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DESCRIPTION

BACKGROUND OF THE INVENTION

[0001] Human pluripotent stem cells (hPSCs) are now widely used to provide a theoretically endless and also large supply of human cardiomyocytes (Kehat et al. J Clin Invest 108, 407-414 (2001); Takahashi et al. Cell 131, 861-872 (2007); Zhang et al., Circ Res 104, e30-41 (2009)). Human cardiomyocytes have been derived from human embryonic stem cells (hESCs) (Thomson et al. Science 282, 1145-1147 (1998)) and induced pluripotent stem cells (hIPSCs) (Takahashi et al., Cell 131, 861-872 (2007)) and have a demonstrated use for multiple purposes including developmental models (Lian et al. Stem Cells 2012 (2012)), drug efficacy and/or safety screening (Schaaf et al. PLoS ONE 6, 20 (2011)), hypertrophy modelling and regenerative applications. Additionally, with recent advances in hIPSC technology, cardiomyocytes exhibiting heritable genetic disease phenotypes can be generated in vitro (Carvajal-Vergara, X. et al. Nature 465, 808-812 (2010); Itzhaki et al., Nature 471, 225-229 (2011); Malan et al. Circ Res (2011); Moretti et al., N Engl J Med 363, 1397-1409 (2010); Yazawa et al. Nature 471, 230-234 (2010)).

[0002] It is now widely accepted that the low density 2D culture of biopsy-derived human cardiomyocytes leads to rapid changes in cardiomyocyte phenotype and morphology (Bird et al. Cardiovasc Res 58, 423-434 (2003)) and making it difficult to extrapolate results to the in vivo situation. In order to obtain a cardiomyocyte phenotype more representative of in vivo conditions, cardiac tissue engineering has been used (Eschenhagen et al. FASEB J 11, 683-694 (1997); Zimmermann et al. Biotechnol Bio-eng 68, 106-114 (2000), Zimmermann et al. Circ Res 90, 223-230 (2002); Tulloch et al. Circ Res 109, 47-59 (2011); Tiburcy et al. Circ Res 109, 1105-1114 (2011); Eschenhagen et al. Am J Physiol Heart Circ Physiol 303, 11 (2012)) to generate constructs with similar properties to the native heart tissue.

[0003] The current ideology of tissue engineering is to generate/isolate the required cell type(s), and seed them into an engineered environment to promote their differentiation and generate in vivo-like tissues. Tissue engineering may therefore be considered as an inefficient process for two reasons, 1) disassociation of a tissue/differentiation culture destroys the extracellular environment thus destroying developmental information (eg. cell-cell interconnectivity, geometric cell positioning, cell-ECM connectivity), this necessitates very large increases in extracellular matrix (ECM) production in order to re-build the environment (Hudson et al. Tissue Eng Part A 17, 2279-2289 (2011)), and 2) the disassociation process is variable between hPSC lines and can lead to considerable cell death.

[0004] Other protocols reported in the literature may require modification of the protocol to enable similar cardiomyocyte efficiencies in multiple hPSC lines. However, the inventor's results demonstrate that changes in differentiation protocol may greatly affect the cardiomyocyte phenotype (e.g. it is shown that dorsomorphin may greatly affect the bioengineered heart muscle (BHM)). This may lead to changes in tissue engineered myocardial properties which may mask the effects of different experimental conditions or genetic disease models, therefore care must be taken when using different protocols in different lines.

[0005] Some recently published protocols may enable the same protocol to be used for multiple lines, they also produce cardiomyocytes with very high purity. However, pure cardiomyocytes do not facilitate the formation of functional tissue engineered myocardium and both cardiomyocytes and stromal cells are required for the formation of functional tissue engineered myocardium (Naito et al. Circulation 114, 172-78 (2006),Hudson et al. Tissue Eng Part A 17, 2279-2289 (2011)).

[0006] WO 2013/056072 A1, WO 2013/111875 A1 and US 2013/177535 disclose methods for producing cardiomyocytes, but not of a bioengineered heart muscle.

[0007] Hence, there is a need in the art for methods for producing bioengineered human myocardium, which are capable of overcoming the above disadvantages.

[0008] The development of a robust differentiation protocol is a very important step allowing the consistent production of BHM. In this study n >140 BHM in >18 independent experiments were produced and every one exhibited spontaneous beating activity. Additionally, the protocol enables to produce BHM from multiple hPSC lines using the same protocol. In addition, all disassociation steps could be eliminated and hPSCs were differentiated directly into bioengineered myocardium, thus retaining the developmental memory of the tissue, prevent any tissue recreation response and provide a more accurate in vitro model of human myocardial development.

SUMMARY OF THE INVENTION

[0009] The present invention relates to a method for producing bioengineered heart muscle from pluripotent stem cells, comprising the steps of

1. (i) cultivating pluripotent stem cells in a basal medium comprising an effective amount of (a) BMP4, Activin A, FGF2, a GSK3-inhibitor, and (b) a serum-free supplement resulting in a final concentration of 0.5-50 mg/ml albumin, 1-100 µg/ml transferrin, 0.1-10 µg/ml ethanol amine, 0.003-0.3 µg/ml sodium selenite, 0.4-40 µg/ml L-Carnitine HCl, 0.1-10 µg/ml Hydrocortisone, 0.05-5 µl/ml Fatty acid supplement, 0.0001-0.1 µg/ml triodo-L-thyronine (T3), thereby inducing mesoderm differentiation of said pluripotent stem cells;
2. (ii) cultivating the cells obtained in step (i) in a basal medium comprising an effective amount of an inhibitor of the Wnt-signaling pathway and a serum-free supplement as defined in step (i), thereby inducing cardiac differentiation of the cells; and
3. (iii) cultivating the cells obtained in step (ii) in a basal medium comprising an effective amount of a serum-free supplement as defined in step (i), under mechanical stimulation, thereby promoting cardiac maturation.

[0010] Carrying out the method disclosed herein human pluripotent stem cell (hPSC)-derived bioengineered heart muscle (BHM) is generated by directed tissue formation of hPSCs in collagen hydrogels. To form BHM, in vivo development was mimicked using a directed serum-free induction protocol causing the tissue to progress through distinct, known developmental stages, through pluripotency, early mesoderm, cardiac progenitor, immature cardiomyocytes and finally to more mature cardiac tissue comprised of 50 % cardiomyocytes, with the rest being predominately a stromal cell fraction. The inventors optimized their serum-free BHM protocol and found that individual BHM properties are highly dependent on particular stimuli, thus indicating that multiple exogenous stimuli are required for optimal BHM properties. In the end rhythmically contractile BHM was produced with measurable contractile force, ability for pacing and inotropy in response to increased resting length, calcium concentration and β-adrenergic stimulation. This BHM protocol, without modification, was capable of consistently producing BHM from multiple hPSC lines (in every BHM in every experiment conducted).

[0011] The present data suggests that the BHM protocol disclosed herein is a robust, serum-free and reproducible way to produce human myocardium for multiple applications. For example, it is also demonstrated that BHM is a potential model of human myocardium development, and shown that inhibition of BMP signalling leads to a more immature cardiac phenotype with reduced contractile strength.

[0012] Accordingly, the present invention is also directed to a BHM produced by the method according to the invention, as further defined in the claims.

[0013] Further contemplated is the use of the BHM according to the invention in an in vitro-model for drug toxicity screening. In other words, the present invention is also directed to a method for screening drug toxicity, comprising the step of contacting a BHM according to the invention with a drug to be screened.

[0014] Moreover, the present invention is directed to the use of the BHM according to the invention in an in vitro method for testing of cardiac function modulation by pharmacological candidate agents. Thus, also described is a method for testing of cardiac function modulation, comprising the step of contacting a BHM according to the invention with a pharmacological candidate agent.

[0015] Finally, the present invention is also directed to the use of the BHM according to the invention as a research tool, as well as to a BHM according to the invention for use in medicine.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0016] A method for producing bioengineered heart muscle from pluripotent stem cells, comprising the steps of

1. (i) cultivating pluripotent stem cells in a basal medium comprising an effective amount of (a) BMP4, Activin A, FGF2, a GSK3-inhibitor, and (b) a serum-free supplement resulting in a final concentration of 0.5-50 mg/ml albumin, 1-100 μg/ml transferrin, 0.1-10 μg/ml ethanol amine, 0.003-0.3 μg/ml sodium selenite, 0.4-40 μg/ml L-Carnitine HCl, 0.1-10

µg/ml Hydrocortisone, 0.05-5 µl/ml Fatty acid supplement, 0.0001-0.1 µg/ml triodo-L-thyronine (T3), thereby inducing mesoderm differentiation of said pluripotent stem cells;

2. (ii) cultivating the cells obtained in step (i) in a basal medium comprising an effective amount of an inhibitor of the Wnt-signaling pathway and a serum-free supplement as defined in step (i), thereby inducing cardiac differentiation of the cells; and
3. (iii) cultivating the cells obtained in step (ii) in a basal medium comprising an effective amount of a serum-free supplement as in (i), under mechanical stimulation, thereby promoting cardiac maturation.

[0017] In a preferred embodiment, the pluripotent stem cells are pluripotent stem cells of primate origin, more preferably the pluripotent stem cells are human pluripotent stem cells. Pluripotent stem cells are able to differentiate into every cell type of the body. As such, human pluripotent stem cells offer the unique opportunity to obtain bona fide human heart cells. Currently, the most utilized pluripotent cells are embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC). Human ESC-lines were first established by Thomson and coworkers (Thomson et al., Science 282: 1145-1147 (1998)). Human ESC research recently enabled the development of a new technology to reprogram cells of the body into a ES-like cell. This technology was pioneered by Yamanaka and coworkers in 2006 (Takahashi & Yamanaka Cell 126: 663-676 (2006)). Resulting induced pluripotent cells (iPSC) show a very similar behavior as ESC and, importantly, are also able to differentiate into every cell of the body. Moreover, it was reported that also parthenogenetic stem cells are likely to be suitable for BHM-production (Didié et al. J Clin Invest. 123, 1285-1298 (2013)). Accordingly, the pluripotent stem cells can be selected from embryonic stem cells, induced pluripotent stem cells, and parthenogenetic stem cells. In the context of the present invention, said pluripotent stem cells are however not produced using a process which involves modifying the germ line genetic identity of human beings or which involves use of a human embryo for industrial or commercial purposes.

[0018] The basal medium used in step (i), can be selected from DMEM/F12, StemPro, Is-cove's medium, αMEM, DMEM, and RPMI. Preferably, the basal medium used in step (i) is RPMI supplemented with pyruvate. However any suitable basal medium may be used in the method. Basal mediums are commercially available or may be prepared according to recipes which are publicly available, e.g. from catalogues of the ATCC.

[0019] If deemed appropriate, the basal medium may be supplemented with non-essential amino acids. If αMEM is used as the basal medium, the basal medium need not be supplemented additionally with non-essential amino acids. The non-essential amino acids are commercially available as a combined supplement. Such a supplement for example comprises 750 mg/L glycine, 890 mg/L L-alanine, 1320 mg/L L-asparagine, 1330 mg/L L-aspartic acid, 1470 mg/L L-glutamic acid, 1150 mg/L L-proline, and 1050 mg/L L-serine.

[0020] As set out above, the basal medium of step (i) comprises an effective amount of BMP4, Activin A, FGF2, and a GSK3-inhibitor. For example, such a basal medium comprises 1-20 ng/ml BMP4, preferably 2-15 ng/ml, more preferably 2.5-10 ng/ml, more preferably 3-8 ng/ml, most preferably 4-6 ng/ml, and even most preferably about 5 ng/ml;

0.1-10 ng/ml FGF2, preferably 1-9 ng/ml, more preferably 2-8 ng/ml, even more preferably 3-7 ng/ml, most preferably 4-6 ng/ml, and even most preferably about 5 ng/ml;
1-20 ng/ml Activin A, preferably 2.5-18 ng/ml, more preferably 5-16 ng/ml, even more preferably 7.5-14 ng/ml, still more preferably 8-12 ng/ml, most preferably 8.5-10 ng/ml, and even most preferably about 9 ng/ml.

[0021] The GSK3-inhibitor in the basal medium of step (i) can be selected, for example, from the group consisting of CHIR99021, CHIR98014, SB216763, TWS119, Tideglusib, SB415286, and LY2090314. However, any GSK3-inhibitor suitable in the method of the invention can be applied. In a preferred embodiment, the GSK3-inhibitor in the basal medium of step (i) is CHIR99021.

[0022] It will be understood by the skilled person that the concentration of an effective amount of a GSK3-inhibitor varies with the availability and inhibition constant of the inhibitor in question. In the context of the present invention, the term "effective amount" as used herein in the context of a GSK3-inhibitor is intended to mean an enzyme inactivating concentration. For example, in case of CHIR99021, the basal medium in step (i) comprises 0.1-10 µM CHIR99021, preferably 0.2-9 µM, more preferably 0.3-8 µM, even more preferably 0.4-7 µM, still more preferably 0.5-6 µM, more preferably 0.6-5 µM, more preferably 0.7-4 µM, more preferably 0.8-3 µM, most preferably 0.9-2 µM, and even most preferably about 1 µM CHIR99021. It will be understood that an effective concentration of any receptor/enzyme agonist or inhibitor varies with the availability and biological activity of the respective compound.

[0023] The serum-free supplement applied in step (i), (ii) and (iii) of the method is formulated to result in a final concentration of 0.5-50 mg/ml albumin (preferably 1-40 mg/ml, more preferably 2-30 mg/ml, still more preferably 3-20 mg/ml, most preferably 4-10 mg/ml, and even most preferably 4.5-7.5 mg/ml such as about 5 mg/ml),
1-100 µg/ml transferrin (preferably 2-90 µg/ml, more preferably 3-80 µg/ml, even more preferably 4-70 µg/ml, still more preferably 5-60 µg/ml, more preferably 6-50 µg/ml, more preferably 7-40 µg/ml, more preferably 8-30 µg/ml, more preferably 9-20 µg/ml, such as about 10 µg/ml),
0.1-10 µg/ml ethanol amine (preferably 0.2-9 µg/ml, more preferably 0.3-8 µg/ml, even more preferably 0.4-7 µg/ml, still more preferably 0.5-6 µg/ml, more preferably 0.6-5 µg/ml, more preferably 0.7-4 µg/ml, more preferably 0.8-3 µg/ml, more preferably 1-2.5 µg/ml, such as about 2 µg/ml),
0.003-0.3 µg/ml sodium selenite (preferably 0.005-0.2 µg/ml, more preferably 0.01-0.1 µg/ml, even more preferably 0.02-0.05 µg/ml, and most preferably about 0.03 µg/ml, such as about 0.032 µg/ml),
0.4-40 µg/ml L-Carnitine HCl (preferably 0.5-30 µg/ml, more preferably 1-20 µg/ml, even more preferably 2-10 µg/ml, most preferably 3-5 µg/ml, and even most preferably about 4 µg/ml),
0.1-10 µg/ml Hydrocortisone (preferably 0.2-9 µg/ml, more preferably 0.3-8 µg/ml, even more preferably 0.4-7 µg/ml, still more preferably 0.5-6 µg/ml, more preferably 0.6-5 µg/ml, more preferably 0.7-4 µg/ml, more preferably 0.8-3 µg/ml, more preferably 0.9-2 µg/ml, such as about 1 µg/ml),
0.05-5 µl/ml Fatty acid supplement (preferably 0.1-4 µl/ml, more preferably 0.2-3 µl/ml, even more preferably 0.3-3 µl/ml, most preferably 0.4-2 µl/ml, and even most preferably 0.45-1 µl/ml, such as about 0.5 µl/ml), and

0.0001-0.1 µg/ml triodo-L-thyronine (T3) (preferably 0.001-0.01 µg/ml, more preferably 0.002-0.0075 µg/ml, even more preferably 0.003-0.005 µg/ml, and most preferably about 0.004 µg/ml).

[0024] In addition, the serum-free supplement may further comprise one or more components selected from the group consisting of vitamin A, D-galactose, L-carnitine, linoleic acid, linolenic acid, progesterone, and putrescine. These components are conducive for the viability of the cells. Suitable concentrations of the respective components are known to the skilled person or can be easily determined using routine measures.

[0025] The serum-free supplement referred to in step (i) is also commercially available. For example, B27® supplement or B27® supplement minus insulin can be used. In a preferred embodiment, the B27® supplement or B27® supplement minus insulin used in step (i) of the above method is applied in an amount of 0.1-10 % B27® or B27® minus insulin, preferably 0.5-8 %, more preferably 1-6 %, even more preferably 1.5-4% , and most preferably about 2% B27® or B27® minus insulin.

[0026] As demonstrated in the examples below, it has been proven advantageous to include an effective amount of ascorbic acid or a salt thereof into the basal medium of step (i). In a preferred embodiment, the basal medium of step (i) comprises 10-1000 µM, preferably 50-400 µM, more preferably 100-300 µM, even more preferably 150-250 µM, and most preferably about 200 µM of ascorbic acid or a salt thereof. The ascorbic acid may be delivered in the free form or as a salt. Since ascorbate is the active ingredient, any salt of ascorbic acid may be used, which provides the ascorbate to the cells, provided the counter ion has no detrimental effect on the cells. As shown in the examples, one suitable salt of ascorbic acid is ascorbate-2-phosphate.

[0027] The length of step (i) and the concentration of factors such as BMP4, Activin A, FGF2, and the GSK3-inhibitor may be optimized by monitoring the efficiency of induction of mesoderm differentiation. This can be achieved by monitoring the expression of cell surface or pluripotency markers, i.e. by (a) a decrease of TRA-1-60 and OCT4 positive cells (pluripotent stem cells) and (b) an increase of MIXL1 and Mesp1 positive cells (mesoderm) (see also Fig 4f herein).

[0028] Briefly, cells are fixed using ethanol, blocked using standard protocols, and then stained with primary antibodies directed against TRA-1-60, OCT4, MIXL1 and/or Mesp1 (cf. Table 2 below) in blocking buffer for 45 min, optionally followed by secondary antibodies (if the primary antibody is not fluorescence labelled) in blocking buffer and Hoechst for 30 min at 4°C (cf. Table 2 below). A BD LSRII is used for flow cytometry analysis (BD Biosystems). For live cells populations are gated based on forward-side scatter profiles. BD FACSDiva Software (BD Bioscience) or Cyflologic v1.2.1 (Cyflo Ltd) are used for analysis. Induction of mesoderm differentiation is indicated if

1. (a) less than 50%, preferably less than 40%, more preferably less than 30%, even more preferably less than 20%, most preferably less than 10%, and even most preferably less than 5% of the cells of the live cells population are positive for TRA-1-60; and/or less than 50%, preferably less than 40%, more preferably less than 30%, even more preferably less than 20%, most preferably less than 10%, and even most preferably less than 5% of the cells of the live cells population are positive for OCT4; and

2. (b) more than 20%, preferably more than 30%, more preferably more than 40%, even more preferably more than 50%, and most preferably more than 60%, of the cells of the live cells population are positiv for MIXL1; and/or more than 20%, preferably more than 30%, more preferably more than 40%, even more preferably more than 50%, and most preferably more than 60% of the cells of the live cells population are positive for Mesp1.

[0029] Usually, step (i) is carried out for 48-96 h. Preferably, step (i) is carried out for 60-84 h, and more preferably step (i) is carried out for 66-78 h.

[0030] The basal medium used in step (ii), can be selected from DMEM/F12, StemPro, Is-cove's medium, αMEM, DMEM, and RPMI. Preferably, the basal medium used in step (ii) is RPMI supplemented with pyruvate. However any suitable basal medium may be used in the method.

[0031] If deemed appropriate, the basal medium of step (ii) may be supplemented with non-essential amino acids. If αMEM is used as the basal medium in step (ii), the basal medium need not be supplemented additionally with non-essential amino acids. The non-essential amino acids are commercially available as a combined supplement. Such a supplement for example comprises 750 mg/L glycine, 890 mg/L L-alanine, 1320 mg/L L-asparagine, 1330 mg/L L-aspartic acid, 1470 mg/L L-glutamic acid, 1150 mg/L L-proline, and 1050 mg/L L-serine.

[0032] The basal medium in step (ii) may be independently selected from the basal medium applied in step (i). However, in a preferred embodiment, the basal medium in steps (i) and (ii) is the same.

[0033] The inhibitor of the Wnt-signaling pathway in the basal medium of step (ii) may be any inhibitor of the Wnt-signaling pathway, which can be suitably applied in the method of the invention. Preferably, said inhibitor of the Wnt-signaling pathway is selected from the group consisting of IWP4, IWP2, IWR-1, IWP1, IWP3, IWR-2, IWR-3, IWR-4, IWR-5, XAV939, DKK1, quercetin, ICG-001, pyrvinium, CCT031374, iCRT-3,5,14, CPG049090, and NC043. More preferably said inhibitor of the Wnt-signaling pathway is selected from the group consisting of IWP4, IWP2, IWR-1, IWP1, IWP3, IWR-2, IWR-3, IWR-4, IWR-5, XAV939, DKK1. As demonstrated in the examples be-IWR-2, IWR-3, IWR-4, IWR-5, XAV939, DKK1. As demonstrated in the examples below, one particularly useful inhibitor of the Wnt-signaling pathway in the basal medium of step (ii) is IWP4.

[0034] The serum-free supplement referred to in step (ii) is as defined for step (i) above. The serum-free supplements applied in step (i) and (ii) may be the same or not. Likewise, B27® supplement or B27® supplement minus insulin can be used in step (ii). In a preferred embodiment, the B27® supplement or B27® supplement minus insulin used in step (ii) of the above method is applied in an amount of 0.1-10 % B27® or B27® minus insulin, preferably 0.5-8 %, more preferably 1-6 %, even more preferably 1.5-4% , and most preferably about 2% B27® or B27® minus insulin.

[0035] It will be understood by the skilled person that the concentration of an effective amount of an inhibitor of the Wnt-signaling pathway varies with the availability and inhibition constant of the inhibitor in question. For example, in case of IWP4, the basal medium of step (ii) may comprise 0.1-10 µM IWP4, preferably 1-9 µM, more preferably 2-8 µM, even more preferably 3-7 µM, still more preferably 4-6 µM, and most preferably about 5 µM IWP4. It will be understood that an effective concentration of any receptor/enzyme agonist or inhibitor varies with the availability and biological activity of the respective compound.

[0036] As demonstrated in the examples below, it has been proven advantageous to include an effective amount of ascorbic acid or a salt thereof into the basal medium of step (ii). In a preferred embodiment, the basal medium of step (ii) comprises 10-1000 µM, preferably 50-400 µM, more preferably 100-300 µM, even more preferably 150-250 µM, and most preferably about 200 µM of ascorbic acid or a salt thereof. The ascorbic acid may be delivered in the free form or as a salt. Since ascorbate is the active ingredient, any salt of ascorbic acid may be used, which provides the ascorbate to the cells, provided the counter ion has no detrimental effect on the cells. As shown in the examples, one suitable salt of ascorbic acid for use in the basal medium in step (ii) is ascorbate-2-phosphate.

[0037] The length of step (ii) and the concentration of the remaining constituents such as the inhibitor of the Wnt-signaling pathway may be optimized by monitoring the efficiency of induction of cardiac differentiation of the cells. This can be achieved by monitoring the expression of differentiation markers, i.e. by an increase of Nkx2.5 and actinin.

[0038] Briefly, cells are fixed using ethanol, blocked, and then stained with primary antibodies directed against Nkx2.5 and/or actinin (cf. Table 2 below) in blocking buffer for 45 min, optionally followed by secondary antibodies (if the primary antibody is not fluorescence labelled) in blocking buffer and Hoechst for 30 min at 4°C (cf. Table 2 below). A BD LSRII is used for flow cytometry analysis (BD Biosystems). For live cells populations are gated based on forward-side scatter profiles. BD FACSDiva Software (BD Bioscience) or Cyflologic v1.2.1 (Cyflo Ltd) are used for analysis. Induction of cardiac differentiation is indicated if more than 20%, preferably more than 30%, more preferably more than 40%, even more preferably more than 50%, and most preferably more than 60%, of the cells of the live cells population are positiv for Nkx2.5; and/or more than 20%, preferably more than 30%, more preferably more than 40%, even more preferably more than 50%, and most preferably more than 60% of the cells of the live cells population are positive for actinin (see also Fig 4d and 4f herein).

[0039] Usually, step (ii) is carried out for 8-12 days. Preferably, step (ii) is carried out for 9-11 days, and most preferably step (ii) is carried out for 10 days.

[0040] The basal medium used in step (iii), can be selected from DMEM/F12, StemPro, Is-cove's medium, αMEM, DMEM, and RPMI. Preferably, the basal medium used in step (iii) is RPMI supplemented with pyruvate. However any suitable basal medium may be used in the method.

[0041] If deemed appropriate, the basal medium of step (iii) may be supplemented with non-essential amino acids. If αMEM is used as the basal medium in step (iii), the basal medium need not be supplemented additionally with non-essential amino acids. The non-essential amino acids are commercially available as a combined supplement. Such a supplement for example comprises 750 mg/L glycine, 890 mg/L L-alanine, 1320 mg/L L-asparagine, 1330 mg/L L-aspartic acid, 1470 mg/L L-glutamic acid, 1150 mg/L L-proline, and 1050 mg/L L-serine.

[0042] The basal medium in step (iii) may be independently selected from the basal medium applied in steps (i) and/or (ii). However, in a preferred embodiment, the basal medium in steps (ii) and (iii) is the same. More preferably, the basal medium in steps (i), (ii) and (iii) is the same.

[0043] As demonstrated in the examples below, it has been proven advantageous to include an effective amount of ascorbic acid or a salt thereof into the basal medium of step (iii). In a preferred embodiment, the basal medium of step (iii) comprises 10-1000 µM, preferably 50-400 µM, more preferably 100-300 µM, even more preferably 150-250 µM, and most preferably about 200 µM of ascorbic acid or a salt thereof. The ascorbic acid may be delivered in the free form or as a salt. Since ascorbate is the active ingredient, any salt of ascorbic acid may be used, which provides the ascorbate to the cells, provided the counter ion has no detrimental effect on the cells. As shown in the examples, one suitable salt of ascorbic acid for use in the basal medium in step (iii) is ascorbate-2-phosphate.

[0044] The serum-free supplement referred to in step (iii) is a serum-free supplement as defined for step (i) above. The serum-free supplements applied in steps (i), (ii) and (iii) may be the same or not. Likewise, B27® supplement or B27® supplement minus insulin can be used in step (iii). In a preferred embodiment, the B27® supplement or B27® supplement minus insulin used in step (iii) of the above method is applied in an amount of 0.1-10 % B27® or B27® minus insulin, preferably 0.5-8 %, more preferably 1-6 %, even more preferably 1.5-4% , and most preferably about 2% B27® or B27® minus insulin.

[0045] The basal medium of step (iii) further comprises an effective amount of TGFβ1. For example, the basal medium of step (iii) may comprise 0.1-10 ng/ml TGFβ1, preferably 0.2-9 ng/ml, more preferably 0.3-8 ng/ml, even more preferably 0.4-7 ng/ml, still more preferably 0.5-6 ng/ml, more preferably 0.6-5 ng/ml, more preferably 0.7-4 ng/ml, more preferably 0.8-3 ng/ml, most preferably 0.9-2 ng/ml, and even most preferably about 1 ng/ml TGFβ1.

[0046] As shown in the examples, it is advantageous for cardiac maturation if the basal medium of step (iii) does not comprise an effective amount of FGF2. In contrast thereto, calcium has been shown to improve cardiac maturation. Accordingly, in a preferred embodiment, the basal medium of step (iii) comprises 0.5-3 mM Ca^{2+}, preferably 0.5-2.75 mM Ca^{2+}, more preferably 1-2.25 mM Ca^{2+}, even more preferably 1-1.5 mM mM Ca^{2+}, and most preferably about 1.2 mM Ca^{2+}.

[0047] Usually, step (iii) of the method of the invention is carried out under mechanical stimulation, e.g. on a stretch device, as generally known in the art. Preferably, the stretch device applies a static, phasic or dynamic stretch to the BHM. More specifically, mechanical stretching can be (a) static, (b) dynamic, or (c) flexible against a resilient load. Preferably, the mechanical stimulation in step (iii) is dynamic mechanical stimulation or static stretch. In a more preferred embodiment, the mechanical stimulation in step (iii) is dynamic mechanical stimulation against a resilient load to facilitate auxotonic contractions.

[0048] Whether cardiac maturation is promoted can be tested by optical inspection for spontaneous or electrically stimulated contractions. Preferably, cardiac maturation is monitored by an isometric contraction experiment, wherein a twitch force development of > 0.01 mN is indicative for cardiac maturation.

[0049] Briefly, contraction experiments are performed in organ baths at 37°C under constant bubbling with 5% CO_2 and 95% O_2 to maintain a physiological pH in Tyrode's solution containing (all in mM): 120 NaCl, 1 $MgCl_2$, 0.2 $CaCl_2$, 5.4 KCl, 22.6 $NaHCO_3$, 4.2 NaH_2PO_4, 5.6 glucose and 0.56 ascorbate. Calcium is adjusted using a 0.2 M calcium chloride solution. All BHM are analysed at 3 Hz with 5 ms square pulses of 200 mA electrical current in order to pace at approximately the embryonic heart rate. Stimulation frequency is altered to confirm proper force-frequency response (Bowditch mechanism). BHM are mechanically stretched at intervals of 125 µm until the maximum twitch force is observed (force-length response; Frank-Starling mechanism).

[0050] Usually, step (iii) is carried out for at least 72 h. Although there is no particular upper limit for the length of step (iii), said step is usually carried out for less than 100 days. In specific embodiments, step (iii) may be carried out for 4-50 days, such as for about 15 days.

[0051] Step (i) of the method of the invention may be preceded by a seeding step, wherein said pluripotent stem cells are seeded in a ratio of (2.5-6 x 10^6 cells /1 mg collagen) / 1 ml medium in a suitable mould. Preferably, the seeding step is carried out 18-30 h prior to step (i).

[0052] The medium used in the seeding step usually comprises 0.2-2 mg/ml collagen (preferably 0.3-1.9 mg/ml, more preferably 0.4-1.8 mg/ml, even more preferably 0.4-1.7 mg/ml, still more preferably 0.5-1.6 mg/ml, more preferably 0.6-1.5 mg/ml, more preferably 0.7-1.4 mg/ml, more preferably 0.8-1.3 mg/ml, more preferably 0.9-1.2 mg/ml, such as about 1 mg/ml). The collagen is preferably of medical grade and selected from the group consisting of collagen type I, collagen type III, collagen type V, and a mixture thereof. In a more preferred embodiment, at least 90% of said collagen is collagen type I. However, said collagen may also further comprises one or more extracellular matrix components selected from the group consisting of elastin, laminin, entactin, nidogen, proteoglycan, and fibronectin. Usually, the exact composition of the collagen will depend on the origin, from where it is derived from. The collagen is preferably of human origin, but bovine or porcine origin, or marine origin, such as from algae or fish origin, is also contemplated. Alternatively, recombinant collagen may also be used.

[0053] In order to achieve suitable cell densities, for some pluripotent cell lines it may be helpful to supplement the medium used in the seeding step with a ROCK-inhibitor. Therefore, in a preferred embodiment, the medium used in the seeding step further comprises a ROCK-inhibitor. The ROCK-inhibitor may be any ROCK-inhibitor, which can be suitably applied in the method of the invention. Preferably, said ROCK inhibitor is selected from Y27632, H-1152P, Thiazovivin, Fasudil, Hydroxyfasudil, GSK429286A, and RKI-1447, preferably selected from Y27632, H-1152P, Thiazovivin, Fasudil, Hydroxyfasudil, and more preferably the ROCK inhibitor is selected from Y27632 or H-1152P. As demonstrated in the examples below, one particularly useful ROCK-inhibitor is Y27632.

[0054] It will be understood by the skilled person that the concentration of an effective amount of a ROCK-inhibitor varies with the availability and inhibition constant of the inhibitor in question. For example, in case of Y27632, the medium used in the seeding step may comprise 1-50 µM, preferably 2.5-40 µM, more preferably 5-30 µM, even more preferably 7.5-20 µM, most preferably 8-12 µM, and most preferably about 10 µM Y27632.

[0055] It will be understood that an effective concentration of any receptor/enzyme agonist or inhibitor varies with the availability and biological activity of the respective compound.

[0056] Apart from the above disclosed method, the invention further relates to a BHM produced by said method, as defined in the claims. Despite the increased maturity observed in our BHM protocol, it should also be noted that the BHM is still a relatively immature tissue. Compared to adult heart tissue the BHM still has an inferior *β-MHC/α-MHC* ratio, and low but still retained expression of progenitor genes (e.g. *ISL1*). However, prolonged culture under appropriate culture conditions with biophysical stimulation may further increase maturity. There is already morphological evidence suggesting that this may also be the case in the BHM system.

[0057] The BHM obtained by the method disclosed herein exhibits the following characteristics: It can be paced at multiple frequencies up to at least 3 Hz, exhibits a calcium EC_{50} of higher than 0.2 mM being preferably in the physiological range 4-8 mM, and a twitch tension of more than 200 µN. The twitch tension is increased in response to increased resting length and resting tension. In response to 1 µM isoprenaline, the BHM exhibits an inotropic response of more than 40 µN under paced conditions at 0.6 mM calcium, preferably more than 45 µN, more preferably more than 50 µN.

[0058] Briefly, all contraction experiments are performed in organ baths at 37°C and physiological pH in Tyrode's solution containing (all in mM): 120 NaCl, 1 $MgCl_2$, 0.2 $CaCl_2$, 5.4 KCl, 22.6 $NaHCO_3$, 4.2 NaH_2PO_4, 5.6 glucose and 0.56 ascorbate. Calcium is adjusted using a 0.2 M calcium chloride solution. All BHM are analysed at 3 Hz with 5 ms square pulses of 200 mA electrical current in order to pace at approximately the embryonic heart rate. Stimulation frequency is altered to confirm proper force-frequency response (Bowditch mechanism). BHM are mechanically stretched at intervals of 125 µm until the maximum twitch force is observed at 2 mM calcium (Frank-Starling mechanism). Subsequently, BHM are subjected to different calcium concentrations (0.2, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4 mM) and the twitch force is recorded. For isoprenaline experiments the calcium concentration is adjusted to 0.6 mM and subsequently the isoprenaline concentration is adjusted to 1 µM.

[0059] Another characteristic of the BHM obtained by the method disclosed herein is that it comprises $CD90^+$ stromal cells. Expression of CD90 can be determined using flow cytometry. Briefly, cells are fixed using ethanol, blocked, and then stained with primary antibodies directed against CD90 (cf. Table 2 below) in blocking buffer for 45 min, optionally followed by secondary antibodies (if the primary antibody is not fluorescence labelled) in blocking buffer and Hoechst for

30 min at 4°C (cf. Table 2 below). A BD LSRII is used for flow cytometry analysis (BD Biosystems). For live cells populations are gated based on forward-side scatter profiles. BD FACSDiva Software (BD Bioscience) or Cyflologic v1.2.1 (Cyflo Ltd) are used for analysis.

[0060] The BHM may provide a good model system for investigating mechanisms driving maturation in a serum-free environment, and we have already demonstrated that with increased culture periods maturity may be increased (we showed increase isoprenaline sensitivity and improve tissue morphology). The capability of long term BHM culture without loss of function (at least 63 days) also suggests that long term pharmacological safety and efficacy experiments are possible. Hence, in a preferred embodiment, the BHM obtained by the method disclosed herein can be maintained for at least 63 days.

[0061] Using the traditional approach of differentiation followed by tissue engineering the differentiation cultures require extensive digestion protocols in order to yield single cell/small clumps required for cardiac tissue engineering applications. These digestion protocols destroy the extracellular environment and spatial distribution formed during development and may hence have a difficult to control inhibitory effect on the cardiac differentiation protocol.

[0062] Using the BHM as a model we demonstrated that factors affecting early development (ASC-2-P, dorsomorphin) and later development (mechanical stimulation, FGF2, TGFβ1, and calcium concentration) had a profound impact on BHM function and properties. Therefore, our BHM protocol may be a useful tool in the study of developmental processes not only governing cardiogenesis, but also tissue formation and properties.

[0063] Accordingly, the BHM obtained by the method disclosed herein can be suitably used as a research tool. For example, the use of the BHM obtained by the method disclosed herein in an in vitro-model for drug toxicity screening is contemplated. In other words, a method for screening drug toxicity is contemplated, comprising the step of contacting a BHM obtained by the method disclosed herein with a drug to be screened. Alternatively, the BHM obtained by the method disclosed herein may be used in an in vitro method for testing of cardiac function modulation by pharmacological candidate agents. Thus, also described is a method for testing of cardiac function modulation, comprising the step of contacting a BHM according to the invention with a pharmacological candidate agent.

[0064] Finally, the BHM obtained by the method disclosed herein can be used in medicine. Merely as an example, it is contemplated that the BHM obtained by the method disclosed herein can be advantageously used in heart repair.

[0065] The invention is further described by the following embodiments:

1. 1. A method for producing bioengineered heart muscle from pluripotent stem cells, comprising the steps of
    1. (i) cultivating pluripotent stem cells in a basal medium comprising an effective amount of (a) BMP4, Activin A, FGF2, a GSK3-inhibitor, and (b) a serum-free supplement resulting in a final concentration of 0.5-50 mg/ml albumin, 1-100 μg/ml

   transferrin, 0.1-10 µg/ml ethanol amine, 0.003-0.3 µg/ml sodium selenite, 0.4-40 µg/ml L-Carnitine HCl, 0.1-10 µg/ml Hydrocortisone, 0.05-5 µl/ml Fatty acid supplement, 0.0001-0.1 µg/ml triodo-L-thyronine (T3), thereby inducing mesoderm differentiation of said pluripotent stem cells;
   2. (ii) cultivating the cells obtained in step (i) in a basal medium comprising an effective amount of an inhibitor of the Wnt-signaling pathway and a serum-free supplement as in (i), thereby inducing cardiac differentiation of the cells; and
   3. (iii) cultivating the cells obtained in step (ii) in a basal medium comprising an effective amount of a serum-free supplement as in (i), under mechanical stimulation, thereby promoting cardiac maturation.
2. 2. The method of embodiment 1, wherein the pluripotent stem cells are pluripotent stem cells of primate origin, preferably human pluripotent stem cells.
3. 3. The method of embodiment 1 or 2, wherein the pluripotent stem cells are selected from embryonic stem cells, induced pluripotent stem cells, and parthenogenetic stem cells.
4. 4. The method of any one of embodiment 1-3, wherein the basal medium of step (i) comprises 10-1000 µM, preferably 50-400 µM, more preferably 100-300 µM, even more preferably 150-250 µM, and most preferably about 200 µM of ascorbic acid or a salt thereof.
5. 5. The method of embodiment 4, wherein the salt of ascorbic acid is ascorbate-2-phosphate.
6. 6. The method of any one of embodiment 1-5, wherein step (i) is carried out for 48-96 h, preferably wherein step (i) is carried out for 60-84 h, most preferably wherein step (i) is carried out for 66-78 h.
7. 7. The method of any one of embodiment 1-6, wherein the basal medium in step (i) comprises 1-20 ng/ml BMP4, preferably 2-15 ng/ml, more preferably 2.5-10 ng/ml, more preferably 3-8 ng/ml, most preferably 4-6 ng/ml, and even most preferably about 5 ng/ml.
8. 8. The method of any one of embodiment 1-7, wherein the basal medium in step (i) comprises 0.1-10 ng/ml FGF2, preferably 1-9 ng/ml, more preferably 2-8 ng/ml, even more preferably 3-7 ng/ml, most preferably 4-6 ng/ml, and even most preferably about 5 ng/ml.
9. 9. The method of any one of embodiment 1-8, wherein the basal medium in step (i) comprises 1-20 ng/ml Activin A, preferably 2.5-18 ng/ml, more preferably 5-16 ng/ml, even more preferably 7.5-14 ng/ml, still more preferably 8-12 ng/ml, most preferably 8.5-10 ng/ml, and even most preferably about 9 ng/ml.
10. 10. The method of any one of embodiment 1-9, wherein the GSK3-inhibitor in the basal medium of step (i) is selected from the group consisting of CHIR99021, CHIR98014, SB216763, TWS119, Tideglusib, SB415286, and LY2090314.
11. 11. The method of embodiment 10, wherein the GSK3-inhibitor in the basal medium of step (i) is CHIR99021.
12. 12. The method of embodiment 11, wherein the basal medium in step (i) comprises 0.1-10 µM CHIR99021, preferably 0.2-9 µM, more preferably 0.3-8 µM, even more preferably 0.4-7 µM, still more preferably 0.5-6 µM, more preferably 0.6-5 µM, more preferably 0.7-4 µM, more preferably 0.8-3 µM, most preferably 0.9-2 µM, and even most preferably about 1 µM CHIR99021.
13. 13. The method of any one of embodiment 1-12, wherein the serum-free supplement in step (i) comprises 0.1-10 % B27 or B27 minus insulin, preferably 0.5-8 %, more preferably

1-6 %, even more preferably 1.5-4% , and most preferably about 2% B27 or B27 minus insulin.

14. 14. The method of any one of embodiment 1-13, wherein the basal medium used in step (i), is DMEM/F12, StemPro, Iscove's medium, αMEM, DMEM, and RPMI.
15. 15. The method of claim 14, wherein the basal medium used in step (i) is RPMI supplemented with pyruvate.
16. 16. The method of any one of embodiment 1-15, wherein the inhibitor of the Wnt-signaling pathway in the basal medium of step (ii) is selected from the group consisting of IWP4, IWP2, IWR-1, IWP1, IWP3, IWR-2, IWR-3, IWR-4, IWR-5, XAV939, DKK1, quercetin, ICG-001, pyrvinium, CCT031374, iCRT-3,5,14, CPG049090, NC043; preferably selected from the group consisting of IWP4, IWP2, IWR-1, IWP1, IWP3, IWR-2, IWR-3, IWR-4, IWR-5, XAV939, DKK1.
17. 17. The method of embodiment 16, wherein the inhibitor of the Wnt-signaling pathway in the basal medium of step (ii) is IWP4.
18. 18. The method of embodiment 17, wherein the basal medium of step (ii) comprises 0.1-10 μM IWP4, preferably 1-9 μM, more preferably 2-8 μM, even more preferably 3-7 μM, still more preferably 4-6 μM, and most preferably about 5 μM IWP4.
19. 19. The method of any one of embodiment 1-18, wherein the basal medium of step (ii) comprises 10-1000 μM, preferably 50-400 μM, more preferably 100-300 μM, even more preferably 150-250 μM, and most preferably about 200 μM of ascorbic acid or a salt thereof.
20. 20. The method of embodiment 19, wherein the salt of ascorbic acid is ascorbate-2-phosphate.
21. 21. The method of any one of embodiment 1-20, wherein step (ii) is carried out for 8-12 days, preferably wherein step (ii) is carried out for 9-11 days, most preferably wherein step (ii) is carried out for 10 days.
22. 22. The method of any one of embodiment 1-21, wherein the serum-free supplement in step (ii) comprises 0.1-10 % B27 or B27 minus insulin, preferably 0.5-8 %, more preferably 1-6 %, even more preferably 1.5-4%, and most preferably about 2% B27 or B27 minus insulin.
23. 23. The method of any one of embodiment 1-22, wherein the basal medium used in step (ii), is DMEM/F12, StemPro, Iscove's medium, αMEM, DMEM, and RPMI.
24. 24. The method of embodiment 23, wherein the basal medium used in step (ii) is RPMI supplemented with pyruvate.
25. 25. The method of any one of embodiment 1-24, wherein the basal medium of step (iii) further comprises 10-1000 μM, preferably 50-400 μM, more preferably 100-300 μM, even more preferably 150-250 μM, and most preferably about 200 μM of ascorbic acid or a salt thereof.
26. 26. The method of embodiment 25, wherein the salt of ascorbic acid is ascorbate-2-phosphate.
27. 27. The method of any one of embodiment 1-26, wherein step (iii) is carried out for at least 72 h, preferably wherein step (iii) is carried out for less than 100 days, more preferably wherein step (iii) is carried out for 4-50 days, and most preferably wherein step (iii) is carried out for about 15 days.
28. 28. The method of any one of embodiment 1-27, wherein the serum-free supplement in

step (iii) comprises 0.1-10 % B27 or B27 minus insulin, preferably 0.5-8 %, more preferably 1-6 %7, even more preferably 1.5-4%, and most preferably about 2% B27 or B27 minus insulin.

29. 29. The method of any one of embodiment 1-28, wherein the basal medium used in step (iii), is DMEM/F12, StemPro, Iscove's medium, αMEM, DMEM, and RPMI.
30. 30. The method of embodiment 29, wherein the basal medium used in step (i) is RPMI supplemented with pyruvate.
31. 31. The method of any one of embodiment 1-30, wherein the basal medium of step (iii) further comprises 0.1-10 ng/ml TGFβ1, preferably 0.2-9 ng/ml, more preferably 0.3-8 ng/ml, even more preferably 0.4-7 ng/ml, still more preferably 0.5-6 ng/ml, more preferably 0.6-5 ng/ml, more preferably 0.7-4 ng/ml, more preferably 0.8-3 ng/ml, most preferably 0.9-2 ng/ml, and even most preferably about 1 ng/ml TGFβ1.
32. 32. The method of any one of embodiment 1-31, wherein the basal medium of step (iii) does not comprise an effective amount of FGF2.
33. 33. The method of any one of embodiment 1-32, wherein the basal medium of step (iii) comprises 0.5-3 mM Ca^{2+}, preferably 0.5-2.75 mM Ca^{2+}, more preferably 1-2.25 mM Ca^{2+}, even more preferably 1-1.5 mM mM Ca^{2+}, and most preferably about 1.2 mM Ca^{2+}.
34. 34. The method of any one of embodiment 1-33, wherein the mechanical stimulation in step (iii) is dynamic mechanical stimulation or static stretch.
35. 35. The method of embodiment 34, wherein the mechanical stimulation in step (iii) is dynamic mechanical stimulation.
36. 36. The method of any one of embodiment 1-35, comprising prior to step (i) a seeding step, wherein said pluripotent stem cells are seeded in a ratio of (2.5-6 x 10^6 cells / 1 mg collagen) / 1 ml medium in a suitable mould.
37. 37. The method of embodiment 36, wherein the collagen is collagen I.
38. 38. The method of any one of embodiment 36-37, wherein said collagen is of human origin, bovine origin, or marine origin.
39. 39. The method of any one of embodiment 36-38, wherein the medium used in the seeding step further comprises a ROCK-inhibitor.
40. 40. The method of embodiment 39, wherein the ROCK inhibitor is selected from Y27632, H-1152P, Thiazovivin, Fasudil, Hydroxyfasudil, GSK429286A, and RKI-1447, preferably selected from Y27632, H-1152P, Thiazovivin, Fasudil, Hydroxyfasudil, and more preferably the ROCK inhibitor is Y27632 or H-1152P, and most preferably the ROCK inhibitor is Y27632.
41. 41. The method of embodiment 40, wherein the medium used in the seeding step comprises 1-50 µM, preferably 2.5-40 µM, more preferably 5-30 µM, even more preferably 7.5-20 µM, most preferably 8-12 µM, and most preferably about 10 µM Y27632.
42. 42. The method of any one of embodiment 36-41, wherein the seeding step is carried out 18-30 h prior to step (i).

DESCRIPTION OF THE FIGURES

[0066]

Figure 1: Optimization of early cardiac differentiation for robust and efficient cardiac differentiation. (A) Schematic of the developed cardiac differentiation protocol. (B,C) Effect of FGF-2 addition on cardiac differentiation in 2D culture. (D,E) Effect of varying BMP4 concentration on cardiac differentiation in 2D culture whilst CHIR is present. (F,G) Effect of removing each factor individually from the 2D cardiac differentiation protocol, all factors were added daily from days 0-3 except IWP4 which was added every 2-3 days from days 3-13. (H,I,J) Assay for the presence of contaminating cell types using qPCR. (K) Immuno-staining for cardiomyocyte markers. (L) Flow cytometry for cardiomyocytes (n = 6 experiments). (M) Immuno-staining for stromal cell markers. (N) Flow cytometry for stromal cells (n = 6 experiments). All data is n = 3 experiments unless otherwise noted. qPCR data (MESP-1, OCT4, SOX17 and NEUROD1) is normalized to GAPDH. * Indicates statistically significant difference ($P < 0.05$) using ANOVA with Tukey's Multiple Comparison Post Hoc test. ** Indicates statistically significant difference from samples supplemented with no factors. *** Indicates statistically significant difference from samples supplemented with no factors, all factors minus BMP4, all factors minus ACT-A and all factors minus IWP-4.

Figure 2: BHM can be formed directly from hPSC. (A) BHM at 22 days of differentiation. (B) Whole-mount immunostaining. (C) Isometric twitch tension (force of contraction) in response to varying calcium concentration, n = 7 from 4 experiments. (D) Flow cytometry profiles of pluripotency (TRA-1-60/OCT4) and cardiac markers (a-actinin), n = 3-4 experiments. (E) Flow cytometry of stromal cell markers at day 22, n = 3 experiments. (F) qPCR expression profiles of markers for pluripotency, mesodermal differentiation, and cardiac differentiation; data is normalized to GAPDH expression, n = 3 experiments. * Indicates statistically significant difference ($P < 0.05$) compared to -1 days using ANOVA with Tukey's Multiple Comparison Post Hoc test.

Figure 3: Optimization of BHM culture condition reveals different parameters respond specifically to different stimuli. (A) ASC-2-P supplementation improves BHM, isometric twitch tension (force of contraction) in response to varying calcium concentration, n =8-9 from 3 experiments, * indicates statistically significant difference ($P < 0.05$) compared to control using two-way ANOVA with Bonferroni post hoc tests. (B) Flow cytometry analysis of ASC-2-P experiments for cardiomyocyte and stromal cell markers, n = 7-8 from 3 experiments. (C) Mechanical stimulation improves BHM function, isometric twitch tension (force of contraction) in response to varying calcium concentration, n =9-11 from 4 experiments, * indicates statistically significant difference ($P < 0.05$) for both mechanical stimulation regimes compared to control using two-way ANOVA Bonferroni post hoc tests. (D) Mechanical stimulation devices. (E) Whole-mount immuno-staining of BHM under control and mechanical stimulation regimes. (F) Growth factors (FGF2:10 ng/mL and TGFb1: 1 ng/mL) added during cardiac maturation regulate BHM function, isometric twitch tension (force of contraction) in response to varying calcium concentration, n =9-11 from 4 experiments. (G) Cardiomyocyte cell size analysis for the growth factor experiments using flow cytometry, n = 6 from 3 experiments, * indicates statistically significant difference ($P < 0.05$) compared to control using Student's t-test. (H) qPCR expression of β-MHC/α-MHC ratio for the growth factor experiments, n = 3-6 experiments, * indicates statistically significant difference ($P < 0.05$) compared to control using ANOVA with Tukey's Multiple Comparison Post Hoc test. (I) qPCR expression of ANP and Sk Act for the growth factor experiments, n = 3 experiments. (J)

Adjusting calcium to 1.2 mmol/L during cariac maturation improves BHM function, isometric twitch tension (force of contraction) in response to varying calcium concentration, n =10-11 from 4 experiments, * indicates statistically significant difference ($P < 0.05$) compared to control using two-way ANOVA Bonferroni post hoc tests. (K) resting tension of BHM for the calcium experiments, n =10-11 from 4 experiments. (L) Elastic modulus of BHM for the calcium experiments, n =10-11 from 4 experiments, * indicates statistically significant difference ($P < 0.05$) compared to control using ANOVA with Tukey's Multiple Comparison Post Hoc test.

Figure 4: BHM produced using the optimized protocol exhibit in-vivo-like properties. (A) BHM can be paced electrically at various rates. (B) BHM respond with increased twitch tension (force of contraction) to increased length (Frank-Starling mechanism). (C) Twitch tension (force of contraction) comparison of 22 day old (from previous data) and 29-30 day old BHM, n = 11 from 4 experiments for 22 days and n = 7 from 2 experiments for 29-30 days. (D) Inotropic response of 22 day old BHM to isoprenaline (1 µmol/L) under paced conditions at 0.6 mM calcium. (E) Inotropic response of 29-30 day old BHM to isoprenaline (1 µmol/L) under paced conditions at 0.6 mM calcium. (F) Comparison of inotropic response to isoprenaline (1 µmol/L) with age, n = 11 from 4 experiments for 22 days and n = 7 from 2 experiments for 29-30 days, * indicates statistically significant difference ($p <0.05$) using Student's t-test.

Figure 5: The BHM protocol can be used in all tested PSC lines. (A,B,C) Data from HES3-BHM; (D,E,F) Data from hIPS-G1-BHM.. (A,D) Isometric twitch tension (force of contraction) in response to varying calcium concentration, n = 4 for each line. (B,E) Whole-mount immuno-staining. (C,F) Flow cytometry analysis of cardimoycytes and stromal cells, n = 3 per line.

Figure 6: 2D cardiac differentiation of multiple hPSC lines. Flow cytometry analysis of cardiac (α-actinin, SIRPA) and stromal cell markers (PDGFRα, α-SMA, collagen I).

Figure 7: The BHM can be used to model developmental processes, using BMP signalling inhibition as an example. (A) qPCR analysis of multiple markers at day 13 of BHM formation. (B) Cell cycle analysis using flow cytometry gating on α-actinin+ cells. (C) Cardiomyocyte number per BHM. (D) Isometric twitch tension (force of contraction) in response to varying calcium concentrations. * indicates statistically significant difference ($P < 0.05$) compared to control using A+B) Student's t-test (n = 3-4) or D) two-way ANOVA Sidak's Multiple Comparison post test.

Figure 8: Summary of protocols used for 2D cardiac differentiation and BHM formation. (A) Protocol used for experiments shown in Figure 1. (B) Protocol used for experiments shown in Figure 2. (C) Protocol used for experiments shown in Figure 3 - addition of ascorbic acid. (D) Protocol used for experiments shown in Figure 3 - mechanical stimulation and growth factors. (E) Protocol used for experiments shown in Figure 3 - addition of calcium. (F) Protocol used for experiments shown in Figure 5. (G) Protocol used for experiments shown in Figure 6. (H) Protocol used for experiments shown in Figure 7.

Figure 9: Custom-made supplement to replace B27®. (A) Contractile force of BHMs (hES2) made with B27® or custom-made supplement (CMS) at 2 mM extracellular calcium, n=2/group. (B) Total CM number in BHM made with B27® or custom-made supplement (CMS), n=2/group.

The following examples are meant to further illustrate, but not limit the invention. The examples comprise various technical features, and it will be appreciated that the invention also relates to combinations of the technical features presented in this exemplifying section.

Figure 10: Supplementation of the culture medium with TGFG-1 during the cardiac maturation phase enhances contractile function of BHM (FOC: force of contraction) in a concentration dependent manner (0.3-10 ng/ml tested; n=5-7BHMs/condition).

EXAMPLES

Cardiac Differentiation Requires Optimization of Early Cardiac Mesoderm Induction

[0067] It has been demonstrated that non-myocyte cell fractions or stromal cells are essential for the function of engineered heart tissues. For this reason a cardiac differentiation protocol was firstly required which consistently produced cardiomyocytes and fibroblasts/stromal cells. The inventors optimized their cardiac differentiation protocol (**Fig. 1a**) for both yield and consistency, based on a previously published serum-free 2D hPSC differentiation protocol (Hudson et al. Stem Cells Dev 21, 1513-1523 (2012)). It was reasoned that robustness and efficiency could be enhanced if WNT activity was stabilized during the mesoderm induction phase. As surrogate marker for mesoderm induction *MESP1* expression was analysed by qPCR on culture day 3; this was followed by flow cytometry for α-actinin (cardiomyocyte marker) at day 16, which were found correlated very well with the amount of beating activity. The most important steps for the progression from the previously published protocol to the new protocol are summarised in **Fig. 1.**

[0068] The earlier protocol used cardiac mesoderm induction with BMP4 and Activin-A for the first 3 days followed by cardiac specification using a WNT inhibitor IWP4 (Hudson et al Stem Cells Dev 21, 1513-1523 (2012)). Consistent with the essential requirement of FGF2 for early mesoderm formation in recent studies using hPSCs and *in vivo* development, the addition of 5 ng/ml FGF2 in during the first 3 days of differentiation resulted in a trend to increased *MESP1* expression (**Fig. 1b**) and subsequently a trend to increased α-actinin (**Fig. 1c**). However, it was enforced WNT signalling that helped improve consistency and differentiation, consistent with its essential role in induction of early mesoderm. To enforce WNT signalling CHIR99021 was used, a small molecule inhibitor of GSK3β which induces WNT signalling even in the presence of canonical WNT inhibitors. CHIR alone or with the differentiation factors used in **Fig. 1b,c** was unable to induce any beating activity. When the BMP4 concentration was varied, optimal and consistent induction of *MESP1* (**Fig. 1d**) and α-actinin expression (**Fig. 1e**) was found at a BMP4 concentration of 5 ng/ml. Each factor from the differentiation protocol was then individually removed to demonstrate its requirement in the efficient and consistent induction of *MESP1* (**Fig. 1f**) and subsequently α-actinin expression (**Fig. 1g**).

[0069] In order to form BHM it is also important that stromal cell populations are present. It was therefore investigated whether stromal cells or other potentially contaminating cell types were present in the optimized differentiation protocol. Very low levels of potentially contaminating cell populations were found, analysed using qPCR for hPSCs (*OCT4* also known as *P0U5F1*) (**Fig. 1h**), endoderm (*SOX17*) (**Fig. 1i**), neural (*NEUROD1*) (**Fig. 1j**), and early mesoderm (*MESP1*) (data not shown). Very high expression of *NKX2-5* and *β-MHC* (also known as *MYH7*) were found in the cardiac differentiation culture of the invention compared to the other conditions (with no factors or without IWP4, data not shown). Additionally it was found that both cardiomyocytes (**Fig. 1k-l**) and different stromal cell types, including: α-smooth muscle actin positive cells (α-SMA$^+$), collagen I positive cells (COLI$^+$) cells, and α-SMA$^+$COLI$^+$ cells were present (**Fig. 1m,n**). Together this data suggests that the cardiac differentiation protocol of the invention efficiently produces cardiomyocytes with the rest of the cells being predominantly stromal cells, thus providing the required cell composition for tissue engineering applications.

Directed Formation of BHM

[0070] After optimization of the cardiac differentiation protocol the hypothesis was tested whether one could form BHM directly from hPSCs. The new serum-free cardiac differentiation protocol (**Fig. 1**) was used with an additional maturation step where the rings were removed from the molds and put onto static stretchers (+10% of slack length) in medium containing 5 ng/ml FGF2 and 200 µM ascorbate-2-phosphate (ASC-2-P). It was found that this protocol was effective in forming BHM (**Fig. 2a**). The BHM started to contract spontaneously in different areas by day 13 and by day 15-17 the contractions became synchronous and rhythmic, and continued until analysis at day 22 (data not shown). The cardiomyocytes in the BHM had an elongated and striated morphology (**Fig. 2b**) and the BHM could be electrically paced and had a measurable force of contraction with responsiveness to calcium concentration (**Fig. 2c**).

[0071] The development of the BHM followed known developmental pathways. The hPSCs were largely differentiated by day 3 indicated by a decrease in TRA-1-60$^+$/OCT4$^+$ cells and *OCT4* expression (**Fig. 2d,f**) and a concomitant expression of early mesodermal markers *MIXL1* and *MESP1* (**Fig. 2f**). By day 8 there was a loss of *MIXL1* and *MESP1* expression (**Fig. 2f**), with a concurrent increase in α-actinin$^+$ cells (**Fig. 2d**) and the expression of cardiomyocyte progenitor cell markers (**Fig. 2f**). There were peaks in the expression of multiple transcription factors involved in cardiogenesis, including: *TBX5* (peak at day 13), *ISL1* (peak at day 8), and *NKX2-5* (peak at day 13) (**Fig. 2f**). This was followed by the expression of more mature cardiac markers *α-MHC* (also known as *MYH6*), *β-MHC*, *ANP* (also known as *NPPA*), and *MLC2v* (also known as *MYL2*) (**Fig. 2f**). Interestingly, the expression of *α-MHC* peaked at day 13, followed by a large increase in *β-MHC* by day 22 and hence an increased *β-MHC*/*α-MHC* ratio (**Fig. 2f**). Furthermore, there was little expression of endodermal and neural markers at day 22 (**Fig. 2f**). Together this data suggests not only that the development of the BHM followed known developmental pathways, but also that cardiac maturation occurs as indicated by the drop in progenitor gene expression, increased *β-MHC* expression and *β-MHC*/*α-MHC* expression ratio, and increased expression of *MLC2v* (indicates maturity, see Tiburcy et al. Circ Res 109, 1105-1114 (2011)). Additionally, at day 22 it was found that the BHM was comprised of 30 ± 6% (n = 4 experiments) cardiomyocytes and a large proportion of stromal cells (**Fig. 2d**). Representative flow cytometry plots are shown in **Fig. 5.**

Optimization of BHM Functionality

[0072] While the BHM protocol outlined in **Fig. 2** represents the first entirely serum-free process of hPSC expansion and formation bioengineered myocardium, the inventors hypothesized that optimization could generate tissues with higher functionality and greater consistency. For these experiments contractile strength (twitch tension/force of contraction) was used as a primary determinant of function as twitch tension depends on a wide variety of myocardial properties including: cardiomyocyte number and phenotype, fibroblast number and phenotype, tissue connectivity, ECM composition, cell-cell connectivity and ECM-cell connectivity. As secondary factors the inventors used, 1) resting tension, because it reflects stroma cell function and extracellular matrix biology, 2) cardiomyocyte size, because of stimuli such as pharmacological stimuli, and 3) cell composition (cardiomyocytes:stroma cells), which is an important determinant for contractile performance. The parameters which changed are shown in **Fig. 3**.

ASC-2-P Enhances BHM Functionality

[0073] Ascorbate (vitamin C) plays a major role in the proper synthesis of collagen and is an antioxidant. It was therefore hypothesized that ascorbate (in the more stable form of ASC-2-P) supplemented during early BHM culture, days 0-13 (it was already added during days 13-22 in **Fig. 2**), would have a positive influence on BHM functionality given the importance of collagens during development. It was found that ASC-2-P significantly improved BHM twitch tension/force of contraction (**Fig. 3a**) and induced a trend to increased cardiomyocyte fraction with no change in the stromal cell fraction (**Fig. 3b**). It was also found that supplementation of ASC-2-P during the entire differentiation protocol improved differentiation in the inventor's 2D protocol by significantly increasing the number of cells without changes in cardiac differentiation efficiency (data not shown). Therefore, in both 2D and BHM formats, ASC-2-P may have increased cell survival and/or progenitor proliferation as proposed in a recent study (Cao et al. Cell Res 22, 219-236 (2012)).

BHM Function is Dependent on Mechanical Stimulation Regime

[0074] Next, it was assessed how static stretch and dynamic mechanical stimulation influenced BHM function. The devices used for static stretch and dynamic mechanical stimulation are shown in **Fig. 3d.** Both static stretch and dynamic mechanical stimulation significantly increased twitch tension/force of contraction of the BHM and both the mechanical stimulation regimes resulted in a similar BHM twitch tension (**Fig. 3c**). Both mechanical stimulation regimes improved the morphology of the cardiomyocytes in the BHM, causing compact, elongated and striated muscle bundles to form (**Fig. 3d**). Dynamic mechanical stimulation were preferred to static stretch, because it facilitates auxotonic contractions (Zimmermann et al, Nat Med 12, 452-458 (2006)).

Cardiomyocyte Properties are Dependant on Exogenous Growth Factors

[0075] There was a trend to decreased twitch tension/force of contraction when adding FGF2 and a trend to increased twitch tension when adding TGFβ1 (**Fig. 3f**). Therefore, in the previous experiments where FGF2 was added during cardiac maturation, this may have actually been detrimental BHM function. It was found that both FGF2 and TGFβ1 induced an increase in cardiomyocyte size (**Fig. 3g**). The addition of TGFβ1 resulted in a more mature *β-MHC/α-MHC* expression ratio (human heart = 9) (**Fig. 3h**) while pathological hypertrophy markers *ANP* (also known as *NPPA*) was decreased (**Fig. 3i**). The addition of FGF2 did not change *β-MHC/α-MHC* expression ratio (**Fig. 3h**), but induced (variably) pathological hypertrophy marker *ANP* (**Fig. 3i**). Together this indicates both factors induce hypertrophy, which is consistent with *in vivo* results. However, FGF2 may be considered as an inducer of pathological hypertrophy, while TGFβ1 may be considered as an inducer of physiological hypertrophy.

[0076] In a further set of experiments the inventors investigated if supplementation of the culture medium with increasing TGFB-1 during cardiac maturation phase has an influence in contractile function of BHM. The inventors observed an enhancement of contractile function of BHM in a concentration dependent manner (**Fig. 10**).

[0077] In the earlier experiments the inventors found a large reduction in α-smooth muscle actin and collagen I positive cells in the BHM when compared with the 2D protocol (**Fig. 1n** vs. **Fig. 2e**). This may be a reflection of subtle differences in myofibro-blast/fibroblast differentiation in 2D and BHM cultures. To more homogeneously detect cardiac-fibroblast-like populations in hPSC-derived 2D and BHM cardiac differentiation cultures antibodies against the canonical CD90 (also known as THY1) were employed in subsequent experiments.

Adjusting Extracellular Calcium to Physiological concentrations Improved BHM Function

[0078] Calcium concentration is tightly regulated in human serum with physiological calcium concentrations of 2.25-2.75 mM and 1.0-1.2 mM, for total calcium and ionized calcium, respectively. Because the concentration of calcium in RPMI medium is quite low (0.42 mM) compared to physiological calcium it was therefore assessed whether adjustment of free calcium concentration improved both BHM maturation and functionality. Adjustment of calcium to 1.2 mM (using a 0.2 M $CaCl_2$ solution) greatly increased the twitch tension of the BHM (**Fig. 3j**). Moreover, an increase in resting tension (**Fig. 3k**) and elastic modulus (**Fig. 3l**) was observed. Optimal elastic modulus has been demonstrated to improve the mechanical work done by cardiomyocytes during contractions which may increase contraction force. The mechanism behind the increased elastic modulus in response to increased calcium is currently unknown. Importantly, it should be noted that there were no changes in calcium handling proteins that were assessed using qPCR (*CASQ2, PLN, ATP2A2,* and *RYR2,* data not shown, n = 3 experiments).

[0079] BHM Produced Using the Optimized Protocol Display *In-Vivo* Like Properties BHM contacted spontaneously and coherently, and could be paced electrically at multiple frequencies up to at least 3 Hz (**Fig. 4a**), covering the range of beating frequency observed during heart development. BHM also increased twitch tension (force of contraction) in response to increased resting length (and resting tension) consistent with the Frank-Starling mechanism (**Fig. 4b**). With increased culture time no change in BHM twitch tension compared to the previous data was found, but an increase in calcium EC_{50} from 0.2 to 0.7 mmol/L was observed (**Fig. 4c**). Extended culture also resulted in an increased inotropic response to 1 μM isoprenaline indicating improved maturity over traditional culture formats where no increase has been observed (**Fig. 4d-f**). Using whole-mount immunostaining, it was found that the BHM also has both stromal cells and endothelial cells present in the muscle bundles (data not shown). Importantly BHM culture could also be maintained for long term (until at least day 63) under the maturation conditions with an observed improvement in morphological appearance (data not shown).

[0080] The Unmodified BHM Protocol Works on Multiple Human Pluripotent Stem Cell Lines Next, it was demonstrated that the optimized BHM (**Fig. 5**) and 2D (**Fig. 6**) protocols work on multiple hPSC lines. For these analyses HES2, HES3 and hIPS-GI lines (reprogrammed dental fibroblasts using vector-free Cytotune reprogramming kit) were used. It is important to note that for both the 2D and BHM protocols it was found that modification of the seeding cell number or the use of a Rho-associated protein kinase inhibitor (10 μM, Y-27632) was required to achieve similar cell densities in all lines after the initial 24 h seeding phase (data not shown). When using the required seeding protocol for a particular cell line the 2D and BHM protocols could be used unmodified for those lines.

[0081] It was found that both the HES3 line and the hIPS line produced BHM with a lower twitch tension (**Fig. 5a,d**) compared to the HES2 line (**Fig. 3j**). However, both the HES3 and hIPS BHM had similar morphologies (**Fig. 5b,e**). The cardiomyocyte fractions for the HES3 BHM was similar (**Fig. 5c**) and for the hIPS BHM was lower (**Fig. 5f**), compared to the HES2 BHM. The decreased functionality in the BHM from these lines is most likely from a lower cell number in the HES3 BHM ($0.50 \pm 0.03 \times 10^6$ cells, n = 3) and hIPS BHM ($0.55 \pm 0.05 \times 10^6$ cells, n = 3), compared to the HES2 BHM ($0.74 \pm 0.13 \times 10^6$ cells, n = 6 from 3 experiments). Therefore, care must be taken to exclude differences caused by changes in cell number and composition when assessing different cell lines rather than different treatments on the same line.

[0082] BHM as a Developmental Model Reveals that BMP Signalling is Required for Terminal Differentiation of Human Cardiomyocytes

Inhibition of BMP signalling is embryonically lethal even when effects are restricted (or at least partially restricted) to the developing heart using CRE driven by various genes (for a review please see Kruithof et al. Differentiation 84, 89-102 (2012)). In these studies there have been multiple processes ascribed to BMP signalling including structural defects, myocardial properties including trabeculae structure and wall thickness, and cell phenotype including dysregulation of progenitor genes and reduced epithelial-to-mesenchymal transformation (EMT). In order to determine the effect of BMP signalling purely on myocardial development without systemic influences and anatomical limitations, BHM was therefore deemed to be a good model system.

In these experiments 2 μmol/L of the BMP receptor signalling inhibitor dorsomorphin were added with each medium exchange from day 6 onwards. At day 13 the dorsomorphin treated BHM failed to down regulate *ISL1* while the expression of other more mature cardiac markers *NKX2-5* and *α*-

MHC were not altered (**Fig. 7a**). When investigating EMT associated genes, it was found that there was no alteration in *CDH1, CHD2, SNAIL1* or *TGFβ2* expression indicating that EMT or factors regulating EMT were not altered in BHM after 13 days (**Fig. 7a**). Using flow cytometry after 22 days it was found that there were more cardiomyocytes in the active cell cycle in the dorsomorphin treated group (**Fig. 7b**). However, it was found that treatment with dorsomorphin did not alter the cardiomyocyte number (**Fig. 7c**). It was also found that the total cell number per BHM and the fractions of cardiomyocytes (α-actinin$^+$) and stromal cells (CD90$^+$) did not change in the dorsomorphin treated group (data not shown). Despite these similarities there was a large reduction in twitch tension/force of contraction to 47 % of the control group in the dorsomorphin treated BHM (**Fig. 7d**). Interestingly, there was neither a change in BHM responsiveness to isoprenaline nor a change in cardiomyocyte size in the dorsomorphin treated BHM (data not shown).

As increased cell cycle activity did not lead to increased cardiomyocyte number and reduced twitch tension, it was calculated whether oxygen concentration may limit cardiomyocyte number in the BHM. To determine if this was the case, mathematically modelled oxygen diffusion profiles were based on models reported in the literature and the parameters in the different BHM conditions (cell number, cardiomyocyte fraction and size). It was found that when using the parameters for the control BHM there was no hypoxic region, even if the number of cardiomyocytes increased to 125 % (data not shown).

Together the present data suggests that BMP inhibition using dorsomorphin results in an increased proliferative state, a result which is consistent with mouse *in viv*o experiments. However, there was no increase in cardiomyocyte number indicating that either there is increased apoptosis or the cardioymocytes are bi-nucleating. Regardless of the mechanism, inhibition of BMP signalling resulted reproducibly in a tissue phenotype that produces less contractile force (and also lower force per cardiomyocyte) and inferior myocardial tissue.

Custom-made supplement to replace B27®

[0083] BHMs were generated from undifferentiated hESC under serum-free conditions according to standard BHM protocol. The standard protocol includes B27® supplement. In this experiment B27® supplement was replaced by a defined, custom-made supplement (CMS, T**able 4**).

[0084] The results show that B27® can be replaced by CMS. The force is similar, also the number of cardiomyocytes generated within the BHM is comparable (**Fig. 9**).

Conclusion

[0085] Using directed differentiation of PSCs in collagen I hydrogels the present application demonstrates that it is possible to guide BHM assembly under serum-free conditions. BHM has multiple applications including pharmacological studies, study of developmental processes, heart maturation processes and also potential regenerative applications.

[0086] In these examples, the robustness of the newly developed protocol was demonstrated by using multiple culture formats and multiple lines for differentiation. It will however be noted that while the experiment-experiment differentiation efficiency was consistent, the efficiency did vary when different batches of reagents were used. It is therefore prudent to establish strict reagent quality control in order to produce BHM with consistent and defined properties for both *in vitr*o and potential therapeutic applications.

Methods

PSC Culture

[0087] HES2-ROSA26-RFP (Irion et al. Nat Biotechnol 25, 1477-1482 (2007)) cells were obtained from Gordon Keller and HES3 cells were obtained from Embryonic Stem Cell International (ESI, Singapore). hIPS were generated from human gingiva biopsy-derived fibroblasts using the Cytotune Reprogramming Kit (Applied Biosystems) following the manufacturer's instructions.

[0088] For IPS generation, 6 days after viral transduction fibroblasts were plated on irradiated mouse embryonic fibroblasts in fibroblast medium (DMEM high glucose, 2 mmol/L glutamine, 10% FBS (PAA), 100 IU/ml Penicillin, 100 µg/ml Streptomycin, all Gibco except where indicated). The next day the medium was exchanged to PSC-medium (Knock-out DMEM (Gibco) supplemented with 20% Knock-Out Serum Replacement (KSR, Gibco), 2 mmol/L glutamine, 100 IU/ml Penicillin, 100 µg/ml Streptomycin, 1% non-essential amino acids (Gibco), and 10 ng/mL FGF2 (Miltenyi Biotec)). Emerging iPS colonies were mechanically picked and expanded by weekly passaging using 1 mg/ml Collagenase NB6 (Cresent Chemical Company).

[0089] For experiments, hPSCs were single cell adapted and cultured on irradiated human foreskin fibroblasts (HFF) in PSC-medium with daily medium changes and weekly passaging using 3 min TrypLE (Gibco) treatment (Ellerstrom et al., Stem Cells 25, 1690-1696 (2007)). Before characterization or differentiation experiments, the hPSCs were plated on 1:30 Matrigel (Millipore) in PBS (Gibco) coated-plates, at 2.5 x 10^4 cells/cm^2 for HES2 or $5x10^4$ cells/cm^2 for HES3 and hIPS lines, and cultured for 3 days in 1:1 PSC-medium minus FGF-2 and HFF-conditioned medium (HFF-CM - harvested from 5 day confluent irradiated HFF cultures) with 10 ng/mL FGF2. The hIPS line also received 10 µmol/L Y-27632 (Stemgent). hPSCs were harvested for experiments by passaging using 3 min TrypLE treatment and then cultured in the appropriate format.

[0090] Pluripotent stem cell lines were regularly tested for mycoplasma using a test kit (Lonza) and characterized using standard assays. Pluripotency markers were assessed via PCR (endogenous *OCT4, SOX2, KLF4, MYC*), qPCR (*OCT4, NANOG, REX1, DNMT3B*) and immunostaining (OCT4, NANOG, TRA-1-60) (Chan et al. Nat Biotechnol 27, 1033-1037 (2009)). Demethylation of the OCT4 promoter was confirmed via bisulfite sequencing (Freberg et al. Mol Biol Cell 18, 1543-1553 (2007)). Karyotyping was used to determine if there were any genetic abnormalities (Campos et al. J Vis Exp 4 (2009)). Pluripotency was confirmed via teratoma formation in SCID mice via flank injection of 4-6 x 10^6 cells.

Differentiation Medium

[0091] For differentiation experiments the hPSCs were then cultured in RPMI 1640 supplemented with 1 mmol/L sodium pyruvate, 100 IU Penicillin, 100 µg/ml Streptomycin and 2% B27 supplement (SF medium, all Gibco) and various factors as indicated. Factors used in this study included: L-ascorbic acid 2 phosphate sesquimagnesium salt hydrate (Sigma), BMP4 (R&D Systems), Activin A (R&D Systems), FGF2 (Miltenyi Biotec), dorsomorphin (Stemgent), CHIR99021 (Stemgent), IWP4 (Stemgent), and TGFβ1 (Peprotech).

2D Cardiac Differentiation

[0092] Cardiac differentiation was optimized on the HES2 line. HES2 hPSCs were plated at $5x10^4$ cells/cm^2 ($1x10^5$ cells/cm^2 for HES3 and hIPS lines) on 1:30 Matrigel/PBS coated-plates and cultured in 1:1 PSC-medium minus FGF-2 and HFF-conditioned medium (HFF-CM - harvested from 5 day confluent irradiated HFF cultures) with 10 ng/mL FGF2. For the hIPS line 10 µM Y-27632 was added to this medium. After 1 day the cells were rinsed with RPMI medium, then differentiated as indicated in each figure with 0.5 ml of medium in each well of a 24 well plate. The protocol details for each figure are outlined in **Fig. 8**.

BHM formation

[0093] BHM formation was optimized on the HES2 line. HES2 hPSCs were suspended 1:1 in PSC-medium minus FGF-2 and HFF-conditioned medium (HFF-CM - harvested from 5 day confluent irradiated HFF cultures) with 10 ng/mL FGF2 and mixed with a collagen I hydrogel. For the HES3, and hIPS lines 10 µM Y-27632 was also added to the medium. The collagen I matrix was formulated with acid-soluabilized bovine collagen I (Devro) with an equi-volume of 2X DMEM (Gibco) and neutralized using 0.1 M sodium hydroxide. The hPSC/collagen I matrix was formulated to give a final collagen I concentration of 1 mg/ml and 5 x 10^5 hPSC per 170 µl. For the HES3 and hIPS lines 1 x 10^6 and 0.5 x 10^6 cells were used respectively per 170 µl. For each BHM, 170 µl of the hPSC/collagen I matrix was pipette into circular molds (i.d. = 4 mm, o.d. = 10 mm) fabricated using poly(dimethylsiloxane) (Sylgard, Dow Corning). After 10 min of culture in the incubator at 37°C the collagen gelled and 1.25 ml of 1:1 human foreskin fibroblast-conditioned medium with 10 ng/ml FGF2 was added per BHM. The following day the BHMs were rinsed with RPMI medium and then differentiated as indicated in each figure, with 1.25 ml of medium per BHM. At day 13 the BHM were transferred onto mechanical stimulators as indicated. The protocol details for each figure are outlined in **Fig. 8.**

Cell Disassociation

[0094] 2D cultures were disassociated by rinsing with PBS followed by incubation for 1 h in 1 mg/ml collagenase type I (Sigma) with 20% fetal bovine serum (FBS, Applied Biosystems) in PBS. The cells were then collected in a tube, rinsed with PBS and incubated with 0.25% Trypsin-EDTA (Applied Biosystems) for 5 min followed by rinsing with FBS containing medium.

[0095] For the initial BHM digestion protocol, BHMs were disassociated in 0.025 mg/ml Liberase TM (Roche), 30 mM 2,3-butanedione monoxime at 37 °C for 60 min in PBS. To preserve cell surface markers, BHM was disassociated using the same protocol as for the 2D digestion.

Quantitative PCR (qPCR)

[0096] Cells, BHMs or human heart biopsies were harvested and stored at -80 °C until RNA extraction using Trizol following manufacturer's instructions (Applied Biosystems). 1 µg of RNA was then treated with DNAse (Roche) followed by cDNA synthesis using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

[0097] qPCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) on a 384-well format AB7900 HT (Applied Biosystems). Gene expression was normalized using $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ using *GAPDH* as the housekeeping gene which we found to be consistently expressed between conditions in all of our experiments. Primer details are given in below **Table 1**.

Table 1

Gene (Acc #)	F	R	Purpose	Size (bp)
GAPDH (NM_002046.3)	CCTCAAGATCATCAGCAATGCC (SEQ ID NO: 1)	ATGTTCTGGAGAGCCCCGC (SEQ ID NO: 2)	qPCR RT-PCR	189
OCT4 (NM_002701, NM_203289, NM_001173531)	CAGTGCCCGAAACCCACAC (SEQ ID NO: 3)	GGAGACCCAGCAGCCTCAAA (SEQ ID NO: 4)	qPCR	161
NANOG (NM_024865)	CAGAAGGCCTCAGCACCTAC (SEQ ID NO: 5)	ATTGTTCCAGGTCTGGTTGC (SEQ ID NO: 6)	qPCR	111
REX1 (NM_174900.3)	CACCGCCTCCCTTGGGAATTCAG (SEQ ID NO: 7)	TGTTCTGTTCACACAGGCTCCAGC (SEQ ID NO: 8)	qPCR	83

Gene (Acc #)	F	R	Purpose	Size (bp)
DNMT3B (NM_175848.1 NM_175849.1 NM_006892.3)	GGCCCAAGTAAAC-	ATGCCTGGTGTCTCCCTT-	qPCR	168
	CTAGCTCGGC (SEQ ID NO: 9)	CATGC (SEQ IOD NO: 10)		
MIXL1 (NM_031944)	CCGAGTCCAG-GATCCAGGTA (SEQ ID NO: 11)	CTCTGACGCCGAGACTTGG (SEQ ID NO: 12)	qPCR	58
NKX2-5 (NM_001166175.1, NM_001166176.1, NM_004387.3)	ACAACTTCGT-GAACTTCGGCG (SEQ ID NO: 13)	GTGGACACTCCCGAGTT-GCTCT (SEQ ID NO: 14)	qPCR	82
TBX5 (NM_000192.3, NM_080717.2, NM_181486.1, NM_080718.1)	TCATAACCAAGGCTG-GAAGG (SEQ ID NO: 15)	GCCCGTCACAGAC-CATTTAT (SEQ ID NO: 16)	qPCR	152
ISL1 (NM_002202.2)	CGCCTTGCAGAGTGACATAG (SEQ ID NO: 17)	GGACTGGCTACCAT-GCTGTT (SEQ ID NO: 18)	qPCR	147
MYH6 (α-MHC) (NM_002471)	CTCCTCCTACGCAACTGCCG (SEQ ID NO: 19)	CGACACCGTCTGGAAG-GATGA (SEQ ID NO: 20)	qPCR	85
MYH7 (β-MHC) (NM_000257)	GACCAGATGAAT-GAGCACCG (SEQ ID NO: 21)	GGTGAGGTCGTT-GACAGAACG (SEQ ID NO: 22)	qPCR	63
MLC2v (NM_000432.3)	GGCGCCAACTCCAACGTGTT (SEQ ID NO: 23)	ACGTTCA-CTCGCCCAAGGGC (SEQ ID NO: 24)	qPCR	149
ACTA1 (Skeletal actin) (NM_001100.3)	ACCCAGATCATGTTT-GAGACC (SEQ ID NO: 25)	TCATAAATGGGCACGTT-GTG (SEQ ID NO: 26)	qPCR	143

Gene (Acc #)	F	R	Purpose	Size (bp)
NPPA (ANP) (NM_006172.3)	TCTGCCCTCCTAAAAAGCAA (SEQ ID NO: 27)	TGTCCTCCCTGGCTGTTATC (SEQ ID NO: 28)	qPCR	156
CASQ2 (NM_001232.3)	TCTTGCAGGGCAGAAGAGGGG (SEQ ID NO: 29)	GGACCTGGGCCACAAGCTCAA (SEQ ID NO: 30)	qPCR	205
NEUROD1 (NM_002500.3)	AGCCAC-GGATCAATCTTCTC (SEQ ID NO: 31)	GCGTGCCTCTAATCATGAAA (SEQ ID NO: 32)	qPCR	143
CDH1 (ECAD) (NM_004360.3)	GAACGCATT-GCCACATACAC (SEQ ID NO: 33)	ATTCGGGCTTGTT-GTCATTC (SEQ ID NO: 34)	qPCR	118
CDH2 (NCAD) (NM_001792.3)	CCTGGAACGCAGTG-TACAGA (SEQ ID NO: 35)	TGGTTTGACCACGGTGAC-TA (SEQ ID NO: 36)	qPCR	104
SNAIL1 (NM_005985.3)	AGCGAGCTGCAG-GACTCTAA (SEQ ID NO: 37)	GGACAGAG-TCCCAGATGAGC (SEQ ID NO: 38)	qPCR	136
MESP1^1 (NM_018670.3)	AGCCCAAGT-GACAAGGGACAACT (SEQ ID NO: 39)	AAGGAACCACTT-CGAAGGTGCTGA (SEQ ID NO: 40)	qPCR	82
SOX17^1 (NM_022454.3)	AGGAAATCCTCA-GACTCCTGGGTT (SEQ ID NO: 41)	CCCAAACTGTTCA-AGTGGCAGACA (SEQ ID NO: 42)	qPCR	111
ATP2A2 (SERCA2) (NM_001681.3 NM_170665,3)	ACCTCATCTCGTCCAACGTC (SEQ ID NO: 43)	TGTCACCAGATTGACCCAGA (SEQ ID NO: 44)	qPCR	110
PLN (NM_002667.3)	ACAGCTGCCAAGGCTACCTA (SEQ ID NO: 45)	TCCATGATACCAGCAGGACA (SEQ ID NO: 46)	qPCR	114
RYR2 (NM_001035.2)	TGCAAGACTCACCGAAGATG (SEQ ID NO: 47)	CCACCCAGACATTAGCAGGT (SEQ ID NO: 48)	qPCR	125

Gene (Acc #)	F	R	Purpose	Size (bp)
COL1A1 (NM_000088.3)	GTGCTAAAGGTGCCAATGGT (SEQ ID NO: 49)	ACCAGGTTCACCGCTGTTAC (SEQ ID NO: 50)	qPCR	128
COL3A1 (NM_000090.3)	CCAGGAGCTAACGGTCTCAG (SEQ ID NO: 51)	CAGGGTTTCCATCTCTTCCA (SEQ ID NO: 52)	qPCR	103
COL5A1 (NM_000093.3)	GACACCTCCAACTCCTCCAA (SEQ ID NO: 53)	AGTGAACTCCCCCTCCAAGT (SEQ ID NO: 54)	qPCR	72
COL4A1 (NM_001845.4)	GTTGGTCTACCGGGACTCAA (SEQ ID NO: 55)	GTTCACCTCTGATCCCCTGA (SEQ ID NO: 56)	qPCR	145
LAMC1 (NM_002293.3)	GTGAGAGGTGCCGAGAGAAC (SEQ ID NO: 57)	GTGCTTAGAGAGCCCACAGG (SEQ ID NO: 58)	qPCR	88
TGFB2 (NM_001135599.2, NM_003238.3)	CGAACCCAAAGGGTACAATG (SEQ ID NO: 59)	TAAGCTCAGGACCCTGCTGT (SEQ ID NO: 60)	qPCR	91
TTN Ex49-50 (ALL TTN)[2] (NM_001267550.1 NM_001256850.1 NM_133437.3 NM_133432.3 NM_003319.4)	GTAAAAAGAGCTGCCCCAGTGA (SEQ ID NO: 61)	GCTAGGTGGCCCAGTGCTACT (SEQ ID NO: 62)	qPCR	68
TTN Ex107-108 (N2BA)[2] (NM_001267550.1 NM_001256850.1 NM_133378.4)	CAGCAGAACTCAGAATCGA (SEQ ID NO: 63)	ATCAAAGGACACTTCACACTC (SEQ ID NO: 64)	qPCR	110
TTN Ex50-219 (N2B)[2] (NM_133437.3 NM_133432.3 NM_003319.4)	CCAATGAGTATGGCAGTGTCA (SEQ ID NO: 65)	TACGTTCCGGAAGTAATTTGC (SEQ ID NO: 66)	qPCR	93
endoOCT4[3] (NM_002701, NM_203289, NM_001173531)	CCTCACTTCACTGCACTGTA (SEQ ID NO: 67)	CAGGTTTTCTTTCCCTAGCT (SEQ ID NO: 68)	RT-PCR	164

Gene (Acc #)	F	R	Purpose	Size (bp)
endoSOX2[3] (NM_003106.3)	CCCAGCAGACTTCA-CATGT (SEQ ID NO: 69)	CCTCCCATTT-CCCTCGTTTT (SEQ ID NO: 70)	RT-PCR	151
endoKLF4[3] (NM_004235.4)	GATGAACTGACCA-GGCACTA (SEQ ID NO: 71)	GTGGGTCATA-TCCACTGTCT (SEQ ID NO: 72)	RT-PCR	145
endoMYC[3] (NM_002467.4)	TGCCTCAAATTG-GACTTTGG (SEQ ID NO: 73)	GATTGAAATTCTGTG-TAACTGC (SEQ ID NO: 74)	RT-PCR	192
SeV	GGATCACTAGGTGA-	ACCAGACAAGAGTT-	RT-PCR	181
	TATCGAGC (SEQ ID NO: 75)	TAAGAGATATGTATC (SEQ ID NO: 76)		
BS OCT4-2[4]	TTAGGAAAATGGG-TAGTAGGGATTT (SEQ ID NO: 77)	TAC-CCAAAAAAACAAATAAAT-TATAAAACCT (SEQ ID NO: 78)	BS	296
BS OCT4-4[4]	GGATGTTATTAAGAT-GAAGATAGTTGG (SEQ ID NO: 79)	CCTAAACTCCCCTTCA-AAATCTATT (SEQ ID NO: 80)	BS	406

1. Kattman, et al. Cell Stem Cell 8, 228-240 (2011).
2. Neagoe, et al. Circulation 106, 1333-1341 (2002).
3. Park, et al. Nature 451, 141-146 (2008).
4. Freberg, et al. Mol Biol Cell 18, 1543-1553 (2007).

Immunostaining

[0098] Digested cardiac differentiation cells were plated on 0.1% gelatin coated glass co-verslips for 24 h in 20% FBS (Gibco) in RPMI 1640 supplemented with 1 mmol/L sodium pyruvate, 100 IU/ml Penicillin and 100 µg/ml Streptomycin. The cells were then fixed in Histofix (Roti) for 10 min at room temperature. The cells were then blocked for 30 min in 5% FBS, 1% bovine serum albumin (Sigma) and 0.5% Triton X-100 (Sigma) in PBS (blocking buffer). The cells were then stained with primary antibodies in blocking buffer for 90 min followed by secondary antibodies in blocking buffer and Hoechst for 60 min at room temperature (**Table 2**). Stained cells were imaged using a Zeiss 710 confocal microscope.

Table 2 Antibodies and stains (FC - Flow cytometry, IF - Immunofluorescence)

Antibody/Stain	Supplier	Cat No.	Marker	Dilution
IgG_1	RnD Systems	MAB002	Control	1:100 FC
				1:100 IF
TRA-1-60-FITC (mouse)	BD Pharmingen	560173	Pluripotency	1:100 FC
				1:100 IF
OCT3/4 (rabbit)	Santa Cruz Biotechnology	sc-9081	Pluripotency	1:200 FC
				1:200 IF
NANOG (goat)	RnD Systems	AF1997	Pluripotency	1:20 IF
α-smooth muscle actin (mouse)	Sigma	A2547	Stromal cell (used predominately in this study)	1:4000 FC
				1:400 IF
α-smooth muscle actin (rabbit)	Abcam	ab32575	Stromal cell (used only with α-actinin co-staining)	1:50 FC
Collagen I (rabbit)	Abcam	ab34710	Stromal cell	1:2000 FC
				1:500 IF
NKX2-5 (rabbit)	Santa Cruz Biotechnology	H-114	Cardiomyocyte/cardiac progenitor	1:200 IF
α-actinin (mouse)	Sigma	A7811	Cardiomyocyte	1:4000 FC
				1:1000 IF
Cardiac Troponin I (mouse)	Millipore	MAB1691	Cardiomyocyte	1:200 IF
SIRPA (mouse)	Biolegend	323802	Cardiomyocyte	1:100 FC
PDGFRα (mouse)	RnD Systems	MAB1264	Mesodermal progenitor	1:25 FC
CD90 (mouse)	RnD Systems	MAB2067	Stromal cell	1:125 IF
				1:500 FC
Donkey anti-goat Alexafluor 546	Invitrogen	A-11056	NA	1:400 IF
Goat anti-mouse Alexafluor 488	Invitrogen	A-11001	NA	1:1000 FC
				1:400 IF
Goat anti-rabbit	Invitrogen	A-11010	NA	1:1000

Antibody/Stain	Supplier	Cat No.	Marker	Dilution
Alexafluor 546				FC
				1:400 IF
Phalloidin Alexafluor 546	Invitrogen	A22283	NA	1:50 IF
Hoechst33342	Invitrogen	H3570	NA	1:1000 FC
				1:1000 IF

Whole-Mount Immuno-Staining

[0099] BHMs were fixed in Histofix for 2-4 h at 4°C. The BHMs were then stained with primary antibodies for 2-3 days followed by secondary antibodies and Phalloidin 546/Hoechst for 2-3 days at 4°C (**Table 2**). Stained BHMs were imaged using a Zeiss 710 confocal microscope.

Flow Cytometry

[0100] Cells were stained either live or fixed using Histofix for 10 min at room temperature or ethanol. The cells were stained in 5% FBS in PBS (membrane blocking buffer) for cell surface markers (excluding TRA-1-60) and blocking buffer for internal markers. The cells were then stained with primary antibodies in blocking buffer for 45 min followed by secondary antibodies in blocking buffer and Hoechst for 30 min at 4°C (**Table 2**). A BD LSRII was used for flow cytometry analysis (BD Biosystems). Live cells populations were gated based on forward-side scatter profiles; fixed cells populations were gated based on Hoechst staining. BD FACSDiva Software (BD Bioscience) or Cyflologic v1.2.1 (Cyflo Ltd) were used for analysis.

Contraction Measurements

[0101] Contraction experiments were performed in organ baths at 37°C and continuous bubbling with 5% CO_2/95% O_2 to maintain a physiological pH in Tyrode's solution containing (all in mM): 120 NaCl, 1 $MgCl_2$, 0.2 $CaCl_2$, 5.4 KCl, 22.6 $NaHCO_3$, 4.2 NaH_2PO_4, 5.6 glucose and 0.56 ascorbate. Calcium was adjusted using a 0.2 M calcium chloride solution.. All BHM were first analysed at 3 Hz with 5 ms square pulses of 200 mA in order to pace at approximately the embryonic heart rate. BHM were mechanically stretched at intervals of 125 µm until Lmax, i.e. the tissue length were maximum twitch tension/force of contraction was recorded in the presence of maxi-mally inotropically active calcium concentrations (2 mmol/L; Frank-Starling mechanism). Subsequently, BHM were subjected to different calcium concentrations (0.2, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4 mM) and the twitch force recorded. For isoprenaline experiments the calcium concentration was adjusted to 0.6 mM and subsequently the isoprenaline concentration was adjusted to 1 µM.

Oxygen Diffusion Profile

[0102] The oxygen diffusion profile was generated using numerical analysis of a pseudo-steady-state approximation of cylinder diffusion with oxygen consumption dependence on concentration (**Eq. 1**). Parameters from the literature (Brown et al. Biotechnol Bioeng 97, 962-075 (2007)) and as determined in previous experiments were used (**Table 3**). Numerical analysis and plotting was performed using MATLAB V12 (Mathworks) using the solver bvp4c and the Singular Term option.

Eq. 1 $$0 = \frac{-D_{O2}}{r}\left[r\frac{dC_{O2}}{dr}\right] + V_{\max}\rho_{cardiomyocyte}e^{\left(\frac{-\alpha}{C_{O2}}\right)}$$

[0103] C_{O2} - oxygen concentration as a function of radial position, r - radial position in cylinder, D_{O2} - oxygen diffusion constant, V_{max} - maximal oxygen generation rate by cardiomyocytes, $\rho_{cardiomyocyte}$ - density of cardiomyocytes, α - constant for oxygen generation rate dependence on oxygen concentration.

Table 3: Oxygen diffusion model parameters

Model Parameter	Value
D_{O2}	2 x 10^5 cm^2/s
$P_{cardiomyocyte}$	$\rho_{cardiomyoc\ yte} = \frac{CellNumber \cdot Cardiomyoc\ ytePurity}{BHMVolume}$
V_{max}	-5.44x10^{-8} nmol/cell/s
α	Calculated to fit oxygen consumption-concentration relationship from [5]; 1.14 µM
Bounding Conditions	
At r = 0 ; $\frac{dC_{O2}}{dr} = 0$	
At r = R ; C_{O2} = C*	R =0.5 mm ; C* = 61 µM

5. Brown, et al. Biotechnol Bioeng 97, 962-975 (2007).

Statistical Analysis

[0104] All data is presented as mean ± s.e.m. Appropriate statistical analyses were used for each data set as indicated in the Figure legends using Graph Pad Prism or Microsoft Excel.

Custom-made supplement to replace B27®

[0105]

Table 4: Custom-made supplement (CMS) to replace B27.

Substance	Final concentration	25x	Supplier
Albumin	5 mg/ml	125 mg/ml	Sigma, A9511
Transferrin	10 µg/ml	250 µg/ml	Sigma, T0665
EthanolamineHCl	2 µg/ml	50 µg/ml	Sigma, E6133
Sodium selenite	0.032 µg/ml	0.8 µg/ml	Sigma, S5361
L-CarnitineHCl	4 µg/ml	100 µg/ml	Sigma, C0283
Hydrocortisone	1 µg/ml	25 µg/ml	Sigma, H2270
Fatty acid supplement	0.5 µl/ml	12.5 µl/ml	Sigma, F7050
Triiodo-L-thyronine	0.004 µg/ml	0.1 µg/ml	Sigma, T 6397
Prepare 25x in cell culture-qualified water.			

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references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

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Patentkrav

1. Fremgangsmåde til at fremstille biomodificeret hjertemuskel fra pluripotente stamceller, omfattende trinnene

(i) dyrkning af pluripotente stamceller i et basalmedium omfattende en effektiv mængde af (a) BMP4, activin A, FGF2, en GSK3-inhibitor, og (b) et serum-frit supplement som resulterer i en slutkoncentration på 0,5-50 mg/ml albumin, 1-100 µg/ml transferrin, 0,1-10 µg/ml ethanolamin, 0,003-0,3 µg/ml natriumselenit, 0,4-40 µg/ml L-carnitin HCl, 0,1-10 µg/ml hydrocortison, 0,05-5 µl/ml fedtsyresupplement, 0,0001-0,1 µg/ml triodo-L-thyronin (T3), for derved at inducere mesoderm differentiering af de pluripotente stamceller;

(ii) dyrkning af cellerne opnået i trin (i) i et basalmedium omfattende en effektiv mængde af en inhibitor af Wnt-signalvejen og et serum-frit supplement som i (i), for derved at inducere hjertedifferentiering af cellerne; og

(iii) dyrkning af cellerne opnået i trin (ii) i et basalmedium omfattende en effektiv mængde af et serum-frit supplement som i (i), under mekanisk stimulering, for derved at fremme hjertemodning.

2. Fremgangsmåde ifølge krav 1, hvor de pluripotente stamceller er valgt fra forster stamceller, inducerede pluripotente stamceller, og partenogenetiske stamceller; og/eller hvor de pluripotente stamceller er pluripotente stamceller af primatoprindelse, fortrinsvis humane pluripotente stamceller.

3. Fremgangsmåde ifølge et hvilket som helst af kravene 1-2, hvor basalmediet i trin (i) omfatter

1-20 ng/ml BMP4, fortrinsvis ca. 5 ng/ml BMP4; og
0,1-10 ng/ml FGF2, fortrinsvis ca. 5 ng/ml FGF2; og
1-20 ng/ml activin A, fortrinsvis ca. 9 ng/ml activin A.

4. Fremgangsmåde ifølge et hvilket som helst af kravene 1-3, hvor GSK3-inhibitoren i basalmediet fra trin (i) er valgt fra gruppen bestående af CHIR99021, CHIR98014, SB216763, TWS119, tideglusib, SB415286, og LY2090314; fortrinsvis hvor basalmediet i trin (i) omfatter 0,1-10 µM CHIR99021.

5. Fremgangsmåde ifølge krav 1, hvor inhibitoren af Wnt-signalvejen i basalmediet fra trin (ii) er valgt fra gruppen bestående af IWP4, IWP2, IWR-1, IWP1, IWP3, IWR-2, IWR-3, IWR-4, IWR-5, XAV939, DKK1, quercetin, ICG-001, pyrvinium, CCT031374, iCRT-3,5,14, CPG049090, NC043; fortrinsvis hvor basalmediet fra trin (ii) omfatter 0,1-10 µM IWP4.

6. Fremgangsmåde ifølge et hvilket som helst af kravene 1-5, hvor basalmediet fra trin (iii)

a) endvidere omfatter 0,1-10 ng/ml TGFβ1, mest fortrinsvis ca. 1 ng/ml TGFβ1; og/eller

b) ikke omfatter en effektiv mængde FGF2; og/eller

c) omfatter 0,5-3 mM Ca^{2+}, mest fortrinsvis ca. 1,2 mM Ca^{2+}.

7. Fremgangsmåde ifølge et hvilket som helst af kravene 1-6, hvor den mekaniske stimulering i trin (iii) er dynamisk mekanisk stimulering eller statisk strækning, fortrinsvis hvor den mekaniske stimulering i trin (iii) er dynamisk mekanisk stimulering.

8. Fremgangsmåde ifølge et hvilket som helst af kravene 1-7, hvor basalmediet fra trin (i), (ii) og/eller (iii) omfatter 10-1000 µM, fortrinsvis 50-400 µM, mere fortrinsvis 100-300 µM, endnu mere fortrinsvis 150-250 µM, og mest fortrinsvis ca. 200 µM ascorbinsyre eller et salt deraf.

9. Fremgangsmåde ifølge et hvilket som helst af kravene 1-8, hvor det serum-frie supplement i trin (i), (ii) og/eller (iii) omfatter 0,1-10 % B27 eller B27 uden insulin, fortrinsvis 0,5-8 %, mere fortrinsvis 1-6 %, endnu mere fortrinsvis 1,5-4%, og mest fortrinsvis ca. 2% B27 eller B27 uden insulin.

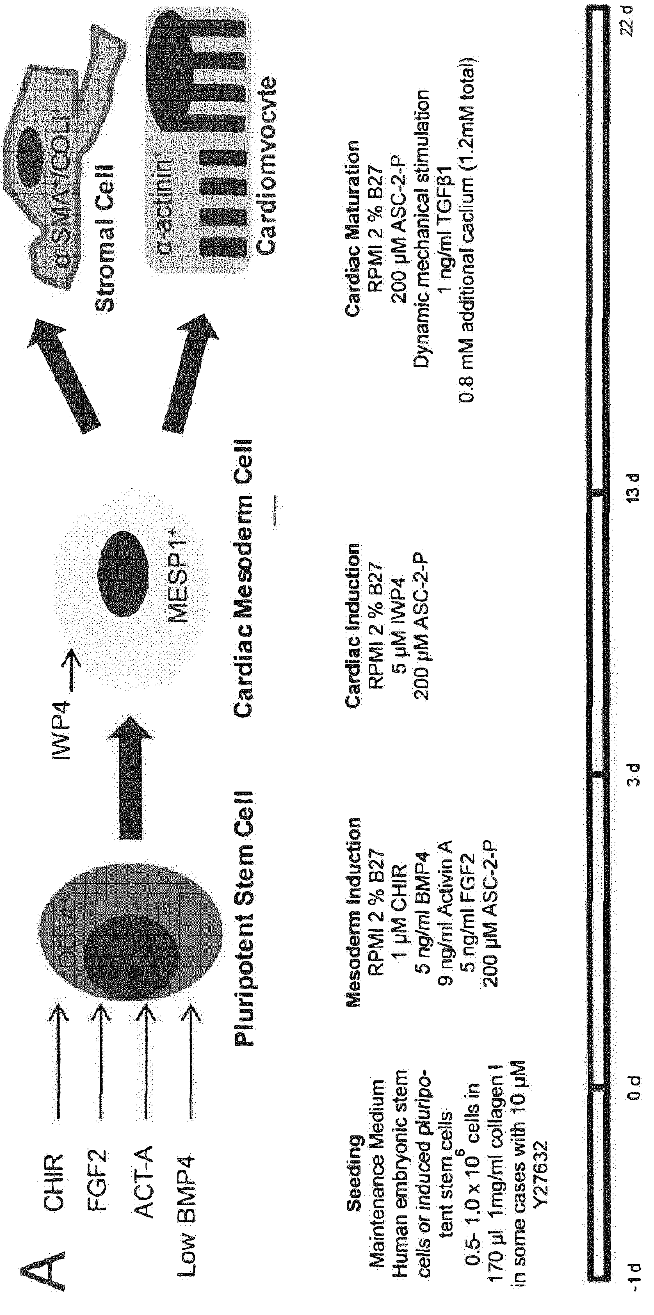
10. Fremgangsmåde ifølge et hvilket som helst af kravene 1-9, hvor basalmediet anvendt i trin (i), (ii) og/eller (iii) er DMEM/F12, StemPro, Iscoves medium, αMEM, DMEM, og RPMI, fortrinsvis hvor basalmediet anvendt i trin (i), (ii) og/eller (iii) er RPMI suppleret med pyruvat.

11. Fremgangsmåde ifølge krav 1, omfattende forud for trin (i) et podningstrin, hvor de pluripotente stamceller podes i et forhold på (2,5-6 x 10^6 celler / 1 mg kollagen) / 1 ml medium i en egnet form; fortrinsvis hvor podningstrinnet udføres 18-30 timer forud for trin (i).

12. Fremgangsmåde ifølge krav 11, hvor mediet anvendt i podningstrinnet endvidere omfatter en ROCK-inhibitor, fortrinsvis valgt fra Y27632, H-1152P, thiazovivin, fasudil, hydroxyfasudil, GSK429286A, og RKI-1447, mere fortrinsvis hvor mediet anvendt i podningstrinnet omfatter 1-50 µM Y27632.

DRAWINGS

Figure 1



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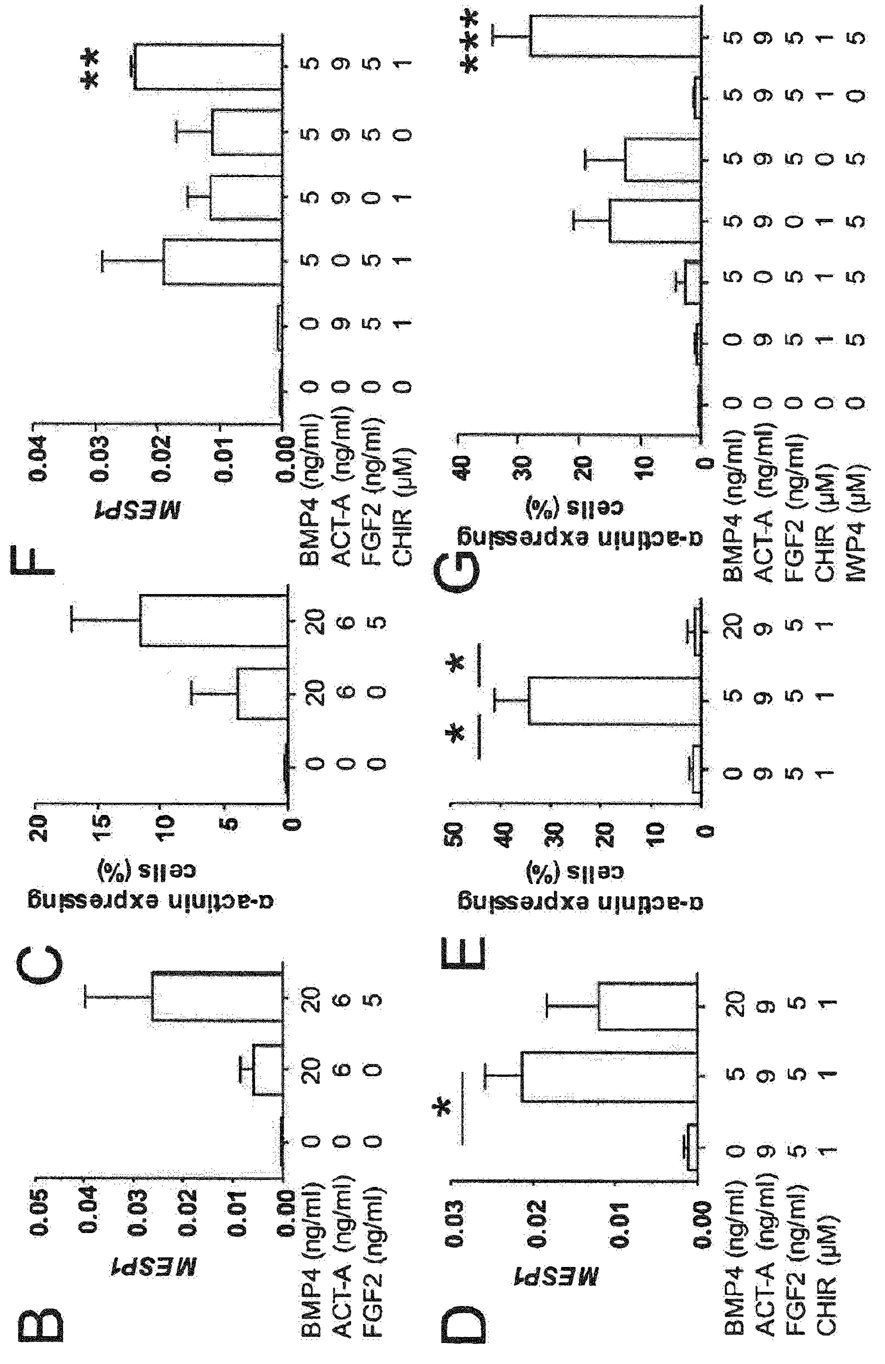

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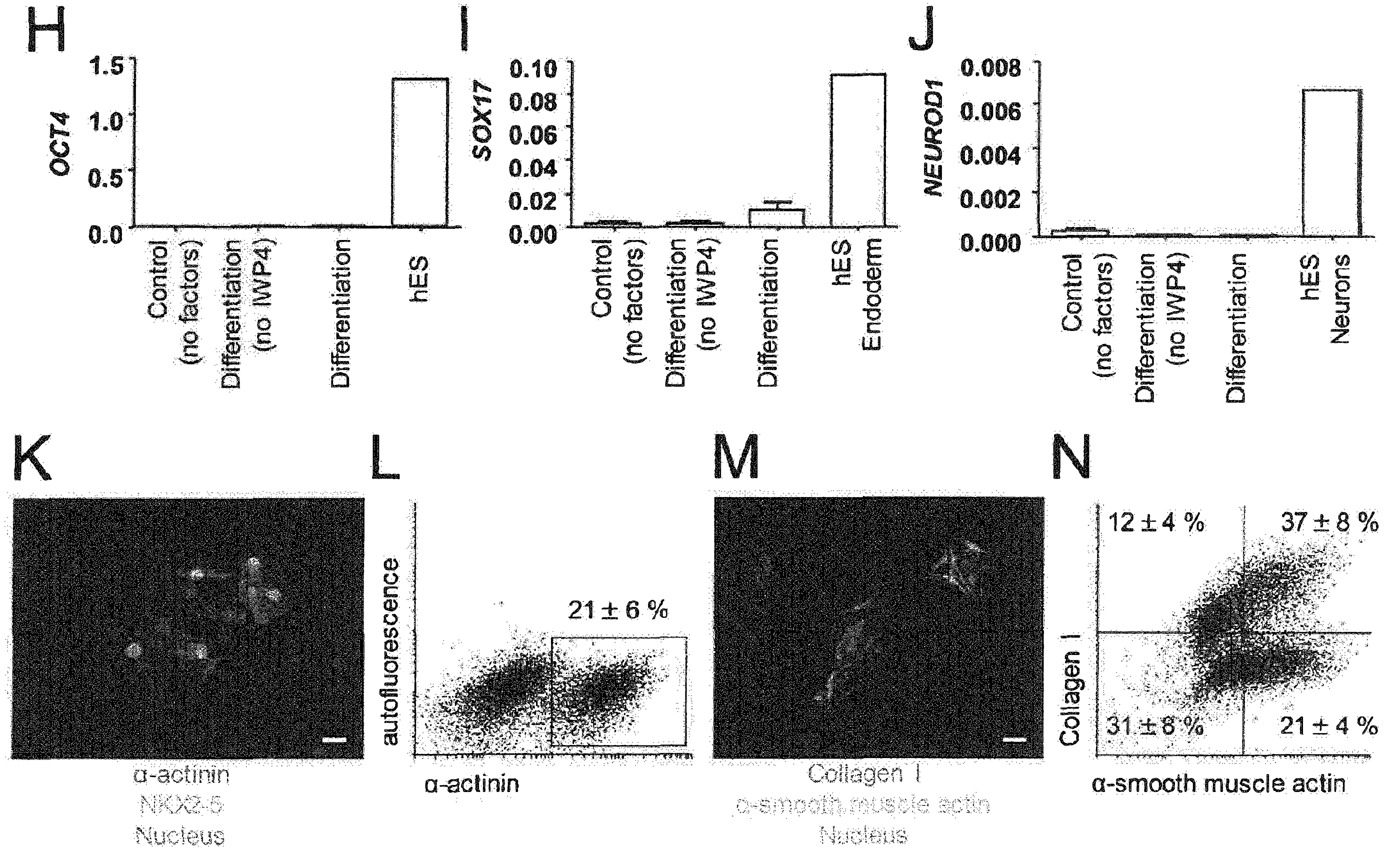

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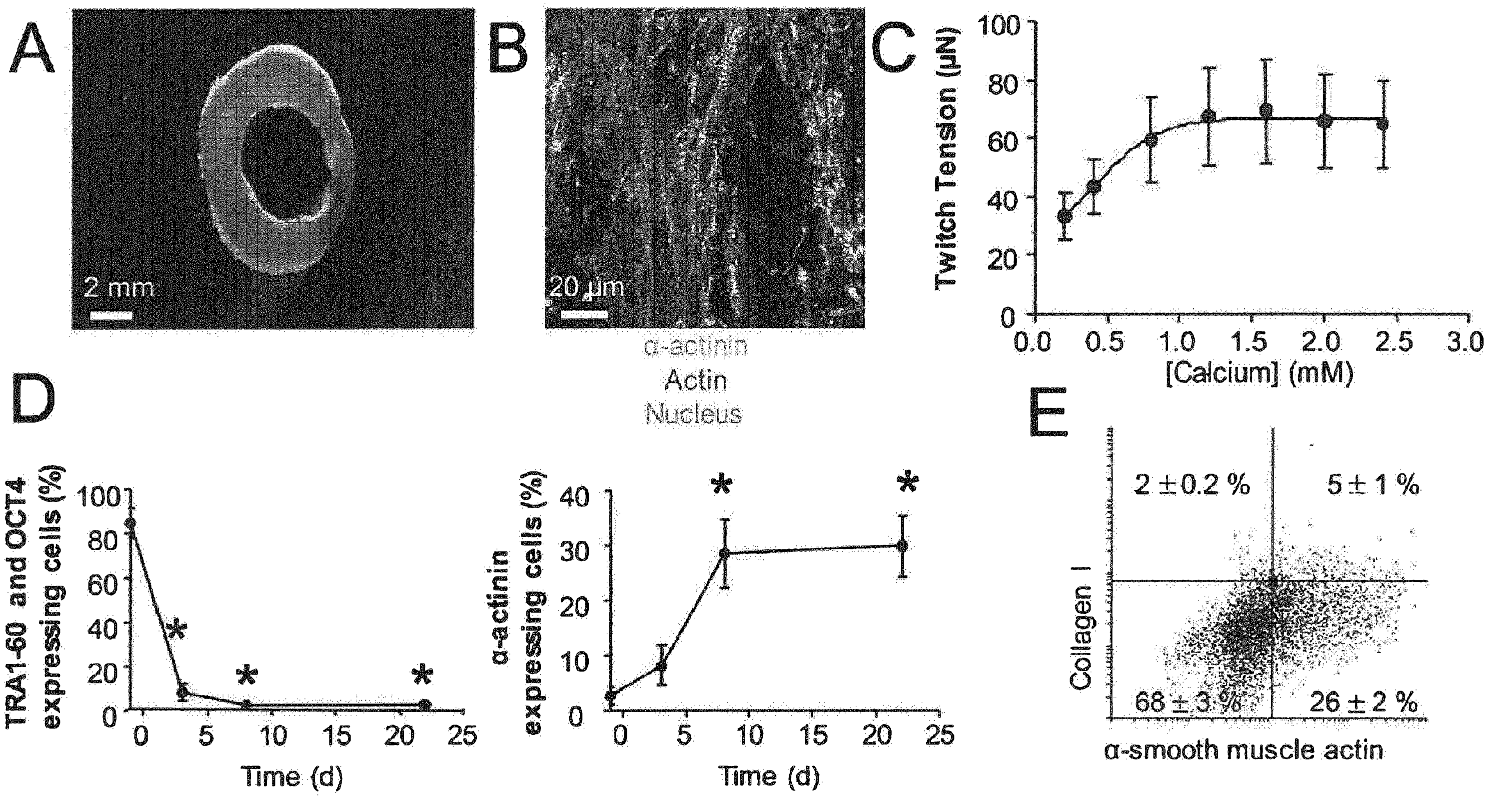
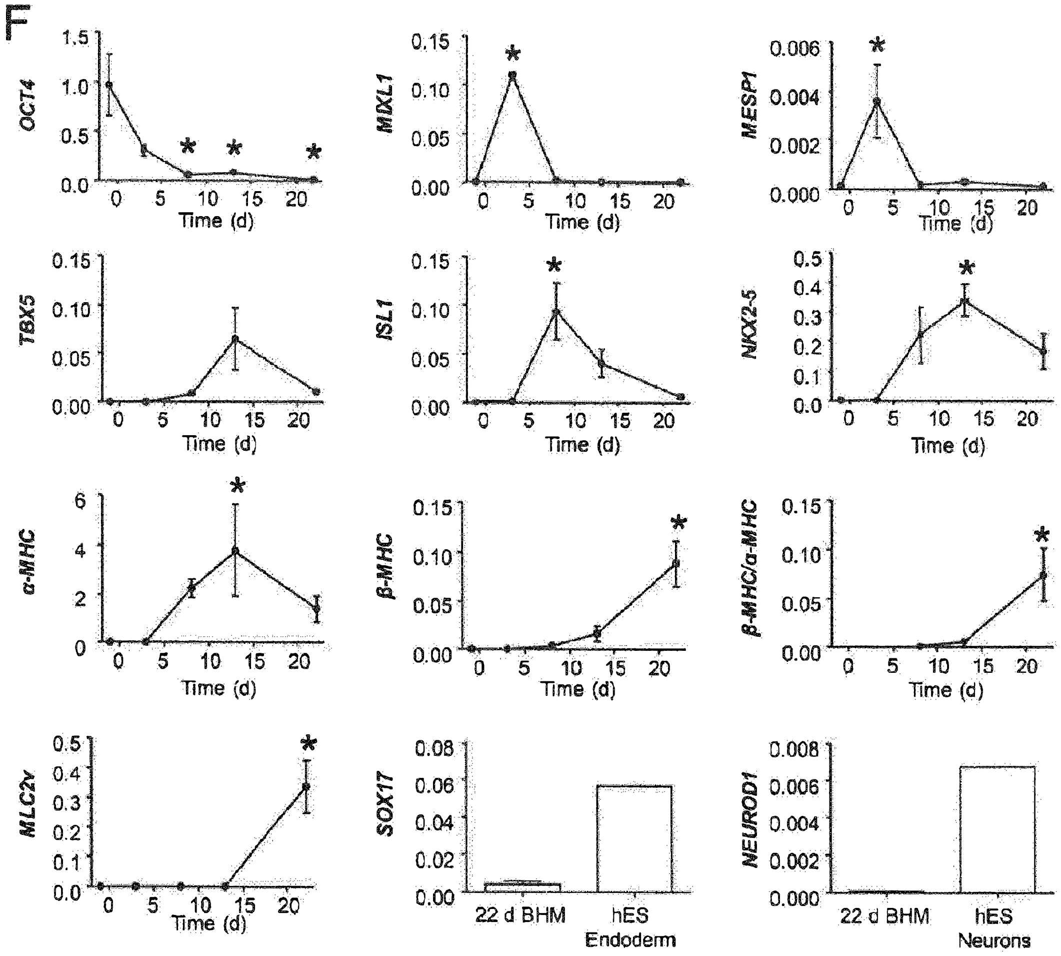

A
2 mm
B
20 µm
α-actinin
Actin
Nucleus
C
Twitch Tension (µN)
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
[Calcium] (mM)
D
TRA1-60 and OCT4 expressing cells (%)
100
80
60
40
20
0
0
5
10
15
20
25
Time (d)
*
*
*
α-actinin expressing cells (%)
40
30
20
10
0
0
5
10
15
20
25
Time (d)
*
*
E
2 ±0.2 %
5 ± 1 %
68 ± 3 %
26 ± 2 %
Collagen I
α-smooth muscle actin

Figure 2 (Continued)



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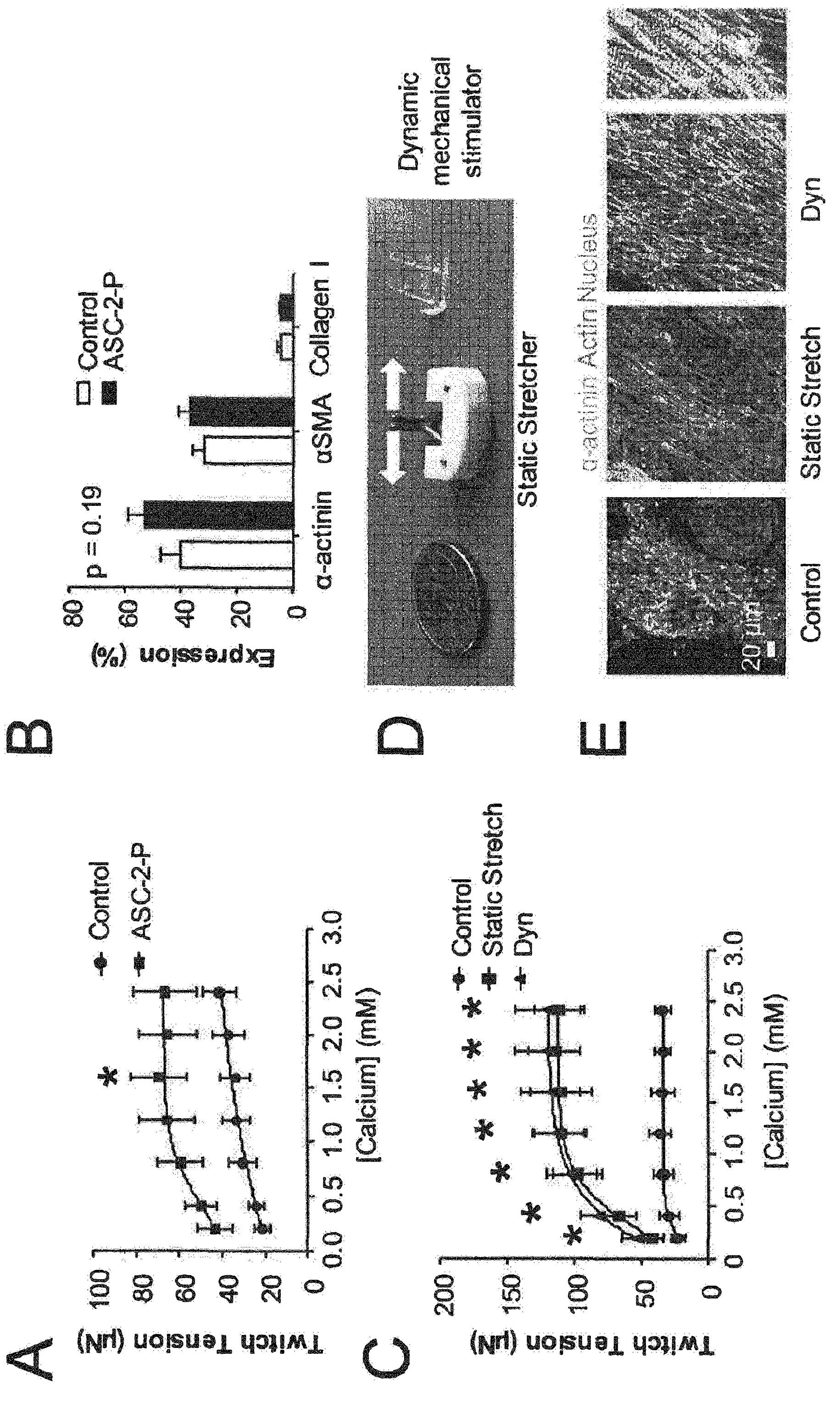
A
Twitch Tension (μN)
100
80
60
40
20
0
0.0 0.5 1.0 1.5 2.0 2.5 3.0
[Calcium] (mM)
*
Control
ASC-2-P
B
Expression (%)
80
60
40
20
0
p = 0.19
α-actinin
αSMA
Collagen I
Control
ASC-2-P
C
Twitch Tension (μN)
200
150
100
50
0
0 0.5 1.0 1.5 2.0 2.5 3.0
[Calcium] (mM)
* * * * * *
Control
Static Stretch
Dyn
D
Static Stretcher
Dynamic mechanical stimulator
E
α-actinin Actin Nucleus
20 μm
Control
Static Stretch
Dyn

Figure 3
(Continued)

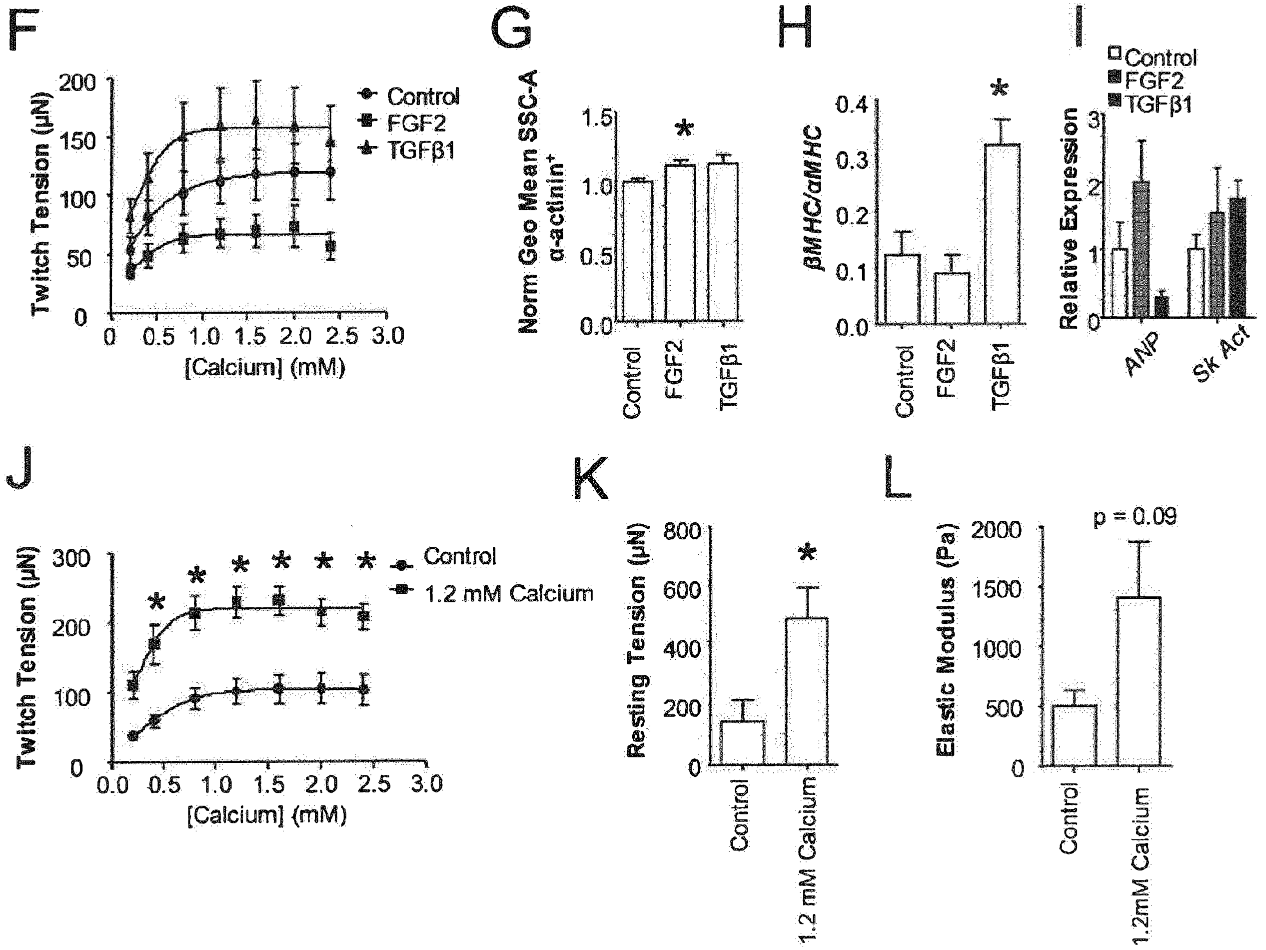

Figure 4

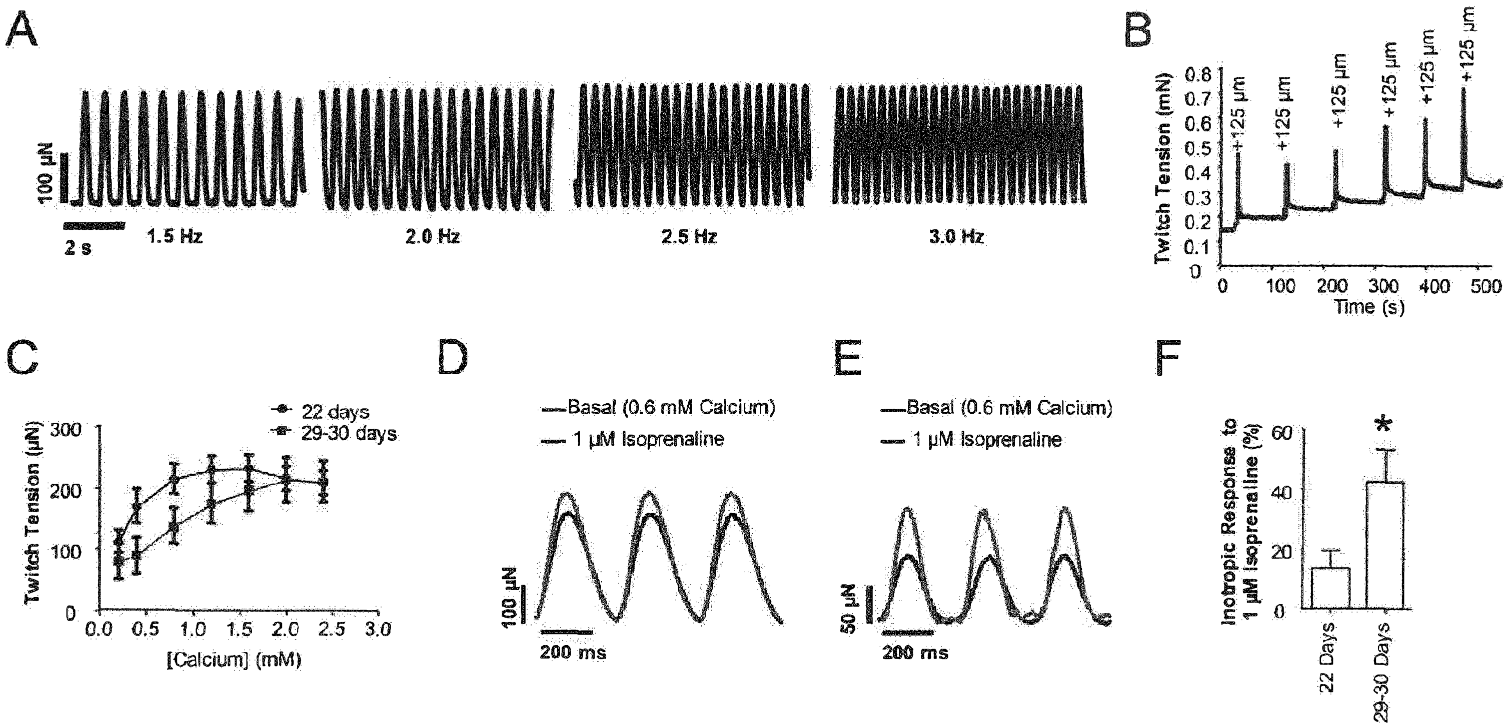
A
100 µN
2 s
1.5 Hz
2.0 Hz
2.5 Hz
3.0 Hz
B
Twitch Tension (mN)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
+125 µm
+125 µm
+125 µm
+125 µm
+125 µm
+125 µm
0
100
200
300
400
500
Time (s)
C
Twitch Tension (µN)
0
100
200
300
22 days
29-30 days
0.0
0.5
1.0
1.5
2.0
2.5
3.0
[Calcium] (mM)
D
Basal (0.6 mM Calcium)
1 µM Isoprenaline
100 µN
200 ms
E
Basal (0.6 mM Calcium)
1 µM Isoprenaline
50 µN
200 ms
F
Inotropic Response to 1 µM isoprenaline (%)
0
20
40
60
*
22 Days
29-30 Days

Figure 5

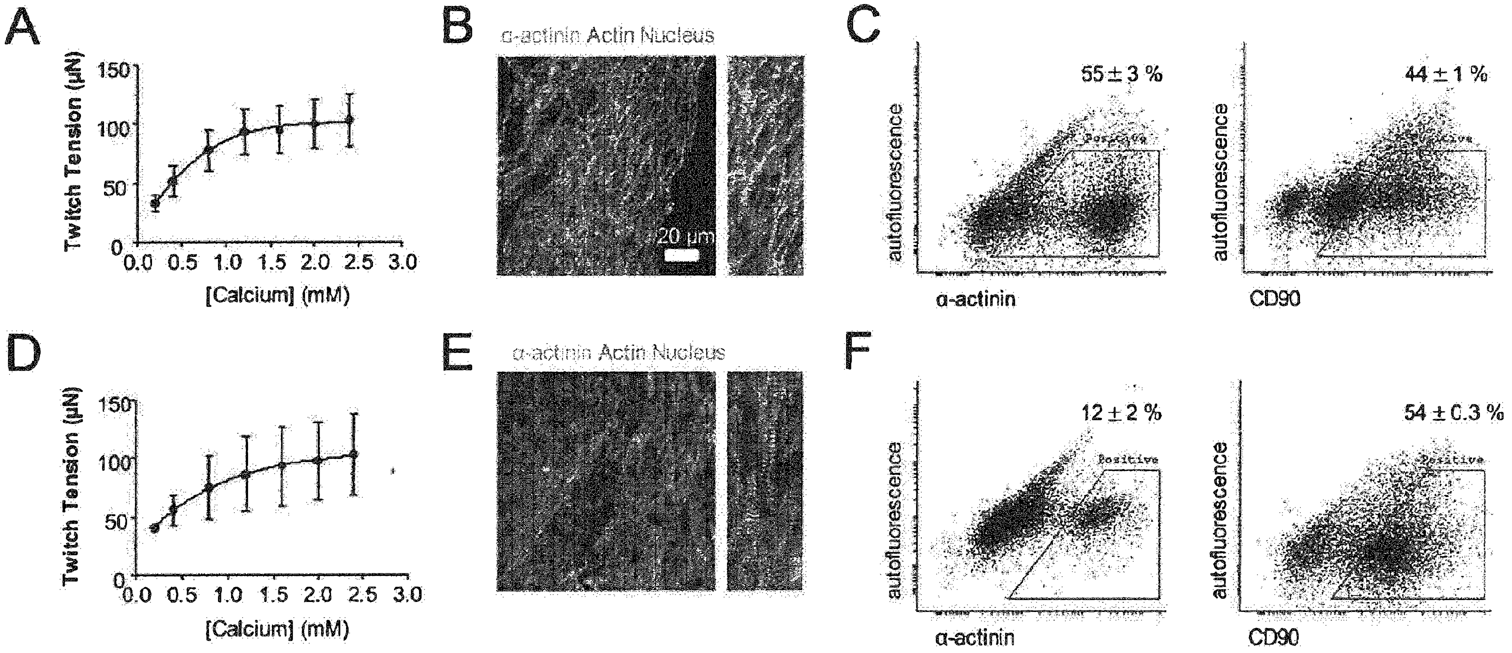
A
Twitch Tension (µN)
150
100
50
0
0.0 0.5 1.0 1.5 2.0 2.5 3.0
[Calcium] (mM)
B
α-actinin Actin Nucleus
20 µm
C
55 ± 3 %
autofluorescence
α-actinin
Positive
44 ± 1 %
autofluorescence
CD90
Positive
D
Twitch Tension (µN)
150
100
50
0
0.0 0.5 1.0 1.5 2.0 2.5 3.0
[Calcium] (mM)
E
α-actinin Actin Nucleus
F
12 ± 2 %
autofluorescence
α-actinin
Positive
54 ± 0.3 %
autofluorescence
CD90
Positive

Figure 6

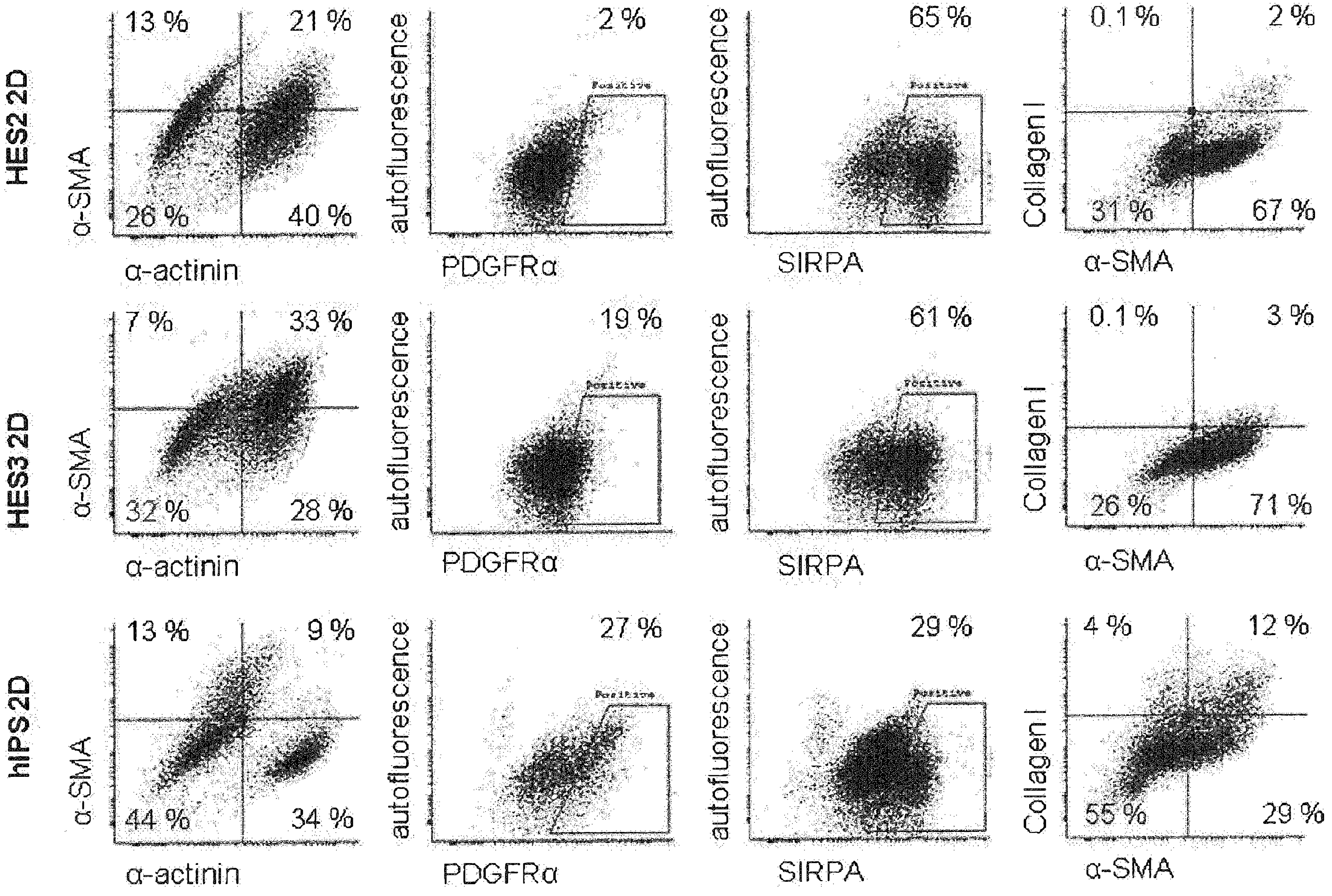
HES2 2D
α-SMA
α-actinin
13 %
21 %
26 %
40 %
autofluorescence
PDGFRα
2 %
Positive
autofluorescence
SIRPA
65 %
Positive
Collagen I
α-SMA
0.1 %
2 %
31 %
67 %
HES3 2D
α-SMA
α-actinin
7 %
33 %
32 %
28 %
autofluorescence
PDGFRα
19 %
Positive
autofluorescence
SIRPA
61 %
Positive
Collagen I
α-SMA
0.1 %
3 %
26 %
71 %
hIPS 2D
α-SMA
α-actinin
13 %
9 %
44 %
34 %
autofluorescence
PDGFRα
27 %
Positive
autofluorescence
SIRPA
29 %
Positive
Collagen I
α-SMA
4 %
12 %
55 %
29 %

Figure 7

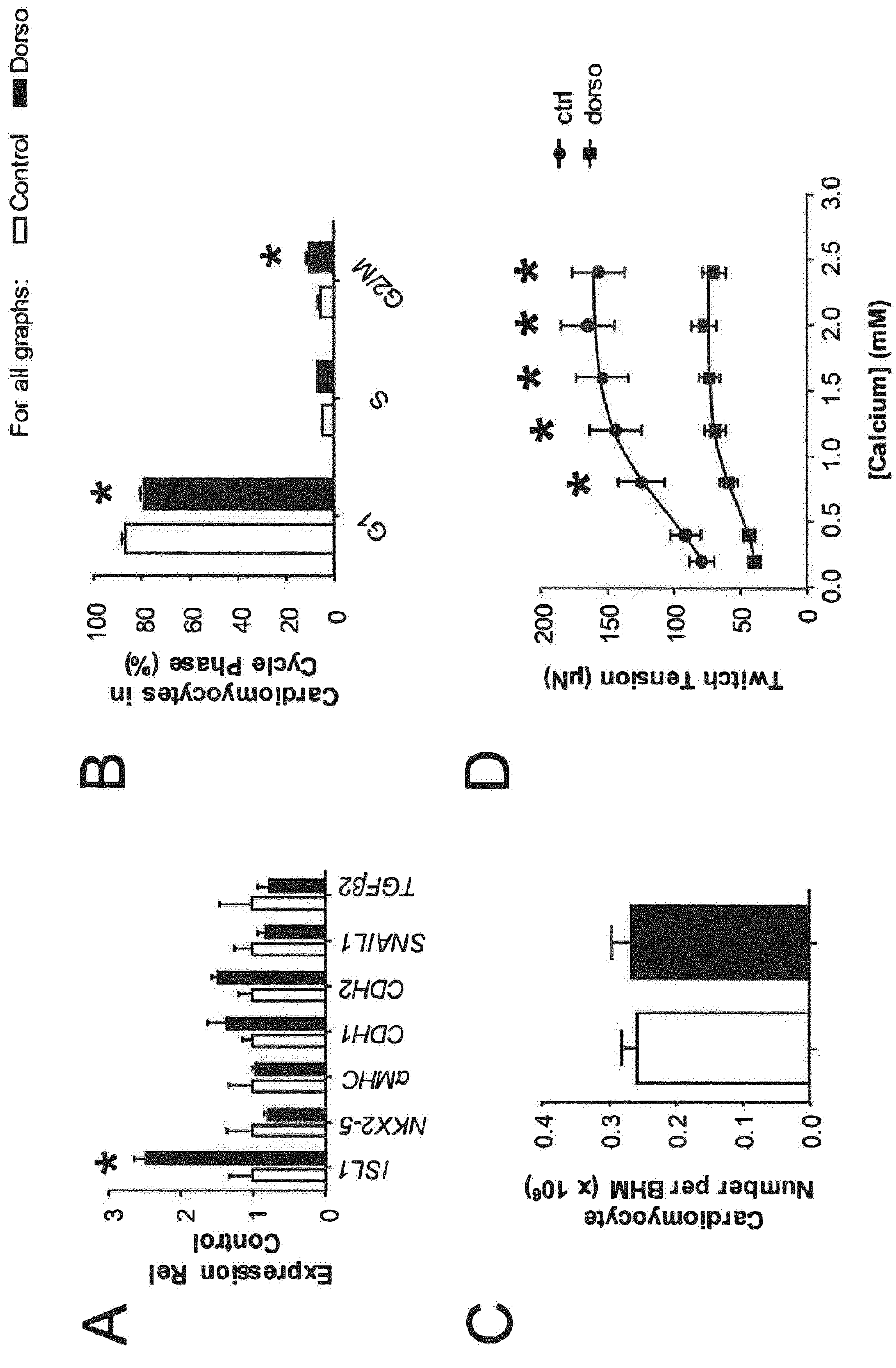
For all graphs:
☐ Control
■ Dorso
A
Expression Rel Control
0
1
2
3
ISL1
NKX2-5
αMHC
CDH1
CDH2
SNAIL1
TGFβ2
*
B
Cardiomyocytes in Cycle Phase (%)
0
20
40
60
80
100
G1
S
G2/M
*
*
C
Cardiomyocyte Number per BHM (x 10⁶)
0.0
0.1
0.2
0.3
0.4
D
Twitch Tension (µN)
0
50
100
150
200
[Calcium] (mM)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
ctrl
dorso
*
*
*
*
*

Figure 8

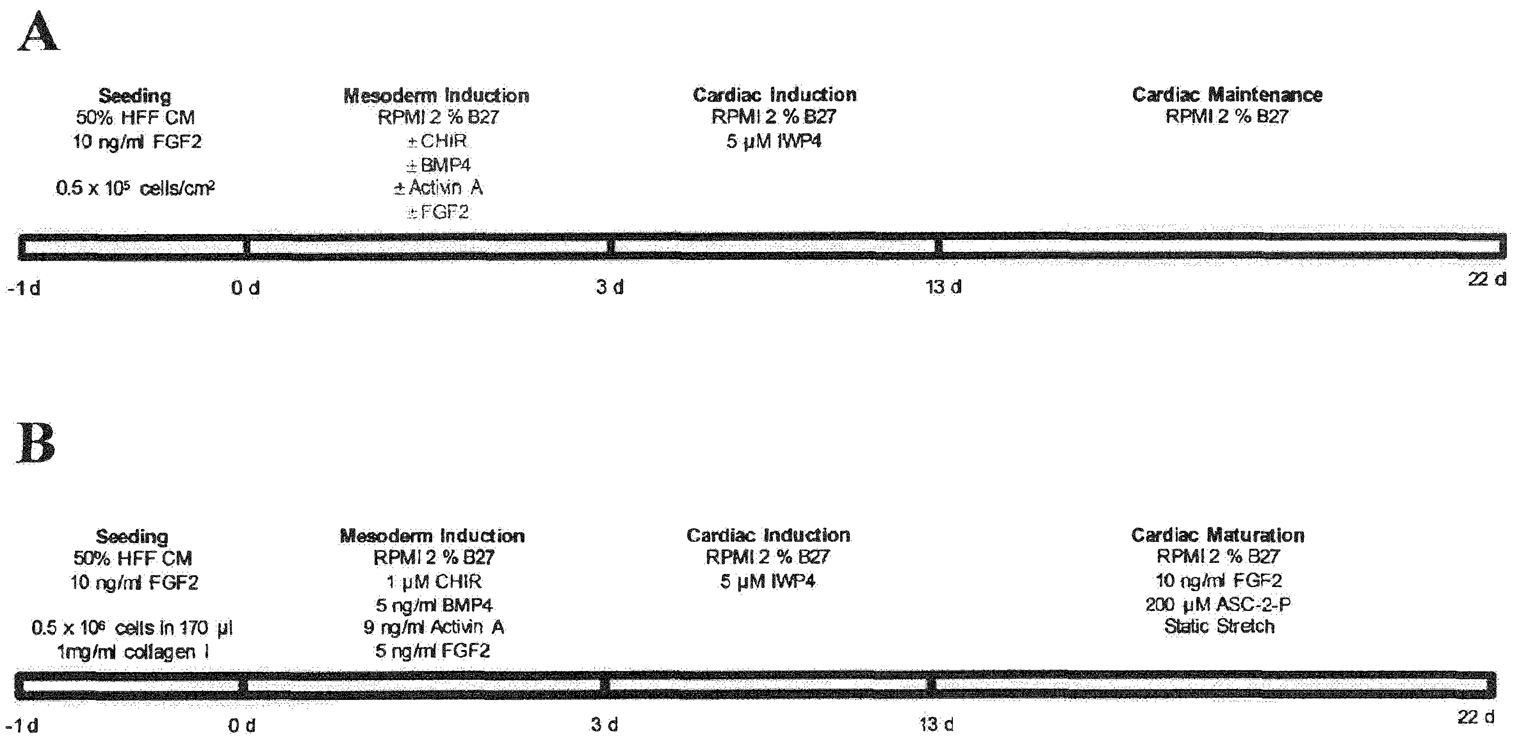
A
Seeding
50% HFF CM
10 ng/ml FGF2
0.5 x 10⁵ cells/cm²
Mesoderm Induction
RPMI 2 % B27
± CHIR
± BMP4
± Activin A
± FGF2
Cardiac Induction
RPMI 2 % B27
5 µM IWP4
Cardiac Maintenance
RPMI 2 % B27
-1 d
0 d
3 d
13 d
22 d
B
Seeding
50% HFF CM
10 ng/ml FGF2
0.5 x 10⁶ cells in 170 µl
1mg/ml collagen I
Mesoderm Induction
RPMI 2 % B27
1 µM CHIR
5 ng/ml BMP4
9 ng/ml Activin A
5 ng/ml FGF2
Cardiac Induction
RPMI 2 % B27
5 µM IWP4
Cardiac Maturation
RPMI 2 % B27
10 ng/ml FGF2
200 µM ASC-2-P
Static Stretch
-1 d
0 d
3 d
13 d
22 d

Figure 8 (Continued)

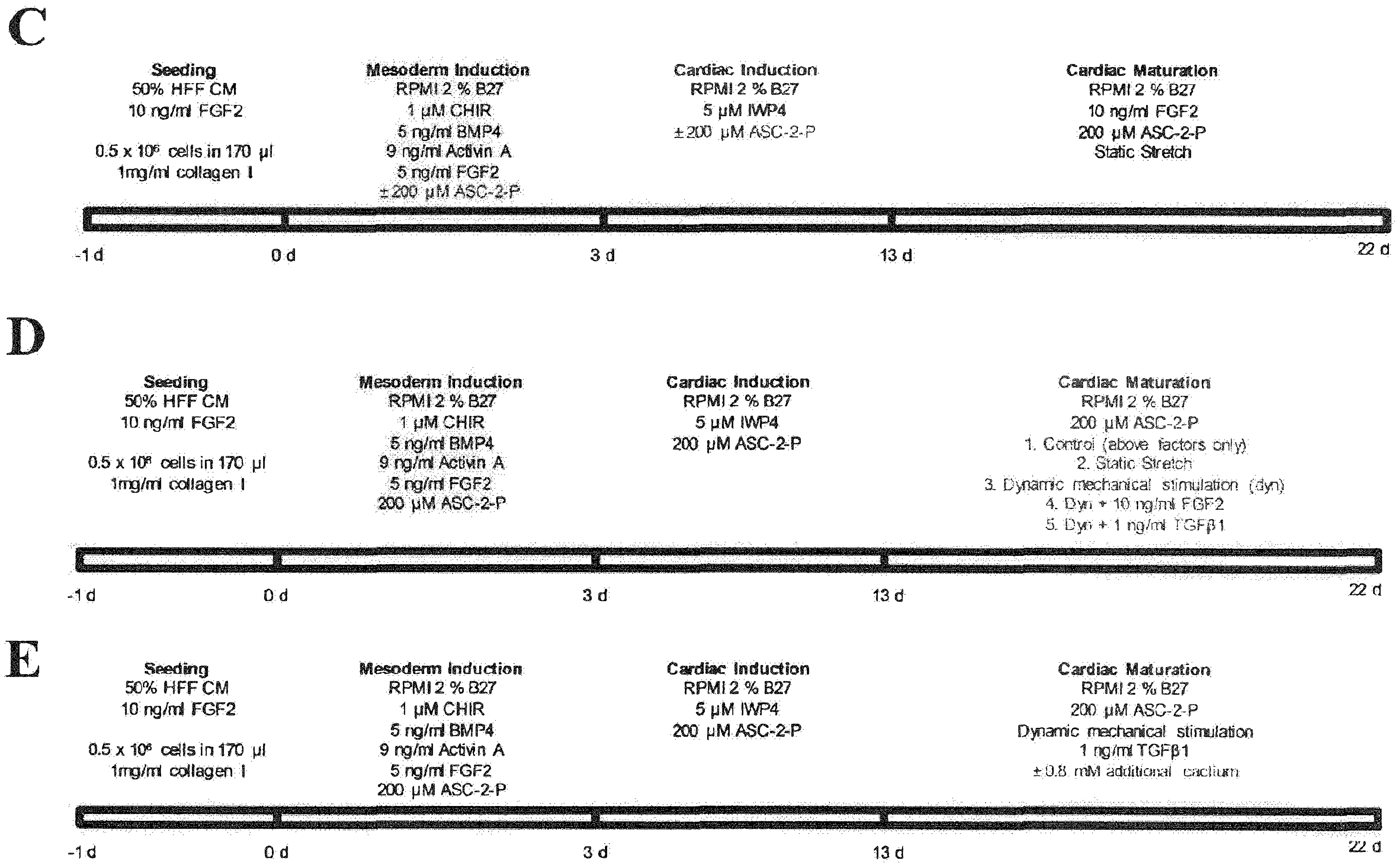

Figure 8 (Continued)

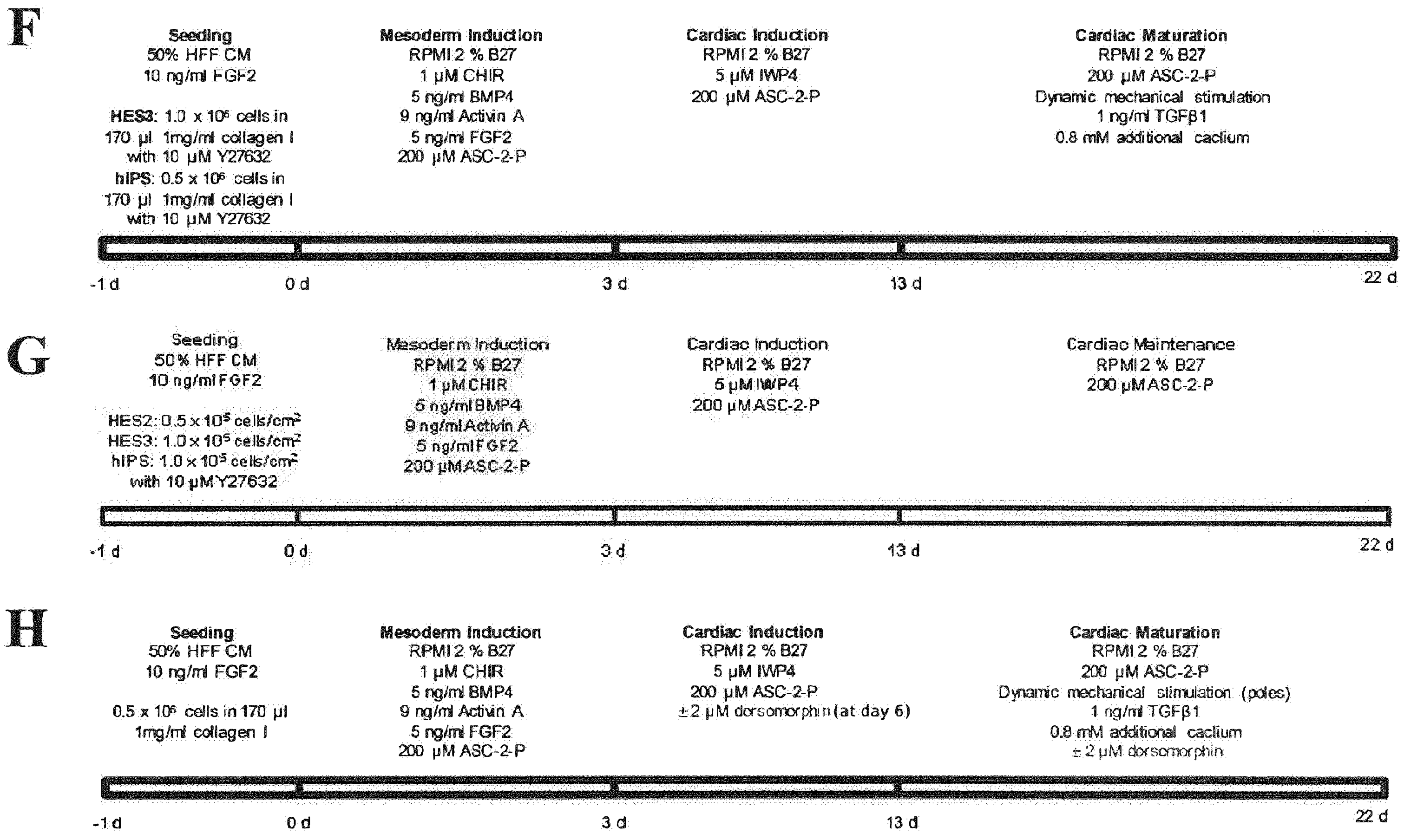

Figure 9

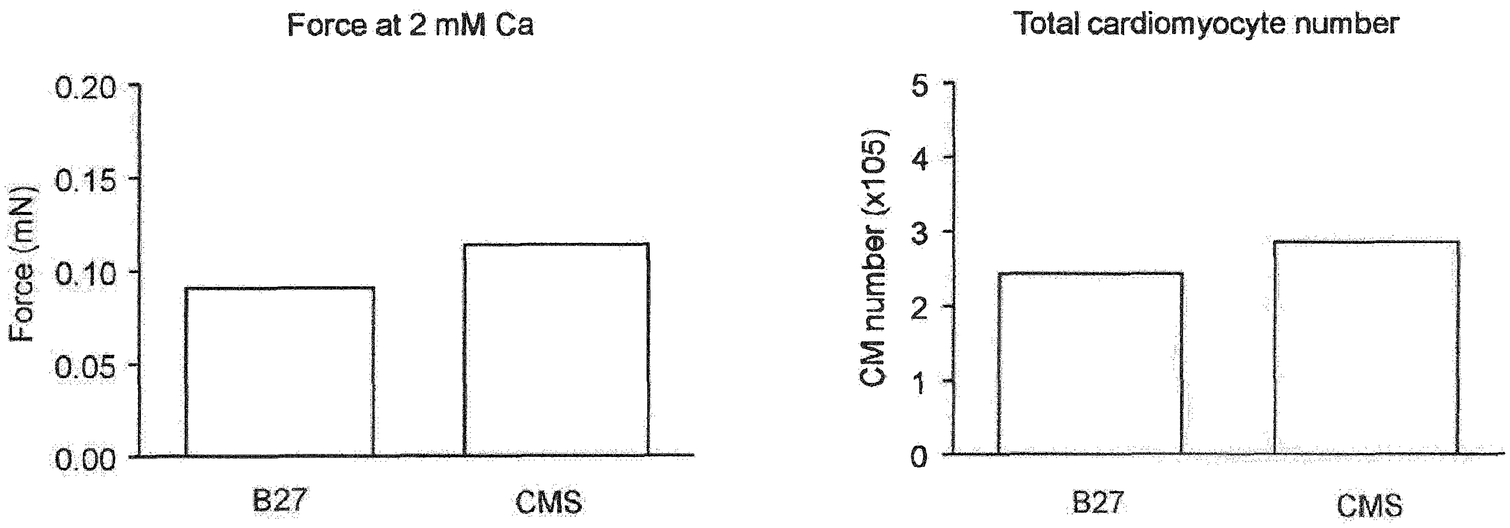
Force at 2 mM Ca
Force (mN)
0.20
0.15
0.10
0.05
0.00
B27
CMS
Total cardiomyocyte number
CM number (x105)
5
4
3
2
1
0
B27
CMS

Figure 10