage is that the particular *in vitro* differentiation scheme used may result in spurious activation of *pdx-1*. Such spurious activation *of pdx-1* may not indicate differentiation along a pancreatic lineage, and may not provide a good predictor of cells capable of further differentiation to insulin expressing, glucose responsive cells. Accordingly, we conducted experiments designed to demonstrate whether that *pdx-1* expression during the directed differentiation of embryonic stem cells using our protocols is physiologically relevant to normal pancreatic development.

We performed an expression time course of genes that are normally activated during formation of the definitive endoderm. As shown in Figures 11 and 12, the *in vitro* expression kinetics of these markers roughly followed the expected *in vivo* activation sequence of gene expression during normal beta cell development. The rapid increase in *Brachyury (Tbra)* (Figure 11) accompanies a drop in the pluripotency markers *Oct4* and *nanog* (Figure 12) and suggests the start of gastrulation-related processes and formation of the embryonic germ layers. This is followed by a more sustained expression of the endodermal genes *Hnβ β and Sox17* that precedes the emergence of *pdx-1* -expressing cells beginning on day 15. On day 20, insulin transcripts were detected, with levels increasing with extended differentiation. Interestingly, *Oct4* expression levels were up-regulated at day 20 to around 60% of undifferentiated levels (day 0). This could be due to the emergence of other Oc^-expressing cell types such as primordial germ cells. In addition, a cursory examination of other mature lineage markers such as *albumin, AFP,* and *Cyp3A4,* normally expressed in forming liver cells, revealed an early expression peak followed by a general decrease (Figure 12).

This analysis indicated that the *in vitro* methods of the invention for directing

the differentiation of embryonic stem cells along a pancreatic lineage induced proper gene expression in a temporally regulated fashion that mimics that observed during normal pancreatic and beta islet cell differentiation.

Example 13: Further Optimization of the 20 Day **3D Differentiation Protocol**

As outlined in detail above, we have developed a 20 day differentiation protocol (initiation protocol) that directs the differentiation of embryonic stem cells along a pancreatic lineage. Specifically, we have developed a protocol involving addition of early factors and late factors that direct the differentiation of embryonic stem cells to pdx-l $^{+}$ cells that can further differentiate to insulin^{+} cells. Although the above early/late factor (EF:LF) differentiation protocol is effective, we conducted additional experiments designed to further optimize this methodology.

We evaluated individual early and late factors. These studies indicated that, of the 8 factors (4 EF and 4 LF) proven effective in our initiation protocol, BMP-4 and Activin A were important components of the early growth factor mix. The efficacy of BMP-4 and Activin A was most dramatic when the late factor mix excluded the poly(ADP-ribose) polymerase inhibitor Nicotinamide. These studies indicated that an initial differentiation protocol based on only 2 early factors and only 3 late factors (rather than 4 early factors and 4 late factors) was highly effective and can be can be readily used to promote the directed differentiation of embryonic stem cells to pdx-l+ cells biased to differentiation along a pancreatic lineage. This revised protocol differs from the initial 20 day protocol only in the nature of the early and late factors used. This 2 EF-3 LF protocol included the early factors ActivinA (50 ng/ml) and BMP-4 (50 ng/ml). The cells were cultured in the early factors as previously described from day 0 to day 10. The 2 EF-3 LF protocol included the late factors HGF (50 ng/ml), exendin-4 (10 ng/ml), and β-cellulin (50 ng/ml).

The 2 EF-3 LF protocol represents an improvement over the previous 4 EF-4 LF protocol because it induced robust pdx-1 expression using a cheaper, faster, and simpler procedure. Figures 13-16 summarize the experiments that led to the development of the 2 EF-3 LF protocol.

Without being bound by theory, since BMP-2 and BMP-4 are two

structurally-related growth factors that bind the same cell surface receptor complex, it was anticipated that the combination of the two growth factors was super-saturating in the 4 EF mix and that one factor alone would be sufficient for *Pdx-1* induction. Figure 13A summarizes data showing that an EF growth cocktail lacking BMP-2 induced pdx-1 expression at levels slightly greater than that induced by all four early EFs (compare G2 to *Gl* in Figure 13A). Somewhat surprisingly, the TGF-β- related ligand Nodal, which is an evohitionarily conserved endoderm inducer, had little effect in the EF cocktail. Note, in Figure 13, "AH" indicates the addition of all four of the EF and/or LF used in the initial 4 EF-4 LF 20 day protocol. Duplicate wells for each experimental condition are shown along with Ct value pdx-1/actin of the best performing conditions. Note that the results depicted in Figure 13A represent normalized expression, and the resulted depicted in Figure 13B represent *pdx-1* expression as % actin input.

Additional experiments demonstrating that Nodal does not significantly improve induction *of pdx-1* expression are summarized in Figure 14. We note, however, that Nodal did not appear to have any adverse effect on pancreatic differentiation, and thus could optionally be included in the initiation protocol. Briefly, the 2 EF-3 LF protocol was performed in the presence or absence of 50 ng/ml recombinant Nodal. On day 20, samples were analyzed by Q-PCR for *Pdx-1* expression, and calculated against a Pdx-1 and actin standard curve. Students T-test established the absence of statistical significance ($p>0.05$) between the two experimental conditions.

Without being bound by theory, one explanation for this result is that differentiating human embryonic stem cells express low levels of Cripto, the requisite co-receptor for Nodal, and that in our assay Activin protein mimics the endogenous Nodal signal. This hypothesis prompted the removal of BMP-2 and Nodal from the early factor mix. Further analysis revealed that the remaining two EFs (BMP-4 and Activin A) in combination yielded *pdx-1* expression levels far greater than those initially observed with the 4 EF-4 LF mix (compare G2 and G8 in Figure 13A). The synergistic effect of BMP-4 and Activin is further supported by single factor experiments (compare G8 through GIl in Figure 13B and G2 through G7 in Figure 15).

In addition, many experiments were performed to determine the optimal concentration of BMP-4 and Activin in combination. We repeatedly found that 50 ng/ml BMP-4 and 50 ng/ml Activin together with the LF mix lacking nicotinamide induced robust *Pdx-1* expression in addition to other markers of the endocrine lineage including insulin, glucagon, Pax4 and somatostatin (Figures 15 and 16).

As mentioned above, these experiments indicated that the presence of nicotinamide in the LF mix inhibited *Pdx-1* expression both in context of the 4 EF (compare lanes G2 with G5 and G6 in Figure 13A) and the streamlined 2 EF mixes (compare G3 through G5 in Fig. 13B). Thus, although pdx-1 expression was achieved using the 4 EF-4 LF initiation protocol that included nicotinamide, this factor was omitted in the 2 EF-3 LF initiation protocol.

Further, the importance of LFs HGF, exendin-4, and beta-cellulin on *pdx-1* expression by pancreatic cells was investigated. During differentiation in BMM marketed as MATRIGEL™, low levels of *pdx-1* are first detected as early as 12 days (see, for example, Figures 4B, 11, 12) and increase gradually over time. The growth factors, HGF, exendin-4 and beta-cellulin, have been extensively characterized for their roles in the maturation, proliferation or modulation of the insulin-secreting kinetics of more specialized islet-derived cell populations, and are thus predicted to provide little instructive signaling to the emerging *pdx-1*-expressing pancreatic progenitors. The contribution of each of the 3 LF as well as nicotinamide toward the expression of *pdx-1* on day 20 was assessed. The results are shown in Figure 29, indicating the removal of the 3LF leads to roughly a 3-fold increase in *pdx-1* levels (second column from the left). These data suggest that the combination of the EFs, Activin A and BMP4, is sufficient to launch pancreatic differentiation within the 3D MATRIGEL™ matrix. No *pdx-1* expression was detected either in the no growth factor control or the 3LF alone (administered beginning on day 10). In Figure 29, Ex-4: exendin-4; Nic: nicotinamide; HGF: hepatocyte growth factor; β-cell: beta-cellulin; 4LF: 3LF plus nicotinamide.

In contrast, these experiments indicated that Beta-cellulin was an important component of the LF mix (compare G2 with G5 and G6 in Figure 13A, and G3 through G5 in Figure 13B). Accordingly, Beta-cellulin was retained as a LF in the 2 EF-3 LF initiation protocol.

In summary, these experiments demonstrated that multiple initiation protocols can be used to help direct the differentiation of embryonic stem cells along a pancreatic lineage. Two representative examples of these initiation protocols are the 4 EF-4 LF initiation protocol and the 2 EF-3 LF initiation protocol described herein.

Example 14: Localization of Pdx-1 Expressing Cells Following Directed Differentiation of Embryoid **Bodies via the Initiation Protocol**

Pdx- 1 immunohistochemistry was performed to corroborate the Q-PCR (quantitative-PCR) data presented above and to allow localization and quantification of Pdx- 1-expressing cells within EBs. This procedure (described at length in the Material and Methods) has been repeated on EBs generated from numerous independent differentiation experiments to eliminate the possibility of artifactual staining. Because the initiation protocol experiments (described above in Example 13) relied on the induction *of pdx-1* mRNA expression as assessed by Q-PCR, it was important to exclude the possibility that BMP-2, Nodal and/or nicotinamide negatively effect the production and accumulation of Pdx-1 protein.

Figure 17 shows immunolocalization *of pdx-1* in embryoid bodies differentiated using the initiation protocol. EBs were differentiated for 20 days using either the 4 EF-4 LF protocol (Figures 17A and 17B) or the 2 EF-3 LF protocol (Figures 17C and 17D). Using either initiation protocol, Pdx-1 -positive cell populations were identified within epithelial ribbons that often enclose lumens and are often confined to the EB periphery (arrows in Figures 17A & 17B). Without being bound by theory, the clusters of Pdx-1 -expressing cells may suggest that a subpopulation of EBs support a pancreatic "niche" that underlies the further development of insulin-producing cells.

The expression of *pdx-1* mRNA was further analyzed by *in situ* hybridization (Figure 22), and shown to precisely correlate with Pdx-1 immunohistochemistry. Briefly, Figures 22A and 22B show *pdx-1* expression by *in situ* hybridization after the 20 day initiation protocol (2 EF-3 LF). The results summarized in Figures 22A and 22B indicated that approximately 1/3 of all EBs harvested from an individual culture well express *pdx-1* (darker staining) after 20 days of differentiation. The

higher magnification view shown in Figure 22B demonstrates that *pdx-1* transcripts localized near the periphery of the EB. Figure 22C indicated that EBs cultured in the absence of growth factors fail to express *pdx-1*. Figure 22D summarizes the results of quantitative PCR of parallel cultures of those cells shown in Figures 22A and 22C, and confirms robust *pdx-1* expression in cultures containing growth factors versus those differentiated in the absence of growth factor.

Additionally, light hematoxylin counterstaining was used in combination with section immunoistochemistry to estimate the number of Pdx-1 -expressing cells in a group of EBs. A field of EBs (a total of 65) was broken into smaller regions for manual counting. Total cell number was determined by hematoxylin staining. We found that at day 20 approximately 1% of the cells per section were *pdx-1* positive. In a field of EBs, around 1/3 of the EBs contained *pdx-1* -positive clusters.

The expression pattern of *pdx-1* in the EBs was further investigated by exploring whether Pdx-1 and C-peptide expressions co-localize. The hES3 cells were directed to differentiate using 2EF-3LF initiation protocol, and a simplified maturation protocol using simply Step 4, as further described in Example 15, "Step-4 only maturation." Sections of the resulting EBs were prepared and immuno-stained using antibodies to Pdx-1 and C-peptide, either as a single or double stained immunohistochemistry samples. The results are shown as Figure 28, wherein the top panels show the high magnification images and the bottom panels show lower magnification images, with DAPI-stained nuclei. Figure 28 shows that Pdx-1-positive cells localized in clusters or epithelial ribbons of the differentiated hES3 cells, and there are C-peptide positive cells among them.

Example 15: Maturation Protocols

Five-step maturation

The. initiation protocols provide a straightforward differentiation regime that directs pluripotent embryonic stem cells toward *pdx-1* -expressing pancreatic progenitors. At the end of the 20-day differentiation protocol *(e.g.,* the initiation protocol), *insulin* expression is still relatively low. Thus, we used a maturation protocol to promote further pancreatic differentiation of the biased cells.

Figure 18A provides a schematic representation of a particular combination

of the 2 EF-3 LF initiation protocol plus a maturation protocol. The maturation protocol depicted in Figure 18A may have as many as 5 steps. The use of any combination of these steps will be referred to as a maturation protocol, and reference to the stage/step, the number of days in culture, and/or the particular factors used will distinguish permutations of the maturation protocol.

Briefly, samples differentiated using the 2 EF-3 LF initiation protocol were further differentiated using this 5 step, 24 day maturation protocol. There was a strong correlation between *pdx-1* expression levels at day 20 (Figure 18B, left panel) and the release of C-peptide into the medium after stage 3 of the maturation protocol (day 36, Figure 18B right panel). Note that C-peptide is the stable by-product released during enzymatic processing of proinsulin, and provides a indirect but reliable measure of insulin secretion in our assays. This finding support the ideas (1) that mid-point (day 20) *pdx-1* expression presages the later emergence of more mature cell types and (2) that the combined *in vitro* protocol approximates the developmental cues that guide pancreatic progenitors toward terminally differentiated endocrine cells.

As shown in Figure 18B (right panel), we detected C-peptide release into the medium after a total of 36 days of extended *in vitro* culture. We began a systematic investigation of the relevance of the five steps of the maturation protocol shown schematically in Figure 18A. We first devised a series of simple process-of-elimination experiments aimed at investigating each of the steps. Day *20 pdx-1*-expressing EBs from the 2 EF-3 LF differentiation protocol were directly shunted into only one step of the multi-step protocol for an additional 24 days of differentiation. In these experiment, the baseline concentration of C-peptide detected after 48 hours of culture (again on day 36) was approximately 0.5 ng/ml, as measured in control cultures that have progressed through the first four steps of the maturation protocol - upper schematics - Figures 18A and 19A.

Figure 19B summarizes the results of these experiments. Interestingly, there was little difference in C-peptide release on day 36 for each of the other conditions aside from Stage 4, which showed a 6-fold increase *in* C-peptide release (Figure 19B). There are many unique aspects to this particular stage: most notably is the continued use of RPMI as the base media, and growth on fibronectin-coated dishes.

Step-4 only maturation

The maturation protocol was further refined as shown in the diagrams of Figure 23. The top diagram is the original 5 step maturation protocol. The middle diagram shows using only Step 4 of the 5-step protocol. Twenty-day old MATRIGEL™ EBs were washed with cold PBS to remove excess BMM marketed as MATRIGEL™, and replated onto fibronectin-coated dishes and cultured directly in Step 4 medium for 4 days. Steps 1-3, as well as Step 5 were omitted.

This simplified protocol was developed based on the observation that Step 4 of the multi-step protocol is the key step to the release of C-peptide from differentiating hES3 cells. As shown in Figure 24, various permutations of the maturation steps were investigated. The arrows at the bottom of the graph show time points when the culture medium was either changed or maintained, according to the permutations shown at the top of the graph. In all variations, a spike in C-peptide release is observed in cultures when they transition to Step 4 culture medium. In fact, in cultures that were shunted from the end of the 20-day MATRIGEL™ protocol directly into Step 4 medium (dashed line in the graph of Figure 24), C-peptide release was accelerated, with a measurable amount released by day 27, and was sustained over the 45-day culture period.

Figure 24 also shows that Step 5 consistently made the C-peptide level to decrease, and the culture exposed only to Step 5 medium showed no C-peptide release (black line).

Maturationfactor investigation

The active maturation component of Step 4 was investigated using the simplified Step 4-only protocol, wherein the differentiating EBs were shunted directly into Step 4 culture conditions after the 20-day MATRIGEL™ protocol. Step 4 medium was modified by removing some of the components, and conditioned medium was collected on day 30 from cultures grown in the modified Step 4 medium. As shown in the diagrams of Figure 23, Step 4 medium is based on RPMI and normally contains 5% FBS, 10 μg forskolin, 40 ng/ml HGF, and 200 ng/ml PYY. The result of the experiment is shown in Figure 25. The removal of all additional growth factors and forskolin, while retaining 5% FBS, causes an approximately two-fold decrease in the levels of C-peptide in the culture medium.

Adding back each of the removed components individually or in combination shows that forskolin contributes to the release of C-peptide (see Figure 25A). The analysis of the aggregate of the experimental results shown in Figure 25A comparing all forskolin-containing conditions and all forskolin-lacking conditions reveals a statistically significant increase in C-peptide release when forskolin is present in the medium. Similar effects are seen on insulin expression (Fig. 25B).

In contrast to forskolin, FBS was shown to be dispensable and not an essential component of Step 4 medium. Briefly, hES3 cells were differentiated using the simplified maturation protocol described above. Step 4 medium was prepared using either RPMI following the standard protocol or CMRL, supplemented with FBS, a commercially available serum replacer (SR) which is chemically defined, or not supplemented at all. C-peptide levels were measured on Day 28. As shown in Figure 26A, there was no statistically significant difference in C-peptide release between culture using medium based on RPMI or CMRJL. Further, as shown in Figure 26B, FBS (center bar) can be omitted from the medium (left bar), and more than adequately compensated by the SR (right bar), for the release of C-peptide.

It was further demonstrated that a low concentration of glucose in the medium abolishes the release of C-peptide and significantly decreases insulin mRNA level in the differentiated hES3 cell population, indicating that the differentiated hES3 cell populations contain cell types capable of glucose-stimulated insulin/C-peptide release. Specifically, hES3 cells were differentiated using the simplified maturation protocol described above. On day 30 of the protocol, the culture medium was removed and replaced by either RPMI or DMEM supplemented by 22 mM glucose, RPMI supplemented with 22 mM glucose, or DMEM supplemented by 5 mM glucose. 5 mM glucose mirrors the physiological concentrations of glucose at which insulin is stockpiled in secretory granules. After 48 hours in the replaced media, C-peptide levels in conditioned medium were assessed by ELISA. As shown in Figure 27, there was little or no statistically significant difference between the media containing 22 mM or 11 mM of glucose, but C-peptide levels were unexpectedly reduced to nearly undetectable levels in the conditioned DMEM with 5 mM glucose (Figure 27B). Similarly, *insulin* mRNA expression remains unchanged at 11 mM and 22 mM glucose concentration, but

drops significantly at 5 mM glucose (Figure 27D). In contrast, the expression level of *pdx-1* mRNA is not significantly affected (Figure 27C). The result is consistent with the well-characterized regulation of *insulin* gene expression by glucose, and indicated the presence of differentiated cells that are responsive to glucose stimulated regulation of insulin.

Example 16: Maturation Protocol Following Modified 10 day Initiation Protocol

We cultured EBs in **a** modified initiation protocol that included only the first 10 days EF phase of the protocol, but omitted the second 10 day LF phase of the protocol. EBs cultured in this manner were then subjected to steps 2 through 4 of the maturation protocol. Cells differentiated in this manner robustly secreted C-peptide (as much as 12-14 ng/ml). Note that in Examples 15 and 16, C-peptide release was assayed by ELISA.

The modified initiation protocol was also used in a simplified multi-step maturation protocol shown at the bottom of Figure 23. Briefly, 10-day old MATRIGEL™ EBs were washed free of BMM marketed as MATRIGEL™ with cold PBS and then replated in suspension culture in Step 2 medium for 8 days, followed by Steps 3 (6 days) and 4 (5 days).

When conditioned medium was sampled on days 26 and 29, some amount of C-peptide was consistently detected (See Figure 3**1).**

Example 17: Cellular Characterization **of Pancreatic** Cell Types **Following Differentiation Using the Initiation and/or Maturation Protocols**

We investigated the distribution and number of insulin/C-peptide synthesizing cells per EB at different time points in the differentiation regime. A full-length *insulin* antisense riboprobe was generated for whole-mount *in situ* hybridization (WISH), a technique that permits the identification of individual cells expressing *insulin* mRNA in 3D cultures. Consistent with the low levels of *insulin* detected by Q-PCR at the end of the 20-day 2 EF-3 LF protocol, WISH reveals very few z«sw/zra-expressing cell clusters on the surface of individual EBs at this stage (Figures 2OA and 20B). At this stage, based on inspection of cryo-sectioned material

(Figure 20E), we estimated that each individual cluster contains at most 4 to 5 cells. In contrast, further differentiation using the maturation protocol stimulated the expansion and formation of numerous sharply defined ;«s«//>z-expressing cell clusters (Figure 20C), with some EBs showing intense surface staining in enlarged patches (higher magnification in Figure 20D) while others show little to no staining. Interestingly, we found that the majority of small to medium-sized EBs contained at least a few *insuli* «-expressing clusters that are roughly confined to one region of an EB, stiggesting both the formation of a pancreatic niche and an EB size for which there exists a propensity for the further maturation of //?.«//w-producing islet-like cells.

These data are further corroborated by C-peptide section immunohistochemistry (Figure 21). Briefly, Figure 2 1 shows paraffin sections of day 45 EBs following immunocytochemistry with C-peptide. C-peptide-positive cells were often distributed at the EB periphery in a manner similar to *insulin*-expressing cell clusters, but were sometimes located in more interior regions (Figure 2 IB-F). Figure 2 1A shows, as a positive control, C-peptide immunolocalization in an adult mouse islet.

Double labeling experiments in day 45 EBs revealed co-localization of insulin and C-peptide in a punctuate pattern that is strikingly reminiscent of secretory granule storage in endogenous beta cells. This provides strong evidence that the insulin-expressing cells induced using the combination of a initiation and maturation protocol stockpile insulin and C-peptide peptides for glucose-stimulated secretion, thus mimicking a biochemical property of endogenous, normal beta cells.

Similar co-localization can be seen when the EBs subject to the simplified protocol without Steps 1 and 5 were immunofluorescent stained. Figure 32 shows high magnification (top panels) and low magnification (bottom panels) images of paraffin-embedded and sectioned EBs harvested on Day 26 from the simplified protocol. At this stage, most insulin positive cells were also reactive for C-peptide, which is the by-product of insulin synthesis. Nuclei are shown by the staining by DAPI.

Figure 33 shows additional evidence of differentiation through the simplified protocol without standard protocol Steps (1) and (5). When EBs subject to the

simplified protocol were examined for Nkx6.I and Pdx- 1 immunoreactivity, the immunofluorescent stain showed efficient formation of cells belonging to the beta-cell lineage (left panels: top, high magnification; bottom, low magnification). These cells were largely confined to epithelial ribbons or tubes that enclose luminal spaces. In addition, *pdx-1*-positive cells clusters are also reactive for glucose transporter Glut2, as can be seen in the right panels. Nuclei are shown by the staining by DAPI.

Unless otherwise specified, the following methods were used for the experiments outlined in Examples 12-17.

Human ES cell culture: hES cells were cultured according to standard procedures. The generation of embryoid bodies (EBs), and the culture of the cells within the 3D MATRIGEL™ during the first 20 days were as follows:

Part 1: Collagenase treatment of hES 1-6

1. Discard central button and cystic parts of the hES colonies under dissecting microscope using glass pipette pump suction. Wash hES plate (PlOO tissue culture plate) with PBS twice. Note: This suction step can be done either before or after collagenase treatment.

2. Add 3 ml of lmg/ml collagenase IV to the hES plate and keep the plate in 37°C CO_2 incubator for 8 min.

3. Aspirate collagenase solution.

4. Wash with 10 ml of PBS once. Aspirate PBS gently. Do not disturb colonies.

5. Add 10 ml of the RPMI/20SR to the plate.

6. Mechanically dissect the hES plate using 2-ml Pasteur pipette.

7. Use cell scraper to dislodge all the dissected pieces.

8. Pipette up and down for a few times to resuspend pellets.

9. Transfer pellets to a 15-ml falcon tube.

10. Wash plate once more with RPMI/20SR to *get all* the residue pellets from the plate.

11. Transfer pellets to the 15-ml falcon tube.

12. Spin down the pellets at 1500rpm, 4min.

13. Aspirate supernatant medium.

Part 2: Make MATRIGEL™ EB

Early **factor** stage

Day O

1. Pre-chill medium on ice or 4^0C. Pre-chill 2 ml, 5 ml and 10 ml pipettes at -20^0C.

2. Prepare 1:6 MATRIGEL™ medium *(e.g.* 1 ml liquefied BMM marketed as MATRIGEL™ + 5 ml RPMI/20SR) using pre-chilled pipettes. Total volume is according to 2 ml for each well.

3. Resuspend hES pellets in MATRIGEL™ medium.

4. Add 2 ml of hES pellet suspension in one well of Ultra-low plate. Note: One PlOO plate of hES colonies can be split into 3 to 5 wells depending on the confluence.

5. For the growth factor group, growth factor cocktail (100 ng Activin A + 100 ng BMP-4 per well) can either be added in the MATRIGEL™ medium before pellet resuspension procedure or directly to the well immediately after cell suspension is plated into the Ultra-low plate.

6. Keep Ultra-low plate in 37°C cell incubator. MATRIGEL™ medium will gel after several hours. And hES pellets will round up and form embedded EBs after overnight.

Day 3 **and** *6*

7. Top up with RPMI/20SR (0.5 ml) + EF (same amount as 1st dose = lOOng Activin A + lOOng BMP-4 per well).

Late factor stage

DlO

1. Remove medium with 1-ml blue pipette under dissecting microscope very slowly and carefully. Do not suck away MATRIGEL™ and EBs.

2. Add 3 ml fresh RPMI/20SR to each well and equilibrate for 1 hr in cell incubator.

3. Remove 3 ml medium with 1-ml blue pipette under dissecting microscope very slowly and carefully.

4. Top up with RPMI/20SR (0.5 ml) + LF (100 ng HGF + 20 ng Exendin-4 + 100 ng B-cellulin per well).

D13 and 16

5. Top up with RPMI/20SR (0.5 ml) + LF (lOOng HGF + 20ng Exendin-4 + lOOng B-cellulin per well).

D20

6. Collect all MATRJGEL™ EB using 2-mI Pasteur pipette to a 15-ml falcon tube. Top up with pre-chilled PBS to 12 ml. Mix well and leave tubes on ice for lOmin.

7. Spin down MATRIGEL™ EB at 2500rpm, 4min in the cool room.

8. Remove supernatant medium very carefully with ImI blue pipette. Do not disturb MATRIGEL™ EB.

9. Top up with pre-chilied PBS to 12 ml.

10. Spin down MATRIGEL™ EB at 2500rpm, 4min in the cool room.

11. Transfer MATRIGEL™ EB to a 1.5-ml EPPENDORF tube using 2 ml Pasteur pipette.

For immunohistochemistry study —**paraffin section**

1) Spin gently to get EB pellets. Note the spin needs to be gentle and brief to keep good morphology of EBs.

2) Fix EBs with 4% PFA (paraformaldyhyde) for 2-4 hours at room temperature in 1.5-ml EPPENDORF tube

3) Wash with PBS 3 times (5 min. each)

4) Gently spin down EB pellets

5) Melt 1.5% agarose at 60^0C

6) Resuspend EBs with 50-100 µl of melted agarose carefully and quickly

7) Leave EPPENDORFF™ tube on ice for a few minutes to solidify agarose

8) Samples are ready for paraffin embedding

For immunohistochemistry study —cryosection

1) Spin gently to get EB pellets. Note the spin needs to be gentle and brief to keep good morphology of EBs.

2) Make mold

3) Resuspend EBs carefully with 50-100 µl of freezing solution carefully. Do not produce bubble.

4) Add EB solution to mold carefully. Do not produce bubble.

5) Freeze samples in liquid nitrogen.

6) Stock samples at -70^{0}C or -20°C

7) Samples are ready for cryosection. Note: the slides need to start from fixation step

For RNA extraction

Use high speed to get EB pellet. (Refer to Qiagen kit protocol or Trizol protocol.)

Note:

1. Some EBs might not be lysed very well by RTL lysis buffer even with vigorous pipetting. In this case, RLT lysate or Trizol lysate is better to be kept at -70°C for at least a few hours before extraction. This freeze and thaw cycle appears to help lysing.

2. Trizol method gives more than double amount higher of the final RNA yield. However, DNA shredding step needs to be thorough and DNAase treatment step is preferred to be 1 hour in order to get rid of the genomic DNA cleanly.

Culture conditions beyond the initial 20 day protocol, in which *pdx-I*-expressing cells were directed toward more mature insulin producing cell populations, will be referred to as the maturation procedure(s). Day 20 EBs were removed from their RPMI- MATRIGEL™ cultures by centrifugation and cold PBS washes, and transferred to a basal medium (DMEM/F12, 17 mM glucose, 2 mM glutamax, 8 mM HEPES, 2% B27 supplement, and 1 **x** pen/strep) thought to promote the recovery and survival of beta cells. On day 26, the cells were cultured for a further 6 days in the same basal media supplemented with 20 ng/ml FGF-18 and 2 μg/ml heparin. Between days 32 and 36, the cells were cultured in basal media supplemented with FGF-18 (20ng/ml), heparin (2 μg/ml), EGF (10 ng/ml), TGFα (4 ng/ml), IGF-I (30 ng/ml), IGF-II (30 ng/ml) and VEGF (10 ng/ml). On day 36, the EBs were plated onto fibronectin-coated (10 μg/ml) tissue culture plates in a new media mix (RPMI + glucose (11 mM), FBS (5%), glutamax (2 mM), HEPES (8 mM), 1 x pen/strep, forskolin (10 μM), HGF (40 ng/ml), and PYY (200 ng/ml)). The final stage (days 40-44) consisted of culture in a CMRL-based media (supplemented with glucose (5 mM), glutamax (2 mM), pen/strep (Ix), exendin-4 (100 ng/ml) and nicotinamide (5 mM). A more detailed ESI 3D MATRIGEL™ Protocol + Differentiation Protocol is as follows:

Cells: hES cells (preferentially hES3) are digested by Collagenase (2 x plOO confluent dishes to one 6 well dish).

Plating: Cells arc first distributed to a 6-well low-attachment plate in BMM marketed as MATRIGEL™ to start the 3D culture, according to QC "MATRIGEL™ EB Protocol" in RPMI-SR. After 20 days, cells are re-plated on low-attachment dishes; after another 16 days cells are plated on Fibronectin-coated standard 6-well plates.

Sampling: (A) collect RNA from one well at day 20 of the 3D MATRIGEL™ protocol; (B) collect supernatant before and 24 h after each medium change from start of the multi-step maturation protocol (10 µl are required for one ELISA well, take 100 µl medium and keep at -20), (C) collect RNA from wells at day 45.

Media used & Growth Factor Treatments:

1. 10-dav 3D MATRIGEL™ Protocol with EFs = Dl-DlO (initiation protocol - EF phase)

2 ml/well 1/6 MATRIGEL™ in RPMI-SR medium: RPMI, 20% SR, Penicillin/Streptomycin 1 x 50 ng/ml each Activin A, BMP-2, BMP-4, Nodal Medium feeding at D3 and D6 (add 500 µl RPMI/20SR with 2 x GF concentrations)

Day 10: Medium Change; remove medium with 1 ml pipette under the dissecting microscope very slow and carefully. Do not suck away MATRIGEL™/cells. Give 3 ml of RPMI-SR, equilibrate 1 hr in the incubator, then remove again and give new medium with late GFs.

2. 10-dav 3D MATRIGEL™ Protocol with LFs = D10-D20 (LF phase)

2 ml/well 1/6 MATRIGEL™ in RPMI-SR medium: RPMI, 20% SR, Penicillin/Streptomycin 1 x 50 ng/ml Betacellulin, 50 ng/ml HGF, 20 nM or 10 ng/mL Exendin-4, 10 mM Nicotinamide Medium feeding at D13 and D16 (add 500 µl with 2 x GF concentrations)

D2 1 - replating on low attachment plates: spin cells at 600 **g** for 4 min (swing bucket); carefully remove BMM marketed as MATRIGEL™ with a pipette; Resuspend in new medium. Collect one RNA sample (= + control for 3D).

3. 6-dav step 1 Multi-step Maturation Protocol = D20-D26

2 ml/well basal medium only, no GFs: DMEM/F12, 17 mM GIc, 2 mM

Glutamax, 8 mM HEPES, 2% B27, Penicillin/Streptomycin 1 x Medium change at D23 (spin cells at 600 g for 4 min in swing bucket, give new medium).

4. 6-day step 2 Multi-step Maturation Protocol = D26-D32

2 ml/well basal medium + 20 ng/ml FGF-18, 2 μg/ml heparin Medium change at D29 (spin cells at 600 g for 4 min in swing bucket, give new medium).

5. 4-day step 3 Multi-step Maturation Protocol = D32-D36

2 ml/well basal medium + 20 ng/ml FGF-18, 2 μg/ml heparin, 10 ng/ml EGF, 4 ng/ml TGF-α, 30 ng/ml IGF-I, 30 ng/ml IGF-II, 10 ng/ml VEGF Medium change at D34 (spin cells at 600 g for 4 min in swing bucket, give new medium)

D36 - replating on Fibronectin: coat 6-well plates for 1 hr with 10 μg/ml Fibronectin in PBS, wash 2 x with RPMI-SR, plate cells in new medium on coated plates.

6. 4-day step 4 Multi-step Maturation Protocol = D36-D40

2 ml/well new RPMI medium: RPMI + 11 mM GIc, 5% FBS, 2 mM Glutamax, 8 mM HEPES, Penicillin/Streptomycin 1 *x*, 10 μM Forskolin, 40 ng/ml HGF, 200 ng/ml PYY.

Medium change at D38 (spin the portion of cells that is not attached to Fibronectin at 600 g for 4 min in swing bucket, while doing that give 1 ml of new medium on the attached cells to avoid drying out, give recovered cells in new medium).

7. 4-day step 5 Multi-step Maturation Protocol = D40-44

2 ml/well new CMRL medium + 5 mM GIc, 5% FBS, 2 mM Glutamax, Penicillin/Streptomycin 100 ng/ml Exendin-4, 5 mM Nicotinamide. Medium change at D42 (as explained for 6).

Tissue processing and embedding: Embryoid bodies were isolated from the 3D MATRIGEL™ by transferring the entire culture to a 15 ml FALCON™ tube and chilling on ice. The EBs were pelleted by centrifugation in a swinging-bucket rotor (1500 rpm). The cell pellet was then rinsed twice in ice-cold PBS and centrifuged in a similar fashion to remove residual BMM marketed as MATRIGEL™. EBs were fixed in 4% paraformaldehyde for 4 hours, rinsed twice in PBS, and then stored at 4°C in 70% ethanol. EBs were then embedded in paraffin using standard dehydration / clearing / paraffin-embedding protocols with a Leica TP1020

automated tissue processor (2 X 1 hr each in 70%, 95%, 100% ethanol followed by 2 x 1 hr in xylenes and 4 x 1 hr in paraffin). Sections were cut at 5 μm, and stored long-term at 4^0C for eventual immunohistochemistry or *in situ* hybridizations. Pancreas tissue was similarly prepared but with extended fixation.

Immunohistochemistry: Tmmunostaining was performed according to standard protocols using the vectorshield colorometric (DAB) detection kit. Slides were first de-waxed 2 x 10 min in xylene, then hydrated in a standard ethanol series (100%, 95%, 90%, 70% ethanol, then 2 times in PBS). For antigen retrieval, slides were slowly heated in 10 mM sodium citrate solution to 95°C (approximately 9 minutes on the defrost setting of a conventional microwave). The slides were then slowly cooled to room temperature (about 30 minutes) and rinsed twice in PBS. Endogenous peroxidase activity was quenched with a 20 min incubation in 3% H_2O_2 followed by 2 rinses in PBS. The slides were blocked for 1 hr in PBS containing 1% BSA and 5% serum (corresponding to the species from which the secondary antibody was derived). The primary antibodies were diluted to the following concentrations: rabbit anti-Pdx-1 (1:30,000), guinea pig anti Pdx-1 (1:2000), and goat anti-Pdx-1 (1:40,000). For C-peptide immunostaining, rabbit anti-human C-peptide (Linco Research, lot#81(lP)) antibodies were used at a 1:5000 dilution. Slides were incubated with the diluted primary antibody overnight. The next day, the slides were rinsed twice in PBS, and a biotinylated goat-anti-rabbit secondary antibody was applied for 1 hr at room temperature. After 3 rinses in PBS, the ABC mixture (Vectashields) was placed on the sections (prepared by mixing 20 μl/ml reagent A and 20 μl/ml reagent B in PBS) for 30 min then rinsed away with 3 x PBS washes followed by addition of the DAB substrate (Vector Laboratories, SK-4100). The color reaction was monitored closely by microscopy, and was stopped by dipping the slide in water and then rinsing once in 1 X PBS. The sections were then dehydrated and cleared in xylenes (Sigma) using standard protocols before mounting in a xylene-based permanent mounting media (DPX neutral mounting media, Sigma).

RNA isolation, cDNA synthesis, **and quantitative PCR:** Total RNA from hESC or EBs at various stages of differentiation was isolated using the Qiagen RNeasy kit or prepared using Trizol reagent (Invitrogen) according to the

manufacturer's instructions. RNA was and quantified by UV absorption. 1 to 5 µg of RNA was DNAse I treated and converted to cDNA using M-MuLV reverse transcriptase (New England Biolabs) using oligo-dT or random hexamer primers according to the manufacturer's instructions. Quantitative PCR was performed according to the manufacturer's instructions using a BioRad iCycler with approximately 50 ng cDNA per reaction containing 250 nM of each primer and Ix SYBR green master mix (Bio-Rad) and analyzed by Bio-RAD thermocycler

The following conditions were used:

Quantitative PCR reaction :

2 X master mix	15 µl
primers (each)	100 nM
template	25 ng
H_2O	to 30 µl

40 Cycles of:

30 s at 95°C - denaturation

30 s at 55^{0}C - annealing

60 s at 72^{0}C - extension

Plate setup:

For each unknown sample, include:

3 replicates from the same RT reaction.

1 sample that was treated identically except that the RT enzyme was not included in the RT reaction (no RT control). This is to control for genomic or other contamination (such as the previous PCR reaction still in your pipette nozzle). This should produce no signal before Ct = 35.

For the genes on the following list, include 2 replicates each of 2 positive controls. Use lOul of each positive control per reaction. These are prepared such that the amount on the label is contained in 10 µl of solution.

Gene	Range				Slope*
	quantity	Ct	quantity	Ct	
Oct4	10 pg	15	1 fg	29	-1.7307
Nanog	10 pg	13	1 fg	28	-1.5994

Sox17	10 pg	15	100 ag	32	-1.5147
glut-2	10 pg	16	100 ag	34	-1.4216
HNF3b	10 pg	13.5	100 ag	31	-1.5167
α Fetal Protein	10 pg	14.5	100 ag	34	-1.7245
Albumin	10 pg	14	1 fg	30	-1.6
Thans-thyretin	10 pg	20	100 ag	37	- 1.7245
Cyt P450-3A4	10 pg	15	100 ag	33	-1.5576
Trp dioxygenase	10 pg	13	1 fg	25	-1.1193
CK19	10 pg	21	1 fg	31	-1.2
GGT	10 pg	20	10 fg	34	-1.8669
Pdx-1	10 pg	15	100 ag	30	-1.2703
Insulin	10 pg	10	1 fg	25	-1.5603
Brachyury	10 pg	15	100 fg	26	-2.4465
tbx6	10 pg	15	10 fg	30	-2.1469
sox-1	10 pg	21	10 fg	34	-1.8892
Neurofilament, HC	10 pg	15	1 fg	29	-1.4708
β-actin	10 pg	15	1 fg	34	-1.3897
GAPDH	10 pg	12	1 fg	28	-1.3978

Analysis **of gene expression.**

1) Cut and paste (or export) data to an excel spreadsheet.

2) Graph Ct (Y) vs. quantity (X) of standard curves. Convert X axis to log scale, Log(X). Get equation Y = slope * Log(X) + Y intercept. Under "options" include equation and R^2 value on chart.

3) Determine input of unknown sample using the following equation (this can be prepared in the excel spreadsheet):

4) Input = 10^((Ct value - y intercept)/slope).

5) Repeat this procedure for the internal control of gene expression (GAPDH or β-actin)

6) Calculate the average input value of the three replicates for the gene and the internal control gene.

7) Calculate normalized expression of your gene using the following

equation: normalized expression = Input value average of gene/Input value average of internal control gene.

8) Calculate the relative expression of your gene: Set one experimental condition as the comparison sample (untreated or time = 0, for example). Relative expression = Normalized expression of unknown/normalized expression of comparison.

Quality controls

(a) The slope of the curve you generate from the positive controls should be roughly equal to the slope in the standard curve chart below. If not, prepare fresh primer mix and standard curve reagents. To compare slopes, the trendlines must be generated in the same way. The slopes generated here use quantity in femtograms.

(b) The Ct value of the unknown sample should be between the Ct values given by the positive controls. Otherwise, the results are outside the sensitive range of the assay.

Standard curves were generated by plotting the log (concentration in fg) of series of 100-fold dilutions of the target PCR amplicon (a range of 10^4 fg to 1 fg per reaction) versus the corresponding threshold Ct value. Normalized expression was determined by the following equation: Normalized expression = (input of target gene/input of actin control), where input is calculated as the inverse log of ((the threshold cycle (Ct value) —Y-intercept of standard curve)/slope of standard curve).

The following specific primer pairs were used:

Gene name	Forward primer	Reverse primer
AFP	GTAGCGCTGCAAACAATGAA (SEQ ID NO: 1)	TCCAACAGGCCTGAGAAATC (SEQ ID NO: 2)
Oct4	GGCAACCTGGAGAATTTGTT (SEQ ID NO: 3)	GCCGGTTACAGAACCACACT (SEQ ID NO: 4)
Nanog	TACCTCAGCCTCCAGCAGAT (SEQ ID NO: 5)	TGCGTCACACCATTGCTATT (SEQ ID NO: 6)
HNF3-b	GGAGCGGTGAAGATGGAA (SEQ ID NO: 7)	TACGTGTTCATGCCGTTCAT (SEQ ID NO: 8)
Sox17	CAGAATCCAGACCTGCACAA	CTCTGCCTCCTCCACGAA

	(SEQ ID NO: 9)	(SEQ ID NO: 10)
Glut-2	CATGTCAGTGGGACTTGTGC (SEQ ID NO: 11)	CTGGCCCAATTTCAAAGAAG (SEQ ID NO: 12)
albumin	TCAGCTCTGGAAGTCGATGA (SEQ ID NO: 13)	TTCACGAGCTCAACAAGTGC (SEQ ID NO: 14)
Pdx-1	CCTTTCCCATGGATGAAGTC (SEQ ID NO: 15)	GGAACTCCTTCTCCAGCTCTA (SEQ ID NO: 16)
Insulin	GGGGAACGAGGCTTCTTCTA (SEQ ID NO: 17)	CACAATGCCACGCTTCTG (SEQ ID NO: 18)
Glucagon	CCAAGATTTTGTGCAGTGGT (SEQ ID NO: 19)	GGTAAAGGTCCCTTCAGCAT (SEQ ID NO: 20)
somatostatin	CCCAGACTCCGTCAGTTTCT (SEQ ID NO: 21)	ATCATTCTCCGTCTGGTTGG (SEQ ID NO: 22)
Pax4	TCTCCTCCATCAACCGAGTC (SEQ ID NO: 23)	GAGCCACTATGGGGAGTGAG (SEQ ID NO: 24)
Cyp450-3A4	ACCGTGACCCAAAGTACTGG (SEQ ID NO: 25)	GTTTCTGGGTCCACTTCCAA (SEQ ID NO: 26)
brachyrury	AATTGGTCCAGCCTTGGAAT (SEQ ID NO: 27)	CGTTGCTCACAGACCACAG (SEQ ID NO: 28)

C-peptide ELISA: C-peptide concentrations in conditioned medium were determined by ELISA with commercially available anti-C-peptide-coated plates (LINCO research) according to the manufacturer's recommendations.

Riboprobe synthesis: Template plasmids were linearized with either *Hind* III (antisense) or *BamHl* (sense), and then purified (Qiagen Qiaquick spin columns). 1.5 µg of recovered DNA template was used in a synthesis reaction containing the corresponding RNA polymerase (Promega), nucleotides (DIG RNA labeling mix- 10 mM ATP, CTP, GTP (each), 6.5 mM UTP, 3.5 mM DIG-1 1-UTP), 1 X Transcription buffer (IX), and 25 Units RNAsin (Promega). RNA probes were precipitated by the addition of 0.1 vol. of 4M LiCl and 2.5 volumes of 100% ethanol and incubated at -20 ^{0}C overnight. Samples were then centrifuged at 13,000 rpm for 30 min. at 4°C. The supernatant was discarded and the pellet was washed with 70% ethanol:30% DEPC-H2O and re-centrifuged for 15 min. The supernatant was removed, and the pelle/‘ α/lowed to dry. Probes were typically resuspended in 50 µl

of DEPC- H_2O, aliquoted and stored at -80°C.

in situ Hybridization: EBs were rehydrated in a descending series of methanohPBT washes (75%, 50%, and 25% methanol). PBT is prepared from 1 X PBS-DEPC plus 0.1% Tween-20. EBs were incubated for 1 hr in 6% H_2O_2 in PBT and then rinsed 3 times in PBT. EBs were then treated for 5 mins in 10 μg/ml proteinase K in PBT, washed in 2 mg/ml glycine in PBT (5 mins), followed by 2 additional PBT washes (5 mins each), and re-fixed in 4% paraformaldehyde (Sigma)/0.2% glutaraldehyde/PBT for 20 min at room temperature. EBs were incubated in hybridization solution (50% deionized formamide (Ambion), 5 X SSC, 0.1% Tween-20 (Sigma), 0.1% SDS (Sigma), 50 μg/ml heparin (Sigma), 50 μg/ml yeast tRNA, 60 mM citric acid in DEPC-treated H_2O) for at least two hours at 70^{0}C. Hybridization solution was then replaced with fresh solution containing 50-100 ng DIG-labeled riboprobe and incubated on a rocking platform overnight at 70^{0}C. The following day, EBs were washed for 5 mins in Solution I (50% formamide, 5 X SSC, 60 mM citric acid, and 1% SDS in DEPC-treated H_2O) prewarmed to 70^{0}C. EBs were washed twice more in solution I for 30 mins each at 70^{0}C, and once in solution I for 30 mins at 65°C. EBs were then washed 3 X in Solution II (50% formamide, 2 X SSC, 24 mM citric acid, 0.2% SDS, and 0.1% Tween-20 in DEPC-treated H_2O) for 30 mins each at 65^{0}C. EBs were cooled to room temperature and washed 3 X (5 min each) in maleic acid buffer (100 mM maleic acid (Sigma), 170 mM NaCl (Sigma), 0.1% Tween-20, and 2 mM levamisole, pH 7.5 with NaOH)(MAB). EBs were then incubated for 90 min at room temperature in blocking solution (MAB, 2% Boehringer Mannheim blocking reagent, 10% heat inactivated sheep serum). Blocking solution was then replaced with fresh blocking solution containing preadsorbed alkaline phosphatase-conjugated anti-digoxygenin antibody (Roche) and incubated on a rocking platform overnight at 4°C. 2μl antibody per ml was preadsorbed by incubation in MAB with 2% Boehringer Mannheim blocking reagent, 1% heat inactivated sheep serum, and 3 mg human EB acetone powder at 4°C for 90 mins and centrifuged for 10 mins at 4°C. EBs were washed 3 X (5 mins each) and 5 X (60-90 mins each) in MAB at room temperature and then incubated overnight in MAB at 4°C. The following day, EBs were washed 3 X (10 mins each) in AP buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween-20,

and 2 mM levamisole). EBs were then incubated in NTMT alkaline phosphatase staining buffer (AP buffer with 3.5 µl/ml NBT and 3.5 µl/ml BCIP) or alkaline phosphatase staining solution (BM Purple, Boehringer Mannheim) until the precipitation reaction was complete. Reaction was arrested with the addition of stop solution (2 mM EDTA in PBT).

Example 18: Three Dimensional Matrices Supplemented with Collagen IV Support Differentiation of Human Embryonic **Stem Cells to Pancreatic Progenitors**

We have found that culturing human ESCs (hESC) embedded within a gel of growth factor reduced (GFR) formulation of BMM marketed as GFR MATRIGEL™ in combination with soluble growth factors enhances the production of cells expressing markers of the pancreatic lineage by day 20 of culture. While not wishing to be bound by theory, we attribute this effect to an increased number of pancreatic progenitor cells expressing markers of the definitive endoderm (DE) by ten days of culture. We show that BMM marketed as GFR MATRIGEL™ is sufficient to stimulate an increase in the number of cells differentiating towards DE. Culturing hESC in matrices of alginate, SPH *(e.g.,* marketed as PURAMATRIX™), or PLGA in combination with soluble collagen IV leads to expression of markers of the pancreatic lineage to levels equal to or greater than the levels seen in cultures grown in BMM marketed as GFR MATRIGEL™. Without being bound by theory, we hypothesize that the properties of BMM marketed as GFR MATRIGEL™ which encourage differentiation of hESC to cells expressing markers of the DE include a hydrated and porous three dimensional support structure coupled with the adhesive and signaling properties of collagen IV. This provides a suitable growth environment for the production of three dimensional tissue structure that limits the formation of the visceral endoderm while stimulating formation of the definitive endoderm. Furthermore, matrices composed of alginate, SPH *(e.g.,* marketed as PURAMATRTX™), and PLGA supplemented with soluble collagen IV encourage the differentiation of hESC clusters towards DE cells with the potential to mature into cells of the pancreatic lineage.

For the purpose of illustration, the examplary results described in this

Example and Example 19 use the directed differentiation protocol described in Figure 23 (lowest scheme, *e.g.*, EF phase only, followed by modified steps 2-4 of the standard 24-day maturation protocol). However, any other protocols can also be used with the subject composition and methods.

Results

BMM marketed as GFR MATRIGEL™ promotes the differentiation of cells expressing markers of the definitive endoderm.

Hallmarks of early mammalian embryogenesis are the formation of the primitive endoderm and epiblast (primitive ectoderm) from cells of the inner cell mass of the blastocyst, and the formation of the primary germ layers from cells of the epiblast through a process of gastrulation. The primitive endoderm further matures into the parietal endoderm, which gives rise to the parietal yolk sac, and the visceral endoderm, which plays a role in patterning of the epiblast and gives rise to the visceral yolk sac. During gastrulation, epiblast cells migrate through a transient structure known as the primitive streak while undergoing a transition from epithelial to mesenchymal cell types (EMT). Cells emerging from this structure are fated to become cells of the mesoderm and definitive endoderm in a pattern predictable by temporal sequence and location along the embryo axis. Of these germ layers, cells of the definitive endoderm give rise to organs of the gut including the pancreas (13).

Human Embryonic stem cells, derived from cells of the inner cell mass of pre-implantation blastocysts, undergo differentiation events reminiscent of primitive endoderm / epiblast formation and gastrulation if allowed to grow as three dimensional aggregates (embryoid bodies - EBs) in suspension culture in the absence of a feeder layer (14). Small clusters of the hESC cell line hES3 were grown as EBs in the presence of serum replacement (SR) growth inducer either free-floating or embedded in semi-solid BMM marketed as GFR MATRIGEL™. RNA was harvested at the indicated time points and analyzed by quantitative RT-PCR for genes which mark developing endodermal tissue in mammalian embryogenesis. By day 3, all cultures showed enhanced expression of genes which mark the formation of a primitive streak and node, gastrulation events that preview formation of definitive endodermal tissues (Figure 34). *Brachyury iTbrd)* is expressed by the entire primitive streak and marks the formation of the axial mesendoderm anterior to

the node (Figure 34A) (15,16). Nodal, first expressed in the epiblast and VE, is restricted to the region that will become the primitive streak and node at gastrulation (Figure 34B)(17, 18). Goosecoid and sonic hedgehog (SHH) mark the node and mesendoderm, while cerberus is expressed by the node and the early definitive endoderm (Figures 34C-E) (17-22). Following this gastrulation event, genes which are expressed in the definitive endoderm and its descendents are enriched in cultures grown in BMM marketed as GFR MATRIGEL™ when compared to free-floating EBs (Figure 35). Soxl7 expression marks the definitive endoderm and primitive foregut, and precedes expression of vHNFl (Figures 35A & 35B)$_5$ whose expression is limited to liver and pancreatic buds which emerge from the epithlium of the DE (18, 22-26). Hex and GATA6 (Figures 35C & 35D) are expressed in the foregut endoderm and the gut endothelium, respectively (23, 27, 29). HNF3beta (Figure 35E) is more widely expressed, appearing first in the anterior primitive streak, followed by expression in the axial mesendoderm and definitive endoderm, the node, and it's descendents the notochord and floor plate (28, 30).

Differentiation of early definitive endoderm and visceral endoderm appear similar (13, 20). Expression of the genes cerberus, Soxl7, Hex, HNF3beta are detected in the anterior portion of the visceral endoderm while vHNFl and GATA6 are expressed more broadly across the parietal and visceral endoderm (29, 31). H19, however, is uniquely expressed in the visceral endoderm during early gastrulation (28). Cultures grown in BMM marketed as GFR MATRIGEL™ have drastically reduced levels of H19 expression, indicating that Soxl7, HNF3beta, Hex, GATA6, and vHNFl are likely marking definitive endoderm in these cultures (Figure 35F). The lack of H19 expression indicates that along with the stimulation of definitive endoderm, the formation of visceral endodermal tissues is repressed in BMM marketed as MATRIGEL™ grown cultures. Alpha feto protein (AFP) and Trans-thyretin (TTR) (Figures 35G & 35H) are expressed in both the visceral endoderm and the prenatal liver (8, 33). The drastic upregulation of these genes only in cultures with presumptive DE one week following the gastrulation event indicates the emergence of prenatal liver. Differentiation of visceral endoderm was not simply delayed in BMM marketed as GFR MATRIGEL™ cultures as expression of Oct4 and Nanog, both markers of ESC pluripotency, are more reduced in these cultures

(Figures 351 & 35J) (34, 35).

In contrast to cultures grown embedded within a three dimensional gel of BMM marketed as GFR MATRIGEL™, cultures grown on a plastic surface coated with a thin layer of BMM marketed as GFR MATRIGEL™ had gene expression patterns similar to free-floating EBs. A three dimensional support, or exposure in three dimensions to adhesive substrates, is therefore a critical component of BMM marketed as GFR MATRIGEL™ DE induction (Figures 34 and 35).

BMM marketed as GFR MATRIGEL™, but not complete BMM marketed as MATRIGEL™, supports differentiation ofhES towards pancreatic lineage.

An early marker of commitment to the pancreatic cell fate is the transcription factor *pdx-I. pdx-J* is expressed concomitantly with specification of pancreatic tissue budding from the foregut and in the endocrine, exocrine, and ductal compartments of the early pancreas. In the adult, *pdx-1* expression is limited to beta cells and a subset of gamma cells and is required for maintenance of adult beta cell function (21, 36, 37). When coupled with a directed differentiation protocol (such as those indicated in Figure 23, lowest scheme), cultures grown embedded in BMM marketed as GFR MATRIGEL™ express significant amounts of *pdx-1* by day 20 of differentiation (Figure 36, lane 2). Cultures grown embedded in complete BMM marketed as MATRIGEL™, however, express significantly less suggesting that at least one growth factor present in complete BMM marketed as MATRIGEL™ suppresses the formation of DE or pancreatic lineages (Figure 36, lane 1).

Alginate, SPH (e.g., marketed as PURAMATRIX™), and PLGA support the differentiation of cells expressing markers of the pancreatic lineage.

Compared to BMM marketed as GFR MATRIGEL™, cultures embedded in SPH *{e.g.,* marketed as PURAMATRIX™) express high levels of *pdx-1* when hydrogel is supplemented with collagen IV or ECM gel, but not collagen I, fibronectin, laminin-1, or heparan sulfate proteoglygcan (Figure 37, lanes 2-7). Cultures embedded in SPH (e.g., marketed as PURAMATRIX™) without any supplement fail to express high levels of *pdx-1* (Figure 37, lane 1). Similarly, cultures grown embedded in alginate express high levels *of pdx-1* when supplmented with collagen IV or ECM gel (Figure 37, lanes 8-10). Cultures grown embedded in PLGA matrix also express high levels of *pdx-1* when supplemented with ECM gel

(Figure 37, lanes 11, 12). Cultures embedded in methylcellulose, or agarose failed to express high levels of *pdx-1* even with supplementation with extracellular matrix. Gels formed entirely from ECM gel, collagen I, or collagen IV also failed to produce high expression of *pdx-1* (Figure 37, lanes 19-21). The failure of methylcellulose, agarose, and extracellular matrix gels was due to poor consistency or integrity of the gel during the course of the experiments.

Methods

Cell culture. hES3 cells were removed from feeder layer by incubation with lmg/ml collagenase for 8 minutes at 37°C. Collagenase solution was aspirated and cells were rinsed with PBS (Gibco). Cystic areas of hES colonies were aspirated and the remaining cells were disrupted into small clusters using a pipette tip. Pellets were transferred to a 15 ml tube (FALCON™) and pelleted by centrifugation (2,500 rpm for 5 minutes). Supernatant was aspirated and cell clumps were transferred to dishes containing mouse embryonic fibroblasts in complete hES media composed of DMEM supplemented with 1 X non-essential amino acids, 1 X Penn/Strep, 2 mM L-Glutamine, 1 X insulin-transferrin-Selenium, beta-mercaptoethanol (all from Gibco), and 20% FBS (Hyclone). Media was changed daily.

Seeding of cells in 3D GFR MATRIGEL™. A comparison of protocols used for seeding cells into 3D GFR MATRIGEL™, alginate, and SPH *(e.g.,* marketed as PURAMATRIX™) is shown in Figure 38.

Growth factor reduced and complete BMM marketed as MATRIGEL™ (Becton Dickinson) was chilled on ice at 4^0C overnight. BMM marketed as MATRIGEL™ was diluted 1:6 in cold RPMI supplemented with 20% serum replacement and penecillin/streptomycin using pre-chilled pipettes. hES3 cells collected using collagenase were resuspended in 2 mis of MATRIGEL™/RPMI solution and plated on 3 cm ultra-low attachment dishes. Cells were incubated in 37°C tissue culture incubators until gel formed (approximately 30 minutes).

Seeding of cells on 2D GFR MATRIGEL™. Plates coated by BMM marketed as MATRIGEL™ were prepared by thawing BMM marketed as MATRIGEL™ overnight at 4^0C. Thawed BMM marketed as MATRIGEL™ was diluted 1:8 in ice cold RPMI medium and added to tissue culture treated dishes. Excess fluid was allowed to evaporate from the dishes for two hours at 37^0C. hES3

cells collected using collagenase were resuspended in 2 mis of RPMI supplemented with 20% serum replacement and plated on the dishes coated by BMM marketed as GFR MATRIGEL™.

Seeding of cells into synthetic peptide hydrogel or SPH (e.g., marketed as PURAMA TRIX™).* Undifferentiated hES3 cells were isolated using collagenase. The cells were pelleted by centrifugation (2,500 rpm for 5 minutes) and resuspended in a 10% sucrose solution two times. 700 µl aliquots of sucrose/cell suspensions were mixed with 700 µl of SPH *{e.g.,* marketed as PURAMATRIX™) (Becton Dickinson) with and without indicated matrix supplements and added in 15 µl drops to wells of six-well ultra-low attachment dishes (Costar) containing 2 mis of RPMI media supplemented with 20% Serum Replacement. The media was replaced three times over the first 30 minutes after plating. Supplements to the Puromatrix were EHS laminin-1, human plasma fibronectin, EHS collagen IV (Sigma C-0543), heparan sulfate proteoglycan (all from Sigma), bovine dermal collagen I (Cohesion Technologies) and BMM marketed as GFR MATRIGEL™ (Becton Dickinson).

Seeding of cells into alginate. A 1% solution of alginate (Sigma) was prepared in water and filter sterilized. The indicated matrices were added to 2 ml aliquots of alginate solution. After collagenase digestion and washing, hES3 were resuspended in the alginate/matrix solutions. This solution was added dropwise in 15 µl drops to wells of a 6-well ultra-low attachment tissue culture dish (Costar) containing 4 mis of 0.1 M $CaCl_2$ and allowed to polymerize for 5 mins. $CaCl_2$ solution was changed for RPMI/20%SR. Droplets were washed an additional 2 X with RPMI/20%SR.

Formation of extra-cellular matrix gels. 1.8 mis of a solution containing 1 mg/ml of purified matrix components were mixed on ice with 200 µl of 10 X PBS. Cell pellets derived from collagenase passaging were re-suspended in this mixture on ice. 76 µl of 0.8 M $NaHCO_3/H_2O$ was added to catalyze gel formation. Mixture was transferred to a well of a six-well ultra-low attachment plate (Costar) and incubated at 37^0C for 30 mins. Media containing growth factors was overlayed on top of the gel.

Seeding cells into agarose. 1 ml of a 0.4% agarose solution of was prepared in 1 X PBS. When the solution reached approximately $4O^0$C, but had not solidified,

it was mixed with 1 ml of RPMl with or without extracellular matrix supplement. Cell pellets derived from collagenase passaging was added and the mixture was quickly transferred to a well of a six-well ultra-low attachment plate (Costar) and incubated at 37^{0}C for 30 mins. Media containing growth factors was overlayed on top of the gel.

Seeding cells into methylcellulose. A 1:1 dilution of methylcellulose (Methocult, Stem Cell Technologies) was prepared at 4°C in RPMI/20%SR and supplemented with 100 μg/ml of either collagen IV (Sigma C-0543), collagen I, or laminin/entactin. A pellet of hES3 cells generated by the collagenase method was resuspended in this solution and transferred to a well of a six-well ultra-low attachment plate (Costar) and incubated at 37°C for 30 mins. Media containing growth factors was overlayed on top of the gel.

Seeding cells into ECM gel. ECM gel (Sigma) was allowed to thaw at 4^{0}C overnight. Undiluted solution was mixed with hES cell pellet harvested by collgenase method and transferred to wells of a six-well ultra-low attachment plate (Costar) and incubated at 37°C for 30 mins. Media containing growth factors was overlayed on top of the gel.

Seeding cells into PLGA matrix. Crystals of NaCl were sifted to produce particles no larger than 30 μm in diameter. A layer of sifted salt approximately 2 mm deep was added to wells of 6-wells dishes coated with aluminum foil. Polylactic-co-glycolic acid (PLGA, Polysciences, Inc, Warrington, PA) was dissolved in a sealed glass bottle in chloroform to a concentration of *2%*. The solution was added to the dish until the salt in each well was completely saturated. The chloroform was allowed to evaporate overnight in a fume hood. The salt was leeched away by repeatedly soaking in DI water for a period of one day. The matrix was sterilized by soaking in 70% ethanol for a period of one hour. The ethanol was rinsed away in DI water and the plate was allowed to dry overnight. The matrix was then removed from the aluminum-coated dish and transferred to a plastic cell culture dish. hES3 cells collected using collagenase were resuspended in 2 mis of RPMI supplemented with 20% serum replacement and plated on a disc of PLGA. A second layer of PLGA was overlayed on top to trap the cells inside the PLGA sandwich.

Differentiation. hES3 cells seeded in BMM marketed as MATRIGEL ™,

synthetic peptide hydrogel *(e.g.*, those marketed as PURAMATRIX™), extra¬ cellular matrix gel, agarose, or methylcellulose were incubated in RPMI media supplemented with 20% serum replacement, penicillin/streptomycin, 50 ng/ml Activin A, and 50 ng/ml BMP-4. Media was changed every 2-3 days for ten days. Cells were then incubated with RPMI media supplemented with 20% serum replacement, penicillin/streptomycin, HGF, exendin4, and betacellulin. Media was changed every 2-3 days for ten days.

RT-PCR. RNA was isolated using the Qiagen RNeasy kit, treated with on-filter DNase and quantified by UV absorption. 1 μg of RNA was converted to cDNA using M-MuLV reverse transcriptase (New England Biolabs) using oligo-dT or random hexamer primers and following manufacturers instructions. Quantitative PCR was performed with 75 ng of each RT reaction, 250 nM of each primer and 1 x SYBR green master mix (Bio-Rad) and analyzed by Bio-RAD iCycler thermocycler. Normalized expression was determined by the following equation: Normalized expression = (input of target gene/input of actin control), where input is calculated as the inverse log of ((the threshold cycle (Ct) —Y-intercept of standard curve)/slope of standard curve). Each standard curve was produced by analyzing a series of 100-fold dilutions of the PCR product made using the identical quantitative PCR from a range of 10 pg to **1** fg per reaction.

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Example 19 Three Dimensional Matrices Supplemented with Collagen IV Support Continued Differentiation of Human ES Cells to Pancreatic Progenitors with Similar Phenotypes as Those Differentiated in BMM Marketed as GFR MATRIGEL™

Examples above demonstrate that hESC clusters embedded in SPH *(e.g.,* marketed as PURAMATRIX™) supplemented with collagen IV, alginate supplemented with collagen IV, or PLGA supplemented with collagen IV and grown according to the culture regime (such as those indicated in Figure 23, lowest scheme) can differentiate towards cells of the endocrine pancreas lineage, which, at day 20, express *pdx-1* (see Figure 37).

This Example shows that cultures embedded in SPH *(e.g.,* marketed as PURAMATRIX™) supplemented with collagen IV and cultured according to the culture regime included in Figure 18A continue to differentiate similarly to cultures embedded in GFR MATRIGEL™. At day 37, both cultures express *pdx-1* and *insulin* mRNA (Figure 39A), and secrete C-peptide into the culture media (Figure 39B). Furthermore, clusters of cells differentiated under these conditions are visually similar. At day 10, EBS generated by the two methods have a similar gross phenotype. Antibody-stained cryosections of EBs differentiated under the two conditions both contain *pdx-1*-positive and insulin-positive cell clusters that are indistinguishable from each other. This data demonstrates that cultures embedded *in* SPH *(e.g.,* marketed as PURAMATRIX™) supplemented with collagen IV are equally as capable as cultures embedded in BMM marketed as GFR MATRIGEL™ to differentiate into mature cells of the endocrine pancreas lineage.

Figure 39 shows that cultures of hESC embedded in SPH *(e.g.,* marketed as PURAMATRIX™) supplemented with collagen IV and differentiated following a culture regime included in Figure 18A express both *pdx-1* and *insulin* mRNA at levels at least as high as BMM marketed as GFR MATRIGEL™-embedded cultures at 37 days of differentiation (Figure 39A). Cultures of hESC embedded in SPH *(e.g.,* marketed as PURAMATRIX™) supplemented with collagen IV and differentiated following a culture regime included in Figure 18A secrete C-peptide into the culture media at levels at least as high as those differentiated in BMM marketed as GFR MATRIGEL™ at 37 days of differentiation (Figure 39B).

Figure 40 shows that hESC cultures embedded in SPH *(e.g.,* marketed as PURAMATRIX™) supplemented with collagen IV and differentiated according to the culture regime included in Figure 18A resemble cultures embedded in BMM marketed as GFR MATRIGEL™. At day 10 of differentiation, EBs formed embedded in SPH *(e.g.,* marketed as PURAMATRIX™) supplemented with collagen IV or EBs formed embedded in BMM marketed as GFR MATRIGEL™ share a similar morphology when visualized by bright field microscopy (Figures 4OA & 40B). At day 34 of differentiation, clusters of cells which contain nuclear Pdx-1 protein and which are double positive for nuclear Pdx-1 and cytoplasmic C-peptide, are evident in both SPH *(e.g.*, marketed as PURAM ATRIX™)-embedded and BMM marketed as GFR MATRIGEL™-embedded cultures.

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and PCT Publication Nos. WO 04/072251; WO 00/70021; WO 02/10347; and WO 02/061033.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS:

1. A composition comprising a chemically defined three-dimensional polymer matrix supplemented with a collagen IV polypeptide, wherein said composition supports directed differentiation of embryonic stem (ES) cells to one or more desired endodermal lineage cells.

2. The composition of claim 1, wherein said polymer matrix comprises one or more synthetic or naturally occurring polymers.

3. The composition of claim 1, wherein said polymer matrix is biodegradable.

4. The composition of claim 3, wherein said polymer matrix comprises poly(lactic acid) (PLA), poly(glycolic acid) (PGA), PLA-PGA co-polymer (PLGA), poly(anhydride), poly(hydroxy acid), poly(orthoester), poly(propylfumarate), poly(caprolactone), polyamide, polyamino acid, polyacetal, biodegradable polycyanoacrylate, biodegradable polyurethane, or polysaccharide.

5. The composition of claim 1, wherein said polymer matrix is non¬ biodegradable.

6. The composition of claim 5, wherein said polymer matrix comprises polypyrrole, polyaniline, polythiophene, polystyrene, polyester, non-biodegradable polyurethane, polyurea, poly(ethylene vinyl acetate), polypropylene, polymethacrylate, polyethylene, polycarbonate, or poly(ethylene oxide).

7. The composition of claim 1, wherein said polymer matrix is a gel.

8. The composition of claim 7, wherein said gel comprises methylcellulose, agarose, or alginate.

9. The composition of claim 1, wherein said polymer matrix is a copolymer, a mixture, or an adduct of one or more of: poly(lactic acid) (PLA), poly(glycolic acid) (PGA), PLA-PGA co-polymer (PLGA), poly(anhydride), poly(hydroxy acid), poly(orthoester), poly(pro ρylfumarate), poly(caprolactone), polyamide, polyamino acid, polyacetal, biodegradable

polycyanoacrylate, biodegradable polyurethane, polysaccharide, polypyrrole, polyaniline, polythiophene, polystyrene, polyester, non-biodegradable polyurethane, polyurea, poly(ethylene vinyl acetate), polypropylene, polymethacrylate, polyethylene, polycarbonate, poly(ethylene oxide), methylcellulose, agarose, peptide hydrogel, or alginate.

10. The composition of claim 1, wherein said polymer matrix is peptide hydrogel, alginate, or PLGA.

11. The composition of claim 1, wherein said polymer matrix excludes one or more components of a solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma.

12. The composition of claim 11, wherein said solubilized basement membrane preparation is a basement membrane matrix or a growth factor-reduced (GFR) basement membrane matrix.

13. The composition of claim 1, wherein said polymer matrix consists essentially of a mixture of:

(1) peptide hydrogel, alginate, or PLGA, and

(2) purified collagen IV.

14. The composition of claim 1, wherein said collagen IV is from mouse or human.

15. The composition of claim 1, wherein said polymer matrix is formed as a particle, a sponge, a tube, or a sphere.

16. The composition of claim 1, further comprising embryonic stem (ES) cells and/or one or more endodermal lineage cells in various stages of differentiation from said ES cells.

17. The composition of claim 1 or 16, wherein said ES cells are human or mouse cells.

18. The composition of claim 1, wherein said composition provides a suitable growth environment for the production of three-dimensional tissue structure that limits the formation of the visceral endoderm while stimulating the formation of the definitive endoderm.

19. The composition of claim 18, wherein cells of the definitive endoderm express one or more of: *cerberus, soxJ7, vHNFl, hex, GATA6, HNF3*β, and substantially lack expression of the visceral endodermal marker *HI9*.

20. The composition of claim 1, wherein said endodermal lineage cells include pancreatic lineage cells that *arepdxl** and/or secrete C-peptide extracellularly.

21. The composition of claim 1, wherein said composition produces at least about 50% as many of a desired differentiated endodermal lineage cells as compared to that produced by solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, or a growth factor-reduced (GFR) version thereof.

22. The composition of claim 1, wherein said composition produces at least about twice as many of a desired differentiated endodermal lineage cells as compared to that produced by solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, or a growth factor-reduced (GFR) version thereof.

23. The composition of claim 1, wherein said composition produces a desired differentiated endodermal lineage cells with similar or substantially the same phenotypes as are produced using solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, or a growth factor-reduced (GFR) version thereof.

24. The composition of claim 23, wherein said phenotypes include one or more of: expression of one or both *of pdxl* and *insulin,* secretion of C-peptide extracellularly, or morphology.

25. The composition of claim 1, wherein said composition produces pancreatic lineage cells that (1) express *both pdxl* and *insulin,* and/or (2) secrete C-peptide, at levels at least as high as, or higher than that produced by cells obtained using solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, or a growth factor-reduced (GFR) version thereof.

26. The composition of claim 1, wherein said composition produces a population of differentiated endodermal lineage cells that are at least about 25% homogeneous without purification with respect to at least one differentiation marker.

27. A method for directed differentiation of an increased number of embryonic stem (ES) cells to endodermal cell types, comprising culturing the ES cells in a composition of any of claims 1-26.

28. The method of claim 27, wherein the ES cells are differentiated into pancreatic lineage cells that express pancreatic lineage marker(s), and/or exhibit a pancreatic lineage function.

29. The method of claim 28, further comprising contacting the ES cells for a sufficient period of time with a sufficient amount of one or more early factors (EFs) selected from activin A, BMP-2, BMP-4, or nodal.

30. The method of claim 29, wherein the pancreatic lineage cells express Pdx-1 and/or insulin, and/or are responsive to glucose, and/or secret C-peptide.

31. The method of claim 30, wherein the pancreatic lineage cells are insulin-producing cells.

32. The method of claim 29, wherein the ES cells are cultured as embryoid bodies (EBs) plated directly onto a support matrix.

33. The method of claim 32, wherein the EBs are cultured in a support matrix only while the EBs are in contact with the EFs.

34. The method of claim 32, wherein the EBs are generated from ES cells grown on mouse embryonic feeder (MEF) or other feeder layers, or from ES cells grown under feeder-free conditions.

35. The method of claim 29, wherein the ES cells are human ES cells.

36. The method of claim 29, wherein the ES cells are mouse ES cells.

37. The method of claim 29, wherein the ES cells are partially or terminally differentiated into the pancreatic lineage.

38. The method of claim 29, wherein the ES cells are contacted with the EFs for about 10 days.

39. The method of claim 29, wherein the EFs comprise activin A and BMP-4.

40. The method of claim 29, wherein the EFs comprise about 50 ng/mL of activin A and about 50 ng/mL of BMP-4.

41. The method of claim 29, further comprising contacting the ES cells, subsequent to contacting the ES cells with the EFs, with a sufficient amount of one or more late factors (LFs) for a second sufficient period of time.

42. The method of claim 41, wherein the one or more LFs are HGF, exendin-4, betacellulin, and nicotinamide.

43. The method of claim 41, wherein the one or more LFs include about 50 ng/mL of HGF, about 10 ng/mL of exendin-4, and about 50 ng/mL of β-cellulin.

44. The method of claim 41, wherein the ES cells are contacted with the EFs for about 10 days, and are subsequently contacted with the LFs for about 10 days.

45. The method of claim 44, wherein the EFs comprise about 50 ng/mL of activin A and about 50 ng/mL of BMP-4, and the LFs include about 50 ng/mL of HGF, about 10 ng/mL of exendin-4, and about 50 ng/mL of β-cellulin.

46. The method of claim 41, further comprising contacting the ES cells, subsequent to the initiation protocol and during a maturation protocol, consecutively with:

(1) a basal medium for about 6 days;

(2) about 20 ng/ml FGF-18, and about 2 μg/ml heparin in the basal medium for about 5-6 days;

(3) about 20 ng/ml FGF-18, about 2 μg/ml heparin, about 10 ng/ml EGF, about 4 ng/ml TGF-α, about 30 ng/ml IGF-I, about 30 ng/ml IGF-II, and about 10 ng/ml VEGF in the basal medium for about 4-5 days;

(4) about 10 μM forskolin, about 40 ng/ml HGF, and about 200 ng/ml PYY for about 3-4 days; and,

(5) about 100 ng/ml Exendin-4, and about 5 mM nicotinamide for about 3-4 days.

47. The method of claim 46, wherein the ES cells are not dissociated by dispase between steps (1) and (2).

48. The method of claim 46, wherein fetal bovine serum (FBS), if any, in the medium is replaced with a chemically defined serum replacer (SR).

49. The method of claim 41, further comprising contacting the ES cells, subsequent to the EF and LF treatment, and during a maturation protocol, with about 10 μM forskolin, about 40 ng/ml HGF_5 and about 200 ng/ml PYY for about 3-4 days.

50. The method of claim 49, wherein the ES cells are grown on fibronectin-coated tissue culture surfaces during the maturation protocol.

51. The method of claim 49, wherein the differentiated cells release C-peptide and/or are responsive to glucose stimulation.

52. The method of claim 49, wherein FBS, if any, in the medium is replaced with a chemically defined serum replacer (SR).

53. The method of claim 29, further comprising contacting the ES cells, subsequent to contacting the ES cells with the EFs and during a maturation protocol, consecutively with:

(1) about 20 ng/ml FGF-18, and about 2 μg/ml heparin in a basal medium for about 8 days;

(2) about 20 ng/ml FGF-18, about 2 μg/ml heparin, about 10 ng/ml EGF, about 4 ng/ml TGFα, about 30 ng/ml IGF-I, about 30 ng/ml IGF-II, and about 10 ng/ml VEGF in the basal medium for about 6 days; and

(3) about 10 μM forskolin, about 40 ng/ml HGF, and about 200 ng/ml PYY for about 5 days.

54. The method of claim 53, wherein the differentiated cells release C-peptide.

55. The method of claim 53, wherein step (1) lasts 6 days, steps (2) and (3) last 4 days each.

56. Differentiated pancreatic lineage cells or cell cultures obtained through the method of claim 27.

57. Differentiated pancreatic lineage cells or cell cultures obtained through the method of claim 30.

58. The differentiated pancreatic lineage cells or cell cultures of claim 57, which are partially differentiated.

59. The differentiated pancreatic lineage cells or cell cultures of claim 57, which are terminally differentiated.

60. The differentiated pancreatic lineage cells or cell cultures of claim 57, which mimic the function, in whole or in part, of insulin-producing cells.

61. Differentiated pancreatic lineage cells or cell cultures obtained through the method of claim 41.

62. Differentiated pancreatic lineage cells or cell cultures obtained through the method of claim 46.

63. Differentiated pancreatic lineage cells or cell cultures obtained through the method of claim 49.

64. Differentiated pancreatic lineage cells or cell cultures obtained through the method of claim 53.

65. A method for the treatment or prophylaxis, in an individual, of diseases, injuries, or conditions of the pancreas characterized by impaired pancreatic function, comprising administering to the individual the differentiated pancreatic lineage cells of claim 56.

66. The method of claim 65, wherein the impaired pancreatic function includes impaired ability to properly regulate glucose metabolism in an affected individual.

67. The method of claim 65, wherein the condition is type I or type II diabetes.

68. The method of claim 65, which is in conjunction with one or more additional therapies effective for the treatment or prophylaxis of the diseases, injuries, or conditions.

			Relative Expression			
Lineage	Marker		Day 0	Day 5	Day 9	Trend
Stem Cell	Oct4	M	1 (1)	0.44 (0.42)	0.11 (0.11)	↓
		C	1 (1)	0.90 (0.20)	0.11 (0.05)	
	Nanog	M	1 (1)	0.31 (0.11)	0.09 (0.03)	↓
		C	1 (1)	0.39 (0.09)	0.07 (0.02)	
Endoderm	AFP	M	1 (1)	445 (478)	7383 (1722)	↑
		C	1 (1)	3565 (2195)	9410 (6208)	
	Prox1	M	1 (1)	6.5 (5.1)	19.0 (13.5)	↑
		C	1 (1)	12.6 (12.6)	14.9 (10.2)	
	Albumin	M	NA (1)	1.0* (416)	6.3* (923)	↑
		C	NA (1)	5.1* (823)	24.3* (1218)	
	Glut2	M	NA (NA)	NA (NA)	NA (+)	↑
		C	NA (NA)	NA (NA)	+ (+)	
Ectoderm	Sox1	M	NA (1)	1.0* (3.4)	4.8* (4.4)	↑
		C	NA (1)	1.5* (2.7)	3.4* (3.6)	
	Nestin	M	1 (1)	13.9 (24.3)	13.0 (32.0)	↑
		C	1 (1)	25.1 (26.0)	15.5 (14.9)	

Figure 1

Figure 2

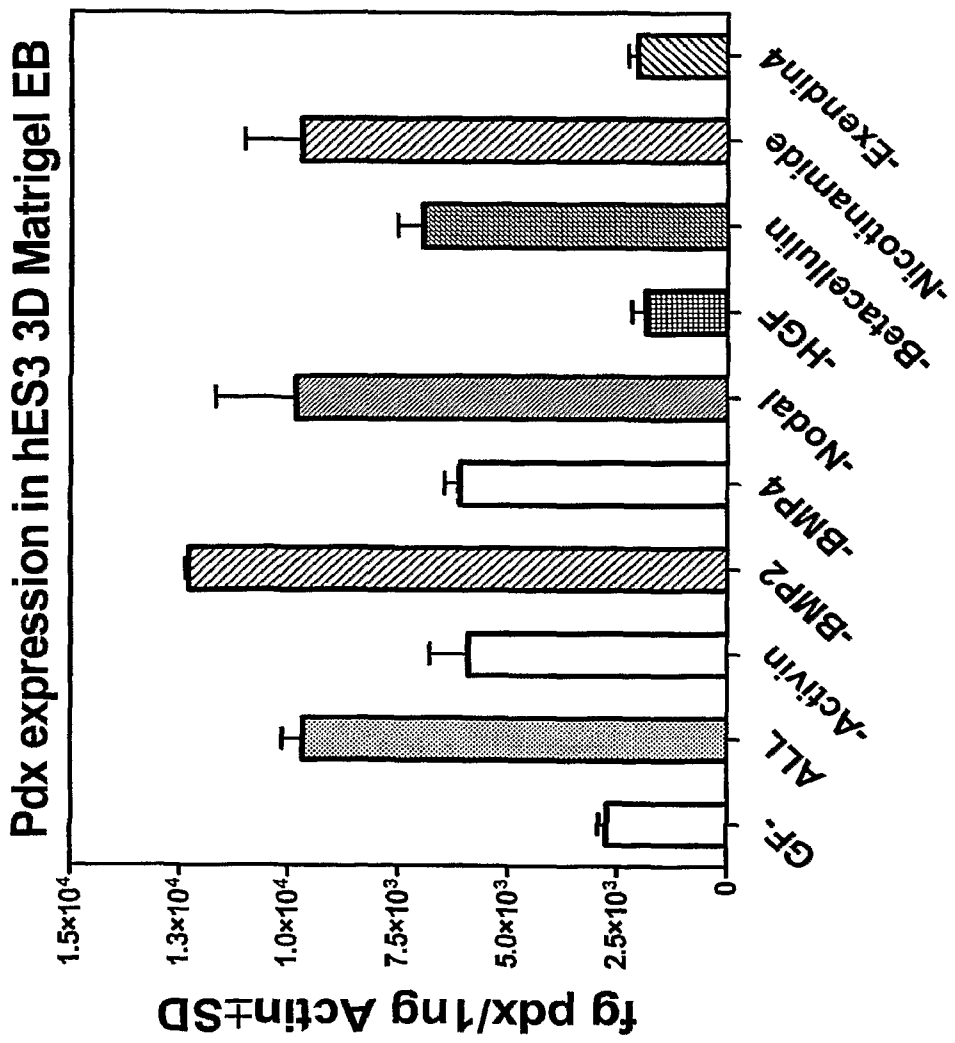
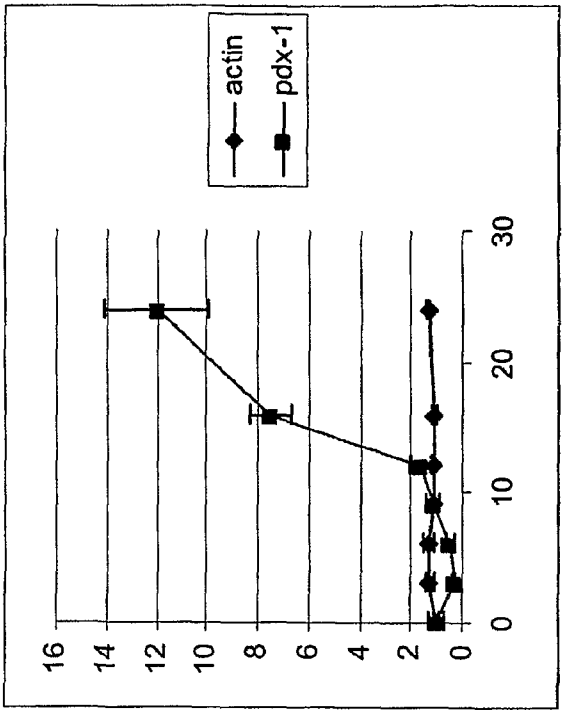

Pdx expression in hES3 3D Matrigel EB
fg pdx/1ng Actin±SD
1.5×10^4
1.3×10^4
1.0×10^4
7.5×10^3
5.0×10^3
2.5×10^3
0
GF-
ALL
-Activin
-BMP2
-BMP4
-Nodal
-HGF
-Betacellulin
-Nicotinamide
-Exendin4

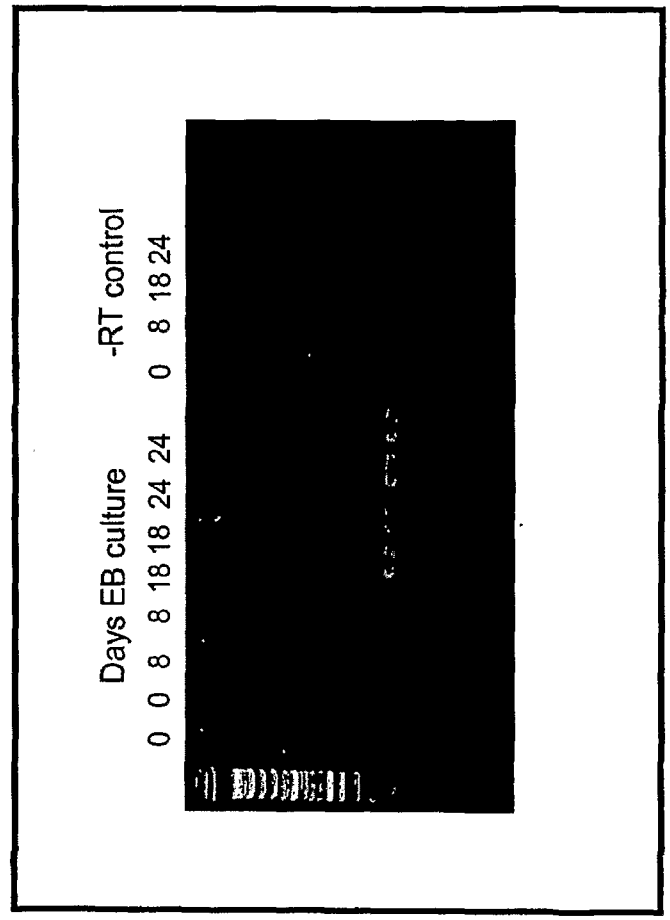
Figure 3



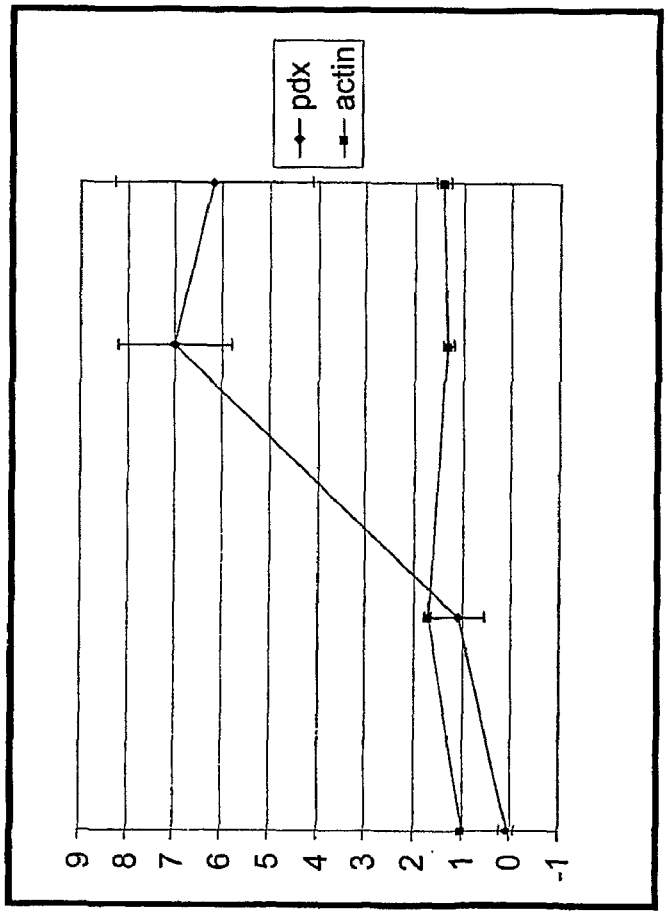
(b)

(a)

Figure 4


Days EB culture
0 0 8 8 18 18 24 24
-RT control
0 8 18 24

(b)


pdx
actin
9
8
7
6
5
4
3
2
1
0
-1

(a)

Figure 5

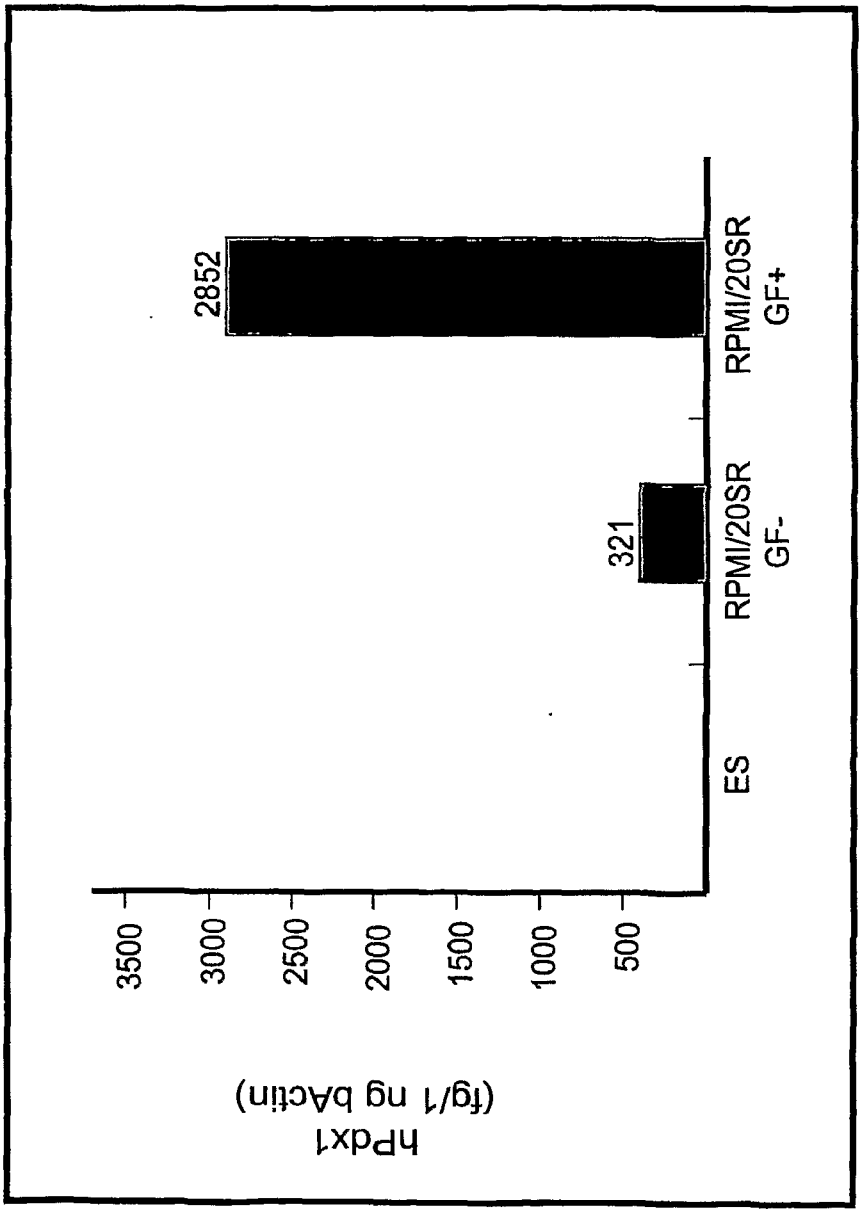
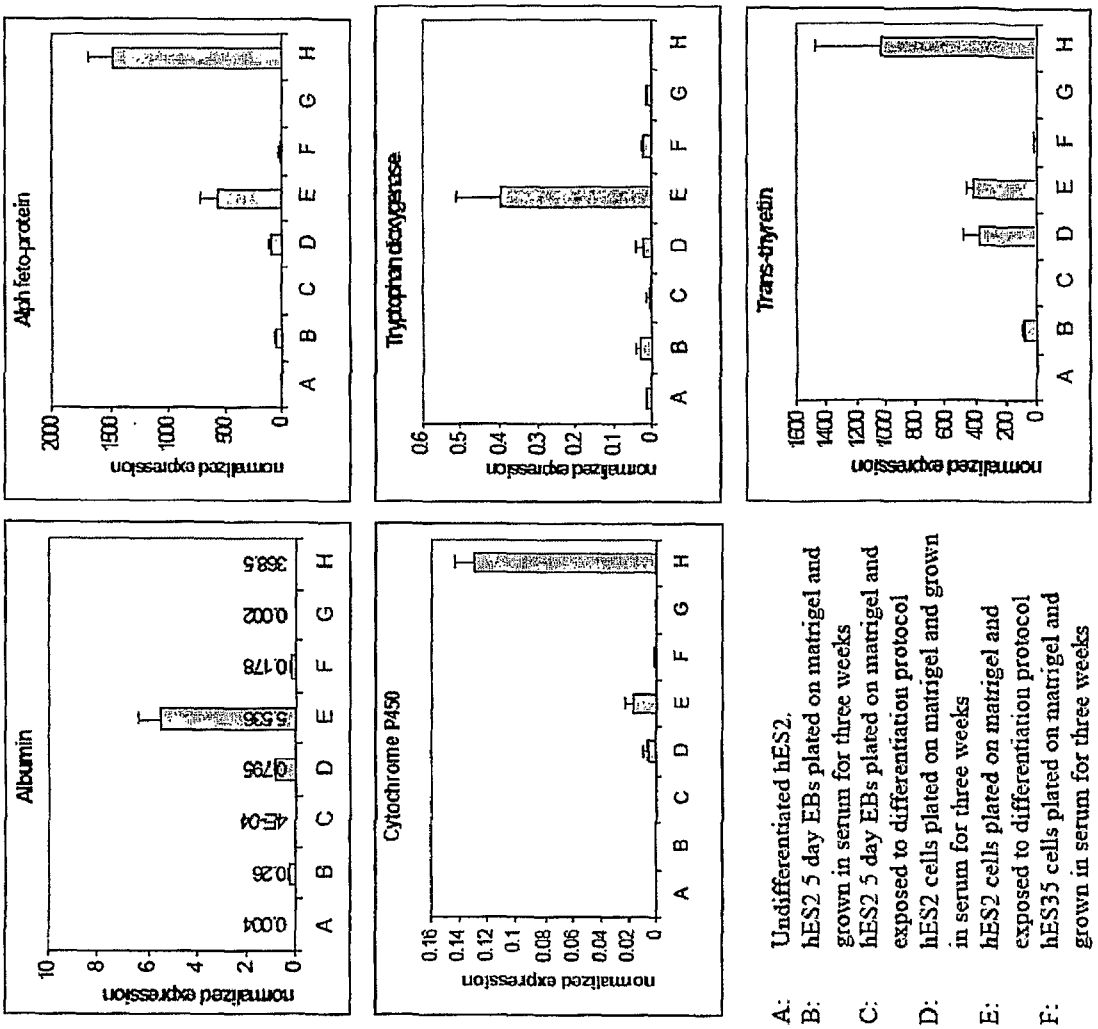
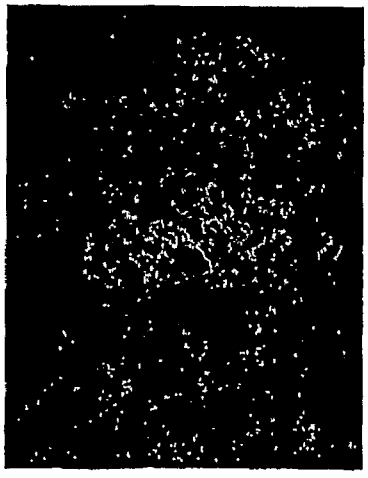

hPdx1
(fg/1 ng bActin)
3500
3000
2500
2000
1500
1000
500
2852
321
ES
RPMI/20SR
GF-
RPMI/20SR
GF+

Figure 6



(b)

A: Undifferentiated hES2.
B: hES2 5 day EBs plated on matrigel and grown in serum for three weeks
C: hES2 5 day EBs plated on matrigel and exposed to differentiation protocol
D: hES2 cells plated on matrigel and grown in serum for three weeks
E: hES2 cells plated on matrigel and exposed to differentiation protocol
F: hES35 cells plated on matrigel and grown in serum for three weeks
G: hES35 cells plated on gelatin and exposed to DMSO and Na-Butyrate
H: HepG2 cells



(a)

Figure 7

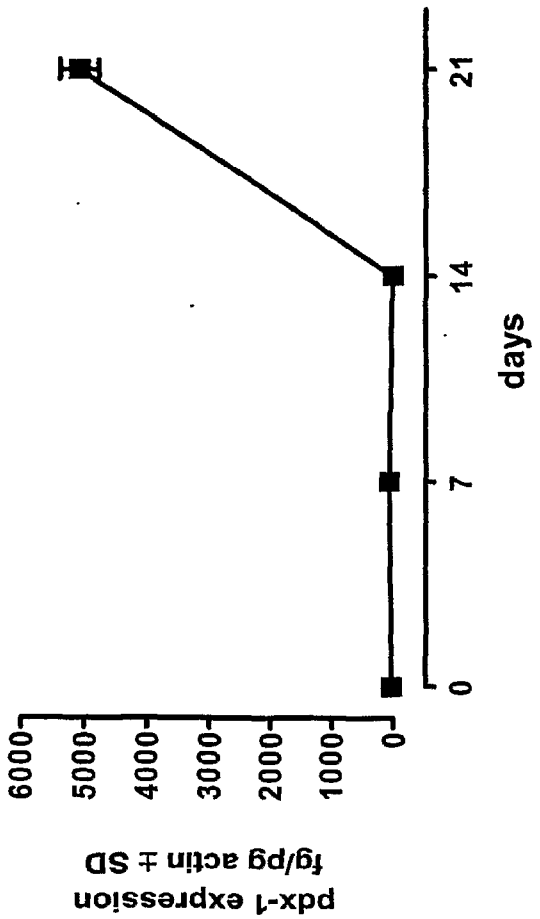
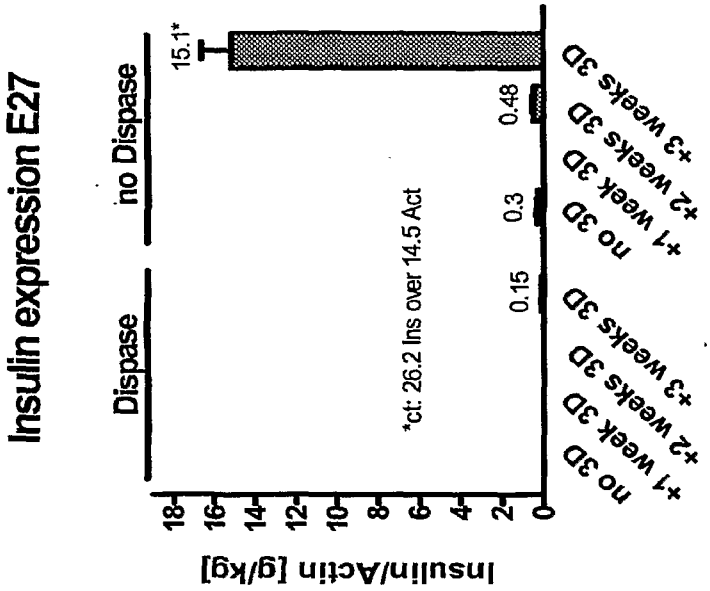

pdx-1 expression
fg/pg actin ± SD
6000
5000
4000
3000
2000
1000
0
0
7
14
21
days

Figure 8


Insulin expression E27
Dispase
no Dispase
15.1*
0.48
0.3
0.15
*ct: 26.2 Ins over 14.5 Act
Insulin/Actin [g/kg]
18
16
14
12
10
8
6
4
2
0
no 3D
+1 week 3D
+2 weeks 3D
+3 weeks 3D
no 3D
+1 week 3D
+2 weeks 3D
+3 weeks 3D
(b)

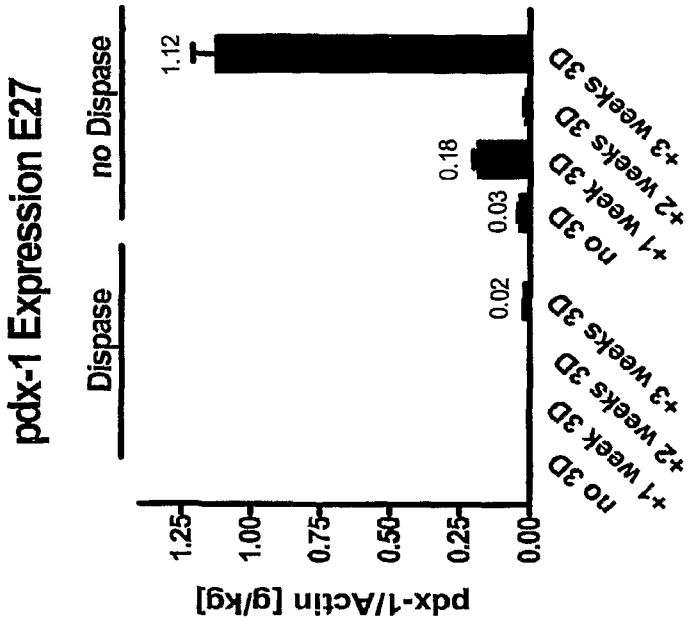

pdx-1 Expression E27
Dispase
no Dispase
1.12
0.18
0.03
0.02
pdx-1/Actin [g/kg]
1.25
1.00
0.75
0.50
0.25
0.00
no 3D
+1 week 3D
+2 weeks 3D
+3 weeks 3D
no 3D
+1 week 3D
+2 weeks 3D
+3 weeks 3D
(a)

Figure 9

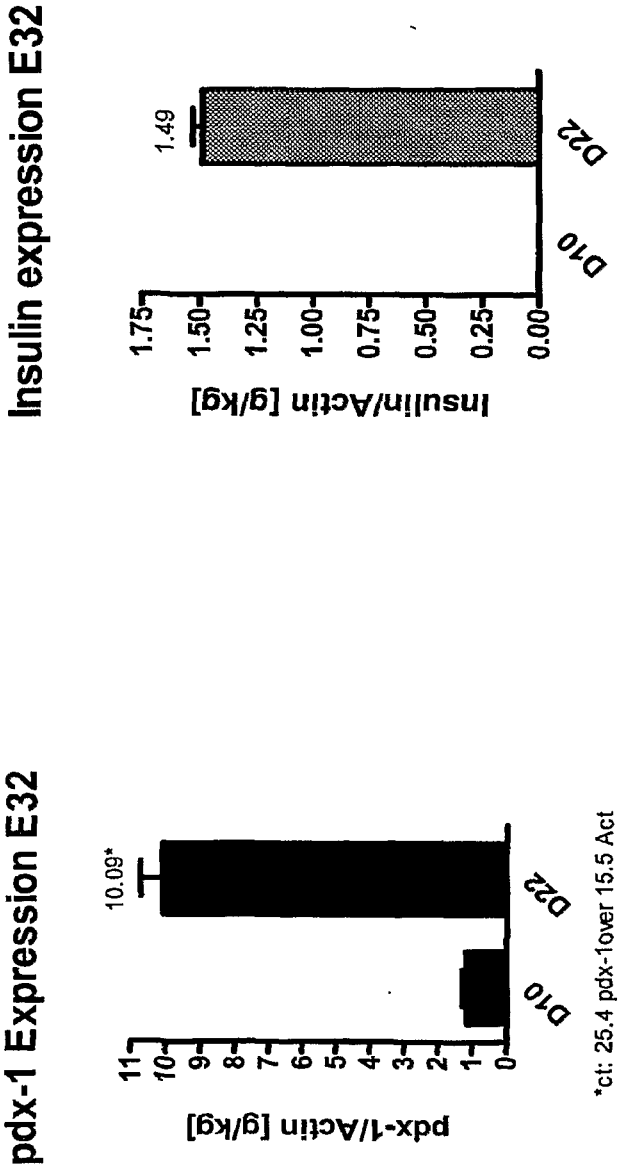

pdx-1 Expression E32
pdx-1/Actin [g/kg]
11
10
9
8
7
6
5
4
3
2
1
0
10.09*
D10
D22
*ct: 25.4 pdx-1over 15.5 Act
(a)
Insulin expression E32
Insulin/Actin [g/kg]
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
1.49
D10
D22
(b)

Figure 10

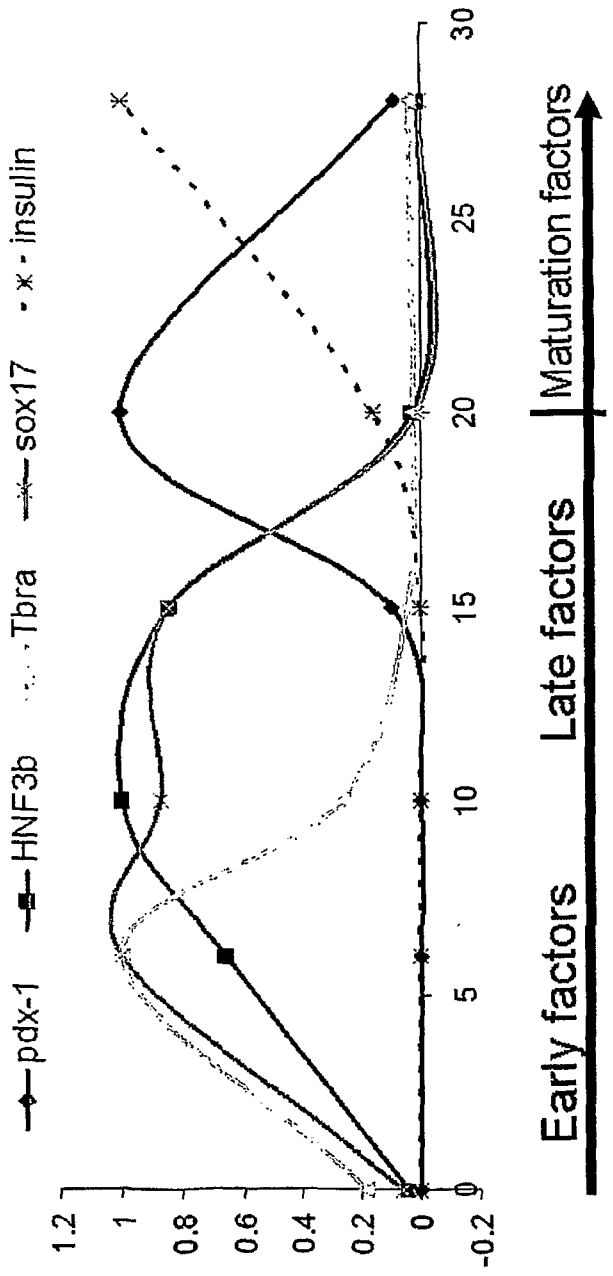

pdx-1
HNF3b
Tbra
sox17
insulin
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
5
10
15
20
25
30
Early factors
Late factors
Maturation factors

Figure 11

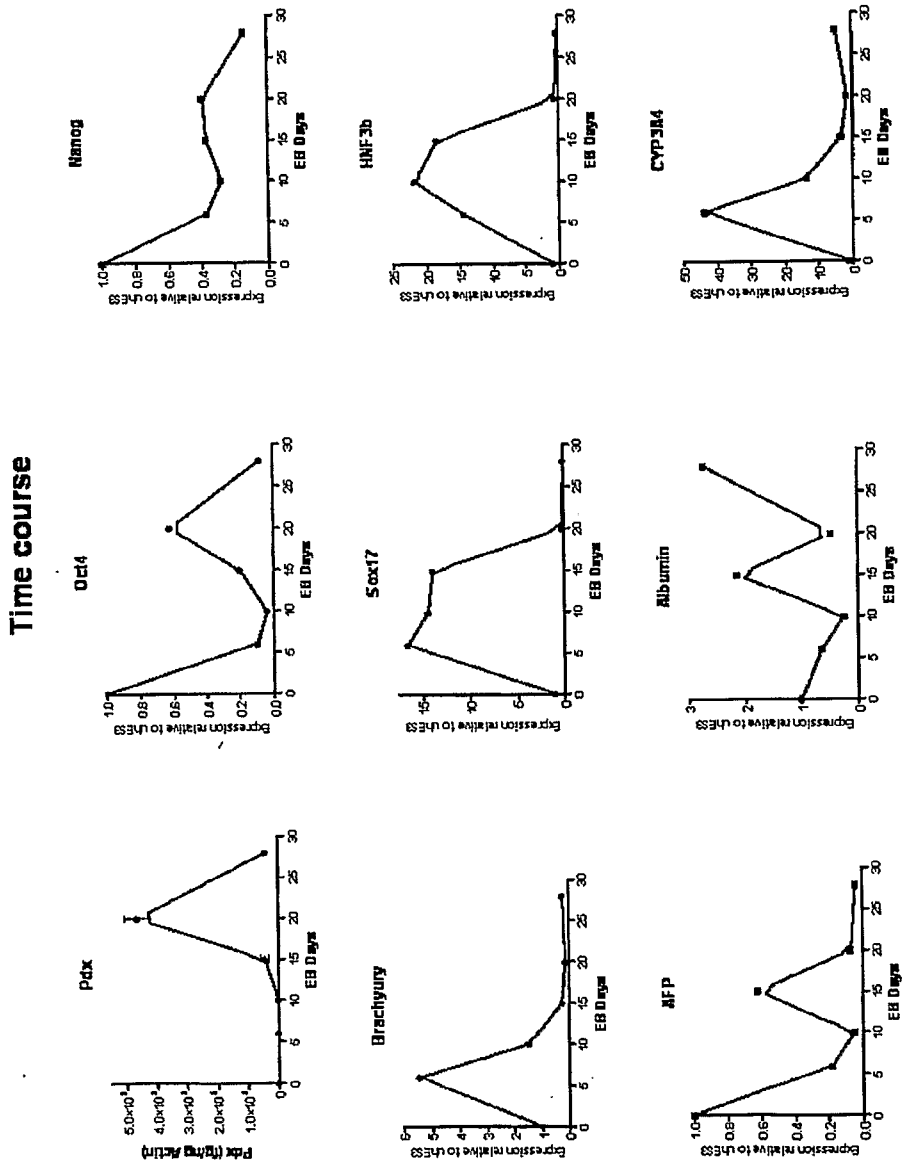

Time course
Pdx
Oct4
Nanog
Brachyury
Sox17
HNF3b
AFP
Albumin
CYP3A4
EB Days
Expression relative to uhES3
Pdx (Hg/ng Actin)

Figure 12

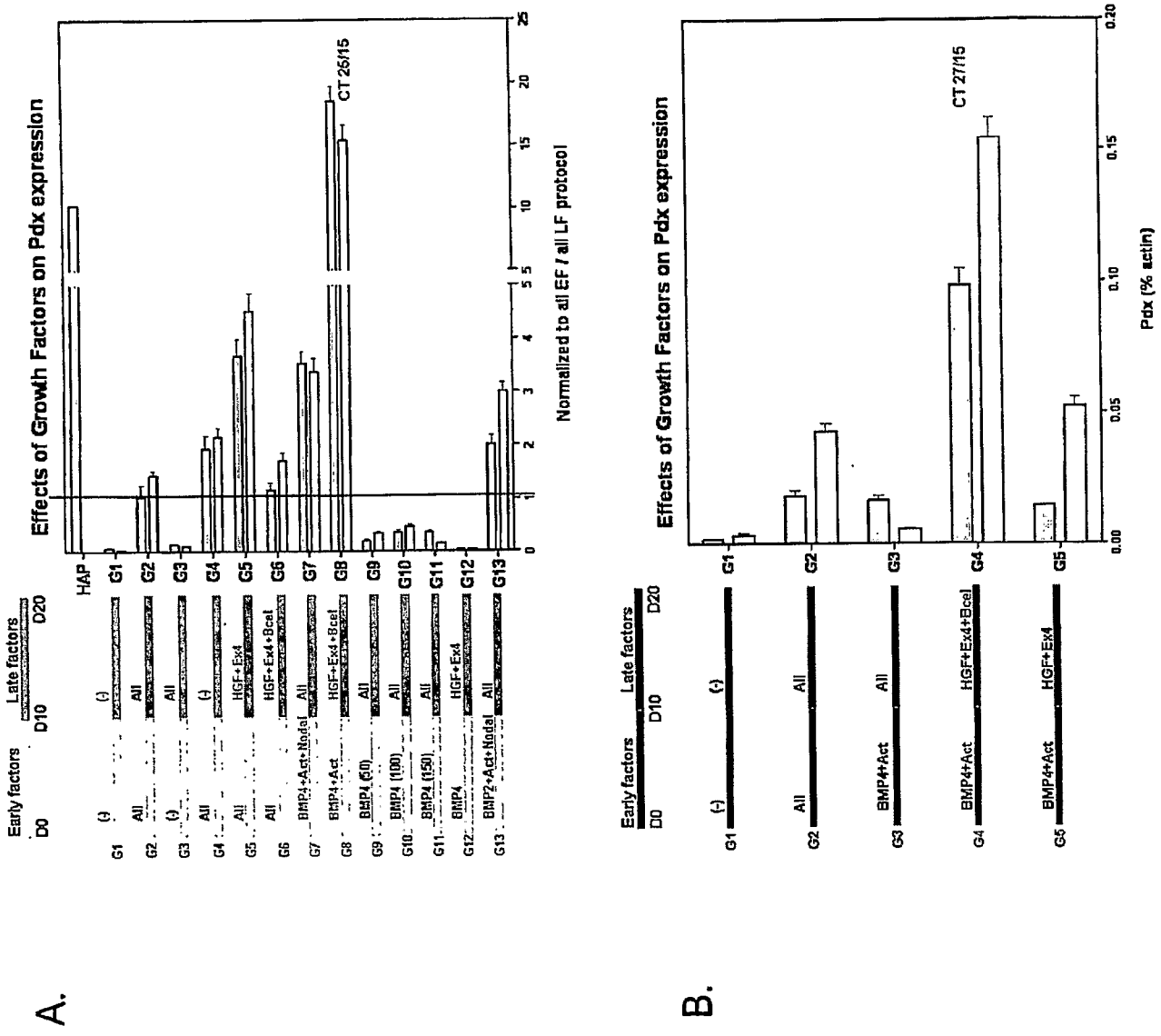

A.
Effects of Growth Factors on Pdx expression
Early factors
Late factors
D0
D10
D20
HAP
G1
G2
G3
G4
G5
G6
G7
G8
G9
G10
G11
G12
G13
(-)
All
HGF+Ex4
HGF+Ex4+Bcel
BMP4+Act+Nodal
BMP4+Act
BMP4 (50)
BMP4 (100)
BMP4 (150)
BMP4
BMP2+Act+Nodal
CT 25/15
Normalized to all EF / all LF protocol
B.
Effects of Growth Factors on Pdx expression
CT 27/15
Pdx (% actin)

Figure 13

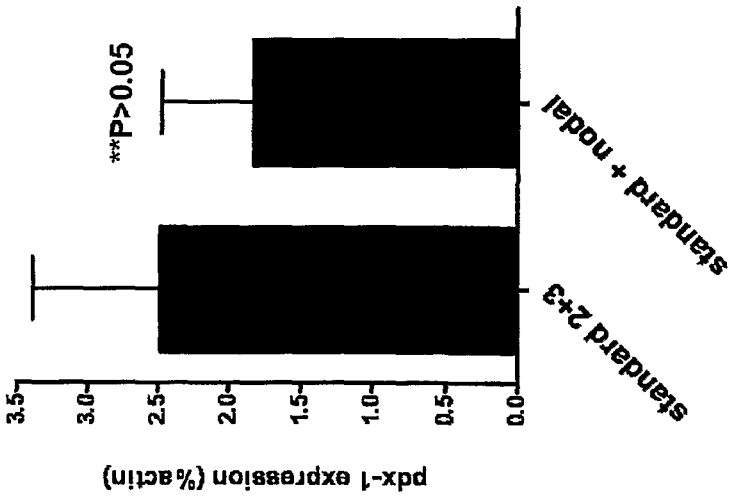

pdx-1 expression (%actin)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
standard 2+3
standard + nodal
**P>0.05

Figure 14

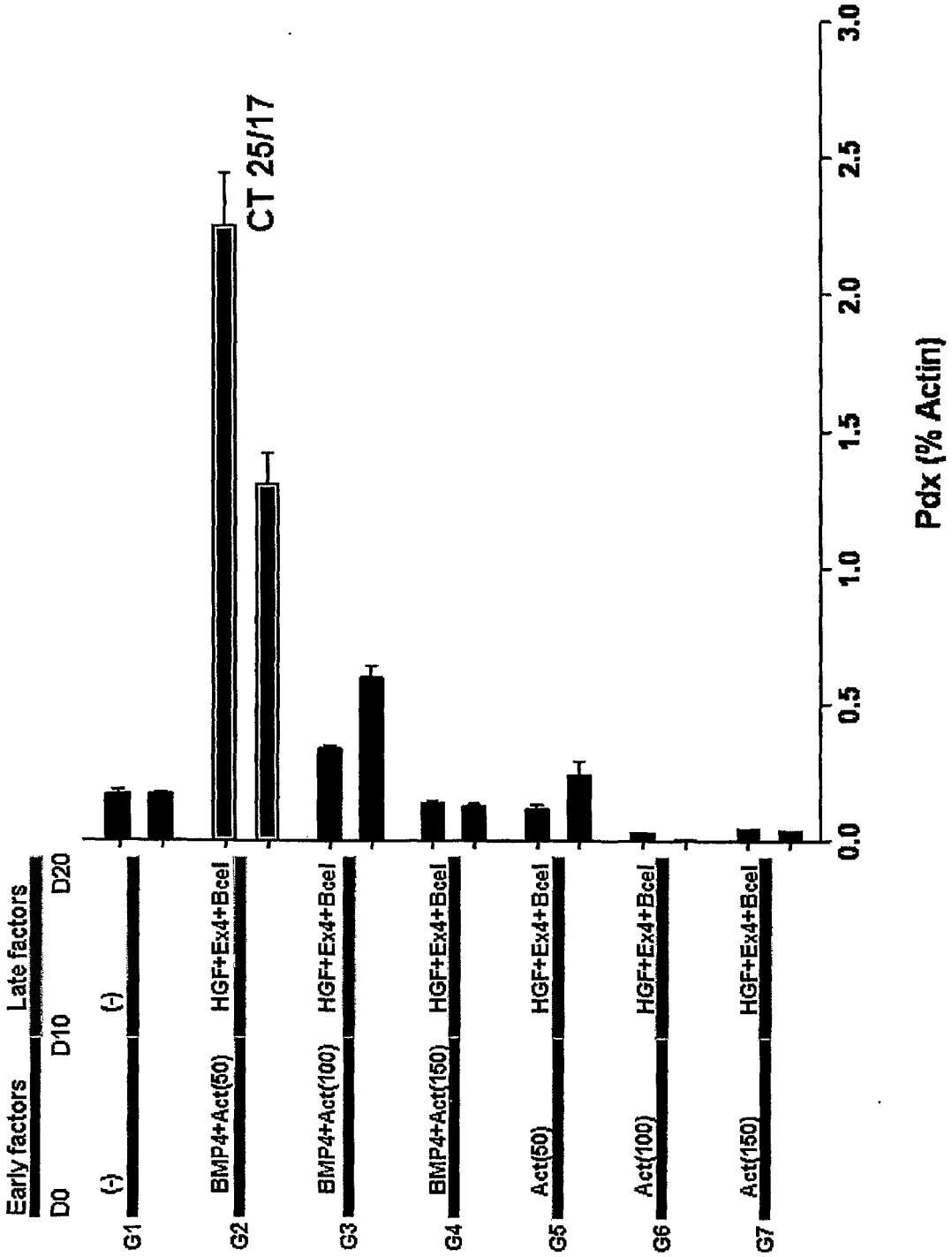

Early factors
Late factors
D0
D10
D20
(-)
(-)
BMP4+Act(50)
HGF+Ex4+Bcel
BMP4+Act(100)
HGF+Ex4+Bcel
BMP4+Act(150)
HGF+Ex4+Bcel
Act(50)
HGF+Ex4+Bcel
Act(100)
HGF+Ex4+Bcel
Act(150)
HGF+Ex4+Bcel
G1
G2
G3
G4
G5
G6
G7
CT 25/17
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Pdx (% Actin)

Figure 15

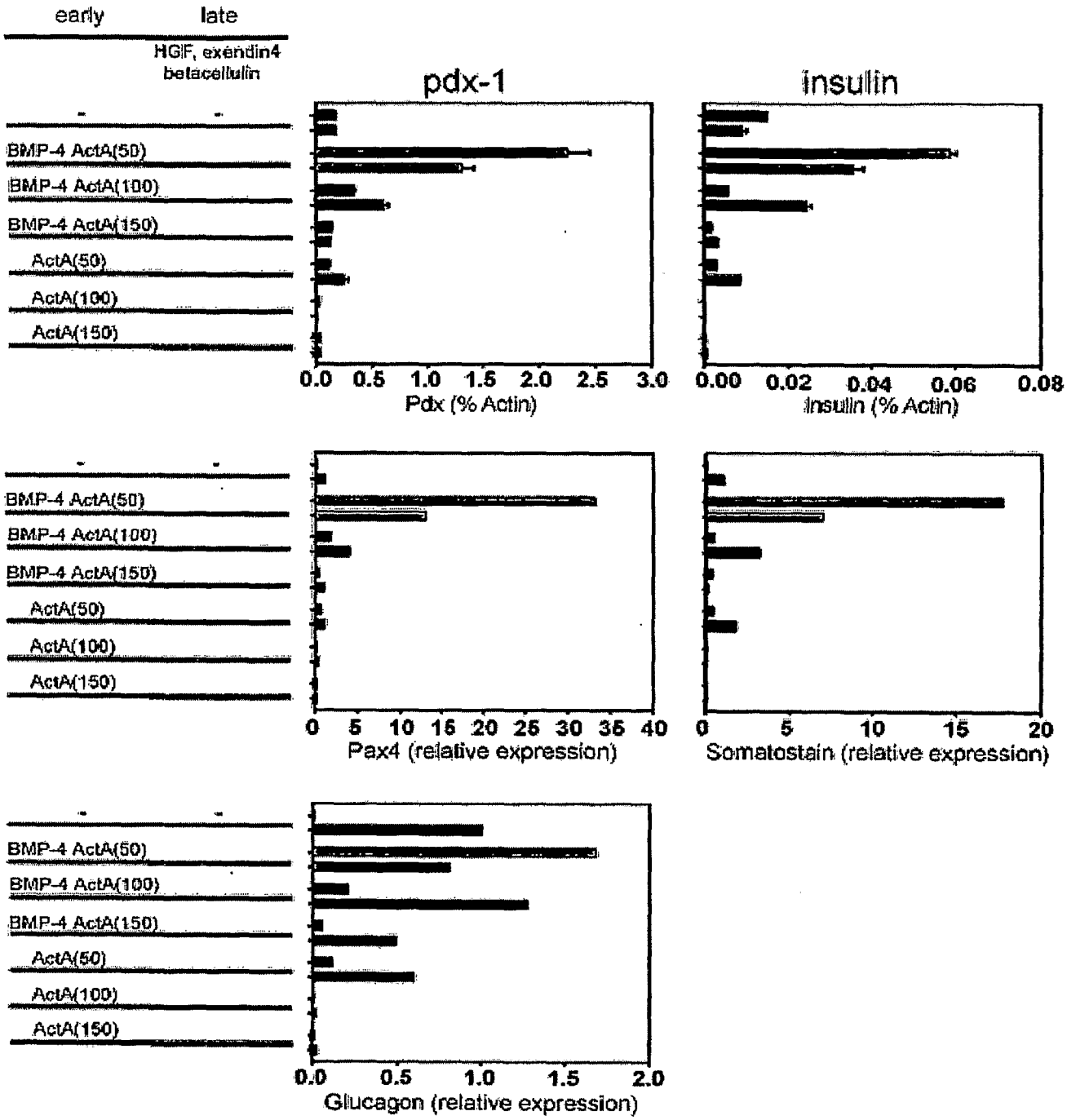

early
late
HGF, exendin4
betacellulin
pdx-1
insulin
BMP-4 ActA(50)
BMP-4 ActA(100)
BMP-4 ActA(150)
ActA(50)
ActA(100)
ActA(150)
0.0 0.5 1.0 1.5 2.0 2.5 3.0
Pdx (% Actin)
0.00 0.02 0.04 0.06 0.08
Insulin (% Actin)
0 5 10 15 20 25 30 35 40
Pax4 (relative expression)
0 5 10 15 20
Somatostain (relative expression)
0.0 0.5 1.0 1.5 2.0
Glucagon (relative expression)

Figure 16

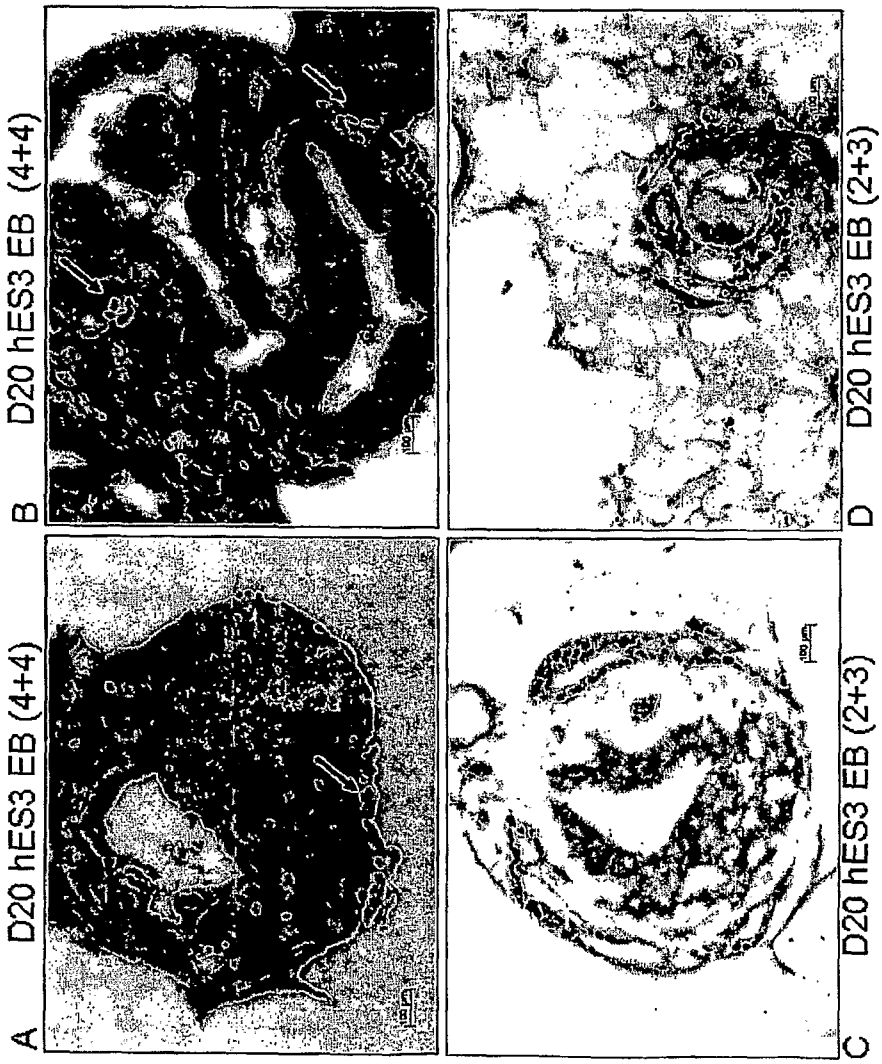


Figure 17

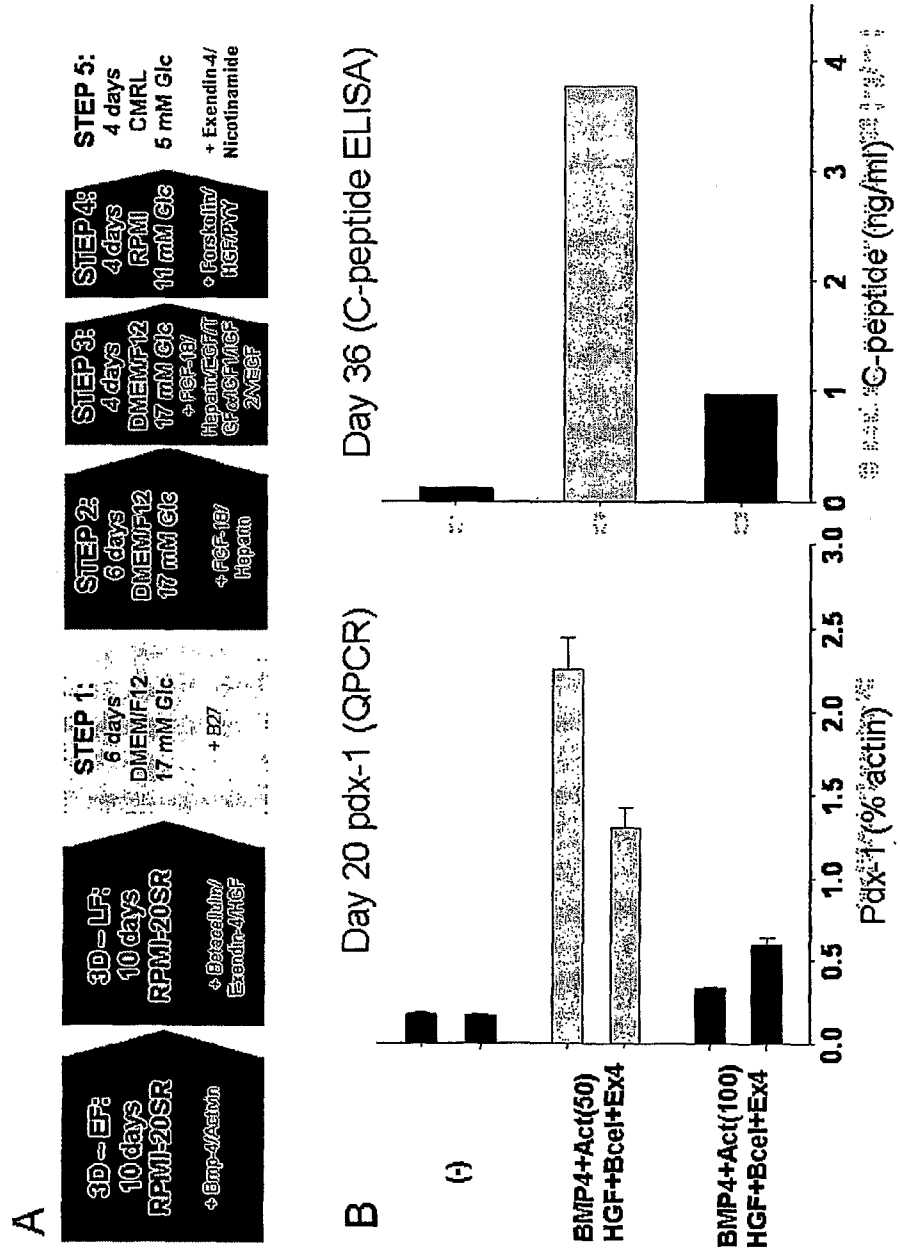

A
3D – EF: 10 days RPMI-20SR
+ Bmp-4/Activin
3D – LF: 10 days RPMI-20SR
+ Betacellulin/ Exendin-4/HGF
STEP 1: 6 days DMEM/F12 17 mM Glc
+ B27
STEP 2: 6 days DMEM/F12 17 mM Glc
+ FGF-10/ Heparin
STEP 3: 4 days DMEM/F12 17 mM Glc
+ FGF-10/ Heparin/EGF/IGF/HGF/VEGF
STEP 4: 4 days RPMI 11 mM Glc
+ Forskolin/ HGF/PYY
STEP 5: 4 days CMRL 5 mM Glc
+ Exendin-4/ Nicotinamide
B
Day 20 pdx-1 (QPCR)
(-)
BMP4+Act(50) HGF+Bcel+Ex4
BMP4+Act(100) HGF+Bcel+Ex4
0.0 0.5 1.0 1.5 2.0 2.5 3.0
Pdx-1 (% actin)
Day 36 (C-peptide ELISA)
0 1 2 3 4
C-peptide (ng/ml)

Figure 18

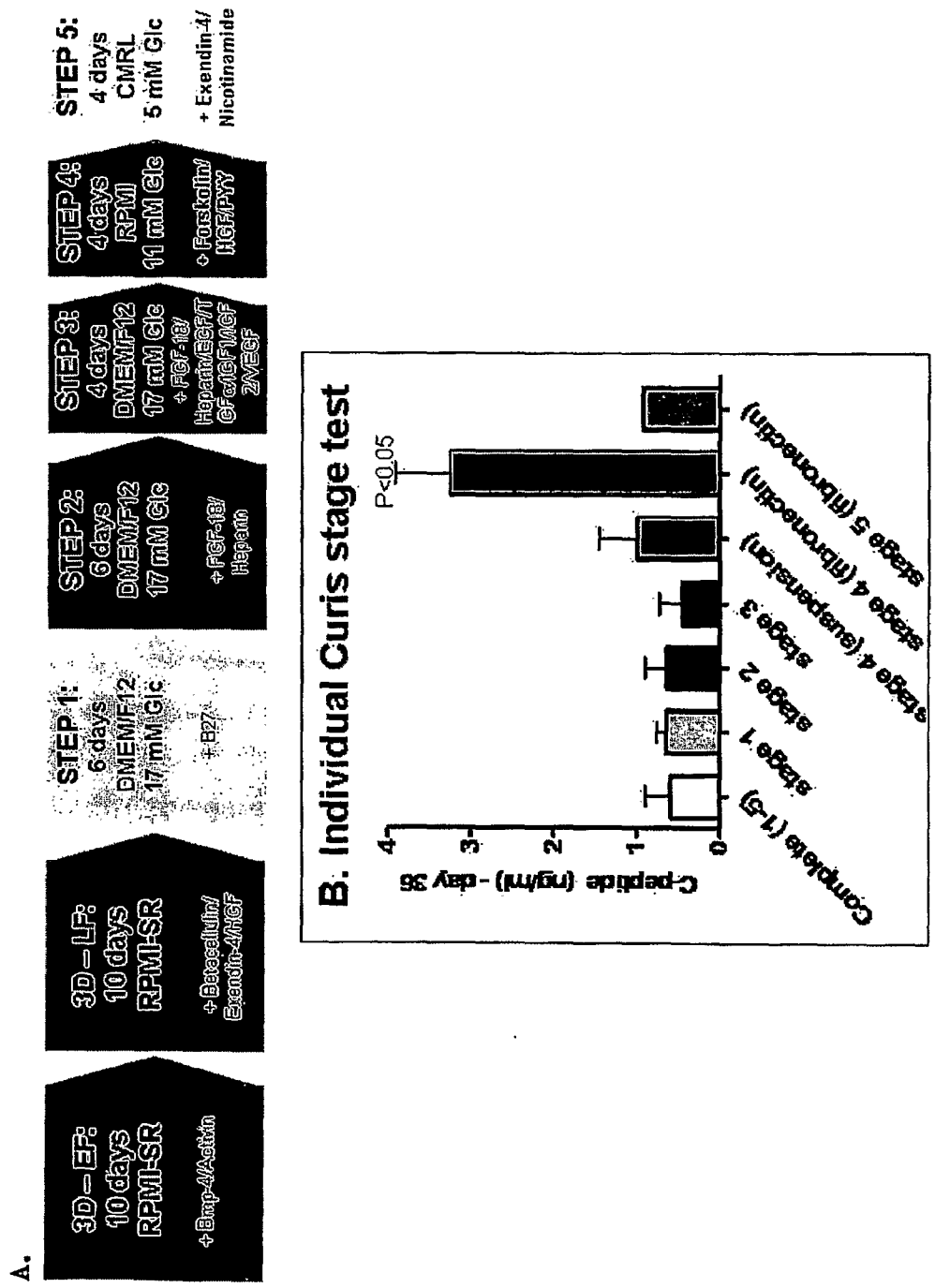

A.
3D – EF:
10 days
RPMI-SR
+ Bmp-4/Activin
3D – LF:
10 days
RPMI-SR
+ Betacellulin/
Exendin-4/HGF
STEP 1:
6 days
DMEMF12
17 mM Glc
+ B27
STEP 2:
6 days
DMEMF12
17 mM Glc
+ FGF-1B/
Heparin
STEP 3:
4 days
DMEMF12
17 mM Glc
+ FGF-1B/
HeparinEGF/TGFα/IGF1/HGF
2/VEGF
STEP 4:
4 days
RPMI
11 mM Glc
+ Forskolin/
HGF/PYY
STEP 5:
4 days
CMRL
5 mM Glc
+ Exendin-4/
Nicotinamide
B. Individual Curis stage test
P<0.05
C-peptide (ng/ml) - day 36
0
1
2
3
4
Complete (1-5)
stage 1
stage 2
stage 3
stage 4 (heparinase)
stage 4 (fibronectin)
stage 5 (fibronectin)

Figure 19

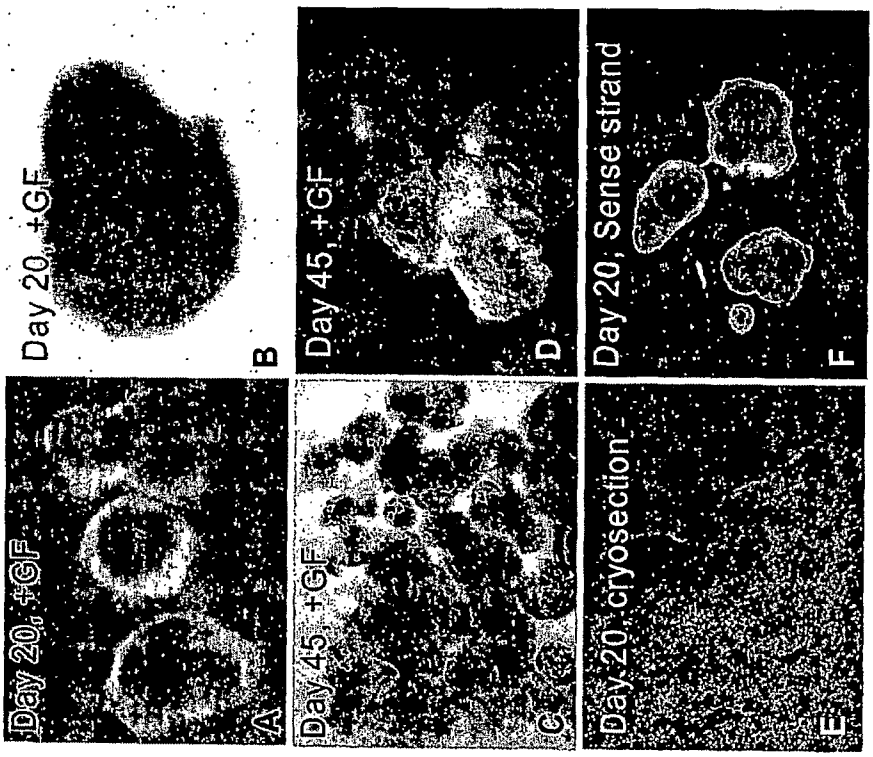

Day 20, +GF
A
Day 20, +GF
B
Day 45, +GF
C
Day 45, +GF
D
Day 20, cryosection
E
Day 20, Sense strand
F

Figure 20

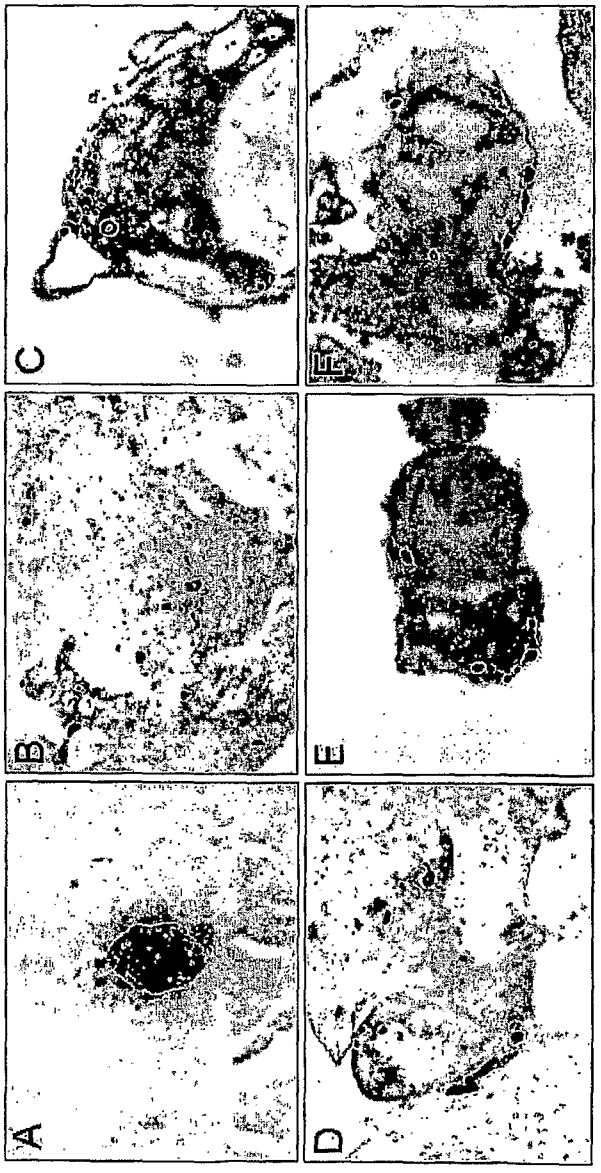


Figure 21

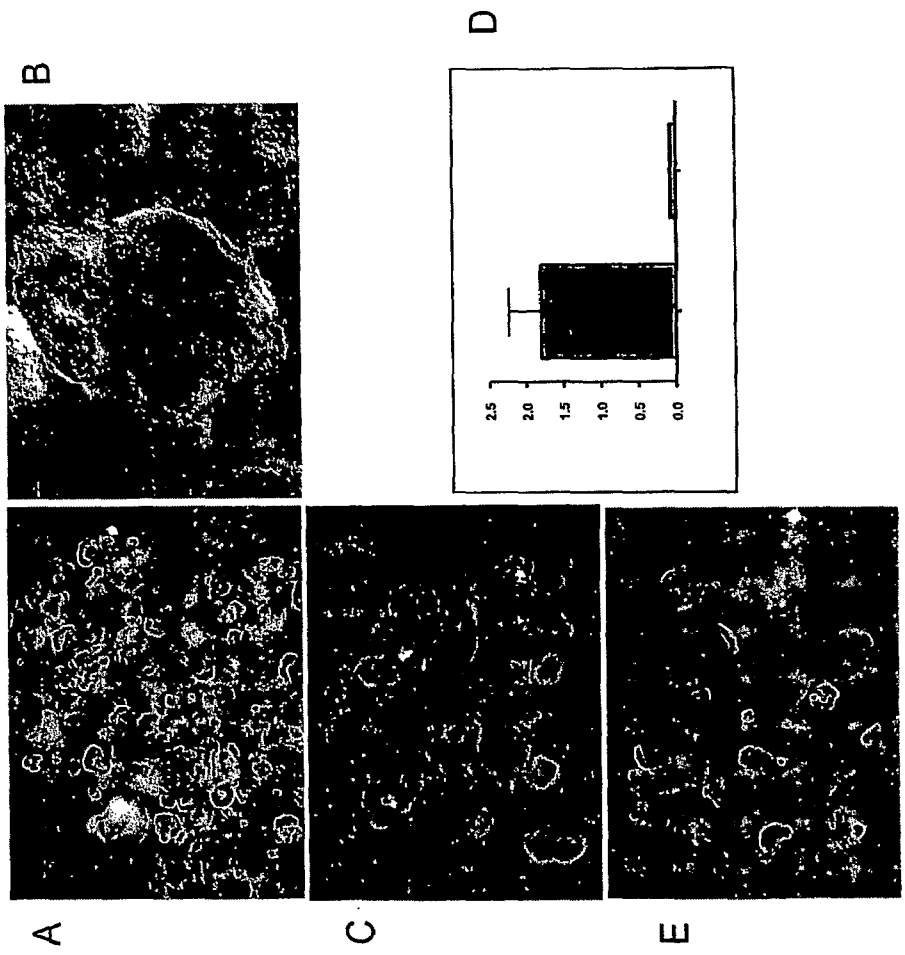


Figure 22

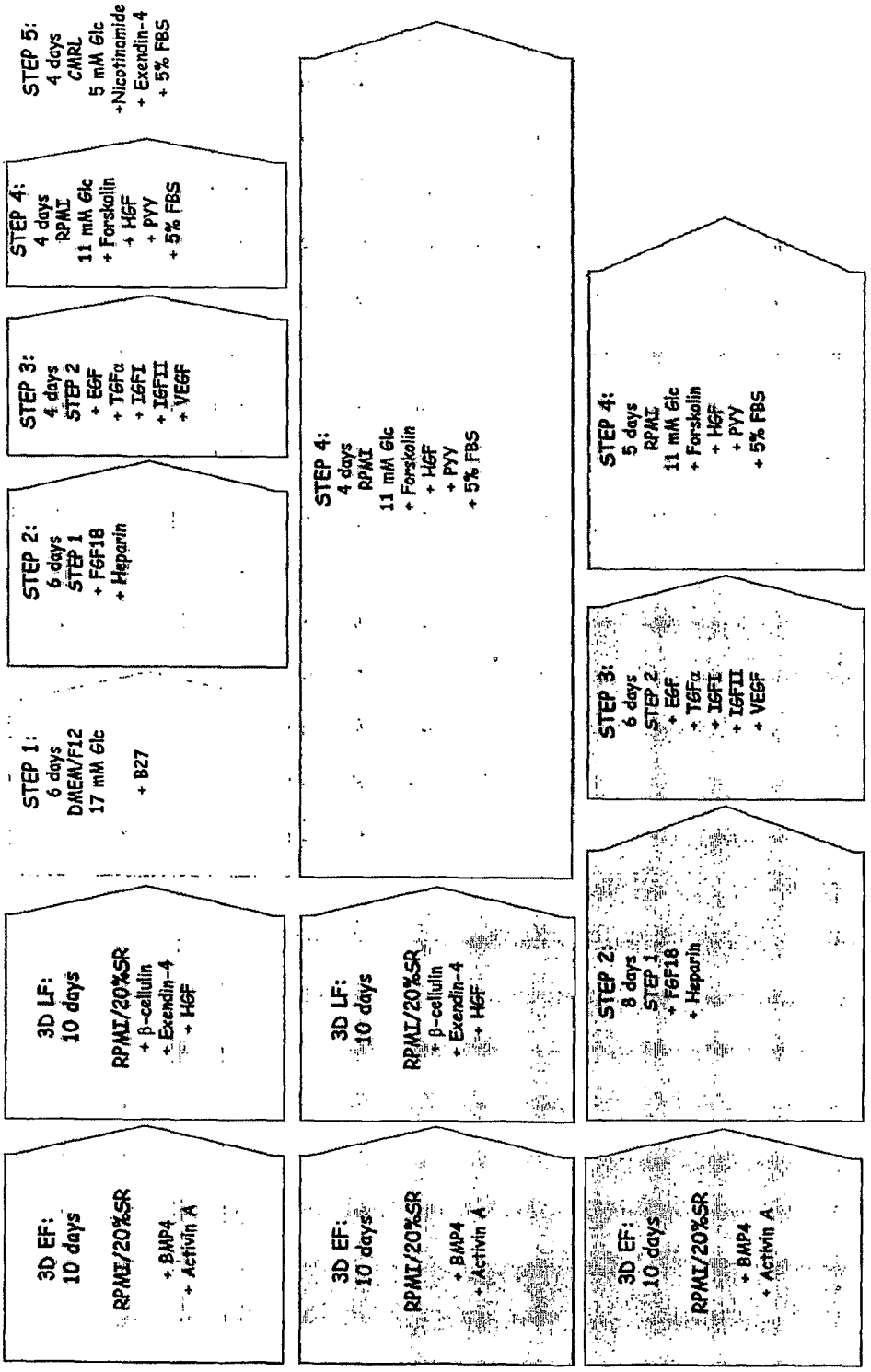

3D EF:
10 days
RPMI/20%SR
+ BMP4
+ Activin A
3D LF:
10 days
RPMI/20%SR
+ β-cellulin
+ Exendin-4
+ HGF
STEP 1:
6 days
DMEM/F12
17 mM Glc
+ B27
STEP 2:
6 days
STEP 1
+ FGF10
+ Heparin
STEP 3:
4 days
STEP 2
+ EGF
+ TGFα
+ IGFI
+ IGFII
+ VEGF
STEP 4:
4 days
RPMI
11 mM Glc
+ Forskolin
+ HGF
+ PYY
+ 5% FBS
STEP 5:
4 days
CMRL
5 mM Glc
+Nicotinamide
+ Exendin-4
+ 5% FBS
3D EF:
10 days
RPMI/20%SR
+ BMP4
+ Activin A
3D LF:
10 days
RPMI/20%SR
+ β-cellulin
+ Exendin-4
+ HGF
STEP 2:
8 days
STEP 1
+ FGF10
+ Heparin
STEP 3:
6 days
STEP 2
+ EGF
+ TGFα
+ IGFI
+ IGFII
+ VEGF
STEP 4:
5 days
RPMI
11 mM Glc
+ Forskolin
+ HGF
+ PYY
+ 5% FBS

Figure 23

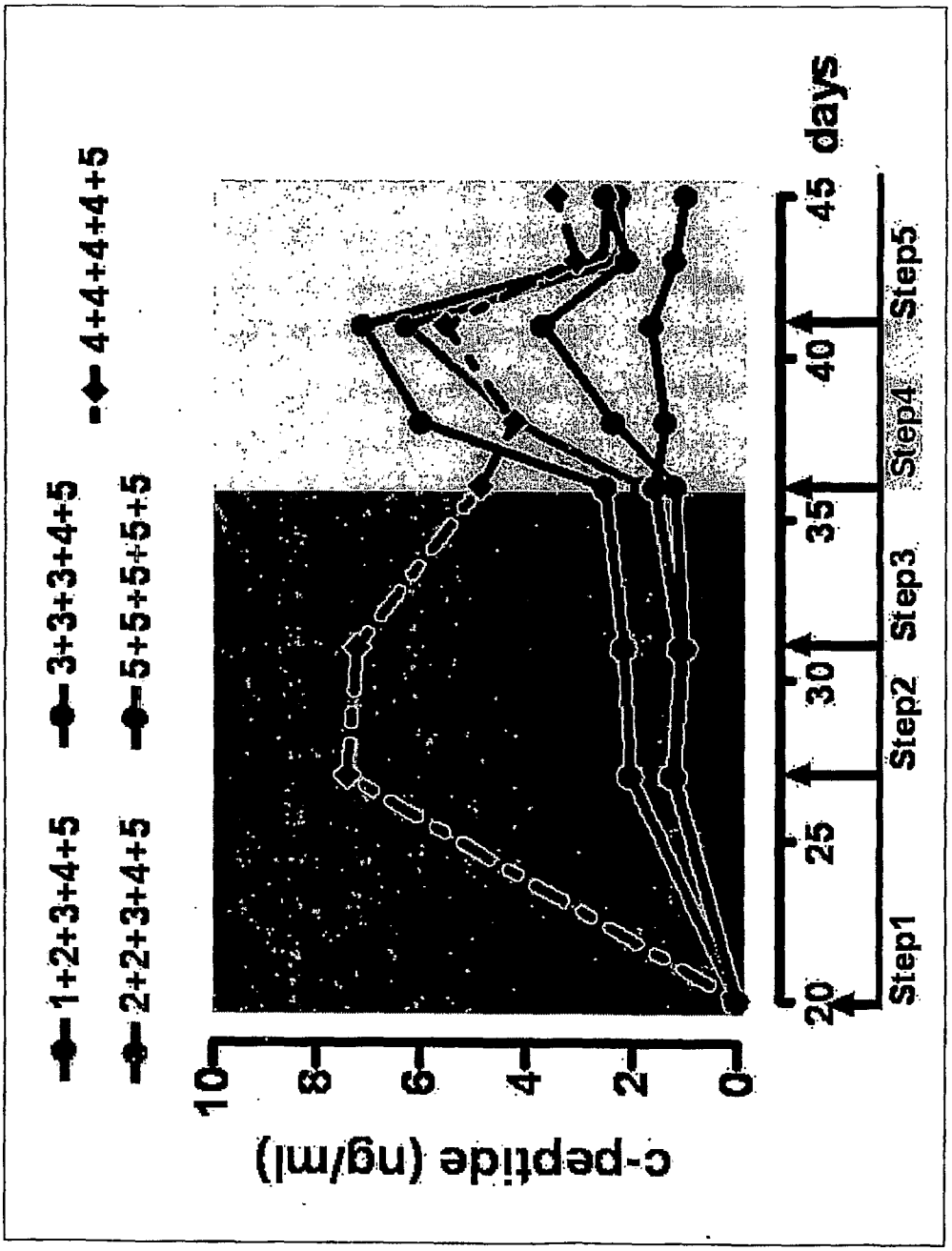

1+2+3+4+5
2+2+3+4+5
3+3+3+4+5
5+5+5+5+5
4+4+4+4+5
c-peptide (ng/ml)
0
2
4
6
8
10
20
25
30
35
40
45 days
Step1
Step2
Step3
Step4
Step5

Figure 24

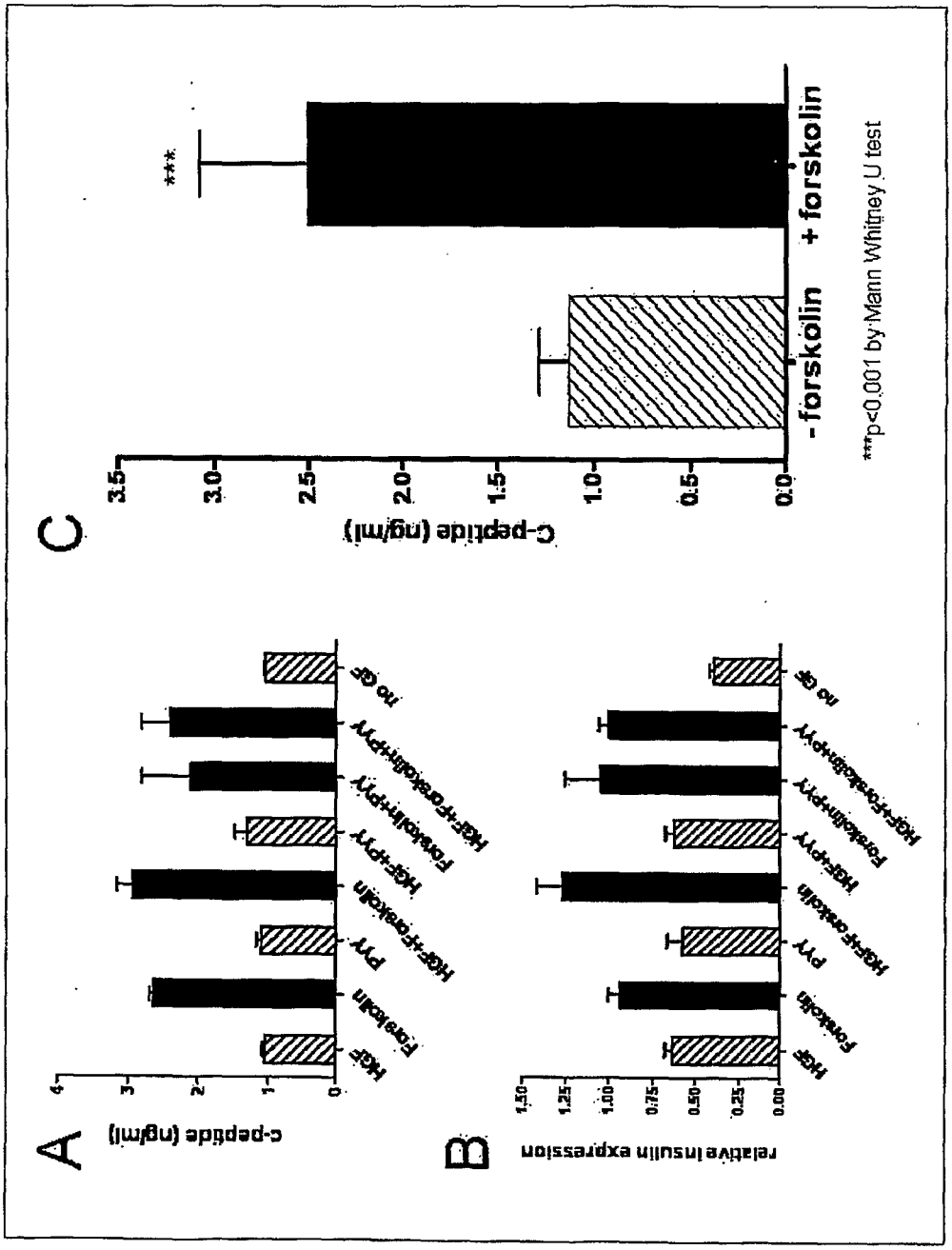

A
c-peptide (ng/ml)
0
1
2
3
4
HGF
Forskolin
PYY
HGF+Forskolin
HGF+PYY
Forskolin+PYY
HGF+Forskolin+PYY
no GF
B
relative insulin expression
0.00
0.25
0.50
0.75
1.00
1.25
1.50
HGF
Forskolin
PYY
HGF+Forskolin
HGF+PYY
Forskolin+PYY
HGF+Forskolin+PYY
no GF
C
C-peptide (ng/ml)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
***
- forskolin
+ forskolin
***p<0.001 by Mann Whitney U test

Figure 25

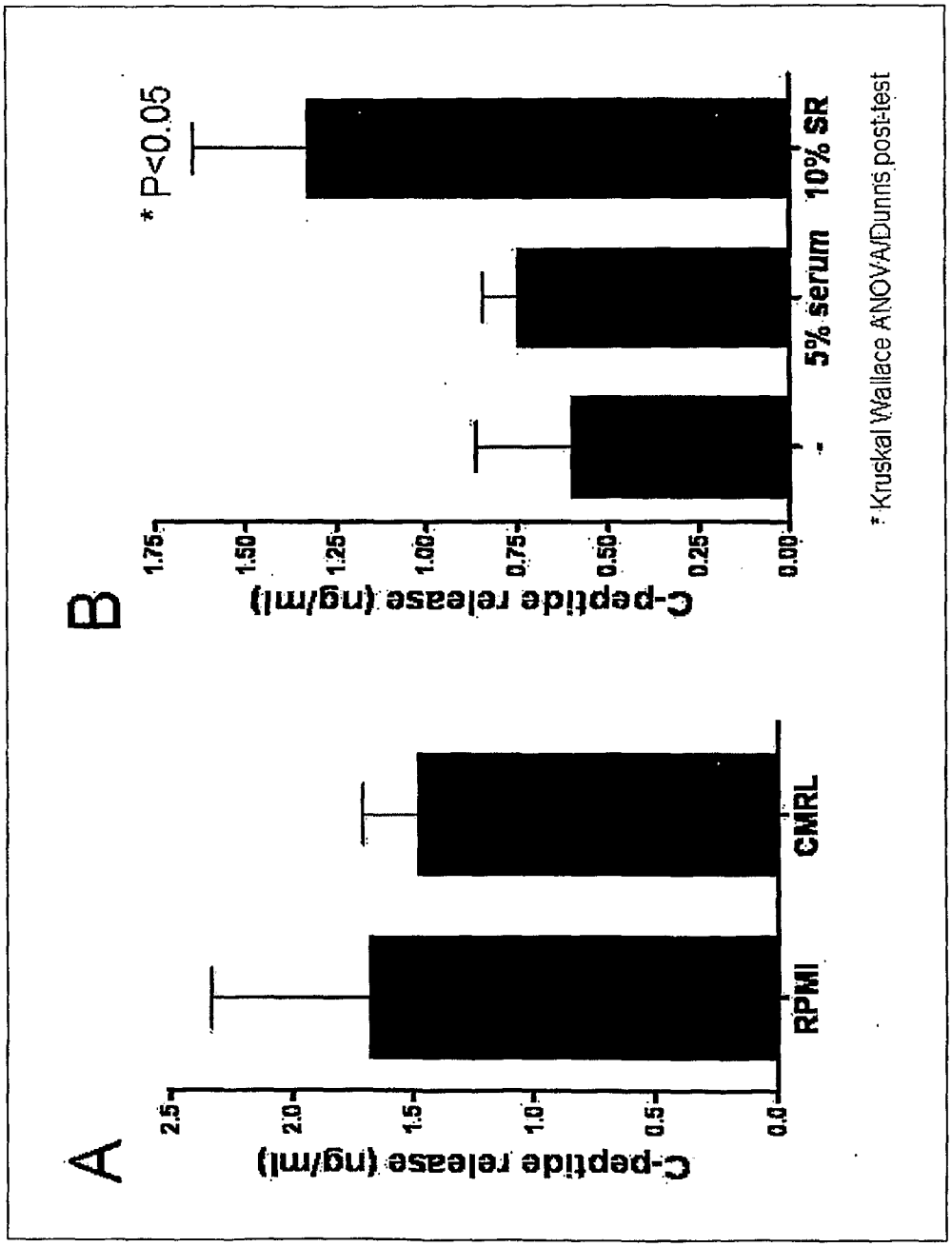

A
C-peptide release (ng/ml)
2.5
2.0
1.5
1.0
0.5
0.0
RPMI
CMRL
B
C-peptide release (ng/ml)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
* P<0.05
-
5% serum
10% SR
* Kruskal Wallace ANOVA/Dunns post-test

Figure 26

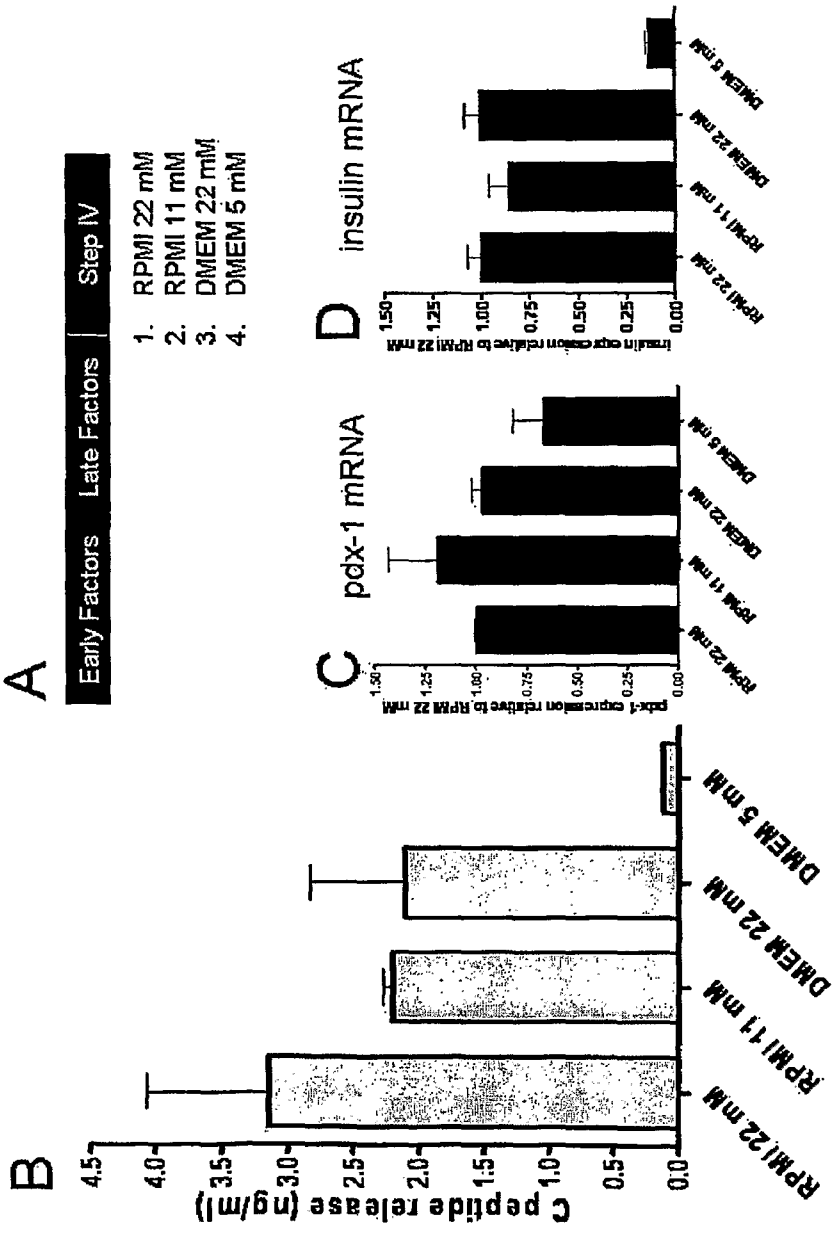

A
Early Factors
Late Factors
Step IV
1. RPMI 22 mM
2. RPMI 11 mM
3. DMEM 22 mM
4. DMEM 5 mM
B
C peptide release (ng/ml)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
RPMI 22 mM
RPMI 11 mM
DMEM 22 mM
DMEM 5 mM
C
pdx-1 mRNA
D
insulin mRNA

Figure 27

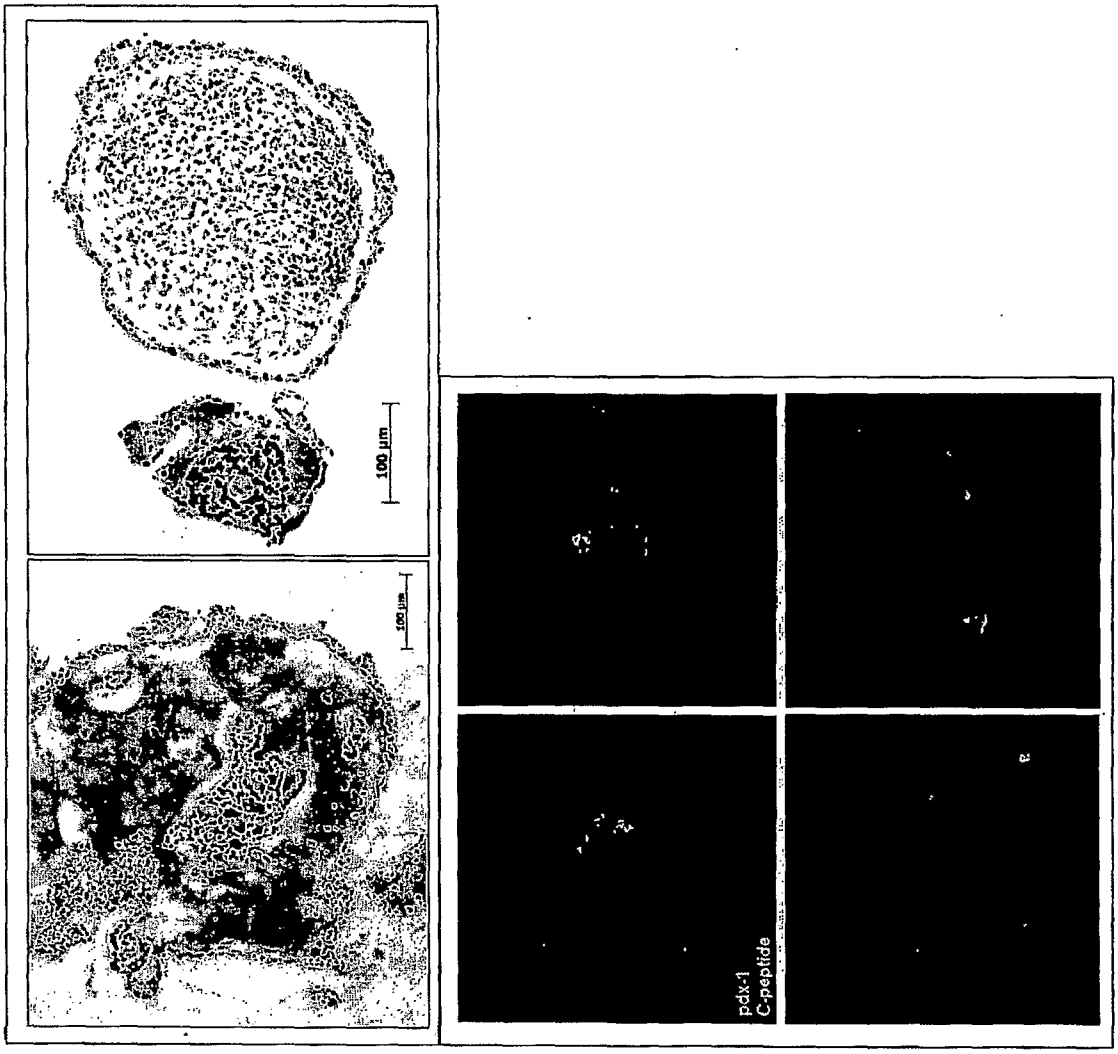


Figure 28

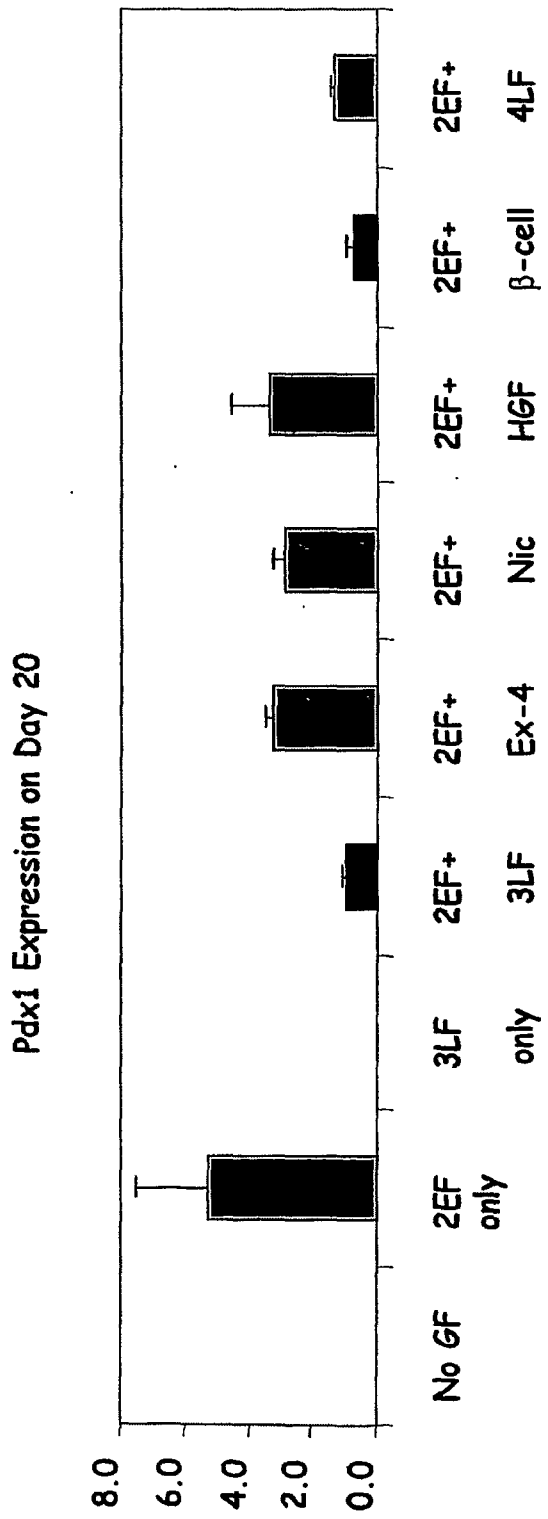

Pdx1 Expression on Day 20
8.0
6.0
4.0
2.0
0.0
No GF
2EF only
3LF
2EF+ 3LF
2EF+ Ex-4
2EF+ Nic
2EF+ HGF
2EF+ β-cell
2EF+ 4LF

Figure 29

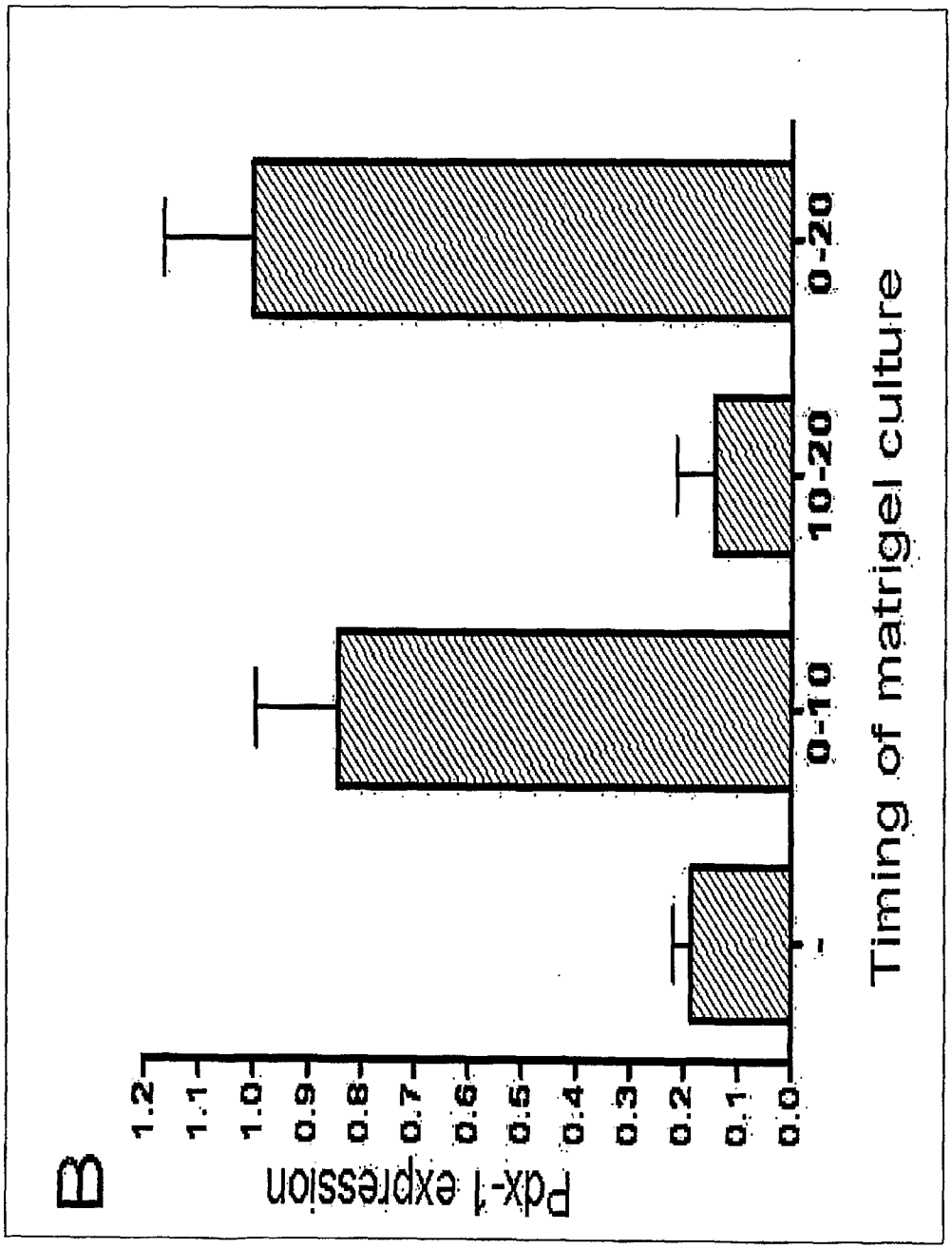

B
Pdx-1 expression
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-
0-10
10-20
0-20
Timing of matrigel culture

Figure 30

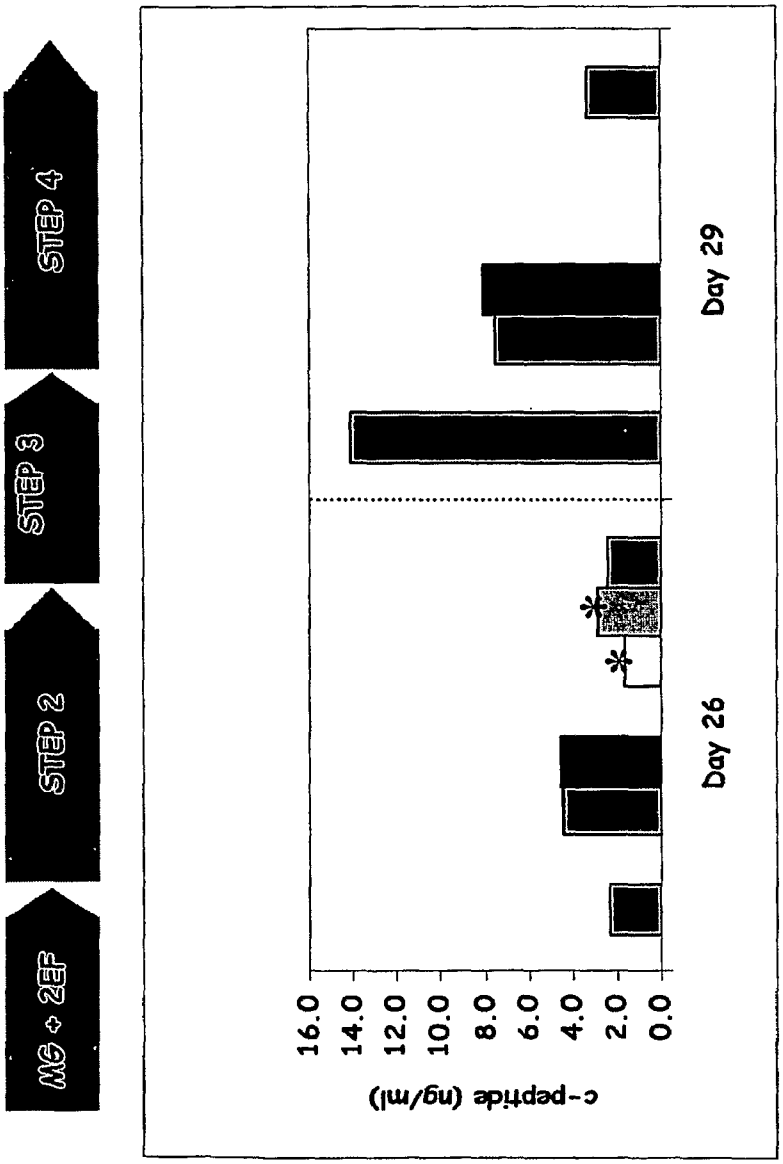

MG + 2EF
STEP 2
STEP 3
STEP 4
c-peptide (ng/ml)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Day 26
Day 29

Figure 31

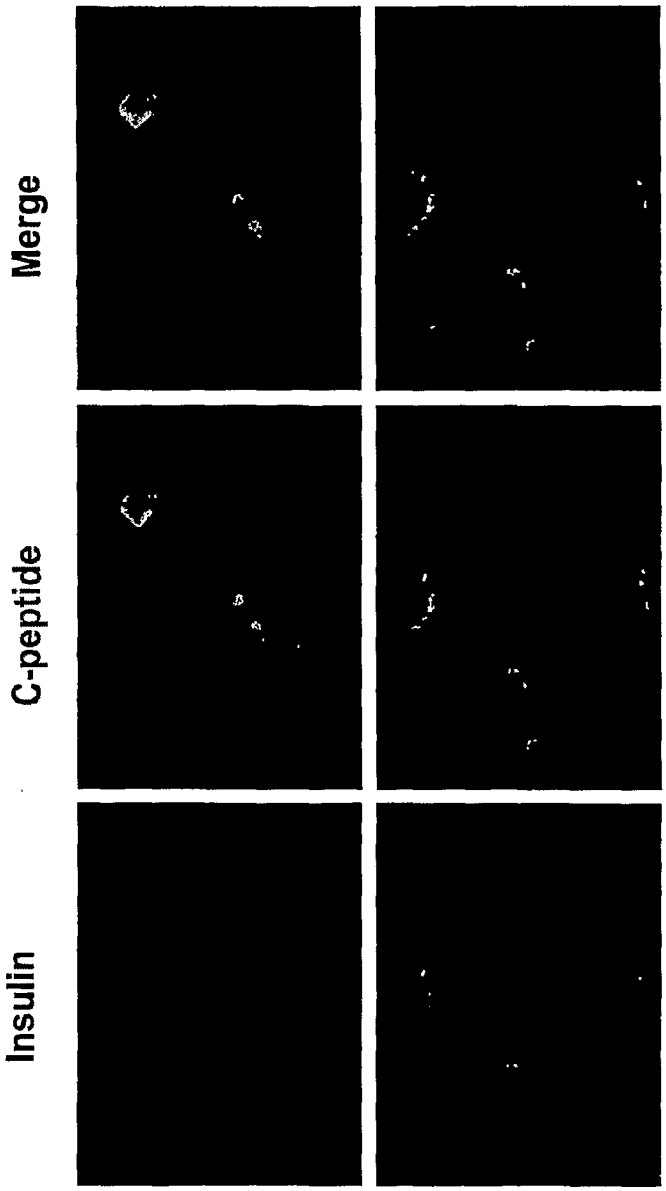


Figure 32

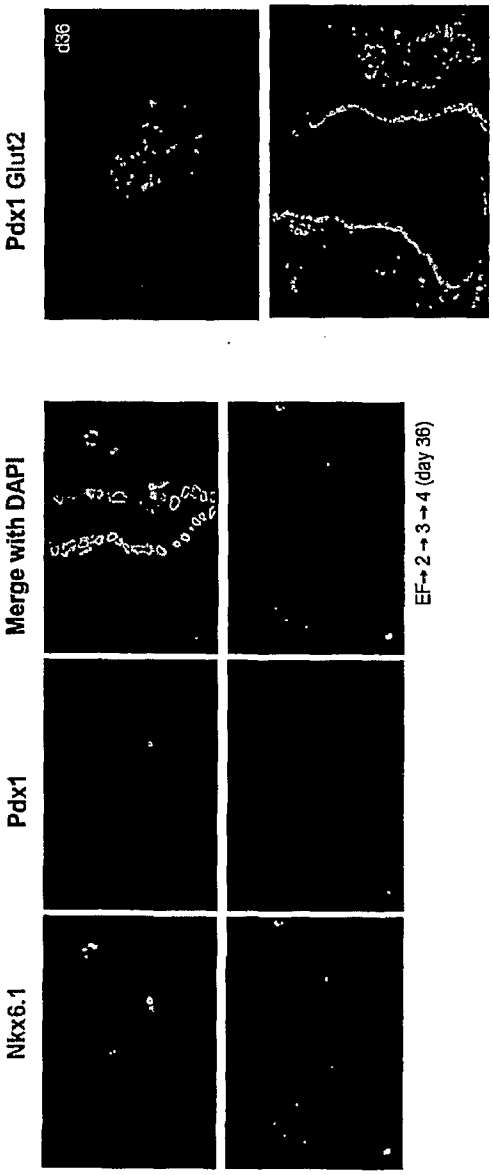


Figure 33

Figure 34

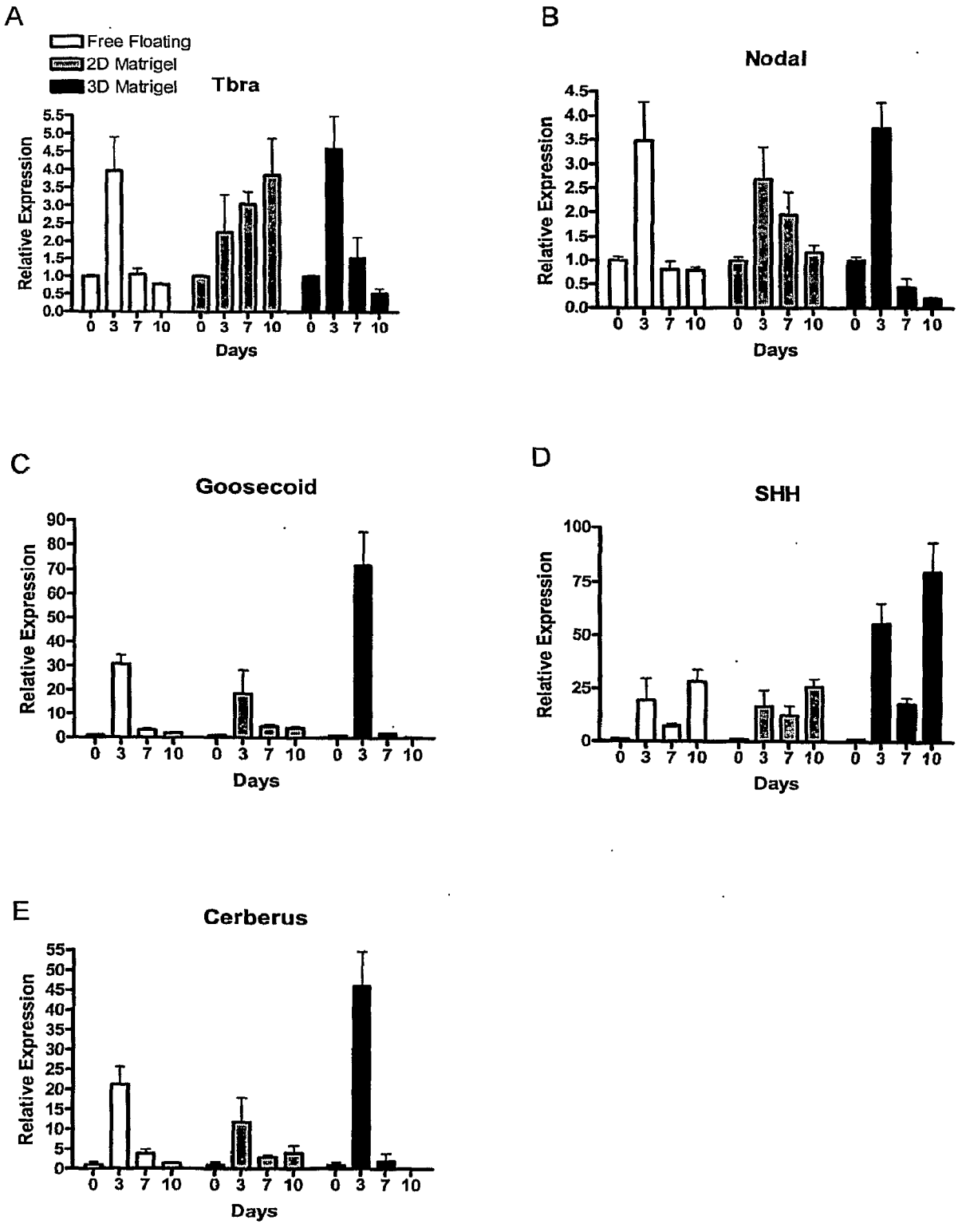

A
Free Floating
2D Matrigel
3D Matrigel
Tbra
Relative Expression
Days
B
Nodal
Relative Expression
Days
C
Goosecoid
Relative Expression
Days
D
SHH
Relative Expression
Days
E
Cerberus
Relative Expression
Days

Figure 35

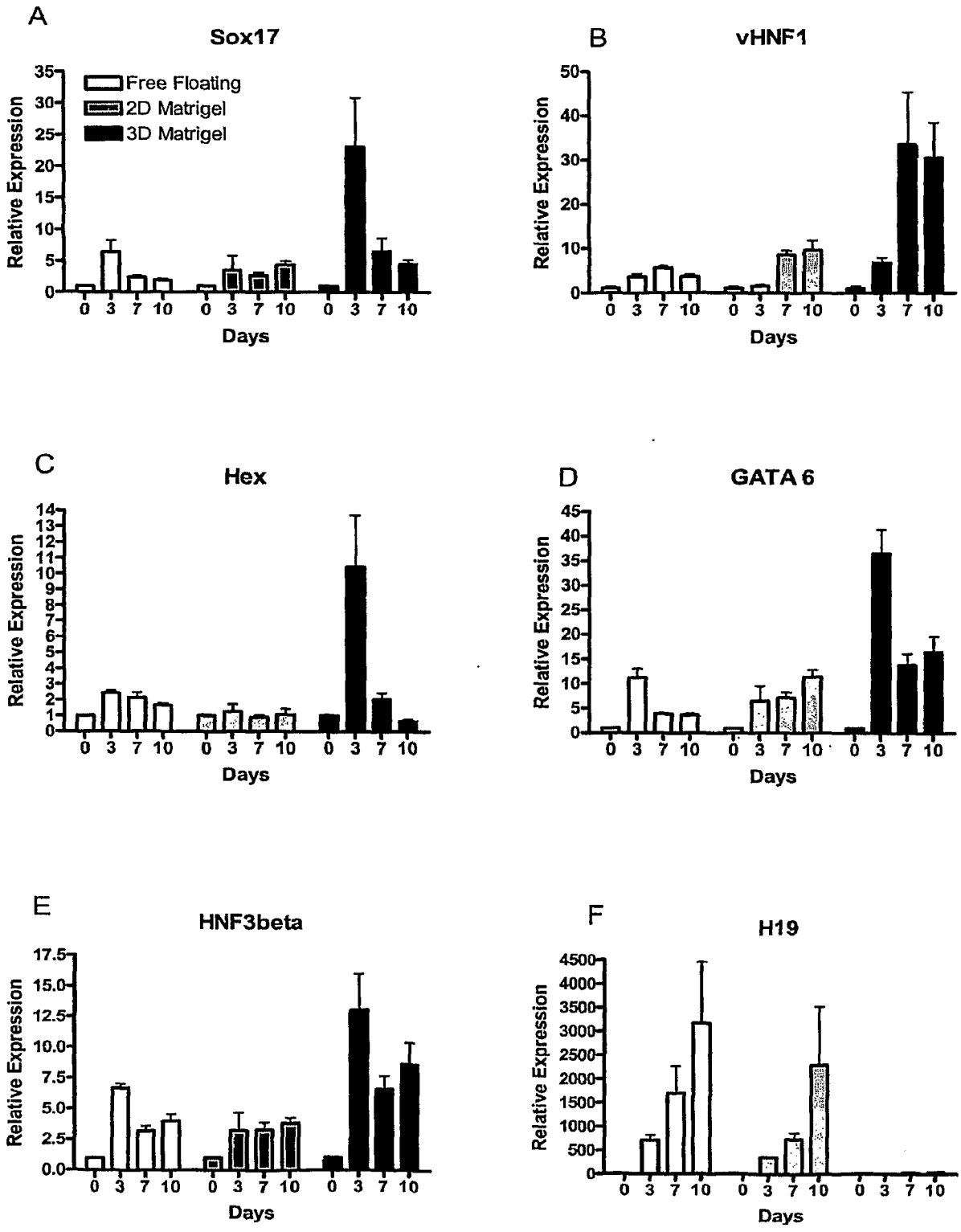
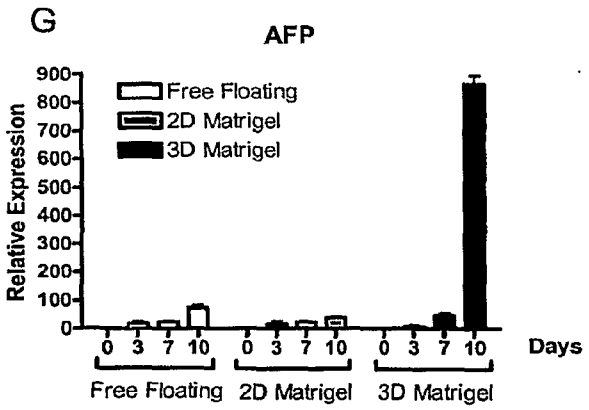
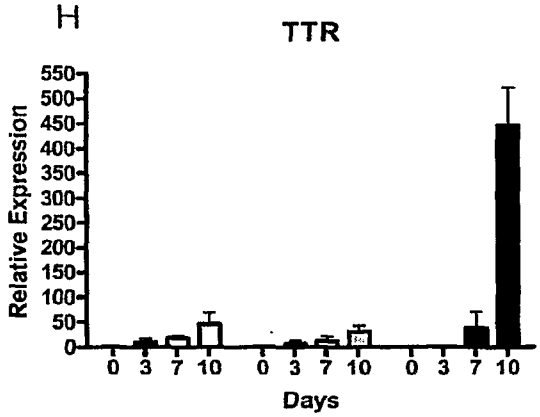
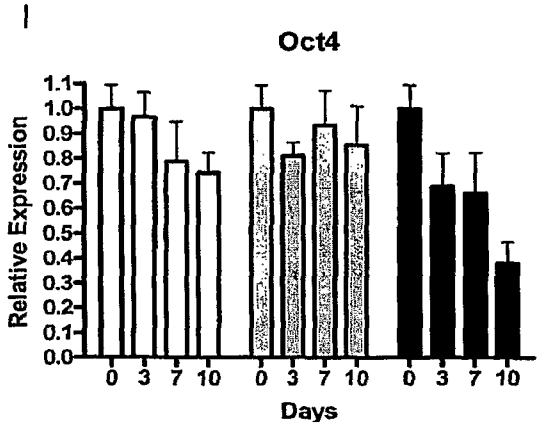


Figure 35, continued.


G
AFP
Relative Expression
900
800
700
600
500
400
300
200
100
0
Free Floating
2D Matrigel
3D Matrigel
0 3 7 10 0 3 7 10 0 3 7 10
Days
Free Floating
2D Matrigel
3D Matrigel


H
TTR
Relative Expression
550
500
450
400
350
300
250
200
150
100
50
0
0 3 7 10 0 3 7 10 0 3 7 10
Days


I
Oct4
Relative Expression
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 3 7 10 0 3 7 10 0 3 7 10
Days

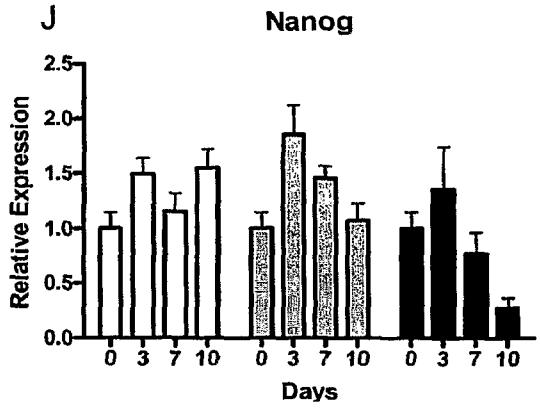

J
Nanog
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
0 3 7 10 0 3 7 10 0 3 7 10
Days

Figure 36.

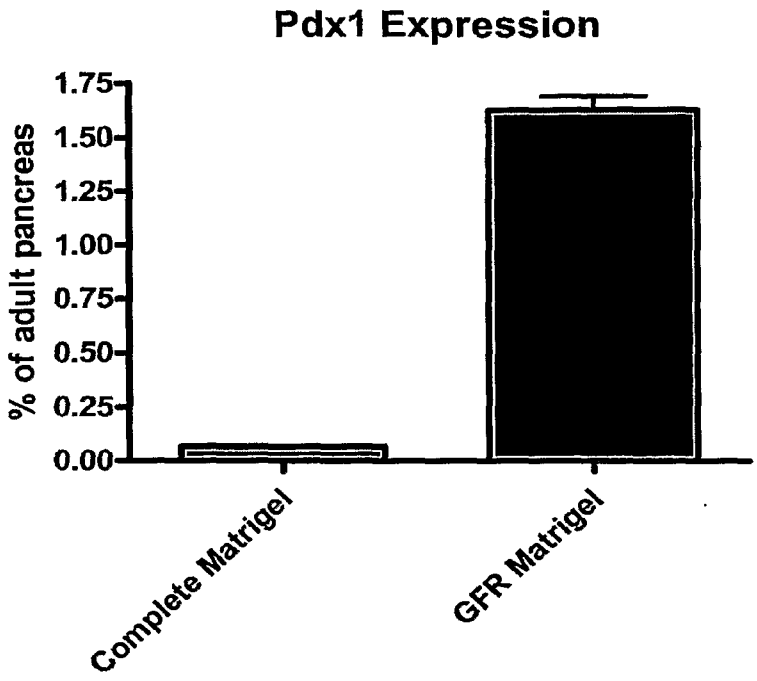

Pdx1 Expression
% of adult pancreas
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Complete Matrigel
GFR Matrigel

Figure 37.

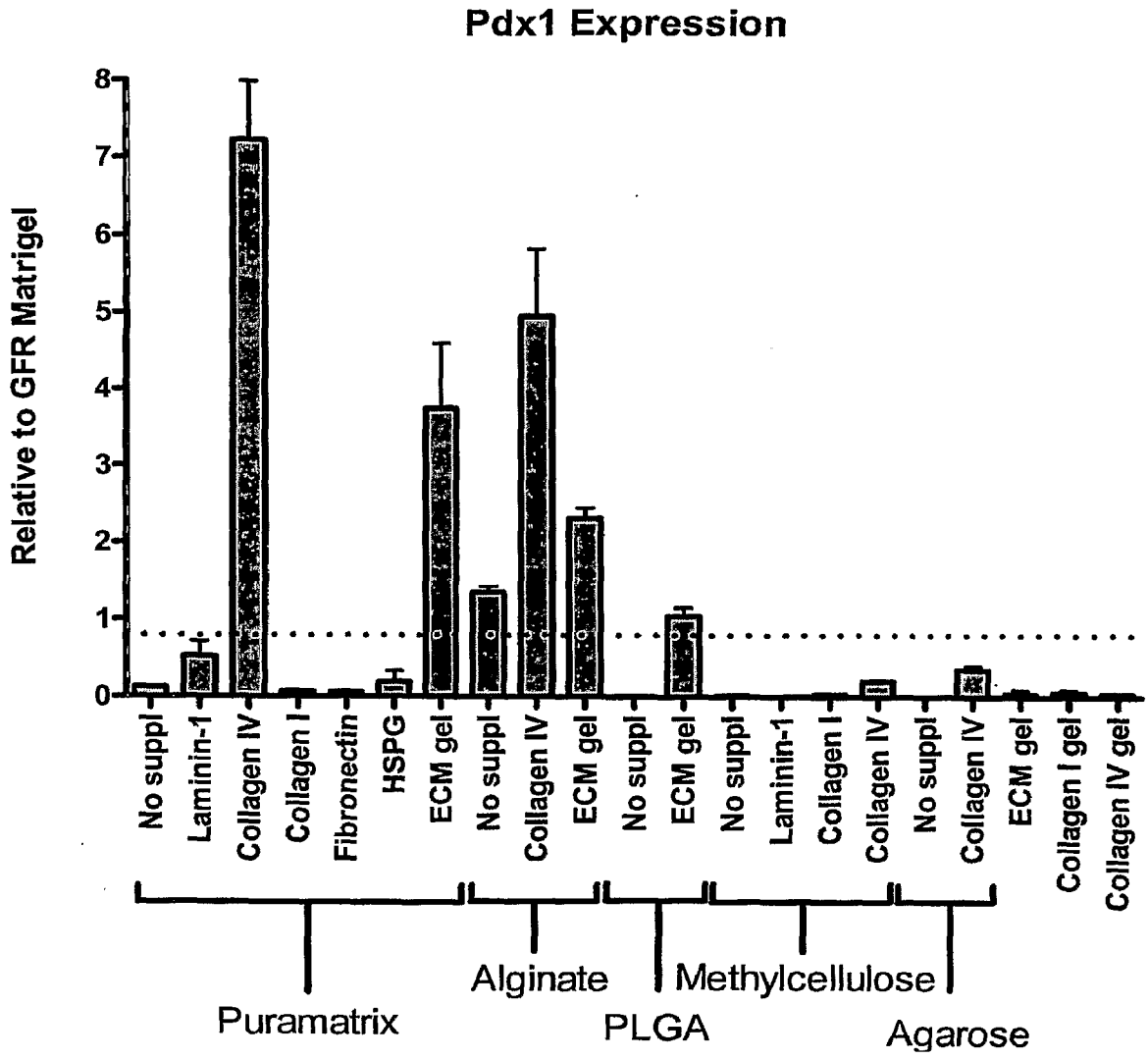

Pdx1 Expression
Relative to GFR Matrigel
8
7
6
5
4
3
2
1
0
No suppl
Laminin-1
Collagen IV
Collagen I
Fibronectin
HSPG
ECM gel
No suppl
Collagen IV
ECM gel
No suppl
ECM gel
No suppl
Laminin-1
Collagen I
Collagen IV
No suppl
Collagen IV
ECM gel
Collagen I gel
Collagen IV gel
Puramatrix
Alginate
PLGA
Methylcellulose
Agarose

Figure 38.

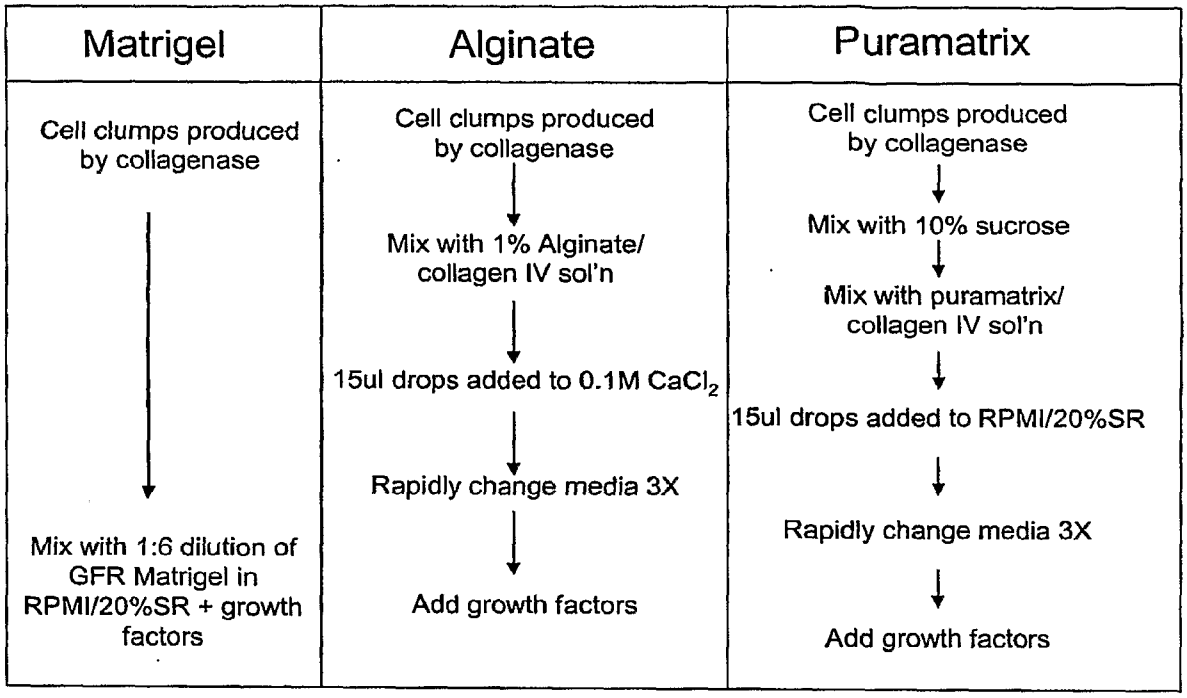

Matrigel
Cell clumps produced by collagenase
Mix with 1:6 dilution of GFR Matrigel in RPMI/20%SR + growth factors
Alginate
Cell clumps produced by collagenase
Mix with 1% Alginate/ collagen IV sol'n
15ul drops added to 0.1M $CaCl_2$
Rapidly change media 3X
Add growth factors
Puramatrix
Cell clumps produced by collagenase
Mix with 10% sucrose
Mix with puramatrix/ collagen IV sol'n
15ul drops added to RPMI/20%SR
Rapidly change media 3X
Add growth factors

Figure 39.

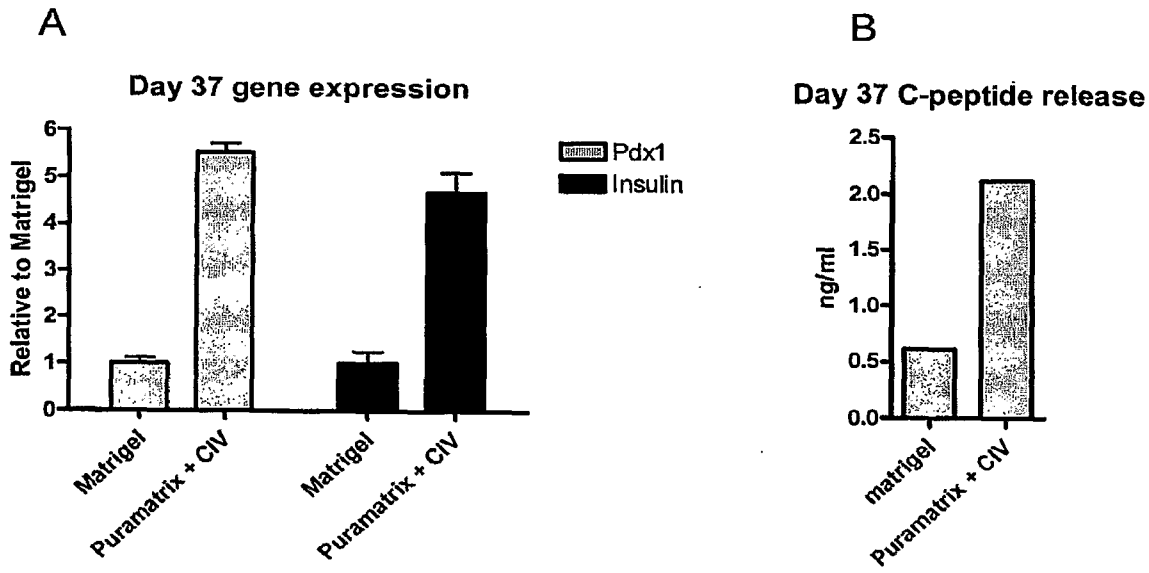
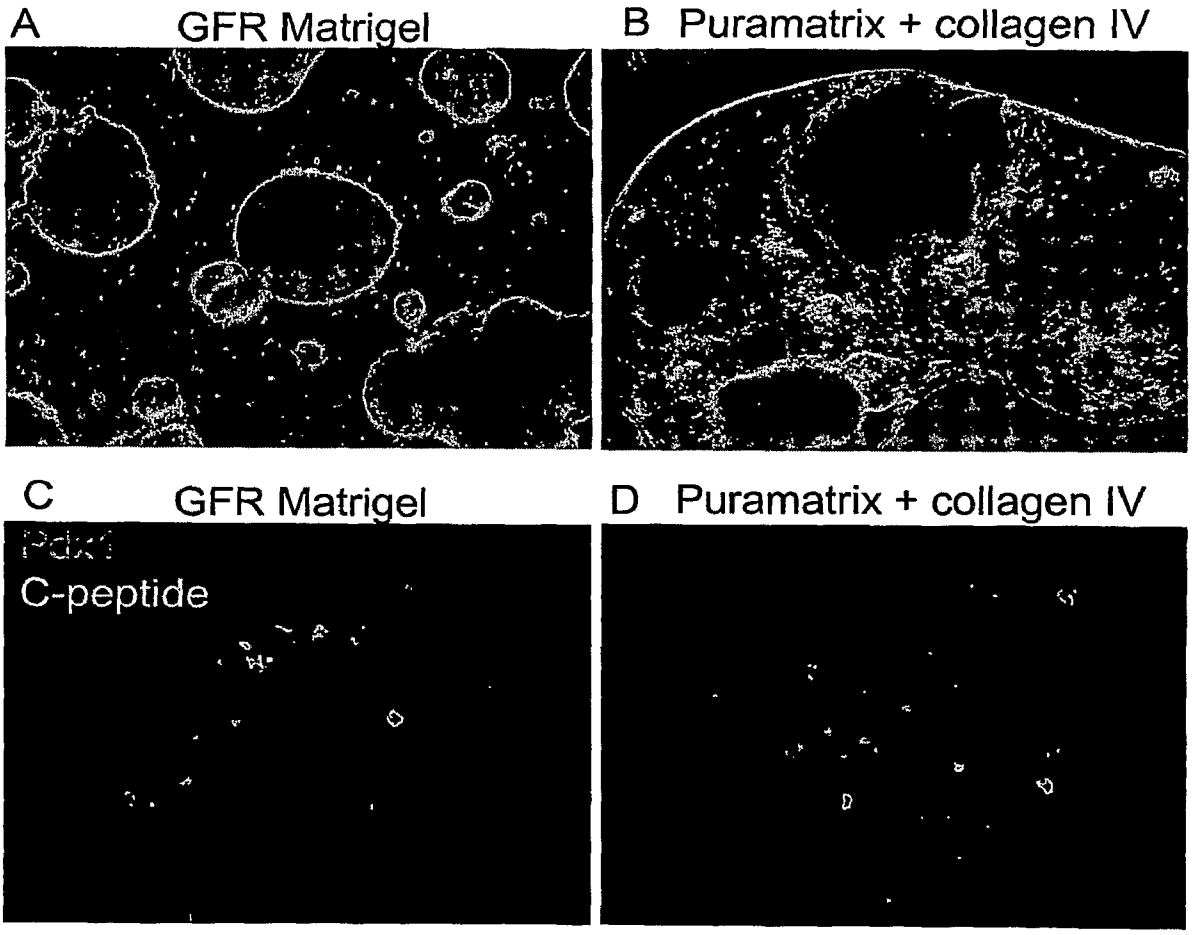

A
Day 37 gene expression
Relative to Matrigel
0
1
2
3
4
5
6
Pdx1
Insulin
Matrigel
Puramatrix + CIV
Matrigel
Puramatrix + CIV
B
Day 37 C-peptide release
ng/ml
0.0
0.5
1.0
1.5
2.0
2.5
matrigel
Puramatrix + CIV

Figure 40.


A
GFR Matrigel
B Puramatrix + collagen IV
C
GFR Matrigel
Pdx1
C-peptide
D Puramatrix + collagen IV