factors) is insufficient to specify homogeneous DE (quantified, by Chetty et al., 2013). BMP, FGF, VEGF and Wnt have also been employed together with TGFβ signals to generate DE (Cheng etal., 2012; Goldman et al., 2013; Green et al., 2011; Kroon et al., 2008; Nostro et al., 2011; Touboul et al., 2010). However, these morphogens are also implicated in mesoderm commitment (Davis et al. , 2008; Gertow et al., 2013) and their precise involvement in DE induction remains to be clarified.

[0006] There is currently no coherent understanding of the signaling logic underlying multiple steps of induction and patterning of the germ layers and differentiation into the various cell lineages.

[0007] It is an aim of the present invention to elucidate the underlying signaling logic of stem cell induction and differentiation in order to unilaterally drive stem cells to a single cell fate with minimal extraneous lineages,

SUMMARY ~~OF THE INVENTION~~

[0007a] In one aspect, provided herein is a method of differentiating pluripotent stem cells into definitive endoderm (DE) lineage comprising

i) contacting said pluripotent stem cells with:

a. one or more activators of TGFβ/Nodal signaling; and

b. one or more activators of Wnt signaling, wherein the one or more activators of Wnt signaling are CHIR99021 or Wnt3a or other family members of the Wnt signaling pathway

to produce cells of the anterior primitive streak (APS) lineage in 24 hours; and

ii) contacting said APS cells with:

a. one or more activators of TGFβ/Nodal signaling; and

b. one or more inhibitors of BMP signaling or one or more inhibitors of Wnt signaling for about 24 hours to about 96 hours

to produce cells of the DE lineage.

[0007b] In another aspect, provided herein is a cell culture medium when used in the method as described herein for differentiating a pluripotent stem cell into APS lineage comprising: a matrix component, one or more activators of TGFβ/Nodal signaling, one or more activators of Wnt/β-catenin signaling.

[0007c] In another aspect, provided herein is a cell culture medium when used in the method as described herein for differentiating an APS cell into DE lineages comprising: one or more activators of TGFβ/Nodal signaling, one or more inhibitors of BMP signaling or one or more inhibitors of Wnt signaling.

[0007d] In another aspect, provided herein is a kit when used in the method as described herein, comprising one or more containers of cell culture medium as described herein, together with instructions for use.

[0008] According to one ~~aspect~~example there is provided a method of differentiating stem cells into one or more cell lineages comprising contacting said cells with one or more activators of TGFβ/Nodal signaling and one or more activators of Wnt signaling.

[0009] According to another example there is provided a method of differentiating anterior primitive streak cells into cells of definitive endoderm (DE) lineage, by contacting said cells of the anterior primitive streak lineage with one or more activators of TGFβ/Nodal signaling, and one or more inhibitors of BMP signaling, or one or more inhibitors of Wnt signaling.

[0010] According to another example there is provided a method of differentiating cells of the DE. into cells of the AFG, by contacting said DE cells with a TGFP inhibitor and a BMP inhibitor.

[0011] According to another ~~aspect~~example there is provided a method of differentiating cells of the DE lineage into cells of the PFG, by contacting said cells of the DE with retinoic acid, a BMP inhibitor, a Wnt inhibitor and a FGF/MAPK inhibitor.

[0012] According to another ~~aspect~~example there is provided a method of differentiating cells of the DE lineage into cells of the MHG, by contacting said DE cells with a BMP activator, a Wnt activator and an PGF activator.

[0013] According to another ~~aspect~~example there is provided a method of inducing pancreatic progenitors of the PFG from the DE within three days by contacting said PFG with one or more FGF/MAPK inhibitors; one or more BMP inhibitors; and retinoic acid (RA).

[0014] According to another ~~aspect~~example there, is provided a method of inducing liver progenitors of the PFG from the DE within four days by contacting said PFG with one or more TGFp inhibitors; one or more BMP activators, retinoic acid and one or more Wnt inhibitors.

[0015] According to another ~~aspect~~example there is provided a cell culture medium for differentiating a stem cell into one or more cell lineages comprising one or more of the following factors: one or more FGF/MAPK inhibitors; one or more Hedgehog inhibitors, one or more BMP inhibitors, one or more Wnt inhibitors, retinoic acid, Activin A, and one or more inhibitors of PI3K/mTOR signaling.

[0016] According to another ~~aspect~~example there is provided a cell culture medium for differentiating a stem cell, into one or more cell lineages comprising one or more of the following factors: one or more activators of TGFβ/Nodal signaling, one or more activators of Wnt/β-catenin signaling, and one or more inhibitors of PI3K/mT0R signaling.

[0017] According to another ~~aspect~~example there is provided a cell culture medium for differentiating a stem cell into one or

more cell lineages comprising one or more of the following factors: one or more activators of TGFβ/Nodal signaling; and one or more inhibitors of BMP signaling.

[0018] According to another ~~aspect~~example there is provided a cell culture medium for differentiating a stem cell into one or more cell lineages comprising a TGFβ inhibitor and a BMP inhibitor.

[0019] According to another ~~aspect~~example there is provided a cell culture medium for differentiating a stem cell into one or more cell lineages comprising one or more of the following factors: retinoic acid, a BMP inhibitor, a Wnt inhibitor and a FGF/MAPK inhibitor.

[0020] According to another ~~aspect~~example there is provided a cell culture medium for differentiating a stem cell into one or more cell lineages comprising one or more of the following factors: BMP4, a Wnt activator and an FGF activator.

[0021] According to another ~~aspect~~example there is provided a cell culture medium for differentiating a stem cell into one or more cell lineages comprising one or more of the following factors: one or more FGF/MAPK inhibitors; one or more Hedgehog inhibitors; one or more BMP inhibitors; one or more WNT inhibitors; retinoic acid (RA), and Activin A.

[0022] According to another ~~aspect~~example there is provided a cell culture medium for differentiating a stem cell into one or more cell lineages comprising one or more of the following factors: one or more TGFβ inhibitors; one or more BMP activators, and one or more Wnt inhibitors.

[0023] According to another ~~aspect~~example there is provided a cell produced according to any of the methods as described herein.

[0024] According to another ~~aspect~~example there is provided a kit for use in any of the methods described herein, comprising one. or more containers of cell culture medium as described herein, together with instructions for use.

Lefty 2. In one embodiment, the modulators of Wnt signaling may include but are not limited to activators such as ~~CHIR99201~~CHIR99021 (6- [[2- [[4- (2, 4-dichlorophenyl) -5- (5-methyl-lH-imidazol-2-yl) -2 pyrimidinyl] amino] ethyl] amino] -3-pyridinecarbonitrile, A1070722 (1-(7-Methoxyquinolin-4-yl)-3-[6-(trifluoromethyl)pyridin-2~yl}urea), Wnt3a, acetoxime or family members of the Wnt signaling pathway, or may include but are not limited to inhibitors such as C5-9 (2-(4--(2-methylpyridin4-yl)phenyl) -N- (4- (pyridin-3-yl)phenyl) acetamide) , IWP2 (N- (6-Methyl-2-benzothiazolyl) -2- [(3,4,6, 7-tetrahydro-4-oxo-3-phenylthieno [3, 2-d] pyrimidin-2-yl) thio] -acetamide) , Dkkl, XAV939 (3,5, 7, 8-Tetrahydro-2- [4- (trif luoromethyl)phenyl] -4H-thiopyrano [4,3-d] pyrimidin-4-one) , IWR1 (4-(1,3,3a,4,7,7aHexahydro-1,3-dioxo-4,7-methano-2H-isoindol-2-yl) -N-8-quinolinyl-Benzamide) FH-535 = (2, 5-Dichloro-N- (2-methyl-4-nitrophenyl)benzenesulfonamide.

[0049] iCRT-14= 5-[[2, 5-Dimethyl-l-(3-pyridinyl)-lK-pyrrol-3-yl] methylene] -3-phenyl-2, 4-thiazolidinedion), JW-55 (N-[4-[[[[Tetrahydro-4- (4-methoxyphenyl) -2H-pyran-4-yl] methyl] amino] carbony] phenyl] -2-furancarboxamide), JW-67(Trispiro[3H-indole-3,2'-[1,3] dioxane-2 ' ' , 3’’’-[3H]indole]-2,2'’’(1H,1'11H)-dione)or Fzd8 (FrizzledS).

[0050] In one embodiment, the modulator of PI3K/mTOR signaling may include but are not limited to inhibitors such as PI-103 (3-[4-(4-morpholinyl) pyrido[3' , 2' : 4 , 5] furo [3 , 2-d] pyrimidin-2-yl]-phenol), PIK-90 (N-(2, 3-dihydro-7,8-dimethoxyimidazo[1,2-c] quinazolin-5-yl)-3-pyridinecarboxamide), or LY294002 (2-(4-morpholinyl)-8-phenyl-4H-l-benzopyran-4-one) , AS-252424 (5-[[5- (4-Fluoro-2-hydroxyphenyl) - 2-furanyl] methylene] -2,4-thiazolidinedione), AS-605240 (5-(6-Quinoxalinylmethylene) -2,4-thiazolidine-2, 4-dione) , AZD-6482 ((-)-2-[[(1R)-1-[7-Methyl-2- (4-morpholinyl) -4-oxo-4H-pyrido [1, 2-a]pyrimidin-9-yl] ethyl] amino] benzoic acid), BAG-956 (α, α, -Dimethyl-4- [2-

[0058] In one embodiment, the one or more modulators of TGFβ/Nodal signaling may be selected from Activin A, TGF-β1, TGF-β2 or nodal. In one embodiment the one or more activators of Wnt signaling may be selected from ~~CHIR99201~~CHIR99021, Wnt3a or other family members of the Wnt signaling pathway.

[0059] In another embodiment, the stem cells are further contacted with one or more inhibitors of PI3K/mTOR signaling. In one embodiment, the one or more inhibitors of PI3K/mTOR signaling may be selected from PI-103, PIK-90 or LY294002.

[0060] In one embodiment the cells may be contacted with Activin A, PI-103 and ~~CHIR99201~~CHIR99021.

[0061] In another embodiment, the stem cells may be contacted with Activin A in an amount from about 1ng/ml to 10/µg/ml, and with ~~CHIR99201~~CHIR99021 in an amount from about InM to lOOmM.

[0062] In another embodiment, the stem cells are contacted with Activin A in an amount of about lOOng/ml, PI-103 in an amount of about 50nM and CH1R99201 in an amount of about 2µM.

[0063] In another embodiment, the stem cells are further contacted with one or more growth factors selected from the group consisting of Adrenomedullin (AM), Angiopoietin (Ang), Autocrine motility factor, Bone morphogenetic proteins (BMPs), Brain-derived neurotrophic factor (BDNF), Epidermal growth factor {EGF), Erythropoietin (EPO), Fibroblast growth factor (FGF), Glial cell line-derived neurotrophic factor (GDNF), Granulocyte colony-stimulating factor (G-CSF), Granulocyte macrophage colony-stimulating factor (GM-CSF), Growth differentiation factor-9 (GDF9), Hepatocyte growth factor (HGF), Hepatoma-derived growth factor (HDGF), Insulin-like growth factor (IGF), Migration-stimulating factor, Myostatin (GDF-8), Nerve growth factor (NGF) and other neurotrophins, Platelet-derived growth factor (PDGF), Thrombopoietin (TPO), Transforming growth - factor alpha(TGF-α) , Tumor necrosis factor-alpha(TNF-α), Vascular endothelial growth factor

[0086] In one embodiment, the BMP inhibitor comprises BMP4, the FGF activator comprises FGF2 and the Wnt activator comprises ~~CHIR99201~~CHIR99021.

[0087] In another embodiment, the definitive endoderm cells are contacted with about lng/ml to lOµg/ml of BMP4, about lng/ml to 10µg/ml of FGF2, and about 1nM to 10µM of ~~CHIR99201~~CHIR99021. In another embodiment, the definitive endoderm cells are contacted with about 10 ng/ml of BMP4, about lOOng/ml of FGF2, and about 3µM of CH1R99201.

[0088] In one embodiment, the cells of the posterior foregut may have elevated gene or protein expression levels of MHG markers including but not limited to CDX2, EVX1, and 5' HOX cluster genes relative to undifferentiated cells.

[0089] In one embodiment, the differentiation of the definitive endoderm cells in to any one of any one of the anterior foregut (AFG), posterior foregut (PFG) or the midgut/hindgut (MHG) may be completed within a period from about 12 to 300 hours, 12 to 280 hours, 18 to 280 hours, 18 to 260 hours, 24 to 260 hours, 24 to 250 hours or 24 to 240 20 hours. In one embodiment, the differentiation of the definitive endoderm cells in to any one of any one of the anterior foregut (AFG), posterior foregut (PFG) or the midgut/hindgut (MHG) will be completed within a period from 24 to 240 hours.

[0090] In another embodiment, the cells of the posterior foregut (PFG) may be induced from the definitive endoderm within three days to further differentiate into pancreatic progenitor cells by contacting said PFG with one or more FGF/MAPK. inhibitors; one or more Hedgehog inhibitors; one or more BMP inhibitors; one or more Wnt inhibitors; retinoic acid (RA), and Activin A.

[0091] In one embodiment, the FGF/MAPK inhibitor comprises PD0325901 or PD173074, the hedgehog inhibitor comprises SANT

Claims

1. A method of differentiating pluripotent stem cells into definitive endoderm (DE) lineage comprising

i) contacting said pluripotent stem cells with:

a. one or more activators of TGFβ/Nodal signaling; and

b. one or more activators of Wnt signaling, wherein the one or more activators of Wnt signaling are CHIR99021 or Wnt3a or other family members of the Wnt signaling pathway

to produce cells of the anterior primitive streak (APS) lineage in 24 hours; and

ii) contacting said APS cells with:

a. one or more activators of TGFβ/Nodal signaling; and

b. one or more inhibitors of BMP signaling or one or more inhibitors of Wnt signaling for about 24 hours to about 96 hours

to produce cells of the DE lineage.

2. The method as claimed in claim 1, wherein the one or more activators of TGFβ/Nodal signaling is Activin A, TGFβ1, TGFβ2 or Nodal.

~~3. The method as claimed in claim 1 or 2, wherein the one or more activators of Wnt signaling are CHIR99201 or Wnt3a or other family members of the wnt signaling pathway.~~

~~4.~~3. The method as claimed in any one of the preceding claims, wherein the one or more inhibitors of BMP signaling is DM3189/LDN-193189, noggin, chordin, or dorsomorphin or DMH1.

~~5.~~4. The method as claimed in any one of the preceding claims, wherein the pluripotent stem cells are contacted with Activin A, and CHIR9902~~20~~1.

~~6.~~5. The method of claim ~~4~~5, wherein the pluripotent stem cells are contacted with about 1ng/ml to 10µg/ml of Activin A and about 1nM to 100mM of CHIR9902~~20~~1.

~~7.~~6. The method of claim ~~5~~6, wherein the pluripotent stem cells are contacted with about 100ng/ml of Activin A and about 2µM of CHIR9902~~20~~1.

~~8.~~7. The method of any one of the preceding claims, wherein the cells of the anterior primitive streak lineage comprise elevated gene expression of anterior streak or pan-streak markers and decreased expression of posterior streak markers relative to undifferentiated cells.

~~9. The method as claimed in any one of the preceding claims, wherein the differentiation of pluripotent stem cells into APS cells is completed within 24 to 60 hours.~~

~~10.~~8. The method of any one of the preceding claims, wherein the APS cells are contacted with about 1ng/ml to 10µg/ml of Activin A and about 1nM to 100mM of LDN-193189.

~~11.~~9. The method of claim ~~8~~10, wherein the APS cells are contacted with about 100ng/ml of Activin A and about 250nM of LDN-193189.

~~12.~~10. The method as claimed in any one of the preceding claims, wherein the one or more inhibitors of Wnt signaling is Iwp2, Dkk1, C-59, or Iwr-1 or XAV-939.

~~13.~~11. The method of claim 10~~2~~, wherein the APS cells are contacted with ~~about~~ 1ng/ml to ~~about~~ 10µg/ml of Activin A and ~~about~~ 1nM to ~~about~~ 100mM of Iwp2 or ~~about~~ 1nM to ~~about~~ 100mM C-59.

~~14.~~12. The method of claim 11~~3~~, wherein the APS cells are contacted with about 100ng/ml of Activin A and about 4µM of Iwp2 or about 1 µM of C-59.

~~15.~~13. The method of any one of the preceding claims, wherein the cells of the definitive endoderm lineage comprise elevated endoderm gene expression and decreased pluripotency gene expression relative to undifferentiated cells.

~~16.~~14. The method of any one of the preceding claims, wherein the cells of the definitive endoderm lineage comprise significantly reduced mesoderm gene expression.

~~17. The method as claimed in any one of the preceding claims, wherein the differentiation of cells from the APS lineage into cells of DE lineage is completed within 24 to 96 hours.~~

~~18.~~15. A cell culture medium when used in the method of any one of claims 1 to 14 for differentiating a pluripotent stem cell into APS lineage comprising ~~one or more of the following factors~~: a matrix component, one or more activators of TGFβ/Nodal signaling, one or more activators of Wnt/β-catenin signaling.

~~19.~~16. A cell culture medium when used in the method of any one of claims 1 to 14 for differentiating an APS cell into DE lineages comprising ~~one or more of the following~~

~~factors~~: one or more activators of TGFβ/Nodal signaling, and one or more inhibitors of BMP signaling or one or more inhibitors of Wnt signaling.

~~20. A cell culture medium for differentiating a DE cell into a AFG cell comprising a TGFβ inhibitor and a BMP inhibitor.~~

~~21. A cell culture medium for differentiating a DE cell into a PFG cell comprising one or more of the following factors: retinoic acid, a BMP inhibitor, a Wnt inhibitor and a FGF/MAPK inhibitor.~~

~~22. A cell culture medium for differentiating a DE stem cell into a MHG cell comprising one or more of the following factors: a BMP activator, a Wnt activator and an FGF activator.~~

~~23. A cell culture medium for differentiating a PFG cell into a pancreatic progenitor cell comprising one or more of the following factors: one or more FGF/MAPK inhibitors; one or more Hedgehog inhibitors; one or more BMP inhibitors; one or more Wnt inhibitors; retinoic acid (RA), and Activin A.~~

~~24. A cell culture medium for differentiating a PFG cell into a liver progenitor cell comprising one or more of the following factors: one or more TGFβ inhibitors; one or more BMP activators, retinoic acid, and one or more Wnt inhibitors.~~

~~25.~~17. A kit when used in the method of any one of claims 1 to 1~~4~~7, comprising one or more containers of cell culture

medium as claimed in any one of claims 15~~8~~ to 16~~24~~, together with instructions for use.