INDUCTION OF PANCREATIC BETA CELLS BY STEM CELL DIFFERENTIATION WITH RNA

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/423,120, filed on November 16, 2016, which is incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The present disclosure relates to directing the induction of pancreatic beta cells from pluripotent stem cells through kinetically controlled cell growth processes utilizing specific combinations and ranges of cell density, reagent concentrations, and specific combinations of mRNAs.

BACKGROUND OF THE INVENTION

[0003] The recent efforts in the generation and consequent differentiation of human stem cells has changed the paradigms concerning the plasticity of cell fate, models for human diseases, and clinical therapeutics. Both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) made from somatic cells can be differentiated into an increasing list of specific cell types indistinguishable from their corresponding primary cells. As a result, stem cells are quite promising for developing new human cell therapies. iPSCs show particular potential in the field of personalized medicine because of the unlimited availability of cells, the noninvasiveness of the procedure to obtain the cells, and the potential to immune-match each treatment to individual patients, granting freedom from immunosuppressive drugs.

[0004] Lots of research dollars are being spent on developing cell replacement therapies to treat or prevent various human diseases. For example, autoimmunity causes patients with type 1 diabetes (T1D) to lose their pancreatic beta cells—and hence their ability to respond to blood glucose levels and produce their own insulin. Therefore, T1D patients can greatly benefit from replacement of their insulin-producing beta cells with an outside source. Transplantation of human islets, which contain insulin-producing beta cells as well as other endocrine cells, have been performed with some success, but finding a reliable supply of donor pancreases remains a significant hurdle to overcome. Now, many academic and industrial groups have developed ways of directing ESCs or iPSCs to become pancreatic progenitor cells, aimed at creating insulin-secreting beta cells in the hopes of eventually using stem cell therapy to with the derived insulin-producing, glucose-responsive cells. However, most of these methods known in the public domain by the time of this filing could not generate fully functional or mature insulin-secreting pancreatic beta cells.

[0005] To alleviate the burden of cost and inconsistency, those of skill in the art often resort to finding small molecules that can influence signal pathways as an agonist or antagonist of growth factor receptors, thereby in a way substituting growth factors. Small molecules are typically much cheaper than growth factors. However, one major disadvantage of small molecules is the non-specific effects they may exert on unintended targets, such as cell membrane-bound receptors, intracellular organelles, or genomic components, etc.

[0006] Another key component of a typical differentiation protocol is the media for culturing cells, which may be composed of nutrients (lipids, amino acids, carbohydrates, vitamins, etc.), proper concentrations of salts, pH buffering agents, critical elements, and common protein factors such as insulin or serum albumin. Different types of cells have different requirements of nutrients and media components and is further complicated by cell type specific growth factors and small molecules for signaling. Therefore, a special "differentiation medium" is often painstakingly tested by removing or adding one component at a time.

[0007] In clinical applications of stem cell derived tissue cells, most components of the established differentiation media require individual certification under the current good manufacturing practice (cGMP) regulations; for example, growth factors need to be produced by special procedures and require individual certification. Likewise, some small molecules added to specific differentiation media are produced through special chemical synthesis processes that vary in purity, stability, and toxicity. In the field of stem cell cultures, some previously published protocols also rely on animal products such as serum or Matrigel.

SUMMARY OF THE INVENTION

[0008] Despite the recent advances in generating iPSC-derived pancreatic beta cells, efficient methods to consistently generate insulin-producing beta cells are still under-developed. Many current protocols require the use of combinations of growth factors, hormones, cytokines, signal peptides and other intercellular signal molecules (collectively referred to growth factors for simplicity in this text) at each step along a differentiation cascade to produce insulin-producing beta cells. Unfortunately, when supplied as purified polypeptides, growth factors are generally expensive, unstable, and inconsistent from batch-to-batch, making them difficult to use. Additionally, because growth factors function in a highly specific yet combinatorial manner, each stage of differentiation is dictated by a different set of growth factors, which is difficult and costly to optimize. One of the goals of the current invention was to fundamentally remove the need for growth factors in guiding the generation of pancreatic beta cells.

[0009] The present disclosure provides methods for inducing stem cell differentiation by modulating cell growth kinetics and associated parameters whereby specific combination of

cells density, reagent concentrations, and combinations of mRNAs are used to control the direction of the differentiation/induction.

[0010] To achieve the object and in accordance with the purpose of the invention, as embodied and broadly described herein, one aspect of the invention relates to a method of inducing differentiation of stem cells into glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of: (a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation; (b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage; (c) directing the differentiating cells towards endoderm cells through culture cell transfection with a first combination of mRNAs at an effective dose and within specific time windows; (d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with a second combination of mRNAs; (e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with a third combination of mRNAs; and (f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response.

[0011] In one embodiment, the invention relates to a method of inducing differentiation of stem cells into glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of: (a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation; (b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage; (c) directing the differentiating cells towards endoderm cells through culture cell transfection with a first combination of mRNAs at an effective dose and within specific time windows; (d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with a second combination of mRNAs; (e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with a third combination of mRNAs; and (f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response, wherein said first combination of mRNAs comprises FoxA2 mRNAs.

[0012] In another embodiment, the invention relates to a method of inducing differentiation of stem cells into glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of: (a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation; (b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage; (c) directing the differentiating cells towards endoderm cells through culture cell transfection with a first combination of mRNAs at an effective dose and within specific time windows; (d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with a second combination of mRNAs; (e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with a third combination of mRNAs; and (f) collecting clusters

enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response, wherein said first combination of mRNAs comprises Sox17 mRNAs.

[0013] In another embodiment, the invention relates to a method of inducing differentiation of stem cells into glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of: (a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation; (b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage; (c) directing the differentiating cells towards endoderm cells through culture cell transfection with a first combination of mRNAs at an effective dose and within specific time windows; (d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with a second combination of mRNAs; (e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with a third combination of mRNAs; and (f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response, wherein said first combination of mRNAs comprises FoxA2 and Sox17 mRNAs.

[0014] In another embodiment, the invention relates to a method of inducing differentiation of stem cells into glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of: (a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation; (b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage; (c) directing the differentiating cells towards endoderm cells through culture cell transfection with a first combination of mRNAs at an effective dose and within specific time windows; (d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with a second combination of mRNAs; (e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with a third combination of mRNAs; and (f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response, wherein said first combination of mRNAs comprises FoxA2, Sox17, GATA4, and GATA6 mRNAs.

[0015] In another embodiment, the invention relates to a method of inducing differentiation of stem cells into glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of: (a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation; (b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage; (c) directing the differentiating cells towards endoderm cells through culture cell transfection with a first combination of mRNAs at an effective dose and within specific time windows; (d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with a second combination of mRNAs; (e) further maturing said pancreatic progenitor cells into

pancreatic endocrine cells with a third combination of mRNAs; and (f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response, wherein said second combination of mRNAs comprises at least one of PDX1, Hlxb9, Ptf1a, ixl1 HNF1a and b, and Sox9 mRNAs.

[0016] In another embodiment, the invention relates to a method of inducing differentiation of stem cells into glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of: (a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation; (b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage; (c) directing the differentiating cells towards endoderm cells through culture cell transfection with a first combination of mRNAs at an effective dose and within specific time windows; (d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with a second combination of mRNAs; (e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with a third combination of mRNAs; and (f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response, wherein said third combination of mRNAs comprises at least one of PDX1, NKX6.1, NKX2.2, Pax6, Pax4, Hlxb9, and Ngn3 mRNAs.

[0017] In another embodiment, the invention relates to a method of inducing differentiation of stem cells into glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of: (a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation; (b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage; (c) directing the differentiating cells towards endoderm cells through culture cell transfection with a first combination of mRNAs at an effective dose and within specific time windows; (d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with a second combination of mRNAs; (e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with a third combination of mRNAs; and (f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response, wherein the mRNA to effect a pancreatic beta cell fate commitment comprises at least one of NKX6.1, MAFA, or MAFA mRNAs.

[0018] In another embodiment, the invention relates to a method of inducing differentiation of stem cells into glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of: (a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation; (b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage; (c) directing the differentiating cells towards endoderm cells through culture cell transfection with a first combination of mRNAs at an effective dose and within specific time windows; (d) further

directing said endoderm cells towards pancreatic progenitor cells through transfection with a second combination of mRNAs; (e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with a third combination of mRNAs; and (f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response, wherein said starting cells are harvested from a body fluid or tissue.

[0019] One aspect of the invention relates to a cell obtained by a method of inducing differentiation of stem cells into glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of: (a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation; (b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage; (c) directing the differentiating cells towards endoderm cells through culture cell transfection with a first combination of mRNAs at an effective dose and within specific time windows; (d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with a second combination of mRNAs; (e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with a third combination of mRNAs; and (f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response.

[0020] One aspect of the invention relates to a composition for treating disease, disorder or malformation comprising a cell obtained by a method of inducing differentiation of stem cells into glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of: (a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation; (b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage; (c) directing the differentiating cells towards endoderm cells through culture cell transfection with a first combination of mRNAs at an effective dose and within specific time windows; (d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with a second combination of mRNAs; (e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with a third combination of mRNAs; and (f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response.

[0021] One aspect of the invention relates to a method of treating disease, disorder, or malformation, comprising the step of administering into the subject in need thereof at least one of a cell obtained by a method of inducing differentiation of stem cells into glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of: (a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation; (b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage; (c) directing the differentiating cells

towards endoderm cells through culture cell transfection with a first combination of mRNAs at an effective dose and within specific time windows; (d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with a second combination of mRNAs; (e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with a third combination of mRNAs; and (f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response and a composition for treating disease, disorder or malformation comprising a cell obtained by a method of inducing differentiation of stem cells into glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of: (a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation; (b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage; (c) directing the differentiating cells towards endoderm cells through culture cell transfection with a first combination of mRNAs at an effective dose and within specific time windows; (d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with a second combination of mRNAs; (e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with a third combination of mRNAs; and (f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response.

[0022] In one embodiment, the invention relates to a method of treating disease, disorder, or malformation, comprising the step of administering into the subject in need thereof at least one of a cell obtained by a method of inducing differentiation of stem cells into glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of: (a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation; (b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage; (c) directing the differentiating cells towards endoderm cells through culture cell transfection with a first combination of mRNAs at an effective dose and within specific time windows; (d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with a second combination of mRNAs; (e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with a third combination of mRNAs; and (f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response and a composition for treating disease, disorder or malformation comprising a cell obtained by a method of inducing differentiation of stem cells into glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of: (a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation; (b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage; (c) directing the differentiating cells towards endoderm cells through

culture cell transfection with a first combination of mRNAs at an effective dose and within specific time windows; (d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with a second combination of mRNAs; (e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with a third combination of mRNAs; and (f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response, wherein said cell is derived from the recipient subject.

[0023] In one embodiment, the invention relates to a method of treating disease, disorder, or malformation, comprising the step of administering into the subject in need thereof at least one of a cell obtained by a method of inducing differentiation of stem cells into glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of: (a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation; (b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage; (c) directing the differentiating cells towards endoderm cells through culture cell transfection with a first combination of mRNAs at an effective dose and within specific time windows; (d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with a second combination of mRNAs; (e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with a third combination of mRNAs; and (f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response and a composition for treating disease, disorder or malformation comprising a cell obtained by a method of inducing differentiation of stem cells into glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of: (a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation; (b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage; (c) directing the differentiating cells towards endoderm cells through culture cell transfection with a first combination of mRNAs at an effective dose and within specific time windows; (d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with a second combination of mRNAs; (e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with a third combination of mRNAs; and (f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response, wherein said starting cells are harvested from the recipient.

[0024] A method of producing induced glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of: (a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation; (b) inducing said starting cells to exit the pluripotent state towards

the mesendoderm lineage; (c) directing the differentiating cells towards endoderm cells through culture cell transfection with a first combination of mRNAs at an effective dose and within the specific time windows; (d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with a second combination of mRNAs; (e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with a third combination of mRNAs; and (f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response.

[0025] Accordingly, the present disclosure also provides novel methods of achieving cell fate determination with no or reduced use of small molecules. A major benefit of the current invention is the simplicity of establishing differentiation medium through use of properly supplied mRNAs of differentiation-directing genes. Optimal combination of mRNAs and appropriate medium, however, can still benefit the process and is an integral part of the current invention. Another incentive behind the current invention is to create a new method that is primarily based on a single type of molecule suitable to uniform certification and quality control processes.

[0026] In the current disclosure, the disclosure describes the novel, enabling processes that involve how cell density and rate of division should be managed to achieve desired differentiation results. Further disclosure teaches the optimization of timing, order of addition, RNA doses and ratios among different RNAs during transfection of RNAs, and their duration or number of repeats. The invention further relates to the choices of surface of culture vessels and environmental conditions such as oxygen concentration. The invention further includes processes and methods of selection of desired cells or enhancement of their percentage in the overall population, and methods of cryopreservation and re-culture of differentiated cells.

[0027] The present invention provides methods for inducing and/or producing stem cells and differentiation by modulating cell growth kinetics and associated parameters whereby specific combination of cells density, reagent concentrations, and combinations of mRNAs are used to control the direction of the differentiation/induction. In one aspect, the current invention provides a newly developed protocol for producing functional and more mature beta cells that function *in vivo*, and methods of using the cells in therapies for indications involving various types of type I and type II diabetes.

[0028] In certain embodiments, the exemplary stem cell induction protocol can be represented by the regimen and steps as described and set forth in the examples below. In some aspects of this invention, very high efficiency was achieved and at lower costs without using large amount of growth factors, by contacting mRNAs with stem cells at critical fate changing points at the

right dose and delivery conditions. Because mRNAs are more specific in directing cellular and developmental events, via encoding functional proteins, the disclosed method is surprisingly more robust than any known methods in creating functional pancreatic beta cells, thereby paving a way for treating diabetes and other diabetes-related conditions in human or mammalian subjects.

[0029] The present disclosure provides differentiation methods that utilize highly efficient and well-controlled expression of master control genes or key transcription factors in tissue specific differentiation. More specifically, these factors are introduced into pluripotent stem cells in the form of properly modified and purified mRNA molecules demonstrated through the provided exemplar.

[0030] In one aspect, the present disclosure provides a method of inducing cell differentiation comprising: utilizing key cell fate factors and fusions between conventional transcription factors (TFs) with transactivation domains, optimized for directing stem cells towards different types of cells; introducing these factors as synthetic messenger RNA (mRNA) into cultured pluripotent stem cells at the preferred density by methods that result in appropriate levels of transgene expression; maintaining cells under optimized conditions to result in high efficiency of specific differentiation whereby the pluripotent state or progenitor state of stem cells or progenitor cells is induced towards a specific lineage or tissue cell type.

[0031] In one aspect, the present disclosure provides a method of producing pluripotent state or progenitor state of stem cells or progenitor cells capable of being induced towards a specific lineage or tissue cell type using a method of cell-induction comprising: utilizing key cell fate factors and fusions between conventional transcription factors (TFs) with transactivation domains, optimized for directing stem cells towards different types of cells; introducing these factors as synthetic messenger RNA (mRNA) into cultured pluripotent stem cells at the preferred density by methods that result in appropriate levels of transgene expression; maintaining cells under optimized conditions to result in high efficiency of specific differentiation.

[0032] In one aspect, the present disclosure provides a method for producing induced glucose-sensing insulin-secreting pancreatic beta cells, the method comprising: a) culturing iPSCs as starting cells under experimentally verified conditions as disclosed herein, prepare the cells as starting cells for differentiation; b) inducing the starting cells to exit the pluripotent state towards the mesendoderm lineage; c) directing the differentiating cells towards endoderm by using endoderm specifying genes' mRNA through culture cell transfection at disclosed dose and within the specific time windows; d) further directing the endoderm cells towards pancreatic

progenitor cells using a further gene's or a combination of genes' mRNA molecules through transfection; e) further maturing the pancreatic progenitor cells into pancreatic endocrine cells with yet another gene's or combination of genes' mRNAs; and f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secret insulin in response.

[0033] In another aspect, the disclosure provides methods for changing the pluripotent state or progenitor state of stem cells or progenitor cells towards a specific lineage or tissue cell type, comprising at least one of: generating stem cells expressing critical cell fate genes (collectively referred as stem cells), including key cell fate factors and fusions between conventional transcription factors (TFs) with transactivation domains, optimized for directing stem cells towards different types of cells; introducing these factors as synthetic messenger RNA (mRNA) into cultured pluripotent stem cells at the preferred density by methods that result in appropriate levels of transgene expression; maintaining cell under optimized conditions to result in high efficiency of specific differentiation.

[0034] Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The objects and advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims.

[0035] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

[0036] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several embodiments of the invention and together with the description, serve to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0037] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0038] The foregoing aspects and advantages of this invention may become apparent from the following detailed description with reference to the accompanying drawings in which:

[0039] FIG. 1A shows endoderm cells from different starting densities and illustrate an exemplary embodiment of endoderm induction.

[0040] FIG. 1B shows endoderm cells induced from iPSCs by using Sox17 mRNA at various densities (e.g. from low to high density as described in the Example) and illustrates an exemplary embodiment of endoderm induction.

[0041] FIG. 2 shows that pancreatic progenitor cells started from different endoderm cell densities and illustrate an exemplary embodiment of pancreatic progenitor induction. FIG. 2A shows one view of exemplary cell density during the induction. FIG. 2B shows one view of exemplary cell density during the induction. FIG. 2C shows one view of exemplary cell density during the induction. FIG. 2D shows one view of exemplary cell density during the induction.

[0042] FIG. 3 shows pancreatic endocrine cells started from different endoderm cell densities forming clusters and illustrate an exemplary embodiment of pancreatic endocrine induction. FIG. 3A shows one view of exemplary cell density/clustering during the induction. FIG. 3B shows one view of exemplary cell density/clustering during the induction. FIG. 3C shows one view of exemplary cell density/clustering during the induction.

[0043] FIG. 4 shows pancreatic endocrine cells matured in culture and produced clusters that floated and illustrates an exemplary embodiment of pancreatic endocrine maturation. FIG. 4A shows one view of exemplary cell maturation during the induction. FIG. 4B shows one view of exemplary cell maturation during the induction. FIG. 4C shows one view of exemplary cell maturation during the induction.

[0044] FIG. 5A shows morphology of beta cell cluster by phase contrast and illustrate an exemplary embodiment of pancreatic beta cell islet-like cluster formation.

[0045] FIG. 5B shows insulin staining (green) of beta cells 10 days post differentiation (Dapi is shown in blue) and illustrate an exemplary embodiment of pancreatic beta cell islet-like cluster formation.

[0046] FIG. 6 shows endoderm and beta cells derived from human iPSCs show specific cell markers. FIG. 6A shows Endoderm cells with the Green: CXCR4 and the Blue: DAPI nuclear reference. FIG. 6B shows Endoderm cells with the Green: FOXA1; the Blue: DAPI; the Red: Phalloidin cell membrane. FIG. 6C shows Beta cell differentiation day 10, Green: insulin; Blue: DAPI Nuclear. FIG 6D shows Beta cell differentiation day 8, Green: Nuclear NKX6.1 Red: Phalloidin cell membrane.

[0047] FIG. 7 shows glucose-stimulated insulin production of chemically derived (Melton protocol) vs. mRNA derived (Allele protocol) beta cells (cells received a series of glucose challenges 1 hour apart) and illustrates pancreatic beta-islets created in vitro display glucose-sensing insulin secretion.

[0048] FIG. 8 shows two million iPSCs in the starting population were transfected using MaxCyte STX, set at Optimization 2, OC-100 processing assembly. Photos were taken 24 hours post-transfection using an EVOS imaging system at 10X. FIG. 8A shows a representative view of the iPSCs during the exemplary transfection protocol. FIG. 8B shows a representative view of the iPSCs during the exemplary transfection protocol. FIG. 8C shows a representative view of the iPSCs during the exemplary transfection protocol. FIG 8D shows a representative view of the iPSCs during the exemplary transfection protocol. FIG 8E shows a representative view of the iPSCs during the exemplary transfection protocol. FIG 8F shows a representative view of the iPSCs during the exemplary transfection protocol.

[0049] FIG 9 shows large beta cell islets formed in 3D from 2D culture at the end of mRNA-mediated pancreatic beta cell differentiation. FIG. 9A shows a representative view of the beta cells associated with the differentiation. FIG. 9B shows a representative view of the beta cells associated with the differentiation.

DETAILED DESCRIPTION OF THE INVENTION

[0050] When describing the present invention, all terms not defined herein have their common meanings recognized in the art. To the extent that the following description is of a specific embodiment or a particular use of the invention, it is intended to be illustrative only, and not limiting of the claimed invention. The following description is intended to cover all alternatives, modifications and equivalents that are included in the spirit and scope of the invention.

[0051] The concept of that a “master control” gene, i.e. one key gene (typically a transcription factor gene, sometimes a small number of genes working together) can decide the fate of cells and tissues and eventually the formation of an entire organ during development, has been generally accepted based on studies in muscle (MyoD), eye (Pax6), and other fields of developmental biology. Shinya Yamanaka’s discovery that differentiated cells can be reverted to a pluripotent state by the expression of a select group of transcription factors expressed in stem cells demonstrated the power of a small number of key transcription factors in driving cells through a lengthy, multi-stage fate change. Work by other groups on iPSC generation expanded the choices of reprogramming factors and showed that some variations can be tolerated in transcription factor choices for the purpose of reprogramming. In Yamanaka’s original work, expression of the reprogramming factors was achieved through the application of viral vectors which integrate into the genome because prolonged expression of these factors is required to effect cell transformation. The attendant modification of the genome represents an important hurdle to therapeutic application of iPSCs, while the possibility of reactivated expression from integrated viral cassettes is a concern even for in vitro studies. The application of mRNA

transfection to reprogramming as most recently disclosed by the current inventor group is particularly appealing as this system allows the expression of reprogramming cocktails and even individual component factors to be modulated in short time frames simply by changing which transcripts are added to the cell culture media. Once transfection of a particular factor is terminated, ectopic expression within the target cells ceases quickly due to the rapid decay of mRNA in the cytoplasm. Even though mRNA does not persist in the target cell, its ability to be directly translated in the cytoplasm, without the need of rate-limiting nuclear translocation as in the case of transfected DNA and integrating viral vectors, more than compensate for mRNA's short half-life to result in highly efficient expression but well within a small time window, which is critical for cell fate determination.

[0052] Long-lasting DNA vectors, such as episomal plasmids, when used for cell fate alteration, require weaning to reduce any risk of random genomic integration. RNA viruses or virus-derivatives, such as the Sendai virus or Venezuelan equine encephalitis (VEE) virus, even after being stripped to be a modified noninfectious RNA replicon, still carries viral elements, prone to recombination with viral elements hidden in the host genome. It is always difficult to be completely sure that the cells are rid of the viral vectors without tedious finding of proof in the form of negative data. The current invention discloses multiple inventive steps aimed at applying the advantages of mRNA-based cell fate determination to directed differentiation. In summary, the current disclosure teaches a single or multiple rounds of ectopic transcription factor expression in a streamlined method to direct cell differentiation.

[0053] Nonetheless, there are technical barriers to mRNA-based stem cell differentiation. Not all stem cell types and culture media are equally conducive to efficient mRNA delivery, and this is currently an impediment to mRNA-based differentiation. It has also been commonly known that stem cells, particularly most human stem cell lines, are rather difficult to culture without forming transfection-resistant patches. It is part of the current invention's teaching that pluripotent stem cells can be grown under conditions that most of the cells can be transfected with modified mRNAs. In another embodiment, the dose of RNA and transfection reagent (both of which have associated toxicities) are to be provided to the cells at levels capable of exerting master control gene effects while supporting the viability of the target cells in the face of the pro-apoptotic and cytostatic forces engendered by the cell fate changing process.

[0054] Accordingly, in view of the problems associated with the previously known stem cell differentiation procedures, the novel methods, materials, and protocols described herein produce different cell types from iPSCs or ESCs with improved efficiency of the process and quality of the resultant cells. The current invention achieved significant improvements through potentiation

of the TF mRNA delivered to the target stem cells. The current invention also provides novel protocols which support the production of footprint-free tissue cells from human stem cells without the use of feeder cells or any other potentially xeno-contaminated reagents. The new protocols extend the benefits of the modified mRNA and help clear remaining roadblocks to the therapeutic application of stem cell derivation technology.

[0055] Given that differentiation from pluripotent to terminally differentiated state often takes multiple steps, requiring a time frame of several weeks to even months, the growth factor-based, stepwise strategy is intrinsically inefficient and tedious. Accordingly, embodiments of the present invention fundamentally remove the need for growth factors in guiding generation of pancreatic beta cells.

[0056] More specifically, this invention relates to changing the pluripotent state or progenitor state of stem cells or progenitor cells towards a specific lineage or tissue cell type by expressing critical cell fate genes (collectively referred as stem cells), including key cell fate factors and fusions between conventional transcription factors (TFs) with transactivation domains, optimized for directing stem cells towards different types of cells; introducing these factors as synthetic messenger RNA (mRNA) into cultured pluripotent stem cells at the preferred density by methods that result in appropriate levels of transgene expression; maintaining cells under optimized conditions to result in previously unattainable efficiency of specific differentiation. Factors expressed through introduction of mRNA can also include growth factors, cytokines, hormones, signal peptides and other cell fate influencing secreted factors or modifying enzymes. Using similar procedure, microRNAs (miRNAs) or other non-protein-coding RNAs can be introduced into cells under cell state transition in order to direct differentiation. Compared to other methods that are known in the art, the current invention dramatically reduces the time, cost, and effort involved in stem cell differentiation into beta cells.

[0057] This invention describes a method of changing the pluripotent state or progenitor state of stem cells or progenitor cells towards a specific lineage or tissue cell type, comprising at least one of: expressing critical cell fate genes, including key cell fate factors, and optimized for directing stem cells towards different types of cells; introducing these factors as synthetic messenger RNA (mRNA) into cultured pluripotent stem cells at the preferred density by methods that result in appropriate levels of transgene expression; maintaining cell under optimized conditions to result in high efficiency of specific differentiation.

[0058] In certain embodiments, the fully stabilized, expanded iPSCs are provided.

[0059] In certain embodiments, there is no need to clear episomes or RNA virus (e.g., Sendai), which can take 10+ passages of iPSCs post-isolation.

[0060] In certain embodiments, the process is feeder-free.

[0061] In certain embodiments, the process is xeno-free, comprising all synthetic or human reagents and no non-human animal-derived components.

[0062] In certain embodiments, the process is footprint-free: having no random integration of DNA into genome (as often happens with episomal).

[0063] In certain embodiments, the process yields a fully-customized genetic background via patient-specific starting tissue and/or genome-editing.

[0064] In another experiment, as an alternative to the process outlined in Table 1, iPSC cells grown as spheres in suspension were transfected directly using electroporation, (for example, using MaxCyte STX electroporator) without plating on the surface of a plate. In one embodiment, 2 million starting iPSCs in spheres were transfected in suspension with different mRNA, e.g. Sox17 or Pax6, or mock transfected. The mRNA amount tested in Figure 8 was 2500ng. Cells were then grown in NBM in the case of Sox17 transfection, or MEMalpha with KSR in the case of Pax6 transfection. Transfection can be repeated 1, 2, 3, 4, 5 or even more times if the transition takes longer period of time. As result, after the 1st transfection of Sox17 mRNA, the cell clusters became significantly smaller and less compact spheres, losing defined "edge" or outer boundary. In contrast, mock-transfected spheres maintain well-defined, showing clearly visible outer "edge" in 2D photos. The smaller spheres of the untransfected or mock-transfected iPSCs have a transparent appearance, whereas the bigger ones look less transparent for being thick in cell layers. For comparison, iPSC spheres transfected with Pax6 (a neural differentiation TF) mRNA progressed towards ectoderm, i.e. neural progenitor cells, of which the spheres became darker and had less sharp "edge" than mock-transfected, but were bigger in size and had more defined boundaries than the Sox17 transfected.

[0065] By the same principle and similar methods, germ layer-specific intermediate cells such as endoderm cells, and more downstream intermediate cells such as hepatocyte progenitor cells, pancreatic progenitor cells, etc., can also be transfected with additional TF mRNAs in spheres. Cells transfected this way are more resistant to toxicity from small molecules, growth factors, or other elements in cell cultures, and should be in general more efficient in differentiation than 2D transfection using chemical reagents. This observation, unseen in scientific publication, was made inadvertently during a testing of an electroporation equipment, and served as an enabling method as part of the current disclosure.

DEFINITIONS

[0066] To facilitate the understanding of this invention, a number of terms are defined below. Terms defined herein have meanings as commonly understood by a person of ordinary skill in

the areas relevant to the present invention. Terms such as "a", "an" and "the" are not intended to refer to only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the invention, but their usage does not delimit the invention, except as outlined in the claims.

[0067] The term “beta cell-like cell” is intended to mean a cell sharing features with a pancreatic beta cell. Beta cell-like cells are further defined by morphological characteristics as well as by specific marker characteristics. As induced pluripotent stem cell-derived beta cell-like cells share similar characteristics (including marker and hormonal characteristics) with pancreatic beta cells, induced pluripotent stem cell-derived beta cell-like cells may be used interchangeably with induced pluripotent stem cell-derived beta cell.

[0068] An “embryoid body” refers to an aggregate of cells derived from pluripotent cells, where cell aggregation can be initiated by any method that prevents the cells from adhering to a surface to form typical colony growth. As used herein, “embryoid body” refers to a three-dimensional spheroid aggregate of pluripotent stem cells, including but not limited to embryonic stem cells derived from the blastocyst stage of embryos from mammalian sources. An embryoid body can be formed from embryonic stem cells derived through any technique generally known in the art, including but not limited to somatic cell nuclear transfer or the reprogramming of somatic cells to yield induced pluripotent stem cells.

[0069] As used herein, the term “induced pluripotent stem cells” refers to a pluripotent stem cell derived from a somatic cell (e.g. an adult somatic cell). Induced pluripotent stem cells are similar to embryonic stem cells in their differentiation abilities to form any adult cell types, but are not derived from an embryo.

[0070] As used herein, “cell,” “cell line,” and “cell culture” include progeny. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Variant progeny that have the same function or biological property, as screened for in the originally transformed cell, are included.

[0071] As used herein, “composition” refers to a combination of active agent and at least one other compound or molecule, inert (for example, a detectable agent or label) or active, such as an adjuvant.

[0072] As used herein, “culturing” refers to maintaining cells under conditions in which they can proliferate and avoid senescence as a group of cells. “Culturing” can also include conditions in which the cells also or alternatively differentiate.

[0073] As used herein, “differentially expressed,” refers to the differential production of RNA, including but not limited to mRNA, tRNA, miRNA, siRNA, snRNA, and piRNA transcribed

from a gene or regulatory region of a genome or the protein product encoded by a gene as compared to the level of production of RNA by the same gene or regulator region in a normal or a control cell. In another context, "differentially expressed," also refers to nucleotide sequences or proteins in a cell or tissue which have different temporal and/or spatial expression profiles as compared to a normal or control cell.

[0074] As used herein, "overexpressed" or "overexpression" refers to an increased expression level of an RNA or protein product encoded by a gene as compared to the level of expression of the RNA or protein product in a normal or control cell.

[0075] As used herein, "underexpressed" or "underexpression" refers to decreased expression level of an RNA or protein product encoded by a gene as compared to the level of expression of the RNA or protein product in a normal or control cell.

[0076] As used herein, "differentiate" or "differentiation," refers to the process by which precursor or progenitor cells (i.e., pancreatic progenitor cells) differentiate into specific cell types, e.g., pancreatic beta cells.

[0077] As used herein, "effective amount" is an amount sufficient to effect beneficial or desired biological, emotional, medical, or clinical response of a cell, tissue, system, animal, or human. An effective amount can be administered in one or more administrations, applications, or dosages. The term also includes, within its scope, amounts effective to enhance normal physiological function.

[0078] As used herein, "expansion" or "expanded" in the context of cells, refers to an increase in the number of a characteristic cell type, or cell types, from an initial population of cells, which may or may not be identical. The initial cells used for expansion need not be the same as the cells generated from expansion. For instance, the expanded cells may be produced by ex vivo or in vitro growth and differentiation of the initial population of cells.

[0079] As used herein, "expression" refers to the process by which polynucleotides are transcribed into RNA transcripts. In the context of mRNA and other translated RNA species, "expression" also refers to the process or processes by which the transcribed RNA is subsequently translated into peptides, polypeptides, or proteins.

[0080] As used herein, "induced pluripotent stem cell" or "iPS cell" or "iPSC" refers to a cell capable of differentiating into multiple cell types that is artificially derived (not naturally derived) from a non-pluripotent cell.

[0081] As used herein, "integration free iPS cell" refers to an iPS cell that does not contain an exogenous transgene integrated into the genome of the non-pluripotent cell.

[0082] As used herein, "isolated" means separated from constituents, cellular and otherwise, in which the polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, are normally associated with in nature. A non-naturally occurring polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, do not require "isolation" to distinguish it from its naturally occurring counterpart.

[0083] As used herein, "concentrated" refers to a molecule, including but not limited to a polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, that is distinguishable from its naturally occurring counterpart in that the concentration or number of molecules per volume is greater than that of its naturally occurring counterpart.

[0084] As used herein, "diluted" refers to a molecule, including but not limited to a polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, that is distinguishable from its naturally occurring counterpart in that the concentration or number of molecules per volume is less than that of its naturally occurring counterpart.

[0085] As used herein, "separated" refers to the state of being physically divided from the original source or population such that the separated compound, agent, particle, or molecule can no longer be considered part of the original source or population.

[0086] As used herein, "mammal," for the purposes of treatments, refers to any animal classified as a mammal, including human, domestic and farm animals, nonhuman primates, and zoo, sports, or pet animals, such as, but not limited to, dogs, horses, cats, and cows.

[0087] As used herein, "stem cell" refers to any self-renewing totipotent, pluripotent cell or multipotent cell or progenitor cell or precursor cell that is capable of differentiating into multiple cell types.

[0088] As used herein, "totipotent" refers cells that can differentiate and give rise to all cells types in an organism, plus the extraembryoinc, or placental, cells.

[0089] As used herein, "pluripotent" refers to cells that can differentiate and give rise to all of the cell types that make up an organism, including any fetal or adult cell type, except for the extraembryonic, or placental, cells.

[0090] As used herein, "multipotent" refers to cells that can develop into more than one cell type, but are more limited than pluripotent cells in the cell types that they can develop into.

[0091] As used interchangeably herein, "subject," "individual," or "patient" refers to a vertebrate organism.

[0092] As used herein, "substantially pure cell population" refers to a population of cells having a specified cell marker characteristic and differentiation potential that is about 50%, preferably about 75-80%, more preferably about 85-90%, and most preferably at least about 95% of the

cells making up the total cell population. Thus, a "substantially pure cell population" refers to a population of cells that contain fewer than about 50%, preferably fewer than about 20-25%, more preferably fewer than about 10-15%, and most preferably fewer than about 5% of cells that do not display a specified marker characteristic and differentiation potential under designated assay conditions.

[0093] As used herein, "pre-differentiation" refers to the process by which precursor or progenitor cells (e.g., pluripotent stem cells) differentiate into intermediate cell types, e.g., pancreatic progenitor cells, which have the potential to differentiate further to final effector cells (e.g. beta cells).

[0094] As used herein, "therapeutic" refers to treating, healing, and/or ameliorating a disease, disorder, condition, or side effect, or to decreasing in the rate of advancement of a disease, disorder, condition, or side effect. The term also includes within its scope enhancing normal physiological function, pallative treatment, and partial remediation of a disease, disorder, condition or side effect.

[0095] The terms "treating" and "treatment" as used herein refer generally to obtaining a desired pharmacological and/or physiological effect. The effect may be prophylactic in terms of preventing or partially preventing a disease, symptom or condition thereof, and/or may be therapeutic in terms of a partial or complete cure of a disease, condition, symptom, or adverse effect attributed to the disease. The term "treatment" as used herein covers any treatment in a mammal, particularly a human, and includes: (a) preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it; (b) inhibiting the disease, i.e., arresting its development; or (c) relieving the disease, i.e., mitigating or ameliorating the disease and/or its symptoms or conditions. The term "treatment" as used herein refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

[0096] As used herein, "preventative" refers to hindering or stopping a disease or condition before it occurs, even if undiagnosed, or while the disease or condition is still in the sub-clinical phase.

[0097] As used herein, "active agent" refers to a substance, compound, or molecule, which is biologically active or otherwise induces a biological or physiological effect on a subject to which it is administered to.

[0098] As used herein, "pharmaceutically acceptable carrier" refers to diluent, adjuvant, excipient, or vehicle with which an active agent, chondrocytes of the present disclosure, or

composition containing chondrocytes of the present disclosure is administered in conjunction with and that is approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals and/or humans.

[0099] Unless otherwise defined herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art.

CELL TYPES

[00100] Exemplary cell types can include, for example, endoderm cells, pancreatic progenitor cells, pancreatic endocrine cells, and beta cells.

[00101] Exemplars of suitable surfaces for culture vessels include but are not limited to Vitronetin, E-cadherin, Corning® Synthemax® II or Matrigel for iPSCs, include but are not limited to Matrigel for endoderm, and include but are not limited to Matrigel or Collagen for pancreatic progenitors and pancreatic endocrine cells.

[00102] In one aspect, an exemplary method for dedifferentiating or reprogramming somatic cells can include the use of any one or more of a synthetic mRNA reprogramming factor selected from Oct4, Sox2, Klf4, cMyc, Nanog, and Lin28 and transactivation domains whereby the somatic cell is reprogrammed or de-differentiated. Methods and compositions for IPSC modulation are described in USSN 13/893,166 and USSN 14/292,317, the contents of which are hereby incorporated by reference.

[00103] In certain embodiments this disclosure provides protocols for the use of suspension cell cultures, and low cell-attachment culture plates and vessels can be used for such suspension cultures.

[00104] In certain embodiments, the environmental conditions such as oxygen concentration can be modulated for optimal induction conditions.

[00105] In certain embodiments, processes and methods of selection of desired cells or enhancement of their percentage confluence or cell density in the overall cell culture population are provided.

[00106] In certain embodiments, methods of producing cryopreserved differentiated beta cell-like cells are provided. In some embodiments differentiated cells are cryopreserved for optimal cell viability during or after storage, for example, by providing culture medium comprising HSA and/or DMSA. In some embodiments culture medium comprising, for example, 2.5% HSA and 10% DMSO in culture medium can be provided. In some embodiments, the cell numbers can be optimized for the further improvement of viability during or after storage.

[00107] Re-culture differentiated cells methods are also provided. Cells can be re-cultured in most culture vessels: e.g., T75 flask, T25 flask, 6-well plate, 96-well plate. Cells can be re-cultured in different cell density for different applications.

[00108] In certain embodiments, the present disclosure also provides methods for managing physical stress on the cells during handling throughout the differentiation process, thereby improving viability. Certain types of cells during the differentiation are very small, like iPSCs. These small cells are very sensitive to centrifugal force. iPSCs are very sensitive to excessive centrifuge force. Some types of cells during the differentiation are very sticky, like iPSCs and endoderm stage cells. These cells are very sensitive to sheer force. When handling these cells, a 10 mL pipet was used to avoid use any small tips and to avoid pipetting the cells up and down repeatedly. For maintenance, these cells can be cultured as colonies and then dissociated as clusters, instead of single cells. For differentiation, if single cells are necessary, one can end the dissociation prior to the cells detaching, remove the dissociation solution, and let the residual dissociation solution further dissociate the cells. This protocol is commonly used in cell culture.

[00109] The specification is most thoroughly understood in light of the teachings of the references cited within the specification. The embodiments within the specification provide an illustration of embodiments of the invention and should not be construed to limit the scope of the invention. The skilled artisan readily recognizes that many other embodiments are encompassed by the invention. All publications and patents cited in this disclosure are incorporated by reference in their entirety. To the extent the material incorporated by reference contradicts or is inconsistent with this specification, the specification will supersede any such material. The citation of any references herein is not an admission that such references are prior art to the present invention.

[00110] Unless otherwise indicated, all numbers expressing quantities of ingredients, reaction conditions, and so forth used in the specification, including claims, are to be understood as being modified in all instances by the term “about.” Accordingly, unless otherwise indicated to the contrary, the numerical parameters are approximations and may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should be construed in light of the number of significant digits and ordinary rounding approaches.

[00111] The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent

with the meaning of "one or more," "at least one," and "one or more than one." The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or."

[00112] Unless otherwise indicated, the term “at least” preceding a series of elements is to be understood to refer to every element in the series. 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.

[00113] 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. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described.

[00114] Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

EXAMPLES

[00115] The invention is now described with reference to the following Examples. These Examples are provided for the purpose of illustration only, and the invention is not limited to these Examples, but rather encompasses all variations that are evident as a result of the teaching provided herein.

EXAMPLE 1: generating endoderm cells from iPSCs

[00116] iPSCs were plated into standard size 6-well cell culture plates (about 9.5cm^2 growth area/well) or standard size 12-well cell culture plates (about 3.8cm^2 growth area/well) to begin differentiation. Other sized culture vessels are optionally applicable as well and sometimes may be more preferred over 6-well or 12-well plates because of higher efficiency of the use of reagents and time. Exemplary conditions for the high efficiency protocol for producing functional differentiated beta cells are provided in the Beta Cell Table, below, including starting cell stage, culture vessels, coating, dissociation agent, medium name and main components, seeding density for an exemplary 6 well plate and oxygen level.

Table 1: Beta Cell Differentiation

	2-6 days	1-3 days	1-3 days	3-6 days
	Stage 1	**Stage 2**	**Stage 3**	**Stage 4**
Starting Cells	iPSCs	Endoderm	Pancreatic Progenitor Cells	Pancreatic Endocrine Cells
Culture Vessels	Ultra-Low Attachment Plate / Flask	Culture Plate / Flask	Culture Plate / Flask	Culture Plate / Flask
Coating	None	Matrigel	Matrigel / Collagen I	Matrigel / Collagen I
Dissociation	EDTA	TypLE	TypLE	TypLE
Medium Names and Main Components	MEMa, DMEM/F12, DMEM B27 10-50 uM Insulin 5% KSR SOX17 mRNA transfection	DMEM/F12, MCDB131 + 8mM D-glucose PDX1 mRNA transfection	DMEM/F12, MCDB131 + 20mM D-glucose PDX1 and NKX6.1 mRNA transfection	CMRL NKX6.1 and MAFA mRNA transfection
Seeding Density (For 6-well Plate)	$1 \times 10^5 - 4 \times 10^5$ cells per well	$1 \times 10^5 - 3 \times 10^5$ cells per well	3×10^5 cells per well	3×10^5 cells per well
Oxygen	Normal Oxygen	Normal Oxygen	Normal Oxygen	Normal Oxygen

[00117] In a 6-well plate, cells of a population size from 1 x 10^5 to 4 x 10^5 per well have been successfully used. iPSCs were considered ready for differentiation when there were enough typical iPSCs colonies with sharp well-defined edges, where the cells are compact, and colonies were not overgrown. The quality of iPSC's of the present invention produced using these criteria proved to be critical for differentiation when the iPSC lines of the present invention were compared with iPSC lines that were produced by others using other methods.

[00118] iPSCs at this stage were induced to differentiate into mesendoderm lineage cells. It was surprisingly determined that suspension culture systems were very useful for scaling up at this stage even though most current protocols for differentiation prefer to use attached monolayer cells. More specifically, iPSCs grown in suspension for induction were more resistant to chemical toxicity and were easier to re-plate in later stages. Ultra-Low Attachment plates (Sigma-Aldrich) or other low attachment plates were used to encourage iPSC suspension cell growth.

[00119] When iPS cells need to be passaged, it was important to dissociate iPSC colonies with a protocol that caused low cytotoxicity and resulted in more small clusters of iPSCs, which can form spheres quickly if suspension culture is desired. iPSCs were dissociated using TripLE™ (ThermoFisher), Accutase (Life Technology), or EDTA by dissociating with EDTA of 0.1 mM, sometimes 0.5 mM, or 1 mM in DPBS (Fisher Scientific), at 37°C for 5 minutes. Various dissociation times were used successfully, including, in some embodiments, 1 to 2 minutes, and sometimes 10 to 20 minutes for this step. In some aspects the dissociation time may be 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 15, 16, 17, 18, 19, or 20 minutes.

[00120] For medium, MEMa, DMEM/F12, and DMEM B27 were tested with 10-50 uM insulin and 5% KSR were suitable to achieve the results as desired at this stage of differentiation. iPSCs were then induced to leave the pluripotent stage and differentiate towards mesendoderm by the presence of GSK3 inhibitors such as CHIR99021, CHIR98014, BIO or GSK inhibitor IX, and SB-216763, which de-repress the functions of the Wnt, BMP4, and Activin A pathway genes. In some embodiments the insulin is present at concentration of about, for example, 10, 11, 12, 13, 14, 15, 16, 17 18, 19, 20, 25, 30, 35, 40, 45 or 50 uM insulin, or in a range between any of the above-recited values. The GSK3 inhibitors were performed in a 1-, 2-, 3-day time windows and used FoxA2, CXCR4 positive cell counts as quality analysis when choosing time and concentrations of the inhibitors of, for example, 5 mM, 8 mM, 10 mM, etc. In some embodiments the inhibitors are be present at 5, 6, 7, 8, 9, or 10 mM, or in a range between any of the above-recited values.

[00121] In one experiment, cells were then transfected with FoxA2, and/or Sox17 mRNA at a dose of 20 ng per well with Stemgent Transfection Reagent (Stemgent). This transfection was also performed including GATA4 mRNA, and/ or GATA6 mRNA, and was repeated for 3, 4, 5 and 6 times, sometimes at a dose of mRNA 10 times higher, using Stemgent Transfection Reagent or other commercially available transfection reagents. Cells at this stage showed morphology that were closer to epithelial cells than mesenchymal cells, derived from iPSCs from different mRNA transfection times and starting density (FIG. 1).

[00122] In another experiment, as an alternative to the process outlined in Table 1, iPSC cells grown as spheres in suspension were transfected directly using electroporation, (for example, using MaxCyte STX electroporator) without plating on the surface of a plate. In one embodiment, 2 million starting iPSCs in spheres were transfected in suspension with different mRNA, e.g. Sox17 or Pax6, or mock transfected. The mRNA amount tested in FIG. 8 was 2500ng. Cells were then grown in NBM in the case of Sox17 transfection, or MEMalpha with KSR in the case of Pax6 transfection. Transfection can be repeated 1, 2, 3, 4, 5 or even more times if the transition takes longer period of time. As result, after the 1st transfection of Sox17 mRNA, the cell clusters became significantly smaller and less compact spheres, losing defined "edge" or outer boundary. In contrast, mock-transfected spheres maintain well-defined, showing clearly visible outer "edge" in 2D photos. The smaller spheres of the untransfected or mock-transfected iPSCs have a transparent appearance, whereas the bigger ones look less transparent for being thick in cell layers. For comparison, iPSC spheres transfected with Pax6 (a neural differentiation TF) mRNA progressed towards ectoderm, i.e. neural progenitor cells, of which the spheres became darker and had less sharp "edge" than mock-transfected, but were bigger in size and had more defined boundaries than the Sox17 transfected.

[00123] By the same principle and similar methods, germ layer-specific intermediate cells such as endoderm cells, and more downstream intermediate cells such as hepatic progenitor cells, pancreatic progenitor cells, etc., can also be transfected with additional TF mRNAs in spheres. Cells transfected this way are more resistant to toxicity from small molecules, growth factors, or other elements in cell cultures, and should be in general more efficient in differentiation than 2D transfection using chemical reagents. This observation, unseen in scientific publication, was made inadvertently during a testing of an electroporation equipment, and served as an enabling method as part of the current disclosure.

EXAMPLE 2: generating pancreatic progenitor cells from endoderm cells

[00124] Endoderm cells are plated on commercial cell culture vessels. 6-well plates were used in experiments shown in FIG. 2, but other standard commercially available well sizes are suitable and applicable. Plates were pre-coated with Matrigel (BD Biosciences), 1 x 10^5 – 1 x 10^6 cells were then plated in DMEM/F12 or MCDB131 supplemented with 8 mM D-glucose.

[00125] In one experiment, cells were then transfected with PDX1 mRNA. Additionally, the cells were transfected or co-transfected, for stronger effects, with mRNA for Hlxb9, Ptf1a, ixl1, HNF1a and b, and Sox9 mRNA at a dose of about 50 ng per well with Stemgent Transfection Reagent (Stemgent) in culture medium, and repeated for 2, 3, or more times, at doses ranging from as low as 10 ng and as high as 200 ng per well, using Stemgent Transfection Reagent or other commercially available transfection reagents. In some embodiments the initial or repeated dose of Stemgent may be 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 1120, 130, 140, 150, 160, 170, 180, 190, or 200 ng per well, or at a range between any of the above-recited values. The cells at this stage appear darker than endoderm cells and tended to form clusters (FIG. 2).

EXAMPLE 3: generating pancreatic endocrine cells from pancreatic progenitor cells

[00126] Pancreatic Progenitor cells were cultured in 6-well or other plates pre-coated with Matrigel (BD Biosciences) or Collagen I (Sigma) in DMEM/F12 or MCDB131 supplemented with 8 mM D-glucose. Other similar attachment cell culture medium is also suitable for use.

[00127] Pancreatic Progenitor cells were transfected with PDX1, NKX6.1, and/or NKX2.2, Pax6, Pax4, Hlxb9 Ngn3, mRNA at a dose of 10-200 ng per well with Stemgent Transfection Reagent (Stemgent) in culture medium supplemented with 200 ng/mL B18R. In some embodiments the dose of Stemgent may be 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 1120, 130, 140, 150, 160, 170, 180, 190, or 200 ng per well, or in a range between any of the above-recited values. Most commercially available transfection reagents are also applicable.

[00128] Pancreatic Progenitor cells were optionally treated at this stage with a series of growth factors for further differentiation. Cells were cultured in S5 media containing MCDB131 + 20mM D-Glucose + 1.754 g/L NaHCO3 + 2% FAF-BSA + ITS-X 1:200 + 2mM Glutamax + 0.25mM Vitamin C + 1% Pen/Strep + Heparin 10 ug/ml (Sigma; H3149). Cells were treated with S5 media with 0.25 uM Sant1 + 100 nM RA + 1 μM XXI (EMD Millipore) + 10 μM Alk5i II (Axxora) + 1 μM T3 (EMD Millipore) + 20 ng/ml Betacellulin (Thermo Fisher Scientific) every other day for four days. On the fifth day, cells were treated with S5 media with 25 nM RA + 1 μM XXI + 10 μM Alk5i II + 1 μM T3 + 20 ng/ml Betacellulin every other day for four days.

[00129] At this stage the cells further clustered and began to float from the monolayer, an early sign of the differentiation into and formation of pancreatic endocrine cells (FIG. 3).

EXAMPLE 4: maturing pancreatic endocrine cells

[00130] Pancreatic endocrine cells were cultured on commercial cell culture vessels. 6-well plates were used in these experiments, but all other types are also applicable. Plates were pre-coated with Matrigel (BD Biosciences) or Collagen I (Sigma) in CMRL (Mediatech). Ultra-Low Attachment plates or flasks were used after the clusters were collected.

[00131] These cells were then transfected with NKX6.1/MAFA or MAFA mRNA at a dose of 10-200 ng per well with Stemgent Transfection Reagent (Stemgent) in culture medium supplemented with 200 ng/mL B18R. Repeated transfections were performed 3 times, and optional additional transfections can be performed, if necessary. Other commercially available transfection reagents are also applicable.

[00132] Pancreatic Endocrine cells were also treated with a series of growth factors for further maturation. Cells were grown in S6 media containing CMRL 1066 Supplemented (Mediatech; 99-603-CV) + 10% FBS (HyClone, VWR; 16777) + 1% Pen/Strep. Cells were treated with S6 with 10 μM Alk5i II + 1 μM T3 every other day for 14 days.

[00133] At this stage, clusters of pancreatic endocrine cells, such as beta cells, form clusters of sizes from a few dozens to thousands of cells self-lifted from a monolayer of maturing pancreatic endocrine cells. The cell cultures at this stage were shown as examples in FIG. 4. These cells were stained with antibodies against cell stage specific protein markers such as NKX6.1 and insulin, the results of which confirmed that some of these cells are indeed pancreatic beta cells (FIG. 5). These cells also demonstrated specific cell fate markers along this artificially induced differentiation pathway to beta cell-like cells (FIG. 6). Furthermore, the beta cell clusters responded to glucose added to the culture medium and the beta cells secreted insulin in response (FIG. 7).

EXAMPLE 5: iPSC-derived beta cells respond to glucose in vitro

[00134] Clusters of cells formed in 3-dementional structures from the monolayer pancreatic endocrine cells, as shown in FIG. 4, were collected and transferred into 15 ml tubes, 2 $x10^5$ to 1 x 10^6 cells per tube, and growth medium was removed by centrifugation (300g for 2 minutes) and washed with 1 ml per sample of Krebs buffer (Melton publication 2014) twice. 3 ml of low glucose (2 mM) Krebs medium was added to the cells and incubated for 2 hours. The medium was removed by centrifugation, and the cells were washed 2 times with 1 ml Krebs buffer per sample. One ml of Krebs low glucose was then added to each tube and incubated for 30 minutes at 37°C in an incubator, with the cap left loose to allow air exchange. Then

supernatant was collected by centrifugation for analysis, the cells were then washed once with 1 ml of Krebs buffer, then incubated in Krebs buffer with high glucose (20 mM) for 30 minutes, then centrifuged to collect supernatant for analysis. The cells were washed twice before going through the low glucose-high glucose cycle again, for a total of 3 times. The supernatants were collected at each step, combining from 4-6 independent repeats, and were assayed for insulin level by using a commercially produced ELISA kit (ALPCO) and plate reader (Tecan or SpectroMax). The assay was also performed with beta cells created by essentially following the Melton protocol (2014) for comparison, and the results showed that beta cells created using the method of the present invention had lower baseline insulin expression, and higher insulin response rate to the increase of glucose in the medium compared to the Melton method (FIG. 7), which demonstrates the unexpected and surprising advantageous characteristics of the embodiments of the present disclosure.

EXAMPLE 6: iPSC-derived beta cells respond to glucose in animal models

[00135] To further validate the functions of the exemplary mature pancreatic beta cells produced according to the present invention, the glucose-responsive insulin-secreting function of the iPSC-derived pancreatic beta cells are tested in diabetes mouse models such as: 1) NOD mouse spontaneous type 1 diabetes model, 2) STZ induced type I diabetes model, 3) ob/ob or db/db type II diabetes model; or non-human primate models such as: STZ induced Macaques/Vervet monkeys or baboon type I diabetes models, or spontaneous or high-fat diets induced Macaques/Vervet monkeys or baboon type II diabetes models.

[00136] Delivery route: exemplary pancreatic beta cell spheres are infused into liver or kidney capsules, or omentum. Testing: serum insulin without glucose stimulation (week 2, 4, 8, 12, 16, 24) is measured; measurement is obtained on the serum insulin without glucose stimulation (week 2, 4, 8, 12, 16, 24); measurement is taken for fasting animals' blood glucose (week 2, 4, 8, 12, 16, 24); ICH of transplant site for Insulin is performed, and staining is performed for C-peptide and glucagon staining. The results demonstrate reversal or control of diabetic symptoms such as high blood glucose and associated effects on organs.

EXAMPLE 7: iPSC-derived beta cells respond to glucose in treating diabetes human patients

[00137] Clinical trials using human iPSC-derived pancreatic beta cells using the disclosed protocols adapted to suit under cGMP procedures are dosed according to animal studies with reference to other cell therapies, even though no iPSC-derived pancreatic beta cell therapy currently ongoing, pancreatic progenitor cells derived from human ESCs are at this moment being used in clinical trials aimed at treating Type I diabetes. Autologous, a preferred type, or allogeneic beta cells derived from iPS cells, are delivered through portal vein, for example, to treat induced type I diabetes, lean type II diabetes, late stage diabetes when a person's own beta cells are weaning or gone, or other types of beta cell malfunction-related disease types. The exemplary manufactured beta cells produced according to the practices of the current invention as described in the present disclosure are delivered to other parts of the human body such as muscles, connective tissues, pancreatic organs or certain sites of other organs.

CLAIMS

1. A method of inducing differentiation of stem cells into glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of:

(a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation;

(b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage;

(c) directing the differentiating cells towards endoderm cells through culture cell transfection with first mRNAs at an effective dose and within specific time windows, wherein the first mRNAs comprise FoxA2 mRNAs or Sox17 mRNAs;

(d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with second mRNAs, wherein the second mRNAs comprise at least one of PDX1, Hlxb9, Ptf1a, ixl1, HNF1a and b, and Sox9 mRNAs;

(e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with third mRNAs, wherein the third mRNAs comprise at least one of PDX1, NKX6.1, NKX2.2, Pax6, Pax4, Hlxb9, and Ngn3 mRNAs; and

(f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secrete insulin in response.

2. The method of claim 1, wherein said first mRNAs comprise FoxA2 mRNAs.

3. The method of claim 1, wherein said first mRNAs comprise Sox17 mRNAs.

4. The method of claim 1, wherein said first mRNAs comprise FoxA2 and Sox17 mRNAs.

5. The method of claim 1, wherein said first mRNAs comprise FoxA2, Sox17, GATA4, and GATA6 mRNAs.

6. The method of claim 1, wherein said second mRNAs comprise at least PDX1 mRNAs.

7. The method of claim 1, wherein said third mRNAs comprise at least PDX1 and NKX6.1 mRNAs.

8. The method of claim 1, further comprising effecting a pancreatic beta cell fate commitment with fourth mRNAs which comprise at least one of NKX6.1, MAFA, or MAFA mRNAs.

9. The method of claim 1, wherein said induced pluripotent stem cells are generated from a body fluid or tissue of a subject.

10. A method of producing induced glucose-sensing insulin-secreting pancreatic beta cells, comprising the steps of:

(a) culturing induced pluripotent stem cells as starting cells under conditions for differentiation;

(b) inducing said starting cells to exit the pluripotent state towards the mesendoderm lineage;

(c) directing the differentiating cells towards endoderm cells through culture cell transfection with first mRNAs at an effective dose and within the specific time windows, wherein the first mRNAs comprise FoxA2 mRNAs or Sox17 mRNAs;

(d) further directing said endoderm cells towards pancreatic progenitor cells through transfection with second mRNAs, wherein the second mRNAs comprise at least one of PDX1, Hlxb9, Ptf1a, ixl1, HNF1a and b, and Sox9 mRNAs;

(e) further maturing said pancreatic progenitor cells into pancreatic endocrine cells with third mRNAs, wherein the third mRNAs comprise at least one of PDX1, NKX6.1, NKX2.2, Pax6, Pax4, Hlxb9, and Ngn3 mRNAs; and

(f) collecting clusters enriched with pancreatic beta cells that are responsive to environment glucose and able to secrete insulin in response.