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(54) **Title:** METHOD FOR CONDITIONING STEM CELLS

(57) **Abstract:** There is provided a method of stimulating an isolated stem cell comprising two stimulation steps: (a) cell mechanical stimulation which comprises the application, to a culture medium comprising the isolated stem cells, of one or more mechanical pulses having a stretching value comprised from 5% to 15%, and a duration of time comprised from 300 to 700 ms, at a frequency comprised from 0.5 to 1.5 Hz; and (b) cell electrical stimulation which comprises the application, to a culture medium comprising the isolated stem cells, of one or more electrical pulses having a potential value comprised from 0mv/cm to 1000mv/cm, and a duration of time comprised from 1 to 3 ms, at a frequency comprised from 0.5 to 1.5 Hz. There is also provided the cell obtainable by the method, further products comprising the cell such as solutions and scaffolds, and its uses in tissue engineering and regenerative therapy.



Method for conditioning stem cells

The present invention relates to an electromechanical method for stimulating and conditioning stem cells, to the cells obtainable by the method and to their
5 uses. The conditioned cells obtainable by the method have multiple applications in tissue engineering and regenerative therapy, especially in the treatment of an infarcted heart.

BACKGROUND ART

10 Cardiovascular diseases remain the most common cause of mortality worldwide. Myocardial infarction (MI) leads to irreversible sequelae such as impaired heart function and in some cases to shorter life span. Heart transplantation is hampered by the low number of donor organs, while
15 mechanical ventricular assist devices are mostly used as a bridge to transplant. With the first clinical trials under way, cardiac tissue engineering is now emerging as a new therapeutic modality. Most cardiac tissue engineering approaches combine cells with biomaterials in a three-dimensional (3D) context.

20 In the developing and adult heart, cardiac cells are constantly subjected to cyclic loading induced by electrical signals. On the one hand, mechanical stretch improves contractility, facilitates secretion of growth factors and calcium handling in cardiomyocytes, modifies extracellular matrix synthesis in
25 cardiac fibroblasts and seems to improve heart muscle survival, cell alignment, elongation, hypertrophy, and differentiation. On the other hand, electrical stimulation is crucial for synchronized contraction of cardiac muscle, enhancing impulse propagation, ultrastructural organization, cell elongation and alignment; and the formation of functional gap junctions.

30 Accordingly, new ways of generating functional tissues from undifferentiated cells with applications in regenerative medicine are gaining momentum, as they could be used as an alternative to transplantation. The application of electrical or mechanical stimuli to a stem cell has the potential to accelerate its
35 differentiation and maturation into a functional cardiomyocyte. A cell conditioned in this way can have several applications in regenerative therapy. One such application might be in direct cell therapy, where stimulus-driven

conditioned cells can be administered directly to damaged cardiac areas with the goal of repairing heart function. This administration can be in different forms such as scaffolds, hydrogels, solutions, and others.

- 5 At a first approach, several mechanical or electrical stimulation processes have been disclosed in an attempt to obtain functional differentiated cells. Govoni and colleagues (cf. Govoni M., et. al. "Mechanostimulation protocols for cardiac tissue engineering" Biomed. Res. Int. 2013 eub), summarizes the progress in the area of dynamic culture devices for mechanical stimulation.
- 10 Tandon and colleagues (Tandon N., et.al. "Optimization of electrical stimulation parameters for cardiac tissue engineering" J. Tissue Eng. Regen. Med. 2011, vol 5, e115-e125) summarizes the optimization of a variety of parameters and conditions involved in the electrical stimulation of neonatal rat cardiomyocytes.
- 15 In an attempt to improve the results obtained with either the mechanical or electrical stimulation alone, a few groups have explored the application of both stimuli combined. Morgan and colleagues (Morgan K., et.al. "Mimicking isovolumic contraction with combined electromechanical stimulation improves the development of engineered cardiac constructs" Tissue Engineering Part A, 2014, vol. 20, pp. 1654-1667), disclosed a method for electro-mechanically stimulating neonatal cardiac cells in a 3D layout. The method comprised mechanical stimulation at 5% stretch with a 50% duty cycle at 1Hz of frequency and electrical stimulation with biphasic pulses of 1ms duration at 1Hz with a voltage of 3V/cm.
- 25 In addition, Pavesi and colleagues (Pavesi A., et.al. "Controlled electromechanical cell stimulation on-a-chip" Scientific Reports 2015, vol. 5, pp. 1-12) discloses the electro-mechanical stimulation of human mesenchymal stem cells (hMSCs). Mechanical stimulation is applied by 3% or 7% strain at 1Hz, whereas electrical stimulation is applied with a biphasic signal of 1ms with an average of 5V/cm at 1Hz. This reference reports changes in morphology, cytoskeletal fiber orientation and expression profile for some markers (such as MYH7) for the stimulated hMSC.
- 30
- 35 Similarly, Wang, B. and colleagues (Wang B. et.al. "Myocardial scaffold-based cardiac tissue engineering: application of coordinated mechanical and electrical stimulations", Langmuir 2013, vol. 29, pp. 11109-11117), also

describes an electro-mechanical stimulation protocol for mesenchymal stem cells in a 3D based bioreactor. However, the whole conditioning strategy described is based on the use of 5-azacytidine, a very potent demethylating agent which induces differentiation by biochemical means. The treatment of cells with chemical reagents could raise safety concerns in ultimate clinical applications.

In spite of the efforts made, there is still the need of further methods for stimulating stem cells for regenerative purposes..

SUMMARY OF THE INVENTION

Inventors have devised a simple and very efficient electromechanical method of stimulating stem cells. Surprisingly, inventors have found that the electrical stimulation with much lower voltages than the ones usually applied (in the field of stem cell priming) lead to a more promising differentiation in terms of expression profile. This finding is unexpected and could be advantageous since submitting the cells to lower voltages which might be less harmful, and yet the differentiation is enhanced as the cells reveal a marker profile with a wider range of upregulated genes which are crucial for the correct functioning of the cardiomyocyte.

In particular, as it is shown below, inventors have applied the method of the invention to a population of human adipose tissue-derived progenitor of cardiac origin (cardiac ATDPCs). Cardiac ATDPCs reside in the epicardial fat and display cardiac and endothelial differentiation potential, as well as beneficial histopathological and functional effects, both when injected as a cell solution into the infarct border zone of a heart and when implanted within a fibrin patch. The electro-mechanical conditioning of cardiac ATDPCs with the method of the invention and subsequent encapsulation in a fibrin scaffold that was implanted onto the ischemic myocardium of infarcted mice, was found to properly boost cardiac function. This is an indication that the method devised leads to a highly functional cell, opening up more reliable cell-based applications in regenerative therapy of the heart.

Thus, a first aspect of the present invention is a method of stimulating an isolated stem cell comprising two stimulation steps: (a) cell mechanical

stimulation which comprises the application, to a culture medium comprising the isolated stem cells, of one or more mechanical pulses having a stretching value comprised from 5% to 15%, and a duration of time comprised from 300 to 700 ms, at a frequency comprised from 0.5 to 1.5 Hz; and (b) cell electrical
5 stimulation which comprises the application, to a culture medium comprising the isolated stem cells, of one or more electrical pulses having a potential value comprised from 10mv/cm to 1000mv/cm, and a duration of time comprised from 1 to 3 ms, at a frequency comprised from 0.5 to 1.5 Hz, for a total period of time from 4 to 10 days, being performed steps a) and b) in any
10 order.

Of note, the method of the invention entails the use of a voltage that is ca. 2 orders of magnitude lower than the usual voltage that is found in several references of the prior art (ca. 50 mV/cm compared to the usual ca. 5V/cm).
15 Unexpectedly, these particular conditions have been found to be advantageous since the stem cell obtained by applying this method reveals a very desirable marker profile, having a wide range of important cardiac markers such as Tbx5 (gene TBX5), GATA-4 (gene GATA4), Cx43 (gene GJA1), BETA-Myosin Heavy Chain (β -MyHC, gene MYH7) or SERCA2 (gene
20 ATP2A2) upregulated as is supported in the experimental section found below. This is an outstanding result when compared to closely related methods such as the one disclosed by Pavesi et.al., or Morgan et. al. (*vide supra*). The method disclosed in the former reference yields a cell whose expression profile lacks upregulation in highly relevant markers for the correct
25 functioning of a cardiomyocyte such as GATA-4 or Cx43. The method disclosed in the latter reference yields a cell that does not seem to lead to an overall change in a wide series of physiologically relevant cardiomyocyte markers.

30 Additionally, it should be emphasized that the method of the invention works well without the need to resorting to biochemical stimulation as the one disclosed in Wang et.al. (*vide supra*), although it could easily be complemented with it.

35 A second aspect of the invention is the stimulated stem cell obtainable by the method of the first aspect of the invention.

A third aspect of the present invention is a veterinary or pharmaceutical composition comprising a therapeutically effective amount of the stimulated stem cell of the second aspect of the invention together with one or more pharmaceutically acceptable vehicles or carriers.

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A fourth aspect of the invention is the stimulated cell of the second aspect of the invention for use as a medicament.

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A fifth aspect of the invention is the stimulated stem cell of the second aspect of the invention or the veterinary or pharmaceutical composition of the third aspect of the invention for use in regenerative therapy.

BRIEF DESCRIPTION OF THE DRAWINGS

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FIG. 1. Electro-mechanical stimulator. (A) SolidWorks design of the printed circuit board used to perform electro-mechanical conditioning on six 3.5-cm culture plates (on the left), and detail of a PDMS silicone substrate (on the right). (B) Electro-mechanical stimulator (left) and side view magnification (right). (C) Diagram showing the stimulation regime applied to the cultured cells: electrical (blue) and mechanical (red) stimulations. Pulse durations and periods, delay between electrical and mechanical pulses, and mechanical pulse shape are programmable through a LabView custom application.

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FIG. 2. Gene and protein analyses after electro-mechanical conditioning of ATDPCs. (A) Real-time PCR of main cardiac genes in cardiac and subcutaneous ATDPCs. Relative expression of cardiomyogenic markers in EMC vs non-conditioned controls is shown for cardiac and subcutaneous ATDPCs. Values were normalized to GAPDH expression and are shown as mean $\pm$ SEM for six independent experiments. $\#P < 0.1$ (trend) and $*P < 0.05$ (significance) vs the subcutaneous ATDPC group. (B-M) Protein expression in cardiac and subcutaneous ATDPCs on a vertical patterned surface, perpendicular to the electric field (E) and stretching (Force, F), as shown on the middle drawing. Phalloidin staining (actin F) and Cx43 expression, SERCA2 and MEF2, and sarcomeric α -actinin and GATA-4 expression in control (B, D, F, H, J, L) and EMC (C, E, G, I, K, M) cardiac (left) and subcutaneous (right) ATDPCs. Nuclei were counterstained with DAPI (B-E, L, M). Scale bars = 50 μ m.

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FIG. 3. Macroscopic evaluation of the 3D engineered construct with cardiac ATDPCs cultured for 21 days prior to *in vivo* grafting. (A) Bright field image composition of the cellular fibrin patch under standard culture conditions. (B) PKH26 cell labeling of cardiac ATDPCs in the fibrin patch and magnification of the core region (B'). (C) Representative image showing cell viability in a fibrin patch loaded with cardiac ATDPCs, as performed by the Live/Dead assay. Magnification of a construct border zone with an abundance of viable cells (light grey) (C'). (D) Representative photograph of an excised heart from a post-infarction animal (visible ligation) 21 days after the cellular fibrin construct implantation (asterisk). (E) Representative image of Masson's trichromic staining of heart cross-section from a MI+EMC treated animal and its magnification (E'). Scale bars = 1 mm (A-E) and 20 μ m (B', C').

FIG. 4. Migration and differentiation of electro-mechanically conditioned cardiac ATDPCs in a mouse model of MI. Immunofluorescence analysis of heart cross-sections at 21 days for cTnI and Cx43 (A-C), GATA-4 and cTnI (C), SERCA2 and MEF2 (D), sarcomeric α -actinin and CD31 (E), vimentin and cTnI (F-H), and SMA (grey) and cTnI (green) (I-K). Cardiac ATDPCs were labeled with PKH26 and nuclei were counterstained with DAPI. Circles indicate the migration of cardiac ATDPCs into murine tissue. Arrowheads show representative expressions of the protein of interest in each panel. Fibrin = F, Myocardium = M, Scar = S. Scale bars = 20 μ m.

FIG. 5. Vascular analysis. (A-C) GSLI B4 isolectin staining showing vessel-like structures within the fibrin construct loaded with cardiac ATDPCs at 21 days post-infarction, as well as in the myocardium-fibrin interphase (A) and the scar (B). An isolectin-positive vessel connects the murine myocardium stained with cTnI and the fibrin patch (C). Nuclei were counterstained with DAPI. White arrowheads indicate vessels and microvessels. Scale bars = 20 μ m. (D-F) MI+EMC heart cross-sections stained with light green Masson's trichromic (D), Gallego's modified trichromic (E), and Movat's pentachromic stainings (F). Functional vessels with erythrocytes are observed in the fibrin construct and connecting the myocardium with the fibrin gel. Scale bars = 20 μ m. (G) Histogram showing the vessel density expressed as the percentage of the area occupied by isolectin-positive cells in control (MI, MI+Fibrin) and cardiac ATDPC-treated (MI+Con, MI+EMC) groups. Sham-operated animals

are also present. Values are mean $\pm$ SEM. * $P_n < 0.05$. All animals were included in the analysis (n=39). (H) Representative GSLI B4 isolectin staining in MI (upper) and MI+EMC (lower) animal heart cross-sections displaying vessel density qualitative differences. Scale bars = 20 μ m.

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FIG. 6. Functional analysis. (A) Left ventricle ejection fraction (LVEF) assessed by echocardiography at baseline, 2 days post-MI, and at 21 days (pre-sacrifice), in the parasternal short-axis view, relative to their LVEF value at baseline. Mean values $\pm$ SEM. All animals were included in the analysis (n=39). (B) Representative M-mode echocardiograms of a control MI animal showing left ventricle anterior wall absence of motility (left), and an MI+Con (centre) and an MI+EMC (right) treated animals exhibiting left ventricle anterior wall motility recovery before sacrifice (please see Table 1 for more data).

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DETAILED DESCRIPTION OF THE INVENTION

For the sake of understanding, the following definitions are included and expected to be applied throughout description, claims and drawings.

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The term "stem cell" as used herein is a cell which is undifferentiated. Such a cell can undergo differentiation when submitted to one or more stimuli. The stimuli might be physical, mechanical, electrical, chemical, biochemical, biological or a combination of any of the latter. By stem cell, it is to be understood here either pluripotent or multipotent cells, and either embryonic or induced.

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The term "mechanical stimulation" as used herein refers to applying a mechanical force to a cell with the goal of inducing a change in its phenotype. One particular form of mechanical stimulation of a cell is stretching, where the cell is elongated by the forces applied, to a higher or lesser degree. Stretching, in the context of the present invention, is applied by the lineal elongation of the substrate where cells are adhered, and is measured by the relative length increment of the substrate. In particular, when it is said that a stretching of 10% is applied to the cell, it is meant that the elongation is homogeneous in all the substrate and is then transferred to the cells.

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The term “electrical stimulation” as used herein refers to applying electrical pulses to a cell with the goal of inducing a change in its phenotype. The current applied can in principle be continuous or alternate, and the electrical pulses can be monophasic and biphasic. By “monophasic” it is here
5 understood that only positive pulses are applied, whereas by biphasic it is understood that both positive and negative pulses are applied, in a way that the average voltage is zero or near zero to avoid electrolysis. The biphasic pulse can take a variety of forms, such as balanced, imbalanced, balanced with delay, balanced with fast reversal and others. In particular, when it is said
10 that 50mV/cm is applied, it is meant that enough voltage is applied to ensure that the electric field in the cell culture is 50 mV/cm, measured as a voltage fall of 50 mV when two auxiliary measurement electrodes are placed at a distance of 1 cm in the region between the two excitation electrodes.

15 When the two stimuli of the invention (mechanical and electrical) are “synchronized” it is herein understood that both operate and work at the same rate or speed, or operate together with a difference in time (between both) that propagates indefinitely. Each stimulus will have a certain periodicity, and the spacing or overlap between both is maintained all along a timeline. If the two
20 stimuli are not overlapped, it means they are not submitted to the cell in an overlapped time window. When they are overlapped, it is understood that the cell receives both stimuli at the same time. The electrical stimulus has a very short time window (ca. 2ms), whereas the mechanical stimulus has a longer time window (ca. 500ms). This means that the overlap of the former on the
25 latter can take many forms. The window of the electrical stimulus can overlap with the much longer window of the mechanical stimulus at its initial, middle or final phase. If they are both started at the same time, the electrical stimulus will overlap in the initial phase of the mechanical stimulus.

30 The terms “priming” and “conditioning” are herein used interchangeably, and refer to a process by which a stem cell is committed to differentiation to a certain cell lineage via a stimulus or a combination of stimuli (in the present case mechanical and electrical). In the experimental data found herein, the lineage is a stem cell of the ATDPCs that is committed to differentiate to a
35 cardiomyocyte.

The term “monolayer” as opposed to 3D, as used herein means that the cells

that are stimulated in a cell culture are arranged in 2D, that is, they are not disposed on top of one another forming piling coats.

5 The term "overexpression" as used herein refers to the stimulated stem cell of the second aspect of the invention and it means that the stimulated cell shows an expression level of a particular marker that is higher than that shown by the same cell prior to the stimulating process.

10 The term "expression level" can be understood as the amount of cardiac gene expression measured using well-established protocols. In the present case, the protocol used was based on quantitative real-time PCR. Total RNA from ATDPCs was used to synthesized the cDNA using random hexamers (Qiagen) and the iScript™ One-Step RT-PCR Kit (BioRad Laboratories). cDNA was preamplified with the TaqMan® PreAmp Master Mix Kit (Applied Biosystems) and then diluted 1:5 with RNase-free water. Real-time PCR
15 amplifications were performed with 2.5 µL cDNA in a final volume of 10 µL, containing 5 µL TaqMan 2× Universal PCR Master Mix, 2 µL RNase-free water, and 0.5 µL FAM-labeled primer/probe (Applied Biosystems). Data were collected and analyzed in duplicate on the Light Cycler® 480 Real-Time PCR
20 System (Roche). The Livak method was used to quantify the absolute ($2^{-\Delta\Delta CT}$) and relative ($2^{-\Delta CT}$) expression of each gene between electro-mechanically conditioned and control samples, using GAPDH as an endogenous reference.

25 The term "therapeutically effective amount" as used herein, refers to the amount of the stimulated cell of the invention that, when administered, is sufficient to regenerate the tissue. The particular dose administered according to this invention will of course be determined by the particular circumstances surrounding the case, including the cell type administered, the route of administration, the particular condition being treated, the recipient subject, and
30 similar considerations.

In the present invention, the term "pharmaceutically acceptable vehicles or carriers" refers to pharmaceutically acceptable materials, compositions or excipients. Each component must be pharmaceutically acceptable in the
35 sense of being compatible with the other ingredients of the pharmaceutical composition. It must also be suitable for use in contact with the tissue or organ of humans and animals without excessive toxicity, irritation, allergic response,

immunogenicity or other problems or complications commensurate with a reasonable benefit/risk ratio. Likewise, the term "veterinary acceptable" means suitable for use in contact with a non-human animal.

5 As mentioned above, a first aspect of the present invention is a method of stimulating an isolated stem cell comprising two stimulation steps: (a) cell mechanical stimulation which comprises the application, to a culture medium comprising the isolated stem cells, of one or more mechanical pulses having a stretching value comprised from 5% to 15%, and a duration of time comprised
10 from 300 to 700 ms, at a frequency comprised from 0.5 to 1.5 Hz; and (b) cell electrical stimulation which comprises the application, to a culture medium comprising the isolated stem cells, of one or more electrical pulses having a potential value comprised from 10mv/cm to 1000mv/cm, and a duration of time comprised from 1 to 3 ms, at a frequency comprised from 0.5 to 1.5 Hz,
15 for a total period of time from 4 to 10 days, being performed steps a) and b) in any order.

In a particular embodiment of the first aspect of the invention, the method is a method, wherein the mechanical stimulation step comprises the application of
20 one or more pulses having a stretching value comprised from 8% to 12% and a time duration comprised from 400 to 600 ms at a frequency of 1 Hz; and the electrical stimulation step comprises the application of one or more pulses having a potential value comprised from 25mv/cm to 99mv/cm and a time duration comprised from 1.5 to 2.5 ms, at a frequency of 1Hz;
25 for a total period of time from 6 to 8 days.

In a particular embodiment of the first aspect of the invention, the method is a method, wherein the mechanical stimulation step comprises the application of
30 one or more pulses having a stretching value comprised from 8% to 12% and a time duration comprised from 400 to 600 ms at a frequency of 1 Hz; and the electrical stimulation step comprises the application of one or more pulses having a potential value comprised from 40mv/cm to 60mv/cm and a time duration comprised from 1.5 to 2.5 ms, at a frequency of 1Hz;
for a total period of time from 6 to 8 days.

35 In another particular embodiment of the first aspect of the invention, the method is a method wherein the mechanical stimulation step comprises the

application of one or more pulses having a stretching value comprised from 8% to 12% and a time duration comprised from 400 to 600 ms at a frequency of 1 Hz; and the electrical stimulation step comprises the application of one or more pulses having a potential value of 50mV/cm and a time duration
5 comprised of 2.0 ms at a frequency of 1Hz;
for a total period of time from 6 to 8 days.

In another particular embodiment of the first aspect of the invention, the method is a method wherein the mechanical stimulation step comprises the
10 application of one or more pulses having a stretching value of 10% and a time duration comprised of 500 ms at a frequency of 1 Hz; and the electrical stimulation step comprises the application of one or more pulses having a potential value comprised from 25mv/cm to 99mv/cm and a time duration comprised from 1.5 to 2.5 ms, at a frequency of 1Hz;
15 for a total period of time from 6 to 8 days.

In another particular embodiment of the first aspect of the invention, the method is a method wherein the mechanical stimulation step comprises the application of one or more pulses having a stretching value of 10% and a time
20 duration of 500 ms the cell at a frequency of 1 Hz and the electrical stimulation step comprises the application of one or more pulses having a potential value of 50mv/cm and a time duration of 2 ms at a frequency of 1Hz;
for a total period of time of 7 days.

25 In another particular embodiment of the first aspect of the invention, the stem cells are in the culture medium in the form of a monolayer.

In another particular embodiment of the first aspect of the invention, the cell electrical stimulation is performed by applying pulses of alternate current.
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In another particular embodiment of the first aspect of the invention, the electrical pulses are biphasic pulses. In another particular embodiment of the invention, the biphasic pulses are balanced. In another particular embodiment of the invention, the biphasic pulses are imbalanced. In another particular
35 embodiment of the invention, the biphasic pulses are balanced with delay. In another particular embodiment of the invention, the biphasic pulses are with slow reversal.

In another particular embodiment of the invention, the biphasic pulses are with fast reversal.

- 5 In another particular embodiment of the first aspect of the invention, the stem cells are in the culture medium in the form of a monolayer and the cell electrical stimulation is performed by applying pulses of alternate current.

10 When applied specifically to the production of functional cardiomyocytes, the invention can be re-phrased as a method for cardiomyocyte production comprising two stimulation steps: (a) cell mechanical stimulation which comprises the application, to a culture medium comprising the isolated stem cells, of one or more mechanical pulses having a stretching value comprised from 5% to 15%, and a duration of time comprised from 300 to 700 ms, at a
15 frequency comprised from 0.5 to 1.5 Hz; and (b) cell electrical stimulation which comprises the application, to a culture medium comprising the isolated stem cells, of one or more electrical pulses having a potential value comprised from 10mv/cm to 1000mv/cm, and a duration of time comprised from 1 to 3 ms, at a frequency comprised from 0.5 to 1.5 Hz, for a total period of time from
20 4 to 10 days, being performed steps a) and b) in any order.

Accordingly, all the embodiments which refer to the method of stimulating an isolated stem cell are also embodiments of the method of production of functional cardiomyocytes from a stem cell.

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In another particular embodiment of the invention, the mechanical stimulation of step a) and the electrical stimulation of step b) are synchronized.

30 In another particular embodiment of the invention, the two stimuli are overlapped.

In another particular embodiment of the invention, the electrical stimulus is overlapped with the initial phase in the time window of the mechanical stimulus.

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In another particular embodiment of the invention, the electrical stimulus is overlapped with the middle phase in the time window of the mechanical

stimulus.

In another particular embodiment of the invention, the electrical stimulus is overlapped with the final phase in the time window of the mechanical stimulus.

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In another particular embodiment of the invention, the two stimuli are not overlapped.

In another particular embodiment of the invention, the stem cell is pluripotent.

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In another particular embodiment of the invention, the stem cell is induced pluripotent.

In another particular embodiment of the invention, the stem cell is multipotent.

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In another particular embodiment of the invention, the stem cell is a mesenchymal stem cell.

In another particular embodiment of the invention, the stem cell is a progenitor cell.

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In another particular embodiment of the invention, the mesenchymal stem cell is a cardiac adipose tissue-derived progenitor cell.

As mentioned above, a second aspect of the present invention is a stimulated stem cell obtainable by the method according to the first aspect of the invention.

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In a particular embodiment of the second aspect of the invention, the stem cell obtained overexpresses at least one marker selected from GATA-4 and Cx43.

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In a particular embodiment of the second aspect of the invention, the stem cell obtained overexpresses both GATA-4 and Cx43. In another embodiment of the second aspect of the invention, the stimulated stem cell overexpresses GATA-4, Cx43, and one or more additional markers selected from the group consisting of: β -MyHC, SERCA2 and Tbx5. In another embodiment of the second aspect of the invention, the stimulated stem cell overexpresses GATA-4, Cx43, β -MyHC, SERCA2 and Tbx5.

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As mentioned above, the third aspect of the present invention is a veterinary or pharmaceutical composition comprising a therapeutically effective amount of the stimulated stem cell of the second aspect of the invention together with
5 one or more pharmaceutically acceptable vehicles or carriers.

The veterinary or pharmaceutical composition of the third aspect can comprise the stimulated cell of the second aspect together with other constituents such as extracellular matrix proteins, growth factors, pro-angiogenic factors, cardiac
10 differentiation factors, and other proteins or metabolites with bioactive activity.

In a particular embodiment of the third aspect of the invention, the composition comprises a biocompatible scaffold or biocompatible matrix comprising the stimulated cell. The matrix could be synthetic or biological, but in any case it
15 must be biocompatible, so the cells are nicely accommodated and the cellular therapeutic effect takes place. The role of the scaffold is to provide not only mechanical support but also the physical and biological cues necessary to direct engraftment, differentiation and maturation of cells and to guide proper alignment to produce a functional tissue. Then, the matrix should support cell
20 migration, proliferation, survival and retention; but also maintain a sufficient flow of nutrients and oxygen. Additionally, the perfect matrix would be biodegradable, nontoxic, absorbable and able to be replaced with the cardiac extracellular matrix secreted by stromal cells/fibroblasts, which equates to viable new tissue. Among the possible matrices, degradable synthetic
25 polymers, biologic materials such as an extracellular matrix (ECM), or hybrid scaffolds stand out.

In a particular embodiment of the third aspect of the invention, the composition comprises a biocompatible hydrogel comprising the stimulated cell. Hydrogels
30 are water-insoluble polymers pre-formed by chemical or physical cross-linking of water-soluble precursors, constituted of either natural or synthetic polymers. The natural polymers may be polysaccharides, proteins and their derivatives, such as collagen, fibrin, alginate, and hyaluronic acid. They possess biological activities that include cell recruiting, modulation of the inflammatory
35 microenvironment and promoting neovascularization. Among the synthetic polymers polyethylene glycol, polylactic acid, polylactic acid-co-glycolic acid, polycaprolactone, polyacrylamide and polyurethane stand out.

In a particular embodiment of the third aspect of the invention, the composition comprises a biocompatible solution comprising the stimulated cell.

- 5 As mentioned above, the fourth aspect of the invention is the stimulated cell of the second aspect of the invention for use as a medicament.

The fourth aspect can be reformulated to the use of the stimulated cell of the second aspect of the invention for the preparation of a medicament.

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The fourth aspect can further be reformulated as a method of treatment which comprises administering a therapeutically effective amount of the stimulated cell of the second aspect of the invention to a subject in need thereof, including a human.

15

As mentioned above, a fifth aspect of the present invention is the stimulated stem cell of the second aspect of the invention or the veterinary or pharmaceutical composition of the third aspect of the invention for use in regenerative therapy.

20

In a particular embodiment of the fifth aspect of the invention, the use is in the treatment of a disease selected from the group consisting of: myocardial infarction, myocarditis, diabetes, neuronal degeneration, spinal cord injury, Crohn's disease, aplastic anemia, rheumatoid arthritis, brain injury, graft
25 versus host disease, liver cirrhosis, fulminant hepatic failure, osteoarthritis, multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer disease, systemic lupus erythematosus, Parkinson's disease, and muscular dystrophy. In another embodiment, the disease is myocardial infarction.

- 30 Throughout the description and claims the word "comprise" and variations of the word, are not intended to exclude other technical features, additives, components, or steps. Furthermore, the word "comprise" and its variations encompass the term "consisting of". Additional objects, advantages and features of the invention will become apparent to those skilled in the art upon
35 examination of the description or may be learned by practice of the invention. The following examples are provided by way of illustration, and they are not intended to be limiting of the present invention. Furthermore, the present

invention covers all possible combinations of particular and preferred embodiments described herein.

EXAMPLES

5

Materials and Methods

Human ATDPCs isolation and culture

Human ATDPCs were isolated from cardiac (cardiac ATDPCs) and
10 subcutaneous (subcutaneous ATDPCs) adipose tissues obtained from
patients undergoing cardiac surgery, under a protocol approved by the
Germans Trias i Pujol University Hospital Ethics Committee. Informed consent
was obtained from all patients, and the study protocol conformed to the
principles of the Declaration of Helsinki. Tissues were obtained from a total of
15 11 patients (cardiac adipose tissue) and 6 patients (subcutaneous adipose
tissue). The cells isolated from each tissue source were pooled and used for
experiments. Subcutaneous ATDPCs were used as control cells for cardiac
ATDPCs *in vitro* experimentation.

20 Adipose tissue biopsy samples were harvested and processed as described in
previous publications of the inventors. Briefly, samples were rinsed with PBS
and cut into small pieces, and visible blood vessels were removed; next, cells
were isolated by collagenase II (Gibco) digestion. Adhered cells were grown in
 α -MEM (Sigma) supplemented with 10% fetal bovine serum (Gibco), 1 mM L-
25 glutamine (Gibco), and 1% penicillin/streptomycin (Gibco), and cultured under
standard conditions (37°C and 5% CO₂).

Electro-mechanical stimulation device

The custom-made electro-mechanical stimulation unit is an improvement of
30 the electrical stimulation set-up already described in WO2013185818A1. This
one consisted in a combination of a monophasic programmable electrical
device, a printed circuit board that facilitated the robust connection of the
electrodes, and a biocompatible polydimethylsiloxane (PDMS) (Sylgard 184,
Dow Corning Corp.) silicone construct designed to provide structural support
35 to cells, electrodes and magnets (FIG. 1A,B). The disposable stimulation
silicone accommodates a cell culture pool in a flexible area (1cm×1cm×2mm)
and holds two electrodes built with platinum wire wrapped around a

Polytetrafluoroethylene core, placed at two opposing sides of the flexible area creating an electric field to induce electrical stimulation. To provide a mechanical stimulation simultaneously and synchronously with the electrical one, two neodymium magnets (Supermagnete, Gottmadingen, Germany) were embedded in the PDMS constructs (Fig. 1A,B). The use of magnets enables the performance of non-invasive mechanical stimulation. Two additional magnets are placed outside the culture plates. One of them is static and fixes one end of the silicone construct and the second one pulls the other end following a programmable pattern thanks to a computer-controlled linear motor (LM 2070-040-11 + MCLM3006S Faulhaber, Schönaich, Germany). The device has six parallel channels to provide stimulation to up to six culture plates and permits the combination of both electrical and mechanical stimulation either independently or synchronously. Pulse amplitudes, durations and periods, delay between electrical and mechanical pulses and mechanical pulse shape are programmable through a LabView (National Instruments) custom application (Fig. 1C).

Cell culture with electro-mechanical conditioning

Concisely, 3×10^4 cells were seeded on each PDMS construct one day before the beginning of the stimulation. The conditioning lasted 7 days and unstimulated cells were used as a control for electro-mechanical conditioning, while subcutaneous ATDPCs were used as a control for cardiac ATDPCs. The electro-mechanical conditioning protocol consisted in: alternating current 2-ms monophasic square-wave pulses of 50 mV/cm at 1 Hz and 10% stretching for 7 days. This was conducted 6 times (with at least 3 replicates each) for the *in vitro* experimentation (gene and protein analyses) and 7 days was the endpoint. Immunostainings were performed on the cells attached to the PDMS construct and fixed with formalin 10% at day 7. Gene analyses were carried out after the trypsinization of the cells attached to the PDMS construct for both control and stimulated groups (n=6).

For the animal studies, cardiac ATDPCs were harvested from stimulated or unstimulated PDMS constructs after 7 days, the fibrin patch was immediately produced and kept under standard culture conditions for less than 24 hours before it was implanted. Briefly, 1×10^5 cells were mixed with 8 μ L of fibrinogen solution (70-110 mg/mL), followed by the addition of 8 μ L of thrombin solution (500 UI/mL) for jellification (Tissucol duo; Baxter). The area of the fibrin patch is $\sim 7 \text{ mm}^2$ and $\sim 1 \text{ mm}$ height. This was carried out 11 times (with 6-12

replicates each) for the whole *in vivo* experimentation (n=39 animals) and 21 days was the endpoint. Echocardiographic measurements were acquired at baseline (2 days before the surgery), post-MI (2 days after the surgery) and at pre-sacrifice (21 days after the surgery) for all animals.

5

Animal studies

The animal study protocol was approved by the Institutional Animal Care and Use Committee and complied with guidelines concerning the use of animals in research and teaching, as defined by the Guide for the Care and Use of
10 Laboratory Animals (NIH Publication No. 80-23). All procedures were performed in accordance with both the national and European legislation (Spanish Royal Decree RD 53/2013 and EU Directive 2010/63/EU) for the protection of animals used for research experimentation.

MI model and fibrin-cell patch delivery

Briefly, mice were anesthetized with a mixture of O₂/isoflurane (2%) (Baxter), intubated, and mechanically ventilated (90 breaths/min, 0.1 mL tidal volume) using a SAR830/AP small animal ventilator (CWE, Inc.). An anterior thoracotomy was performed and the proximal left anterior descending (LAD)
20 coronary artery was occluded using a 7-0 silk suture. Sham animals were operated in the same manner with no occlusion of the LAD coronary artery before implantation of the fibrin-cell patches. To generate the adhesive construct, Tissucol solution (8 µL) with 1×10^5 cells or culture medium was mixed with 8 µL of thrombin solution for jellification (Tissucol duo; Baxter).
25 Fibrin patches with or without cardiac ATDPCs were implanted using Glubran® surgical glue (Cardiolink), which fulfils the required safety and compatibility standards for use in experimental animals and humans, to seal the edge of the patch to the myocardium. The animals were sacrificed 21 days after the operation. Using cardioplegic solution, hearts were arrested in
30 diastole and then excised, fixed in 10% formalin solution (Sigma), cryopreserved in 30% sucrose in PBS, embedded in OCT (Sakura Finetek Europe B.V.), and snap-frozen in liquid nitrogen-cooled isopentane for histological analysis (16).

Experimental groups

The study was performed on 39 female SCID mice (11-15 weeks old and weighing 20-25 g; Charles River Laboratories) using cardiac ATDPCs. Cells

were labeled prior to fibrin patch inclusion using the PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labelling (Sigma) following manufacturer's protocol. Mice were distributed randomly into the following groups: MI alone (MI) (n = 8), MI with cell-free fibrin implants (MI+Fibrin) (n = 6), MI with implantation of fibrin loaded with naïve -control- cardiac ATDPCs (MI+Con) (n = 8), and MI with implantation of fibrin loaded with electro-mechanically conditioned (EMC) cardiac ATDPCs (MI+EMC) (n = 5). Sham groups that lacked MI and underwent implantation of control fibrin-cell patches (Sham+Con) (n = 7) and EMC fibrin-cell patches (Sham+EMC) (n=5) served as control groups. The global mortality in the experiment was only 7.14%.

Quantitative real-time PCR

Total RNA was isolated from cardiac and subcutaneous ATDPCs using the AllPrep RNA/Protein Kit (Qiagen). cDNA was synthesized using random hexamers (Qiagen) and the iScript™ One-Step RT-PCR Kit (BioRad Laboratories) according to the manufacturer's protocol. cDNA was preamplified with the TaqMan® PreAmp Master Mix Kit (Applied Biosystems) and then diluted 1:5 with RNase-free water.

Real-time PCR amplifications were performed with 2.5 µL cDNA in a final volume of 10 µL, containing 5 µL TaqMan 2× Universal PCR Master Mix, 2 µL RNase-free water, and 0.5 µL FAM-labeled primer/probe (Applied Biosystems), including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1), T-box transcription factor (Tbx5) (Hs00361155_m1), myocyte-specific enhancer factor 2A (MEF2A) (Hs01050409_m1), GATA-binding protein 4 (GATA-4) (Hs00171403_m1), α-actinin (Hs00241650_m1), cardiac Troponin I (cTnI) (Hs00165957_m1), connexin43 (Cx43) (Hs00748445_s1), sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA2) (Hs00544877_m1), and β-myosin heavy chain 7 (β-MyHC) (Hs00165276_m1).

The following cardiac markers were evaluated: transcription factors (Tbx5, MEF2A, GATA-4), structural genes (α-actinin, cTnI, β-MyHC), and calcium-handling related genes (Cx43, SERCA2).

Data were collected and analyzed in duplicate on the Light Cycler® 480 Real-Time PCR System (Roche). The Livak method was used to quantify the absolute ($2^{-\Delta\Delta CT}$) and relative ($2^{-\Delta CT}$) expression of each gene between electro-

mechanically conditioned and control samples, using GAPDH as an endogenous reference.

Immunofluorescence

- 5 Cells attached to the PDMS construct were fixed with 10% formalin, permeabilized, blocked in 10% normal horse serum for 1h, and incubated for 1h at room temperature with primary antibodies raised against Cx43 (6.4 µg/mL; Sigma), sarcomeric α-actinin (11.5 µg/mL ascites fluid; Sigma), GATA-4 (4 µg/mL; R&D), MEF2 (4 µg/mL; Santa Cruz), and SERCA2 (4 µg/mL; Santa Cruz). Secondary antibodies were conjugated with Cy2 and Cy3 (7.5 µg/mL; Jackson ImmunoResearch), and actin fibers (actinF) were stained with Phalloidin Alexa 568 (0.161 µM; Invitrogen). Nuclei were counterstained with DAPI (0.1 µg/mL; Sigma). Images were acquired with the Axio Observer Z1 inverted microscope (Zeiss).
- 10
- 15 Eight serial cryosections per sample were stained with light green Masson's trichromic (Collagen: green; Myocardial fibers: red; Nuclei: black or brown; Cytoplasm: pink), Gallego's modified trichromic (Collagen: blue; Myocardium: yellow-pink; Elastic fibers: purple; Nuclei: fuchsia) and Movat's pentachromic (Nuclei: black; Collagen: yellow; Ground substance: blue; Muscle: purple; Elastic fibers: brownish grey) staining from all groups.
- 20 Further immunoanalyses were performed on cryosections using specific monoclonal antibodies against Cx43 (Sigma), sarcomeric α-actinin (Sigma), GATA-4 (R&D), MEF2 (Santa Cruz), SERCA2 (Santa Cruz), cTnI (10 µg/mL; Abcam), phospho-histone 3 (PH3) (1 µg/mL; Cell Signaling), CD31 (4 µg/mL; Abcam), SMA (1:50 ascites fluid; Sigma) and vimentin (10 µg/mL; Abcam).
- 25 The vessel area was assessed in sections stained with biotinylated GSLI B4 isolectin (10 µg/mL; Vector Labs). Secondary antibodies conjugated with Cy2 and Cy3 (Jackson ImmunoResearch). Nuclei were counterstained with DAPI (Sigma). Images were captured under a laser confocal microscope (Axio-Observer Z1, Zeiss). Quantitative histological measurements were made using ImageJ analysis software (NIH).
- 30

Cell viability analysis

- To determine cell viability in the fibrin patches, the LIVE/DEAD® viability/cytotoxicity kit (Invitrogen) was used according to the manufacturer's instructions. Fibrin patches loaded with 1×10^5 cells were cultured for 3 weeks under standard culture conditions, and then washed in PBS prior to staining.
- 35

The stained patch constructs were analyzed and quantified using the confocal microscope (Axio-Observer Z1, Zeiss), and Maximum Projection Intensity plus Tiles-stitching image post-processing were applied (Zen Blue software, Zeiss).

5 Analysis of cardiac function

Cardiac function was assessed by echocardiography using an 18- to 38-MHz linear-array transducer with a digital ultrasound system (Vevo 2100 Imaging System, Visual Sonics). Measurements were made at baseline, 2 days post-MI, and 21 days after surgery (pre-sacrifice) for all 39 animals. The
10 investigators were blinded to the treatment groups. Standard parasternal short-axis views were obtained in B-Mode and M-Mode. Functional parameters were measured, including left ventricle (LV) fractional shortening (LVFS), LV ejection fraction (LVEF), LV anterior wall thickness (LVAWT), LV posterior wall thickness (LVPWT), LV end-diastolic dimension (LVEDD), and
15 LV end-systolic dimension (LVESD).

Statistical analyses

Relative fold changes of cardiac and subcutaneous ATDPCs gene expressions were compared using Student's *t*-test, and the statistical
20 difference was determined for the samples from 6 separate experiments. Vessel density was assessed using one-way ANOVA and Tukey post-hoc analysis for multiple comparisons. Greenhouse-Geisser analysis was used for LVEF repeated measures (baseline and post-MI) to confirm homogeneity of surgical procedure. Paired samples *t*-test was also used to compare
25 differences between baseline and pre-sacrifice echocardiographic parameters in each experimental group. The LVEF differentials (Δ LVEF) between pre-sacrifice and baseline were evaluated using one-way ANOVA and Tukey post-hoc analysis for multiple comparisons.
All the results are presented as the mean $\pm$ SEM. **P* < 0.05 was considered
30 statistically significant. Statistical analyses were performed using SPSS Statistics software (version 21, IBM SPSS Inc.).

Results and Discussion

35 Electro-mechanical conditioning

Electrical and mechanical conditionings were first applied individually in order to optimize each protocol. Electrical conditioning was based on a previous

works of the inventors in which alternating current 2-ms monophasic square-wave pulses of 50 mV/cm at 1 Hz were found optimal. Synchronous mechanical conditioning was performed with an *ad-hoc* custom magnet-driven system (see Methods) operated at a frequency of 1 Hz and a strain of 10% for 7 days. Briefly, 3×10^4 cells were seeded on the silicone surface subjected to stimulation, with changes of culture medium twice a week. Unstimulated cells were used as a control for electro-mechanical conditioning. Subcutaneous ATDPCs were used as a control for cardiac ATDPCs.

- 10 Physiological conditioning promotes expression of cardiac genes and proteins
Electro-mechanical conditioning modulated gene expression in both cell types, with cardiac ATDPCs showing a stronger upregulation of cardiac genes (FIG. 2A). In cardiac ATDPCs, Tbx5 ($\#P = 0.081$), GATA-4 ($*P = 0.050$), and Cx43 ($*P = 0.025$) increased ~2-fold compared to subcutaneous ATDPCs, in
15 response to electro-mechanical conditioning. Additionally, significant and higher increases were also observed on key structural and calcium-related genes, such as β -MyHC (5-fold, $*P=0.000$) and SERCA2 (2.8-fold, $\#P=0.076$) in cardiac ATDPCs EMC samples compared to controls.
- 20 The protein expression of main cardiac markers in non-conditioned controls and EMC ATDPCs is shown in FIG. 2B-M. Cx43 was mostly distributed in the cytoplasm and at the plasma membrane to allow cell connections through gap junctions (FIG. 2B-E). MEF2 was expressed in the nuclei, and SERCA2 and α -actinin were observed in the cytoplasm without mature sarcomere
25 organization nor synchronous beating (FIG. 2F-M). GATA-4 protein expression was detected only in the nuclei of control and EMC cardiac ATDPCs (FIG. 2J,K).

30 Viability and migration of physiologically trained cardiac ATDPCs in 3D fibrin gel

- Cardiac ATDPCs were labeled with PKH26 and cultured *in vitro* in a 3D fibrin gel to assess cell tracking and viability prior to *in vivo* implantation. After 21 days of *in vitro* culture, the fibrin patch maintained its initial morphology and size (FIG. 3A), and the cells retained the PKH26 labeling (FIG. 3B).
35 Importantly, ~84% of cells (green fluorescence) remained viable (FIG. 3C). The engineered fibrin construct was implanted over the infarcted area in the murine model of acute myocardial infarction MI (FIG. 3D) and over healthy

myocardium (sham animals). After 21 days, the physiologically engineered construct (MI+EMC) was nicely attached to the myocardium and almost indiscernible through macroscopic observation (FIG. 3D-E). Construct's adaptation to the murine myocardium surface is shown in different heart cross-sections of MI+EMC animals (FIG. 3E).

Cell migration to underlying ischemic myocardium was observed only occasionally in both control and EMC cardiac ATDPCs fibrin constructs (FIG. 4A,I). *De novo* expression of cTnI was identified in some of the implanted cells (FIG. 4A). Cardiac ATDPCs within the fibrin patch also contained Cx43, GATA-4, SERCA2, MEF2, and α -actinin (FIG. 4A-E), suggesting that the cardiomyogenic lineage gained *in vitro* persisted *in vivo*. Additional proof of cell phenotype is the absence of PH3, associated with proliferation, from cardiac ATDPCs (data not shown).

Remarkably, cell migration between the host tissue and the engineered construct was observed in all treated groups, and was most obvious in animals in which a cell-free fibrin hydrogel was implanted. Analysis of the colonized spindle-shaped cell population observed in the fibrin patch was positive for vimentin and smooth muscle actin (SMA), suggesting the presence of fibroblasts and myofibroblasts (FIG. 4F-K).

Neovascularization of engineered fibrin constructs and underlying myocardium

Fluorescence microscopy demonstrated neovascularization of the fibrin patches in all groups (FIG. 5A-F). Remarkably, colocalization of the GSLI B4 isolectin endothelial marker and PKH26 within the fibrin construct and the underlying scar suggests that EMC cardiac ATDPCs were integrated into vascular structures (FIG. 5A,B). Moreover, inventors also observed vessel connections between the murine myocardium and the engineered fibrin construct (FIG. 5C,F). Refined histological analysis confirmed the presence of erythrocytes inside the vessels within the fibrin construct (FIG. 5D,E), demonstrating the functionality and interconnectivity of these construct neovessels with host tissue circulation (FIG. 5F). SMA was also abundant around scar vessels and engineered fibrin construct neovessels (FIG. 4I-K).

Vessel density was measured in the border zone of the infarcted tissue. Isolectin staining showed ~14% greater vessel density in the subjacent

myocardium that received fibrin patches loaded with both control and EMC cardiac ATDPCs than from MI controls ($P=0.007$ and $P=0.03$ for MI+Con and MI+EMC *versus* MI, respectively) (FIG. 5G). A trend of increase in infarct border zone neovascularization was also observed in the MI+Fibrin group, suggesting that fibrin alone may have angiogenic potential in the ischemic myocardium, an effect that was significantly enhanced when the construct was embedded with cardiac ATDPCs.

Implantation of a physiologically conditioned engineered 3D patch prevents ventricular remodeling and drives cardiac function recovery post-MI

Echocardiographic analyses were conducted to determine whether EMC cardiac ATDPCs exerted a beneficial effect on the restoration of cardiac function after MI (FIG. 6). Statistical analysis confirmed a similar reduction in cardiac function assessed by left ventricle ejection fraction (LVEF) in all infarct groups ($P=0.14$). Significant adverse remodeling assessed by increased ventricular diameters (left ventricle end diastolic diameter and left ventricle end systolic diameter) and depressed function (LVEF and left ventricle shortening fraction) was observed in MI and MI+Fibrin groups; no such ventricular remodeling was present in the MI+Con and MI+EMC groups (Table 1, below). The difference in left ventricle ejection fraction ($\Delta\text{LVEF}_{\text{sacrifice-baseline}}$) between the baseline and pre-sacrifice values was calculated (Table 2, below).

Remarkably, 80% of MI+EMC animals presented a $\Delta\text{LVEF}_{\text{sacrifice-baseline}} \geq -5\%$, which is considered clinically relevant. In contrast, only 0% (MI), 33% (MI+Fibrin) and 50% (MI+Con) of the animals not treated with EMC cells presented clinically relevant $\Delta\text{LVEF}_{\text{sacrifice-baseline}} (\geq -5\%)$. The LVEF trend among the studied groups is shown in FIG 6A. Assessment of $\Delta\text{LVEF}_{\text{sacrifice-MI}}$ (pre-sacrifice – post-MI) in MI+EMC animals showed mean values 5%, 11%, and 12% higher than those observed for MI+Con, MI+Fibrin and MI, respectively (FIG. 6A).

To conclude, inventors have devised a method for synchronous electro-mechanical conditioning of stem cells from heart tissue, and have tested the obtained cells in an engineered 3D fibrin patch for treating infarcted myocardium in a murine model with very promising results. This study provides evidence that the electro-mechanically conditioned ATDPCs

obtained by the method of the invention maintain their cardiomyogenic potential within the *in vivo* environment, migrate to the murine myocardium and scar, improve cardiac function after MI, and increase vessel density. This in vivo data clearly point to the promising character of the stimulated stem cell

5 obtained with the electro-mechanical method herein disclosed.

Table 1. Cardiac function parameters.

	Sham+Con			Sham+EMC			MI			MI+Fibrin			MI+Con			MI+EMC		
	Baseline	Pre- Sacrifice	P value	Baseline	Pre- Sacrifice	P value	Baseline	Pre- Sacrifice	P value	Baseline	Pre- Sacrifice	P value	Baseline	Pre- Sacrifice	P value	Baseline	Pre- Sacrifice	P value
LVAWTd (mm)	0.9±0.1	0.9±0.05	0.64	0.8±0.05	0.8±0.1	0.68	0.9±0.1	0.8±0.1	0.18	0.9±0.05	0.8±0.05	0.31	0.9±0.1	0.8±0.1	0.55	0.7±0.1	0.8±0.1	0.28
LVAWTs (mm)	1.0±0.1	1.2±0.1	0.36	1.0±0.1	1.2±0.1	0.46	1.1±0.1	1.1±0.1	0.70	1.2±0.1	1.1±0.1	0.31	1.1±0.1	1.1±0.1	0.52	1.0±0.1	1.1±0.05	0.20
LVPWTd (mm)	0.8±0.1	0.8±0.05	0.41	1.0±0.1	1.0±0.1	0.69	0.8±0.04	1.0±0.1	0.09	0.7±0.1	0.9±0.1	0.18	0.7±0.05	0.8±0.2	0.45	0.7±0.1	0.8±0.1	0.10
LVPWTs (mm)	1.2±0.1	1.0±0.1	0.06	1.3±0.1	1.3±0.1	0.96	1.1±0.1	1.1±0.1	0.58	1.1±0.1	1.0±0.1	0.77	1.0±0.1	1.1±0.2	0.59	0.9±0.05	1.1±0.1	0.02*
LVEDD (mm)	3.4±0.1	3.5±0.1	0.74	3.3±0.1	3.5±0.1	0.19	3.5±0.02	3.9±0.1	0.03*	3.5±0.1	3.8±0.1	0.03*	3.5±0.1	3.9±0.2	0.11	3.7±0.03	3.8±0.3	0.68
LVEDS (mm)	2.3±0.1	2.4±0.1	0.60	2.2±0.1	2.3±0.1	0.48	2.3±0.05	2.9±0.2	0.01*	2.4±0.1	2.9±0.1	0.02*	2.5±0.1	2.8±0.3	0.20	2.6±0.1	2.7±0.2	0.76
LVFS (%)	33±2	31±3	0.70	33±3	33±3	0.96	34±2	24±2	0.01*	30±1	24±2	0.06	29±1	29±3	0.76	28±2	27±2	0.78
LVEF (%)	64±3	58±8	0.55	62±4	62±5	0.87	61±2	46.7±3	0.01*	58±1	47±4	0.05*	56±1	55±5	0.62	55±3	55±3	0.97

Values are shown as mean $\pm$ SEM. LVAWTd: left ventricle anterior wall thickness diastole; LVAWTs: left ventricle anterior wall thickness systole; LVPWTd: left ventricle posterior wall thickness diastole; LVPWTs: left ventricle posterior wall thickness systole; LVEDD: left ventricle end-diastolic dimension; LVESD: left ventricle end-systolic dimension; LVFS: left ventricle fractional shortening; LVEF: left ventricle ejection fraction.

Table 2. Left ventricle ejection fraction calculated as differentials between values at 21 days post-operation and baseline.

	Δ LVEF (%)	<i>P</i> values
MI	-15 $\pm$ 8	
MI+Fibrin	-11 $\pm$ 8	0.56
MI+Con	-3 $\pm$ 8	0.23
MI+EMC	0 $\pm$ 7	0.03*

Values are shown as the differentials in percentage $\pm$ SEM. LVEF: left ventricle ejection fraction; *P* values are referred to MI group.

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CLAIMS

1. A method of stimulating an isolated stem cell comprising two stimulation steps:

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(a) cell mechanical stimulation which comprises the application, to a culture medium comprising the isolated stem cells, of one or more mechanical pulses having:

- a stretching value comprised from 5% to 15%, and
- 10 - a duration of time comprised from 300 to 700 ms, at a frequency comprised from 0.5 to 1.5 Hz;

and

(b) cell electrical stimulation which comprises the application, to a culture medium comprising the isolated stem cells, of one or more electrical pulses

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having:
- a potential value comprised from 10mv/cm to 1000mv/cm, and
- a duration of time comprised from 1 to 3 ms, at a frequency comprised from 0.5 to 1.5 Hz;

for a total period of time from 4 to 10 days, being performed steps a) and b) in
20 any order.

2. The method of stimulating stem cells according to claim 1, wherein:

the mechanical stimulation step comprises the application of one or more pulses having a stretching value comprised from 8% to 12% and a time
25 duration comprised from 400 to 600 ms at a frequency of 1 Hz; and

the electrical stimulation step comprises the application of one or more pulses having a potential value comprised from 25mv/cm to 99mv/cm and a time duration comprised from 1.5 to 2.5 ms, at a frequency of 1Hz;
for a total period of time from 6 to 8 days.

30

3. The method of stimulating stem cells according to any of the previous claims, wherein the mechanical stimulation step comprises the application of one or more pulses having a stretching value of 10% and a time duration of 500 ms the cell at a frequency of 1 Hz and the electrical stimulation step
35 comprises the application of one or more pulses having a potential value of 50mv/cm and a time duration of 2 ms at a frequency of 1Hz;
for a total period of time of 7 days.

4. The method of stimulating stem cells according to any one of claims 1-3, wherein the stem cells are in the culture medium in the form of a monolayer.
- 5 5. The method of stimulating stem cells according to any one of claims 1-4, wherein the electrical pulses are biphasic pulses.
6. The method of stimulating stem cells according to any one of claims 1-5, wherein the mechanical stimulation of step a) and the electrical stimulation of
10 step b) are synchronized.
7. The method of stimulating stem cells according to claim 6, wherein the two stimuli are overlapped.
- 15 8. The method of stimulating stem cells according to claim 7, wherein the two stimuli are performed simultaneously.
9. The method of stimulating stem cells according to any one of claims 1-8, wherein the stem cell is a mesenchymal stem cell.
20
10. The method of stimulating stem cells according to claim 9, wherein the mesenchymal stem cell is a cardiac adipose tissue-derived cell.
11. A stimulated stem cell obtainable by the method according to any one of
25 claims 1-10.
12. The stimulated stem cell according to claim 11, which overexpresses at least one biomarker selected from GATA-4 and Cx43.
- 30 13. A veterinary or pharmaceutical composition comprising a therapeutically effective amount of the stimulated stem cell of any one of claims 11-12 together with one or more pharmaceutically acceptable vehicles or carriers.
14. The stimulated stem cell of any one of claims 11-12 for use as a
35 medicament.
15. The stimulated stem cell of any one of claims 11-12 or the pharmaceutical

composition of claim 13 for use in regenerative therapy.

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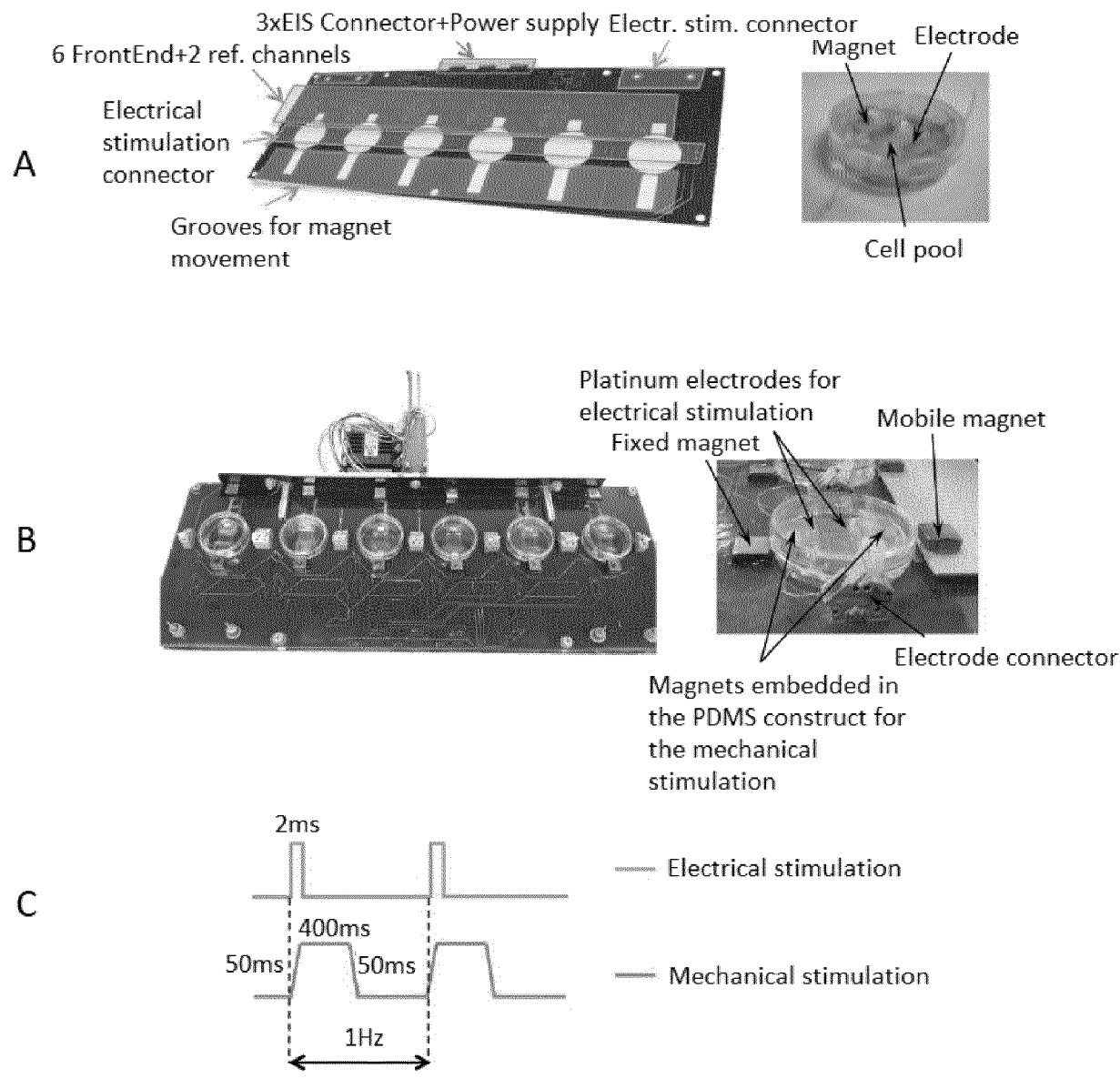


FIG.1

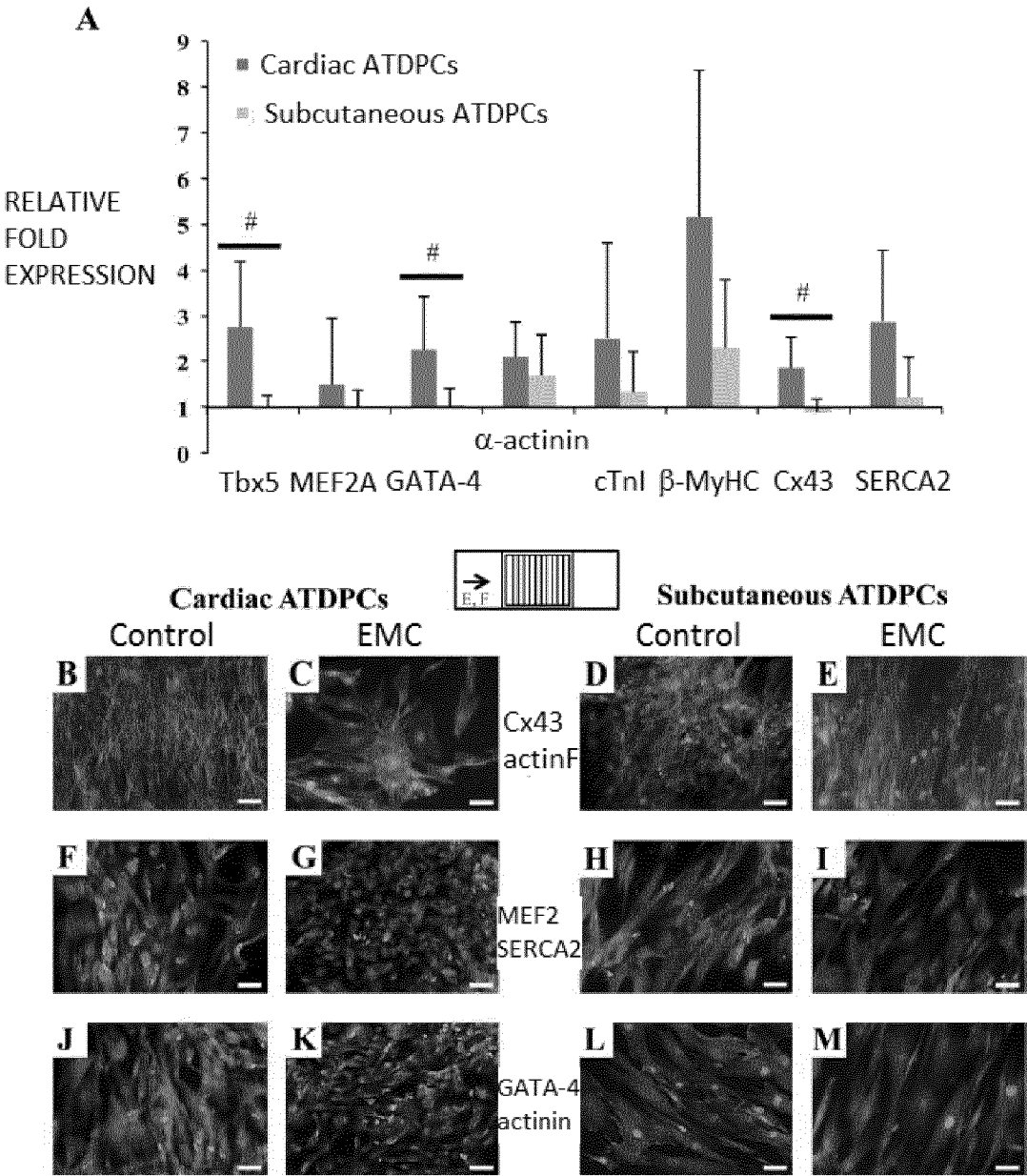


FIG.2

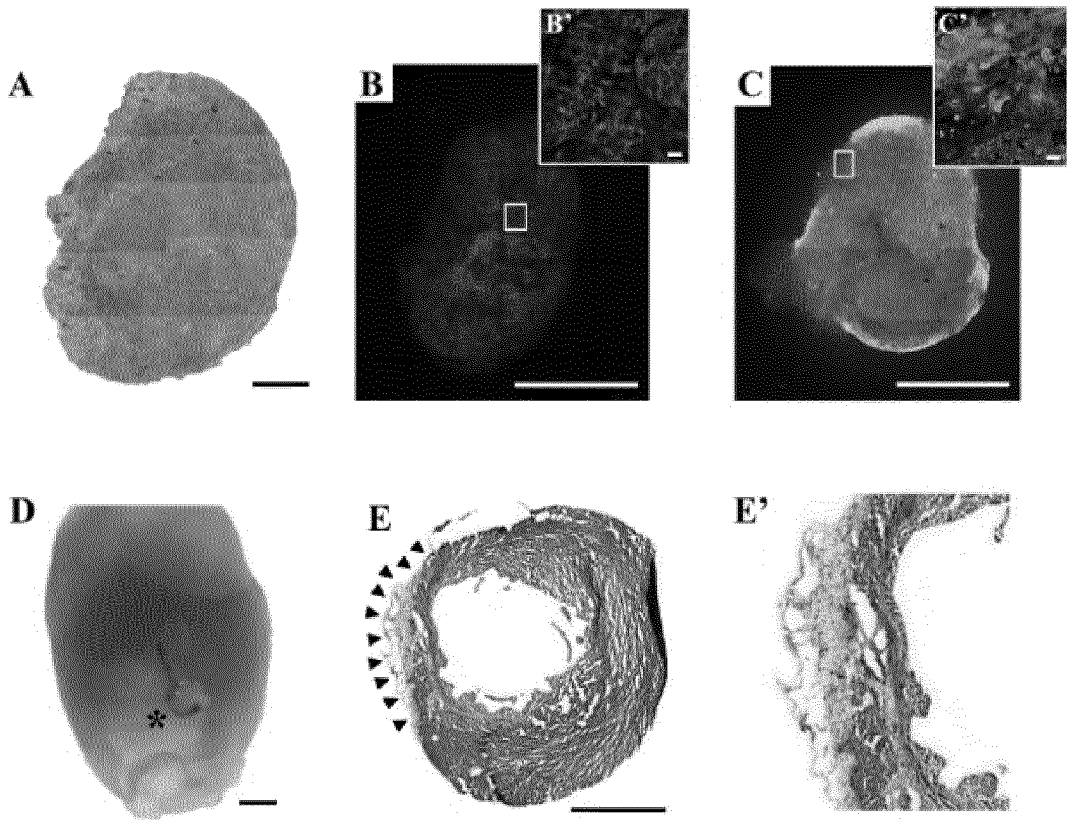


FIG.3

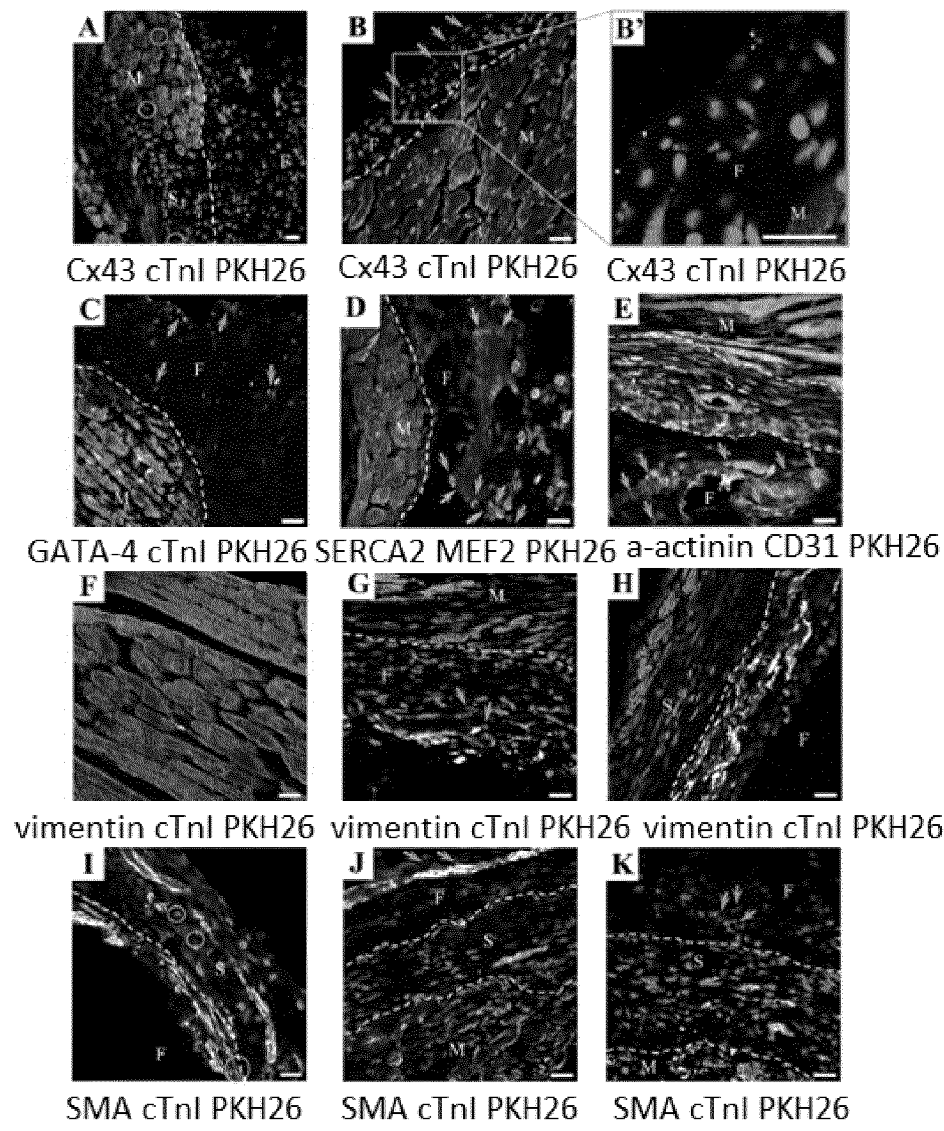


FIG. 4

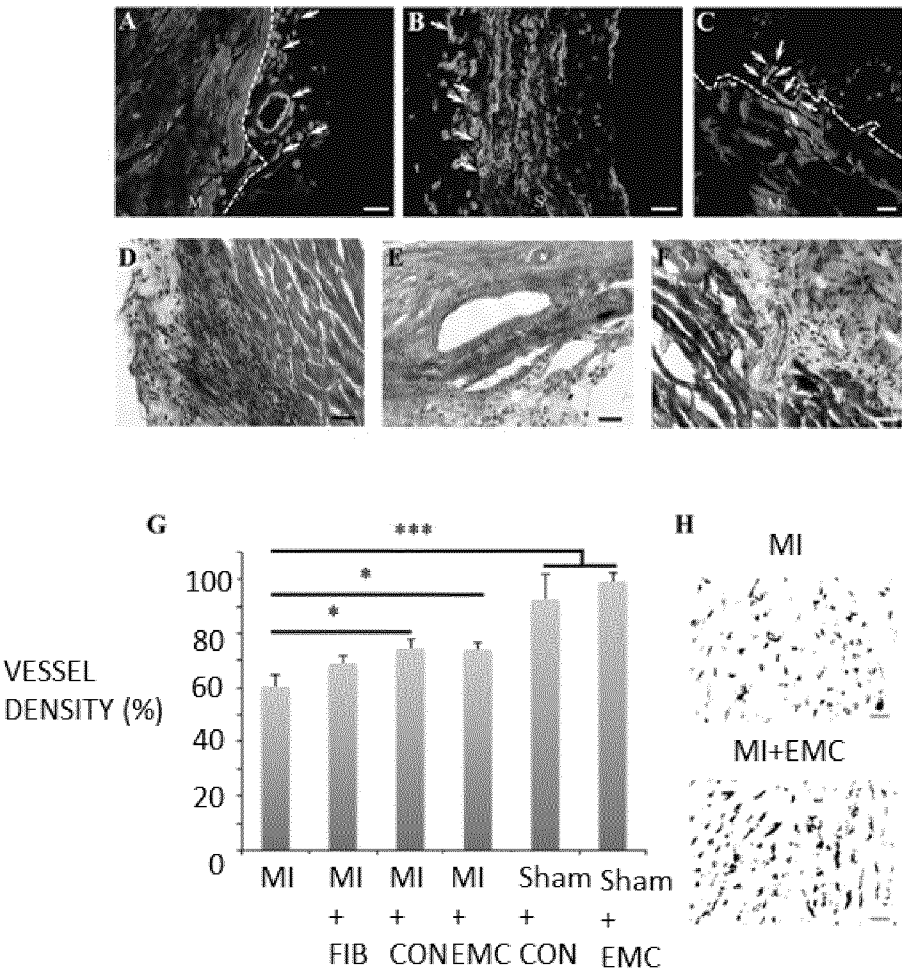


FIG. 5

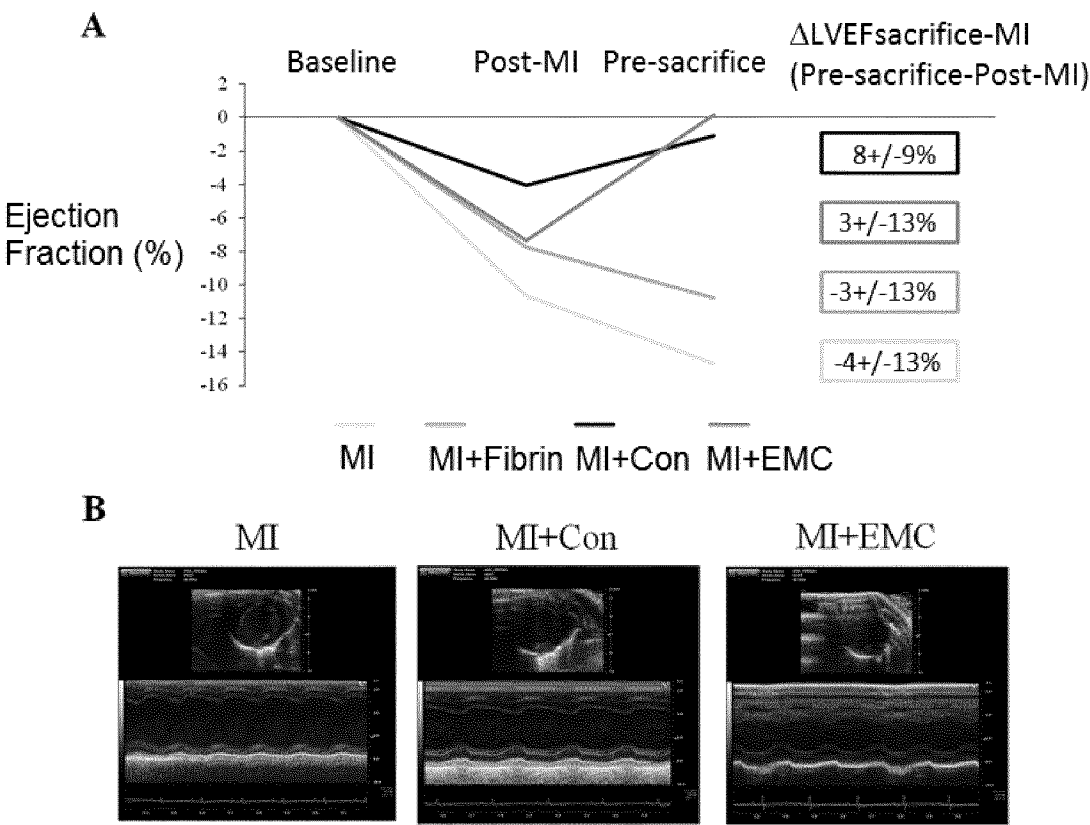


FIG.6

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/051258

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12M1/42 C12N5/0775
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12M C12N

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

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

EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2014/113345 A1 (LEE PETER G W [CA] ET AL) 24 April 2014 (2014-04-24) paragraphs [0056], [0093]; claims 10-12 -----	1-15
Y	WO 2013/185818 A1 (UNIV CATALUNYA POLITECNICA [ES]; FUNDACIO INST D INVESTIGACIO EN CIENC) 19 December 2013 (2013-12-19) claim 15; figures 3,4 ----- -/-	1-15



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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Date of the actual completion of the international search

8 March 2016

Date of mailing of the international search report

17/03/2016

Name and mailing address of the ISA/

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Authorized officer

Novak-Giese, Sabine

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/051258

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AIDA LLUCIA-VALLDEPERAS ET AL: "Physiological conditioning by electric field stimulation promotes cardiomyogenic gene expression in human cardiomyocyte progenitor cells", STEM CELL RESEARCH & THERAPY, BIOMED CENTRAL LTD, LONDON, UK, vol. 5, no. 4, 4 August 2014 (2014-08-04), page 93, XP021193953, ISSN: 1757-6512, DOI: 10.1186/SCRT482 page 94, column 1, last paragraph; figure 1	1-15
Y	----- ANDREA PAVESI ET AL: "Controlled electromechanical cell stimulation on-a-chip", SCIENTIFIC REPORTS, vol. 5, 2 July 2015 (2015-07-02), page 11800, XP055255659, DOI: 10.1038/srep11800 page 11801, paragraph 2	1-15
Y	----- KATHY YE MORGAN ET AL: "Mimicking Isovolumic Contraction with Combined Electromechanical Stimulation Improves the Development of Engineered Cardiac Constructs", TISSUE ENGINEERING PART A, vol. 20, no. 11-12, 1 June 2014 (2014-06-01), pages 1654-1667, XP055255661, US ISSN: 1937-3341, DOI: 10.1089/ten.tea.2013.0355 page 1655, column 2, last paragraph; figures 1,4,5	1-15
Y	----- BO WANG ET AL: "Myocardial Scaffold-Based Cardiac Tissue Engineering: Application of Coordinated Mechanical and Electrical Stimulations", LANGMUIR, vol. 29, no. 35, 3 September 2013 (2013-09-03), pages 11109-11117, XP055255662, NEW YORK, NY; US ISSN: 0743-7463, DOI: 10.1021/la401702w page 11113, column 2, paragraph 1; figure 1	1-15
Y	----- US 2007/238169 A1 (ABILEZ OSCAR J [US] ET AL) 11 October 2007 (2007-10-11) paragraphs [0175], [0176], [0194] -----	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/051258

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