Method for producing Engineered Heart Muscle (EHM)

The present invention provides a new method for producing Engineered Heart Muscle (EHM) under chemically fully defined conditions all compatible with GMP regulations. The resulting human myocardium generates force and shows typical heart muscle properties.

BACKGROUND OF THE INVENTION

Heart disease is the number one cause of death in industrialized countries and its prevalence is expected to rise despite refined pharmacological and interventional treatment. Consequently, novel pharmacological and non-pharmacological treatment modalities are inevitably called for. Tissue engineered myocardium can on the one hand be used to identify new drugs or drug targets for the treatment of heart disease (substance screening/target validation) and may on the other hand be directly applied in cardiac repair (regenerative/reparative medicine) (Eschenhagen & Zimmermann Circ Res 97: 1220-1231 (2005); Zimmermann et al. Cardiovasc Res 71: 419-429 (2006)). A main prerequisite of functional engineered myocardium, as of native heart muscle, is the ability to generate force.

Several myocardial tissue engineering modalities have been established throughout the past decade. However, reliable force-generation has only been demonstrated using hydrogel-cell-entrapment (Eschenhagen et al. Faseb J 11: 683-694 (1997); Kofidis et al. J Thorac Cardiovasc Surg 124: 63-69 (2002); Morritt et al. Circulation 115: 353-360 (2007); Zimmermann et al. Biotechnol Bioeng 68: 106-114 (2000); Tulloch et al. Circ Res 109: 47-59 (2011)) or cell-sheet technologies (Shimizu et al. Circ Res 90: e40 (2002)). The inventors and others have provided evidence that myocyte entrapment in collagen-hydrogels offer a three-dimensional growth milieu that can on the one hand facilitate assembly of multicellular, anisotropic cardiac muscle and on the other hand support advanced maturation of immature cardiomyocytes (Tiburcy et al. Circ Res 109: 1105-1114 (2011)). Resulting engineered heart muscle (EHM) preparations (formerly described as engineered heart tissue: EHT) ultimately facilitate the formation of contractile myocardial constructs with properties of postnatal heart muscle (Radisic *et al. Proc Natl Acad Sci U S A* 101: 18129-18134 (2004); Tiburcy *et al. Circ Res* 109: 1105-1114 (2011); Zimmermann *et al. Circ Res* 90: 223-230 (2002)). Proof-of principle animal studies have shown that after implantation onto diseased hearts EHMs not only electrically integrate but also improve heart function (Zimmermann et al. Nat Med 12: 452-458 (2006)).

In principle, the inventors have shown that tissue engineered myocardium may be a novel treatment modality for diseased heart. However, all published cardiac tissue engineering approaches so far rely on the use of undefined animal components, mostly animal matrix (e.g. rat collagen, bovine fibrin, mouse tumor-derived extracellular matrix [Matrigel®]), and animal serum (Tulloch et al. Circ Res 109: 47-59 (2011), Zimmermann et al. Circ Res 90: 223-230 (2002), Zimmermann et al. Nat Med 12: 452-458 (2006), Schaaf et al. PLoS One 6: e26397 (2011); Soong et al. Curr Prot Cell Biol 23.8.1-23.8.21 (2012); WO 01/55297, WO 2007/054286, and WO 2008/058917). In the rat EHM model the inventors have performed first studies to replace animal serum with a serum-free medium. While the inventors were able to achieve a comparable force production in resulting tissues, the inventors could not take out animal components during the initial phase of tissue formation in the first seven days (Naito *et al. Circulation* **114**: I72-78 (2006); Zimmermann, Universitätsklinikum Hamburg Eppendorf, Habilitation (2006); Schneiderbanger, Universität Hamburg, Dissertation (2006)).

Recently, several serum-free, cytokine-directed protocols for more efficient cardiac differentiations have been described (Burridge et al. *Cell Stem Cell* 10: 16-28 (2012)) yielding cultures containing up to 98% cardiomyocytes (Lian *et al. Proc Natl Acad Sci U S A* (2012)). Importantly, these serum-free differentiation protocols offer potential clinical applicability as defined substances without animal products are utilized. Whilst scaling of human heart cells under GMP conditions appears to be a resolvable caveat, the generation of human force-generating myocardium still remains a challenge. It remains a pivotal issue to support organotypic organization and advanced maturation of ESC-derived myocytes under defined, serum-free culture conditions.

SUMMARY OF THE INVENTION

Here, the inventors report a protocol to engineer human myocardium (Human Engineered Heart Muscle: hEHM) using all defined components. These components include a hydrogel matrix, human cells and serum-free culture medium conditions all compatible with GMP regulations. The resulting human myocardium generates force and shows typical heart muscle properties.

More specifically, a method for producing engineered heart muscle (EHM) is provided, the method comprising the steps of:

i) providing a serum-free reconstitution mixture in one or more moulds, said reconstitution mixture comprising (a) a serum-free minimum essential medium; (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) and 0.2-2 mg/ml collagen; and (c) a mixture of human cardiac myocytes and human non-myocytes, wherein 20 to 80% of the total cell mixture are cardiac myocytes; wherein the reconstitution mixture has a pH of 7.2 to 7.6;

ii) culturing the serum-free reconstitution mixture in said one or more moulds, whereby the serum-free reconstitution mixture is allowed to condense for at least 15 min;

iii) culturing the mixture obtained in step (ii) in said one or more moulds in a serum-free EHM culture medium until the mixture condenses to at least 50% of its original thickness, wherein said EHM culture medium comprises (a) a basal medium comprising 0.5-3 mmol/L Ca^{2+}; (b) a serum-free supplement as defined in (i)(b); (c) 0.5-10 mmol/L L-glutamine; (d) 0.01-1.0 mmol/L ascorbic acid; (e) 1-100 ng/ml IGF-1; and (f) 1-10 ng/ml TGFβ1;

iv) culturing the mixture obtained in step (iii) under mechanical stretching in a serum-free EHM culture medium as defined in step (iii) (a)-(f), whereby force-generating EHM is formed.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

More specifically, the invention provides a method for producing engineered heart muscle (EHM), the method comprising the steps of:

(i) providing a serum-free reconstitution mixture in one or more moulds, said reconstitution mixture comprising (a) a serum-free minimum essential medium; (b) a serum-free supplement resulting 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),

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), and

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);

and (c) a mixture of human cardiac myocytes and human non-myocytes, wherein 20 to 80% of the total cell mixture are cardiac myocytes;

wherein the reconstitution mixture has a pH of 7.0 to 7.8, preferably 7.1 to 7.7, more preferably 7.2 to 7.6, even more preferably 7.3 to 7.5, and most preferably about 7.4;

(ii) culturing the serum-free reconstitution mixture in said one or more moulds, whereby the serum-free reconstitution mixture is allowed to condense for at least 15 min, preferably 0.25-3h, and more preferably for 0.5-1.5h,

(iii) culturing the mixture obtained in step (ii) in said one or more moulds in a serum-free EHM culture medium until the mixture condenses to at least 50% (preferably at least 55%, more preferably at least 60%, even more preferably at least 70%, and most preferably at least 75%) of its original thickness,

wherein said EHM culture medium comprises

(a) a basal medium comprising 0.5-3 mmol/L Ca^{2+} (preferably 1-2.5 mmol/L, more preferably about 2 mmol/L);

(b) a serum-free supplement as defined in (i)(b);

(c) 0.5-10 mmol/L L-glutamine (preferably 1-7 mmol/L, more preferably 2-6 mmol/L, even more preferably 3-5 mmmol/L, still more preferably about 2 mmol/L);

(d) 0.01-1.0 mmol/L ascorbic acid (preferably 0.05-1.0 mmol/L, more preverably 0.1-0.5 mmol/L, even more preferably 0.2-0.4 mmol/L, still more preferably about 0.3 mmol/L);

(e) 1-100 ng/ml IGF-1 (preferably 2-90 ng/ml, more preferably 3-80 ng/ml, even more preferably 4-70 ng/ml, still more preferably 5-60 ng/ml, more preferably 6-50 ng/ml, more preferably 7-40 ng/ml, more preferably 8-30 ng/ml, more preferably 9-20 ng/ml, such as about 10 ng/ml); and

(f) 1-10 ng/ml TGFβ1 (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);

(iv) culturing the mixture obtained in step (iii) under mechanical stretching in a serum-free EHM culture medium as defined in step (iii) (a)-(f), whereby force-generating EHM is formed.

The minimum essential medium in step (i) may be selected from Iscove's medium, αMEM, DMEM, and RPMI. In a preferred embodiment, the basal medium is Iscove's medium or αMEM. In a more preferred embodiment, the basal medium is Iscove's medium. However any suitable minimal medium may be used in the method. Recipes of suitable minimum essential mediums are provided herein or are publicly available, e.g. from catalogues of the ATCC.

Preferably, the serum-free supplement of step (i) further comprises one or more components selected from the group consisting of vitamin A, D-galactose, 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.

For the serum-free supplement referred to in component (b) of step (i), commercially available B27® supplement or B27® supplement minus insulin can be used. Alternatively, the custom made supplement as shown in Table 2 below can be used. In a preferred embodiment, the B27® supplement or B27® supplement minus insulin used as component (b) of step (i) of the above method is applied in an amount of 2-6% (v/v). More preferably,

the B27® supplement or B27® supplement minus insulin used as component (b) of step (i) of the above method is applied in an amount of 4% (v/v).

Further, said reconstitution mixture of step (i) preferably comprises 0.3-0.5 mg collagen per 1.5 x 10^6 cardiac myocyte and non-myocyte cell mixtures. More preferably, said reconstitution mixture of step (i) comprises about 0.4 mg collagen per 1.5 x 10^6 cardiac myocyte and non-myocyte cell mixtures.

The collagen in component (c) of the reconstitution mixture of step (i) 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, component (c) of the reconstitution mixture of step (i) comprises 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 origin, or marine origin, such as from algae or fish origin, is also contemplated.

The development of functional (i.e force-generating), defined, serum-free human tissue engineered myocardium for potential regenerative heart therapy requires a number of hurdles to be overcome. Of paramount importance is a reliable source of human heart cells. To date, human pluripotent stem cells have emerged as the major source of human heart cell. 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); incorporated herein in its entirety by reference). 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); incorporated herein in its entirety by reference). 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. Cardiac differentiation of ESCs and iPSCs occurs in embryoid body (Schroeder *et al.* Biotechnol Bioeng 92: 920-933 (2005); incorporated herein in its entirety by reference) cultures as a more or less stochastic event yielding cell populations containing 5-20% *bona fide* cardiomyocytes (Kehat *et al. J Clin Invest* 108: 407-414 (2001); Mummery *et al. Circulation* 107: 2733-2740 (2003); Xu et al. *Circ Res* 91: 501-508 (2002); all incorporated herein by reference). Moreover, it was reported that also parthenogenetic stem

cells are likely to be suitable for EHM-production (Didié et al. J Clin Invest. doi:10.1172/JCI66584; incorporated herein in its entirety by reference). Accordingly, in a preferred embodiment, the cardiac myocytes are human cardiac myocytes. Preferably, said cardiac myocytes are derived from embryonic stem cells, wherein the cell is 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. In an alternative embodiment, the cardiac myocytes are derived from induced pluripotent cells, parthogenetic stem cells, or adult stem cells, as described above.

Recently, several serum-free, cytokine-directed protocols for more efficient cardiac differentiations have been described (Burridge et al. *Cell Stem Cell* 10: 16-28 (2012); incorporated herein in its entirety by reference) yielding cultures containing up to 98% cardiomyocytes (Lian *et al. Proc Natl Acad Sci U S A* (2012); incorporated herein in its entirety by reference). Accordingly, in another preferred embodiment, the cardiac myocytes can be obtained by serum-free differentiation. On the other hand, the cardiac myocytes may also be derived from non-human primate stem cells, fetal or neonatal cardiac myocytes.

It has been demonstrated that it is advantageous to provide the cardiac myocytes in admixture with cells of one or more class of cells selected from the group of non-myocytes such as fibroblasts, endothelial cells, smooth muscle cells, and mesenchymal stem cells. Hence, preferably the cardiac myocytes admixture contains 20-80% cardiac myocytes, more preferably 30-70% cardiac myocytes, even more preferably 40-60% cardiac myocytes, and most preferably about 50% cardiac myocytes, wherein the non-myocytes are fibroblasts or endothelial cells. Indeed, it is a particularly preferred embodiment that the non-myocytes are fibroblasts. Suitable non-myocytes may be identified by expression of e.g. the CD90 surface marker. Suitable cells can be identified, for example, using techniques such as immune staining or fluorescence activated cell sorting (FACS). EHMs resulting from such an admixture usually generate a higher force.

Preferably, the cardiac myocytes are provided in step (i) in a cell concentration of at least 2.7-20 x 10^6 per ml. However, in a more preferred embodiment, the cardiac myocytes are provided in step (i) in a cell concentration of at least 2.9-10 x 10^6 per ml, even more preferably in a cell concentration of at least 3.1-5 x 10^6 per ml, and in a most preferred embodiment in a cell concentration of at least 3.3-3.4 x 10^6 per ml.

The mould referred to in the method may have any suitable form allowing incorporation of the EHM in a host in need thereof. However, in a preferred embodiment, the mould is ring-, multiangular-, disc- or pouch-shaped.

Cell culturing is carried out using common procedures and equipment generally known in the art. Usually, culturing conditions comprise a temperature in the range of 30-40 °C, preferably 36-38 °C, and most preferably at about 37 °C, using a humidified cell culture incubator in the presence of 5-10% CO_2.

For the serum-free supplement referred to in component (b) of step (iii), commercially available B27® supplement or B27® supplement minus insulin can be used. Alternatively, the custom made supplement as shown in Table 2 below can be used. In a preferred embodiment, the B27® supplement or B27® supplement minus insulin used as component (b) of step (iii) of the above method is applied in an amount of 2-6% (v/v). More preferably, the B27® supplement or B27® supplement minus insulin used as component (b) of step (i) of the above method is applied in an amount of 4% (v/v).

In addition, the serum-free supplement of step (iii) 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. As noted above, 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.

The basal medium comprised in said EHM culture medium in step (iii) may be selected from Iscove's medium, αMEM, DMEM, and RPMI. Since RPMI usually has a lower concentration of calcium, it may be necessary to supplement the RPMI basal medium accordingly. If deemed appropriate, the basal medium may be supplemented with non-essential amino acids. If αMEM is used as the basal medium, the EHM culture 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.

In a preferred embodiment, the basal medium comprised in said EHM culture medium in step (iii) is Iscove's medium or αMEM. In a more preferred embodiment, the basal medium comprised in said EHM culture medium in step (iii) is Iscove's medium. However any basal medium may be used in the method. Recipes of suitable minimum essential mediums are provided herein or are publicly available, e.g. from catalogues of the ATCC.

As demonstrated in the Examples below, the serum-free EHM culture medium advantageously further comprises VEGF, FGF, or both VEGF and FGF. Addition of VEGF and/or FGF has been shown to result in EHM exhibiting a higher force.

Typically, VEGF is added in a concentration of about 5-20 ng/ml VEGF, preferably 6-18 ng/ml, more preferably 7-16 ng/ml, even more preferably 8-14 ng/ml, most preferably 9-12 ng/ml, and even most preferably in a concentration of about 10 ng/ml.

FGF is added in a concentration of about 5-20 ng/ml FGF, preferably 6-18 ng/ml, more preferably 7-16 ng/ml, even more preferably 8-14 ng/ml, most preferably 9-12 ng/ml, and even most preferably in a concentration of about 10 ng/ml.

In principle, any type of VEGF, FGF, IGF1 and TGFβ1 can be used, as long as these growth factors are capable of signalling via their corresponding receptors on the cell surface of the cardiac myocytes of the EHM. However, in a preferred embodiment the VEGF is human VEGF. In another preferred embodiment, the FGF is human FGF. In still another preferred embodiment IGF1 is human IGF1. In still another embodiment, the TGFβ1 is human TGFβ1. In a most preferred embodiment, all of VEGF, FGF, IGF1, and TGFβ1 are human.

Usually, culturing in step (iii) is carried out for at least 3 days, preferably for about 3 to about 7 days.

In principal, the further culturing in step (iv) may be carried out for any suitable period of time. However, usually, the further culturing in step (iv) is carried out for a period of at least 3-60 days, preferably for 4-30 days, more preferably for 5-20 days. In a most preferred embodiment, the further culturing is carried out for 7 days, since this time period represents an optimal balance of a preferably short culturing time and a time period which is sufficient to result in force-generating EHMs.

Usually, step (iv) of the above method is carried out on a stretch device, as generally known in the art. Preferably, the stretch device applies a static, phasic or dynamic stretch to the EHM. More specifically, mechanical stretching can be (i) static, (ii) dynamic, or (iii) flexible against a resilient load.

As will be further demonstrate below, the EHM produced by the above method generates more than 0.01 mN force upon induction with 3 mM calcium as determined using the method described in Zimmermann et al. Circ. Res. 90, 223-230 (2002), preferably more than 0.1 mN force, more preferably more than 0.2 mN force, and most preferably more than 0.3 mN force upon induction with 3 mM calcium as determined using the method described in Zimmermann et al. Circ. Res. 90, 223-230 (2002).

Another detailed prior art protocol which is suitable to serve as a basis for the improved method disclosed herein is described by Soong et al. Curr Prot Cell Biol. 55: 23.8.1-23.8.21

(2012), which is incorporated herewith in its entirety, and in particular reference is made to the "Basic Protocol 2", and the "Support Protocol 2".

Component	Basic protocol	Matrix protocol	Serum-free protocol
Matrix	Rat tail collagen I (0.4 mg/EHM)	Medical grade bovine collagen (0.4 mg/EHM)	Medical grade bovine collagen (0.4 mg/EHM)
	Matrigel (10% v/v)		
Concentrated medium (2x)	2x DMEM	2x DMEM	2x DMEM
	20% horse erum	40% FBS	8% B27 or 8% B27 minus insulin or custom made serum supplement (2x)
	4% chick embryo extract		
	200 U/ml Penicillin 200 mg/ml Streptomycin	200 U/ml Penicillin 200 mg/ml Streptomycin	200 U/ml Penicillin 200 mg/ml Streptomycin
Culture medium	Iscove's	Iscove's	Basal medium (Iscove's, αMEM, RPMI) comprising 1-2 mmol/L Ca^{2+}
	20% FBS	20% FBS	4% B27 or 4% B27 minus insulin or custom made serum supplement
	1% non-essential amino acids	1% non-essential amino acids	1% non-essential amino acids, omit if αMEM
	2 mmol/L L-glutamine	2 mmol/L L-glutamine	2 mmol/L L-glutamine, omit if αMEM or if RPMI
	0.3 mmol/L ascorbic	0.3 mmol/L ascorbic	0.3 mmol/L ascorbic

	acid	acid	acid, omit if αMEM
	100 µmol/l β-mercaptoethanol	100 µmol/l β-mercaptoethanol	0.05% Fatty acids supplement
			20 ng/ml IGF
			10 ng/ml VEGF
			10 ng/ml FGF
			5 ng/ml TGFb1 day 0-3
	100 U/ml Penicillin 100 mg/ml Streptomycin	100 U/ml Penicillin 100 mg/ml Streptomycin	100 U/ml Penicillin 100 mg/ml Streptomycin

Table 1: Comparison of protocols for human EHM generation.

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

Table 2: Custom-made supplement to replace B27.

Substance	Concentration (mg/l)	Substance	Concentration (mg/l)
NaCI	4505	L-serine	42
KCI	330	L-valine	94
NaH2PO4	125	L-cystine	70
MgSO4·7H2O	200	L-asparagine	25
CaCI2·2H2O	218,6	L-aspartic acid	30
KNO3	0.076	L-alanine	25
Na2SeO3	0.0173	L-glutamic acid	75
D-glucose	4500	L-proline	40
HEPES	5958	Biotin	0.013
NaHCO3	3024	Vitamin B12	0.013
Phenol red	15	Nicotin acid amide	4
Na-pyruvate	110	Cholin chloride	4
L-arginine·HCI	84	D-Ca-pantothenate	4
L-histidine·HCI·H2O	42	Pyridoxal·HCI	4
Glycine	30	Thiamine·HCI	4
L-isoleucine	105	Riboflavin	0.4
L-lysine·HCI	146	Folic acid	4
L-methionine	30	Myo-inositol	7.2
L-phenylalanine	66		
L-leucine	105		
L-threonine	95		
L-tryptophane	16		
L-tyrosine·2Na	104.2		

Table 3: Iscove's basal medium formulation without glutamine (Biochrom).

Components	Molecular Weight	Concentration (mg/L)
AminoAcids		
Glycine	75	10
L-Alanyl-Glutamine	217	446
L-Arginine	174	200
L-Asparagine	132	50
L-Aspartic acid	133	20
L-Cystine	240	50
L-Glutamic Acid	147	20
L-Histidine	155	15
L-Hydroxyproline	131	20
L-Isoleucine	131	50
L-Leucine	131	50
L-Lysine hydrochloride	183	40
L-Methionine	149	15
L-Phenylalanine	165	15
L-Proline	115	20
L-Serine	105	30
L-Threonine	119	20
L-Tryptophan	204	5
L-Tyrosine	181	20
L-Valine	117	20
Vitamins		
Biotin	244	0.2
Choline chloride	140	3

D-Calcium pantothenate	477	0.25
Folic Acid	441	1
Niacinamide	122	1
Para-Aminobenzoic Acid	137	1
Pyridoxine hydrochloride	206	1
Riboflavin	376	0.2
Thiamine hydrochloride	337	1
Vitamin B12	1355	0.005
i-Inositol	180	35
InorganicSalts		
Calcium nitrate ($Ca(NO_3)_2$ $4H_2O$)	236	100
Magnesium Sulfate ($MgSO_4$-$7H_2O$)	246	100
Potassium Chloride (KCl)	75	400
Sodium Bicarbonate ($NaHCO_3$)	84	2000
Sodium Chloride (NaCl)	58	6000
Sodium Phosphate dibasic (Na_2HPO_4) anhydrous	142	800
Other Components		
D-Glucose (Dextrose)	180	2000
Glutathione (reduced)	307	1
Phenol Red	376.4	5

Table 4: RPMI basal medium (Invitrogen,)

Components	Molecular Weight	Concentration (mg/L)
AminoAcids		
Glycine	75	50
L-Alanine	89	25
L-Alanyl-L-Glutamine	203	406
L-Arginine	211	105
L-Asparagine-H2O	132	50
L-Aspartic acid	133	30
L-Cysteine hydrochloride	158	100
L-Cystine	240	24
L-Glutamic Acid	147	75
L-Histidine	155	31
L-Isoleucine	131	52.4
L-Leucine	131	52.4
L-Lysine	146	58
L-Methionine	149	15
L-Phenylalanine	165	32
L-Proline	115	40
L-Serine	105	25
L-Threonine	119	4
L-Tryptophan	204	10
L-Tyrosine	181	36
L-Valine	117	46
Vitamins		
Ascorbic Acid	176	50
Biotin	244	0.1

Choline chloride	140	1
D-Calcium pantothenate	477	1
Folic Acid	441	1
Niacinamide	122	1
Pyridoxal hydrochloride	204	1
Riboflavin	376	0.1
Thiamine hydrochloride	337	1
Vitamin B12	1355	1.36
i-Inositol	180	2
InorganicSalts		
Calcium chloride ($CaCl_2$-$2H_2O$)	147	264
Magnesium Sulfate ($MgSO_4$-$7H_2O$)	246	200
Potassium Chloride (KCl)	75	400
Sodium Bicarbonate ($NaHCO_3$)	84	2200
Sodium Chloride (NaCl)	58	6800
Sodium Phosphate monobasic (Na_2HPO_4-$2H_2O$)	156	158
Other Components		
D-Glucose (Dextrose)	180	1000
Lipoic Acid	206	0.2
Phenol Red	376.4	10
Sodium Pyruvate	110	110

Table 5: αMEM basal medium formulation (Invitrogen).

Substance	Concentration	Supplier
Iscove's		Biochrom, F0465
B27 supplement or B27 supplement minus insulin or custom-made supplement	4%	Invitrogen, 17504044 or 0050129SA
Non-essential amino acids	1%	Invitrogen, 11140035
L-Glutamine	2 mmol/L	Invitrogen 25030-081
Penicillin/Streptomycin	100 U/ml / 100 mg/ml	Invitrogen, 15070-063
Ascorbic acid	0.3 mmol/L	Sigma, A8960
hFGF	10 ng/ml	Peprotech, AF-100-18B
hIGF	20 ng/ml	Peprotech, AF-100-11
hVEGF	10 ng/ml	Peprotech, AF-100-20
hTGF-β1, day 0-3	5 ng/ml	Peprotech, 100-21
Bovine collagen, acid soluble DM6	0.8 mg/ml	Devros Medical

Table 6: Serum-free, defined medium for EHM generation.

Substance	Concentration	Supplier
αMEM		Invitrogen, 32561-029
B27 supplement or B27 supplement minus insulin or custom-made supplement	4%	Invitrogen, 17504044 or 0050129SA
Penicillin/Streptomycin	100 U/ml / 100 mg/ml	Invitrogen, 15070-063
hFGF	10 ng/ml	Peprotech, AF-100-18B
hIGF	20 ng/ml	Peprotech, AF-100-11
hVEGF	10 ng/ml	Peprotech, AF-100-20

hTGF-β1, day 0-3	5 ng/ml	Peprotech, 100-21
Bovine collagen, acid soluble DM6	0.8 mg/ml	Devros Medical

Table 7: Alternative serum-free, defined medium for EHM generation.

The invention is further described by the following embodiments:

1 A method for producing engineered heart muscle (EHM), the method comprising the steps of:

(i) providing a serum-free reconstitution mixture in one or more moulds, said reconstitution mixture comprising (a) a serum-free minimum essential medium; (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) and 0.2-2 mg/ml collagen; and (c) a mixture of human cardiac myocytes and human non-myocytes, wherein 20 to 80% of the total cell mixture are cardiac myocytes; wherein the reconstitution mixture has a pH of 7.2 to 7.6;

(ii) culturing the serum-free reconstitution mixture in said one or more moulds, whereby the serum-free reconstitution mixture is allowed to condense for at least 15 min;

(iii) culturing the mixture obtained in step (ii) in said one or more moulds in a serum-free EHM culture medium until the mixture condenses to at least 50% of its original thickness, wherein said EHM culture medium comprises (a) a basal medium comprising 0.5-3 mmol/L Ca^{2+}; (b) a serum-free supplement as defined in (i)(b); (c) 0.5-10 mmol/L L-glutamine; (d) 0.01-1.0 mmol/L ascorbic acid; (e) 1-100 ng/ml IGF-1; and (f) 1-10 ng/ml TGFβ1;

(iv) culturing the mixture obtained in step (iii) under mechanical stretching in a serum-free EHM culture medium as defined in step (iii) (a)-(f), whereby force-generating EHM is formed.

2 The method of embodiment 1, wherein the minimum essential medium in step (i) is selected from Iscove's medium, αMEM, DMEM, and RPMI.

3 The method of embodiment 2, wherein the basal medium is Iscove's medium or αMEM.

4 The method of embodiment 2, wherein the basal medium is Iscove's medium.

5 The method of any one of embodiment 1-4, wherein the serum-free supplement of step (i) further comprises one or more components selected from the group consisting of vitamin A, D-galactose, linoleic acid, linolenic acid, progesterone, and putrescine.

6 The method of any one of embodiment 1-5, wherein the serum-free supplement in component (b) of step (i) is B27® supplement or B27® supplement minus insulin.

7 The method of embodiment 6, wherein the serum-free supplement in component (b) of step (i) is 2-6% (v/v) B27® supplement or B27® supplement minus insulin.

8 The method of embodiment 6, wherein the serum-free supplement in component (b) of step (i) is 4% (v/v) B27® supplement or B27® supplement minus insulin.

9 The method of any one of embodiment 1-8, wherein said reconstitution mixture of step (i) comprises 0.3-0.5 mg collagen per 1.5 x 10^6 cardiac myocyte and non-myocyte cell mixtures.

10 The method of embodiment 9, wherein said reconstitution mixture of step (i) comprises about 0.4 mg collagen per 1.5 x 10^6 cardiac myocyte and non-myocyte cell mixtures.

11 The method of any one of embodiment 1-10, wherein in component (c) of the reconstitution mixture of step (i) said collagen is selected from the group consisting of collagen type I, collagen type III, collagen type V, and a mixture thereof.

12 The method of any one of embodiment 1-11, wherein in component (c) of the reconstitution mixture of step (i) at least 90% of said collagen is collagen type I.

13 The method of any one of embodiment 1-12, wherein in component (c) of the reconstitution mixture of step (i) said collagen is of medical grade.

14 The method of any one of embodiment 1-13, wherein in component (c) of the reconstitution mixture of step (i) said collagen is of human origin, bovine origin, or marine origin.

15 The method of any one of embodiment 1-14, wherein in component (c) of the reconstitution mixture of step (i) said collagen further comprises one or more extracellular matrix components selected from the group consisting of elastin, laminin, entactin, nidogen, proteoglycan, and fibronectin.

16 The method of any one of embodiment 1-15, wherein the reconstitution mixture of step (i) has a pH of 7 to 7.8.

17 The method of embodiment 13, wherein the reconstitution mixture of step (i) has a pH of about 7.4.

18 The method of any one of embodiment 1-17, wherein the cardiac myocytes are human cardiac myocytes.

19 The method of any one of embodiment 1-18, wherein the cardiac myocytes are derived from embryonic stem cells, wherein the cell is 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.

20 The method of any one of embodiment 1-19, wherein the cardiac myocytes are derived from induced pluripotent cells, parthogenetic stem cells, or adult stem cells.

21 The method of any one of embodiment 1-20, wherein the cardiac myocytes are obtained by serum-free differentiation.

22 The method of any one of embodiment 1-21, wherein the cardiac myocytes are non-human primate stem cell-derived, fetal or neonatal cardiac myocytes.

23 The method of any one of embodiment 1-22, wherein the cardiac myocytes are provided in admixture with cells of one or more class of cells selected from the group of non-myocytes such as fibroblasts, endothelial cells, smooth muscle cells, and mesenchymal stem cells.

24 The method of embodiment 23, wherein the cardiac myocytes admixture contains 20-80% cardiac myocytes.

25 The method of embodiment 23, wherein the cardiac myocytes admixture contains 30-70% cardiac myocytes.

26 The method of embodiment 23, wherein the cardiac myocytes admixture contains 40-60% cardiac myocytes.

27 The method of embodiment 23, wherein the cardiac myocytes admixture contains 50% cardiac myocytes.

28 The method of any one of embodiment 1-27, wherein the non-myocytes are fibroblasts or endothelial cells.

29 The method of any one of embodiment 1-28, wherein the non-myocytes are fibroblasts.

30 The method of any one of embodiment 1-29, wherein the non-myocytes express CD90.

31 The method of any one of embodiment 1-30, wherein the cardiac myocytes are provided in step (i) in a cell concentration of at least 2.7-20 x 10^6 per ml.

32 The method of embodiment 31, wherein the cardiac myocytes are provided in step (i) in a cell concentration of at least 2.9-10 x 10^6 per ml.

33 The method of embodiment 31, wherein the cardiac myocytes are provided in step (i) in a cell concentration of at least 3.1-5 x 10^6 per ml.

34 The method of embodiment 31, wherein the cardiac myocytes are provided in step (i) in a cell concentration of at least 3.3-3.4 x 10^6 per ml.

35 The method of any one of embodiment 1-34, wherein in step (ii) the mould is ring-, multiangular-, disc- or pouch-shaped.

36 The method of any one of embodiment 1-35, wherein culturing in step (ii) is carried out for 0.25-3h.

37 The method of embodiment 36, wherein culturing in step (ii) is carried out for 0.5-1.5h.

38 The method of any one of embodiment 1-37, wherein culturing is carried out at a temperature range of 30-40 °C.

39 The method of embodiment 38, wherein culturing is carried out at a temperature range of 36-38 °C.

40 The method of embodiment 39, wherein culturing is carried out at about 37 °C.

41 The method of any one of embodiment 1-40, wherein culturing is carried out in a humidified cell culture incubator in the presence of 5-10% CO_2.

42 The method of any one of embodiment 1-41, wherein the serum-free supplement in component (b) of step (iii) is B27® supplement or B27® supplement minus insulin.

43 The method of embodiment 42, wherein the serum-free supplement in component (b) of step (iii) is 2-6% (v/v) B27® supplement or B27® supplement minus insulin.

44 The method of embodiment 43, wherein the serum-free supplement in component (b) of step (iii) is 4% (v/v) B27® supplement or B27® supplement minus insulin.

45 The method of any one of embodiment 1-44, wherein said serum-free supplement of step (iii) further comprises one or more components selected from the group consisting of vitamin A, D-galactose, L-carnitine, linoleic acid, linolenic acid, progesterone, and putrescine.

46 The method of any one of embodiment 1-45, wherein the basal medium comprised in said EHM culture medium in step (iii) is selected from Iscove's medium, αMEM, DMEM, and RPMI.

47 The method of embodiment 46, wherein the basal medium is Iscove's medium or αMEM.

48 The method of embodiment 47, wherein the basal medium is Iscove's medium.

49 The method of any one of embodiment 1-48, wherein the serum-free EHM culture medium comprises about 20 ng/ml IGF1.

50 The method of any one of embodiment 1-49, wherein the IGF1 is human IGF1.

51 The method of any one of embodiment 1-50, wherein the serum-free EHM culture medium comprises about 5 ng/ml TGFβ1.

52 The method of any one of embodiment 1-51, wherein the TGFβ1 is human TGFβ1.

53 The method of any one of embodiment 1-52, wherein the serum-free EHM culture medium further comprises VEGF, FGF, or both VEGF and FGF.

54 The method of any one of embodiment 1-53, wherein the VEGF is human VEGF.

55 The method of any one of embodiment 1-54, wherein the FGF is human FGF.

56 The method of embodiment 53, wherein the serum-free EHM culture medium comprises about 5-20 ng/ml VEGF.

57 The method of embodiment 56, wherein the serum-free EHM culture medium comprises about 10 ng/ml VEGF.

58 The method of embodiment 53, wherein the serum-free EHM culture medium comprises about 5-20 ng/ml FGF.

59 The method of embodiment 58, wherein the serum-free EHM culture medium comprises about 10 ng/ml FGF.

60 The method of any one of embodiment 1-59, wherein the serum-free EHM culture medium in step (iii) additionally 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.

61 The method of any one of embodiment 1-60, wherein culturing in step (iii) is carried out for at least 3 days.

62 The method of embodiment 61, wherein culturing in step (iii) is carried out for about 3 to about 7 days.

63 The method of any one of embodiment 1-62, wherein the culturing in step (iv) is carried out for a period of at least 3-60 days.

64 The method of embodiment 63, wherein the further culturing is carried out for 4-30 days.

65 The method of embodiment 63, wherein the further culturing is carried out for 5-20 days.

66 The method of embodiment 63, wherein the further culturing is carried out for 6-10 days.

67 The method of embodiment 63, wherein the further culturing is carried out for 7 days.

68 The method of any one of embodiment 1-67, wherein step (iv) is carried out on a stretch device.

69 The method of embodiment 68, wherein the stretch device applies a static, phasic or dynamic stretch.

70 The method of any one of embodiment 1-69, wherein said EHM generates more than 0.01 mN force upon induction with 3 mM calcium as determined using the method described in Zimmermann et al. Circ. Res. 90, 223-230 (2002).

71 The method of embodiment 70, wherein said EHM generates more than 0.1 mN force upon induction with 3 mM calcium as determined using the method described in Zimmermann et al. Circ. Res. 90, 223-230 (2002).

72 The method of embodiment 70, wherein said EHM generates more than 0.2 mN force upon induction with 3 mM calcium as determined using the method described in Zimmermann et al. Circ. Res. 90, 223-230 (2002).

73 The method of embodiment 70, wherein said EHM generates more than 0.3 mN force upon induction with 3 mM calcium as determined using the method described in Zimmermann et al. Circ. Res. 90, 223-230 (2002).

DESCRIPTION OF THE FIGURES

Figure 1. hEHM formation. hEHM after 10 days in culture (3 days in the casting mold followed by 7 days on a custom made spacer; culture day 3 + 7). Bar: 1 mm (.

Figure 2. Characterization of hEHM contractile function. (**A**) Chronotropic response after stimulation with cAMP-elevating compounds (Iso: beta-adrenergic stimulation; IBMX: phosphodiesterase inhibition, n=6/group). (**B**) Inotropic response of hEHMs to increasing concentrations of extracellular calcium, comparison of hES2 (n=12), hES3 (n=12) and iPS I2 (n=3) (**C**) Inotropic response of hEHMs (hES2) to isoprenaline (triangles) and carbachol (squares) stimulation. Inset: normalized twitches at low calcium (lowest graph), stimulation with isoprenaline (highest graph) and carbachol (middle graph). (**D**) Relaxation of hEHMs with isoprenaline (triangles) and carbachol (squares) stimulation. Inset: normalized twitches $*P < 0.05$ vs. baseline values (**A**: paired two-tailed Student's *t*-test; **C, D**: ANOVA followed by Dunnet's *post hoc* test).

Figure 3. Importance of non-myocytes for EHM formation. (**A**) Normalized force compared to cardiomyocyte percentage (actinin+ cells) of EHM generated from hES2 line (circles: monolayer cardiac differentiation protocol (Hudson, *et al. Stem Cells Dev* 21: 1513-1523 (2012)), -squares: EB cardiac differentiation protocol (Yang *et al. Nature* 453: 524-528 (2008))) and iPS BJ line (triangles). (**B**) Contractile force of hEHM generated with 100%

cardiomyocytes (CM 100%) and a mixture of 70% cardiomyocytes and 30% cardiac fibroblasts (CM/CF 70/30%), n=1.

Figure 4. GMP-compatible hydrogel matrix. (**A**) Contractile force of hEHMs made with rat collagen (0.4 mg/EHM, n=12) or bovine collagen (0.4 mg/EHM, n=14) at an extracellular calcium concentration of 2 mmol/L. (**B**) Contractile force at 2 mmol/L extracellular calcium of hEHMs made with the "Original protocol" (rat collagen, Matrigel®, n=12) and the "Matrix protocol" (bovine collagen, no Matrigel®, n=9). Please refer also to **Table 1**. (**C**) Contractile force at 2 mmol/L extracellular calcium of hEHMs made with bovine (bov.) collagen only, bovine collagen plus Matrigel (10% v/v), bovine collagen plus fibronectin (5 µg/EHM), and bovine collagen plus fibronectin (5 µg/EHM) plus laminin (5 µg/EHM), n=4/group.

Figure 5. Screening of basal media and serum-free growth supplement vs. Iscove with 20% serum. (**A**) F.l.t.r.: Change in cell death, cardiomyocyte percentage (CM percentage), cardiomyocyte mean actinin fluorescence (CM maturation), cardiomyocyte size based on sideward scatter area (CM size) and non-myocyte size based on sideward scatter area (NM size) of serum free media based on Iscove's, RPMI and aMEM basal media (see also **Table 2-4**) compared to serum containing EHM medium (see also **Table 1**).

(**B**) F.l.t.r.: Change in cell death, cardiomyocyte percentage (CM percentage), cardiomyocyte mean actinin fluorescence (CM maturation), cardiomyocyte size based on sideward scatter area (CM size) and non-myocyte size based on sideward scatter area (NM size) of serum free media with 2% and 4% B27 plus insulin (B27+) and minus insulin (B27-) compared to serum containing EHM medium (see also **Table 1**).

Figure 6. Screening of peptide growth factors and fatty acid supplement. (**A**) Factors considered for serum-free, defined EHM medium. F.l.t.r.: Change in cell death , cardiomyocyte percentage (CM percentage), cardiomyocyte mean actinin fluorescence (CM maturation), cardiomyocyte size based on sideward scatter area (CM size) and non-myocyte size based on sideward scatter area (NM size) by indicated growth factors and fatty acid supplement compared to serum free medium without the factor. (**B**) Factors not considered for serum-free, defined EHM medium. F.l.t.r.: Change in cell death, cardiomyocyte percentage (CM percentage), cardiomyocyte mean actinin fluorescence (CM maturation), cardiomyocyte size based on sideward scatter area (CM size) and non-myocyte size based on sideward scatter area (NM size) by indicated growth factors and fatty acid supplement compared to serum free medium without the factor.

Figure 7. Serum-free, defined EHM generation from hESC cells. (**A**) Contractile force of hEHMs (hES2) with increasing concentrations of extracellular calcium, comparison of serum-containing medium (Serum), and serum-free Iscove's-based medium (SF-IMDM, see also **Table 5**), serum-free aMEM-based medium (SF-aMEM, see also **Table 6**), and serum-free RPMI-based medium (SF-RPMI). Respective EC_{50} for calcium, n=15/5/6/7 for Serum/SF IMDM, SF-aMEM, SF-RPMI, n.d. not determined. (**B**) Immunostaining of serum-free hEHM, showing typical heart muscle properties (muscle bundles). Staining was performed with an antibody against α-sarcomeric actinin and with DAPI for nuclear staining. (**C**) Percent change in force after adrenergic stimulation with 1 µM isoprenaline of serum-containing EHM (Serum), and serum-free Iscove's-based EHM (SF-IMDM), serum-free aMEM-based EHM (SF-aMEM), n=15/5/6, *p<0.05 (**D**) Percent change in force after muscarinic stimulation with 10 µM carbachol of serum-containing EHM (Serum), and serum-free Iscove's-based EHM (SF-IMDM), serum-free aMEM-based EHM (SF-aMEM), n=15/5/6. Bar: 20 µm

Figure 8. Effect of calcium on serum-free hEHM force. A) Contractile force of hEHMs (hES2) with increasing concentrations of extracellular calcium, comparison of RPMI medium (~0.4 mM calcium, n=2), and RPMI medium with addition of 0.8 mM CaCl (final free calcium concentration ~1.2 mM calcium, n=2), **B)** Response to 1 µM isoprenaline of serum-free hEHM cultured in RPMI or RPMI+calcium medium.

Figure 9. Effect of TGFb1 on hEHM force. (**A**) Contractile force of hEHMs (iPS BJ) with increasing concentrations of extracellular calcium, comparison of serum-free medium (SF), and serum-free medium with 5 ng/ml TGFb1 from culture day 0-3 (n=4/group, *p<0.05). (**B**) Comparison of contractile force of human EHM (hES2) in serum-free medium with TGFb1 treatment from culture 0-3 (SF+TGFb1 day 0-3) and culture day 0-10 (SF+TGFb1 day 0-10, n=5/group).

Figure 10. Effect of FGF and VEGF on hEHM force. Contractile force of hEHMs (hES2) with increasing concentrations of extracellular calcium, comparison of serum-free medium (SF, n=3), serum-free medium with 10 ng/ml FGF-2 (SF+FGF, n=3), and serum-free medium with 10 ng/ml VEGF (SF+VEGF, n=3), *p<0.05 SF+FGF vs SF.

Figure 11. Effect of B27 supplement concentration on hEHM force. Contractile force of hEHMs (hES2) with increasing concentrations of extracellular calcium, comparison of serum-containing medium (Serum), and serum-free medium with 2% B27 (SF-2% B27) and serum-free medium with 4% B27 (SF-4% B27), n=17/9/11, *p<0.05 vs 2% B27.

Figure 12. Effect of B27 composition on hEHM force. Contractile force of hEHMs (hES2) with increasing concentrations of extracellular calcium, comparison of serum-containing medium (n=9), serum-free medium with full B27 (n=5), serum-free medium with B27 minus antioxidants (n=5), and serum-free medium with B27 minus insulin (n=5).

Figure 13. Replacement of B27 by custom-made supplement in hEHM from hES and iPS cells. (**A**) Contractile force of hEHMs (hES2) with increasing concentrations of extracellular calcium, comparison of serum-containing medium (n=3), serum-free medium with B27 minus insulin (B27-insulin, n=5), and serum-free medium with custom-made supplement (CMS; n=3). (**B**) Contractile force of hEHMs from iPS cells (BJ) with increasing concentrations of extracellular calcium, comparison of serum-containing medium (n=8), serum-free medium with B27-insulin (n=8), and serum-free medium with custom-made supplement (CMS; n=4).

Figure 14. Maturation of serum-free hEHM with prolonged culture time. (**A**) Contractile force of hEHMs (hiPS-G1) at 2 week (lower graph), 4 week (middle graph), and 8 week (upper graph) time point of culture under serum-free conditions (n=4-6 per condition). (**B**) Brightfield image of individual cardiomyocyte from 8 week old serum-free EHM. Bar: 20 µm.

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.

EXAMPLES

Materials

The materials used herein are commercially available. For example, DMEM, RPMI, αMEM (cat. No. 32561-029), streptomycin, penicillin, and B27 are obtainable from Invitrogen; bovine collagen of medical grade is available from Devros Medical; fatty acid supplement can be ordered from Sigma (cat. No. F7050); and the various growth factors are available from Peprotech (FGF2, AF-1ßß-18B; IGF-1, AF-100-11; TGFβ1, 100-21).

Methods

Human ESC and iPS-lines and culture

The inventors utilized H9.2 (Technion, Haifa, Israel), hES3 (Embryonic Stem Cell International, Singapore) and transgenic hES3-ENVY (Costa, M., *et al. Nat Methods* 2: 259-260 (2005)) as well as hES2 line (McEwen Centre for Regenrative Medicine, Toronto, Canada; Yang *et al. Nature* 453: 524-528 (2008)) in the present study (approval by the Robert-Koch-Institute to W.-H.Z.: permit #12; reference number: 1710-79-1-4-16). Differentiated EBs were shipped to Hamburg/Goettingen at room temperature and arrived within 72-96 hrs. iPS lines were from Toronto (iPS BJ) and Goettingen (iPS I2, Streckfuss-Bomeke et al. Eur Heart J (2012) doi: 10.1093/eurheartj/ehs203, and iPS Sendai).

EBs were digested with collagenase B (1 mg/ml; H9.2), collagenase I (2 mg/ml) and/or trypsin/EDTA (0.25%/1 mmol/l; hES3, hES3-ENVY, hES2, iPS NJ, iPS I2) as described elsewhere (Kehat *et al. J Clin Invest* 108: 407-414 (2001); Mummery *et al. Circulation* 107: 2733-2740 (2003); Xu et al. *Circ Res* 91: 501-508 (2002); Yang *et al. Nature* 453: 524-528 (2008); Passier *et al. Stem Cells* 23: 772-780 (2005); each incorporated herein by reference). Cardiomyocytes were counted in representative aliquots of enzymatically dispersed cells after staining of tropomyosin or sarcomeric actinin.

Basic human engineered heart muscle (hEHM) construction

The inventors constructed hEHMs using a modified EHM-engineering protocol (Zimmermann *et al. Circ Res* 90: 223-230 (2002), incorporated herein by reference). Briefly, EHMs (reconstitution volume: 450 µl) were prepared by pipetting a mixture containing freshly dispersed ESC-derivatives ($1x10^4$ – $15x10^6$ cells in Iscove-Medium with 20% fetal calf serum, 1% non-essential amino acids, 2 mmol/l glutamine, 100 µmol/l β-mercaptoethanol, 100 U/ml penicillin, and 100 mg/ml streptomycin), pH-neutralized collagen type I from rat tails (0.4 mg/EHM), Matrigel™ (10% v/v; Becton Dickenson or tebu), and concentrated serum-containing culture medium (2x DMEM, 20% horse serum, 4% chick embryo extract, 200 U/ml penicillin, and 200 mg/ml streptomycin) in circular molds (inner/outer diameter: 2/4 mm; height: 5 mm) (**Table 1**). hEHM condensed quickly within the casting molds and were transferred onto static stretch devices (110% of slack length) (Zimmermann *et al. Nat Med* 12: 452-458 (2006), incorporated herein by reference) on culture day 3. Medium was changed every other day. hEHM culture under stretch was performed for 7 days.

Another detailed prior art protocol which is suitable to serve as a basis for the improved method disclosed herein is described by Soong et al. Curr Prot Cell Biol. 23.8.1-23.8.21 (2012), which is incorporated herewith in its entirety, and in particular reference is made to the "Basic Protocol 2", and the "Support Protocol 2".

Withdrawal and replacement of xenogenic matrix components

A protocol with reduced xenogenic components (Matrix protocol, **Table 1**) was established to enable pre-GMP hEHM. Cells were reconstituted in a mixture of pH-neutralized bovine collagen (Devros Medical, 0.4 mg/EHM), concentrated serum-containing culture medium (2x DMEM, 40% fetal calf serum, 200 U/ml penicillin, and 200 mg/ml streptomycin) and cultured in Iscove-Medium with 20% fetal calf serum, 1% non-essential amino acids, 2 mmol/l glutamine, 0.3 mmol/l ascorbic acid, 100 µmol/l β-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Withdrawal and replacement of xenogenic medium components

To generate fully defined, serum-free EHM cells were reconstituted in a mixture of pH-neutralized bovine collagen (Devros Medical, 0.4 mg/EHM), concentrated serum-free medium medium (2x DMEM, 8% B27, 200 U/ml penicillin, and 200 mg/ml streptomycin) and cultured in Iscove-Medium with full 4% B27, 1% non-essential amino acids, 2 mmol/l glutamine, 0.3 mmol/l ascorbic acid, 20 ng/ml IGF-1, 10 ng/ml FGF2, 10 ng/ml VEGF, 5 ng/ml TGFb1 (culture day 0-3 only), and 100 U/ml penicillin, and 100 µg/ml streptomycin (Serum-free protocol, **Table 1**). B27 supplement contains vitamins (Biotin, DL Alpha Tocopherol, Acetate DL Alpha-Tocopherol, Vitamin A), proteins and enzymes (BSA, fatty acid free Fraction V, Catalase, Human Recombinant Insulin, Human Transferrin, Superoxide Dismutase), and other cell-supporting components (Corticosterone, D-Galactose, Ethanolamine, Glutathione (reduced), L-Carnitine, Linoleic Acid, Linolenic Acid, Progesterone, Putrescine, Sodium Selenite, and T3 (triodo-I-thyronine). Where indicated full B27 (Invitrogen, A1486701) was compared to B27 without antioxidants (Invitrogen, #10889038) and B27 without insulin (Invitrogen, #0050129SA). B27 supplement was replaced by a custom-made supplement consisting of Albumin, Transferrin, Ethanolamine, Sodium selenite, L-Carnitine HCl, Hydrocortisone, Fatty acid supplement, and Triiodo-L-thyronine (**Table 2**).

Analyses of contractile function

The inventors analyzed force of contraction and twitch kinetics (contraction time: time from 50% to maximal contraction; relaxation time: time from maximal contraction to 50%

relaxation) under isometric conditions as described before (Zimmermann *et al. Circ Res* 90: 223-230 (2002), incorporated herein by reference). Contraction frequency was assessed by light microcopy (unstimulated spontaneous contractions) immediately after removing EHMs from the incubator.

Flow cytometry

EBs cultured in different medium conditions were made into a single cell suspension as described above. Cells were fixed in 70% ice cold ethanol under constant mixing. The cells were stained for sarcomeric actinin (Sigma) to label cardiomyocytes and DAPI to analyze nuclear DNA content and to exclude cell doublets. Cells were run on a LSRII Cytometer (BD). At least 10,000 live cells were analyzed. The following parameters were then analyzed, (1) cell death (percentage of cells in the sub-G1 fraction), (2) cardiomyocyte and non-myocyte percentage (Actinin-positive and negative cells, respectively) (3) Cardiomyocte maturation (mean actinin fluorescence), (4) cardiomyocyte and (5) non-myocyte size (based on sideward scatter area, SSC-A).

Morphological analyses

hEHMs were fixed in neutral buffered 4% formaldehyde/1% methanol, pH 7.4 for confocal laser scanning microscopy (CLSM; Zeiss 510 Meta LSM system or Zeiss 710 LSM) respectively as described earlier (Zimmermann *et al. Circ Res* 90: 223-230 (2002), incorporated herein by reference). For CLSM, the inventors prepared vibratome sections (100 µm; Leica VT1000 S) and subjected them to immune fluorescent labeling with antibodies directed against α-sarcomeric actinin (Sigma clone EA-53, 1:800; with appropriate secondary antibodies). Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole; 1 µg/ml).

Statistical analysis

Data are presented as mean ± standard error of the mean. Statistical differences were determined using paired and unpaired two-tailed Student's *t*-tests or ANOVA followed by Dunnett's *post hoc* test as indicated. A P value < 0.05 was considered statistically significant.

Results

Generation of human engineered heart muscle (hEHM)

EBs - prepared in Haifa (H9.2;), Singapore (hES3 and hES3-ENVY; Costa *et al. Nat Methods* 2: 259-260 (2005)), and Toronto (hHES2; iPS) – were sent to Hamburg/Göttingen by express

mail at room temperature in an air-tight container filled with culture medium. Delivery was ensured within 72-96 h. After arrival, EBs were transferred into fresh culture medium and allowed to recover for 24-48 h. Within that time EBs regained spontaneous contractile activity. EBs were enzymatically dispersed EBs and the resulting single cell suspensions allocated to hEHM generation or cytohistology. An initial series of experiments explored the number of necessary cell quantity per hEHM ($1x10^4$ — $15x10^6$ cells) and utility of different ESC-lines (H9.2, hES2, hES3, hES3-ENVY) and iPS lines (I2, BJ, Sendai) for hEHM construction (n = 67). Spontaneous beating of variably sized areas could be observed in all cultures within 48 h of hEHM casting. However, force-generating hEHMs formed only if 1.25 - $15x10^6$ cells/EHM were utilized (**Fig. 1**). Depending on the size of the EHM, the cell density can easily be adapted.

Organotypic function of hEHM

hEHMs contracted stably and rhythmically (0.8 ± 0.05 Hz at 37°C; n = 14) for at least 3 weeks in culture. The inventors performed a detailed functional characterization at 10 days. Incubation with isoprenaline increased spontaneous beating frequency to 1.2 ± 0.1 Hz (n = 6; P < 0.01 **Fig. 2A**). Additional inhibition of phosphodiesterase with 3-isobutyl-1-methylxanthine (IBMX; 100 µmol/l) caused a further increase in spontaneous beating rate to 1.6 ± 0.1 Hz (n = 6; P < 0.001 **Fig. 2A**). hEHM developed maximal twitch mean forces of 130 ± 13 µN at 2.4 mmol/l calcium (EC_{50}: 0.8 ± 0.04 mmol/l, n = 17; **Fig. 2B**). β-Adrenergic stimulation with 1 µmol/l isoprenaline at sub-EC_{50} extracellular calcium (0.4 mmol/l) increased force of contraction by 47 ± 12% (n = 12; **Fig. 2C**) and shortened relaxation to 113 ± 2.3 ms (n = 12; **Fig. 2D**).

Importance of non-myocytes in EHM formation

All ESC-lines and iPS lines utilized here appeared to be suited for hEHM generation. To test which cardiomyocyte content is optimal for a force generating tissue, the inventors plotted developed force against the cardiomyocyte percentage. Interestingly, a bell-shaped distribution with highest forces developed at a cardiomyocyte percentage of 40-80% was found (**Fig. 3A**).

This observationsuggests a critical role of non-myocytes for proper tissue formation. To investigate this the inventors performed experiment with human cardiomyocytes that were purified by the surface marker CD172a (SIRPα) (Dubois *et al. Nat Biotechnol* 29: 1011-1018 (2011)). EHMs generated from purified cardiomyocytes did not form force-generating tissue

(**Fig. 3B**). Supplementation with 30% human cardiac fibroblasts, however, yielded a stiffer tissue with good force production. These results emphasize that non-myocytes are vital to cardiac tissue formation and that these cells also need to be supported under serum-free conditions.

Generation of hEHM with GMP-compatible matrix

The inventors initially constructed hEHMs based on a protocol that the inventors had developed in a neonatal rat heart cell model (Zimmermann *et al. Biotechnol Bioeng* 68: 106-114 (2000)). This protocol includes several non-human components (including rat collagen, Matrigel, horse serum, fetal calf serum, and chick embryo extract) that are incompatible with a "therapeutic application" *in vivo*. To address this caveat, a series of experiments directly testing whether non-human components of the hEHM-matrix could be reduced were first conducted. Rat collagen was replaced with medical grade (GMP) bovine collagen without loss of performance (**Fig. 4A**). Also, Matrigel®, horse serum and chick embryo extract could be left out without negative impact on hEHM formation and function ("Matrix protocol", **Fig. 4B, Table 1**). Complementing bovine collagen with other extracellular matrix proteins such as fibronectin and laminin, one of the major components of Matrigel®, did not yield additional benefit (**Fig. 4C**).

Definition of serum-free medium to support hEHM formation

To further define the human EHM culture and make it GMP compatible, the inventors sought to replace all undefined serum component with chemically defined supplements. To screen for these supplements the inventors introduced a simplified screening algorithm based on 3D-human embryoid body (EB) cultures. ESCs for this screen were cultured under serum-free conditions (Yang *et al. Nature* 453: 524-528 (2008); Kattman *et al. Cell Stem Cell* 8: 228-240 (2011)). The reference for the screens was our serum-containing EHM medium (**Table 1**): (1) Iscove's, (2) 2 mmol/L L-glutamine, (3) 20% FBS, (4) 1% non-essential amino acids, (5) 0.3 mmol/L ascorbic acid, (6) 100 µmol/l β-mercaptoethanol, (7) 100 U/ml Penicillin/100 mg/ml Streptomycin. As read outs for the beneficial or detrimental role of basal culture medium and supplements a flow cytometry-based protocol (Tiburcy *et al. Circ Res* 109: 1105-1114 (2011); incorporated herein by reference) was established to determine (1) cell death (based on sub-G1 DNA content), (2) cardiomyocyte content (based on actinin expression), (3) cardiomyocyte maturation (based on actinin mean fluorescence per cardiomyocyte), (4) cardiomyocyte size, and (5) non-myocyte size (based on sideward scatter area).

The inventors first screened three basal media formulations (Iscove's, RPMI, αMEM: **Table 3-5**) with and without B27 supplementation. B27 has been used by several groups for differentiation of human ESCs and iPSCs (Burridge et al. *Cell Stem Cell* 10: 16-28 (2012)). The screen demonstrated that B27 was essential for EB formation irrespective of the tested basal medium. Iscove's and RPMI showed comparable results while aMEM appeared to cause slightly higher cell death. On the other hand, αMEM was superior for cardiomyocyte actinin expression (**Fig. 5A**).

Given own preliminary findings showing suboptimal performance of EHMs cultured in the presence of basal RPMI (**Fig. 7**), the inventors continued the screen with Iscove's or aMEM as basal medium. The inventors next scrutinized the utility of B27 in its standard formulation with and without insulin. 4% B27 supplementation with insulin (10 µg/ml final concentration) showed lowest cell death and possibly greater non-myocyte support (lower cardiomyocytes percentage suggest a shift to more non-myocytes) as compared to B27 without insulin and lower B27 (2%) supplement concentration (**Fig. 5B**). The inventors next screened the utility of 6 peptide growth factors at above EC50 concentrations and fatty acid supplements. PDGF-BB, CT-1 and Neuregulin-1 caused substantial cell death whereas IGF-1 appeared to protect from cell death **(Fig. 6A,B)**. TGFβ1, VEGF and fatty acid supplements enhanced individual cardiomyocyte actinin expression. FGF-2 supported non-myocyte growth and increased cardiomyocyte cell size (**Fig. 6A**). TGFβ2 had no beneficial effect (**Fig. 6B**). All of the tested compounds or combinations thereof were implicated in cardiac development and EHM generation (Naito *et al. Circulation* 114: I72-78 (2006); Shimojo *et al. Am J Physiol Heart Circ Physiol* 293: H474-481 (2007); Vantler*et al. J Mol Cell Cardiol* 48: 1316-1323 (2010); Odiete et al. *Circ Res* 111: 1376-1385 (2012); Wollert & Chien *J Mol Med (Berl)* 75: 492-501 (1997); Price *et al. Anat Rec A Discov Mol Cell Evol Biol* 272: 424-433 (2003); Molin *et al. Dev Dyn* 227: 431-444 (2003); Corda *et al. Circ Res* 81: 679-687 (1997); Lopaschuk & Jaswal *J Cardiovasc Pharmacol* 56: 130-140; all incorporated herein by reference).

EHM development is characterized by two stages. Initially there is a "condensation phase" where the isolated cells are "settling in" the matrix, reorganizing themselves and the matrix which may also be accompanied by substantial cell death. This stage is greatly influenced by the non-myocytes. The second stage is the maturation of the tissue under mechanical load. This phase is characterized by hypertrophic growth and maturation of cardiomyocytes, alignment, increasing force development, and matrix stabilization (Tiburcy *et al. Circ Res* 109: 1105-1114 (2011)).

The inventors reasoned that according to the stage different medium conditions may be required. To prevent cell death during the condensation phase the inventors chose a combination of medium components that were neutral or even reducing cell death in the initial screen. This is Iscove's basal medium, 4% B27, and IGF-1. Also, the matrix reorganisation and condensation through non-myocytes was supported by factors that increase number and/or size of non-myocytes (IGF-1, TGF-beta1, FGF-2, in the first stage). VEGF is added for support of cardiomyocyte maturation (**Table 5**). This medium was then tested for its ability to support the formation of force-generating EHM. Serum-free EHM were beating coherently at a spontaneous beating frequency of 113±12 bpm, n=7. Serum containing EHM were beating significantly faster (199±8 bpm, n=8). The inventors found a similar maximal force development and calcium sensitivity compared to the serum containing control **(Fig. 7A)**. Both, Iscove's and αMEM basal medium supported EHM formation with comparable results in contractile force. RPMI did not support force-generating tissue (**Fig. 7A, Table 5, 6**). Morphologically, serum-free EHM contained well developed muscle bundles with anisotropically aligned cardiomyocytes (**Fig. 7B**). Importantly, the serum-free EHM responded to adrenergic and muscarinic stimulation as expected for heart muscle. In fact the αMEM EHM responded significantly better than serum-containing controls. (**Fig. 7 C, D**).

The inventors hypothesized that the insufficient performance of RPMI medium was due to the lower than physiological free calcium concentration of RPMI medium (0.424 mmol/L, **Table 4**). To test if this was true the inventors performed an additional experiment to compare RPMI medium with RPMI medium with 0.8 mM CaCl added (final free calcium concentration 1.242 mmol/L). While EHM with RPMI hardly contracted supplementation of calcium lead to measurable maximal force and better responsiveness to isoprenalin (**Fig. 8**). In fact maximal force production with supplemented RPMI was comparable to IMDM or αMEM medium (**Fig. 7A**) suggesting that a range of ~1 – 2 mmol/L calcium is required for proper tissue function.

To verify the results from the initial screen, the inventors additionally tested the influence of critical factors on the formation of functional EHM. TGFβ1 addition from day 0 to 3 was essential, but prolonged TGFβ1 treatment did not yield an additional benefit (**Fig. 9A, B**). Both FGF and VEGF were able to enhance force-generation confirming an important contribution to tissue formation (**Fig. 10**).

Increasing the B27 supplement concentration to 4% was superior to 2% B27 (**Fig. 11**). To test which components of B27 are essential for EHM function the inventors first performed

an experiment with different commercially available B27 formulations. the inventors compared full B27 with B27 minus insulin, and B27 minus antioxidants. B27 minus antioxidants performed comparable to full B27 suggesting that antioxidants are not required. Surprisingly, B27 minus insulin performed better than full B27 implying that insulin supplementation is also not required (**Fig. 12**).

Based on these results the inventors then developed a custom-made serum supplement to replace B27 (**Table 7**). When the inventors tested this custom-made serum supplement against serum-containing medium and serum-free medium with B27 minus insulin the inventors found comparable maximal force development suggesting that B27 can be omitted from the serum-free EHM culture and replaced by custom-made serum supplement (**Fig. 13A**). These findings were confirmed with hEHM from iPSC demonstrating utility for generation of defined engineered heart muscle from different pluripotent stem cell sources. (**Fig. 13B**).

To investigate if serum-free hEHM supports long-term culture and maturation of cardiomyocytes the inventors tested force production of serum-free hEHM from hIPS-G1 at week 2, week 4, and week 8 of culture. The inventors observed a strong increase in force production (**Fig. 14A**) and rod-shaped morphology with regular cross striation of individual hEHM-derived cardiomyocytes (**Fig. 14B**) indicating well advanced maturation under serum-free conditions.

Conclusion

This study demonstrates for the first time that differentiated, force-generating human heart muscle can be generated *in vitro* under fully defined, serum-free conditions. The protocol works for embryonic (ESC) and induced pluripotent (iPS) stem cells-derived heart muscle.

This is a major breakthrough which enables future *in vitro* studies to investigate e.g. maturation and hypertrophy without confounding serum factors but also potential *in vivo* applications and therapeutic approaches under GMP regulations.

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Claims

1. A method for producing engineered heart muscle (EHM), the method comprising the steps of:
   (i) providing a serum-free reconstitution mixture in one or more moulds, said reconstitution mixture comprising (a) a serum-free minimum essential medium; (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) and 0.2-2 mg/ml collagen; and (c) a mixture of human cardiac myocytes and human non-myocytes, wherein 20 to 80% of the total cell mixture are cardiac myocytes;
   wherein the cardiac myocytes are 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;
   wherein the reconstitution mixture has a pH of 7.2 to 7.6;
   (ii) culturing the serum-free reconstitution mixture in said one or more moulds, whereby the serum-free reconstitution mixture is allowed to condense for at least 15 min;
   (iii) culturing the mixture obtained in step (ii) in said one or more moulds in a serum-free EHM culture medium until the mixture condenses to at least 50% of its original thickness, wherein said EHM culture medium comprises (a) a basal medium comprising 0.5-3 mmol/L Ca^{2+}; (b) a serum-free supplement as defined in (i)(b); (c) 0.5-10 mmol/L L-glutamine; (d) 0.01-1.0 mmol/L ascorbic acid; (e) 1-100 ng/ml IGF-1; and (f) 1-10 ng/ml TGFβ1;
   (iv) culturing the mixture obtained in step (iii) under mechanical stretching in a serum-free EHM culture medium as defined in step (iii) (a)-(f), whereby force-generating EHM is formed.

2. The method of claim 1, wherein the minimum essential medium in step (i) and/or in step (iii) is selected from Iscove's medium, αMEM, DMEM, and RPMI,

preferably wherein the basal medium is Iscove's medium or αMEM, more preferably wherein the basal medium is Iscove's medium.

3. The method of claim 1 or 2, wherein the serum-free supplement of step (i) and/or step (iii) further comprises one or more components selected from the group consisting of vitamin A, D-galactose, linoleic acid, linolenic acid, progesterone, and putrescine.

4. The method of any one of claims 1-3, wherein the serum-free supplement in component (b) of step (i) and/or in component (b) of step (iii) is B27® supplement or B27® supplement minus insulin, preferably wherein the serum-free supplement in component (b) is 2-6% (v/v) B27® supplement or B27® supplement minus insulin, more preferably wherein the serum-free supplement in component (b) is 4% (v/v) B27® supplement or B27® supplement minus insulin.

5. The method of any one of claims 1-4, wherein said reconstitution mixture of step (i) comprises 0.3-0.5 mg collagen per 1.5 x 10^6 cardiac myocyte and non-myocyte cell mixtures, preferably wherein said reconstitution mixture of step (i) comprises about 0.4 mg collagen per 1.5 x 10^6 cardiac myocyte and non-myocyte cell mixtures.

6. The method of any one of claims 1-5, wherein in component (c) of the reconstitution mixture of step (i) said collagen is of medical grade and selected from the group consisting of collagen type I, collagen type III, collagen type V, and a mixture thereof, preferably wherein in component (c) of the reconstitution mixture of step (i) at least 90% of said collagen is collagen type I, and preferably wherein in component (c) of the reconstitution mixture of step (i) said collagen further comprises one or more extracellular matrix components selected from the group consisting of elastin, laminin, entactin, nidogen, proteoglycan, and fibronectin.

7. The method of any one of claims 1-6, wherein the reconstitution mixture of step (i) has a pH of 7 to 7.8, preferably wherein the reconstitution mixture of step (i) has a pH of about 7.4.

8. The method of any one of claims 1-7, wherein the cardiac myocytes are human cardiac myocytes.

9. The method of any one of claims 1-8, wherein the cardiac myocytes are provided in admixture with cells of one or more class of cells selected from the group of non-myocytes such as fibroblasts, endothelial cells, smooth muscle cells, and mesenchymal stem cells, wherein the cardiac myocytes admixture contains 20-80% cardiac myocytes, preferably wherein the cardiac myocytes are provided in step (i) in a cell concentration of at least 2.7-20 x 10^6 per ml.

10. The method of any one of claims 1-9, wherein culturing in step (ii) is carried out for 0.25-3h, preferably for 0.5h - 1.5h.

11. The method of any one of claims 1-10, wherein the serum-free EHM culture medium comprises (i) about 20 ng/ml human IGF1; (ii) about 5 ng/ml human TGFβ1; and/or (iii) about 5-20 ng/ml human VEGF and/or about 5-20 ng/ml human FGF.

12. The method of any one of claims 1-11, wherein the serum-free EHM culture medium in step (iii) additionally 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.

13. The method of any one of claims 1-12, wherein culturing in step (iii) is carried out for at least 3 days, preferably for about 3 to about 7 days.

14. The method of any one of claims 1-13, wherein the culturing in step (iv) is carried out for a period of at least 3-60 days, preferably for 4-30 days, more preferably for 5-20 days, even more preferably 6-10 days, and most preferably for 7 days,
wherein step (iv) is carried out on a stretch device, preferably wherein the stretch device applies a static, phasic or dynamic stretch.

15. The method of any one of claims 1-14, wherein said EHM generates more than 0.01 mN force upon induction with 3 mM calcium as determined using the method described in Zimmermann et al. Circ. Res. 90, 223-230 (2002), preferably wherein said EHM generates more than 0.1 mN force, more preferably whereis said EHM generates more than 0.2 mN force, and most preferably wherein said EHM generates more than 0.3 mN force upon induction with 3 mM calcium.