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(54) **Title**: METHOD FOR DIFFERENTIATION OF PLURIPOTENT STEM CELLS INTO CARDIOMYOCYTES

(57) **Abstract**: This application relates to a method for differentiating pluripotent stem cells (PSCs) into cardiomyocytes. Moreover this application relates to a method for differentiating human embryonic stem cells (h ESCs) and induced pluripotent stem cells (i PSCs) into defined cardiomyocytes based on linked steps of chemically defined medium inductions.

METHOD FOR DIFFERENTIATION OF PLURIPOTENT STEM CELLS INTO CARDIOMYOCYTES

FIELD OF THE INVENTION

This application relates to a method for differentiating pluripotent stem cells (PSCs) into cardiomyocytes. Moreover this application relates to a method for differentiating human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) into proliferating cardiomyocytes based on linked steps of chemically defined medium inductions.

BACKGROUND

For many years, various cell culture systems have been used in preclinical drug development. However, established cell models only partially reflect pharmaceutically relevant disease-specific physiology because they are either derived from tumorigenic tissue or from transformed and immortalized cells. In particular, because terminally differentiated cardiomyocytes have been shown to possess limited proliferative potential, they do not have the capacity to effectively generate cell models for drug development. Hence there is a need for more disease relevant human cell types that can be used as reliable cell models in research and drug development.

Human embryonic stem cell (hESC) and induced pluripotent stem cells (iPSC) provide researchers with immense opportunities for generating functional human cell types such as cardiomyocytes, neuronal cells, pancreatic cells, etc.. Robust protocols for in vitro differentiation of pure hESC and iPSC derived human cardiomyocyte (hESCM) cultures would present a powerful tool, not only to advance the understanding of early human cardiogenesis, but also to use the cardiomyocytes as a non-transformed human cell model to test drug efficacy in preclinical stages of drug development and to assess cardiac toxicity before entering the clinic. Additionally, hESC derived human cardiomyocytes could open opportunities for identifying pathways critical to cardiac regeneration and ultimately lead to clinical applications supporting stem-cell based therapy.

For developing cell assay models for pharmaceutical research and development such differential protocols need to generate cells that ideally fulfill the following criteria: a) are robust with a high level of reproducibility; b) generate large numbers of highly pure cell types; c) can be differentiated in a short time; d) generate cells that can be frozen to ensure batch conformity for

multiple screening campaigns; e) provide functionality and physiology relevant for modeling disease-specific readouts. P. W. Burridge et al review prior art approaches to differentiate pluripotent cells into cardiomyocytes (P. Burridge, Keller, Gold, & Wu, 2012). So far none of the known protocols fulfil the criteria above. In particular, cardiomyocytes obtained through the known protocols are difficult to freeze and thaw without losing any functional properties.

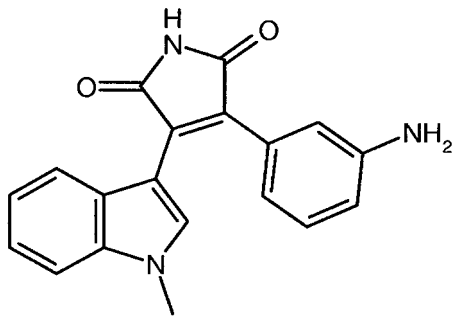
To fulfill these requirements, we developed a novel differentiation method that generates large numbers of highly pure cardiomyocytes (up-to 95%). The differentiation protocol is using defined small molecules to direct differentiation towards the cardiac lineage in a time span of 10 days. To further increase their purity, the cardiomyocytes are enriched by replating them using conditions that are preferential for cardiomyocytes. Furthermore the cardiomyocytes can afterwards be frozen, stored under liquid nitrogen and thawed again. The cardiomyocytes have been tested to compliant with several screening formats used in pharmaceutical research and development. The present invention provides an improved method for differentiating pluripotent stem cells into cardiomyocytes in a shorter amount of time and with a significantly increased yield compared to prior art protocols. The new method alleviates the necessity of obtaining embryoid bodies or small cell clumps from pluripotent stem cells and removes the major drawback of low reproducibility and standardization of methods known so far. Moreover, the high efficiency allows the use of these defined cardiomyocytes in large scales in drug discovery and safety assessments, in regenerative medicine applications, and in in vitro disease modeling in the pharmaceutical industry.

SUMMARY OF THE INVENTION

1. Provided herein is a method for differentiating pluripotent stem cells into cardiomyocytes, said method comprising the steps of:

   a) providing pluripotent cells at a density of 3 - $7x10^5/cm^2$

   b) incubating said cells in an insulin free medium comprising a compound of formula



In one embodiment the cells are incubated in an insulin free medium comprising 0.3-10μM of said compound.

In one embodiment step b) comprises incubating the cells for 12-48 hours.

In one embodiment the method additionally comprises step c) incubating said cells in an insulin free medium comprising Wnt- C59.

In one embodiment step c) comprises incubating said cells in an insulin free medium comprising 1-10 μM Wnt- C59.

In one embodiment step c) comprises incubating the cells for 24-72 hours.

In one embodiment the cells are incubated for 24-48 hours in insulin free medium in between the steps.

In one embodiment the method additionally comprises step d) incubating said cells in a medium comprising insulin.

In one embodiment the medium of step b), c) and d) comprises ascorbic acid.

In one embodiment the pluripotent stem cell is an induced pluripotent stem cell.

In one embodiment the induced pluripotent stem cell is a human cell.

In one embodiment the induced pluripotent stem cell is obtained from a subject suffering from a disease caused by dysfunction of heart cells.

In one embodiment cardiomyocytes obtained by a method according to any of the above embodiments are provided.

In one embodiment a biobank of cardiomyocytes obtained by a method according to any of the above embodiments are provided.

In one embodiment the cardiomyocytes obtained by a method according to any of the above embodiments or of the biobank of cardiomyocytes are used as an in vitro model for diseases caused by dysfunction of heart cells.

In one embodiment a therapeutic composition comprising cardiomyocytes obtained by a method according to any of the above embodiments or of the biobank of cardiomyocytes.

Any of the above embodiments may be present singly or in combination.

SHORT DESCRIPTION OF THE FIGURES

Figure 1: FACS Analysis Identifies a High Concentration of Cardiomyocytes at Differentiation Day 14. Both hESC and iPSC produce similar results. A: Human embryonic stem cell derived cardiomyocytes. B: Human induced pluripotent stem cell derived cardiomyocytes.

Figure 2: FACS Analysis of multiple cardiomyocyte differentiation proves robustness of the Protocol.

Figure 3: FACS Analysis Shows that Purification Method Improves Purity of Cardiomyocytes. A: 60 % purity with $5.5x10^5/cm^2$ cardiomyocytes on day 14. B: 98 % purity with $4.4x10^5/cm^2$ cardiomyocytes after additional purification step.

Figure 4: Immunofluorescence Staining - Confocal Microscope Analysis Reveals in Cells a Striation Pattern by alpha Actinin and Troponin T that is Typical for Cardiomyocytes. Green (*): Alpha Actinin, Red (#): Troponin T, Blue (+): Nuclei.

Figure 5: xCELLigent Analysis – Isoproterenol Increases Beating Frequency in Pluripotent Stem Cell Derived Cardiomyocytes.

Figure 6: Pluripotent stem cell derived cardiomyocytes show a high rate of survival Cardiomyocyte number after thawing at differentiation day 14 and day 18. In each experiment $4x10^6$ cells were frozen.

DETAILED DESCRIPTION OF THE INVENTION

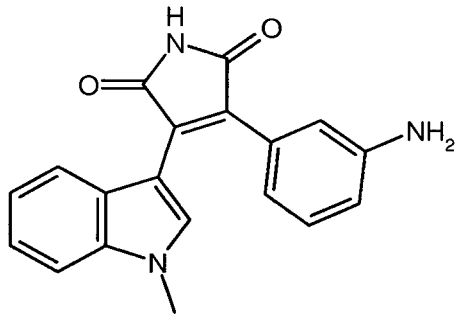
The present invention provides an improved method for differentiating pluripotent stem cells into cardiomyocytes in a shorter amount of time and with a significantly increased yield of proliferating cardiomyocytes compared to prior art protocols.

The novel method for differentiating human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) into defined cardiomyocytes disclosed herein is based on linked steps of chemically defined medium inductions, generating beating cells after only ten days (or earlier: eight days) after the differentiation was initiated.

In one embodiment a method for differentiating pluripotent stem cells into cardiomyocytes is provided, said method comprising the steps of:

a) providing pluripotent cells at a density of 3 - 7 x 10^5 cells/ cm^2

b) incubating said cells in an insulin-free medium comprising a compound of formula



3-(3-Amino-phenyl)-4-(1-methyl-1H-indol-3-yl)-pyrrole-2,5-dione (CP21)

The pluripotent stem cells are provided at a density of 3- 7 x 10^5 cells/ cm^2, i.e. a very high density. In one embodiment the cells are provided at a density of $5.5x10^5$ cells/cm^2. Surprisingly the inventors of the present method found that providing the cells at a high density is increasing the differentiation efficiency and cardiomyocyte yield.

In one embodiment the cells provided at high density are washed with a suitable buffer or medium prior to initializing differentiation with step a), to remove any dead cells.

The medium of step b) is an insulin-free medium. The lack of insulin in the early differentiation medium of step b) is important since earlier reports have shown that an insulin containing differentiation medium blocks cardiogenesis (Lian u. a., 2013).

To initialize differentiation, the cells are incubated in insulin-free medium comprising compound 21 (3-(3-Amino-phenyl)-4-(1-methyl-1H-indol-3-yl)-pyrrole-2,5-dione, also referred to as "compound 21" or "CP21" herein; see e.g. L. Gong et al; Bioorganic& Medicinal Chemistry Letters 20 (2010), 1693-1696) to activate the wnt-pathway. The optimal concentration of compound 21 to induce cardiomyocyte differentiation is dependent on the cell density of the pluripotent cells that are attached to the cell vessel. In several parallel differentiation experiments using different cell densities (1.8-11x10^5/cm2 hESC or iPSC) and various CP21 concentrations (0-10µM) it was found that using a cell density of 5.5x10^5/cm^2 and a CP21 concentration of 2µM resulted in the most efficient differentiation of pluripotent stem cells into cardiomyocytes. CP21 concentrations above 5µM showed decreased cell viability. This is surprising as prior art protocols require higher concentrations of other modulators of the Wnt pathway for efficient differentiation.

In one embodiment, step b) of the differentiation method comprises incubating the cells in a medium comprising 0.3 – 10 µM CP21, preferably 0.5 – 5 µM CP21. In one preferred embodiment step b) of the differentiation method comprises incubating the cells in a medium comprising 2 µM CP21.

After 24h CP21 incubation the cells show strong cell death. Testing various incubation times of CP21 showed that 24h was optimal for cardiogenesis and longer or shorter incubation times resulted in less efficient differentiation.

In one embodiment step b) comprises incubating the cells for 12-48 hours, preferably for 18- 24 hours, in an insulin free medium comprising CP21.

In one preferred embodiment step b) comprises incubating the cells for 24 hours in an insulin free medium comprising CP21.

In one embodiment the medium of step b) comprises Ascorbic Acid. The addition of Ascorbic acid to the basic medium has been shown to improve cardiomyocyte differentiation (Cao u. a., 2012).

Hence in one embodiment the medium of step b) is a, insulin-free medium comprising CP21 and Ascorbic Acid. In one such embodiment the medium comprises 0.5 – 5 μM CP21 and Ascorbic acid.

In one embodiment said method further comprises step c) incubating said cells in an insulin-free medium comprising Wnt-C59.

Wnt-C59 is a small molecule that blocks the Wnt signaling pathway (WO2010101849, 2-(4-(2-methylpyridin-4-yl)phenyl)-N-(4-(pyridin-3-yl)phenyl)acetamide). Wnt-C59 is a very potent and highly selective Wnt signaling antagonist. It prevents palmitylation of Wnt proteins by Porcupine (a membrane-bound O-acyltransferase), thereby blocking Wnt protein secretion and activity.

Using different concentrations of the wnt repressor Wnt-C59 (1-10μM) resulted in a significant increase in cardiomyocytes. The optimal concentration was identified at 2μM. In cases where no Wnt-C59 was added, the differentiation did not result in cardiomyocytes. Concentrations of more than 5μM Wnt-C59 showed increased cell death. In one embodiment, step c) of the differentiation method comprises incubating the cells in a medium comprising 1 -10 μM Wnt-C59. In one preferred embodiment step c) of the differentiation method comprises incubating the cells in a medium comprising 2 μM Wnt-C59.

Since the wnt pathway is highly complex other Wnt inhibitors with a different mode of action were tested.

Anthelmintic niclosamide (Chen et al, Biochemistry. 2009 Nov 3;48(43):10267-74.) promotes Frizzled1 endocytosis, downregulates Dishevelled-2 protein, and inhibits Wnt3A-stimulated beta-catenin stabilization and LEF/TCF reporter activity.

Pyrvinium is a potent inhibitor of Wnt signaling by binding all casein kinase 1 (CK1) family members in vitro and selectively potentiating casein kinase 1α (CK1α) kinase activity resulting in stabilization of Axin and increased β-catenin turnover (Thorne et al, Nat Chem Biol. 2010 Nov;6(11):829-36.).

Anthelmintic niclosamide and Pyrvinium were tested for their ability to induce cardiomyocyte differentiation. Contrary to WntC-59 both other Wnt inhibitors did not result in a successful generation of cardiomyocytes. The different efficacy of the tested Wnt inhibitors to differentiate pluripotent stem cells into cardiomyocytes suggests that the specific inhibition of the wnt pathway by blocking wnt secretion seems to be a key mechanism.

In one embodiment step c) comprises incubating the cells for 24-72 hours, preferably for 48 hours in an insulin free medium comprising Wnt-C59.

In one embodiment said insulin-free medium of step b) and c) is a serum-free medium. In one embodiment said insulin-free medium is RPMI1680 (Gibco).

In one embodiment the cells are incubated in an insulin free medium for 24 hours to 48 hours, preferably 48 hours between each step b) and c). In one embodiment said medium is a serum-free medium. In another embodiment said medium comprises Ascorbic Acid.

In one embodiment the cells are incubated in a serum-free, insulin-free medium comprising Ascorbic Acid for 24 hours to 48 hours, preferably 48 hours between each step, b) and c).

In one embodiment the method for differentiation of pluripotent cells into cardiomyocytes as described by any of the embodiments above additionally comprises step d) incubating the cells in medium comprising insulin. At this later stage, insulin promotes proliferation of cardiomyocytes and their cardiac precursor cells.

In one embodiment step d) comprises incubating the cells for 36- 60 hours, preferably for 48 hours in a medium comprising insulin. In one embodiment said medium is a serum-free medium. In another embodiment said medium comprises Ascorbic Acid.

Suitable media to be used in the expansion step d) are for example DMEM, high glucose + L-glutamine + pyruvate and Carnitine, Taurine, Creatine, BSA, Vitamin C or iCell Cardiomyocytes Maintenance Medium from Cellular Dynamics international.

Preferably the media are changed in between each step, e.g. the medium is removed e.g. by aspiration or centrifuging the cells and discarding the supernatant and then the medium used in the subsequent step is added to the cells. In one embodiment the cells are washed with a suitable buffer or medium prior to adding the medium of the subsequent step to remove any dead cells.

Buffers or media useful for washing the cells are known in the art. One example of a suitable buffer for washing the cells is e.g. phosphate buffered saline (PBS).

In one embodiment, the pluripotent cells useful in the method for differentiation are cultivated under conditions permitting stable growth and / or duplication times. For example, the cells are grown in pluripotency medium and passaged several times. "Pluripotency medium" as used herein refers to any chemically defined medium useful for the attachment of the pluripotent stem cells as single cells on a monolayer while maintaining their pluripotency and are well known in the art. In one embodiment, the pluripotency medium is a serum free medium comprising a small molecule inhibitor of the Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK) family of protein kinases (herein referred to as ROCK kinase inhibitor).

In one embodiment the ROCK kinase inhibitor is selected from the group of 1-(5-Isoquinolinesulfonyl) homopiperazine), N-Benzyl-2-(pyrimidin-4-ylamino) thiazole-4-carboxamide) and (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclo-hexanecarboxamide dihydrochloride).

Examples of ROCK kinase inhibitor useful herein are Fasudil (1-(5-Isoquinolinesulfonyl)homopiperazine), Thiazovivin (N-Benzyl-2-(pyrimidin-4-10 ylamino)thiazole-4-carboxamide) and Y27632 ((+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclo-hexanecarboxamide dihydrochloride, e.g. Catalogue Number: 1254 from Tocris bioscience). In one preferred embodiment the ROCK kinase inhibitor is Y27632. In one embodiment, the pluripotency medium is a serum free medium comprising 2-20 μM Y27632, preferably 5-10 μM Y27632. In another embodiment the pluripotency medium is a serum free medium comprising 2-20 μM Fasudil. In another embodiment the pluripotency medium is a serum free medium comprising 0.2-10 μM Thiazovivin.

With the new method presented herein it is now possible to differentiate cardiomyocytes expressing Alpha Actinin and Troponin T from pluripotent stem cells with a yield of up to 60-98%.

In one embodiment said method further comprises step e) replating the cells and incubating them in insulin free medium. This step further increases the purity of the cardiomyocytes. In one embodiment the cells are replated and incubated in insulin free medium supplemented with fetal bovine serum for 18- 32 hours, preferably for 24 hours. In one such embodiment, the medium further comprises a ROCK inhibitor. In one embodiment the ROCK inhibitor is Y-27632.

The cardiomyocytes obtained by the method described herein can be expanded for several passages and retain their functional properties after freezing and thawing.

As used herein the term "differentiating", "differentiation" refers to one or more steps to convert a less-differentiated cell into a somatic cell, for example to convert a pluripotent stem cell into cardiomyocytes. Differentiation of a pluripotent stem cell to cardiomyocytes is achieved by the method described herein.

The term "stem cell" as used herein refers to a cell that has the ability for self-renewal. An "undifferentiated stem cell" as used herein refers to a stem cell that has the ability to differentiate into a diverse range of cell types. As used herein, "pluripotent stem cells" as used herein refers to a stem cell that can give rise to cells of multiple cell types. Pluripotent stem cells (PSCs) include human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs). Human induced pluripotent stem cells can be derived from reprogrammed somatic cells, e.g. by transduction of four defined factors (Sox2, Oct4, Klf4, c-Myc) by methods known in the art. The human somatic cells can be obtained from a healthy individual or from a patient. These donor cells can be easily obtained from any suitable source. Preferred herein are sources that allow isolation of donor cells without invasive procedures on the human body, for example human skin cells, blood cells or cells obtainable from urine samples. Although human pluripotent stem cells are preferred, the method is also applicable to non-human pluripotent stem cells, such as primate, rodent (e.g. rat, mouse, rabbit) and dog pluripotent stem cells.

As used herein, "cardiomyocytes" are cells that express at least the cellular marker Troponin T (Troponin T Type 2 (Cardiac), gene symbol TNNT2, Entrez Gene: 7139, UniProtKB: P45379), and in a preferred embodiment also the cellular marker Alpha Actinin (ACTN2 actinin, alpha 2, gene symbol ACTN2, Entrez Gene: 88, UniProtKB: P35609). Expression of Troponin T and/ or Alpha Actinin can be assessed by methods known in the art, for example by FACS analysis as described in the example section. Cardiomyocytes can express spontaneous periodic contractile activity ("beating"). This means that when the cardiomyocytes obtained by the method of the invention are cultured in a suitable tissue culture environment with an appropriate Ca++ concentration and electrolyte balance, the cells can be observed to contract in a periodic fashion across one axis of the cell, and then release from contraction, without having to add any additional components to the culture medium. In addition the cells obtained by the method disclosed herein can express other characteristics of cardiomyocytes, such as ion channel or appropriate electrophysiology.

As used herein, "proliferating cardiomyocytes" are cells that express expressing Alpha Actinin and Troponin T and which proliferate by cell division.

"Expression of marker" means that a certain gene is transcribed into mRNA and usually is subsequently translated into a protein (its gene product) which exerts a certain function in a cell. The expression of a marker can be detected and quantified on the RNA level or on the protein level by methods known in the art. Preferred herein is the detection of the expression of a marker on the protein level, e.g. by testing for the presence of a certain protein with antibodies binding to the marker.

Any of the above embodiments may be present singly or in combination.

In one embodiment of the present invention a method for generating patient specific or healthy individual specific cardiomyocytes is provided. Towards this end, human induced pluripotent stem cells (iPSCs) obtained from a patient or healthy individual are differentiated into cardiomyocytes with the method described herein. The patient-specific human iPSCs can be obtained by methods known in the art by reprogramming somatic cells obtained from the patients or healthy individuals to pluripotent stem cells. For example, fibroblast cells, keratinocytes or adipocytes may be obtained by skin biopsy from the individual in need of treatment or from a healthy individual and reprogrammed to induced pluripotent stem cells by the methods known in the art. Other somatic cells suitable as a source for induced pluripotent stem cells are leucocytes cells obtained from blood samples or epithelial cells or other cells obtained from urine samples. The patient specific induced pluripotent stem cells are then differentiated to patient specific or healthy individual specific cardiomyocytes by the method described herein. In another aspect of the invention, a population of cardiomyocytes produced by any of the foregoing methods is provided. Preferably, the population of cardiomyocytes is patient specific, i.e. derived from iPSCs obtained from diseased individuals. In another embodiment the population of cardiomyocytes is obtained from a healthy individual.

Patient derived cardiomyocytes represent a disease relevant *in vitro* model to study the pathophysiology of diseases like Dilated cardiomyopathy, Hypertrophic cardiomyopathy, Restrictive cardiomyopathy, Arrhythmogenic right ventricular cardiomyopathy, coronary heart disease. In one embodiment the cardiomyocytes obtained by this method are used for screening for compounds that reverse, inhibit or prevent diseases caused by dysfunction of heart cells, e.g. Cardiac hypertrophy, decreased beating efficiency, disorganized striation of the cardiomyocyte, insufficient calcium handling. Preferably, the cardiomyocytes obtained by the method of the invention described herein are derived from diseased subjects. In another embodiment the cardiomyocytes obtained by this method are used for screening and evaluating new targets and compounds for treatment of heart diseases, e.g those mentioned above. Preferably, the

cardiomyocytes obtained by the method of the invention described herein are derived from individuals affected by diseases like for example Dilated cardiomyopathy, Hypertrophic cardiomyopathy, Restrictive cardiomyopathy, Arrhythmogenic right ventricular cardiomyopathy, coronary heart disease. Differentiating cardiomyocytes from diseased subjects represents a unique opportunity to early evaluate drug safety in a human background paradigm. In another embodiment the cardiomyocytes obtained by this method are used as an *in vitro* model of the heart.

The present invention provides a highly efficient method to supply patient specific cardiomyocytes or compatible cells from healthy individuals with the same HLA type suitable for transplantation, both derived in xeno-free conditions. "Xeno-free culture conditions" refers to a medium and a substrate for attachment that comprising components only of human and recombinant origin. Thus the risk of contamination with xenopathogens is circumvented and the renal cells are safe for use in regenerative medicine. Differentiation of patient specific induced pluripotent stem cells (iPSCs) into patient specific cardiomyocytes with the method described herein represents an easy accessible and reproducible technology to generate autologous sources of cardiomyocytes. The use of autologous and/or compatible cells in cell therapy offers a major advantage over the use of non-autologous cells, which are likely to be subject to immunological rejection. In contrast, autologous cells are unlikely to elicit significant immunological responses.

In a further preferred aspect of the invention the generation of a BioBank of patient specific cardiomyocytes is envisaged. In one embodiment, a BioBank comprising different populations of cardiomyocytes obtained from healthy individuals and / or patients is generated. The term "BioBank" as used herein means a library of biological samples taken from different individuals or species. The archived collection of specimen and associated data is intended for research purposes with the aim of addressing diseases associated with Dilated cardiomyopathy, Hypertrophic cardiomyopathy, Restrictive cardiomyopathy,Arrhythmogenic right ventricular cardiomyopathy, coronary heart disease. In another embodiment, the BioBank is used for vascular regenerative medicine approaches.

In another aspect, the invention provides a therapeutic composition comprising cardiomyocytes produced by any of the foregoing methods or comprising any of the foregoing cell populations. Preferably, the therapeutic compositions further comprise a physiologically compatible solution including, for example, a phosphate-buffered saline with 5% human serum albumin. The therapeutic composition can be used to treat, prevent, or stabilize diseases such as for example, Dilated cardiomyopathy, Hypertrophic cardiomyopathy, Restrictive cardiomyopathy, Arrhythmogenic right ventricular cardiomyopathy, coronary heart disease. For example, fibroblast cells, keratinocytes or adipocytes may be obtained by skin biopsy from the individual in need of treatment or from a healthy individual and reprogrammed to induced

pluripotent stem cells by the methods known in the art ("Induction of pluripotent stem cells from adult human fibroblasts by defined factors." Takahashi et al., 2007, Cell 131, 861-72). Other somatic cells suitable as a source for induced pluripotent stem cells are leucocytes cells obtained from blood samples or epithelial cells or other cells obtained from urine samples. The patient specific induced pluripotent stem cells are then differentiated to cardiomyocytes by the method described herein, harvested and introduced into the individual to treat the condition. The cardiomyocytes produced by the method of the invention may be used to replace or assist the normal function of diseased or damaged tissue.

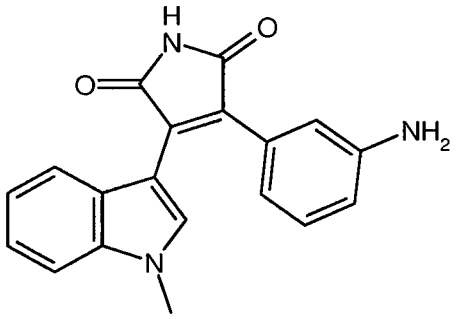
Another embodiment of the invention is the use of BioBanks of cardiomyocytes for therapy of diseases associated with Dilated cardiomyopathy, Hypertrophic cardiomyopathy, Restrictive cardiomyopathy, Arrhythmogenic right ventricular cardiomyopathy, coronary heart disease. The BioBanks preferably comprise cardiomyocytes obtained from patients or healthy individuals with several HLA types. Transplanting cells obtained from a healthy donor to an individual in need of treatment with a compatible HLA type obviates the significant problem of rejection reactions normally associated with heterologous cell transplants. Conventionally, rejection is prevented or reduced by the administration of immunosuppressants or anti-rejection drugs such as cyclosporine. However, such drugs have significant adverse side-effects, e.g., immunosuppression, carcinogenic properties, kidney toxicity as well as being very expensive. The present invention eliminates, or at least significantly reduces, the need for anti-rejection drugs, such as cyclosporine, imulan, FK-506, glucocorticoids, and rapamycin, and derivatives thereof.

With respect to the therapeutic methods of the invention, it is not intended that the administration of cardiomyocytes to a mammal be limited to a particular mode of administration, dosage, or frequency of dosing; the present invention contemplates all modes of administration, including intramuscular, intravenous, intrarticular, intralesional, subcutaneous, or any other route sufficient to provide a dose adequate to prevent or treat a disease. The cardiomyocytes may be administered to the mammal in a single dose or multiple doses. When multiple doses are administered, the doses may be separated from one another by, for example, one week, one month, one year, or ten years. One or more growth factors, hormones, interleukins, cytokines, small molecules or other cells may also be administered before, during, or after administration of the cells to further bias them towards a particular cell type.

EXAMPLES

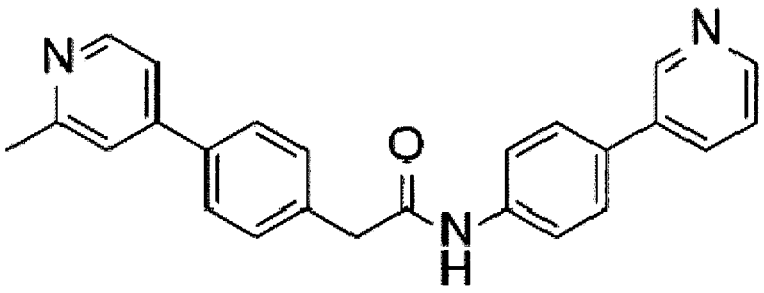
Materials and Methods

CP21R7: 3-(3-Amino-phenyl)-4-(1-methyl-1H-indol-3-yl)-pyrrole-2,5-dione (also referred to as "compound 21" or "CP21" herein; see e.g. L. Gong et al; Bioorganic& Medicinal Chemistry Letters 20 (2010), 1693-1696).



CP21R7

Wnt-C59 : 2-(4-(2-methylpyridin-4-yl)phenyl)-N-(4-(pyridin-3-yl)phenyl)acetamide (Cellagen Technology, Cat. C7641-2s, WO2010101849):



Human ESCs: SA001, LOT CA001 were isolated on March 20, 2001 at Göteborg University and Cellartis AB Arvid Wallgrens Backe 20, SE-413 46 Göteborg, SWEDEN follows all applicable laws in Sweden and is approved by the Local Research Ethics Committees at Göteborg University and Uppsala University. Embryo source: Frozen, surplus from IVF. Donor confidentiality: In order to protect the privacy and the confidentiality of the donors, all identifiers associated with the embryo donors have been removed. Thus, no information about the donors is accessible. Notably, the donation did not result in any financial gain for the donors. We have the approval to work with hESCs and to derive different cell lines. The responsible ethical committee (Ethikkommission beider Basel) and the Federal office of public health have approved our research project. (Ref-No: R-FP-S-1-0002-0000).

Human iPSCs: Catalogue Number: SC101A-1 Lot. Number 110218-FF from SBI System Biosciences / Catalogue Number: A13777 from Life technologies Gibco® Episomal hiPSC Line.

Human pluripotent stem cells are routinely cultured on hESC-qualified Matrigel (BD Bioscience) in TeSR1 medium (Stem cell Technologies). Cultures are passaged every 4-6 days using StemPro Accutase (Invitrogen). For an increased viability TeSR1 medium is comprising 10 μM ROCK-inhibitor one hour prior enzymatic dissociation.

500ml Differentiation medium

RPMI1680 + Glutamax	481ml	GIBCO#61870
Ascorbic Acid (10mg/ml)	4ml	Sigma#A4544
(final concentration: 80 μg/ml)		
B27 - Insulin (50x)	10ml	Invitrogen#05-0129SA
PenStrep	5ml	GIBCO#15140-122
(final concentration: 50U/ml)		

500ml Expansion medium

RPMI1680 + Glutamax	481ml	GIBCO#61870
Ascorbic Acid (10mg/ml)	4ml	Sigma#A4544
(final concentration: 80 µg/ml)		
B27 + Insulin (50x)	10ml	Invitrogen#12587-01
PenStrep	5ml	GIBCO#15140-122
(final concentration: 50U/ml)		

Further reagents and materials useful herein:

Matrigel (BD Bioscience, Cat.354277)
mTeSR1 medium (Stemcell Technologies, Cat.05850)
Accutase (Innovative Cell Technologies, Cat.AT-104)
Rock inhibitor, Y-27632 (Millipore, Cat.SCM075)
RPMI medium (Gibco by Life Technologies, Cat.61870)
Ascorbic Acid (Sigma, Cat.A4544)
50xB-27® Supplement Minus Insulin (Gibco by Life Technologies, Cat.0050129SA)
Penicillin-Streptomycin (Gibco by Life Technologies, Cat.15070)
50xB27 plus Insulin, minus Vitamin A (Gibco by Life Technologies, Cat.12587)
0.05% Trypsin/EDTA, 1x (Gibco by Life Technologies, Cat.25300)
autoMACS Running Buffer (Miltenyi, Cat.130-091-221)
Inside Perm + InsideFix (Miltenyi, Inside Stain Kit, Cat.130-090-477)
0.1% Gelatine (Millipore, Cat.ES-006-B)
Cryogenic vial (Corning#430659)
Mr.Frosty Freezing Container (Thermo Scientific#5100-0001)
DMSO (Sigma#D2438)
Fetal Bovine Serum (Invitrogen#16000044)
Falcon Cell Culture Dishes 35x10mm (BD#353001)
Falcon Cell Culture Dishes 100x20mm (BD#353003)
6-well- plates Corning Costar (Sigma#CLS3516)
Anti-Sarcomeric Alpha Actinin [EA-53] antibody (Abcam, Cat.ab9465)
Anti-Cardiac Troponin T antibody (Abcam, Cat.ab45932)
Alexa Fluor® 488 and Donkey Anti-Mouse IgG (H+L) (Invitrogen, Cat.A21202)
Alexa Fluor® 647 Donkey Anti-Rabbit IgG (H+L) (Invitrogen, Cat.A31573)
Alexa Fluor® 555 Donkey Anti-Rabbit IgG (H+L) (Invitrogen, Cat.A31572)
Hoechst 33258, Pentahydrate (bis-Benzimide) (Molecular Probes, Cat.H3569)

Differentiation of cardiomyocytes from human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC)

Human embryonic stem cells (hESC) or induced pluripotent stem cells (iPSC) were cultured in 56cm^2 dishes coated with Matrigel (BD Bioscience, Cat.354277) at 37°C and 5% CO2 in 10 ml mTeSR1 medium (Stemcell Technologies, Cat.05850).

Before starting the cardiomyocyte differentiation the cells were passaged for 3-4 times to ensure that the pluripotent stem cells showed stable growth and duplication times.

To propagate pluripotent stem cells by conserving their pluripotent state, hESC or iPSC were treated the following: The cells were washed once with 10 ml PBS -/-, and afterwards incubated with 3 ml Accutase (Innovative Cell Technologies, Cat.AT-104) for 2-3 minutes at 37°C and 5% CO_2, to detach the cells.

The enzymatic reaction of Accutase was stopped with 7 ml mTeSR1 and followed by centrifugation of the cells for 3 minutes at 500xg.

The cells were resuspended in 10ml mTeSR1, and counted. For further cultivation, 2x10^6 cells were plate on 56cm^2 dishes with fresh coated Matrigel. Further the hESC or iPSC were cultivated in 10 ml mTeSR1 and 10 µM Rock inhibitor, Y-27632 (Millipore, Cat.SCM075) at 37°C and 5% CO2. Subsequently, 10 ml mTeSR1 medium was changed daily and the pluripotent stem cells were cultivated to a density of 80% before passaging.

For successful differentiation into cardiomyocytes pluripotent stem cells were plated at high density using 5.5x10^5/cm^2 of hESC or iPSC. Passaging and cultivation were performed as described above for pluripotent stem cells.

After 24 hours (day 1) the hESC or iPSC were washed once with 180µl/cm^2 PBS -/- and the cultivation medium was changed to 180µl/cm^2 differentiation medium.

To initiate the differentiation of the pluripotent stem cells towards the cardiac lineage, the medium was comprising 2µM compound 21 (CP21,) a small molecule and highly selective inhibitor of glycogen synthase kinase 3 (GSK3β).

After 24 hours (day 2) incubation with CP21, the cells were washed with PBS -/- as described above and cultivated for 48 hours in 220µl/cm2 differentiation medium.

After 48 hours (day 4) the cells were washed with PBS -/- as described above and cultivated for 48 hours in 220 µl/cm^2 differentiation medium comprising 2µM Wnt-C59 (Cellagen Technology, Cat. C7641-2s, WO2010101849), a potent wnt signaling inhibitor, by blocking the wnt secretion.

After 48 hours (day 6) the cells were washed with PBS -/- as described above and cultivated for 48 hours in in 220 µl/cm^2 differentiation medium.

After 48 hours (day 8) the cells were washed with PBS -/- as described above and cultivated for 48 hours in 220 µl/cm^2 RPMI medium comprising Ascorbic Acid, Penicillin-Streptomycin but now including B27 plus Insulin, minus Vitamin A (= expansion medium)

First cardiomyocytes visible by beating cells were observed at day 8 of differentiation further increasing until day 14.

Subsequent medium changes were performed every 48 hours using 220 µl/cm^2 expansion medium.

Cell Characterization

To test the efficiency of the differentiation process the cardiomyocytes were characterized at differentiation day 14 by cell immunohistochemistry and Fluorescence Activated Cell Sorting (FACS) using antigens specific to cardiomyocytes.

Fluorescence Activated Cell Sorting (FACS) Analysis

Cells were washed with 180 µl/cm^2 PBS -/- and dissociated 5-10 minutes with 100 µl/cm^2 0.05% 1x Trypsin/EDTA (Gibco by Life Technologies, Cat.25300) at 37°C and 5% CO_2.

If necessary, the cells were gently scraped from the cultivation vessel, pipetted up and down and subsequently incubated 5-10 minutes at 37°C and 5% CO_2.

Afterwards threefold expansion medium and 10% fetal bovine serum (FBS) was added.

Then, cells were filtered through 100 µm cell strainer and counted.

For analysis, 1x10^6 cells in suspension were transferred into 1.5 ml tube. After 3 minutes centrifugation at 500xg, supernatant was removed and cells were fixed with 50 µl of Inside Fix

(Miltenyi, Inside Stain Kit, Cat.130-090-477) and 50 µl PBS -/- for 15 minutes at room temperature in the dark.

Afterwards 100 µl autoMACS Running Buffer (Miltenyi, Cat.130-091-221) was added and centrifuged. Supernatant was removed and cells were washed with 100 µl Inside Perm (Miltenyi, Inside Stain Kit, Cat.130-090-477), centrifuged and supernatant was removed. Cells were incubated with Anti-Sarcomeric Alpha Actinin [EA-53] antibody (Abcam, Cat.ab9465) and Anti-Cardiac Troponin T antibody (Abcam, Cat.ab45932), 1:100 diluted in Inside Perm for 1 hour at 4°C.

Afterwards cells were washed with 500 µl Running Buffer, centrifuged and supernatant was removed. Cells were incubated with secondary antibodies (1:1000 in Inside Perm) for 10 minutes at room temperature. The following secondary antibodies were used: Alexa Fluor® 488 Donkey Anti-Mouse IgG (H+L) (Invitrogen, Cat.A21202) and Alexa Fluor® 647 Donkey Anti-Rabbit IgG (H+L) (Invitrogen, Cat.A31573).

Subsequently, cells were washed with 500µl Running Buffer, after centrifugation resuspended cells in 500µl Running Buffer and measured by fluorescence activated cell sorting (FACS) system.

Differentiation of cardiomyocytes from human embryonic stemcells (hESC) and induced pluripotent stemcells (iPSC) using different CP21 concentrations

The protocol as described above was repeated with different CP21 concentrations. The results are shown in the table below: (-) No cardiomyocytes obtained, (+) – (+++): Amount of cardiomyocytes obtained.

Cp21 conc. in µM	0	0.3	1	2	3	5	10
Experiment I	-	-	+	++	++	+	-
Experiment II	-	-	+	+++	+	-	-
Experiment III	-	-	+	++	++	-	-

Purification

To increase purity of the cardiomyocytes an enrichment step was developed.

As described above cells were washed with 180 µl/cm^2 PBS -/- and dissociated 5-10 minutes with 100 µl/cm^2 0.05% 1x Trypsin/EDTA (Gibco by Life Technologies, Cat.25300) at 37°C and 5% CO_2.

If necessary, the cells were gently scraped from the cultivation vessel, pipetted up and down and subsequently incubated 5-10 minutes at 37°C and 5% CO_2.

Afterwards threefold expansion medium and 10% fetal bovine serum (FBS) was added.

Then, cells were filtered through 100µm cell strainer and counted.

Fresh plates coated with 130 µl/cm^2 0.1% Gelatine (Millipore, Cat.ES-006-B) were incubated for 1 hour at 37°C.

2.7x10^5/cm^2 cells were plated in 180µl/cm^2 expansion medium 10% fetal bovine serum (FBS). In addition 10 µM Rock inhibitor was added. After 24 hours 220 µl/cm^2 medium was changed expansion medium without FBS and Rock inhibitor. The medium was changed every 48 hours.

At day 18-21 cells were analysed with FACS and again replated as described above in different formats for following analysis: Immunoflourescence stainings, xCELLigence to detect Beating Rhythm and Proarrhythmic Effects of Compounds in Stem Cell-Derived Cardiomyocytes.

Cells were transferred to plate formats compliant with assay conditions. Cells were allowed to attach for 24 hours in 200 µl/cm^2 expansion medium plus 10% fetal bovine serum (FBS). In addition 10 µM Rock inhibitor was added. After 24 hours 220 µl/cm^2 expansion medium was changed without FBS and Rock inhibitor. The medium was changed every 48 hours.

Freezing and Thawing of Cardiomyocytes

At day 14 cardiomyocytes were replated as describes for the purification method. On day 18 cells were dissociated as outlined above and subsequently analyzed by FACS for their alpha-actinin and troponin T expression. Cultures with 80% and above cardiomyocytes were subjected to the freezing protocol. Culture with less than 80% cardiomyocytes were discarded.

Cells were counted and $4x10^6$ cells were frozen with 1 ml of cooled FBS comprising 10% DMSO and 10µM Y-27632 per cryogenic vial.

Cells were centrifuged for 3 minutes at 500xg and subsequently resuspended carefully in FBS supplemented 10% DMSO and 10µM Y-27632. 1 ml aliquots of the cardiomyocytes cell suspensions were filled into 4°C pre-chilled cryogenic vials and frozen for 24 hours at -80°C. Afterwards cryovials were stored in liquid nitrogen.

To thaw the cardiomyocytes a vial was incubated for 1-2 minutes at 37°C in a waterbath and the cells were carefully transferred in 10ml expansion medium plus 10% fetal bovine serum. Cells were centrifuged for 2 minutes at 300xg. Afterwards the pellet was resuspended in 6ml expansion medium plus 10% fetal bovine serum and 10µM Y-27632 and plated onto 3 wells of 6-well-plate coated with 0.1% gelatin. After 24 hours cell were changed to 220 $\mu l/cm^2$ expansion medium without FBS and Y-27632. Subsequently the medium was changed every 3 days and after 5-7 days the cells were plated onto plate formats compliant with assay conditions (e.g. Assay for detecting disorganization of cardiac striation: 96 well format; Assay for recording beating frequency: 96 well format).

xCELLigent Cardiomyocyte Beating Analysis

Isoproterenol increases the heart rate and myocardial contractility by stimulating cardiac beta-1 receptors. To detect this proarrhythmic effect in the stem cell derived cardiomyocytes, $7x10^4/cm^2$ cells were plated on special E-Plate Cardio 96 (Roche, Cat. No. 05232368001) coated with 130 $\mu l/cm^2$ 0.1% Gelatine for 1 hour at 37°C. After cells attached to the plate and recovered for 2 days as described above, medium was changed to iCell Cardiomyocytes Maintenance Medium (Cellular Dynamics, Cat. No.CMM-100-120-005). Cells were measured using the xCELLigence RTCA Cardio System (Roche Applied Science). 7 days after plating the cells were treated with 3µM Isoproterenol and measured directly. Each 96-well plate was measured at a resolution of 12,9 ms. The first 3 minutes were measured without interruption and over the next 24 hours the cells were measured every 15 minutes for 1 minute duration.

Immunofluorescence Staining

For immunofluorescence staining, cells were fixed with 4% Paraformaldehyd for 15 minutes at room temperature.

After washing cells with PBS -/-, the cells were blocked and permeabilized for 20 minutes at room temperature with 10% donkey serum in PBS -/- and 0.1% Triton (Blocking Buffer). Afterwards the cells were stained overnight in blocking buffer at 4°C with 1:100 diluted primary antibodies Anti-Sarcomeric Alpha Actinin [EA-53] antibody (Abcam, Cat.ab9465) and Anti-Cardiac Troponin T antibody (Abcam, Cat.ab45932).

Cells were washed with PBS -/- and stained 1:1000 in blocking buffer with secondary antibodies Alexa Fluor® 488 and Donkey Anti-Mouse IgG (H+L) (Invitrogen, Cat.A21202) and Alexa Fluor® 555 Donkey Anti-Rabbit IgG (H+L) (Invitrogen, Cat.A31572) for one hour at room temperature in blocking buffer. Nuclei were stained after several PBS -/- washing steps with 1:1000 diluted Hoechst 33258, Pentahydrate (bis-Benzimide) (Molecular Probes, Cat.H3569) in PBS -/-.

Results

After differentiation, the cells were analyzed for their cardiomyocyte content. Fig. 1 depicts a FACS analysis quantifying cardiomyocytes on differentiation day 14.

An average of 80-90% cardiomyocytes characterized by Alpha Actinin and Troponin T double positive cells was obtained. In Fig. 1, a subpopulation of the cells stained single positive for Alpha Actinin (5-10%). This is an indication of more immature cardiomyocytes and for this reason this population was not included for scoring. This result was independent of using hESC (Fig.1A) or iPSC (Fig. 1B) as a source of pluripotent stem cells. Starting with $5.5x10^5/cm^2$ pluripotent stem cells the differentiation protocol generated an average of $4\text{-}5x10^5/cm^2$ Alpha Actinin and Troponin T positive cardiomyocytes.

To demonstrate robustness of the differentiation protocol we performed several experiments and analyzed the content of cardiomyocytes in each culture. Fig. 2 depicts 10 independent experiments showing differentiation efficacies towards cardiomyocytes ranging between 95 and 40%. However, the majority of the experiments (7 out of 10) showed a cardiomyocyte content over 75%, which is an . acceptable ratio. Experiments generating 60 % cardiomyocytes and more

were progressed. Differentiations with less than 60% cardiomyocytes were discarded. The variability between experiments is most likely caused by the quality and cultivation state of the pluripotent stem cells at the beginning of the differentiation.

To further increase purity towards cardiomyocytes, an additional purification step was established. At differentiation day 14, the cells were detached and analysed by FACS. Figure 3 A shows that the culture counts $9x10^5/cm^2$ cells containing 60% ($5.4x10^5/cm^2$) cardiomyocytes at day 14. For the purification method to be successful, the minimum percentage of Alpha Actinin positive cells should be 60% and more. The dissociated cells are replated ($2.7x10^5/cm^2$) and cultivated in expansion medium. After 7 days cells were harvested, $4.5x10^5/cm^2$ cells were counted and analyzed. Figure 3 B shows that after the purification step the cardiomyocyte content in the culture increases from 60 to 98%, demonstrating the efficient generation of $4.4x10^5/cm^2$ highly enriched cardiomyocytes by using this method. Afterwards cells were transferred to cultivation formats compliant with assay conditions.

The cardiomyocytes were analysed by immunofluorescence for further characterization. Fig.4 shows an immunofluorescence stain of cardiomyocytes at day 27 using antibodies against Alpha Actinin (green), Troponin T (red) and the nuclei specific Hoechst stain (blue). The resulting immunofluorescence in Fig. 4 shows cells with alpha actinin and troponin T specific striation that is characteristic for cardiomyocytes.

Activation of β-receptors on the heart induces positive chronotropic effects in cardiomyocytes. To confirm that the pluripotent stem cell derived cardiomyocytes respond to β-receptor activation, the cardiomyocytes were incubated with the β-receptor agonist isoproterenol and subsequently analysed using the xCELLigence system. Fig. 5 shows that after incubating the pluripotent stem cell derived cardiomyocytes with 3μM isoproterenol the beating rate increased to 60 beats a minute from 45 when compared to untreated control. This experiment further demonstrated that the pluripotent stem cell derived cardiomyocytes generated by this differentiation protocol resemble functional human cardiomyocytes.

Freezing and thawing of cardiomyocytes has been traditionally difficult due to the low level of cell recovery after thawing.

Since it is important for assay development to have large batches of identical cells, we tested if the pluripotent stem cell derived cardiomyocytes can be stored in a freezer and afterwards

thawed. We tried to freeze the differentiated cardiomyocytes at different ages (day 14, 18 and 32). As can be seen in fig. 6, cardiomyocytes frozen at earlier differentiation stages show a higher cell survival rate after thawing. However, cell thawed after purification on day 18 of differentiation provided the best conditions for using the cells for pharmaceutical assays. At this stage cells show a much higher purity after thawing and cardiomyocytes could be directly transferred onto cell culture vessels that are compliant with assay formats.

When thawing cardiomyocytes frozen at differentiation day 32, the survival rate was very low and many cells were lost. This is due to the low proliferation rate of the cells at this stage resulting in low recovery of cardiomyocytes after thawing.

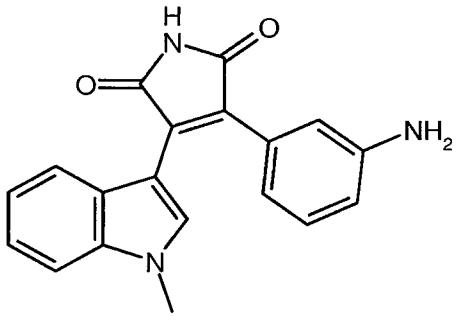
We determined that the optimal time for freezing pluripotent derived cardiomyocytes was after purification at differentiation day 18. At this stage recovery rate is on average more than 85% Alpha Actinin and Troponin T positive cells and cardiomyocytes are still proliferating providing optimal conditions for using the cells further for assay development.

Claims

1. A method for differentiating pluripotent stem cells into cardiomyocytes, said method comprising the steps of:

a) providing pluripotent cells at a density of 3 - $7x10^5/cm^2$

b) incubating said cells in an insulin free medium comprising a compound of formula



2. The method of claim 1, wherein the cells are incubated in an insulin free medium comprising 0.3-10μM of said compound.

3. The method of claim 1 or 2, wherein step b) comprises incubating the cells for 12-48 hours.

4. The method of any of claims 1 to 3, additionally comprising step c) incubating said cells in an insulin free medium comprising Wnt- C59.

5. The method of claim 4, wherein step c) comprises incubating said cells in an insulin free medium comprising 1-10 μM Wnt- C59.

6. The method of claims 4 or 5, wherein step c) comprises incubating the cells for 24-72 hours.

7. The method of any of claims 1 to 6, wherein the cells are incubated for 24-48 hours in insulin free medium in between the steps.

8. The method of any of claims 1 to 7, additionally comprising step d) incubating said cells in a medium comprising insulin.

9. The method of any of claims 1 to 8, wherein the medium of step b), c) and d) comprises ascorbic acid.

10. The method of any of claims 1 to 9 wherein said pluripotent stem cell is an induced pluripotent stem cell.

11. The method of claim 10, wherein said induced pluripotent stem cell is a human cell.

12. The method of claims 10 or 11, wherein said induced pluripotent stem cell is obtained from a subject suffering from a disease caused by dysfunction of heart cells.

13. Cardiomyocytes obtained by a method according to any of claims 1 to 12.

14. A biobank of cardiomyocytes obtained by a method according to any of claims 1 to 12.

15. Use of the cardiomyocytes obtained by a method according to any of claims 1 to 12 or of the biobank of claim 14 as in vitro model for diseases caused by dysfunction of heart cells.

16. A therapeutic composition comprising cardiomyocytes obtained by a method according to any of claims 1 to 12 or the biobank of claim 14.

17. The methods and uses essentially as herein described.

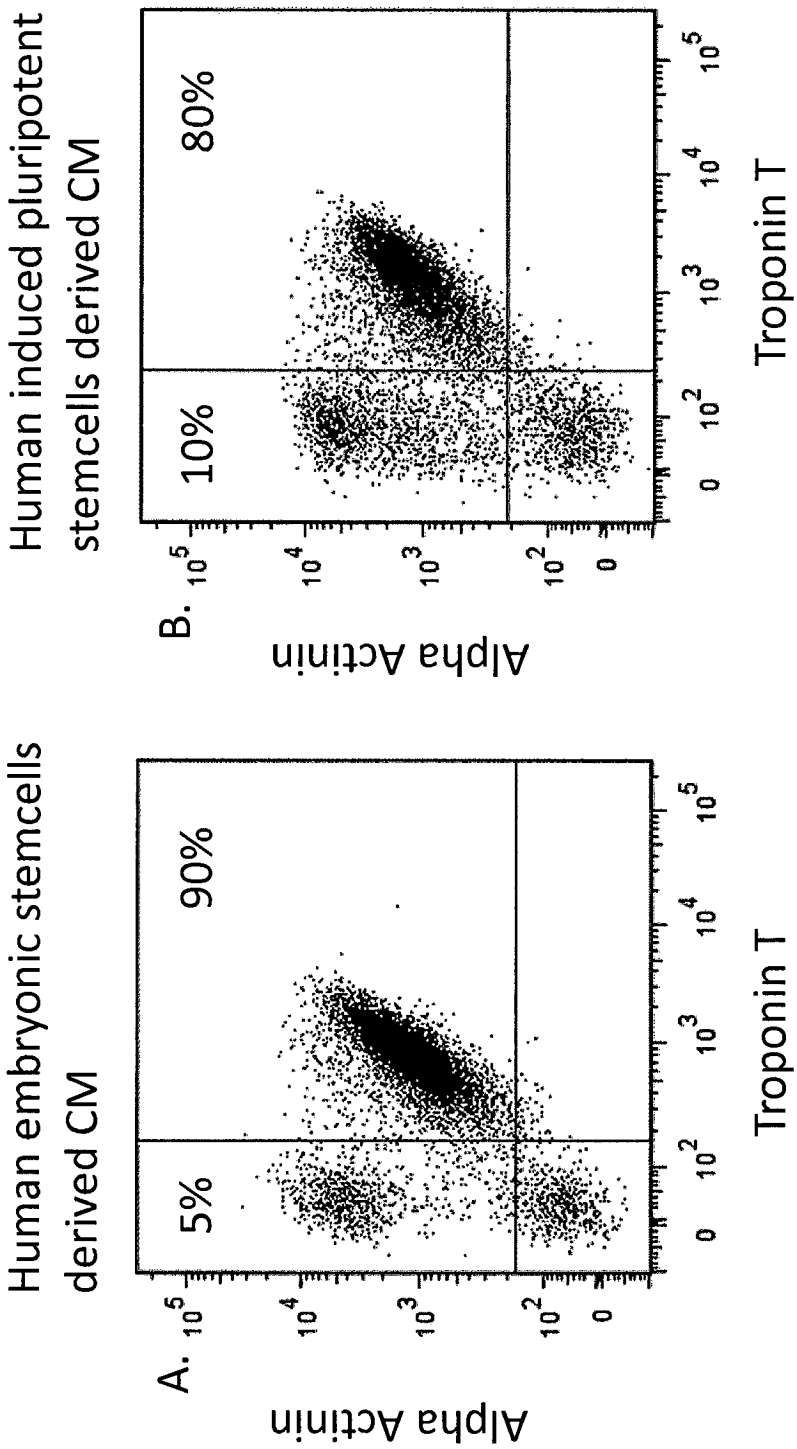

Human embryonic stemcells derived CM
A.
5%
90%
Alpha Actinin
Troponin T
Human induced pluripotent stemcells derived CM
B.
10%
80%
Alpha Actinin
Troponin T

Fig. 1

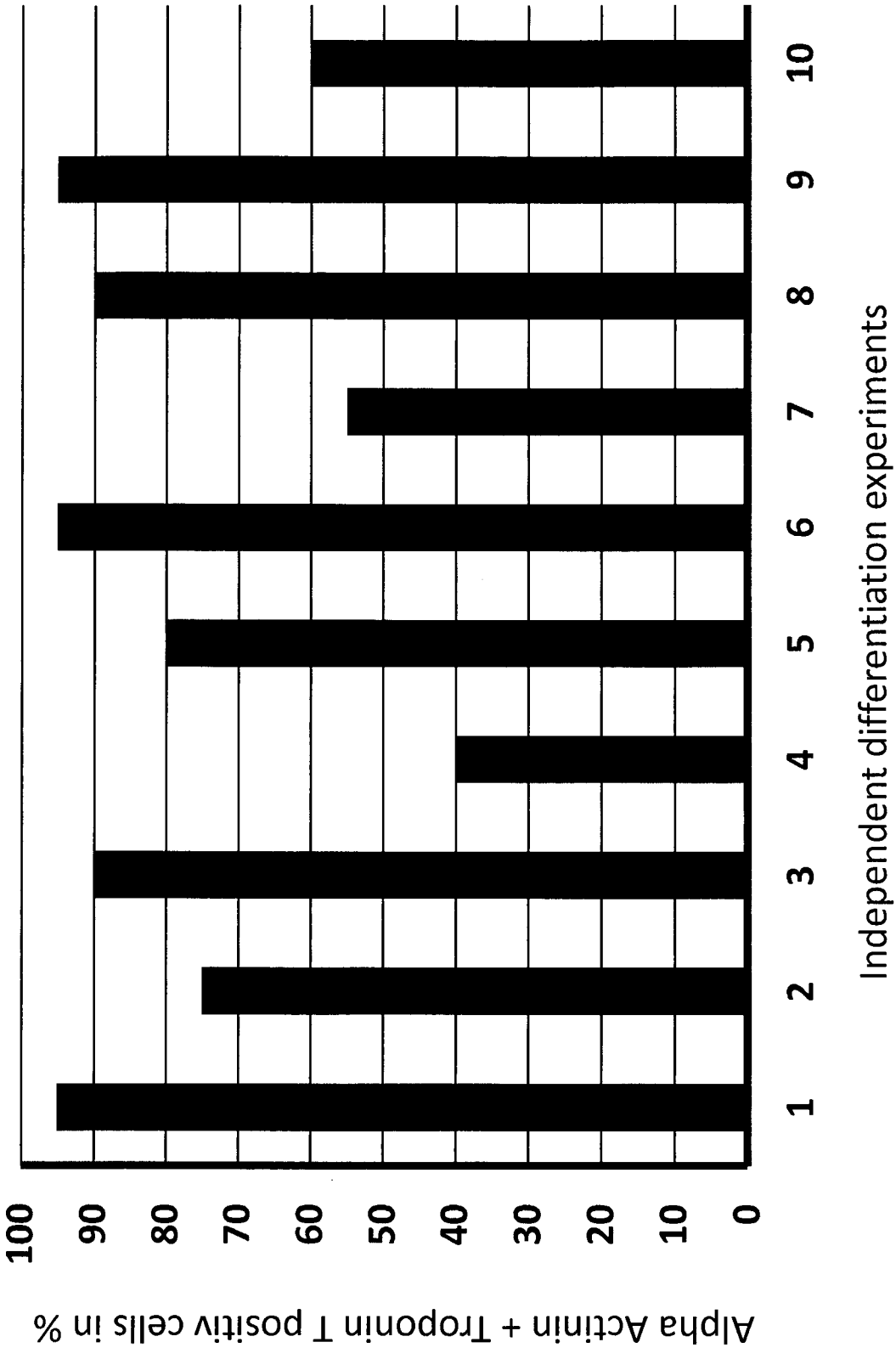
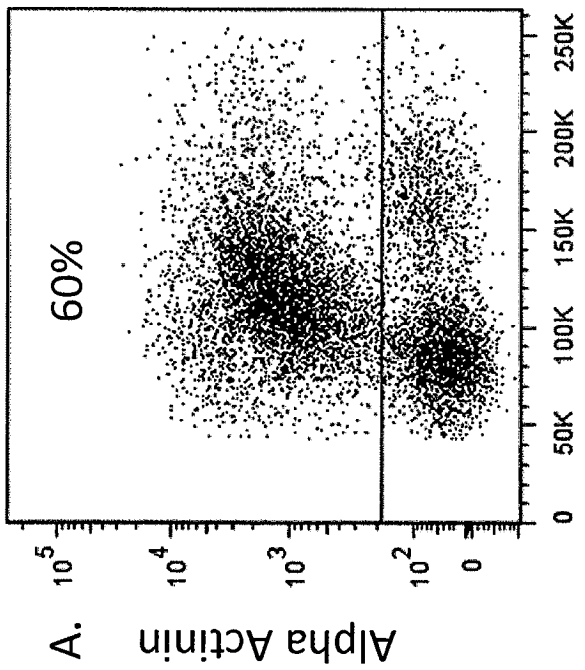

Alpha Actinin + Troponin T positiv cells in %
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
Independent differentiation experiments

Fig. 2


A.
Alpha Actinin
60%
FSC
0
50K
100K
150K
200K
250K
Differentiation on day 14
60 % purity
5.5x10^5/cm^2 cardiomyocytes

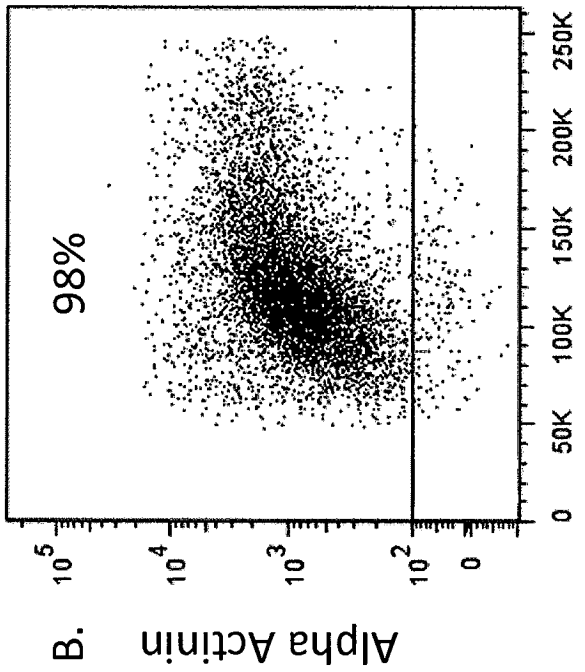

B.
Alpha Actinin
98%
FSC
Purification on day 21
98 % purity
4.4x10^5/cm^2 cardiomyocytes

Fig. 3

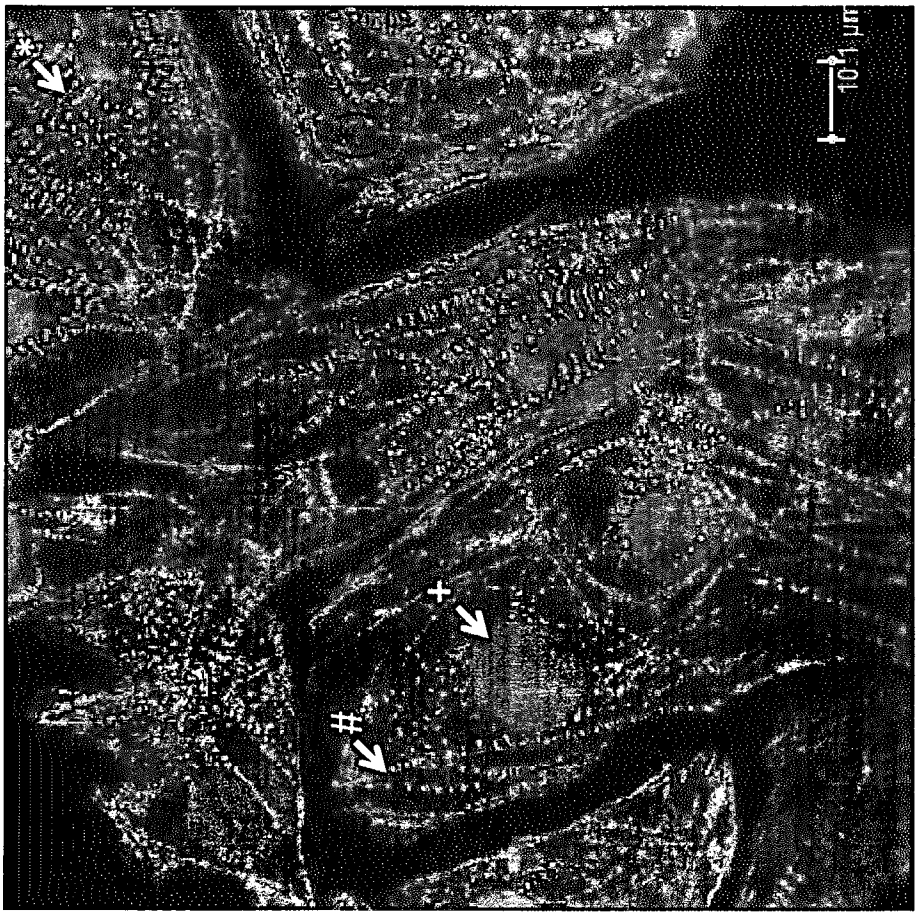
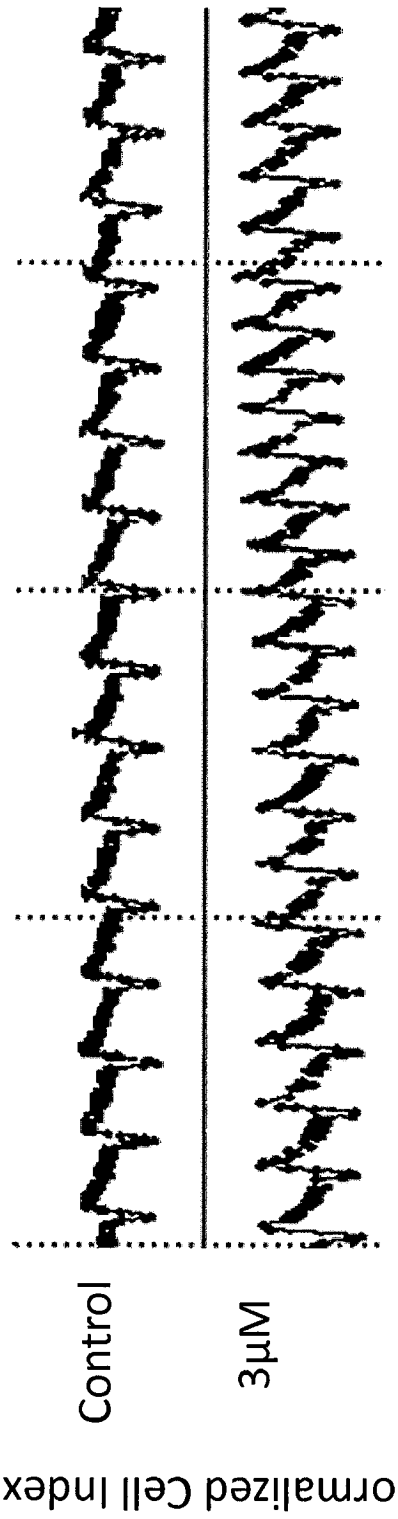


Fig. 4



Control	45 beats/minute
3µM Isoproterenol	60 beats/minute

Fig. 5

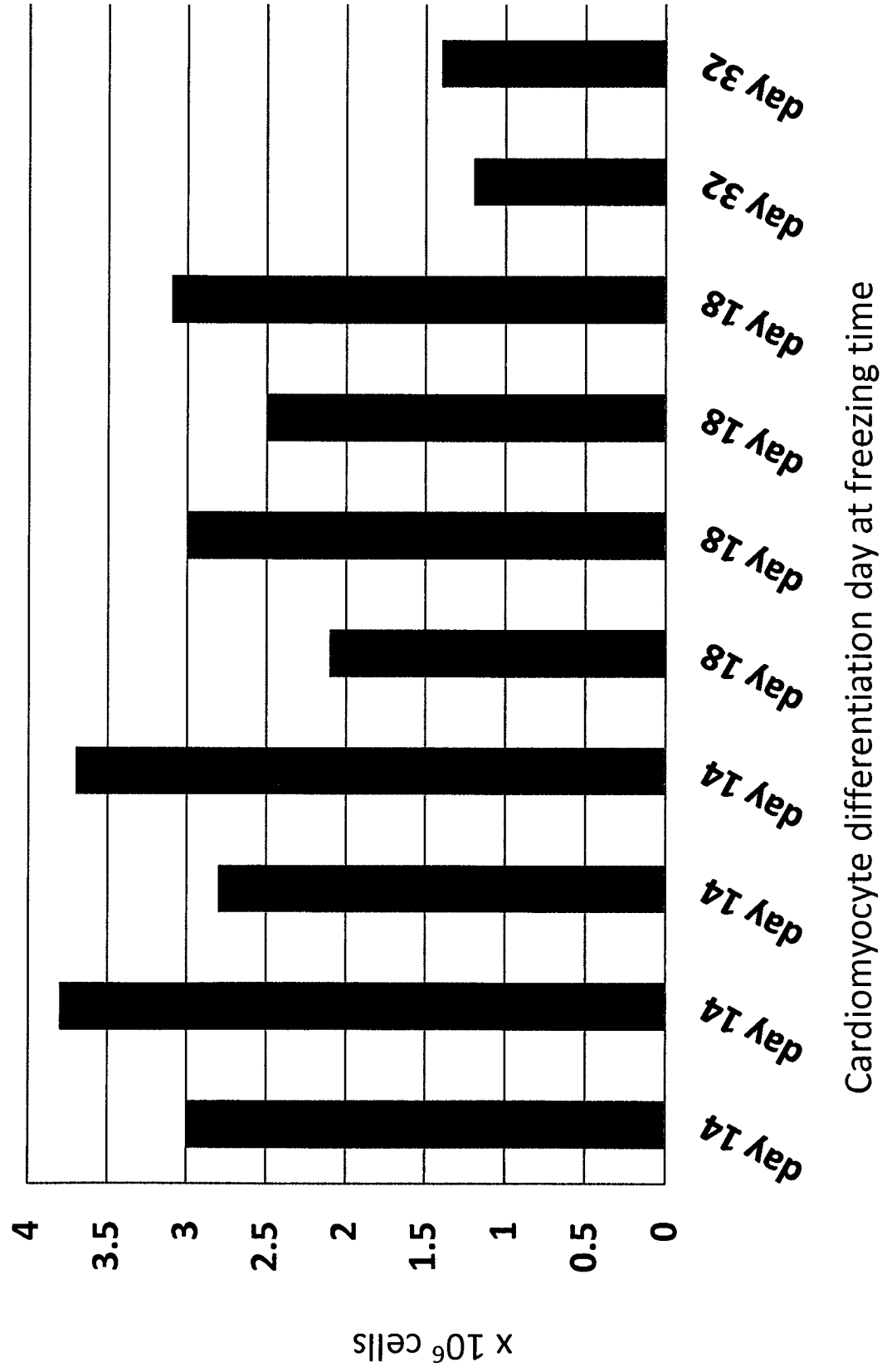

x 10⁶ cells
4
3.5
3
2.5
2
1.5
1
0.5
0
day 14
day 14
day 14
day 14
day 18
day 18
day 18
day 18
day 32
day 32
Cardiomyocyte differentiation day at freezing time

Fig. 6

INTERNATIONAL SEARCH REPORT

international application No
PCT/EP2015/059745

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/077
ADD.

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)
C12N

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)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/056072 A1 (WISCONSIN ALUMNI RES FOUND [US]; PALECEK SEAN [US]; KAMP TIMOTHY [US];) 18 April 2013 (2013-04-18)	13-17
Y	claims 1-40 examples 1-8	1-17

X	XIAOJUN LIAN ET AL: "Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/[beta]-catenin signaling under fully defined conditions", NATURE PROTOCOLS, vol. 8, no. 1, 20 December 2012 (2012-12-20), pages 162-175, XP055053767, ISSN: 1754-2189, DOI: 10.1038/nprot.2012.150	13-17
Y	the whole document	1-17

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[X] Further documents are listed in the continuation of Box C.

[X] 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
7 July 2015	20/07/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bayer, Martin

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHANG DONGHUI ET AL: "Tissue-engineered cardiac patch for advanced functional maturation of human ESC-derived cardiomyocytes", BIOMATERIALS, ELSEVIER SCIENCE PUBLISHERS BV., BARKING, GB, vol. 34, no. 23, 2 May 2013 (2013-05-02), pages 5813-5820, XP028541480, ISSN: 0142-9612, DOI: 10.1016/J.BIOMATERIALS.2013.04.026	13-17
Y	the whole document	1-17
Y	WO 2012/168167 A1 (HOFFMANN LA ROCHE [CH]; CHRISTENSEN KLAUS [CH]; GRAF MARTIN [CH]; IACO) 13 December 2012 (2012-12-13) example 1 page 6, paragraph 3	1-17

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2015/059745

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013056072 A1	18-04-2013	US 2013189785 A1	25-07-2013
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(54)发明名称

用于使多能干细胞分化为心肌细胞的方法

(57)摘要

本申请涉及用于使多能干细胞(PSC)分化为心肌细胞的方法。此外,本申请涉及用于使人胚胎干细胞(hESC)和诱导型多能干细胞(iPSC)分化为确定的心肌细胞的方法,其基于化学成分确定的培养基诱导的连接步骤。

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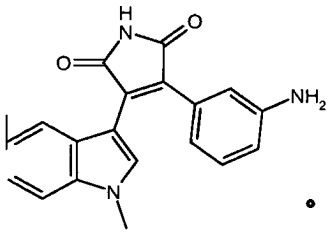
1.用于使多能干细胞分化为心肌细胞的方法，所述方法包括步骤：

a)提供3-7x10^5/cm^2密度的多能细胞；

b)在含有下式化合物的无胰岛素培养基中孵育所述细胞：



2.权利要求1的方法，其中在含有0.3-10μM所述化合物的无胰岛素培养基中孵育细胞。

3.权利要求1或2的方法，其中步骤b)包括孵育细胞12-48小时。

4.权利要求1至3中任一项的方法，其还包括步骤c)在含有Wnt-C59的无胰岛素培养基中孵育所述细胞。

5.权利要求4的方法，其中步骤c)包括在含有1-10μM Wnt-C59的无胰岛素培养基中孵育所述细胞。

6.权利要求4或5的方法，其中步骤c)包括孵育细胞24-72小时。

7.权利要求1至6中任一项的方法，其中在步骤之间在无胰岛素培养基中孵育细胞24-48小时。

8.权利要求1至7中任一项的方法，其还包括步骤d)在含有胰岛素的培养基中孵育细胞。

9.权利要求1至8中任一项的方法，其中步骤b)、c)和d)的培养基包含维生素C。

10.权利要求1至9中任一项的方法，其中多能干细胞是诱导型多能干细胞。

11.权利要求10的方法，其中诱导型多能干细胞是人细胞。

12.权利要求10或11的方法，其中诱导型多能干细胞获自罹患由心脏细胞功能障碍引起的疾病的个体。

13.心肌细胞，其通过权利要求1至12中任一项的方法获得。

14.心肌细胞的生物银行，其通过权利要求1至12中任一项的方法获得。

15.通过权利要求1至12中任一项的方法获得的心肌细胞或权利要求14的生物银行的用途，用作由心脏细胞功能障碍引起的疾病的体外模型。

16.治疗组合物，其包含通过权利要求1至12中任一项的方法获得的心肌细胞或权利要求14的生物银行。

17.方法和用途，其基本如本文所述。

用于使多能干细胞分化为心肌细胞的方法

技术领域

[0001] 本申请涉及用于使多能干细胞(PSC)分化为心肌细胞的方法。此外，本申请涉及用于使人胚胎干细胞(hESC)和诱导型多能干细胞(iPSC)分化为增殖心肌细胞的方法，该方法基于化学成分确定的培养基诱导的连接步骤。

背景技术

[0002] 许多年以来，多种细胞培养系统已用于临床前药物开发。但是，已建立的细胞模型仅部分反映药物相关疾病特异性生理学，因为它们源自致瘤组织或源自转化和无限增殖化细胞。具体而言，因为终末分化的心肌细胞显示具有有限的增殖潜能，所以它们不具有有效产生用于药物开发的细胞模型的能力。因此，存在对可在研究和药物开发中用作可靠的细胞模型的更多疾病相关人细胞类型的需要。

[0003] 人胚胎干细胞(hESC)和诱导型多能干细胞(iPSC)为研究人员提供了产生功能性人细胞类型(如心肌细胞、神经元细胞、胰细胞等)的巨大机会。用于体外分化纯hESC和iPSC来源的人心肌细胞(hESCM)培养物的稳健流程将是强大的工具，不仅增进对早期人心脏发生(cardiogenesis)的理解，还用心肌细胞作为非转化人细胞模型来在药物开发的临床前阶段测试药物功效，及在进入临床之前评估心脏毒性。此外，hESC来源的人心肌细胞为鉴定对心脏再生至关重要的途径打开了机会，并最终导致支持基于干细胞的疗法的临床应用。

[0004] 为了发展用于药物研究和开发的细胞测定模型，这类分化流程需产生理想地满足以下标准的细胞：a)稳健，具有高水平的可重复性；b)产生大量高度纯化的细胞类型；c)可在短时间内分化；d)产生可冷冻的细胞，以保证多种筛选活动的批次一致性；e)提供功能性和生理学相关性用于模拟疾病特异性读出。P.W.Burridge等综述了使多能细胞分化为心肌细胞的现有技术方法(P.Burridge,Keller,Gold,&Wu,2012)。迄今没有一种已知的流程满足以上标准。具体而言，通过已知流程获得的心肌细胞难以冷冻和解冻而不丧失任何功能特性。

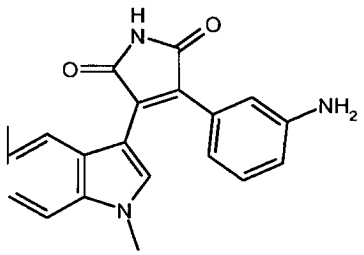
[0005] 为了满足这些需要，我们发展了产生大量高度纯化的心肌细胞(至多95％)的新的分化方法。该分化流程是用确定的小分子来在10天的时间跨度内指导向心肌细胞系分化。为了进一步提高其纯度，通过用心肌细胞偏好的条件重新接种(replating)它们来富集心肌细胞。此外，随后可以冷冻心肌细胞，在液氮下保存，并再次解冻。已测试该心肌细胞符合几种用于药物研究和开发的筛选型式。本发明提供与现有技术流程相比在较短时间内和以显著提高的产率使多能干细胞分化为心肌细胞的改进方法。该新方法减轻了从多能干细胞获得拟胚体或小细胞团的必要性，并去除了迄今已知的方法的低可重复性和标准化的主要缺点。此外，高效率允许在药物产业中将这些确定的心肌细胞大规模用于药物发现和安全性评估、用于再生医学应用及用于体外疾病模拟。

[0006] 发明概述

[0007] 1.本文提供用于使多能干细胞分化为心肌细胞的方法，该方法包括步骤：

[0008] a)提供密度为3-7x10^5/cm^2的多能干细胞；

[0009] b)在含有下式的化合物的无胰岛素培养基中孵育该细胞：

[0010] 

[0011] 在一个实施方案中，在含0.3-10μM该化合物的无胰岛素培养基中孵育该细胞。

[0012] 在一个实施方案中，步骤b)包括孵育该细胞12-48小时。

[0013] 在一个实施方案中，该方法还包括步骤c)在含Wnt-C59的无胰岛素培养基中孵育该细胞。

[0014] 在一个实施方案中，步骤c)包括在含1-10μM Wnt-C59的无胰岛素培养基中孵育该细胞。

[0015] 在一个实施方案中，步骤c)包括孵育该细胞24-72小时。

[0016] 在一个实施方案中，在步骤之间在无胰岛素培养基中孵育该细胞24-48小时。

[0017] 在一个实施方案中，该方法还包括步骤d)在含胰岛素的培养基中孵育该细胞。

[0018] 在一个实施方案中，步骤b)、c)和d)的培养基包含维生素C。

[0019] 在一个实施方案中，该多能干细胞是诱导型多能干细胞。

[0020] 在一个实施方案中，该诱导型多能干细胞是人细胞。

[0021] 在一个实施方案中，该诱导型多能干细胞获自患有由心脏细胞功能异常引起的疾病的个体。

[0022] 在一个实施方案中，提供通过任意以上实施方案的方法获得的心肌细胞。

[0023] 在一个实施方案中，提供通过任意以上实施方案的方法获得的心肌细胞的生物银行(biobank)。

[0024] 在一个实施方案中，通过任意以上实施方案的方法获得的心肌细胞或心肌细胞的生物银行用作由心脏细胞功能障碍引起的疾病的体外模型。

[0025] 在一个实施方案中，提供包含通过任意以上实施方案的方法获得的心肌细胞或心肌细胞的生物银行的治疗组合物。

[0026] 任意以上实施方案可以单独存在或组合存在。

[0027] 附图简述

[0028] 图1:FACS分析鉴定处于分化第14天的高浓度心肌细胞。hESC和iPSC都产生相似的结果。A:人胚胎干细胞来源的心肌细胞。B:人诱导型多能干细胞来源的心肌细胞。

[0029] 图2:多次心肌细胞分化的FACS分析证明流程的稳健性。

[0030] 图3:FACS分析显示纯化方法改善心肌细胞的纯度。A:第14天$5.5x10^5/cm^2$心肌细胞的60%纯度。B:附加纯化步骤后$4.4x10^5/cm^2$心肌细胞的98%纯度。

[0031] 图4:免疫荧光染色——共焦显微镜分析显示心肌细胞典型的细胞中的α辅肌动蛋白和肌钙蛋白T条纹模式。绿色(*):α辅肌动蛋白;红色(#):肌钙蛋白;蓝色(+):细胞核。

[0032] 图5:xCELLigent分析——异丙基肾上腺素提高多能干细胞来源的心肌细胞的跳动频率。

[0033] 图6:多能干细胞来源的心肌细胞在分化第14天和第18天解冻后显示高比例的存活心肌细胞数。在每个实验中,冷冻4x10^{6}个细胞。

[0034] 发明详述

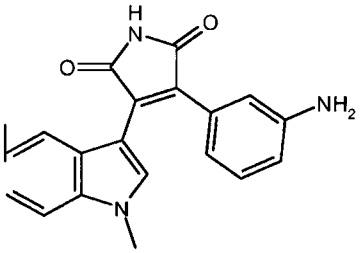
[0035] 本发明提供与现有技术流程相比,在较短时间内和以显著提高的增殖心肌细胞产率,使多能干细胞分化为心肌细胞的改进方法。

[0036] 本文公开的用于使人胚胎干细胞(hESC)和诱导型多能干细胞(iPSC)分化为确定的心肌细胞的新方法基于化学成分确定的培养基诱导的连接步骤,在起始分化后仅10天(或更早:8天)后产生跳动细胞。

[0037] 在一个实施方案中,提供用于使多能干细胞分化为心肌细胞的方法,该方法包括步骤:

[0038] a)提供密度为3-7x 10 ^{5}细胞/cm^{2}的多能细胞;

[0039] b)在含有下式的化合物的无胰岛素培养基中孵育该细胞:

[0040] 

[0041] 3-(3-氨基-苯基)-4-(1-甲基-1H-吲哚-3-基)-吡咯-2,5-二酮(CP21)

[0042] 按3-7x 10 ^{5}细胞/cm^{2}的密度(即非常高的密度)提供多能干细胞。在一个实施方案中,按5.5x10^{5}细胞/cm^{2}的密度提供细胞。令人惊奇地,本方法的发明人发现,按高密度提供细胞提高了分化效率和心肌细胞产率。

[0043] 在一个实施方案中,在以步骤a)起始分化之前,用适宜的缓冲液或培养基洗涤按高密度提供的细胞,以去除任何死细胞。

[0044] 步骤b)的培养基是无胰岛素培养基。步骤b)的早期分化培养基中缺乏胰岛素很重要,因为之前的报道已显示,含有胰岛素的分化培养基阻断心脏发生(Lian u.a.,2013)。

[0045] 为了起始分化,在含有化合物21,(3-(3-氨基-苯基)-4-(1-甲基-1H-吲哚-3-基)-吡咯-2,5-二酮,本文中也称为"化合物21"或"CP21";参见例如L.Gong等;Bioorganic& Medicinal Chemistry Letters 20(2010),1693-1696)的无胰岛素培养基中孵育细胞,以激活wnt途径。化合物21诱导心肌细胞分化的最适浓度依赖于附着于细胞容器的多能细胞的细胞密度。在使用不同细胞密度(1.8-11x10^5/cm2hESC或iPSC)和多种CP21(0-10μM)浓度的几个平行实验中,发现使用5.5x10^5/cm^{2}的细胞密度和2μM的CP21浓度导致多能干细胞最有效地分化为心肌细胞。高于5μM的CP21浓度显示降低的细胞活率。这是令人惊奇的,因为现有技术流程需要更高浓度的其他Wnt途径调节剂进行有效的分化。

[0046] 在一个实施方案中,分化方法的步骤b)包括在含有0.3-10μM CP21、优选0.5-5μM CP21的培养基中孵育细胞。在一个优选实施方案中,分化方法的步骤b)包括在含有2μM CP21的培养基中孵育细胞。

[0047] CP21孵育24小时后,细胞显示强烈的细胞死亡。测试多种CP21孵育时间显示,24小时对心脏发生而言最适,更长或更短的孵育时间导致低效的分化。

[0048] 在一个实施方案中，步骤b)包括在含有CP21的无胰岛素培养基中孵育细胞12-48小时，优选18-24小时。

[0049] 在一个优选实施方案中，步骤b)包括在含有CP21的无胰岛素培养基中孵育细胞24小时。

[0050] 在一个实施方案中，步骤b)的培养基包含维生素C。已显示向基础培养基中加入维生素C改善心肌细胞分化(Cao u.a.,2012)。

[0051] 因此，在一个实施方案中，步骤b)的培养基是含有CP21和维生素C的无胰岛素培养基。在一个这种实施方案中，培养基包含0.5-5μM CP21和维生素C。

[0052] 在一个实施方案中，该方法进一步包括步骤c)在含有Wnt-C59的无胰岛素培养基中孵育该细胞。

[0053] Wnt-C59是阻断Wnt信号传导途径的小分子(WO2010101849,2-(4-(2-甲基吡啶-4-基)苯基)-N-(4-(吡啶-3-基)苯基)乙酰胺)。Wnt-C59是非常有效和高选择性的Wnt信号发放拮抗剂。它阻止Porcupine(膜结合O-酰基转移酶)对Wnt蛋白质的棕榈酰基化(palmitylation)，从而阻断Wnt蛋白质分泌和活性。

[0054] 使用不同浓度的wnt阻遏物Wnt-C59(1-10μM)导致心肌细胞显著增加。最适浓度鉴定为2μM。在不加入Wnt-C59的情况下，分化不产生心肌细胞。超过5μM Wnt-C59的浓度显示增加的细胞死亡。在一个实施方案中，分化方法的步骤c)包括在含有1-10μM Wnt-C59的培养基中孵育细胞。在一个优选实施方案中，分化方法的步骤c)包括在含有2μM Wnt-C59的培养基中孵育细胞。

[0055] 由于wnt途径高度复杂，测试了其他具有不同作用方式的Wnt抑制剂。

[0056] 抗蠕虫药氯硝柳胺(niclosamide)(Chen等,Biochemistry.2009年11月3日;48(43):10267-74.)促进Frizzled1胞吞作用，下调Dishevelled-2蛋白质，并抑制Wnt3A刺激的β-联蛋白稳定化和LEF/TCF报道活性。

[0057] 扑蛲灵(Pyrvinium)是Wnt信号发放的有效抑制剂，在体外结合所有酪蛋白激酶1(CK1)家族成员并选择性增强酪蛋白激酶1α(CK1α)激酶活性，导致Axin的稳定化和β-联蛋白转换增加(Thorne等,Nat Chem Biol.2010年11月;6(11):829-36.)。

[0058] 针对其诱导心肌细胞分化的能力测试了抗蠕虫药氯硝柳胺和扑蛲灵。与Wnt-59不同，其他两种Wnt抑制剂都不导致心肌细胞的成功产生。所测试的Wnt抑制剂对多能干细胞分化为心肌细胞的不同功效表明，通过阻断wnt分泌而特异性抑制wnt途径似乎是关键机制。

[0059] 在一个实施方案中，步骤c)包括在含有Wnt-C59的无胰岛素培养基中孵育细胞24-72小时，优选48小时。

[0060] 在一个实施方案中，步骤b)和c)的该无胰岛素培养基是无血清培养基。在一个实施方案中，该无胰岛素培养基是RPMI1680(Gibco)。

[0061] 在一个实施方案中，在每个步骤b)和c)之间在无胰岛素培养基中孵育细胞24小时至48小时，优选48小时。在一个实施方案中，该培养基是无血清培养基。在另一实施方案中，该培养基包含维生素C。

[0062] 在一个实施方案中，在每个步骤b)和c)之间在含有维生素C的无血清、无胰岛素培养基中孵育细胞24小时至48小时，优选48小时。

[0063] 在一个实施方案中，本文所述通过以上任意实施方案使多能细胞分化为心肌细胞的方法还包括步骤d)在含有胰岛素的培养基中孵育细胞。在此后期阶段，胰岛素促进心肌细胞及其心脏前体细胞的增殖。

[0064] 在一个实施方案中，步骤d)包括在含有胰岛素的培养基中孵育细胞36-60小时，优选48小时。在一个实施方案中，该培养基是无血清培养基。在另一实施方案中，该培养基包含维生素C。

[0065] 适合用于扩增步骤d)的培养基是例如DMEM，高葡萄糖+L-谷氨酰胺+丙酮酸和肉碱、牛磺酸、肌酸、BSA、维生素C，或来自Cellular Dynamics international的iCell心肌细胞维持培养基。

[0066] 优选地，在每个步骤之间更换培养基，例如，例如通过吸取或离心细胞并弃去上清来去除培养基，然后将用于随后的步骤的培养基加至细胞。在一个实施方案中，在加入随后的步骤的培养基之前用适宜的缓冲液或培养基洗涤细胞，以去除任何死细胞。用于洗涤细胞的缓冲液或培养基为本领域已知。适合用于洗涤细胞的缓冲液的一个实例是例如磷酸缓冲盐溶液(PBS)。

[0067] 在一个实施方案中，在允许稳定的生长和/或倍增时间的条件下培养用于分化方法的多能细胞。例如，将细胞培养在多能性培养基中并传代几次。本文所用的“多能性培养基”指任何化学成分确定的培养基，其用于将多能干细胞作为单细胞附着于单层同时保持其多能性，且为本领域公知。在一个实施方案中，该多能性培养基是含有Rho相关卷曲螺旋形成蛋白丝氨酸/苏氨酸激酶(ROCK)家族蛋白激酶的小分子抑制剂(本文中称为ROCK激酶抑制剂)的无血清培养基。

[0068] 在一个实施方案中，该ROCK激酶抑制剂选自1-(5-异喹啉磺酰基)高哌嗪)、N-苄基-2-(嘧啶-4-基氨基)噻唑-4-甲酰胺)和(+)-(R)-反式-4-(1-氨乙基)-N-(4-吡啶基)环己甲酰胺二盐酸盐)。

[0069] 本文所用的ROCK激酶抑制剂的实例是Fasudil(1-(5-异喹啉磺酰基)高哌嗪)、Thiazovivin(N-苄基-2-(嘧啶-4-10基氨基)噻唑-4-甲酰胺)和Y27632((+)-(R)-反式-4-(1-氨乙基)-N-(4-吡啶基)环己甲酰胺二盐酸盐，例如来自Tocris bioscience的目录号1254)。在一个优选实施方案中，该ROCK激酶抑制剂是Y27632。在一个实施方案中，该多能性培养基是含有2-20μM Y27632、优选5-10μM Y27632的无血清培养基。在另一实施方案中，该多能性培养基是含有2-20μM Fasudil的无血清培养基。在另一实施方案中，该多能性培养基是含有0.2-10μM Thiazovivin的无血清培养基。

[0070] 使用本文中所呈现的新方法，现在可能以至多60-98％的产率从多能干细胞分化表达α辅肌动蛋白和肌钙蛋白T的心肌细胞。

[0071] 在一个实施方案中，该方法进一步包括步骤e)重新接种细胞，并在无胰岛素培养基中孵育它们。此步骤进一步提高心肌细胞的纯度。在一个实施方案中，重新接种细胞，并在补充了胎牛血清的无胰岛素培养基中孵育18-32小时，优选24小时。在一个这种实施方案中，该培养基进一步包含ROCK抑制剂。在一个实施方案中，该ROCK抑制剂是Y-27632。

[0072] 通过本文所述方法获得的心肌细胞可以扩增几代，并在冷冻和解冻后保持其功能特性。

[0073] 本文所用的术语“分化”指低分化细胞转变为体细胞，例如多能干细胞转变为心肌

细胞的一个或多个步骤。通过本文所述的方法达到多能干细胞向心肌细胞的分化。

[0074] 本文所用的术语“干细胞”指具有自我更新能力的细胞。本文所用的“未分化的干细胞”指具有分化为多种细胞类型的能力的干细胞。如本文所用，本文所用的“多能干细胞”指可以产生多种细胞类型的细胞的干细胞。多能干细胞(PSC)包括人胚胎干细胞(hESC)和人诱导型多能干细胞(hiPSC)。人诱导型多能干细胞可以源自重编程体细胞，例如，通过本领域已知的方法转导四种确定的因子(Sox2、Oct4、Klf4、c-Myc)。人体细胞可以获自健康个体或获自患者。这些供体细胞可以容易地从任意适宜的来源获得。本文优选的是允许分离供体细胞而不在人体上进行侵入性操作的来源，例如人皮肤细胞、血细胞或可从尿液样品获得的细胞。虽然优选人多能干细胞，但该方法也适用于非人多能干细胞，如灵长类、啮齿类(例如大鼠、小鼠、兔)和狗多能干细胞。

[0075] 本文所用的“心肌细胞”是这样的细胞，其至少表达细胞标志肌钙蛋白T(2型肌钙蛋白T(心脏)，基因符号TNNT2，Entrez Gene:7139，UniProtKB:P45379)，且在优选实施方案中还表达细胞标志α辅肌动蛋白(ACTN2辅肌动蛋白，α2，基因符号ACTN2，Entrez Gene:88，UniProtKB:P35609)。肌钙蛋白T和/或α辅肌动蛋白的表达可以通过本领域已知的方法，例如通过实施例部分中所述的FACS分析来评估。心肌细胞可以表现自发性周期性收缩活动(“跳动”)。这意味着，在将通过本发明的方法获得的心肌细胞培养在具有适当Ca++浓度和电解质平衡的适宜组织培养环境中时，可以观察到细胞以周期性方式跨细胞的一个轴收缩，然后从收缩释放，而无需向培养基中加入任何附加成分。此外，通过本文公开的方法获得的细胞可以表现心肌细胞的其他特征，如离子通道或适当的电生理学。

[0076] 本文所用的“增殖心肌细胞”是表达α辅肌动蛋白和肌钙蛋白T且通过细胞分裂增殖的细胞。

[0077] “标志的表达”指某个基因转录为mRNA，且随后通常翻译为在细胞中发挥某种功能的蛋白质(其基因产物)。标志的表达可以通过本领域已知的方法在RNA水平或在蛋白质水平检测和定量。本文优选的是例如通过用结合标志的抗体测试某种蛋白质的存在来在蛋白质水平检测标志的表达。

[0078] 任意以上实施方案可以单独存在或组合存在。

[0079] 在本发明的一个实施方案中，提供用于产生患者特异性或健康个体特异性心肌细胞的方法。为此，用本文所述的方法使获自患者或健康个体的人诱导型多能干细胞(iPSC)分化为心肌细胞。可以通过本领域已知的方法重编程获自患者或健康个体的体细胞为多能干细胞，来获得患者特异性人iPSC。例如，可以通过皮肤活检从需要治疗的个体或从健康个体获得成纤维细胞、角质形成细胞或脂肪细胞，并通过本领域已知的方法重编程为诱导型多能干细胞。适合作为诱导型多能干细胞的来源的其他体细胞是从血液样品获得的白细胞，上皮细胞，或从尿液样品获得的其他细胞。然后可以通过本文所述的方法使患者特异性诱导型多能干细胞分化为患者特异性或健康个体特异性心肌细胞。在本发明的另一方面，提供通过任意前述方法产生的心肌细胞群体。优选地，心肌细胞群体为患者特异性的，即源自从患病个体获得的iPSC。在另一实施方案中，该心肌细胞群体获自健康个体。

[0080] 患者来源的心肌细胞是研究如扩张型心肌病、肥厚型心肌病、限制型心肌病、致心律失常型右室心肌病、冠心病的疾病的病理生理学的疾病相关体外模型。在一个实施方案中，通过此方法获得的心肌细胞用于筛选逆转、抑制或预防由心脏细胞功能障碍引起的疾

病(例如心脏肥大、跳动效率降低、心肌细胞条纹错构、钙处理不足)的化合物。优选地,通过本文所述的本发明的方法获得的心肌细胞源自患病个体。在另一实施方案中,通过此方法获得的心肌细胞用于筛选和评价用于治疗心脏病(例如上文提到的那些)的新靶标和化合物。优选地,通过本文所述的本发明的方法获得的心肌细胞源自患有疾病(例如扩张型心肌病、肥厚型心肌病、限制型心肌病、致心律失常型右室心肌病、冠心病)的个体。从患病个体分化心肌细胞是在人背景模式中早期评价药物安全性的唯一机会。在另一实施方案中,通过此方法获得的心肌细胞用作心脏的体外模型。

[0081] 本发明提供高效的方法来提供患者特异性心肌细胞或来自具有适合用于移植的相同HLA类型的健康个体的相容细胞,二者均在无异种条件下衍生。"无异种培养条件"指仅包含人和重组来源的成分的用于附着的培养基和底物。因此,避免了受异种病原体污染的风险,肾细胞可安全地用于再生医学。用本文所述的方法使患者特异性诱导型多能干细胞(iPSC)分化为患者特异性心肌细胞是可容易地进行和重现的产生自体来源的心肌细胞的技术。自体和/或相容细胞在细胞治疗中的使用提供了超过使用非自体细胞的巨大优势,非自体细胞可能经受免疫学排斥。相反,自体细胞不可能引出显著的免疫学反应。

[0082] 在本发明的另一优选方面,设想产生患者特异性心肌细胞的生物银行。在一个实施方案中,产生包含获自健康个体和/或患者的不同心肌细胞群体的生物银行。本文所用的术语"生物银行"指从不同个体或物种采集的生物样品的文库。样品和相关数据的存档汇编旨在用于研究目的,目的是研究与扩张型心肌病、肥厚型心肌病、限制型心肌病、致心律失常型右室心肌病、冠心病相关的疾病。在另一实施方案中,该生物银行用于血管再生医学方法。

[0083] 在另一方面,本发明提供包含通过任意前述方法产生的心肌细胞或包含任意前述细胞群体的治疗组合物。优选地,该治疗组合物进一步包含生理学上相容的溶液,包括例如含5%人血清白蛋白的磷酸缓冲盐溶液。该治疗组合物可以用于治疗、预防或稳定疾病,例如扩张型心肌病、肥厚型心肌病、限制型心肌病、致心律失常型右室心肌病、冠心病。例如,可以通过皮肤活检从需要治疗的个体或从健康个体获得成纤维细胞、角质形成细胞或脂肪细胞,并通过本领域已知的方法("Induction of pluripotent stem cells from adult human fibroblasts by defined factors."Takahashi等,2007,Cell 131,861-72)重编程为诱导型多能干细胞。适合作为诱导型多能干细胞的来源的其他体细胞是从血液样品获得的白细胞、上皮细胞,或从尿液样品获得的其他细胞。然后通过本文所述的方法使患者特异性诱导型多能干细胞分化为心肌细胞,收集,并引入该个体来治疗病症。通过本发明的方法产生的心肌细胞可以用于替换或辅助患病或受损组织的正常功能。

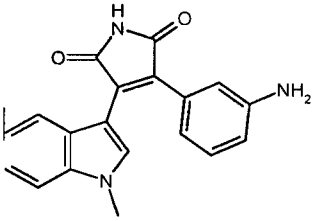
[0084] 本发明的另一实施方案是心肌细胞的生物银行在治疗与扩张型心肌病、肥厚型心肌病、限制型心肌病、致心律失常型右室心肌病、冠心病相关的疾病中的用途。该生物银行优选包含获自具有几个HLA类型的患者或健康个体的心肌细胞。将从健康供体获得的细胞移植至具有相容HLA类型的需要治疗的个体避免了通常与异源细胞移植相关的排斥反应的问题。通常,通过施用免疫抑制剂或抗排斥药物如环孢霉素来预防或减轻排斥。但是,这类药物具有显著的副作用,例如免疫抑制、致癌特性、肾脏毒性以及非常昂贵。本发明消除或至少显著减少了对抗排斥药物(如环孢霉素、imulan、FK-506、糖皮质激素和雷帕霉素,及其衍生物)的需要。

[0085] 对于本发明的治疗方法，对哺乳动物施用心肌细胞并非旨在限于具体的施用方式、剂量、或给药频率；本发明考虑所有施用方式，包括肌内、静脉内、关节内、病灶内、皮下、或足以提供足以预防或治疗疾病的剂量的任意其他途径。心肌细胞可以以单个剂量或多个剂量对哺乳动物施用。在施用多个剂量时，剂量可以相互间隔例如一周、一个月、一年或十年。在施用细胞之前、期间或之后，也可以使用一种或多种生长因子、激素、白介素、细胞因子、小分子或其他细胞，以进一步使它们偏向特定细胞类型。

实施例

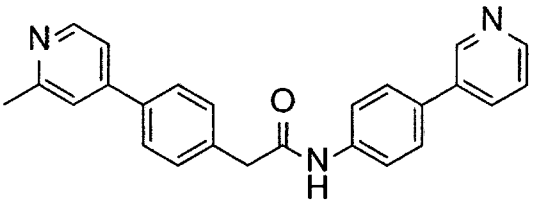
[0086] 材料和方法

[0087] CP21R7：3-(3-氨基-苯基)-4-(1-甲基-1H-吲哚-3-基)-吡咯-2,5-二酮(本文中也称为“化合物21”或“CP21”；参见例如L.Gong等；Bioorganic&Medicinal Chemistry Letters 20(2010),1693-1696)

[0088] 

[0089] CP21R7

[0090] Wnt-C59：2-(4-(2-甲基吡啶-4-基)苯基)-N-(4-(吡啶-3-基)苯基)乙酰胺(Cellagen Technology,目录号C7641-2s,WO2010101849)：

[0091] 

[0092] 人ESC:SA001、LOT CA001于2001年3月20日在**Göteborg** University和Cellartis AB Arvid Wallgrens Backe 20,SE-413 46**Göteborg,**瑞典，按照瑞典所有适用法律分离，并由**Göteborg** University和Uppsala University的地方研究伦理委员会(Local Research Ethics Committees)批准。胚胎来源：冷冻，来自IVF的剩余物。供体保密性：为了保护供体的隐私和保密，所有与胚胎供体相关的标识都已去除。因此，不能得到关于供体的信息。应指出，捐献未使供体获得任何经济利益。我们获批进行hESC研究和衍生不同细胞系。责任伦理会员会(Ethikkommission beider Basel)和联邦公共卫生办公室已批准了我们的研究项目。(Ref-No:R-FP-S-1-0002-0000)。

[0093] 人iPSC：来自SBI System Biosciences的目录号SC101A-1批号110218-FF/来自Life technologies**Gibco®**Episomal hiPSC Line的目录号A13777。

[0094] 人多能干细胞常规培养在TeSR1培养基(Stem cell Technologies)中的hESC-qualified Matrigel(BD Bioscience)上。培养物每4-6天用StemPro Accutase(Invitrogen)传代。为了提高活率，TeSR1培养基在酶解离之前1小时包含10μM ROCK抑制剂。

[0095] 500ml分化培养基

[0096] RPMI1680+Glutamax 481ml GIBCO#61870

[0097] 维生素C(10mg/ml) 4ml Sigma#A4544

[0098] (终浓度:80μg/ml)

[0099] B27-胰岛素(50x) 10ml Invitrogen#05-0129SA

[0100] 青霉素/链霉素 5ml GIBCO#15140-122

[0101] (终浓度:50U/ml)

[0102] 500ml扩增培养基

[0103] RPMI1680+Glutamax 481ml GIBCO#61870

[0104] 维生素C(10mg/ml) 4ml Sigma#A4544

[0105] (终浓度:80μg/ml)

[0106] B27+胰岛素(50x) 10ml Invitrogen#12587-01

[0107] 青霉素/链霉素 5ml GIBCO#15140-122

[0108] (终浓度:50U/ml)

[0109] 本文中所用的其他试剂和材料:

[0110] Matrigel(BD Bioscience,目录号354277)

[0111] mTeSR1培养基(Stemcell Technologies,目录号05850)

[0112] Accutase(Innovative Cell Technologies,目录号AT-104)

[0113] Rock抑制剂Y-27632(Millipore,目录号SCM075)

[0114] RPMI培养基(Life Technologies的Gibco,目录号61870)

[0115] 维生素C(Sigma,目录号A4544)

[0116] **50xB-27®**补充剂不含胰岛素(Life Technologies的Gibco,目录号0050129SA)

[0117] 青霉素-链霉素(Life Technologies的Gibco,目录号15070)

[0118] 50xB27加胰岛素不含维生素A(Life Technologies的Gibco,目录号12587)

[0119] 0.05%胰蛋白酶/EDTA,1x(Life Technologies的Gibco,目录号25300)

[0120] autoMACS运行缓冲液(Miltenyi,目录号130-091-221)

[0121] Inside Perm+InsideFix(Miltenyi,Inside Stain Kit,目录号130-090-477)

[0122] 0.1%明胶(Millipore,Cat.ES-006-B)

[0123] 冻存管(Corning#430659)

[0124] Mr.Frosty冷冻容器(Thermo Scientific#5100-0001)

[0125] DMSO(Sigma#D2438)

[0126] 胎牛血清(Invitrogen#16000044)

[0127] Falcon细胞培养皿35x10mm(BD#353001)

[0128] Falcon细胞培养皿100x20mm(BD#353003)

[0129] 6孔板Corning Costar(Sigma#CLS3516)

[0130] 抗肌节α辅肌动蛋白[EA-53]抗体(Abcam,目录号ab9465)

[0131] 抗心脏肌钙蛋白T抗体(Abcam,目录号ab45932)

[0132] Alexa**Fluor®**488和驴抗小鼠IgG(H+L)(Invitrogen,目录号A21202)

[0133] Alexa**Fluor®**647驴抗兔IgG(H+L)(Invitrogen,目录号A31573)

[0134] AlexaFluor®555驴抗兔IgG(H+L)(Invitrogen,目录号A31572)

[0135] Hoechst 33258,五水合物(bis-Benzimide)(Molecular Probes,目录号H3569)

[0136] 从人胚胎干细胞(hESC)和诱导型多能干细胞(iPSC)分化心肌细胞

[0137] 人胚胎干细胞(hESC)或诱导型多能干细胞(iPSC)在37℃和5%CO2下培养在Matrigel(BD Bioscience,目录号354277)包被的56cm^{2}平皿中的10ml mTeSR1培养基(Stemcell Technologies,目录号05850)中。

[0138] 在开始心肌细胞分化之前,传代细胞3-4次,以确保多能干细胞显示稳定的生长和倍增时间。

[0139] 为了繁殖多能干细胞而保持其多能状态,按以下处理hESC或iPSC:用10ml PBS-/-洗涤一次,然后用3ml Accutase(Innovative Cell Technologies,目录号AT-104)在37℃和5%CO2下孵育2-3分钟,以脱附细胞。

[0140] 用7ml mTeSR1终止Accutase的酶促反应,然后500xg离心细胞3分钟。

[0141] 将细胞重悬在10ml mTeSR1中,并计数。为了进一步培养,将2x10^{6}细胞接种在现包被Matrigel的56cm^{2}平皿上。此外,在37℃和5%CO2下在10ml mTeSR1和10μM Rock抑制剂Y-27632(Millipore,目录号SCM075)中培养hESC或iPSC。随后,每天更换10ml mTeSR1培养基,培养多能干细胞至80%密度,然后传代。

[0142] 为了成功分化为心肌细胞,用5.5x10^5/cm^{2}的hESC或iPSC按高密度接种多能干细胞。按上文针对多能干细胞所述进行传代和培养。

[0143] 24小时(1天)后,用180μl/cm^{2}PBS-/-洗涤hESC或iPSC一次,将培养基换为180μl/cm^{2}分化培养基。

[0144] 为了起始多能干细胞向心脏系的分化,培养基包含2μM化合物21(CP21),CP21是糖原合酶激酶3(GSK3β)的小分子高选择性抑制剂。

[0145] 用CP21孵育24小时后(第2天),用上文所述PBS-/-洗涤细胞,并在220μl/cm^{2}分化培养基中培养48小时。

[0146] 48小时后(第4天),用上文所述PBS-/-洗涤细胞,并在含有2μMWnt-C59(Cellagen Technology,目录号C7641-2s,WO2010101849)的220μl/cm^{2}分化培养基中培养48小时,Wnt-C59是阻断wnt分泌的有效的wnt信号发放抑制剂。

[0147] 48小时后(第6天),用上文所述PBS-/-洗涤细胞,并在220μl/cm^{2}分化培养基中培养48小时。

[0148] 48小时后(第8天),用上文所述PBS-/-洗涤细胞,并在含有维生素C、青霉素-链霉素但现在包括加胰岛素而不含维生素A的B27的220μl/cm^{2}RPMI培养基(=扩增培养基)中培养48小时。

[0149] 可见为跳动细胞的第一批心肌细胞在分化的第8天观察到,并进一步增加至第14天。

[0150] 随后每48小时用220μl/cm^{2}扩增培养基进行培养基更换。

[0151] 细胞表征

[0152] 为了测试分化方法的效率,在分化第14天用对心肌细胞特异的抗原通过细胞免疫组织化学和荧光激活细胞分选(FACS)表征心肌细胞。

[0153] 荧光激活细胞分选(FACS)分析

[0154] 用180μl/cm^{2}PBS-/-洗涤细胞，用100μl/cm^2 0.05%1x胰蛋白酶/EDTA(Life Technologies的Gibco，目录号25300)在37℃和5%CO_2下解离5-10分钟。

[0155] 根据需要，轻轻从培养容器刮下细胞，上下吹吸，随后在37℃和5%CO_2下孵育5-10分钟。

[0156] 然后加入三倍扩增培养基和10%胎牛血清(FBS)。

[0157] 然后，通过100μm细胞滤过器过滤细胞，并计数。

[0158] 为了分析，将1x10^{6}细胞悬液转入1.5ml管。500xg离心3分钟后，弃上清，用50μl Inside Fix(Miltenyi，Inside Stain Kit，目录号130-090-477)和50μl PBS-/-在室温下避光固定细胞15分钟。

[0159] 然后加入100μl autoMACS运行缓冲液(Miltenyi，目录号130-091-221)，并离心。弃上清，用100μl Inside Perm(Miltenyi，Inside Stain Kit，目录号130-090-477)洗涤细胞，离心，并弃上清。用1:100稀释在InsidePerm中的抗肌节α辅肌动蛋白[EA-53]抗体(Abcam，目录号ab9465)和抗心脏肌钙蛋白T抗体(Abcam，目录号ab45932)4℃孵育细胞1小时。

[0160] 然后用500μl运行缓冲液洗涤细胞，离心，并弃上清。用第二抗体(1:1000于Inside Perm中)在室温下孵育细胞10分钟。使用了以下第二抗体：Alexa**Fluor®** 488驴抗小鼠IgG(H+L)(Invitrogen，目录号A21202)和Alexa**Fluor®**647驴抗兔IgG(H+L)(Invitrogen，目录号A31573)。

[0161] 随后，用500μl运行缓冲液洗涤细胞，离心后，将细胞重悬在500μl运行缓冲液中，并通过荧光激活细胞分选(FACS)系统测量。

[0162] 用不同CP21浓度从人胚胎干细胞(hESC)和诱导型多能干细胞(iPSC)分化心肌细胞

[0163] 用不同CP21浓度重复上述流程。结果显示在下表中：(-)未获得心肌细胞，(+)-(+++)：所获得的心肌细胞的量。

[0164]

Cp21浓度μM	0	0.3	1	2	3	5	10
实验I	-	-	+	++	++	+	-
实验II	-	-	+	+++	+	-	-
实验III	-	-	+	++	++	-	-

[0165] 纯化

[0166] 为了提高心肌细胞的纯度，发展了富集步骤。

[0167] 如上文所述，用180μl/cm^{2}PBS-/-洗涤细胞，并用100μl/cm^2 0.05%1x胰蛋白酶/EDTA(Life Technologies的Gibco，目录号25300)在37℃和5%CO_2下解离5-10分钟。

[0168] 根据需要，轻轻从培养容器刮下细胞，上下吹吸，随后在37℃和5%CO_2下孵育5-10分钟。

[0169] 然后加入三倍扩增培养基和10%胎牛血清(FBS)。

[0170] 然后，通过100μm细胞滤过器过滤细胞，并计数。

[0171] 将130μl/cm^2 0.1%明胶(Millipore，目录号ES-006-B)现包被的平板37℃孵育1小时。

[0172] 将2.7x10^5/cm^{2}细胞接种在180μl/cm^{2}扩增培养基10%胎牛血清(FBS)中。此外，加入10μM Rock抑制剂。24小时后，用不含FBS和Rock抑制剂的扩增培养基更换培养基。每48小时更换培养基。

[0173] 在第18-21天，用FACS分析细胞，并按上文所述再次重新以不同型式接种进行以下分析：免疫荧光染色、xCELLigence，以检测跳动节律和化合物在干细胞来源心肌细胞中的致心律失常效应。

[0174] 将细胞转移至符合测定条件的平板型式。使细胞在200μl/cm^{2}扩增培养基加10%胎牛血清(FBS)中附着24小时。此外，加入10μM Rock抑制剂。24小时后，更换为不含FBS和Rock抑制剂的220μl/cm^{2}扩增培养基。每48小时更换培养基。

[0175] 心肌细胞的冷冻和解冻

[0176] 在第14天，按上文所述重新接种心肌细胞用于纯化方法。在第18天，按上文所述解离细胞，然后通过FACS分析其α辅肌动蛋白和肌钙蛋白T表达。对含有80%及以上心肌细胞的培养物进行冷冻流程。低于80%心肌细胞的培养物废弃。

[0177] 计数细胞，每个冻存管(cryogenic vial)用1ml含10%DMSO和10μMY-27632的冷FBS冷冻4x10^{6}个细胞。

[0178] 细胞500xg离心3分钟，然后小心地重悬在补充了10%DMSO和10μM Y-27632的FBS中。将心肌细胞悬液的1ml整分试样装入4℃预冷的冻存管中，并在-80℃冷冻24小时。然后将冻存管保存在液氮中。

[0179] 为了解冻心肌细胞，在37℃水浴中孵育管1-2分钟，小心地将细胞转入10ml扩增培养基加10%胎牛血清中。300xg离心细胞2分钟。然后将沉淀重悬在6ml扩增培养基加10%胎牛血清和10μM Y-27632中，接种在0.1%明胶包被的6孔板的3个孔中。24小时后，将细胞更换为220μl/cm^{2}不含FBS和Y-27632的扩增培养基。然后每3天更换培养基，5-7天后将细胞接种在符合测定条件的平板型式中(例如，用于检测心脏条纹错构的测定：96孔型式；用于记录跳动频率的测定：96孔型式)。

[0180] xCELLigent心肌细胞跳动分析

[0181] 异丙基肾上腺素通过刺激心脏β-1受体来提高心率和心肌收缩力。为了在干细胞来源心肌细胞中检测此致心律失常效应，将7x10^4/cm^{2}细胞接种在用130μl/cm^{2}0.1%明胶37℃包被1小时的特殊E-Plate Cardio 96(Roche，目录号05232368001)上。按上文所述使细胞附着于平板并恢复2天后，将培养基换为iCell心肌细胞维持培养基(Cellular Dynamics，目录号CMM-100-120-005)。用xCELLigence RTCA Cardio System(Roche Applied Science)测量细胞。接种7天后，用3μM异丙基肾上腺素处理细胞，并直接测量。按12.9ms的分辨率测量每块96孔板。前3分钟不间断测量，随后的24小时每15分钟测量1分钟持续时间。

[0182] 免疫荧光染色

[0183] 对于免疫荧光染色，用4%多聚甲醛在室温下固定细胞15分钟。

[0184] 用PBS-/-洗涤细胞后，用含10%驴血清和0.1%Triton的PBS-/-(封闭缓冲液)在室温下封闭和透化细胞20分钟。然后，用1:100稀释的一抗抗肌节α辅肌动蛋白[EA-53]抗体(Abcam，目录号ab9465)和抗心脏肌钙蛋白T抗体(Abcam，目录号ab45932)在封闭缓冲液中4℃过夜染色细胞。

[0185] 用PBS-/-洗涤细胞，并用1:1000稀释在封闭缓冲液中的二抗Alexa**Fluor®** 488和驴抗小鼠IgG(H+L)(Invitrogen,目录号A21202)和Alexa**Fluor®** 555驴抗兔IgG(H+L)(Invitrogen,目录号A31572)在室温下，在封闭缓冲液中染色。几个PBS-/-洗涤步骤后用1:1000稀释在PBS-/-中的Hoechst 33258五水合物(bis-Benzimide)(Molecular Probes,目录号H3569)染色细胞核。

[0186] 结果

[0187] 分化后，针对其心肌细胞含量分析细胞。图1显示定量分化第14天的心肌细胞的FACS分析。

[0188] 获得了表征为α辅肌动蛋白和肌钙蛋白T双阳性细胞的平均80-90％心肌细胞。在图1中，一个细胞亚群染色为α辅肌动蛋白单阳性(5-10％)。这指示更不成熟的心肌细胞，由于此原因，此群体未包括在评分中。此结果独立于用hESC(图1A)还是iPSC(图1B)作为多能干细胞的来源。开始于$5.5x10^5/cm^2$多能干细胞，分化流程产生平均$4-5x10^5/cm^2$α辅肌动蛋白和肌钙蛋白T阳性心肌细胞。

[0189] 为了证明分化流程的稳健性，我们进行了几次实验，并分析了每个培养物中心肌细胞的含量。图2显示10次独立的实验，显示向心肌细胞的分化效率在95％和40％之间。但是，大多数实验(10次实验中的7次)显示超过75％的心肌细胞含量，这是可接受的比例。产生60％心肌细胞及以上的实验继续进行。低于60％心肌细胞的分化废弃。实验间的变异性最有可能由分化开始时多能干细胞的质量和培养状态引起。

[0190] 为了进一步提高心肌细胞的纯度，建立了附加的纯化步骤。在分化第14天，脱附细胞，并通过FACS分析。图3A显示，培养物计数$9x10^5/cm^2$细胞在第14天包含60％($5.4x10^5/cm^2$)心肌细胞。为使纯化方法成功，α辅肌动蛋白阳性细胞的最小百分比应为60％及以上。重新接种解离的细胞($2.7x10^5/cm^2$)，并培养在扩增培养基中。7天后，收集细胞，计数$4.5x10^5/cm^2$细胞，并分析。图3B显示，纯化步骤后，培养物中心肌细胞含量从60％提高至98％，证明通过使用此方法有效地产生了$4.4x10^5/cm^2$高度富集的心肌细胞。然后，将细胞转移至符合测定条件的培养型式。

[0191] 通过免疫荧光分析心肌细胞进行进一步表征。图4显示使用抗α辅肌动蛋白(绿色)、肌钙蛋白T(红色)的抗体及细胞核特异的Hoechst染料(蓝色)的第27天的心肌细胞免疫荧光染色。所得到的图4中的免疫荧光显示心肌细胞特征性的α辅肌动蛋白和肌钙蛋白T特异性条纹。

[0192] 心脏上β受体的激活诱导心肌细胞中的正性变时作用(positive chronotropic effect)。为了确认多能干细胞来源的心肌细胞响应β受体激活，用β受体激动剂异丙基肾上腺素孵育心肌细胞，然后用xCELLigence系统分析。图5显示，与未处理的对照相比，用3μM异丙基肾上腺素孵育多能干细胞来源的心肌细胞后，跳动速率从45提高至60次跳动/分钟。此实验进一步证明，通过此分化流程产生的多能干细胞来源的心肌细胞类似于功能性人心肌细胞。

[0193] 由于解冻后低水平的细胞恢复，心肌细胞的冷冻和解冻通常很困难。

[0194] 由于具有大批相同的细胞对测定开发很重要，我们测试了多能干细胞来源的心肌细胞是否可以保存在冰箱中，然后解冻。我们尝试在不同时间(第14、18和32天)冷冻分化的心肌细胞。如图6中可见，在较早分化阶段冷冻的心肌细胞在解冻后显示较高的细胞存活

率。但是，在分化第18天纯化后解冻的细胞提供了将细胞用于药物测定的最佳条件。在此阶段，细胞在解冻后显示高得多的纯度，心肌细胞可直接转移至符合测定型式的细胞培养容器上。

[0195] 当解冻在分化第32天冷冻的心肌细胞时，存活率非常低，许多细胞丢失。这是由于处于此阶段的细胞的低增殖速率导致心肌细胞解冻后的低恢复。

[0196] 我们确定，冷冻多能细胞来源的心肌细胞的最适时间是在分化第18天纯化之后。在此阶段，恢复率平均超过85％α辅肌动蛋白和肌钙蛋白T阳性细胞，心肌细胞仍在增殖，提供了将细胞进一步用于测定开发的最适条件。

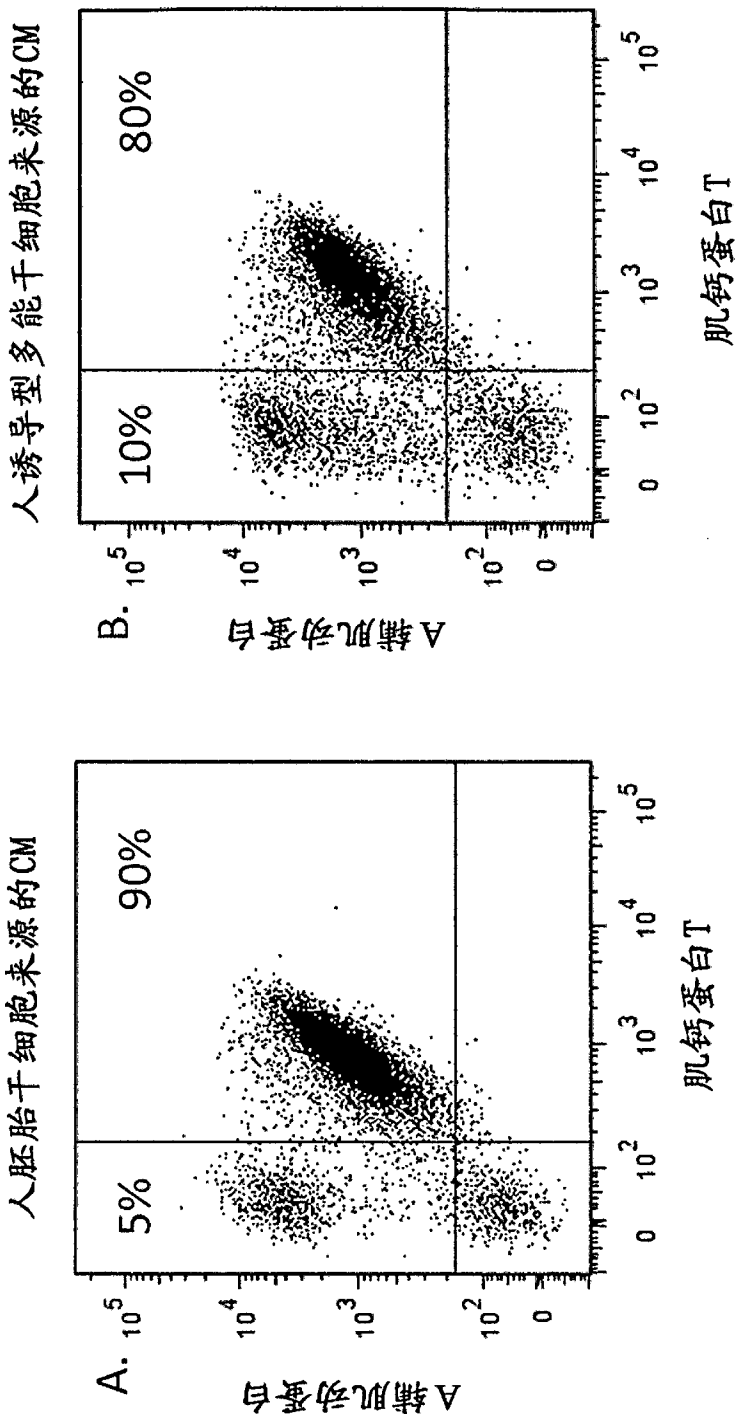

人胚胎干细胞来源的CM
5%
90%
A.
A辅肌动蛋白
肌钙蛋白T
人诱导型多能干细胞来源的CM
10%
80%
B.
A辅肌动蛋白
肌钙蛋白T

图1

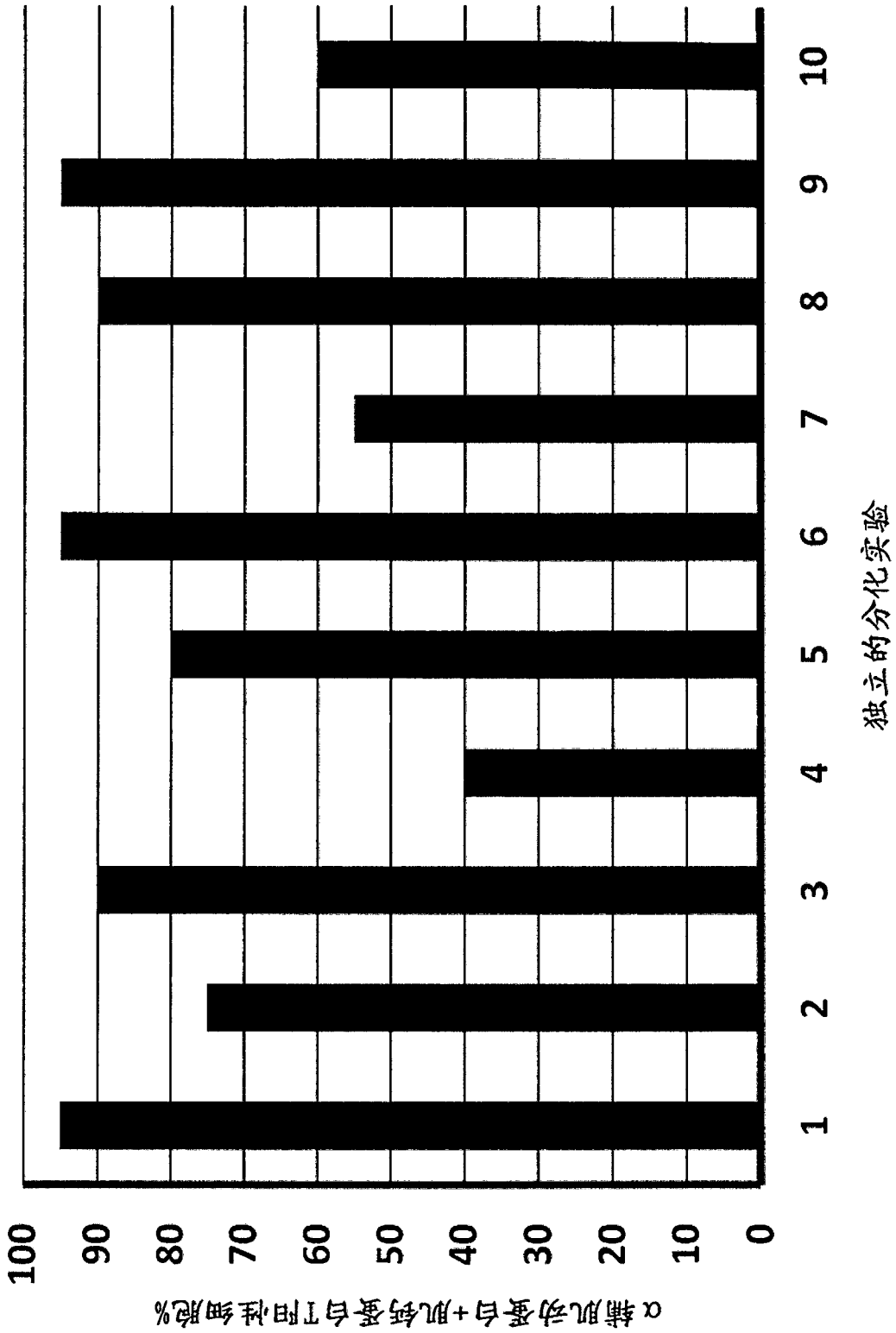

α辅肌动蛋白+肌钙蛋白T阳性细胞%
0
10
20
30
40
50
60
70
80
90
100
1
2
3
4
5
6
7
8
9
10
独立的分化实验

图2

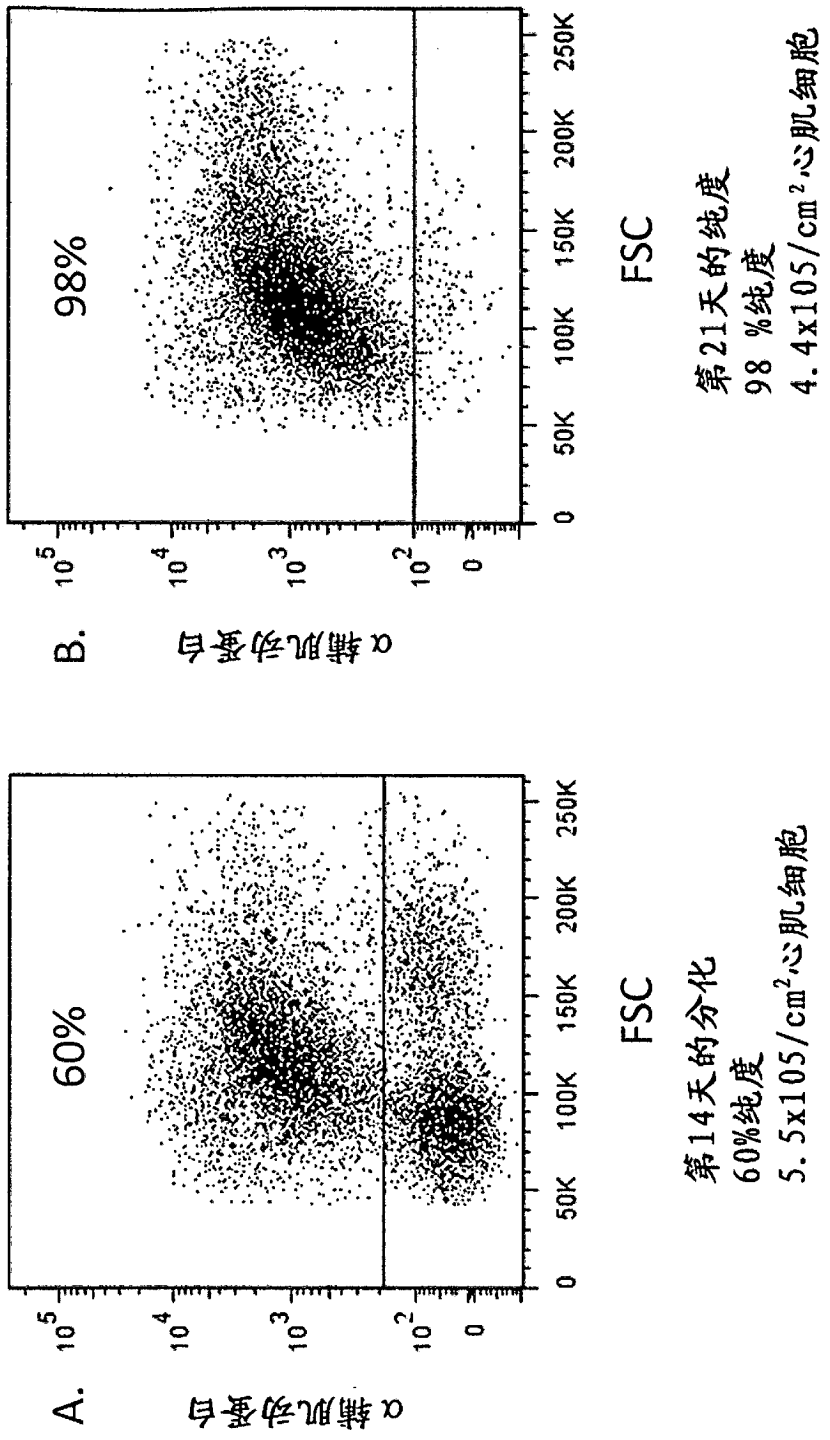

A.
α辅肌动蛋白
60%
FSC
第14天的分化
60%纯度
5.5x105/cm²心肌细胞
B.
α辅肌动蛋白
98%
FSC
第21天的纯度
98 %纯度
4.4x105/cm²心肌细胞

图3

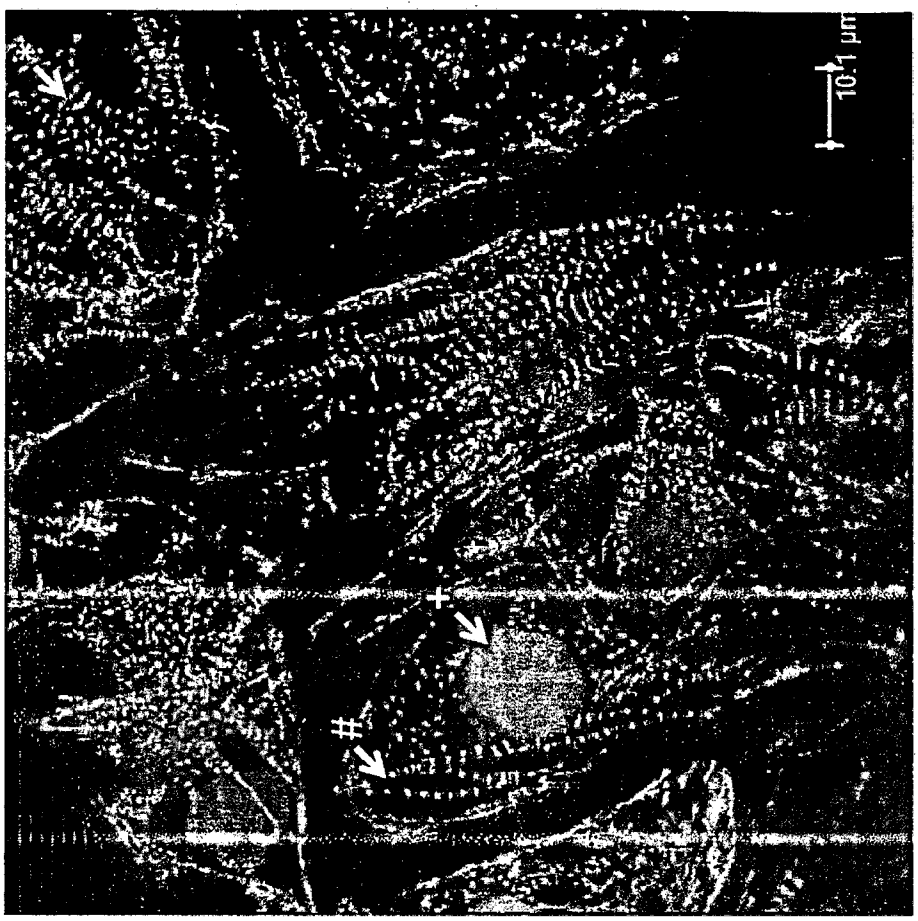
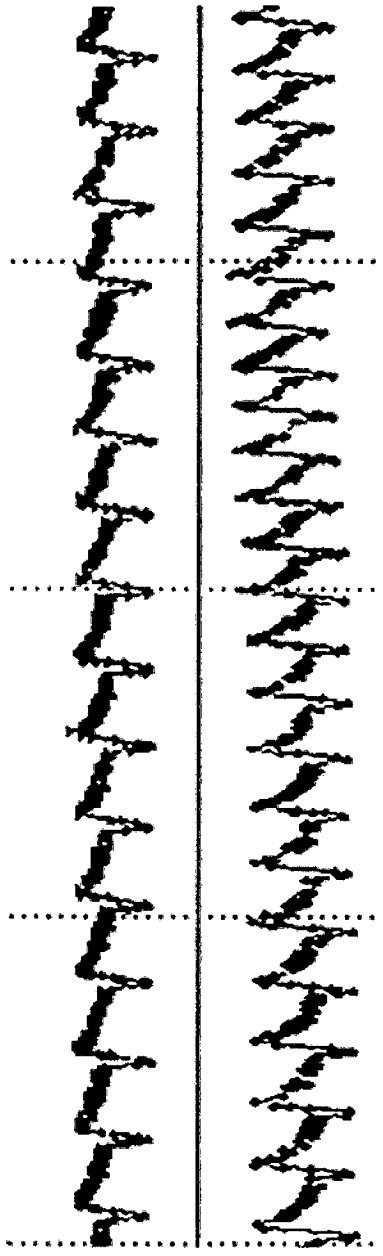


图4



对照	45次跳动/分钟
3μM 异丙基肾上腺素	60次跳动/分钟

图5

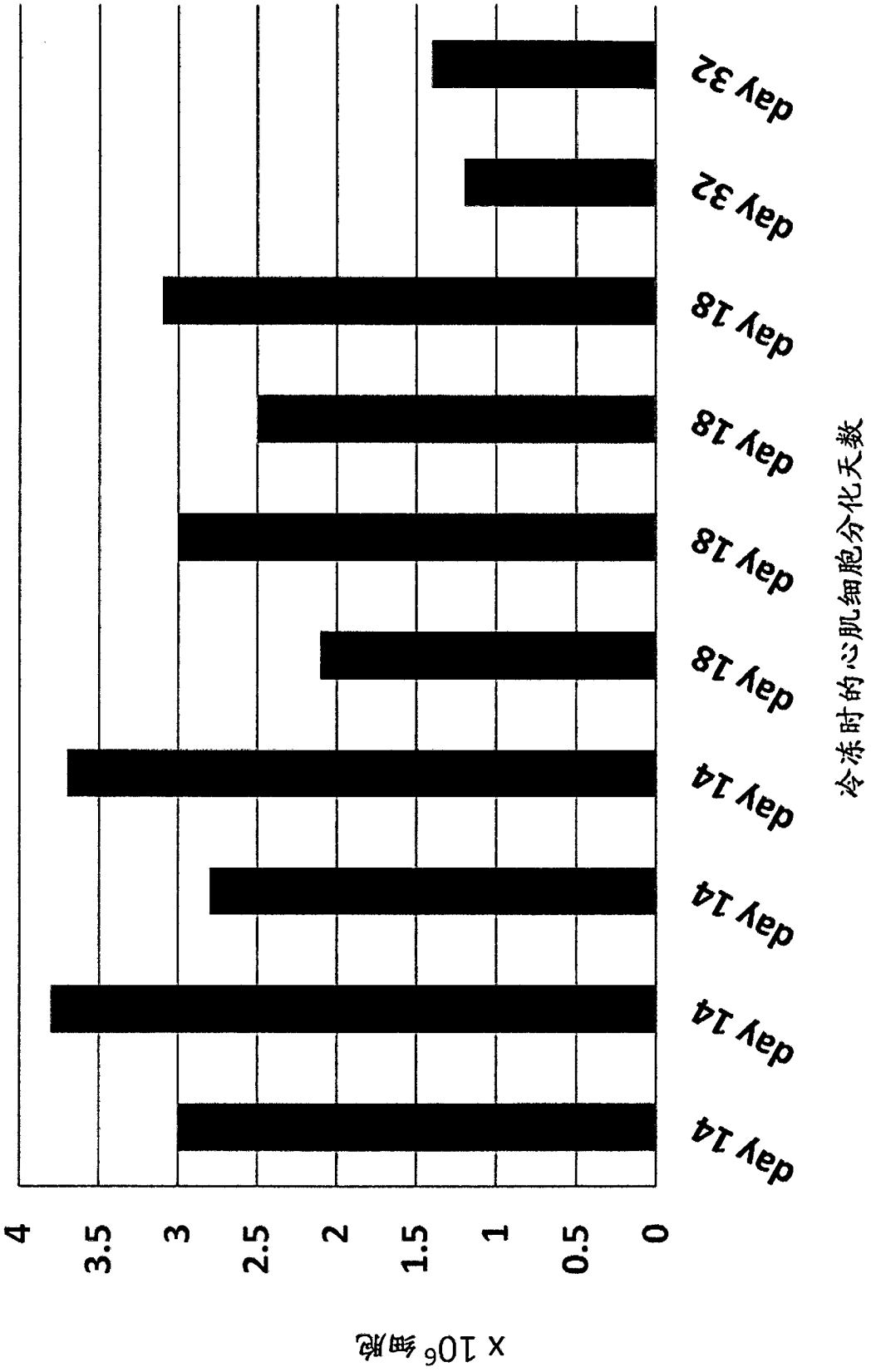

day 32
day 32
day 18
day 18
day 18
day 18
day 14
day 14
day 14
day 14
0
0.5
1
1.5
2
2.5
3
3.5
4
x 10⁶ 细胞
冷冻时的心肌细胞分化天数

图6

Abstract

This application relates to a method for differentiating pluripotent stem cells (PSCs) into cardiomyocytes. Moreover this application relates to a method for differentiating human embryonic stem cells (h ESCs) and induced pluripotent stem cells (i PSCs) into defined cardiomyocytes based on linked steps of chemically defined medium inductions.