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(54) **Title**: METHOD FOR PRODUCING CARDIOMYOCYTES

(57) **Abstract**: The present invention provides a method for producing human cardiomyocytes in a culture system selectively and efficiently from pluripotent stem cells by using activators and inhibitors of the Wnt signaling pathway. The present invention also provides use of an inhibitor in the production.

METHOD FOR PRODUCING CARDIOMYOCYTES

Field of the Invention

The present invention is generally related to the field of stem cells. More specifically, it concerns the production of cardiomyocytes from pluripotent stem cells.

Background

Human adult cardiomyocytes are terminally differentiated. Although a small percentage of cardiomyocytes may have proliferative capacity, it is not sufficient to adequately replace injured or dead cardiac tissue during myocardial infarction after a coronary heart event. Loss of functional cardiomyocytes leads to chronic heart failure.

Therapeutic methods for restoring heart function replace injured or dead cardiomyocytes by new functional cardiomyocytes. Pluripotent stem cells, such as human embryonic stem (ES) cells or induced pluripotent stem (iPS) cells, are potential sources of cardiomyocytes. Differentiation of pluripotent stem cells into cardiomyocytes can be achieved upon induction in vitro.

Progress has been made in the differentiation of pluripotent stem cells into cardiomyocytes. However, technical challenges remain for cardiomyocyte differentiation in order to obtain sufficient numbers and substantially enriched populations of cardiomyocyte lineage cells from pluripotent stem cells. The low efficiency and wide variation of differentiation methods are some of the most critical issues. Thus, there is a need to improve the differentiation efficiency of pluripotent stem cells into cardiomyocytes, especially for large-scale production.

The low efficiency of the current differentiation methods is perhaps the result of the commonly-used method of induction, the embryoid body (EB) method. In the EB method, ES cells cultured in suspension culture as single cells form a structure called embryoid bodies (EBs) which are similar to the early embryonal structures.

Cardiomyocytes with spontaneous beating ability appear when these EBs are cultured in suspension. Cardiomyocytes prepared by the EB method vary in their purity and exhibit very similar properties to those of immature cardiomyocytes in fetal hearts.

Methods of purifying only cardiomyocytes from a mixture of various kinds of cells from EBs include a method of density gradient separation and a method of labeling cardiomyocyte with a mitochondria staining dye and sorting through FACS. However, these methods are inefficient and time-consuming.

In order to address the efficiency of pluripotent stem cell differentiation, addition of differentiation factors to modulate developmental signaling pathways has been tested. Examples include: 1) the addition of antagonists to reduce the total signal in certain pathways, 2) the addition of agonists to increase the total signal in certain pathways or 3) combinations of agonists and antagonists to optimize the signal.

Methods of increasing the efficiency of cardiogenesis include treating the differentiating cells with various cytokines or small molecule compounds. Such methods have various degrees of success with some of the most efficient methods derived from the biological principles guarding the development of cardiomyocytes during embryogenesis.

It is known that TGFβ signaling pathways regulate cardiac differentiation. In particular, BMP and Activin are two exemplary TGFβ signaling pathways that can be optimized. Another pathway that governs cardiogenesis is the Wnt signaling pathway.

Roles of Wnt Signaling During Development of Cardiomyocytes

Wnt secretory proteins are widely found not only in vertebrate but also in invertebrate animals. There are 19 Wnt genes (Wnt-1, 2, 2b/13, 3, 3a, 4, 5a, 5b, 6, 7a, 7b, 8a, 8b, 9a, 9b, 10a, 10b, 11, 16) in humans and mice. Wnt proteins bind to the seven-transmembrane Frizzled (abbreviated as Fzd) family of receptors present on the cell membrane. Such binding triggers signaling pathway involving accumulation and nuclear migration of β-catenin, which is called the “classical” Wnt pathway or the “canonical” Wnt signaling pathway. It is also known that activation of the canonical Wnt signaling pathway is induced by treatment with GSK-3β inhibitors of small molecules.

Wnt ligands are known to activate other signaling pathways through Fzd receptors. Such signaling pathways include the planar cell polarity (PCP) pathway which activates JNK (Jun N-terminal kinase). These pathways are called “non-classical” Wnt pathways or “non-canonical” Wnt signaling pathways, in contrast to the canonical Wnt signaling pathway.

Wnt proteins are involved in a wide variety of biological functions during development, growth and differentiation of various cells and tissues. Cardiomyocytes develop from a part of the lateral plate mesoderm at the early stage of development, and then repeatedly divide and grow to form a heart. The presence or absence of Wnt signals plays an important role in this process. For example, in the early stage of avian or *Xenopus laevis* development, ectopic and/or forced expression of the Wnt-3a or Wnt-8a (which activates the canonical Wnt signaling) significantly inhibits heart. On the other hand, so-called Wnt antagonists (e.g., Frzb, Dkk-1) which bind to Wnt-3a or Wnt-8a to inhibit its signaling promote heart formation, thus suggesting that canonical Wnt signals act to inhibit myocardial development. On the other hand, activation of non-canonical Wnt signaling pathways which antagonize canonical Wnt signals is known to positively induce differentiation of cardiomyocytes. It has been shown that Wnt-11 which activates non-canonical pathways is essential for heart development in *Xenopus laevis* and cultured mouse ES cells.

Summary of the Invention

The present invention provides a method for producing cardiomyocytes from pluripotent stem sells, especially, a method for increasing the efficiency of differentiation of pluripotent stem cells into cardiomyocytes by selectively activating or inhibiting the Wnt signaling pathways. Furthermore, the present invention provides the use of a compound in producing cardiomyocytes.

In the first aspect, the present invention provides a method for producing cardiomyocytes, comprising the following steps:

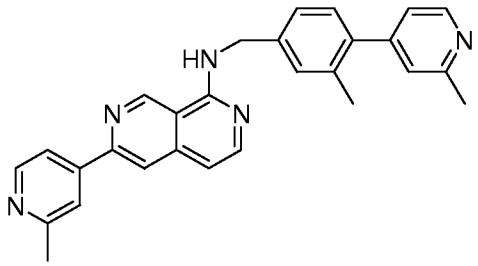
1) Culturing pluripotent stem cells in a medium containing a Wnt activator for 48-120 hrs, to induce mesoderm differentiation;

2) Culturing the differentiating cells obtained from step 1 in a medium containing a Wnt inhibitor for 48-144 hrs, to induce cardiac mesoderm;

3) Culturing the differentiating cells obtained from step 2 in a medium containing a Wnt inhibitor for 48-120 hrs, to induce cardiac progenitor cells;

4) Culturing the differentiating cells obtained from step 3 in a medium containing a Wnt activator for 48-240 hrs, to maintain the survival of the cardiomyocytes.

In another aspect, the present invention provides the use of a compound named CGX307 in producing cardiomyocytes as a Wnt inhibitor, wherein the compound is N-(3-methyl-4-(2-methylpyridin-4-yl) benzyl)-6-(2-methylpyridin-4-yl)-2,7-naphthyridin-1-amine and the structure thereof is



In still another aspect, the present invention provides the use of the compound in the preparation of a therapeutic drug for heart conditions.

Brief Description of Drawings

Figure 1 shows that Wnt activators, recombinant Wnt-3a protein or small molecule compound CHIR99021, increase the expression of mesodermal marker Brachyury in differentiating human iPS cells.

Figure 2 shows that canonical Wnt pathway inhibitors, recombinant Wnt-5a protein or small molecule compounds XAV939 or CGX307, increase the expression of cardiac mesodermal marker Flk-1/KDR in differentiating human iPS cells.

Figure 3 shows that canonical Wnt pathway inhibitors, recombinant Wnt-5a protein or small molecule compounds XAV939 or CGX307, increase the expression of cardiac progenitor marker Isl-1 in differentiating human iPS cells.

Figure 4 shows that canonical Wnt pathway activator, recombinant Wnt-3a protein, increases the survival of differentiated cardiac cells in the presence of Thymosin β-4.

Figure 5 shows the enhancing effects of using Wnt activator and inhibitor (the groups labeled “Wnt”) on the number of beating cells.

Figure 6 shows the increased expression cardiac-specific protein α-actinin after the entire procedure described above.

Left panel: Control without Wnt activator and inhibitor, right panel: Wnt group. Green (strips): α-actinin, blue (oval): DAPI.

Embodiments

Abbreviations:
BMP: bone morphologic protein
TGF: transforming growth factor
bFGF: basic fibroblast growth factor
FACS: fluorescence activated cell sorting
GSK: glycogen stimulating kinase
Dkk: dickhorf
ES: embryonic stem cells
iPS: induced pluripotent stem
qRT-PCR: quantitative reverse-transcription polymerase chain reaction
ROCK: Rho kinase

Pluripotent stem cells that can be used in the present invention include ES cells and iPS cells derived from mammals such as mice, monkeys and humans, as well as all pluripotent stem cells that are characteristically similar to ES cells. In a certain aspect, the pluripotent stem cells, which may be clonally derived from a single pluripotent stem cell, may comprise a substantial portion of cells clonally derived from a single cell, or may be a pool of multiple populations of cells, wherein each population of cells is clonally derived from a single cell. An example for obtaining pluripotent stem cells from a single cell may comprise incubating a single pluripotent stem cell in medium comprising a ROCK inhibitor under conditions to promote cell growth, such as under adherent culture conditions. In another aspect, certain chemicals such as ascorbic acid can be added at a concentration from 1mg/L to 1000mg/L to maintain pluripotency and increase cardiogenesis.

As used herein, human iPS cells are obtained by transduction of human foreskin fibroblasts with retroviruses containing Oct4, Sox2 and Klf4 following published protocol (Takahashi et al., 2007). Transfected cells are plated on mouse fibroblast cells in medium PSGen (System Biosciences Inc., Mountain View, CA) supplemented with 100 ng/mL Wnt-3a (StemRD Inc., Burlingame, CA) for about 4 weeks. Colonies resembling

ES cell colonies are picked and expanded in a commercially-available iPSC medium PSGro (StemRD Inc., Burlingame, CA). The colonies are further characterized by immunocytochemical staining of Oct4 expression to ensure majority of the cells continue to express the pluoripotency marker.

As used herein, the compound N-(3-methyl-4-(2-methylpyridin-4-yl) benzyl)-6-(2-methylpyridin-4-yl)-2,7-naphthyridin-1-amine is designated as CGX307 for simplification.

Substances that activate or inhibit the Wnt signaling pathway (herein referred to as "Wnt activator" or "Wnt inhibitor") can be added to the medium during a certain stage of culture. Specific examples of the substance that activates the canonical Wnt signaling pathway include various canonical Wnt proteins, small molecule GSK-3β inhibitors such as CHIR99021. Specific examples of the substance that inhibits the canonical Wnt signaling pathway include various non-canonical Wnt proteins such as Wnt-5a, secreted Wnt antagonist such as Dkk-1, and small molecule inhibitors such as inhibitors of Porcupine such as IWP-1 or CGX307 and Tankyrases inhibitor such as XAV939.

In some steps of the present method, the pluripotent stem cells are transiently treated with a Wnt activator, including but not limited to recombinant Wnt proteins, for example, a recombinant protein obtained from Wnt gene expression. The concentration of the recombinant Wnt protein used is from 0.1 ng/mL to 1000 ng/mL, preferably 1 ng/mL to 200 ng/mL.

GSK-3β inhibitors are defined as substances that inhibit the kinase activity of GSK-3β protein (e.g., the ability to phosphorylate β-catenin). Known examples include an indirubin derivative 6-bromoindirubin 3′-oxime (BIO), a maleimide derivative SB216763 (3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione), or CHIR99021. These compounds are commercially available from a number of suppliers including StemRD Inc. In a case where these GSK-3β inhibitors are used, their optimal concentrations are dependent on differences in the potencies of particular compounds.

In some other steps of the present method, the pluripotent stem cells are transiently treated with a Wnt inhibitor, including but not limited to small molecule inhibitors such as inhibitors of Porcupine or Tankyrases. The concentrations of such inhibitors used vary from 0.001 nM to 1000 nM, preferably 1 nM to 100 nM.

In one aspect, the present invention provides a method for producing cardiomyocytes, comprising the steps of:

1) Culturing pluripotent stem cells in a medium containing a Wnt activator for 48 to 120 hrs to induce mesoderm differentiation, wherein the Wnt activator is Wnt-3a or GSK-3β inhibitor 6-((2-((4-(2,4-Dichlorophenyl)-5-(4-methyl-1H-imidazol-2-yl)pyrimidin-2-yl)amino)ethyl)amino) nicotinonitrile (also named CHIR99021). The concentration of the Wnt activator is from 0.1 ng/mL to 1000 ng/mL, preferably 1 ng/mL to 200 ng/mL. Furthermore, additional factors comprised in the medium for mesoderm differentiation include BMP-4 at a concentration from 0.1 ng/mL to 100 ng/mL, and Activin A at a concentration from 0.1 ng/mL to 100 ng/mL.

2) Culturing the differentiating cells obtained from step 1 in a medium containing a Wnt inhibitor for 48 to 144 hours, to induce cardiac mesoderm, wherein the Wnt inhibitor is Wnt-5a, XAV939 or CGX307. The concentration of the inhibitor is from 0.001 nM to 1000 nM, preferably 1 nM to 100 nM. Additional factors comprised in the medium for cardiac mesoderm differentiation include inhibitors of BMP signaling such as noggin at a concentration from 10 ng/mL to 1000 ng/mL, Apelin at a concentration from 0.1 nM to 100 nM, and a small molecule inhibitor for BMP receptor such as LDN-193189 or SB431542 at a concentration from 1 nM to 1000 nM.

3) Culturing the differentiating cells obtained from step 2 in a medium containing a Wnt inhibitor for 48 to 120 hours to induce cardiac progenitor cells, wherein the Wnt inhibitor is Wnt-5a, XAV939 or CGX307. The concentration of the inhibitor is from 0.001 nM to 1000 nM, preferably 1 nM to 100 nM. Additional factors comprised in the medium for cardiac progenitor differentiation include bFGF at a concentration from 0.1 ng/mL to 100 ng/mL and VEGF at 0.1 nM to 100 nM.

4) Culturing the differentiating cells obtained from step 3 in a medium containing a Wnt activator for 48 to 240 hours to maintain the survival of the cardiomyocytes, wherein the activator can be recombinant Wnt-3a or inhibitor CHIR99021. The concentration of the Wnt activator is from 0.1 ng/mL to 1000 ng/mL, preferably 1 ng/mL to 200 ng/mL. Additional factors comprised in the medium for cardiac progenitor differentiation is Thymosin β-4 at a concentration from 0.1 nM to 100 nM.

In a preferred embodiment, the pluripotent stem cells are embryonic stem cells or induced pluripotent stem cells.

In a preferable embodiment, the method further comprises measuring differentiation efficiency of each step.

The measurement of differentiation efficiency may comprise measuring expression of mesoderm markers at 5, 6, 7, 8, 9, 10 days, cardiac mesoderm markers at 11 to 14 days, cardiovascular progenitor markers at 11 to 21 days, and cardiomyocyte markers at 14 to 28 days after the initial differentiation by immunocytochemistry well known in the art. Examples of markers are mesoderm marker Brachyury, cardiac mesoderm marker Flk-1/KDR or Nkx2-5, cardiovascular progenitor marker Isl-1, and cardiomyocyte-specific marker protein α-actinin.

In still another embodiment, cardiomyocytes derived from pluripotent stem cells by the aforementioned method can be further collected, isolated and purified by known methods to efficiently obtain large quantities of pure cardiomyocytes. Cardiomyocytes prepared according to the present invention exhibit morphological, physiological and immunocytological characteristics of cardiomyocytes. In terms of physiological and/or immunocytological characteristics, cells prepared according to the present invention may express one or more markers specific to cardiomyocytes, for example, markers mentioned above.

Furthermore, cardiomyocytes prepared by the aforementioned method of the present invention can be used in methods of screening compounds which promote the development, differentiation, regeneration, survival of cardiomyocytes.

Also, cardiomyocytes prepared by the aforementioned method can be used in methods for treating hearts suffering from cardiac disorders. Examples of cardiac disorders include myocardial infarction, ischemic heart disease, congestive heart failure, hypertrophic cardiomyopathy, dilative cardiomyopathy, myocarditis, chronic heart failure and the like.

In another aspect, the present invention provides the use of the compound CGX307 in producing cardiomyocytes. CGX307 is used as a Wnt inhibitor, and acts to induce differentiation of stem cells into cardiomyocytes.

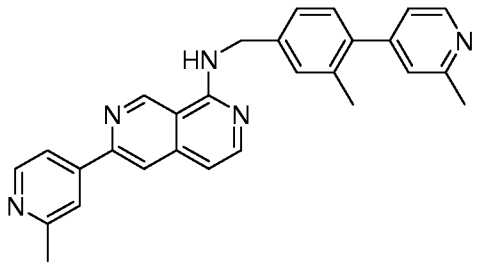
In an embodiment of this aspect, CGX307 can be used as a therapeutic drug for heart conditions including myocardial infarction, ischemic heart disease, congestive heart failure, hypertrophic cardiomyopathy, dilative cardiomyopathy, myocarditis, chronic heart failure and the like.

Examples

The present invention will be explained in more detail below with the examples and figures. It should be understood that the examples are given just for exemplification, which are not intended to limit the scope of the present invention.

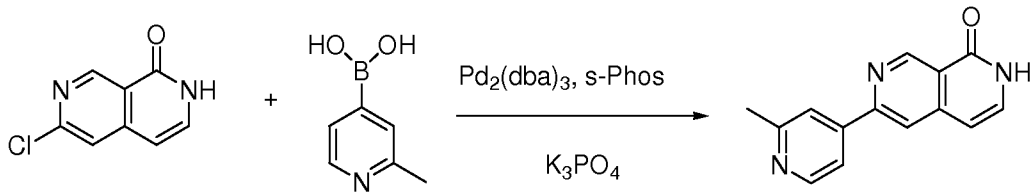
Example 1: Structure and synthesis of CGX307

As designated above, CGX307 is N-(3-methyl-4-(2-methylpyridin-4-yl) benzyl)-6-(2-methylpyridin-4-yl)-2,7-naphthyridin-1-amine, and the structure is listed as below. It is synthesized by the present inventor.



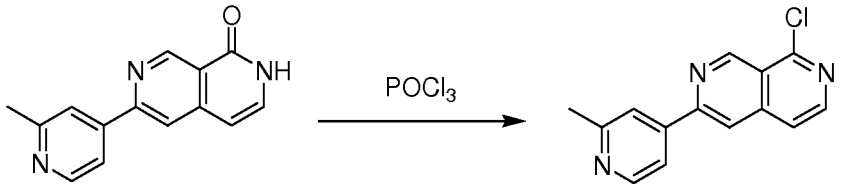
The synthesis process comprises the following steps.

Step 1:



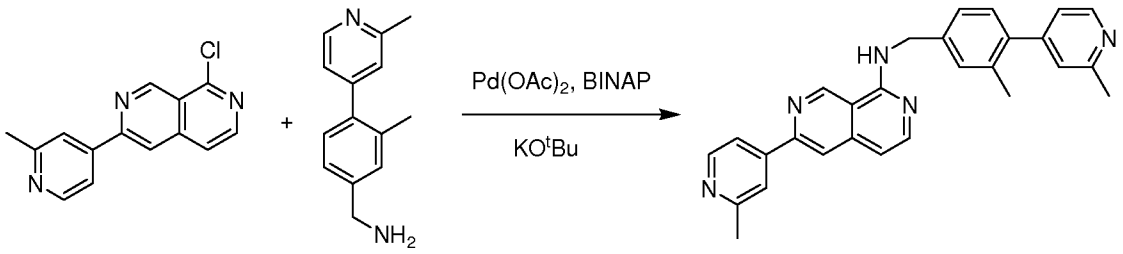
6-chloro-2,7-naphthyridin-1(2H)-one (200 mg, 1.10 mmol) and 2-methylpyridin-4-yl-4-boronic acid (227.60 mg, 1.66 mmol) are dissolved in BuOH (5.0 mL) and water (1.0 mL). K_3PO_4 (705.20 g, 3.32 mmol), $Pd_2(dba)_3$ (49.60 mg, 0.22 mmol) and S-phos (91.00 mg, 0.11 mmol) are added under N_2. The reaction mixture in the pressure tube is heated up to 130℃ for 1h. After cooling down the reaction to room temperature (RT), poured the mixture into the water, extracted by EA for three times. The combined organic layer is washed with brine, dried over Na_2SO_4, concentrated under the vacuum to get the crude. The crude product is purified by column with 5% MeOH in DCM to get the final compound 6-(2-methylpyridin-4-yl)-2,7-naphthyridin-1(2H)-one (yield ~ 61%). MS *m/z* 238.1 (M + 1).

Step 2:



6-(2-methylpyridin-4-yl)-2,7-naphthyridin-1(2H)-one (150 mg, 0.63 mmol) is dissolved in $POCl_3$ (15.0 mL), the pressure tube is sealed and heated up to 160℃ for 4 h. After cooling down the reaction to RT, excessive $POCl_3$ is removed under vacuum. Crushed ice is slowly added into the mixture, and then added into $NaHCO_3$ to neutralize until pH ~7.5. Extracted the solution by EA three times, the combined organic layer is washed with brine, dried over Na_2SO_4, and concentrated under vacuum. The crude is purified by column with EA/Hexane (1:1) to get the compound 1-chloro-6-(2-methylpyridin-4-yl)-2,7-naphthyridine (yield ~55%). MS *m/z* 256.1 (M + 1).

Step 3:



1-chloro-6-(2-methylpyridin-4-yl)-2,7-naphthyridine(10.00 mg, 0.039 mmol) and (3-methyl-4-(2-methylpyridin-4-yl)phenyl)methanamine (10.00 mg, 0.047 mmol) are dissolved in Toluene (1.0 mL). KO^tBu(8.80 mg, 0.078 mmol), $Pd(OAc)_2$ (0.90 mg, 0.0039 mmol) and BINAP (4.90 mg, 0.0078 mmol)is added into the mixture under N_2. The reaction is heated up to 100℃ for overnight. After cooling down the reaction to RT, poured the mixture into the water, extracted by EA for three times. The combined organic layer is washed with brine, dried over Na_2SO_4, then concentrated under vacuum. The

crude product is purified by prep-TLC by EA/Hexane (4:1) to get N-(3-methyl-4-(2-methylpyridin-4-yl)benzyl)-6-(2-methylpyridin-4-yl)-2,7-naphthyridin-1-amine (8.8mg, yield ~52%). 1H NMR (300 MHz, CDCl3): δ2.31 (s, 3H), 2.63 (s, 3H), 2.70 (s, 3H), 4.91 (d, J = 5.10 Hz, 2H), 5.88 (br, 1H), 7.00 (d, J = 5.40 Hz, 1H), 7.08 (d, J = 5.10 Hz, 1H), 7.12 (s, 1H), 7.22 (d, J = 7.50 Hz, 1H), 7.36 (m, 2H), 7.77 (d, J = 4.50 Hz, 1H), 7.88 (s, 1H), 7.98 (s, 1H), 8.24 (d, J = 6.00 Hz, 1H), 8.53 (d, J = 4.80 Hz, 1H), 8.64 (d, J = 5.40 Hz, 1H), 9.31 (s, 1H). MS *m/z* 432.2 (M + 1).

Example 2

Human iPS cells prepared from foreskin fibroblasts with lentiviral Oct-4, Sox-2 and Klf-4 in PSGen (System Biosciences Inc., Mountain View, CA) supplemented with 100 ng/mL Wnt-3a (StemRD Inc., Burlingame, CA) are maintained in the PSGro Human iPSC/ESC Growth Medium (StemRD Inc.) on 6-well plates coated with Matrigel. Cells passaged under ordinary culture conditions are washed twice with phosphate-buffered saline (hereinafter referred to as PBS) and treated with Accutase solution (Millipore, SCR005) to obtain small clusters of 5-20 cells. Differentiation is initiated by changing the medium to the PSGro Basal Medium (StemRD Inc.) with Wnt activitor, Activin A and BMP-4. Unless otherwise stated, the same condition described below is used for differentiation in all experiments. The colonies were further characterized by immunocytochemical staining of Oct4 expression to ensure majority of the cells continue to express the pluoripotency marker.

Example 3

Wnt Activator Enhances Mesoderm Differentiation from iPS Cells

Induction of iPS cell differentiation is accomplished in the same manner as used in Example 2 in PSGro Basal Medium (StemRD Inc.) containing either 50 ng/mL recombinant Wnt-3a (StemRD Inc.), 100 ng/mL Wnt-5a (StemRD Inc.), or 100nM CHIR99021. The medium also contains 10 ng/mL Activin A (StemRD Inc.) and 10 ng/mL BMP-4 (StemRD Inc.). After 48 hrs to 120 hrs treatment, cells are harvested for the analysis of gene expression of mesodermal marker Brachyury by using qRT-PCR.

As shown in Fig.1, after 48 hrs treatment, expression of Brachyury is significantly higher in groups treated with Wnt activator Wnt-3a or CHIR99021. These results indicate that Wnt activator significantly induces mesodermal differentiation from human iPS cells. This effect is observed as long as a Wnt activator is present for the first 48 hours.

Optimal concentrations of the Wnt activators are determined. When the recombinant Wnt-3a protein is added at concentrations of 1 ng/mL to 100 ng/mL, optimal Brachyury expression is obtained at a concentration from 10 ng/mL to 50 ng/mL.

Example 4

Wnt Inhibitor Enhances Cardiac Mesoderm Differentiation from iPS Cells

Induction of cardiac mesodermal cell differentiation is accomplished in the same manner as used in Example 2 in PSGro Basal Medium containing Wnt inhibitor either 100 ng/mL recombinant Wnt-5a (StemRD Inc.), 100 nM of XAV939, or 100 nM of CGX307. The PSGro Basal Medium also contains 200 ng/mL Noggin (StemRD Inc.) or a small molecule inhibitor for BMP receptor such as LDN-193189 or SB431542 both at 1 nM to 1000 nM. In the example, the medium contains 500 nM of LDN-193189. After 48 hrs to 144 hrs treatment, cells are harvested for the analysis of gene expression of cardiac mesodermal marker Flk-1/KDR by using qRT-PCR.

As shown in Fig. 2, expression of Flk-1/KDR is significantly higher in groups treated with Wnt inhibitors for 96 hrs. These results indicate that Wnt inhibitors significantly induce cardiac mesodermal differentiation from human iPS cells.

Example 5

Wnt Inhibitor Enhances Cardiac Progenitor Differentiation from iPS Cells

Differentiating cells are cultured in the medium containing an aforementioned Wnt inhibitor for 48 to 120 hours to induce cardiac progenitor cells. The medium also contains factors known for cardiac progenitor differentiation such as bFGF at a concentration from 0.1 ng/mL to 100 ng/mL and VEGF at 0.1 nM to 100 nM. After treatment, cells are harvested for the analysis of gene expression of cardiovascular progenitor marker Isl-1.

In the example, differentiating cells are cultured in the PSGro Basal medium containing 100 ng/mL Wnt-5a, 100 nM of XAV939, or 100 nM CGX307 for 96 hours to induce cardiac progenitor cells. The medium also contains bFGF at a concentration of 10 ng/mL and VEGF at 10 nM.

As shown in Fig. 3, expression of Isl-1 is significantly higher in groups treated with Wnt inhibitors for 96 hrs. These results indicate that Wnt inhibitors significantly induce cardiac progenitor cell differentiation from human iPS cells.

Example 6

Wnt Activator Enhances Cardiac Progenitor Cell Survival from iPS Cells

The resulting differentiated cells from aforementioned procedures are cultured in a medium containing a Wnt activator selected from aforementioned group for 48 to 240 hours to maintain the survival of the cardiomyocytes. The medium also contains Thymosinβ-4, a factor known to increase the survival of cardiac cells, at 0.1 nM to 100 nM. Live cell numbers are measured by the CellTiter Glow assay (Promega).

In the example, the differentiated cells are cultured in the PSGro Basal medium containing 50 ng/mL Wnt-3a or CGX307 for 120 hours. Two groups also contain Thymosin β-4 at 30 nM.

As shown in Fig. 4, survival of cells as measured by CellTiter Glo assay (Promega) is significantly higher in groups treated with Wnt activator. These results indicate that Wnt activators significantly increase survival of cardiac progenitor cell differentiated from human iPS cells.

Example 7

Combined Treatment by Wnt Activator and Inhibitor Increases Number of Beating Cells

At the end of the aforementioned 4-step procedure, spontaneous beating colonies are counted. The Control group that does not have any Wnt activator or inhibitor treatment is counted at Day 16, whereas the groups with sequential treatment of Wnt activator and inhibitor are counted at Day 10 and Day 16.

As shown in Fig. 5, in the group without any Wnt activator or inhibitor treatment, about 5% or less of colonies exhibit beating at about 16 days after induction of differentiation. In contrast, in the group with sequential treatment of Wnt activator and inhibitor, spontaneous beating started about 10 days after induction of differentiation, and almost 50% of colonies exhibit beating at 16 days after induction of differentiation.

Example 8

Combined Treatment by Wnt Activator and Inhibitor Increases Cardiomyocyte Protein Markers

At the end of the aforementioned 4-step procedure, immunocytochemical staining of cardiomyocyte-specific marker protein α-actinin using a monoclonal antibody against α-actinin (1:250, Abcam) is carried out, to confirm that beating cells express cardiomyocytes specific marker on protein level.

As shown in Fig. 6, cells in the Wnt activator- and inhibitor-treated group at 16 days after induction of differentiation increase in number for positive staining of cardiomyocyte-specific marker protein α-actinin.

These results further indicate that the combined treatment by Wnt activator and inhibitor has a significant promoting effect on induction of myocardial differentiation.

CLAIMS:

1. A method for producing cardiomyocytes, comprising the following steps:

1) Culturing pluripotent stem cells in a medium containing a Wnt activator for 48 to 120 hrs, to induce mesoderm differentiation;

2) Culturing the differentiating cells obtained from step 1 in a medium containing a Wnt inhibitor for 48 to 144 hrs, to induce cardiac mesoderm;

3) Culturing the differentiating cells obtained from step 2 in a medium containing a Wnt inhibitor for 48 to 120 hrs, to induce cardiac progenitor cells;

4) Culturing the differentiating cells obtained from step 3 in a medium containing a Wnt activator for 48 to 240 hrs, to maintain the survival of the cardiomyocytes;

wherein the Wnt activator in steps 1 and 4 is Wnt-3a or CHIR99021, and the Wnt inhibitor in steps 2 and 3 is Wnt-5a, XAV939 or N-(3-methyl-4-(2-methylpyridin-4-yl) benzyl)-6-(2-methylpyridin-4-yl)-2,7-naphthyridin-1-amine.

2. The method of claim 1, wherein the concentration of the Wnt inhibitor is from 0.001 nM to 1000 nM, preferably 1 nM to 100 nM.

3. The method of claim 1, wherein the concentration of the Wnt activator is from 0.1 ng/mL to 1000 ng/mL, preferably 1 ng/mL to 200 ng/mL.

4. The method of claim 1, wherein the medium in step 1 further comprises BMP-4 at a concentration from 0.1 ng/mL to 100 ng/mL, and Activin A at a concentration from 0.1 ng/mL to 100 ng/mL.

5. The method of claim 1, wherein the medium in step 2 further comprises noggin at a concentration from 10 ng/mL to 1000 ng/mL, Apelin at a concentration from 0.1 nM to 100 nM, or a small molecule inhibitor for BMP receptor such as LDN-193189 or SB431542 at a concentration from 1 nM to 1000 nM.

6. The method of claim 1, wherein the medium in step 3 further comprises bFGF at a concentration from 0.1 ng/mL to 100 ng/mL and VEGF at a concentration from 0.1 nM to 100 nM.

7. The method of claim 1, wherein the medium in step 4 further comprises hymosin β-4 at a concentration from 0.1 nM to 100 nM.

8. The method of any one of claims 1-7, wherein the pluripotent stem cells are embryonic stem cells or induced pluripotent stem cells.

9. Use of the compound N-(3-methyl-4-(2-methylpyridin-4-yl) benzyl)-6-(2-methylpyridin-4-yl)-2,7-naphthyridin-1-amine in producing cardiomyocytes from pluripotent stem cells.

10. The use of claim 9, wherein the compound is used as a Wnt inhibitor and acts to induce differentiation of stem cells into cardiomyocytes.

11. Use of the compound N-(3-methyl-4-(2-methylpyridin-4-yl) benzyl)-6-(2-methylpyridin-4-yl)-2,7-naphthyridin-1-amine in the preparation of a therapeutic drug for heart conditions including myocardial infarction, ischemic heart disease, congestive heart failure, hypertrophic cardiomyopathy, dilative cardiomyopathy, myocarditis and chronic heart failure.

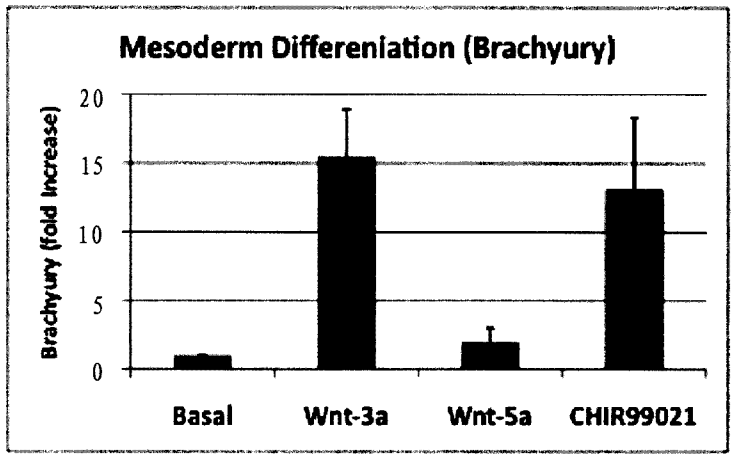

Mesoderm Differeniation (Brachyury)
Brachyury (fold increase)
20
15
10
5
0
Basal
Wnt-3a
Wnt-5a
CHIR99021

Fig 1

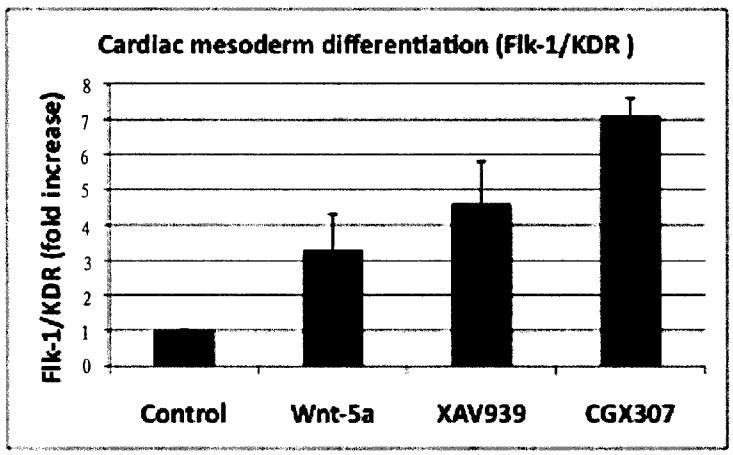

Cardiac mesoderm differentiation (Flk-1/KDR)
Flk-1/KDR (fold increase)
8
7
6
5
4
3
2
1
0
Control
Wnt-5a
XAV939
CGX307

Fig 2

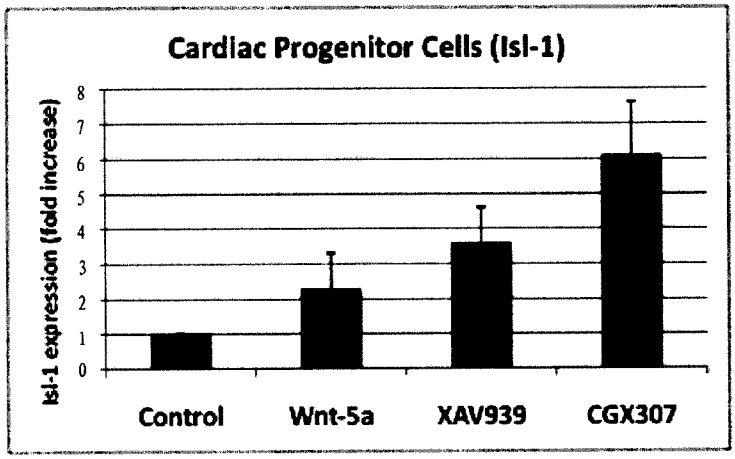

Cardiac Progenitor Cells (Isl-1)
Isl-1 expression (fold increase)
8
7
6
5
4
3
2
1
0
Control
Wnt-5a
XAV939
CGX307

Fig 3

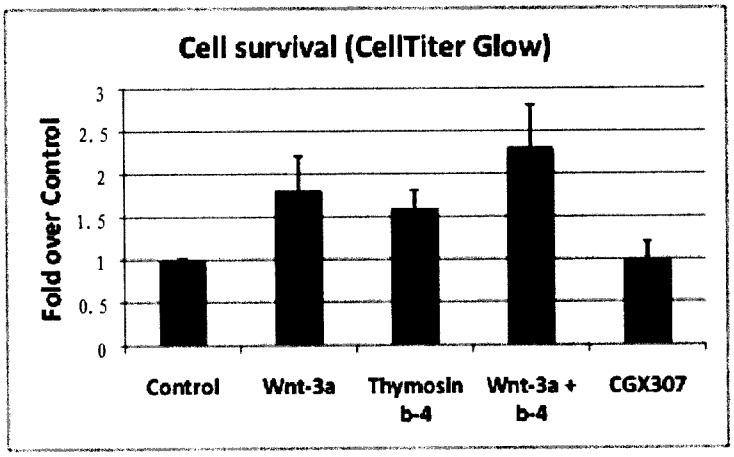

Cell survival (CellTiter Glow)
Fold over Control
3
2. 5
2
1. 5
1
0. 5
0
Control
Wnt-3a
Thymosin b-4
Wnt-3a + b-4
CGX307

Fig 4

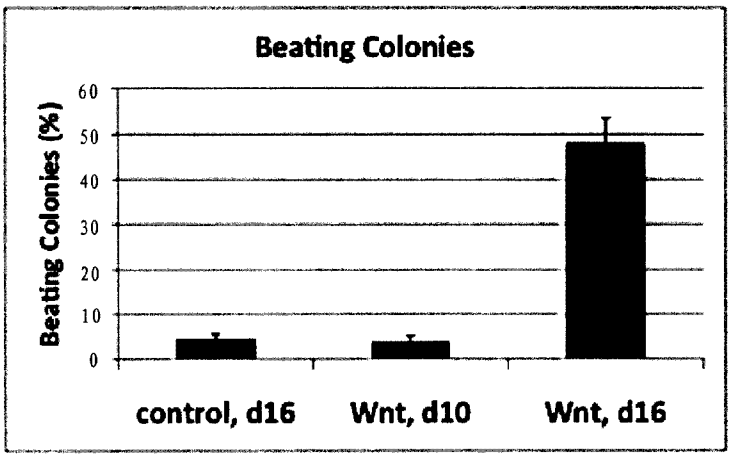

Beating Colonies
Beating Colonies (%)
60
50
40
30
20
10
0
control, d16
Wnt, d10
Wnt, d16

Fig 5

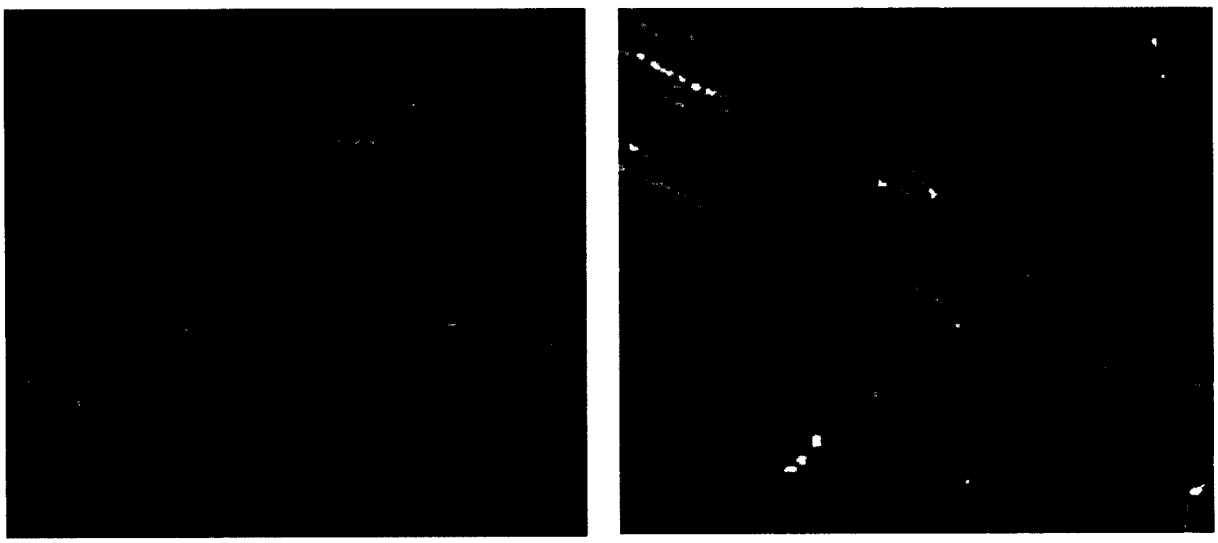


Fig 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2012/074852

A. CLASSIFICATION OF SUBJECT MATTER

See the extra sheet

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C12N5/-; C07D401/-; A61P9/-

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC, CNKI, CPRS, Google： stem cell?, embryonic, pluripotent, ips, es, cardiomyocyte?, myocardial cell?, cardiac cell?, wnt???, cardiac progenitor cell?, cardiac mesoderm?, wnt, activator?, inhibitor?, heart?, methyl, methylpyridin, naphthyridin, chir99021, xav939

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CN101426902A(ASUBIO PHARMA CO., LTD.)06 May 2009 (06.05.2009) See claims1-16	1-11
A	CN101573442A(THE J. DAVID GLADSTONE INSTITUTES)04 Nov. 2009 (04.11.2009) See claims1-22	1-11
A	CN101365784A(AGENCY FOR SCIENCE TECHNOLOGY AND RESEARCH)11 Feb. 2009 (11.02.2009) See claims1-41	1-11
A	US2004/0014209A1 (LASSAR, A. B. ET AL.) 22 Jan. 2004 (22.01.2004) See claims1-61	1-11

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim (S) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 Jan. 2013 (07.01.2013)	**07 Feb. 2013 (07.02.2013)**
Name and mailing address of the ISA/CN The State Intellectual Property Office, the P.R.China 6 Xitucheng Rd., Jimen Bridge, Haidian District, Beijing, China 100088 Facsimile No. 86-10-62019451	Authorized officer YAO,Jinxiao Telephone No. (86-10)62414345

Form PCT/ISA /210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2012/074852

Patent Documents referred in the Report	Publication Date	Patent Family	Publication Date
CN101426902A	06.05.2009	WO2007126077A1	08.11.2007
		EP2014766A1	14. 01.2009
		KR20090009267A	22. 01.2009
		AU2007244226A1	08.11.2007
		CA2650685A1	08.11.2007
		INCHENP200806526E	27. 03.2009
		JP2008513304T2	10. 09.2009
		US2009325288A1	31. 12.2009
		RU2008146991A	10. 06.2010
		RU2433174C2	10. 11.2011
		EP2457994A1	30. 05.2012
CN101573442A	04.11.2009	WO2008060446A2	22.05.2008
		WO2008060446A9	10.07.2008
		WO2008060446A3	13.11.2008
		EP2084263A2	05.08.2009
		JP2010508846A	25.03.2010
		US2010129915A1	27.05.2010
		INCHENP200902753E	25.06.2010
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		EP1941029A2	09.07.2008
		AU2006306809A1	03.05.2007
		JP2009512458A	26.03.2009
		CA2626152A1	03.05.2007
		INCHENP200802009E	06.02.2009
		US2009304642A1	10.12.2009
		SG166174A1	29.11.2010
		WO2007050043A3	02.08.2007
US2004/0014209A1	22.01.2004	NONE	NONE

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2012/074852

Continuation of: Second Sheet: A.CLASSIFICATION OF SUBJECT MATTER:

C12N5/0735 (2010.01)i

C12N5/07(2010.01)i

C07D401/00 (2006.01)i

C07D401/12 (2006.01)i

C07D401/14 (2006.01)i

A61P9/00 (2006.01)i